MOLECULAR CHARACTERIZATION OF ACID PHOSPHATASE IN THE LICHEN *CLADONIA PORTENTOSA*

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FACULTY OF SCIENCE AND AGRICULTURE

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Molecular Characterization of acid phosphatase in the lichen *Cladonia portentosa*

Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the Faculty of Science and Agriculture Higher Degrees Office for examination by the University appointed Examiners.

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Purification and biophysical characterization of secreted acid phosphatase from lichen *Cladonia portentosa* (in preparation)

N.P. Mtshali[1], P. Crittenden[2*], P. Dyer[2], D. Archer[2], J.F. Finnie[1], E.J. Hogan[2], A. Plumridge[2], C. Lounds[2] and J. van Staden[1]

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Cytochemical localization of acid phosphatase and its regulation by inorganic phosphate from the mycobiont *Cladonia portentosa* (in preparation)

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Gene expression profiling of phosphorus starvation and menadione in the mycobiont of *Cladonia portentosa* (in preparation)

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ABSTRACT

Acid phosphatases (apase) are important hydrolytic enzymes that function in the acquisition, production; transport and recycling of inorganic phosphate (Pi), thus making a significant contribution towards nutrients dynamic of many ecological niches. The aim of this study was to characterize the apase enzyme found in the lichen *Cladonia portentosa* at the molecular level. The initial experiment entailed cloning the apase gene by PCR using degenerate primers designed from close relatives of *C. portentosa* from the Ascomycete family. The isolation of apase gene from *Cladonia portentosa* using PCR was not successful.

Attempts were then made to purify the secreted apase and to determine its biochemical and molecular properties and to allow comparison with already characterized secreted phosphatases from other fungal sources existing in the NCBI database. It was anticipated that the partial sequence of the purified enzymes would provide a corresponding apase gene. The acid phosphatase enzyme was partial purified to 45 fold by a gel filtration with a yield of 18%. It gave a single, broad glycoprotein band on native PAGE and SDS-PAGE corresponding in size to 250 and 148 kDa, respectively.

Under reducing conditions, the purified enzyme migrated as two bands of 116 and 32 kDa, indicating the heterodimer nature of this enzyme. Only one distinct band, (p*I* 6.4) was observed after electrofocusing. The optimum temperature for the enzyme was 65 °C where an optimal pH was detected at 2.5. The enzyme was inhibited by known acid phosphatase inhibitors (fluoride, molybdate, orthovanadate and tartrate) and the metals (Cu^{2+} and Zn^{2+}). The purified enzyme demonstrated broad substrates selectivity and had a K_M of 31.2±0.25 µM for phytic acid. Peptide analysis by Mass Spectrometry (MS) MALDI-TOF indicated the presence of two apase proteins. The amino sequences of purified apase/s from *Cladonia portentosa* were FLAETNPAPFGH, AVGLGYVEELLAR and AQGLGYVQEVLAR. Comparing the amino acids of the sequenced protein with that of already known proteins confirmed the enzyme to be a secreted histidine acid phosphatase, resembling other acid phosphatases and phytase from several filamentous fungi with respect to amino acid composition.

To investigate the effect of phosphorus on *C. portentosa* apase, the mycelium was grown under different concentrations of Pi [0.05, 1.0, 3.0, 10 and 100 mM (KH_2PO_4)]. The aim was to localize the apase enzyme and to screen for the occurrence of the gene coding for the acid phosphatase enzyme. A treatment of 3.0 mM Pi induced high levels of apase compared to all other treatments. In addition, cultures of *C. portentosa* were grown in axenic cultures to study the effect of pH and Pi versus menadione on the production of acid phosphatase and mycelia growth. A culture media of pH 4.8 and 6.0 resulted in higher apase secretion than when compared with pH 2.5 medium. The presence of 2.0 µM menadione marginally increased levels of the apase compared to the control treatment. Apase was further localized cytochemically using fluorescent substrate-enzyme-labelled fluorescence (ELF-97) which forms a fluorescent crystalline precipitate at the site of phosphate activity. Fluorescent microscope revealed that the enzyme was present in all treatments, irrespective of Pi concentration, however, the fluorescence signals were intense in low Pi concentrations (0.05 and 1.0 and 3.0 mM Pi). Ultrastructure localization using live mycelium under confocal microscopy using Vector blue III substrate revealed that the enzyme was localized in the cytoplasm, cell membrane, vacuole and small organelles, presumed to be endosomes. Co-staining with FM4-64, confirmed the punctuate structure to be secretory vesicles or a vacuolar network.

To investigate the effect of P starvation on *C. portentosa* at a molecular level, the effect of Pi on the gene expression profile was examined. The generation of a cDNA library from axenic grown mycelium treated with P provided a foundation for the identification and characterization of genes expressed in the P treated mycelium through expressed sequence tags (ESTs). Several genes were identified whose transcriptional profiles have been significantly changed by phosphorus treatment and menadione. They include genes required for signal transduction and vesicular transport, cell biosynthesis and protein metabolism and stress response. In conclusion, this study constitutes the first step towards understanding the molecular mechanism governing acid phosphatase in *C. portentosa*.

PREFACE

My journey on this project started like the adventure of Alice in Wonderland, I thought I was just chasing a rabbit around the corner, a relative short journey but those few steps became a journey lasting a few years. My dreams became my very own nightmares that kept me in suspense until the very end. Through the years, I've come to learn that Science is like an infinite pit, where today's discovery becomes a foundation of tomorrow's progress. I hope my research can be a step to such findings for tomorrow's gateway. With limited years of study, one can only go a certain distance before the resources and oil wells run dry. Disappointments and unexpected results are part of the game. My subject of study became so personal, a beloved baby, that kept me awake at night and would not rest during the day. At the end, I was disappointed with some of my expected results from this project but in retrospect I've learnt much more and discovered things I never knew. I'm grateful to be given the opportunity to study this beautiful Reindeer's lichen; I could not have chosen a better subject.

I'm grateful for the guidance and mentoring from South Africa and England and all interesting mentors that I met along the way. I'm grateful to those individual who took care of me and my well-being at home and abroad. There were times when circumstances pushed me over the edge, but I was given a pair of wings that took me to unknown territories. I was reminded of the words of Christopher Logue, which resonated louder than ever. Upon my landing I still encountered challenges, and I kept falling. I remain forever grateful to all those hands that picked me up when I was down. Yes, Angels exist but they do not fly nor do they have wings. It is true: "*Umuntu ngumuntu ngabantu*"- Zulu proverbs "you are a human because of others"- we rely on others for our success.

INSPIRATION

La quête scientifique a cela de remarquable qu'elle presse
sans cesse l'homme à se dépasser-**Blaise Pascal**
(What is striking in the scientific quest, is that it pushes
Continuously the man to surpass himself)

You may write me down in history, with your bitter, twisted lies...
I rise, up from a past that's rooted in pain. I rise.
I'm a black ocean, leaping and wide.
I am the dream and the hope of the slave. I rise, I rise, and I rise
—Maya Angelou (extracted from 'Still I Rise')

Come to the edge. We might fall. Come to the edge.
It's too high!-COME TO THE EDGE!
And they came. And he pushed. And they flew.
—Christopher Logue-New Numbers

Weil ein Schwarzer hässlich ist.
Ist mir den kein Herz gegeben?
Bin ich micht von Fleisch und Blut?
—Mozart, *Die Zauberflöte*

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I dedicate this thesis to my beloved parents: Mpumelelo and Irene Mtshali. "I am blessed to have such amazing individuals to be my parents on this planet." ***UNkulunkulu anigcine nina boma Galela-Gasa njengengonyama!" Ngiyabonga baba nomama!***

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LIST OF ABBREVIATIONS

AA	amino acid(s)
AAC	ADP/ATP carrier
ACM	*Aspergillus* Complete Medium
ADP	Adenosine 5'-Diphosphate
ATP	Adenosine 5'-Triphosphate
BCA	Bicinchoninic acid
Bp	Base pair
BSA	bovine serum albumin
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate
DCW	Dry cell weight
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	Ethylenediaminetetra–acetic acid
ER	endoplasmic reticulum
GF	Gel filtration
GPI	glycosyl phosphatidylinositol-linked protein
HMW	high molecular weight
IEF	Isoelectric focusing
IPTG	Isopropyl ß-D-thiogalactopyranoside
IUPAC-IUB	International Union of Pure and Applied/ Applied Chemistry-International
Kb	Kilobase (pair)
kDa	kilodaltons
K_M	inverse of enzyme affinity (Michaelis constant)
Lag	longevity-assurance gene
LMW	Low molecular weight
MOPS	3-(N-Morpholino) propanesulfonic acid sodium
Mr	Molecular weight
ORF	open reading frame
P	Phosphorus
PCR	Polymerase Chain Reaction

Pi	inorganic phosphate
p*I*	isoelectric points
RPM	revolutions per minute
RSt	Stokes radius of solute
RT	Room Temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacryl Amide Gel Electrophoresis
SOC	Super Optimium Broth
SEM	standard error of the mean
Taq polymerase	*Thermus aquaticus* polymerase
TCA	Trichloroacetic acid
TEMED	N,N,N,N'N'-Tetramethylethylenediamine
Thr	Threonine
UORF	upstream open reading frame
V	elution volume
v/v	volume/volume
V_0	void volume
Vc	geometric column volume
V_{max}	maximum reaction rate
Vt	total volume
w/v	weight /volume

1. LITERATURE REVIEW

1.1 INTRODUCTION AND RATIONALE FOR THE STUDY

Lichens are particularly sensitive to anthropogenic activities which pollute the environment. This makes them ideal biomarkers in assessing the effect of air pollution, ozone depletion, metal contamination and nutrient concentrations in the environment **(FERRY *et al.*, 1976; HAWKSWORTH and ROSE 1976; ROSE and HAWKSWORTH, 1981; VOKOU *et al.*, 1999)**. Most species live for decades thus they can provide a long term reflection of local environmental conditions such as elemental composition of plant matrices and the fluxes of elements between biota, the lithosphere and atmosphere.

In the British Isles, agriculture-based pollution includes ammonium depositions, herbicides and pesticides, has been implicated in the unprecedented decrease in grassland communities (Bryophytes, grass and lichens) over the past years **(PHOENIX *et al.*, 2003)** reducing species richness **(BROWN, 1992; ALSTRUP, 1992; MODENESI, 1993; DAVIES *et al.*, 2007; RIDDELL *et al.*, 2008)**. Anthropogenic nitrogen deposition is a major threat to the long-term stability and floristic diversity of natural ecosystems such as lichen heaths **(BOBBINK *et al.*, 1998)**. The reason for this is that these ecosystems are adapted to nutrient-poor conditions and have extremely slow growth rates i.e. very slow vegetation dynamics and correspondingly low regeneration capacity. In the long term, this means that species with a higher demand for nutrients and a higher competitive capacity under the present eutrophic conditions may replace the original lichen heath species **(LEE *et al.*, 1992; AERTS and HEIL, 1993)**.

Recent studies by **HOGAN *et al.* (2010a; 2010b)** revealed that mat-forming terricolous lichens provide coherent biomarkers for N enrichment which respond to deposition in a consistent and predictable manner. Interestingly, these studies also revealed that nitrogen enrichment changes lichen N:P ratios from values of N limitation to those indicative of P limitation, thus driving the up-regulation of phosphomonoesterase (PME, i.e. phosphatase) enzymes.

The upregulation of phosphatase (PME) in phosphorus-deficient soils (i.e. natural and agricultural ecosystems) is a common phenomenon. Rapid adaptation of soil organisms to fluctuating nutrient supply especially P is essential for their survival, since this macroelement has profound consequences for fungal growth and physiology. To cope with low P availability, many soil microbes have evolved sophisticated metabolic and developmental strategies to enhance phosphate acquisition and remobilization. Research progress in P starvation in soil microorganisms have emerged from mycorrhizal fungi and also some general free-living fungi. Despite a large body of data on metabolic and developmental acclimations of free-living fungi to P starvation, little is known about the underlying molecular processes and P sensory mechanisms operating in lichens. The molecular mechanisms that monitor phosphate availability and integrate the nutritional signal in lichens are essentially unknown.

In the physiological characterization study conducted by **HOGAN *et al.*, 2010a,** *Cladonia portentosa* was demonstrated to be an ideal model species for several reasons: (i) it has a wide distribution in Europe and constitutes the dominant vegetation of much of the arctic tundra and Calluna-dominated heathlands (including upland moorlands in the British Isles) (Figure 1.1); (ii) *Cladonia portentosa* is a typical heathland species, heathland soils are poor in nutrients (such as P and N) and acidic in nature; (iii) this species is mat-forming, thus relies on atmospheric deposition for nutrients, providing a good indicator of atmospheric chemistry, and it is ecologically successful as it forms large cushions (Figure 1.2A). More importantly, PME activity measured in *Cladonia portentosa* was the highest reported in lichens and several fungi, thus, unusually high levels of PME found in this lichen have prompted interest to study their secreted PME. To date, no molecular characterization of PME have been reported for this lichen.

Cladonia portentosa (Dufour) Coem is a fruticose lichen, commonly known as "Reindeer Moss", belonging to the family *Cladoniaceae*, a division of the Ascomycota **(DOBSON, 2005)**. This lichen belongs to a class of *Lecanoromycetes.* It is intrinsically branched, compact and interwoven, and forms large mats up to 6 cm in height (Figure 1.2A-C). The internal structure is radial with a dense outer cortex, a thin algal layer, a medulla and a more or less hollow centre or dense central cord (Figure 1.2B). Most typical fructicose lichens are anchored by basal rhizoidal

strands derived from the cortex although many species are without any attachment to the substrate.

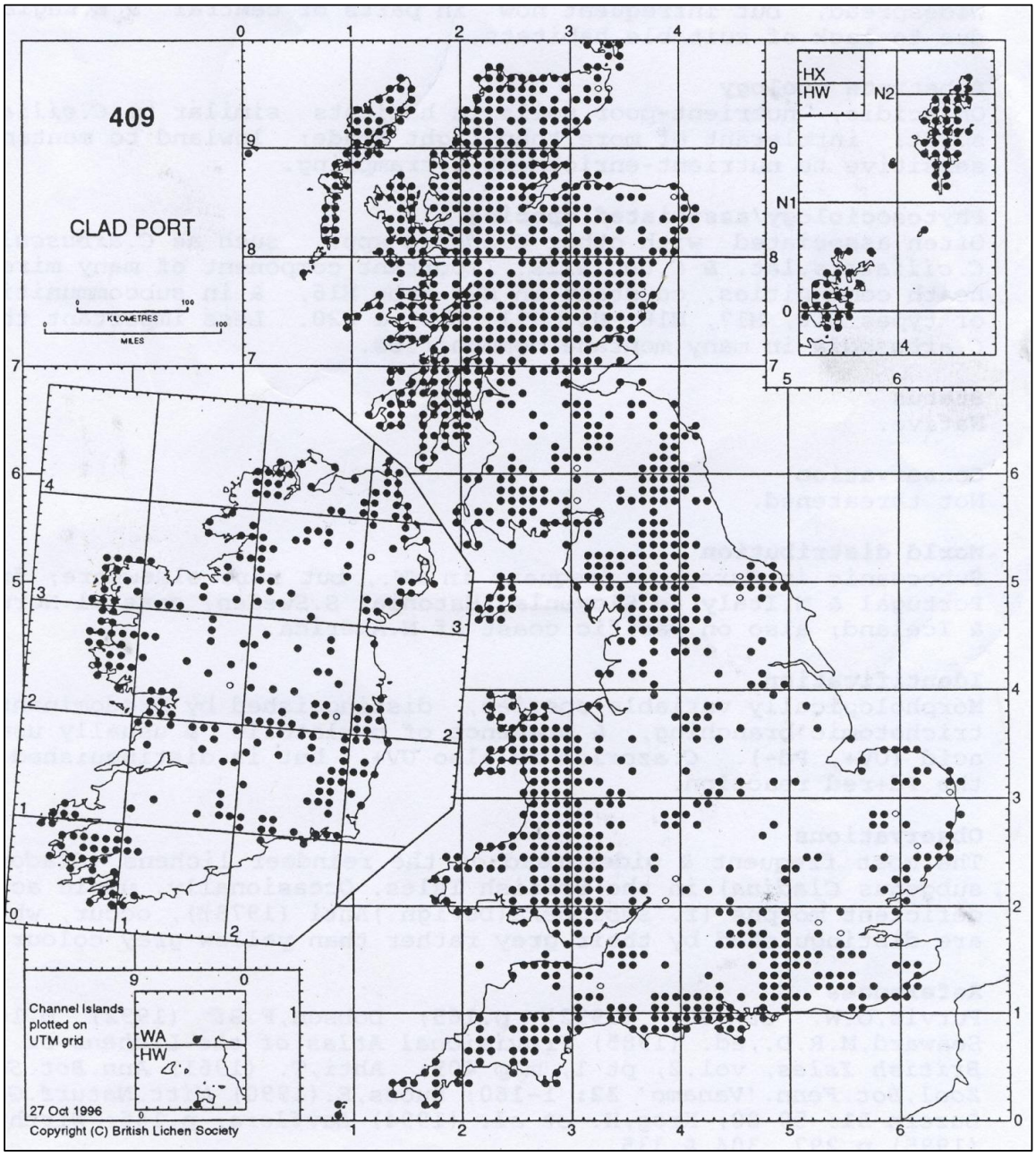


Figure 1.1: Map of distribution of *Cladonia portentosa* across the British Isle.

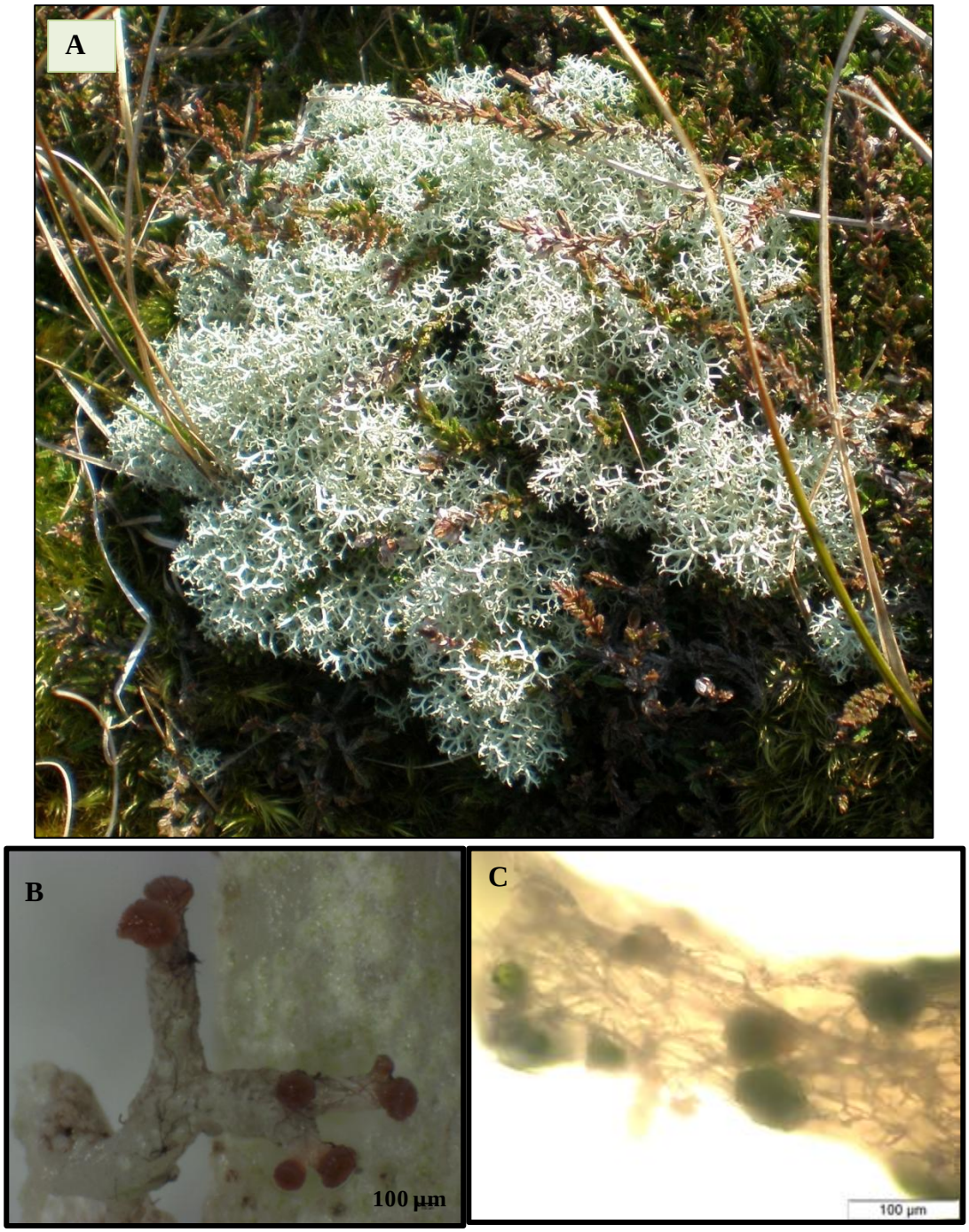


Figure 1.2: (A-C) *Cladonia portentosa* forms large mats. (A) Portion of podetium showing brown-reddish discocarps (B). Lichen under bright field light, where the clumps of algal photobiont (*Trebouxia)* can be seen (C).

1.2 AIM/S OF THE PRESENT STUDY

The present study was aimed at molecular characterization of acid phosphatases from *Cladonia portentosa*. To date, there has been no molecular characterization of acid phosphatase from lichens reported in the literature. Specific aims of the study were:-

Cloning the acid phosphatase gene by PCR;

I. Purifying the acid phosphatase protein, and to study its biophysical characteristic properties;
II. Cytochemical localization of secreted acid phosphates in the mycelium of *Cladonia portentosa;* and
III. Assessing the influence of inorganic phosphorus (Pi) on acid phosphatase biosynthesis under limiting and sufficient conditions.

1.3 INTRODUCTION TO LICHENS

Lichens exist as discrete thalli and are implicitly treated as individuals in many studies, even though they may be a symbiotic entity involving two or three Kingdoms. The partnership may be between heterotrophic fungi (mycobionts) and photosynthetic prokaryotic cyanobacteria (phycobiont) or eukaryotic (algae) organisms (photobionts). They are important constituents of many vegetative ecosystems in the world, from the tropics to polar regions **(HALE, 1983; NASH, 1996)**. Approximately 8% of terrestrial ecosystems are lichen-dominated and include around 17 000 species **(HALE, 1983; NASH, 1996)**. They occur in some of the most extreme environments on earth such as arctic tundra, hot deserts, rocky coasts and toxic slag heaps, deep sea, wood and sediments (**KOHLMEYER and KOHLMEYER, 1977; RAGHUKUMAR *et al*., 2004; SELBMANN *et al*., 2005)**. In addition to their presence in most natural, industrial and agricultural settings, they can also be found as epiphytes on leaves and branches in rain forests and mesic habitats **(KOHLMEYER and KOHLMEYER, 1977; RAGHUKUMAR *et al*., 2004; SELBMANN *et al*., 2005)**.

A lichen comprises of a 'vegetative plant-like body' thallus which is remarkably complex, having little resemblance externally to either non-lichenized fungi or algae **(HALE, 1974)**. The basic building blocks of fungi are elongated cellular threads called hyphae. Masses of hyphae form a vegetative thallus or mycelium, which is usually inconspicuous in non-lichenized fungi but often quite elaborate and durable in lichens. The lichen thallus is a relatively stable and well-balanced symbiotic system with both heterotrophic and autotrophic components, making these organisms classic examples of symbiosis **(NASH, 2008)**. Even though lichens are an association of different organisms, they are named after the fungal partner, which dominate the symbiosis in most cases **(VERMA *et al*., 2008)**. Lichens can be considered as ecosystems where the interaction of partners results in behaviour and life forms that are not found in the isolated partners **(NASH, 1996; KRANNER *et al*., 2005; VRÁBLÍKOVÁ *et al*., 2006; OKSANEN, 2006)**. The spectrum of interaction, remain an enigma to biology, which range from mutualism,

through commensalism to situations where the associates are doomed by antagonistic activities of an invading fungus **(RICHARDSON, 1999)**.

The discovery of nitrogen fixation by various lichens has given birth to the concepts of parasymbiosis. Parasymbiosis is where parasymbionts such as Discomycete or Pyrenomycete fungi infest lichen thalli **(HALE, 1974)**. Parasymbionts derive nutrients from the algae and maintain a balanced state of symbiosis within them and with the composite thallus. These parasitic symbiotic fungi may cause extensive damage, resulting in localized necrotic patches or in the complete death of the thallus **(RAMBOLD and TRIEBEL, 1992; NASH, 2008**). Alternatively, these secondary fungi do not appear to cause damage but they are assumed not to benefit their hosts either. However, their competitive presence often makes it difficult to isolate the mycobiont **(CRITTENDEN *et al*., 1994)**.

Lichens are an ancient group of fungi **(TEHLER *et al*., 2003)** and 98% of them belong to the Ascomycota phylum. This is the largest phylum of fungi (~64 000 known species) and one of the most diverse and ubiquitous phyla of eukaryotes **(KIRK *et al.*, 2001; 2008)**. They function in the decay of organic substrates and act as mutualists, parasites and pathogens of animals, plants and other fungi since they occur in numerous ecological niches and virtually all terrestrial and aquatic ecosystems. More than 40% of all named Ascomycota are lichenized, covering approximately 8% of the Earth's landmasses **(BRODO *et al*., 2001)**. A small percentage of lichens belong to the Basidiomycota and Deuteromycota (known as Fungi imperfecti or mitosporic fungi) **(HAWKSWORTH *et al*., 1995; TEHLER, 1996)**. The latter group is an artificial class, in which sterile species are placed.

Approximately 40 genera of algae and cyanobacteria have been reported as photobionts in lichens **(TSCHERMAK-WOESS, 1988; BÜDEL, 1992)**. It is estimated that about 85% of lichen-forming fungi are symbiotic with green algae of which about 1500 species are known **(TSCHERMAK-WOESS, 1988; BÜDEL, 1992; HONEGGER, 1996)**. In comparison, approximate 10% are symbionts with cyanobacteria (blue green algae)

(HONEGGER, 1996). Whereas ~3-4% are cephalodiate species which associate simultaneously with green algae and cyanobacteria **(TSCHERMAK-WOESS, 1988; HAWKSWORTH *et al*., 1995; HONEGGER, 1996)**.

The most frequent photobionts belong to three genera, *Trebouxia, Trentepohlia* and *Nostoc* **(FRIEDL and BÜDEL, 2008)**. The two genera *Treboxia* and *Trentepohlia* are of eukaryotic nature, thus referred to as photobionts and belong to the green algae. Conversely, the genus *Nostoc* belongs to oxygenic photosynthetic bacteria thus is refered to as phycobionts or cyanobionts. The photobionts belongs to the green algae (phylum *Chlorophyta*) **(BOLD and WYNNE, 1985; VAN DEN HOEK *et al*., 1993)**. Tripartite lichen thalli consist of lichen fungi and green alga (*Chlorophyta*, **LEWIS and MCCOURT, 2004)** while the cyanobacteria are spatially separated from algae in internally or externally occurring fungal compartments called cephalodia **(BÜDEL and SCHEIDEGGER, 1996)**.

At different stages, some mycobionts can change their photosynthesizing partner from green algae to cyanobacteria and *vice versa*, resulting in a change of thallus morphology **(OKSANEN, 2006)**. This behaviour was suggested to be due to an environmental adaptation and related to ecological compatibility of the photobiont **(HONEGGER, 1996; STENROOS *et al*., 2003)**. Naturally, isolated mycobionts grow so slowly that they are unlikely to survive well in the free-living state due to competition with other fungi or consumption by other organisms. Thus, most mycobionts are assumed to have an obligate relationship to lichenization, although the specificity of the mycobiont for a particular photobiont may not be as great as one might assume **(BECK *et al*., 1998; RAMBOLD *et al*., 1998, NASH, 2008)**.

Numerous studies have revealed that both bionts appear in nature among a mixture of millions of non-symbiotic microorganisms, and many mechanisms of compatibility combination are required **(GALUN and KARDISH, 1995; LEGAZ *et al*., 2004)**. Thus, specificity is required for the lichen association **(LEGAZ *et al*., 2004)**. In this context, specificity can be defined as ‘preferential, but not exclusive, association of one biont with

another' **(BUBRICK, 1988)**. This is illustrated by *Cladonia cristitella*, which produce squamules with different species of *Trebouxia*, displaying a selective behaviour **(AHMADJIAN and JACOBS, 1981)**. However, the mycobiont of *C. cristatella* cannot form squamules with green algae other than *Trebouxia*, showing high specificity **(AHMADJIAN, 1993)**. Furthermore, culture experiments by **SCHAPER and OTT (2003)** on selectivity of the mycobiont of *Fulgensia bracteata* towards potential photobionts provide evidence for fungal selectivity and varying compatibility of the respective symbionts, which can be interpreted as a cascade of interdependent processes of specific and non-specific reactions of symbionts involved.

Elaborative work by **AHMADJIAN (1993)**, demonstrated that specificity needs mechanism of recognition to be finely tuned. When the algal cells multiply inside a growing thallus, daughter cells are enveloped by fungal hyphae which recognize new cells as compatible. Thus, recognition mechanisms are paramount not only for *de novo* formation of new associations but also for the maintenance of the symbiotic equilibrium in lichen symbiosis **(AHMADJIAN, 1993)**. For such a relationship to function, cell surface recognition factors have been proposed which include phytohaemagglutinins, algal binding protein (ABP), legands and cell enzymes such as arginases **(AHMADJIAN *et al*., 1978; BUBRICK *et al*., 1984; LEGAZ *et al*., 2004)**.

1.3.1 Reproduction in Lichens

The upper surface of many lichens bears fruiting bodies know as apothecium (Figure 1.1B), which can forcibly discharge tiny spores to a height of a few millimeters where they have a good chance of becoming airborne. Their survival and germination depends on the spore landing on a suitable photobiont partner before they can form new lichens **(BÜDEL and SCHEIDEGGER, 1996)**. Thus, many lichens increase their chance of successful reproduction by producing vegetative propagules such as isidia and soredia, which contain both a fungal and photosynthetic partner which becomes detached and grow into a new thallus **(OTT, 1987a; 1987 b; BÜDEL and SCHEIDEGGER, 1996)**. Soredia typically consist of photobiont cells enclosed in a loose network of hyphae; these

structures can develop diffusely over the surface of the thallus or in specialized areas called soralia **(SEYMOUR *et al*., 2005)**. Isidia are small, smooth, cylindrical peg-like structures that project outwards from the outer cortex of the thallus **(SEYMOUR *et al*., 2005)**. Soredia and isidia can be dispersed by wind, rain or small animals **(BÜDEL and SCHEIDEGGER, 1996)**. Lichens can also multiply by thallus fragmentation **(HONEGGER *et al*., 1996)**.

Non-lichenized and lichen-forming Ascomycetes are neither female nor male. Each haploid mycelium is theoretically capable of differentiating both gametangia (ascogonia) and gametes (microconidia=spermatia). Their sexual reproduction is regulated by mating type (MAT) genes **(DEBUCHY and TURGEON, 2006; TURGEON and YODER, 2000)**. In contrast, Basidiomycetestes have severals MAT loci and thus very complex mating systems. Filamentous Ascomycetes have one MAT locus, which is completely different in haploid mycelia carrying one out of the two MAT alleles of the same heterothallic cross-fertilised species. These are referred to as MAT1-1 and MAT1-2 **(TURGEON and YODER, 2000)**.

The majority of lichen forming Ascomycete and their photobionts can be cultured in single cultures and the lichen can be resynthesized from these **(MURTAGH *et al.*, 1999; BRUNAUER and STOCKER-WÖRGÖTTER, 2005)**. However, cultures in the aposymbiotic state have failed to differentiate sexual reproductive stages under these conditions, thus classical genetic crossing experiments cannot be conducted. Another set-back is that lichen mycobionts grow extremely slowly compared to their photobiont partner **(FRIEDL and BÜDEL, 1996)**. Improvements in nutrient media have resulted in faster growing rates but still unmatchable to their non-lichenized fungal counterparts **(STOCKER-WÖRGÖTTER, 2005)**.

1.3.2 Uses and application of lichens

Throughout the centuries lichens have been used for various purposes such as food, dyes and as medicinal remedies. Due to their bitter taste, lichens have not been used as sources of food by humans to a great extent, although there are several cases where they are used

in various cultures (e.g. Japan) as delicacies. Lichens are, however, a source of food for animals in the arctic regions such as deer and caribou, who supplement their normal diet with lichens such as *Cladonia* and *Cetraria*s during the winter **(HALE, 1974)**. Sheep in the Libyan desserts are reported to graze on the subfoliose lichen *Lecanora esculanta*. A number of invertebrates secrete lichenases (break down lichen material to glucose) thus they can utilize lichen as a food source **(HALE, 1974)**.

Lichens have been used for medical purposes since the middle ages. The use of lichens in folklore medicine has been world-wide: (India, China, Japan, Europe and America) **(HALE, 1974)**. Modern studies have shown that a number of lichens contain active compounds such as usnic acid from *Cladonia* species. Lichens were used extensively as a source of dyes before the discovery of coal-tar dyes. Species such as *Rocella, Parmelia* and *Evernia* have been extensively used for this purpose.

1.3.3 Ecology of lichens

Lichens display a variety of morphologies, with three major forms: crustose (crust-like biofilm), foliose (leaf-like) and fruticose (branched tree-like, shrubby, pendulous) types **(HAWKSWORTH *et al.*, 1995; BÜDEL and SCHEIDEGGER 1996; OKSANEN, 2006)**. Foliose lichens are characterized by a more or less flattened thallus with easily distinguishable upper and lower surfaces and are attached to the substrate either directly by the hyphae of the lower cortex or medulla **(THORMANN, 2006)**. In comparison, fructicose lichens grow erect or are pendant and without distinguishable upper and lower surfaces on their thalli. These lichens are attached to the substrate at one or very few points. Crustose lichens form crusts over their substrates. Their lower surfaces grow on and among the particles that constitute the substrates; hence they cannot be removed from the substrates in one piece **(BRODO *et al.*, 2001)**.

Lichens possess a number of morphological and chemical adaptations enabling them to survive stressful conditions (hot deserts, artic tundras, heaths, and tree canopies) and to quickly restore their metabolic activity **(ARKIN *et al.*, 2000; BECKETT *et al.*, 2003)**.

They are prominent members of poikilohydric organisms, whose water status varies passively with surrounding environmental conditions **(NASH, 1996)**. They are able to exist in an extreme dry state with very low levels of metabolic activity **(SUNDBERG *et al*., 1997),** but can rapidly resume normal physiological activities upon rehydration. It was shown that water loss from lichen thalli is accompanied by almost total inactivation of photosynthetic gas exchange and loss of variable chlorophyll fluorescence **(SCHROETER *et al*., 1991)** whereas re-wetting of the thalli with liquid water normally restores photosynthetic activity within minutes **(WEISSMAN *et al*., 2005)**. Metabolic activities, mainly respiration and photosynthesis frequently result in the production of reactive oxygen species (ROS) **(FRIDOVICH, 1999; KOHEN and NYSKA, 2002)**. These are enhanced during stress such as nutrition limitation, exposure to xenobiotics or desiccation and/ or rehydration.

Lichenization is a strategy for the mycobiont (heterotrophic organism) to acquire fixed carbon from the alga. In addition, the presence of a photosynthesizing partner allows the lichen to inhabit substrates that are virtually devoid of organic matter. These organisms can efficiently extract nutrients (phosphorus, magnesium, calcium, potassium, sulphur and iron) from recalcitrant surfaces **(RICHARDSON, 1975)**. Rhizinae on the lichen thalli may have a function in the uptake of nutrients. The alliance between these organisms (fungi and algae/cyanobacteria) enables them to live together in inhospitable areas (oligotrophic habitats and severe climatic zones), where they could not do so independently irrespective of habitat extremity, such as light, dryness and temperatures which are less favourable for higher plants **(KERSHWA, 1985; VRÁBLÍKOVÁ *et al*., 2006; ZAVARZINA and ZAVARZIN, 2006)**. In addition, both mycobiont and algal photobiont may participate in seasonal photoacclimation in green algal lichens **(VRÁBLÍKOVÁ *et al*., 2006)**. The light and desiccation is greater in lichen symbiosis than in its isolated partners **(KRANNER *et al*., 2005; VRÁBLÍKOVÁ *et al*., 2006)**. Lichens adapted to open habitats tolerate extreme desiccation and UV exposure via their screening cortical pigment by preventing the formation of scavenging free radicals **(NYBAKKEN *et al*., 2004; GAUSLAA, 2005; VRÁBLÍKOVÁ *et al*., 2006)**.

The existence of the lichens in these oligotrophic habitats and severe climatic zones contribute substantially to biomass and support a high biodiversity of micro-and macro organisms, creating complex food webs and adding significantly to energy flow **(SEAWARD, 2004)**. In addition, lichen associations, especially the phycolichens play an important role in soil fertility and ecological energetic due to their N-fixing abilities. The disappearance of lichens, due to many aspects of human interference in the natural world, has therefore led inexorably to environmental impoverishment. Lichens are natural sensors of our changing environment. Lichens are therefore used increasingly in evaluating threatened habitats, in environmental impact assessments, and in monitoring environmental perturbations, particularly those resulting from a disturbingly large and growing number of chemical pollutants.

1.3.4 Using lichens to monitor pollution

Lichens are particularly sensitive to anthropogenic activities which pollute the environment making them ideal biomarkers in assessing the effect of air pollution (chemical deposits), ozone depletion, metal contamination and nutrient concentration in the environment **(FERRY *et al*., 1976; HAWKSWORTH and ROSE, 1976; ROSE and HAWKSWORTH, 1981; VOKOU *et al*., 1999)**. Lichens have no vascular system for conducting water or nutrients; thus they depend on atmospheric sources (e.g. fog and dew). However, major water resources for lichens often have much higher pollutant concentrations than precipitation. The lichens nutrient concentration mechanism also will concentrate pollutants **(NASH, 2008)**. Unlike many vascular plants, lichens have no deciduous parts, and hence cannot avoid pollutant exposure by shedding such parts. Furthermore, the lack of stomata and cuticle in lichens means aerosols may be absorbed over the entire thallus surface, thus lichens have little biological control over gas exchange, and air pollutant gases are assumed to readily diffuse down the photobiont layer **(NASH, 2008)**. Although dehydration allows lichens to survive dry periods, it also concentrates solutions to the point that toxic concentrations may occur. Finally alteration of the symbiotic balance between the photobiont and mycobiont may readily lead to a breakdown of the lichen association **(FEIGE and JENSEN, 1992; NASH, 2008)**.

Most lichen species live for decades or hundreds of years and a few even longer: thus as perennials they are subject to the cumulative effects of pollutants. Lichens can provide a long term reflection of local environmental conditions such as elemental composition of plant matrices and the fluxes of elements between biota, lithosphere and atmosphere. In general the elemental composition of plants reflects the chemical composition of their growth media such as soil, air and nutrient solutions **(FARAGO, 1994)**. This was demonstrated in the study by **DILLMAN (1992)** where he reported a significant accumulation of elements such as Cu, Ca, Mg and K in lichens as a function of distance and direction from the refineries nearby.

Classic studies on lichens show that (sulphur dioxide) SO_2 pollution resulted in the decline of many lichens especially around big cities such as London. Improvement of air quality and reinforcement of the clean air legislation in many European countries (e.g. 1956, 1968; clean air acts in UK) has resulted in dramatic reduction in pollution especially SO_2 levels. This improvement was evident by the recovery of the lichen flora in the 1980's **(VAN DOBBEN and DE BAKKER, 1996)**.

In recent years, agriculture-based pollution which includes ammonium depositions, herbicides and pesticides have been shown to have had a negative effect on lichen species richness **(BROWN, 1992; ALSTRUP, 1992; MODENESI, 1993)**. The major sources of deposited atmospheric N are ammonia (NH_3) and nitrogen oxides (NO and NO_2) on a global scale, especially in Europe. Emission of reduced N, mainly resulting from livestock management and fertilizer application exceeds those of oxidised N **(PITCAIRN *et al.*, 2003)**. Acid and oxidising forms of N are broadly toxic and tend to reduce species richness **(DAVIES *et al.*, 2007)**. The supply of reactive nitrogen to global terrestrial systems has doubled and when released on land, they result in eutrophication of both fresh water and ground water, while emission to the atmosphere results in regional eutrophication and acidification, crop damage and impact on human health **(GALLOWAY, 1998; ERISMAN *et al.*, 2003)**.

Lichens are taxonomically diverse **(TEHLER, 1996)**. Among lichens, oligotrophs (acidophytes) are adapted to environments with low nutrient availability compared to mesotrophs (neutrophytes) which require moderate N and eutrophs that thrive in nutrient rich environments **(GEISER *et al.*, 2010)**. Their relative dominance shifts with nutrient N deposition, allowing characterization of community effects and ecological harm **(VAN HERK *et al.*, 2003; MITCHELL *et al.*, 2005; SPARRIUS, 2007; SUTTON *et al.*, 2008)**. Due to their diversity, other species can survive increased N loads either by avoiding excessive assimilation **(HYVÄRINEN and CRITTENDEN, 1998; DAHLMAN *et al.*, 2002)** or an ability to store surplus N in the form of amino acids such as arginine **(SILBERSTEIN *et al.*, 1986)**. Such increases in tissue N concentrations are likely to cause an increased C demand, both to provide C skeletons for amino acid synthesis **(NORDIN and NÄSHOLM, 1997)** and energy for increased respiration **(CHAPIN *et al.*, 1987)**.

Several lichens have declined or even disappeared from habitats with elevated N levels **(DAHLMAN *et al.*, 2002)** which might reflect uncontrolled N assimilation leading to toxic levels of NH_4^+ in the tissue **(GAIO-OLIVEIRA *et al.*, 2001)**. In the Netherlands, during the last 10 years an increase in nitrophytic lichen species, paralleled by a decrease in acidophytic ones, has occurred in areas with high cattle density **(VAN DOBBEN and TER BRAAK, 1998)**. This phenomenon was especially apparent on acid-barker trees, on which nitrophytes were previously scarce or absent **(VAN DOBBEN and TER BRAAK, 1998)**. A similar shift in species composition was also observed in the UK **(WOSELEY and JAMES, 2002)** and Switzerland **(RUOSS and VONARBURG, 1995)**. Comprehensive lichen mapping programmes for the British Isles has shown that some species have extended their ecological and geographical range by exploitation of acidified substrata **(SEAWARD, 2004)**. Similar mapping programmes have been established in many other European countries **(VOKOU *et al.*, 1999)**.

British heaths consist of a large proportion of the remaining European heathlands, where mat-forming lichens are major vegetation components **(FARRELL, 1989; HYVÄRINEN and CRITTENDEN, 1998)**. Genera such as *Cladonia, Cetraria, Flavocetraria* and

Sterecaulon grow as extensive carpets in the vegetation of oligotrophic tundra, sub-artic taiga and heath and ombrotrophic peabogs **(RODWELL, 1991)**. They are important contributors to the functional ecology of these habitats, in terms of biomass and carbon storage **(LANGE *et al.*, 1998)**.

The need to study acid load in these fragile heathland ecosystems has prompted several studies. **CRITTENDEN (1989)** demonstrated the efficient uptake by *Cladonia stellaris* and *Stereocaulon paschale* of NH_4^+ and NO_3^- delivered in rainfall (80%) and suggested that carpets of mat-forming lichens are significantly ombrotrophic in nature thus possess enormous potential as indicators of N deposition, particularly across large areas of remote northern terrain in which they are often abundant and for which measurement of N load are generally sparse. Mat- forming lichens occur typically in open situations where they intercept rainfall that is largely unmodified by overlapping plant canopies **(HYVÄRINEN and CRITTENDEN, 1998**). Recent studies by **HOGAN *et al*. (2010a)** revealed that mat-forming terricolous lichens provide coherent biomarkers for N enrichment. Interestingly, nitrogen enrichment also induces P-limitations in *C. portentosa* with attendant changes in chemical and physiological characteristics that could be used as a sensitive biomarker with which to detect low levels of N pollution **(HOGAN *et al*., 2010a; 2010b)**.

1.3.5 Nutrient acquisition in fungi/lichens

Lichens like many other organisms, need nutrients to survive. There is a lack of factual information relating to food webs and nutrient recycling in most lichens, mainly because whole lichens cannot be readily grown in culture; therefore, precise concentrations of nutrients necessary for lichens growth remain undefined **(NASH, 2008)**. It is well-established that lichen carbohydrate demands are supplied by the photobionts. The flux of carbohydrates, such as polyols in the case of green algae lichens and glucose in the case of cyanolichens, from the photobiont to the mycobiont have been demonstrated in several studies **(SMITH and DOUGLAS, 1987)**. The preference of carbohydrates that are sugar-alcohols seem to be common in lichens with green algal photobionts, whereas in lichens with cyanobacteria, glucose is often preferred **(FIEGE and JENSEN, 1992)**.

Studies involving nutrient acquisition in lichens are still in its infancy. Other symbiotic organisms such as mycorrhizae and rhizospheric fungi have shed light on the ability of the symbiotic fungus to acquire nutrients. Vesicular-arbuscular mycorrhizal (VA) fungi colonize plant roots and transport water and mineral nutrients from the soil to the plant while the fungus is benefiting from carbon compounds provided by the host plant **(TURK *et al*., 2006)**. VA-fungi are associated with improved growth of plant species due to increased nutrient uptake, production of growth promoting substances, tolerance to drought, salinity and transplant shock and synergistic interaction with other beneficial soil microorganism such as N-fixers and P-solubilizers **(SREENIVASA and BAGYARAJ, 1989)**. The benefits of symbiotic association of plant roots with VA fungi are well-known. Enhanced growth because of increased acquisition of phosphorus and other low mobile mineral nutrients have been reported **(KWAPATA and HALL, 1985; TURK *et al*., 2006)**. Ectomycorrhizal (ECM) trees benefit from association with Basidiomycetes that possess several high-affinity P transporters that are expressed in extra-radical hyphae and whose expression is enhanced by P deficiency **(PLASSARD and DELL, 2010)**.

To date no comparable flux of nutrients from the mycobiont to the photobiont has been demonstrated. Questions whether the fungus serves as the reservoir of inorganic nutrients for the photobionts through haustoria are being raised and need further investigation. In mycorrizal fungi, this seems to be the case. For instance, involvement of cytoplasmic streaming in the translocation of phosphate in arbuscular mycorrhizal fungi has been suggested based on calculations of the energy required for the high flux rates of phosphorus in hyphae **(TINKER, 1975; HALEY and SMITH, 1983)**. The relationship between tubular-form vacuoles and cytoplasmic streaming implies the importance of this form in transport inside hyphae **(UETAKE *et al*., 2002)**.

Nutrient acquisition in lichens can also be gleaned from field studies. The addition of nutrients often stimulates growth and various metabolic processes, thereby demonstrating nutrient requirements. For example, when lichens were fertilized, they exhibited a varying response. For instance *Cladonia stellaris* did not increase its growth whereas cyanobacteria species *S. paschal* and *Peltigera aphthosa* responded positively

(KYTÖVIITA, 1993; HYVÄRINEN *et al.*, 2003). These results suggest that the impact of enhanced N on lichen growth may be biased towards the growth of the photobionts rather than the mycobionts. The consequence of increasing the photobiont's growth may inevitably lead to breakdown of the symbiosis.

SUN and FRIEDMANN (2005) found a positive relationship between alga to fungus ratio and habitat summer temperature in *Cladina rangiferina*. They suggested that regulation of the ratio of the producer (alga to fungus) directly contributes to adaptation to a wide range of thermal regimes and to the distribution of lichens. The differential responses of fungal and algal growth to N and P fertilization observed in their study suggested that the tissue nutrient contents, and particularly the nutrient balance, affect resource allocation in the lichen thallus **(MAKKONEN *et al.*, 2007)**.

The distribution and dominance of *Cladonia portentosa* in acidic and nutrients-poor soil, demonstrates the ability of this lichen to efficiently acquire nutrients. Several studies have demonstrated limiting effects of nitrogen (N) on lichen productivity but phosphorus (P) limitation deserves much more consideration. Unlike nitrogen, P has no gas phase, and frequently P may be lost from ecosystems by sedimentation and secondary mineral formation **(NASH, 2008)**.

It is well known that the presence of an array of hydrolases such as PME helps these fungi to acquire nutrients. This is common in other symbiotic organisms like mycorrhizal fungi where phosphorus deficiency increase PME up-regulation. The importance of secreted acid phosphatase is better recognised in plant-fungus symbiotic (mycorrhizal) relationships, as their production by soil microorganisms increases the amount of phosphate available to plants **(YADAV and TARAFDAR, 2003)**, contributing significantly to the nutrient dynamics of most ecological niches where phosphorus is deficient **(MOLLA *et al.*, 1984; JAYACHANDRAN *et al.*, 1992; TURNER *et al.*, 2001)**.

Phosphate (P) is one of the essential elements but least available nutrients in many natural ecosystems **(HALSTEAD and MCKERCHER, 1975; SHIMOGAWARA *et al*., 1999; ABEL *et al.*, 2000; BOZZO *et al*., 2002; GEORGE *et al*., 2011)**. Paradoxically, soils usually contain a relatively large amount of P, in the form that is not directly available for use. Up to 80% occurs in organic forms **(RICHARDSON *et al*., 2005)** of which inositol phosphates constitute the largest (~50%) fraction **(TURNER *et al*., 2002)**. Another contributing factor to low phosphate availability is mainly due to its insoluble precipitation with cations or its conversion into organic complexes **(WYKOFF *et al*., 1999; CHEN *et al*., 2000; RAGHOTHAMA, 2000)**. To cope with low phosphate availability, many plants and microbes have evolved numerous physiological (inorganic phosphate (Pi) transporters), biochemical (secreted enzymes) and molecular (multi-genes) adaptations to scavenge traces of usable phosphorus from the environment **(FURIHATA *et al*., 1992; SHIMOGAWARA and USUDA, 1995; JESCHKE *et al*., 1997; SCHACHTMAN *et al*., 1998; RAGHOTHAMA, 1999; ABEL, 2002)**.

A central component in this situation is a class of enzymes, capable of acting on the ester bond (Enzyme Commission number, EC 3.1) and catalysing the cleavage of phosphate esters (EC 3.1.3), constituting the subclass of phosphohydrolases, i.e. phosphatases (phosphomonoesterases, PME).

1.4 ACID PHOSPHATASE

1.4.1 General characterization of acid phosphatase

Phosphatases are a diverse class of enzymes which are generally characterized as being either acidic or alkaline phosphatase depending whether their optimal pH catalysis is below or above pH 7.0 **(VINCENT *et al.*, 1992)**. Non-specific phosphatases can be either alkaline or acid phosphatases. Traditionally, phosphatases have been characterized into at least five classes: (i) alkaline phosphatase, (ii) purple acid phosphatase, (iii) low molecular phosphatase, (iv) high molecular acid phosphatases; and (v) protein phosphatase. Their classification can be further extended to include several frameworks such as substrate type

and inhibition by tartrate as tartrate-sensitive and tartrate resistant **(VINCENT *et al.*, 1992)**. These classes differ in their pH optima, substrate specificities and possibly even reaction mechanisms **(VINCENT *et al.*, 1992)**. The focus of this study is on acid phosphatases.

Acid phosphatases [Apase, orthophosphoric-monoester phosphohydrolase, (EC 3.1.3.2)] are a group of enzymes responsible for the transfer of phosphoryl groups from phosphomonoesters to water **(VINCENT *et al.*, 1992)**. These enzymes are ubiquitous and abundant in most cells of animal tissue, plants and several microbes with different degrees in enzyme activity and substrate specificity. According to **VINCENT *et al.* (1992)** these enzymes achieve the biological hydrolysis of single phosphate groups from a variety of substrates in a thermodynamically favourable process ($\Delta G^{o'} \leq$-9kJ mole^{-1}).

High weight molecular phosphatase can be further classified according to substrate specificity, such as subclass-phytases. Phytases (*myo*-inositol hexakisphosphate phosphohydrolase) are unique phosphatases which catalyses the stepwise hydrolysis of phytic acid to orthophosphate, a series of lower phosphate esters of *myo*-inositol and eventually *myo*-inositol **(BRINCH-PEDERSEN *et al.*, 2002)**. The International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC-IUB, 1975) classified this group of enzymes as 3-phytase (EC 3.1.3.8) or 6-phytase (EC 3.1.3.26), based on the position-specificity of the initial hydrolysis from phytate **(BRINCH-PEDERSEN *et al.*, 2002)**. Three structurally distinct classes of enzymes have been described as phytases: histidine acid phosphatase (HAPs), beta-propeller phytase (βPP) and purple acid phosphatases (PAP) **(MULLANEY and ULLAH, 2003)**.

1.4.2 Occurrence and cellular location

Acid phosphatases are abundant in plants, bacteria, fungi and some algae. They have also been found in mammalian body fluid and tissue such as bone, kidney, placenta and spleen **(HOLLANDER, 1971)**. In plants, acid phosphatases have been found in the cell wall,

vacuole, roots and suspension medium **(KANEKO *et al.*, 1990; DUFF *et al.*, 1991; LEBANSKY *et al.*, 1992; MIERNYK, 1992)**.

Although it is apparent that apases are distributed in a wide array of organisms only yeast, bacteria, fungi and some algae have been reported to secrete apase outside their cells **(ADKINS and LEDUC, 1980)**. In bacteria, acid phosphatases have been mainly localized in the cell membrane and cytoplasm **(VOELZ and ORTIGOZA, 1964)**. *Escherichia coli* apase (EcAp) has been localized in the periplasmatic space **(OSTANIN *et al.*, 1992)**. In yeasts such as *Saccharomyces cerevisiae* and *Rhodoturula glutinis*, these enzymes are extracytoplasmic, localized in the cell wall of yeast or in the periplasmic space **(TONINO and STEYN-PARVE, 1963; WEIMBERG and ORTON, 1965; TRIMBLE *et al.*, 1981)**. Acid phosphatase in different fungi has been reported in the periplasmic space, vacuole, cell wall and cell membrane and secretory vesicles **(ARNOLD and LACY, 1977; HÄNSSLER *et al.*, 1975; WEBER and PITT, 1997a; ALEKSIEVA *et al.*, 2003)**. Secreted repressible apase enzyme in *Botrytis cinerea* was noted for having high affinity for p-nitrophenyl, and the histochemical localization showed that the enzyme was mainly in the apical secretory vesicle of hyphae **(WEBER and PITT, 1997a)**. In the fungus, *Humicola lutea*, apase under (Pi) inorganic phosphate-sufficient medium was found in the cytoplasmic membrane whereas under Pi-deficient medium, the apase deposits were found outside the cell wall and in the intracellular space **(ALEKSIEVA *et al.*, 2003)**.

1.4.3 Properties of acid phosphatase

1.4.3.1 Physical properties

Most acid phosphatases are glycoproteins and display varying molecular weight sizes, P*I* and subunit structure depending on the source of isolation. Resistance to inhibition by tartrate also distinguishes type 5 acid phosphatase from acid phosphatase of lysosomal **(VON FIGURA and WEBER, 1978)** or prostatic **(VIHKO *et al.*, 1978)** origin. The animal enzymes are also distinguished from lysosomal and prostatic acid phosphatases by their resistance to inhibition by L (+) tartrate, and are commonly referred to TRAPS. Mammalian tartrate resistant acid phosphatase (TRACP, TRAP or TR-AP) also known as

type 5 acid phosphatase (AcP5, E.C 3.1.3.2) **(VIHKO *et al.*, 1978)**. Together with similar enzymes isolated from animals, plants and fungi, it belongs to the group of purple acid phosphatases (PAP).

PAPs from mammalian sources (human, cow, pig and mouse) are reported to be monomeric with a molecular size of approximately 35 kDa. These enzymes contain an Fe (III-Fe (II) binuclear metal centre and exhibit high sequence identity (>80%) **(KLABUNDE and KREBS, 1997)**. On the other hand, plant PAPS (red kidney bean, sweet potato and soybean) are homodimeric and are composed of a subunit molecular mass of approximately 55-110 kDa. Thus, they are generally characterized as high molecular weight acid phosphatases. These plants' PAPs are more diverse with respect to metal centres Fe (III)-Zn (II) or Fe (III)-Mn (II) **(BECK *et al.*, 1986; LEBANSKY *et al.*, 1992; SCHENK *et al.*, 1999)**. A number of plant genes putatively encoding low molecular weight (M_r) PAPs have been identified **(SCHENK *et al.*, 2000)**.

The PAP enzymes have been reported to be present in the cyanobacterium *Synechocytistis* sp as well as in *Mycobacterium tuberculosi*s and *M. leprae* **(SCHENK *et al.*, 2000)**. Until recently, *Aspergillus ficuum* was the only fungal species reported to have a monomeric PAP with a molecular mass of approximately 85 kDa **(ULLAH and CUMMINS, 1988)**. Since the completion and sequencing of the genome of several *Aspergillus* sp, other PAPs such as *Aspergillus oryzae* **(MACHIDA *et al.*, 2005)** and *Aspergillus flavus* **(**NCBI Reference Sequence: XP_002376348.1, deposited by **NIERMAN, 2007)** have been reported. Mammalian PAPS are highly conserved with 80% amino acid homology **(SCHENK *et al.*, 2000**). Interestingly, low sequence identity (20%) has been found between plant and mammalian PAPs, except for the metal-ligating amino acid residues which are identical **(KLABUNDE *et al.*, 1995; SCHENK *et al.*, 2000)**.

Most acid phosphatases of yeasts and fungi are glycoproteins, like most proteins that work in the extracellular *milieu* (Table 1.1). For instance, in yeast apases, the sugar component represents 50% of the enzyme in *Saccharomyces cerevisiae* **(SHNYREVA *et al.*, 1992)**

and 40% of the enzyme in *Rhodoturula glutinis* **(TRIMBLE *et al.*, 1981)**. It has been estimated that about a third of all proteins that enter secretory pathways in eukaryotic cells may be N-glycosylated and tens of thousands of glycoprotein variants may coexist in eukaryotic cells **(WALSH *et al.*, 2005)**. In addition, these proteins have a tendency to form tetramers in solution. *Aspergillus niger* pH 2.5 acid phosphatase has an apparent native molecular mass of 269 kDa with a glycosylated subunit of approximately 65 kDa and an unglycosylated form of 50.8 kDa. It forms a tetramer in solution **(KOSTREWA *et al.*, 1999)**. In *Penicillium funiculosun* the phosphatase appear to be a 76 kDa heterodimer composed of 51 and 26 kDa subunits quantified on SDS-PAGE.

All apases (mammalian, plants and microorganisms) have pH optima below 7.0 (Table 1.1). However, the ranges vary immensely from species to species ranging from 2.5-6.0. For example, in *Aspergillus ficuum* three apases with pH optima between 2.2, 2.5, and 5.5 were isolated by **ULLAH and CUMMINS (1987)**. Some apases have a wide pH optima, for example the apase found in *A. fumigatus*, is active at pH 3.0 to 7.0, with the optimum activity occurring between pH 4.0 and 6.0 **(BERNARD *et al.*, 2002)**.

The conserved active site (N-terminal) RHGXRXP, (where X represents any amino acid) is a hallmark feature of all high molecular acid phosphatases as well as R and HD motifs located at almost identical positions in the active sites **(OSTANIN *et al.*, 1992; SCHNEIDER *et al.*, 1993; KOSTREWA *et al.*, 1997; CHI *et al.*, 1999; MULLANEY *et al.*, 2000)** (Table 1.2). In contrast, low molecular apases are devoid of this motif.

Table 1.1: Physical properties of various acid phosphatases, isolated from different organisms. T= Optimal temperature. P*I*= isoelectric point.

Sources	Protein's name	Forms	Native (kDa)	Mr	P*I*	pH	T (°C)	Glycoprotein	References
MAMMALIAN:									
Homo sapiens	TRACP	I	174.40	3.8-4.1		4.5	-	yes	**ROBINSON and GLEW, 1980**
Rat	PAP	II	150	-			-	yes	**VIHKO *et al*., 1993**
Homo sapiens	TRACP	I	35-37	-		2.5	-	yes	**VIHKO *et al*., 1978**
PLANTS :									
Tomatoes (L*ycopersicon esculentum*	6-phyt	-	4.3	-		4.3	-	yes	**LI *et al*., 1997**
Potato	Apase	II	100	-		5.8	-	yes	**GELLATLY *et al*., 1994**
Wheat	Apase	III	55	-		4.5-5.5	-	yes	**VERJEE, 1969**
BACTERIA:									
Escherichia coli	Phyt	II	44.7	6.3-6.5		4.5	60	yes	**GOLOVAN *et al*., 2000**
Bacillus subtilis (natto)	Phytase	-	36-38	-		6.0-6.5	60	-	**SHIMIZU, 1992**
FUNGI & YEAST:									
Pichia pastoris	apase	-	-	-		-	-	yes	**HAN and LEI, 1999**
Saccharomyces cerevisiae	Phyt	I	100	2.0-2.5		3.5	55-60	yes	**HAN *et al*., 1999**
Aspergillus niger	3Phyates	I	100	5.0		5.0	-	yes	**DVOŘÁKOVÁ *et al*., 1997**

Table 1.2: Amino acid sequence alignments of the conserved region where acid phosphatase from different organisms share a high degree of amino acid conservation "RHGXRXP". PAP= purple acid phosphatases.

Organisms	Acc. No	Sequences	protein	Location	References
Homo sapiens	NP_001601	RFVTLLY**RHGDRSP**VK	PAP	Lysosomal	**POHLMANN *et al*., 1988; WANG *et al*., 2008**
Rattus norvegicus	P20611	RFVTLLY**RHGDRS**PVK-	PPAL	Lysosomal	**HIMENO *et al* ., 1989**
Aspergillus niger	A2QSK3	DQVIMIK**RHGER**Y**P**SP	3-phytase B	Cell membrane	**PEL *et al*., 2007**
Saccharomyces cerevisiae	P24031	KQLQMLA**RHGERYP**TY	*PHO3*	Cell membrane	**BAJWA *et al*., 1984**
Schizosaccharomyces pombe	CAB68657	KQVHTLQ**RHGSR**NPTG	apase	Vacuole	**ELLIOTT *et al*., 1986**
Bacillus cereus	Q633T0	RFVTLLY**RHGDSRSP**VK	PPA	Periplasmic space	**HAN *et al*., 2006**

The high molecular weight (histidine) acid phosphatase family is functionally conserved from prokaryotes to higher eukaryotes **(CHI *et al.*, 1999)**. Thus, several members of this enzyme family can be found in bacteria such as *Bacillus cereus* **(HAN *et al.*, 2006)**; fungi such as *Aspergillus ficuum* and *A. niger* **(KOSTREWA *et al.*, 1997; PEL *et al.*, 2007)** as well as in rats **(HIMENO *et al.*, 1989; SCHNEIDER *et al.*, 1993)** and the human prostate **(POHLMANN *et al.*, 1988)** (Table 1.2).

1.4.3.2 Enzymatic properties

The most common substrates for phosphatases are para-nitrophenyl phosphate (p-NNP), *β*-glycerophosphate (*β*-GLOB) and α-naphthyl phosphate. Most apases are considered to be non-specific phosphoesterases since they hydrolyze phosphate mono-esters, with the general formula R-O-P, at similar rates regardless of the sites or chemical nature of the R-group.

Another interesting substrate for most HAPs is phytic acid. On the basis of this substrate, two classes of HAPs known as phytase A (PhyA) and phytase B (PhyB) which prefer phytic acid as substrate have been characterized **(WYSS *et al.*, 1999)**. PhyB typically exhibits considerable activity with a broad range of phosphate compounds [phenyl phosphate, *para*-nitrophenyl phosphate, sugar phosphates, *β*-glycerophosphates, phosphoenolpyruvate, 3-phosphoglycerate, ADP and ATP] **(WYSS *et al.*, 1999; TOMSCHY *et al.*, 2002)**. Enzymes which displayed PhyB behaviour have been isolated from *Aspergillus fumigates*, *Emericella nidulans*, and *Myceliophthora thermophila* **(WYSS *et al.*, 1999)**. On the other hand, PhyA have narrow substrate specificity but, higher specificity for phytic acid. PhyA has been isolated in organisms such as *Aspergillus niger*, *Aspergillus terreus* 9A1 and in particular *E. coli* **(WYSS *et al.*, 1999)**. *A. niger* NRRL 3135 produced both PhyA and PhyB, operating at pH 2.5 and 5.0, respectively **(ULLAH and CUMMINS, 1987)**. PhyB was first characterized as on apase having an optimum pH 2.5 but substrate-specificity and sequencing identified it as a phytase **(WYSS *et al.*, 1999)**.

Comparison of the structural differences of the two enzymes could explain the enzyme's catalytic activity and structural specificity. When these two enzymes were compared, no

variation was found in their activities, however the amino acids comprising the substrate specificity site were completely different **(MULLANEY *et al.*, 2002)**. When the three dimensional model of the PhyA molecules were examined, the six amino acids in its substrate specificity sites K9, K94, E228, D262, K300 and K301 were found to encircle the cavity containing the HAP active center **(MULLANEY *et al.*, 2002)**. Further inspection of the two enzymes revealed the presence of helix 209-228 in *E. coli* phytase, but it is not present in *A. fumagatus* phytase. It takes up space at the active site (acting as a 'gate keeper' for any substrate access to the HAP active site), thus preventing non-phytate substrate from entering. **KOSTREWA *et al.* (1999)** examined the substrate specificity site of PhyB from *A. fumagatus* and *E. coli* and found them to be different. Phy B is composed of two amino acids at D75 and E272. This means that the PhyB substrate specificity site (SSS) is more electrostatically neutral and therefore can accommodate a broader substrate spectrum than *A. niger* PhyA. *Aspergillus fumagatus* phytase has a larger catalytic pocket than *E. coli* phytase, which may be the reason why *A. fumagatus* has a broader substrate specificity **(WYSS *et al.*, 1999; TOMSCHY *et al.*, 2002)**. Another interesting, difference between *A. niger* PhyA and PhyB is that the active form of PhyA is a monomer, whereas the active form of PhyB is a tetramer **(WYSS *et al.*, 1999)**. This tetrameric structure initially provides PhyB with thermostability, but it also explained why it is incapable of proper refolding after it has been denatured by heating.

Most acid phosphatases (non-specific and phytases) are generally inhibited by fluoride, molybdate and vanadate. Fluoride and tartrate differentially inhibit certain apases and may be used to discriminate between apases. Molybdate and vanadate serves as a transition-state analogue of the pentavalent phosphate reaction intermediating the mode of fluoride inhibition of secreted apase, shown to be pH-dependent. Several metal ions have been shown to modulate phytase activity, for instance phytase from *Enterobacter* sp and *Bacillus subtilis* were inhibited by Zn^{2+}, Cu^{2+} and Al^{3+} **(SHIMIZU, 1992; YOON *et al.*, 1996)**. Cu^{2+} was also reported to depress the enzyme activities in *A. nidulans* and *A. terreus* phytases **(WYSS *et al.*, 1999)**.

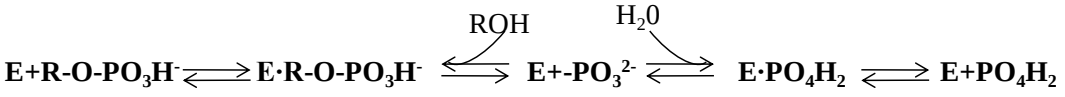
Most fungal apases do not require metal ion cofactors thus they are not stimulated by metallic ions with the exception of some bacterial and purple acid phosphatases **(WANNET *et al.*, 2000; KNEIPP *et al.*, 2003; GUIMARÃES *et al.*, 2004)**. Most purple apases isolated from

plants and mammalian sources are binuclear metalloproteins, which catalyze the hydrolysis of a wide range of phosphate and anhydrides **(SCHENK *et al.*, 2000)**. The characteristic purple colour is due to tyrosine → Me (III) charge-transfer transition **(KLABUNDE and KREBS, 1997)**. The major feature of these enzymes is their insensitivity towards inhibition by tartrate. Thus PAPS are also known as tartrate-resistant acid phosphatases (TRAPS) **(VINCENT *et al.*, 1991)**.

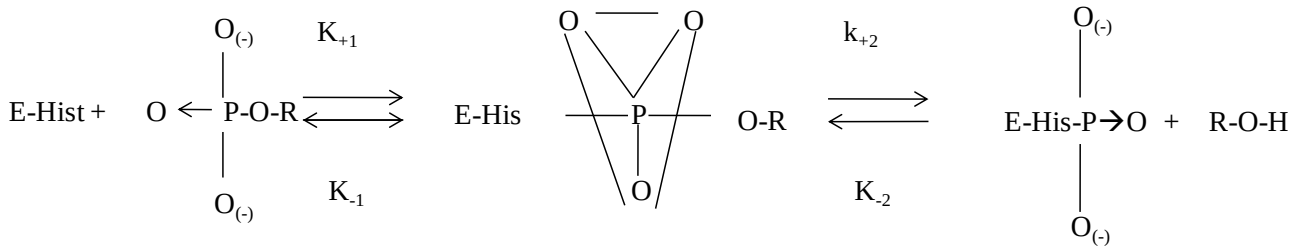
Bacterial phytases (E*nterobacter and Bacillus*) are inhibited by EDTA, indicating that a metal ion (calcium) is needed for activity. On the other hand EDTA had no effect on *A. nidulans* and *A. terreus* **(WYSS *et al.*, 1999)**. Reducing agents such as 2-mercaptoethanol and reduced glutathione have been reported to have no major effects on microbial phytases **(WYSS *et al.*, 1999)**.

1.4.4 Mechanism of action

The catalytic mechanism of several high molecular weight apase enzymes (mammalian, bacteria and fungi) have been studied including numerous mechanical studies **(OSTROWKI, 1978; VAN ETTEN, 1982; BUCHWALD *et al.*, 1984)**. Biochemical experiments demonstrated that most apases utilize a common catalytic mechanism involving the formation of a covalent thiophosphate intermediate that is subsequently hydrolyzed by an activated water molecule. The reaction proceeds via the classic double displacements pathway in accordance with Figure 1.3, summarized in step I and II **(VAN ETTEN, 1982; VINCENT *et al.*, 1992)**.



Step I:



StepII:

Figure 1.3: Kinetic mechanism of acid phosphatase

Apases hydrolyze phosphate esters via the two step mechanism elaborated in Steps I and II (adapted from **TOSOMBA, 1997)**. In the first step of the reaction, the enzyme reacts with the phosphate ester to form the Michaelis complex. In the case of histidine phosphatases, the first step of the reaction involves nucleophilic attack on the phosphate group by histidine and protonation of the leaving group by another group on the enzyme to produce a covalent phosphoenzyme intermediate and an alcohol molecule. In the next step, the phosphoenzyme intermediate is hydrolyzed, leading to the formation of inorganic phosphate **(MCTIGUE and VAN ETTEN, 1978; ZHANG and VAN ETTEN, 1991; ISHIKAWA *et al.*, 2000)**.

In the second step, the phosphate group is transferred to water or some other neucleophilic acceptor and the phosphohistidyl enzyme intermediate is hydrolyzed forming the non-covalent enzyme/phosphate complex (Step II). The transition state for the breakdown of the phosphoryl intermediate has been suggested to be a trigonal bipyramidal species with the nucleophilic enzyme group and attacking water molecule at the axial position **(VAN ETTEN *et al.*, 1974)**.

The catalytic mechanism of phosphomonoester hydrolysis by HAPs was further elucidated by site directed mutagenesis **(OSTANIN *et al.*, 1992; OSTANIN and VAN ETTEN, 1993)** and by the crystal structure of transition state analogue complexes **(LIM *et al.*, 2000)**. On the basis of these results, the following catalytic mechanism for phosphomonoester hydrolysis was proposed: the histidine residue in the conserved motif, RHGXRXP, serves as a nucleophile in the formation of a covalent phosphohistidine intermediate **(OSTANIN *et al.*, 1992; LINDQVIST *et al.*, 1994)** and the aspartic acid residues of the C-terminal conserved HD motif serve as a proton donor to the oxygen atom of the scissile phosphomonoester bond **(LINDQVIST *et al.*, 1994; PORVARI *et al.*, 1994)**.

Most phytases hitherto studied also follow Michaelis Menten behaviour. Phytic acid has six phosphate groups that may be released by phytases at different rates and in a different order **(WYSS *et al.*, 1999)**. All members of the HAP class share two conserved active site motifs (RHGXRXP and HD **(VAN ETTEN *et al.*, 1991)** and hydrolyze metal-free phytate in the acidic pH range. HAPs can initiate hydrolysis of phytic acid on either the C3 or the C6 position of the inositol ring and produce myo-inositol monophosphate as the final product **(MULLANEY and ULLAH, 2003; GREINER and CARLSSON, 2006)**. As a well-accepted process of acid phosphatase catalysis, the removal of phosphate from phytate requires the stabilization of the reaction intermediate to allow the release of non-phosphate products and the recruiting of a water molecule for the following hydrolysis of the intermediate. Without proper orientation of the transient intermediate, the water molecule cannot attack the phosphamide bond properly and thus the reaction will be disrupted. In *Aspergillus fumagatus* phytase, the reaction intermediate contains a phosphohistidine residue stabilized by intensive hydrogen bond interaction between three oxygen atoms of the phosphate and five residues (Arg58, Arg62, Arg142, Hist 338, and Asp339) together with water molecules **(LIU *et al.*, 2004)**. After X-ray analysis of crystallized phytases from *Aspergillus niger* var. *ficuum*, a model for substrate binding attack was proposed to involve the formation of hydrogen bonds to the 3-phosphate group of phytic acid and attack by His59 **(KOSTREWA *et al.*, 1997)**. Site directed mutagenesis studies have shown that H12 and R11 are essential for catalysis, while substitution of residues corresponding to R15, R79, H257, and D258 severely impair catalytic activity **(OSTANIN *et al.*, 1994)**.

1.4.5 Factors that induce the biosynthesis of acid phosphatase

1.4.5.1 Regulation of apase by inorganic phosphate (Pi) concentration

The induction of apase activity under phosphorus starvation is the phenomenon observed under spectrum of organisms and systems (plants, bacteria and fungi). In several plant species such as *Arabidopsis thaliana*, *Lycopersicon esculentum* and *Brassica nigra*, phosphorus starvation has been shown to induce the biosynthesis of multiple apases **(DUFF *et al*., 1991; HARAN *et al*., 2000; BOZZO *et al*., 2004)**. In tomato, three PSI apase isozymes were isolated in suspension culture **(BALDWIN *et al*., 2001; BOZZO *et al*., 2004)**. Two secreted monomeric apases have subunit molecular masses of 84 and 57 kD, respectively, whereas the third is a heterodimeric intracellular apase composed of a 1:1 ratio of 63 and 57 kD subunits, with a molecular mass of 142 kD **(BOZZO *et al*., 2002; 2004a)**.

The regulation of repressible acid phosphatase in many fungi is also negatively controlled by inorganic phosphate (Pi) levels present in the growth medium **(SHIEH *et al*., 1969; YOSHIDA and TAMIYA, 1971; HAN *et al*., 1994; MORALES *et al*., 2000)**. Repressible acid phosphatases are induced under Pi starvation whereas constitutive acid phosphatases are not influenced by the Pi concentration in the medium **(WEBER and PITT, 1997b)**. In a wide range of filamentous fungi, alkaline and acid phosphatase including phytases have been reported to be induced and secreted in the medium when grown under low or Pi limiting conditions **(SHIEH *et al*., 1969; YOSHIDA and TAMIYA, 1971; HAN *et al*., 1994; MORALES *et al*., 2000)**. The control of acid phosphatase synthesis by the concentration of inorganic phosphate in the medium has been reported for the extracellular enzyme of *Aspergillus ficuum*, *Aspergillus awamori* and *Rhizopus microsporus* **(SHIEH *et al*., 1968; TSEKOVA *et al*., 2000; 2002)**. These studies, of fungi grown as stationary cultures in a mineral medium have shown that the type and the amount of phosphatase synthesized is dependent on the concentration of inorganic phosphate in the medium **(CASIDA, 1959; BENNUM and BLUM, 1966)**. For example, *Cladosporium cucumerinum* produced multiple isozymes of extracellular acid phosphatase, in response to phosphate concentration in the medium. This was also related to growth phase **(PEDREGOSA *et al*., 1991)**.

High levels of Pi are known to repress the biosynthesis of the enzyme. For example, *Botrytis cinera* secreted low levels of a constitutive apase into the liquid medium when grown under P starvation or in the presence of high concentrations (4.0 mM) of organic phosphate or inorganic phosphate **(WEBER and PITT, 1997a)**. Under P starvation the repressible enzyme secreted acid phosphatase in the medium was increased up to 80-fold **(WEBER and PITT, 1997a)**. Phosphorus concentration in the medium is not the only factor that induces phosphatases. Age, carbon and as well as the pH of the medium are some of the physiological factors known to induce apases.

1.4.5.2 The effect of pH, carbon source and culture age in the regulation of apases

Several studies have shown that pH regulation of gene expression is a general phenomenon across a wide spectrum of species and organisms. pH control ensures that gene regulation can respond appropriately to ambient pH, for example, in response to changes in extracellular pH: - bacteria control the expression of many genes involved in processes including amino acid degradation and virulence **(OLSON, 1993; BLANKENHORN *et al.*, 1999)**. Genes whose expression is likely to be influenced by ambient pH include those involved in the provision of secreted enzymes, permeases and exported metabolites, all of which must function at ambient pH, and probably also those involved in internal pH homeostasis **(NAHAS *et al.*, 1982; FOSTER, 1999)**.

D'SOUZA and VOLFOVÁ (1982) showed that the excretion of an extracellular cellulolytic enzyme into the culture medium by *Aspergillus terreus* was increased when pH was maintained constant at 5.0. *Rhodoturula rubra* excreted three phosphates into the medium when the cells were grown without control of pH, but excreted only one phosphatase (pH 6.0 optimum) when the cells were grown at a constant pH of 4.5 **(ALTIKRETE *et al.*, 1984)**. Furthermore, under the same conditions of pH control the excretion of acid phosphatase by *Saccharomyces* and *Kluyveromyces* was not stimulated **(WELLER *et al.*, 1981; ALTIKRETE *et al.*, 1984)**. However, it was observed that *Saccharomyces cerevisiae* grown at pH 3.0 released more apase than at pH 5.5. The excretion of acid phosphates by *Rhodotorulla glutinis* is related to the pH of

the medium, for instance, no release of apase was observed at pH 3.0 but only at pH 4.5 and 6.5 **(LEELASART and BONALY, 1988)**. During growth, the phosphatase excretion into the medium at a constant pH of 4.5 was 5 times higher than that observed at variable pH's **(LEELASART and BONALY, 1988)**.

Biosynthesis of phosphatase enzymes is under pH regulation; such genes include the pacA encoding apase and those encoding for acid phosphodiesterase **(CADDICK *et al*., 1986; 1986b)**. All these genes are expressed preferentially under acid growth conditions whereas those encoding alkaline phosphatase (palD) all expressed under alkaline growth conditions **(CADDICK *et al*., 1986b)**. Biosynthesis of phosphatase enzymes under pH regulation is useful in organisms such as *Aspergillus nidulans*, which can grow under a wide range of pH's (2.5-9.0) **(CADDICK *et al*., 1986b; 1986c)**. Such a pH regulatory system ensures that extracellular enzymes as well as permeases and exported metabolites are produced under conditions of pH where they can function: for example, acid phosphatase under acid conditions and alkaline phosphatase under alkaline conditions **(CADDICK *et al*., 1986b; DENISON, 2000)**.

Different carbon sources are known to induce apases. The fungus *Aspergillus caespitosus*, release high levels of alkaline phosphatase into the medium when grown with xylan or wheat, raw and sugar cane bagasse in the culture medium **(GUIMARÃES *et al*., 2003)**. In contrast, activity was found only intracellularly when the fungus was cultured in glucose–supplemented media. In addition, to the pH values and level of Pi, the growth and excretion of apase enzymes into the medium of yeast and fungi depends on the type of yeast cell, the media, temperature and stage of growth **(D'SOUZA and VOLFOVÁ, 1982; LEELASART and BONALY, 1988)**. Studies by **TOH *et al*. (1978)** showed that when *Saccharomyces cerevisiae* was grown at a pH below 4.0 in low Pi medium containing $(NH_4)_2SO_4$ as the nitrogen source, derepression of acid phosphatase did not occur.

1.4.6 Genetic responses to phosphorus deficiency in microorganisms

All living organisms (plants, yeasts, fungi and bacteria) have evolved complex signal transduction networks that enable them to sense the availability of nutrients and modulate metabolic activities for their acquisition. Molecular studies on Pi starvation conducted on plants (lupin, tomatoes and Arabidopsis) revealed a complex pattern of induction of a number of enzymes and genes in response to Pi starvation, suggesting that there is a highly regulated molecular network in plants to alleviate Pi deficiency **(RAGHOTHAMA, 1999)**. There are similarities in the response of higher plants and yeasts to Pi starvation **(RAGHOTHAMA, 1999)**. These include structural genes such as Pi transporters and phosphatases which are induced in a similar manner in both organism types.

Studies on genetic dissection of microbial Pi starvation using *Escherica coli, Saccharomyces cerevisiae* and *Neurospora crassa* have revealed complex regulatory networks in these organism and related species **(LENBURG and O'SHEA, 1996; WANNER, 1996)**. In *E. coli*, the *Pho* regulon comprises a set of at least 15 genes involved in the acquisition of Pi **(TORRIANI, 1990)**. The availability of extracellular Pi is monitored by a Pi-specific transporter that regulates the activity of a two-component signalling system. This dual system consists of a transmembrane Pi sensor and a response regulator which is a transcription activator of the *PHO* gene **(WANNER, 1996)**. These genes are controlled by a two component regulatory system encoded by the *PhoB-PhoR* operon. *PhoR* is a histidine protein kinases located in the inner membrane and acts as a Pi sensor **(LENBURG and O'SHEA, 1996)**. Under Pi deprivation, *PhoR* autophosphorylates itself, which in turn leads to phosphorylation of *PhoB*, the positive regulatory protein **(LENBURG and O'SHEA, 1996)**. The phosphorylated *PhoB* binds to a specific region (the pho Box) of the promoter of each gene of the *Pho* regulon and activates transcription.

In yeast, that expression of structural genes encoding repressible phosphatase and high affinity, Pi uptake is controlled at transcriptional level by an intricate cascade involving both positive and negative regulatory proteins **(OSHIMA, 1982; TOH-E *et al.*, 1988; OSHIMA *et al.*, 1996)**. At least 30 genes are coordinately derepressed by Pi starvation and are likely to play a role in phosphorus assimilation. The Pi responsive genes are also under the same physiological

and genetically known *PHO*-regulon, which encodes Pi acquisition enzymes and regulatory proteins, such as nucleases, both alkaline and acid phosphatases, Pi transporters, Pi binding proteins or Pi sensor protein kinases **(OSHIMA, 1997)**. The *PHO*-regulon of yeast which responds to changes in Pi concentration, includes genes coding for (a) structural proteins such as acid and alkaline phosphatases (PHO5, PHO8, PHO10, PHO11) and high affinity Pi transporters (PHO84), (b) positive regulators, and (c) negative regulators **(OSHIMA, 1997)**. PHO5 encodes the major secreted acid phosphatases and is induced up to 1000 fold in response to Pi starvation. Simple genetic screens based on Pi–responsive acid phosphatase secretion have identified positive (PHO2, PHO4, PHO81) and negative (PHO80, PHO85) regulators of the signalling pathway that controls induction and secretion of PHO5 **(OSHIMA, 1997)**. PHO2 and PHO4 are transcription factors that bind to the PHO5 promoter **(LENBURG and O'SHEA, 1996)**.

In the filamentous fungus *Neurospora crassa*, four regulatory genes *nuc-2, preg, pgov* and *nuc-1* have been identified to regulate Pi surplus **(METZENBERG, 1979)**. The availability of Pi is sensed by the *nuc-2* gene, whose transcription is regulated in response to the levels of Pi **(METZENBERG, 1979; PELEG *et al*., 1996)**. The NUC-2 protein transmits the metabolic signal downstream of the regulatory pathway, inhibiting the functioning of the PREG-PGOV complex under limiting Pi, which allows the activation of the wide domain transcription regulatory NUC-1 **(KANG and METZENBERG, 1990; PELEG *et al*., 1996)**. This allows the translocation of the transcriptional regulator NUC-1 into the nucleus **(PELEG *et al*., 1996)**.

PREG and PGOV are cyclin-like and a mitogen-activated protein kinases, respectively, whereas NUC-1 is a member of the basic helix-loop-helix (bHLH) family of proteins **(KANG and METZENBERG, 1993)** which includes a large number of transcriptional regulators **(ROBINSON and LOPES, 2000)**. A dosage titration and hierarchical regulatory network activates the derepression of nucleases, phosphatases and transporters necessary for fulfilling the cell's Pi requirements **(LEAL *et al*., 2009**). NUC-2 is an ankyrin-like repeat protein, which carries one of the common protein sequence motifs **(PELEG *et al*., 1996)**; it regulates important biological functions of the cell cycle and cytoskeleton organization **(MICHAELY and BENNET, 1992; CAI and ZHANG, 2006)**. The NUC-2, PREG, PGOV and NUC-1

proteins have homology, respectively to PH081, PHO80, PHO85 and PHO4 from *Saccharomyces cerevisiae*. PHO80 and PHO85 form a cyclin-CDK complex which phosphorylates *PHO4* **(KNIGHT *et al*., 2004; WATERS *et al*., 2004)**.

Out of four structural genes (*PHO3, PHO5, PHO10, PHO11*) in yeast encoding apases only constitutive and repressible acid phosphatases genes (*PHO3* and *PHO5*) have been shown to have a high homology with apases in *Schizosaccharomyces pombe* acid phosphatase (gene *pho1*); *Aspergillus awamorii* phytases A and B (gene *phyA* and *phyB*); mammalian lysosomal and prostatic acid phosphatase; and several *Caenorhabditis elegans* hypothetical proteins.

1.4.7 Function of acid phosphatases

The overall function of apases remains somewhat obscure. They are believed to function in the production, transport and recycling of Pi. Several studies have elucidated the role of these enzymes in generation, acquisition and mobilization of inorganic phosphate and their significant contribution to the nutrient dynamics of most ecological niches **(MOLLA *et al.*, 1984; DUFF *et al*., 1994)**. PME present in soil, mainly from microbes, have been reported capable of hydrolyzing mono-ester soil organic P and thereby increasing orthophosphate availability **(LEFEBVRE *et al*., 1990; DUFF *et al*., 1991)**.

The biological functions of PAP in plants are unknown, a role in phosphorus acquisition have been proposed since the enzyme is repressed by inorganic phosphate starvation **(DUFF *et al*., 1994)**. Furthermore, other studies show that they exist as multifunctional proteins that display alkaline peroxidase activity, hypothesized to function in the metabolism of reactive oxygen species (ROS) during senescence or pathogen infection **(HAYMAN and COX, 1994; BOZZO *et al*., 2002; 2004)**.

The physiological functions of mammalian tartrate resistant acid phosphatases (TRACP or TRAP) also known as purple acid phosphatases in biological systems are not yet known **(VIHKO *et al.*, 1978)**. These enzymes have been implicated in bone resorption **(HAYMAN *et***

***al*., 1996)** and iron transport **(NUTTLEMAN and ROBERTS, 1990)** thus, TRAP activity is the most commonly used histochemical marker for osteoclasts.

The high level of non-specific phosphatase activity in microbial cells suggest that these enzymes have a nutritional role, providing orthophosphate from phosphorylated substances present in the medium **(GUIMARÃES and JORGE, 2003)**. Phosphorylated compounds are widely distributed in living systems. They serve as storage forms for energy (ATP and phosphocreatine), as components of information macromolecules (nucleotides and deoxynucleotides), as allosteric effectors of certain enzymes (fructose-1-6-bisphosphate) and second messengers (e.g. cAMP, cGMP, inositol phosphates) **(VINCENT *et al.*, 1992)**. A number of processes in yeast and filamentous fungi such as cell cycle, transcription, signal transduction, infection-related morphogenesis and mating have been shown to require dephosphorylation events **(OSHIMA, 1997; DICKMAN and YARDEN, 1999)**. Hence, the hydrolysis of phosphate esters is an important process in energy metabolism, metabolic regulation and a wide variety of cellular signal transduction pathways of cells **(VINCENT *et al.*, 1992)**.

Phytic acid is the primary phosphate storage compound in seeds, and typically ±70% of the phosphate reserves is sequested in this compound **(LOTT, 1984)**. Phytic acid is largely indigestible by monogastric animals because they have no or limited phytases in their digestive tract. Since monogastric animals such as pigs, poultry and fish are not able to metabolise phytate, phytase is normally added to animal feed to reduce phosphorus excretion in order to reduce phosphate pollution in the environment **(SHIN *et al.*, 2001; BRINCH-PEDERSEN *et al.*, 2002)**.

2. PROSPECTING FOR THE ACID PHOSPHATASE GENE FROM THE MYCO-BIONT *CLADONIA PORTENTOSA* BY PCR

2.1 GENERAL INTRODUCTION

The isolation of apase genes has been of great interest with regards to their important industrial and agricultural applications. The use of acid phosphatase enzymes especially phytase in animal feed supplementation has escalated the number of studies conducted in this enzyme family **(WYSS *et al.*, 1998)**. A survey of the literature revealed that various acid phosphatase genes have been cloned and sequenced in several filamentous fungi e.g. *phoA* **(HAAS *et al.*, 1992; EHRLICH *et al.*, 1994)**, *aph* and *phy* **(MACRAE *et al.*, 1988; PIDDINGTON *et al.*, 1993)**, *phyA* **(VAN HARTINGVELDS *et al.*, 1993)** and *Af* from *Aspergillus ficuum* **(ULLAH and CUMMINS, 1988)**. The most studied acid phosphatases are those belonging to the genus *Aspergillus* **(EHRLICH *et al.*, 1993; PIDDINGTON *et al.*, 1993; VAN HARTINGSVELDT *et al.*, 1993)**, several yeast acid phosphatases especially from *Saccharomyces cerevisiae* **(BAJWA *et al.*, 1984; ELLIOTT *et al.*, 1986)** and other industrial yeasts such as *Kluyveromyces lactis* **(FERMIÑÁN and DOMÍNIGUEZ, 1997)**. So far, several methods have been employed for the purpose of gene cloning such as PCR and RT-PCR. These are considered as the simplest procedures for the isolation of homologous genes from genomes or transcriptomes **(MITCHELL *et al.*, 1997; PASAMONTES *et al.*, 1997a; 1997b; WYSS *et al.*, 1998)**.

The cloning of genes or gene fragments is a fundamental part of molecular biology. In order to study a particular DNA sequence, one must be able to manipulate that sequence. Polymerase Chain Reaction (PCR) is a useful tool to quickly and easily amplify the desired sequences. Given the successful sequencing of whole and partial genomes of organisms from all biological kingdoms, DNA cloning by PCR has become an easy attainable option. The PCR process uses multiple cycles of template denaturation; primer annealing and primer elongation to amplify DNA sequences **(SAIKI *et al.*, 1985)**. Since amplified products from the previous cycle serve as templates for the next cycle of amplification, PCR is an exponential process and a highly

sensitive technique for nucleic acid detection. Typically, enough amplified product is generated after 20 to 30 cycles of PCR so that it can be visualized using ethidium bromide stain.

The template can include purified genomic or plasmid DNA, RNA converted to cDNA or unpurified, crude biological samples such as bacterial colonies or phage plaques. However, when deriving nucleic acid from lichens there are several major problems. The existence of several genomes in the lichen is usually resolved by separate cultures of the symbiont, using algal-free fungal parts of the thallus or discriminative techniques such as specific primers in PCR, or hybridization with specific probes. The drawback of having a small quantity of sample availability is overcome by using amplification techniques. Nucleic acid purification presents an additional problem due to the persistence of cellular contaminants **(CUBERO and CRESPO, 2002)**. Lichen-forming fungi usually produce phenolic compounds that inhibit protein catalysis, thus, inhibiting polymerase and restriction enzymes **(CUBERO and CRESPO, 2002)**. In addition, they contain large quantities of polysaccharides, which at high concentrations also inhibit enzyme activity. The major problem with polysaccharide elimination is that they co-precipitate with nucleic acid when using alcohol precipitation **(CUBERO and CRESPO, 2002)**.

The primers determine the length of the amplified product. The most frequently used thermostable polymerase is *Taq* DNA polymerase **(SAIKI *et al.*, 1985; INNIS and GELFAND, 1990)**. The amplification solution also contains buffer, deoxynucleotide triphosphates and magnesium. The magnesium ion concentration affects enzyme activity, primer annealing, and melting temperature of the template and PCR product, fidelity and primer dimer-formation (**INNIS and GELFAND, 1990)**. Public DNA databases (NCBI) allow researchers to design primers to amplify their DNA fragments of interest directly from the genomic DNA of the desired organisms. Degenerate primers are a mix of different sequences representing all possible bases coding for a single amino acid. The PCR products can be ligated into a suitable vector which can then be transformed into and replicated by *E. coli.*

Advancement in cloning and sequencing of the genus *Aspergillus* and other filamentous fungi has been a useful tool since most genes of these species can be found in the NCBI database and

Aspergillus Broad Institute databases. The lichen genome is lagging behind as little or no lichen sequences have been deposited in the gene bank database. *Cladonia portentosa* belongs to the Ascomycete family, sharing several characteristics with many filamentous fungi (*Aspergillus*, *Scleretonia* and *Botryotinia*). Given the above information attempts were made to: clone acid phosphatase from *Cladonia portentosa* by PCR using degenerate primers, designed using homologous sequences from close relatives of this species.

2.2 MATERIALS AND METHODS

2.2.1 Database search and clustering of acid phosphatases

All amino acid (AA) sequences were searched in non-redundant genebank using the NCBI database, and a key word such as "acid phosphatase." Initially, all AA sequences available at the time this analysis was initiated (2/02/2007) were included. More than 20 000 hits were established, comprising of acid phosphatases from different organisms (mammalian, fungi, plants and bacteria). The list was refined to include only fungal acid phosphatases, comprising mainly of filamentous fungi from the Ascomycete family. The protein sequences were used to infer the phylogenetic relationships.

Further refinement was made by selecting few apase protein sequences from Ascomycete fungi. Selected proteins were divided into five groups: (a) pH 2.5 optimum acid phosphatase (*Aspergillus niger*), (b) pH 5.0 optimum acid phosphatase (*Aspergillus niger*), (c) pH 6.0 optimum acid phosphatase (*Aspergillus niger*), (d) those encoding 'secreted acid phosphatase' isolated mainly from soil fungi, plant fungi and pathogenic fungi and (e) the repressible acid and constitutive acid phosphatase (*PHO5* and *PHO3* gene, respectively) from *Saccharomyces cerevisiae*. Sequences of each 'classified' apase gene was then searched using the BLAST search engine (www.uniprot.org/BLASTP) to find homologous sequences to that particular selected protein. All protein sequences used in this study are listed with their accession number in Table 2.1.

Table 2.1: BLAST output obtained using the sequence of *Saccharomyces cerevisiae* gene *PHO3*, accession number CAA25557.1. Note that this protein sequence was related to most apases from the different groups: (yeast apases, fungal apases and secreted apases from soil and pathogenic fungi).

Sequences producing significant alignments:				
Accession No.	**Enzyme description**	**Organism**	**Query coverage**	**E value**
CAA25557.1	constitutive acid phosphatase (PH03)	*Saccharomyces cerevisiae*	100%	0.0
NP_009651.1	Pho5p Repressible acid phosphatase	*Saccharomyces cerevisiae*	100%	0.0
NP_009434.1	PHO11-acid phosphatase	*Saccharomyces cerevisiae*	100%	0.0
NP_012087.1	Pho12p	*Saccharomyces cerevisiae*	100%	0.0
EDN59799.1	acid phosphatase	*Saccharomyces cerevisiae*	98%	0.0
XP_453063.1	hypothetical protein	*Kluyveromyces lactis*	93%	7e-144
XP_002421811.1	constitutive acid phosphatase precursor, putative	*Candida dubliniensis*	97%	3e-113
XP_002546108.1	hypothetical protein	*Candida tropicalis*	97%	6e-112
XP_002615703.1	hypothetical protein	*Clavispora lusitaniae*	97%	5e-110
XP_713452.1	hypothetical protein	*Candida albicans*	92%	5e-110
XP_002546120.1	hypothetical protein	*Candida tropicalis*	98%	4e-109
XP_713478.1	hypothetical protein	*Candida albicans*	92%	2e-108
XP_001527604.1	hypothetical protein	*Lodderomyces elongisporus*	91%	1e-107
XP_718379.1	hypothetical protein	*Candida albicans*	92%	2e-107
EEQ43330.1	hypothetical protein	*Candida albicans*	92%	2e-107
XP_002421792.1	acid phosphatase	*Candida dubliniensis*	92%	2e-107
ABN04184.1	phytase	*Schwanniomyces capriottii*	97%	4e-106
XP_451007.1	hypothetical protein	*Kluyveromyces lactis*	98%	2e-98
EDK37345.2	hypothetical protein	*Pichia guilliermondii*	92%	9e-98
XP_451542.1	Repressible acid phosphatase	*Kluyveromyces lactis*	91%	2e-97
XP_001385026.2	acid phosphatase, secreted	*Scheffersomyces stipitis*	92%	2e-97
CAB46490.1	acid phosphatase	*Kluyveromyces lactis*	91%	8e-97

ABU53001.1	histidine acid phosphatase	*Kodamaea ohmeri*	92%	9e-96
CBI71332.1	phytase	*Wickerhamomyces anomalus*	92%	9e-96
XP_002999366.1	hypothetical protein	*Kluyveromyces lactis*	91%	1e-95
CAA83964.1	acid phosphatase	*Kluyveromyces lactis*	91%	1e-95
XP_002419861.1	acid phosphatase, putative	*Candida dubliniensis*	98%	2e-95
XP_460696.1	hypothetical protein	*Debaryomyces hansenii*	97%	4e-95
EEQ45271.1	hypothetical protein	*Candida albicans*	98%	2e-94
XP_722801.1	hypothetical protein	*Candida albicans*	98%	4e-94
XP_001385108.1	secreted acid phosphatase	*Scheffersomyces stipitis*	97%	2e-93
XP_002545353.1	hypothetical protein	*Candida tropicalis*	97%	4e-90
XP_001385109.2	acid phosphatase	*Scheffersomyces stipitis*	98%	1e-88
XP_002770768.1	hypothetical protein	*Debaryomyces hansenii*	92%	1e-84
ABU49229.1	acid phosphatase	*Kodamaea ohmeri*	73%	2e-81
XP_001528805.1	hypothetical protein	*Lodderomyces elongisporus*	92%	1e-78
AAC62537.1	repressible acid phosphatase	*Pichia angusta*	98%	2e-72
BAH58739.1	phytase	*Pichia fabianii*	90%	9e-65
ADF49635.1	phytase B	*Aspergillus ficuum*	90%	9e-64
P34754.1	3-phytase B-	*Aspergillus niger*	90%	6e-63
P34755.1	pH 2.5 optimum acid phosphatase	*Aspergillus niger*	90%	9e-62
BAB84518.1	acid phosphatase	*Monascus purpureus*	90%	8e-61
CAJ77470.1	secretory acid phosphatase	*Arxula adeninivorans*	96%	4e-57
XP_001267206.1	3-phytase B precursor,	*Neosartorya fischeri*	90%	9e-56
XP_001401628.2	3-phytase B	*Aspergillus niger*	89%	1e-55
XP_002490985.1	repressible acid phosphatase	*Pichia pastoris*	97%	4e-54
XP_002836377.1	hypothetical protein	*Tuber melanosporum*	86%	4e-54
XP_001271709.1	3-phytase B precursor,	*Aspergillus clavatus*	91%	1e-53
CBX93196.1	hypothetical protein	*Leptosphaeria maculans*	86%	1e-52
XP_002566223.1	hypothetical protein	*Penicillium chrysogenum*	86%	8e-51
XP_001823	multiple inositol polyphosphate	*Aspergillus oryzae*	89%	3e-50

915.1	phosphatase			
XP_002380978.1	multiple inositol polyphosphate phosphatase, putative	*Aspergillus flavus*	89%	4e-50
CBF78112.1	TPA: conserved hypothetical protein	*Aspergillus nidulans*	89%	3e-48
XP_682014.1	hypothetical protein	*Aspergillus nidulans*	84%	9e-48
XP_001209864.1	conserved hypothetical protein	*Aspergillus terreus*	85%	3e-46
XP_002485322.1	acid phosphatase	*Talaromyces stipitatus*	92%	3e-45
XP_002148872.1	repressible acid phosphatase precursor	*Penicillium marneffei*	84%	1e-44
XP_001935660.1	3-phytase B precursor	*Pyrenophora tritici-repentis*	89%	3e-42
XP_002849736.1	phytase	*Arthroderma otae*	89%	5e-39
XP_001797928.1	hypothetical protein	*Phaeosphaeria nodorum*	79%	3e-37
XP_658168.1	histidine acid phosphatase	*Aspergillus nidulans*	86%	6e-36
XP_001214216.1	hypothetical protein	*Aspergillus terreus*	89%	1e-35

2.2.2 Phylogenetic analyses

The phylogenic tree was produced using BLAST outputs from Table 2.1, comprising of selected protein sequences from five groups, which were pooled together to established the phylogenetic relationship. Ten sequences were selected from each group. The phylogenetic tree was constructed with the neighbour–joining method and a bootstrap value was calculated for 33 runs using Phylip.3.64 software. Phylogeny was displayed via tree view.

2.2.3 Multiple sequence alignments

Multiple sequence alignments of apases were performed using CLUSTALW using the default alignment parameters **(THOMPSON *et al*., 1997)**. Suitable parameters for all partial alignments were gap opening penalty, 10.0; gap extension penalty, 0.2; and gap separation distance, 8. The Blosum 62 series protein-weight matrix was used in all cases. These parameters were the same for the first alignment of different classes of the apase family. The protein used in this study included those available in Swiss-PROT, UniProt and genebank NCBI and are listed in Table 2.1 together with their accession number and the organisms from which they

originate. The sequence alignments were displayed with vector NTI and refined manually with respect to know structural homology.

2.2.4 Isolation of lichen mycobionts of *Cladonia portentosa*

Cladonia portentosa was collected at various sites in the UK: Peak District National Park, Derbyshire, England and in Thurso, Scotland. All material was air dried at room temperature for 12 h and then stored at -20 °C. Axenic cultures of the lichen mycobiont were obtained from single ascospores, isolated following the method of **AHMADJIAN (1993)**. The thallus was screened under a binocular microscope to determine if it was fertile. Fertile thalli were placed in Petri dishes containing distilled water and allowed to soak for 4 h. The spore-bearing structures were blotted gently to remove excess water. The apothecia were dissected from a single podetium and separately attached to the underside of Petri dish lids with petroleum jelly (Vaseline) to allow ascospores to be discharged downwards onto the agar. This was done at 15 °C in the dark. Precautions were taken to ensure that the apothecium was not in contact with the agar below. The culture media were prepared as described by formulae of culture media and for descriptions of culture techniques for fungi **(LILLY and BARNETT, 1951)**. The ingredients consisted of malt extract (20 g), yeast extract (2.0 g), agar (20 g) and distilled water (1.0 L).

The plates were incubated at 15 °C for 24 h, after which the lids with Vaseline were discarded and replaced by clean ones. The plates were monitored for contamination until spores were observed on the agar plates. The cultures were subcultured as required. In these lichen samples, viable spores typically formed colonies (2-5 mm in diameter) after 1-2 months. When colonies had reached 10 mm in diameter (3-5 months) they were excised from the agar surface using a scalpel. For stock, the colonies were stored as slopes consisting of the culture media described above. To increase biomass of the mycelium, cultures were grown for 3-4 months in liquid medium using the culture media described above.

2.2.5 Culturing of filamentous fungi using positive controls

Positive controls from *Aspergillus niger* and *Neosartorya fischeri* were grown in Petri dishes with *Aspergillus* Complete Medium (ACM; *Aspergillus* minimal medium supplemented with casamino acids (1.5 g), bactopeptone (2.0 g) and yeast extract (1.5g) at 28 °C). *Aspergillus* minimal medium [AMM: (all l^{-1}) $NaNO_3$ (6.0 g), KCl (0.52 g), $MgSO_4.7H_2O$ (0.52 g), KH_2PO_4 (1.52 g), $FeSO_4.7H_2O$ (0.5 mg), $ZnSO_4.7H_2O$ (0.5 mg), glucose (20 g) and agar (20g)]. For easy extraction, a sterile cellophane disc was placed over ACM dishes before inoculating. The plates were left for 3 days at 28 °C in an incubator room. The mycelia were harvested and freeze-dried overnight. The mycelium was kept in the freezer at -70 °C until used for genomic extraction.

2.2.6 Genomic DNA extraction

Two conventional protocols were used in this study for obtaining nucleic acids from lichens or lichen symbionts. One optimized by **MURTAGH *et al*. (1999)** involved the use of phenol extraction and the second one as modified by **CUBERO *et al*. (1999),** involved the application of cetyl-trimethyl ammonium bromide (CTAB), based on that of **ROGERS and BENDICH (1988)** similar to that described by **ARMALEO and CLERC (1995)**. It exploits the ability of CTAB to prevent the co-precipitation of polysaccharides and polyvinyl polypyrrolidone (PVPP) to eliminate polyphenolic compounds **(PICH and SCHUBERT, 1993)**.

2.2.6.1 Phenol extraction- method A

Mycobiont samples weighing between 3.0-100 mg were freeze-dried overnight. After freeze-drying, the mycelium was ground under liquid nitrogen using a mortar and pestle. The ground mycelium was re-suspended in 3.0 ml extraction buffer (200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM EDTA), and 0.5% (w/v) SDS and extracted twice with phenol/chloroform after treatment with RNAse for 30 min and 5.0 mg/ml proteinase K at 37 °C for 30 min respectively. After centrifugation at 9700x g for 30 min at 4°C, DNA was precipitated by adding 0.7 ml volumes of isopropanol. The pellet was washed with 70% (v/v) ethanol, air dried and re-suspended in TE buffer (10 mM Tris, 1.0 mM EDTA, pH 8.0). The presence of DNA and the integrity of DNA in the extracted sample was checked by electrophoresis (0.8 % (w/v) and

quantified by NanoDrop ND-1000 spectrophotometer (Thermo-Fisher Scientific, MA, USA). DNA concentration was quantified by lambda DNA markers (Roche).

2.2.6.2 CTAB extraction-method B

After freeze-drying, the mycelium samples were placed in liquid nitrogen and ground to fine powders using a mortar and pestle. A volume of 400 µl of CTAB extraction buffer (100 mM Tris/HCl, pH 8.0, 30 mM EDTA, 1.0 M NaCl, 1.0% w/v CTAB) was added to each tube and gently mixed by tapping with fingers. PVPP was added to tubes directly at a concentration of 1.0% (w/v). The tubes were placed in the water bath at 60-70 ºC for 30 min, where they were inverted several times. After incubation, 500 µl of CI (chloroform: isoamyl alcohol 24:1 (v/v)) was added to each tube and mixed vigorous by shaking. The mixture was centrifuged at 10 000x g, for 5 min at room temperature. The upper aqueous phase was transferred to a new tube where it was diluted by CTAB precipitation buffer (40 mM NaCl, 0.5 % (w/v) CTAB) and then centrifuged at 10 000x g for 5 min. If the pellet was visible at the bottom of centrifuge tube, the liquid was discarded or else the samples were placed at 4 ºC for 5 min to help CTAB-DNA complex to be formed. The mixture was centrifuged as mentioned above. The pellet was resuspended in 0.4 ml pre-warmed 1.2 M NaCl solution and 3.0 µl (10X) RNAse buffer and 2.0 µl RNAse A (10 mg ml^{-1}) was added. The mixture was incubated for 30 min at 37 ºC. The remaining pre-warmed NaCl was added (370 µl). The second step involved adding 500 µl CI and repeating the steps already described above.

The volume recovered after centrifugation was measured and an equal volume of isopropanol was added and centrifuged at 13 000x g for 15 min at 4 ºC. Upon the formation of a transparent-whitish pellet, the aqueous phase was discarded. A volume of 500 µl 70% ethanol was added to the pellet and centrifuged at 13 000x g at 4 ºC. After draining the ethanol, the pellet was dried and re-suspended in 50 µl pre-warmed TE buffer.

2.2.6.3 Commercial Kits-method C

Two commercial kits were used to extract DNA from mycobionts and lichen samples. After freeze-drying, the mycelium was ground under liquid nitrogen using a mortar and pestle, followed by total genomic extraction using either Qiagen Plant DNA extraction kit or Wizard® Genomic DNA purification kit (Promega) (as per the manufacture's protocol).

2.2.7 Design of degenerate PCR primers

The protein alignments were used to identify the conserved motifs, which were selected to design the degenerate primers. For isolation of acid phosphatase genes, PCR primers were designed, based on protein sequence comparisons of several acid phosphatases from related filamentous fungal yeasts and fungi as mentioned in Section 2.2.1. Protein sequences were selected (Table 2.1) which would allow the specific isolation of acid phosphatase. Conserved regions were used to design a 20 to 30-mer primer sequence. The sequence only included the first two bases of the terminal aspartic acid codon as this allows annealing with either an aspartic or glutamic acid codon. Several pair of primers were designed for this experiment (Table 2.2).

Table 2.2: List of degenerate primers used for gene cloning of acid phosphatase

Primer name	**Protein motif**	**Annealing temperature (°C)**
For_aph1	5'NCAYGGNGARCGNTAYCC3'	56.5
For_aph2	5'GNCAYGGNGARAGRTAYCC3'	51.2
Rev_aph1	5'RCANGARWANCCNGGNCC3'	55.4
Rev_aph2	5'RCARCTRWANCCNGGNCC3'	54.6
FAPH1	5'TTYGGIGARGGITTYTTYGG3'	56.0
FAPH2	5'ATNGGNGTHATYTRNTYTCYTCNGC3'	60.4
RAPH	5'ATIGGIGTDATRTYIGTRTCRTGNGC3'	60.4
Fapase1	5'ATGYTIGCIGARASIAAYCCNGC3'	61.5
Rapase2	5'NGMNCGNTYNTYGARNTGYATNGGNTYNGGNTGNCG3'	61.5

2.2.8 PCR optimization

All PCR reaction preparations were carried out on ice. The PCR mixture was prepared as follows: each reaction mixture volume (25 µl) contained 50 ng DNA template, 40 pmol of both primers (forward and reverse) (Table 2.2), 200 µM of each dNTP (New England Biolab), and 1 U of hot start DNA polymerase (Roche). Cycle parameters were 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 52 °C and 1 min at 72 °C before the final 7 min at 72 °C (all at ramp rate 60 °C min^{-1}). The PCR was performed using a Gene Amp kit (Perkin-Elmer Cetus) according to the manufacturer's instructions. Where no results were obtained, alternative sets of primers were used (Table 2.2). The elongation temperature was optimized according to the thermodynamic set of degenerate primers. The product was loaded to 1.0 or 1.5% agarose gel, using a Tris-Borate-EDTA buffer (TBE) [89 mM Tris base, 89 mM of boric acid and 2 mM EDTA (pH 8.0)] and the products were visualized by staining with ethidium bromide. A DNA Marker ladder of 100 bp and 1 kb served as molecular markers (New England Biolab and MBI Fermentas).

Two positive controls, *Aspergillus niger* and *Neosartorya fischeri* were used to optimize the PCR. The sequences of these two organisms were included in the alignments used to design degenerate primers. The genomic DNA template used ranged from 5.0, 50 and 100 ng. Different PCR parameters such as DNA template concentration, primer concentration, annealing temperature and $MgCl_2$ concentrations were tested. Initially low stringent conditions were used to detect PCR products (Table 2.3).

Table 2.3: Experiment design showing a typical gradient PCR experiment design using various parameters (temperature, primer concentration and DNA templates concentration).

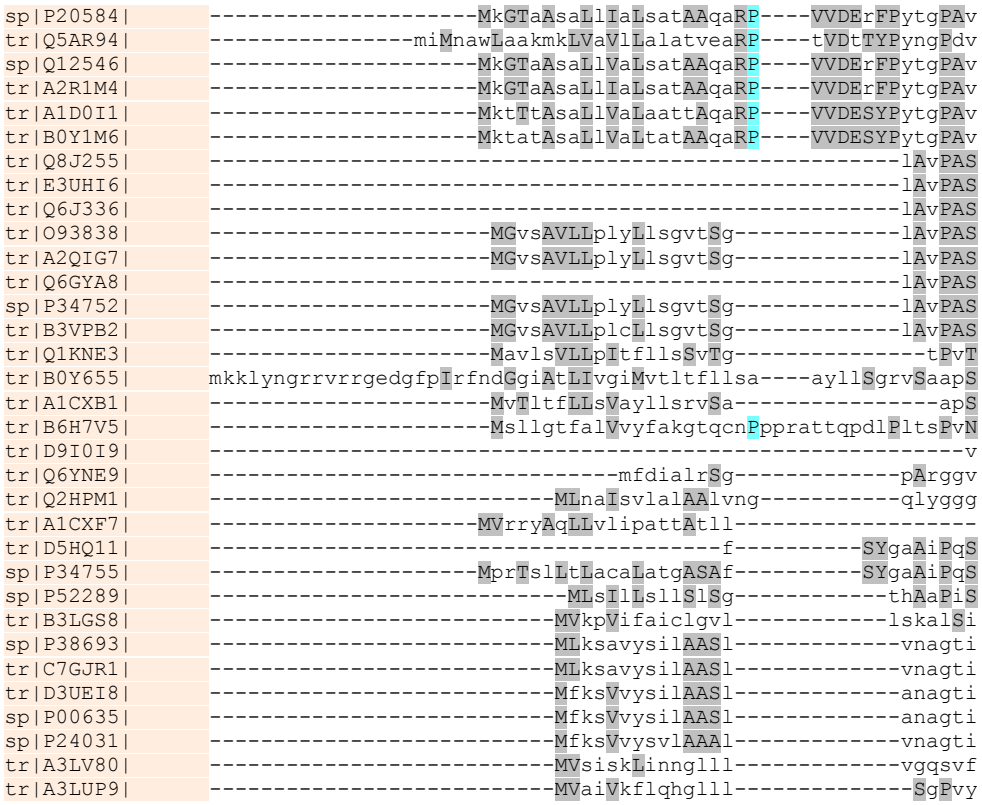
Concentration of genomic DNA	**Concentration of primers**	**Temperature (**ºC)
50 ng	40 pmol	48
50ng	80 pmol	48
50 ng	40 pmol	50
50 ng	80 pmol	50
50 ng	40 pmol	52
50 ng	80 pmol	52
5 ng	40 pmol	48
5ng	80 pmol	48
5 ng	40 pmol	50
5 ng	80 pmol	50
5 ng	40 pmol	52
5 ng	80 pmol	52

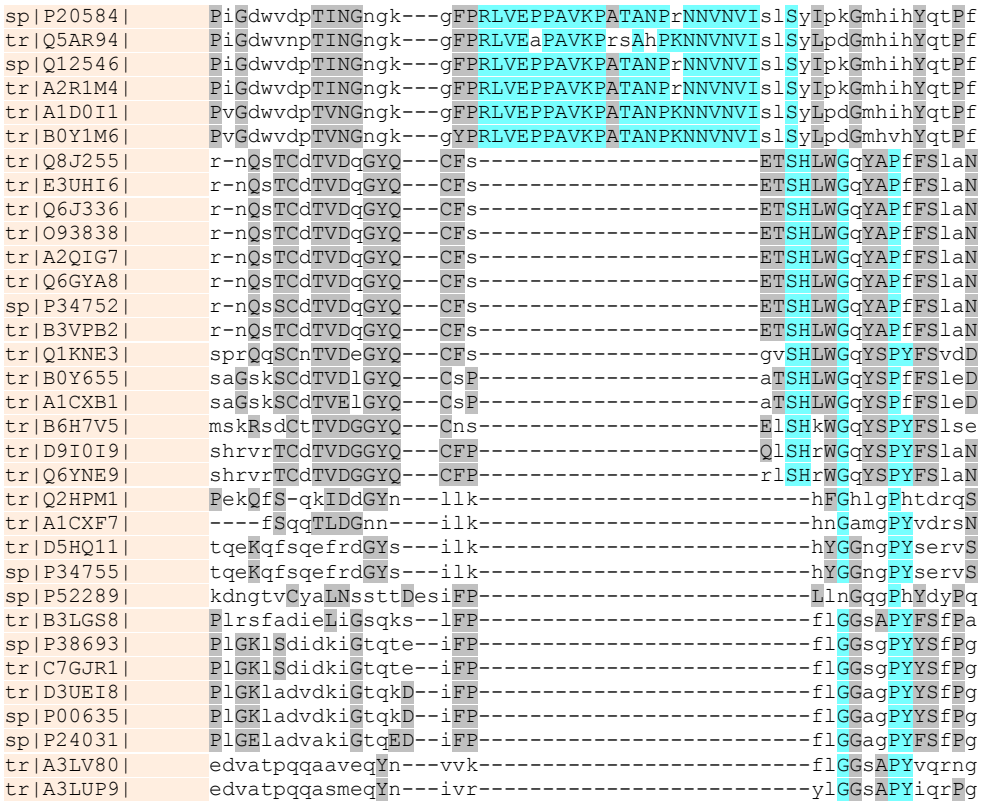
Gradient PCR was conducted using different concentrations of primers ranging from 40, 80 and 100 pmol. In addition low temperatures such 42 ºC, 48 ºC, 50 ºC and 52 ºC were initially compared. In addition, $MgCl_2$ concentrations (1.0, 1.5 and 2.5 mM) were also assessed.

2.3 RESULTS

2.3.1 Conserved regions and typical motifs in the sequences of apases

In the initial attempt, many apase protein sequences were retrieved. At this time, the main goal was to identify the conserved motifs in fungal apases for subsequent degenerate primer design. The unsummonable amount of data retrieved made it impractical for sequence alignments and also for phylogenetic analysis. More than 27 000 hits were obtained using the search words 'acid phosphatase.' After clustering of apase proteins and conducting multiple sequence alignment it became apparent that the protein sequences were highly divergent and homology was poor (Figure 2.1). To overcome the problem, the gene were separated into different groups. 'Selected' sequences used for the BLAST search are highlighted (Table 2.1). The greatest sequence conservation was achieved in the area with histidine regions ('RHGXRXP', R and HD motifs) (Figure 2.1).

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sp|Q12546|
tr|A2R1M4|
tr|A1D0I1|
tr|B0Y1M6|
tr|Q8J255|
tr|E3UHI6|
tr|Q6J336|
tr|O93838|
tr|A2QIG7|
tr|Q6GYA8|
sp|P34752|
tr|B3VPB2|
tr|Q1KNE3|
tr|B0Y655|
tr|A1CXB1|
tr|B6H7V5|
tr|D9I0I9|
tr|Q6YNE9|
tr|Q2HPM1|
tr|A1CXF7|
tr|D5HQ11|
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sp|P52289|
tr|B3LGS8|
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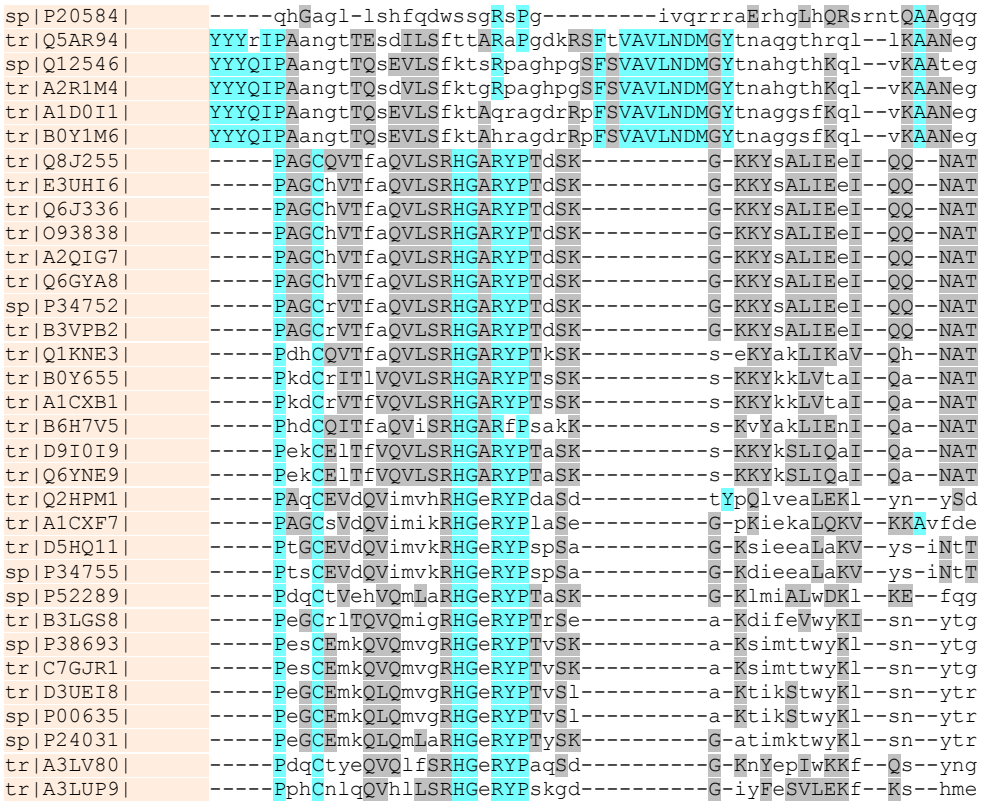
tr|A3LV80|
tr|A3LUP9|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tr|Q5AR94| YYYrIPAangtTEsdILSfttARaPgdkRSFtVAVLNDMGYtnaqgthrql--lKAANeg
sp|Q12546| YYYQIPAangtTQsEVLSfktsRpaghpgSFSVAVLNDMGYtnahgthKql--vKAAteg
tr|A2R1M4| YYYQIPAangtTQsdVLSfktgRpaghpgSFSVAVLNDMGYtnahgthKql--vKAANeg
tr|A1D0I1| YYYQIPAangtTQsEVLSfktAqragdrRpFSVAVLNDMGYtnaggsfKql--vKAANeg
tr|B0Y1M6| YYYQIPAangtTQsEVLSfktAhragdrRpFSVAVLNDMGYtnaggsfKql--vKAANeg
tr|Q8J255| -----PAGCQVTfaQVLSRHGARYPTdSK----------G-KKYsALIEeI--QQ--NAT
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tr|Q6GYA8| -----PAGChVTfaQVLSRHGARYPTdSK----------G-KKYsALIEeI--QQ--NAT
sp|P34752| -----PAGCrVTfaQVLSRHGARYPTdSK----------G-KKYsALIEeI--QQ--NAT
tr|B3VPB2| -----PAGCrVTfaQVLSRHGARYPTdSK----------G-KKYsALIEeI--QQ--NAT
tr|Q1KNE3| -----PdhCQVTfaQVLSRHGARYPTkSK----------s-eKYakLIKaV--Qh--NAT
tr|B0Y655| -----PkdCrITlVQVLSRHGARYPTsSK----------s-KKYkkLVtaI--Qa--NAT
tr|A1CXB1| -----PkdCrVTfVQVLSRHGARYPTsSK----------s-KKYkkLVtaI--Qa--NAT
tr|B6H7V5| -----PhdCQITfaQViSRHGARfPsakK----------s-KvYakLIEnI--Qa--NAT
tr|D9I0I9| -----PekCElTfVQVLSRHGARYPTaSK----------s-KKYkSLIQaI--Qa--NAT
tr|Q6YNE9| -----PekCElTfVQVLSRHGARYPTaSK----------s-KKYkSLIQaI--Qa--NAT
tr|Q2HPM1| -----PAqCEVdQVimvhRHGeRYPdaSd----------tYpQlveaLEKl--yn--ySd
tr|A1CXF7| -----PAGCsVdQVimikRHGeRYPlaSe----------G-pKiekaLQKV--KKAvfde
tr|D5HQ11| -----PtGCEVdQVimvkRHGeRYPspSa----------G-KsieeaLaKV--ys-iNtT
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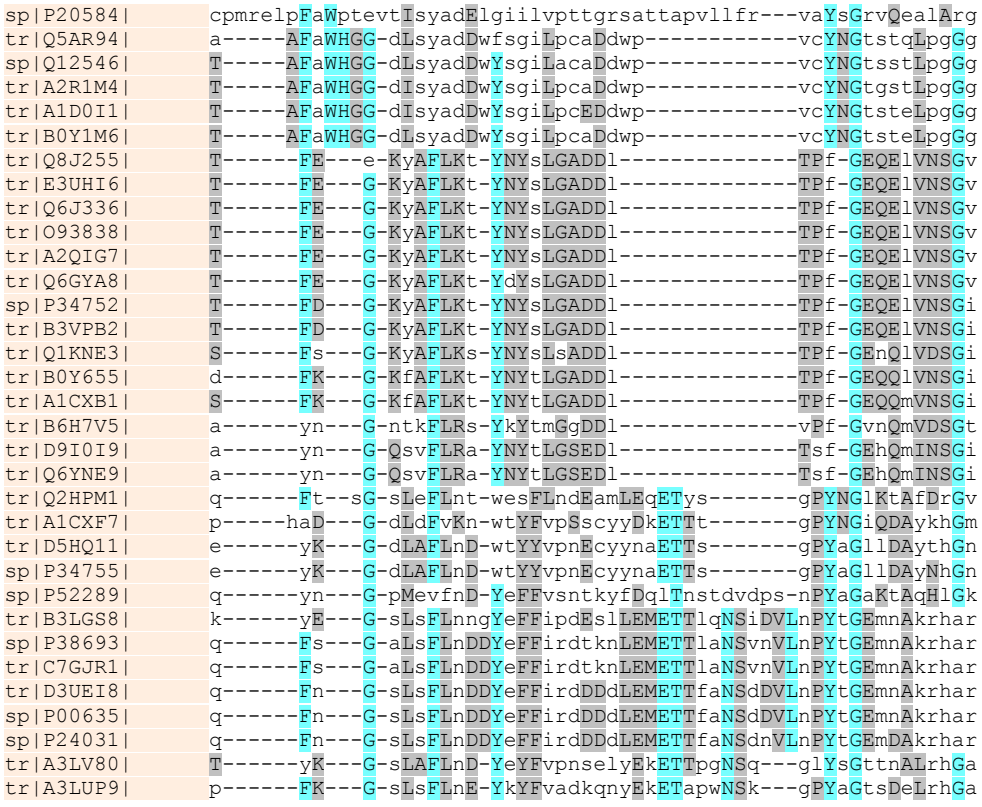
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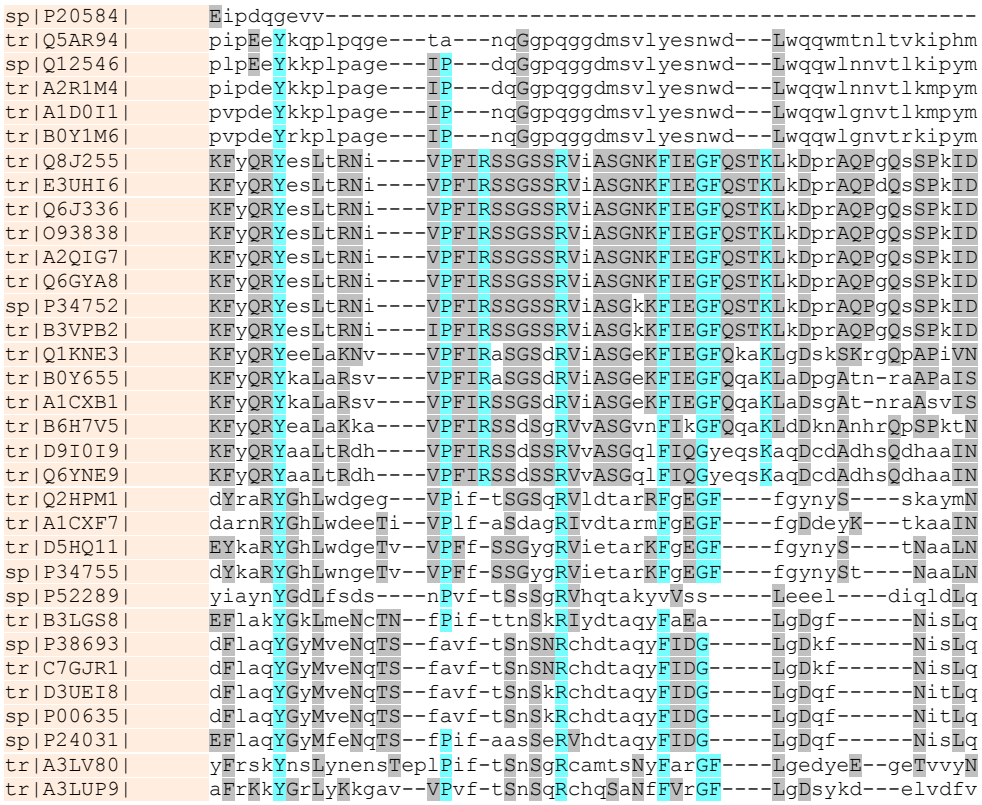
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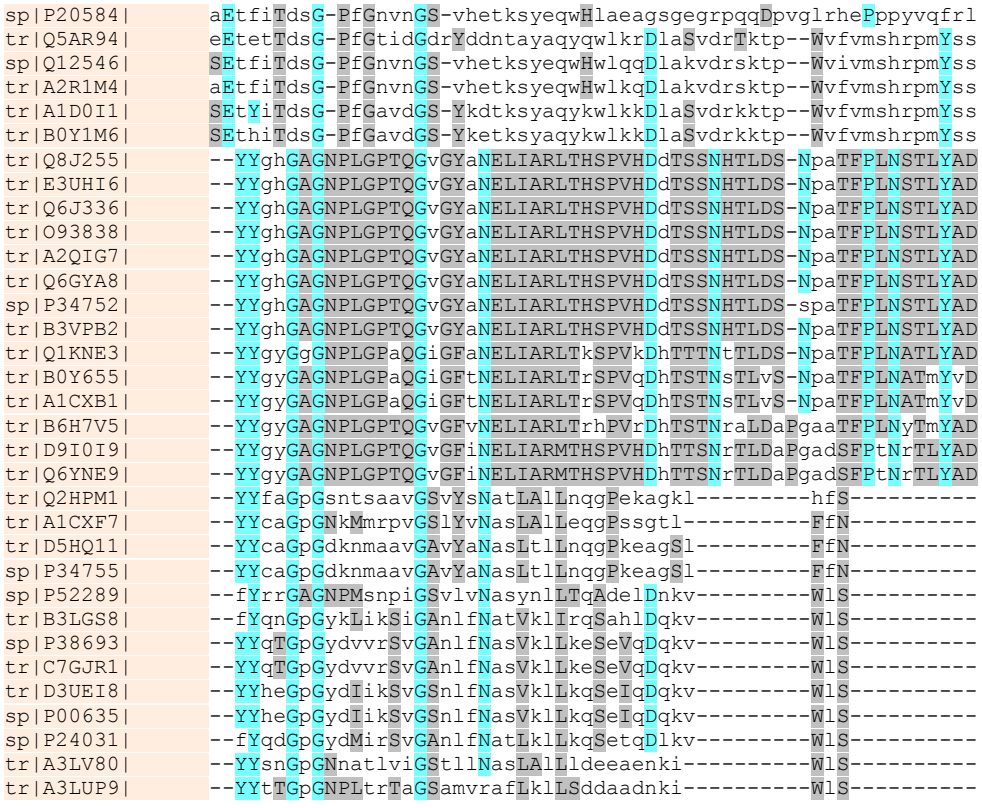
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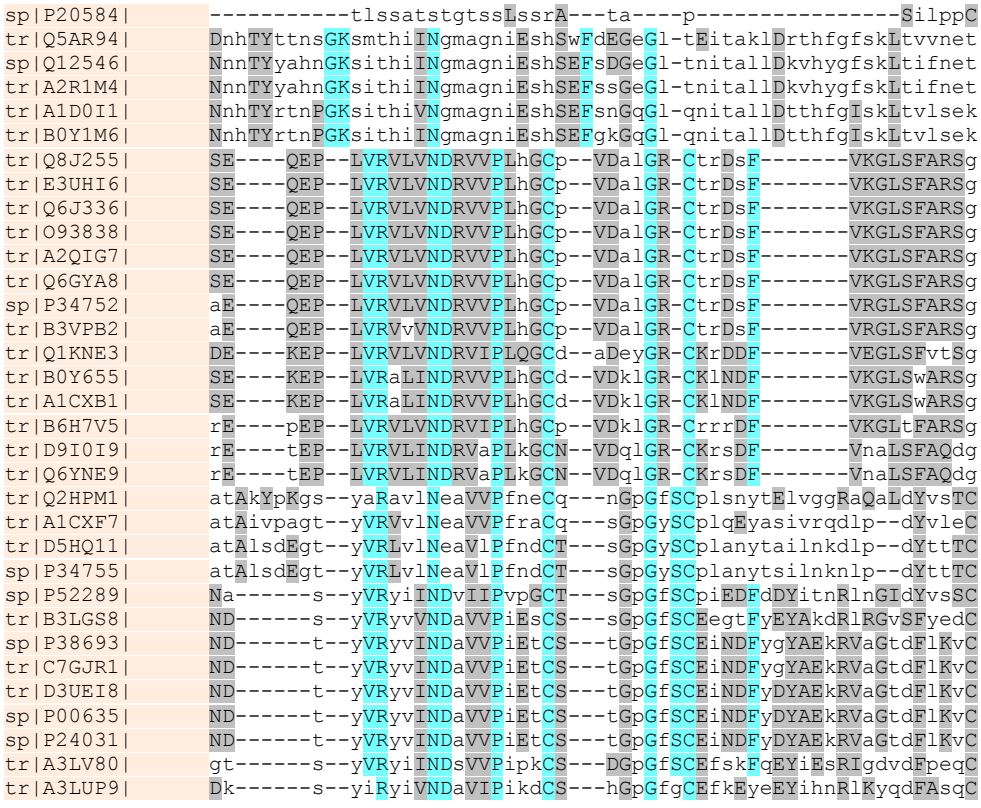
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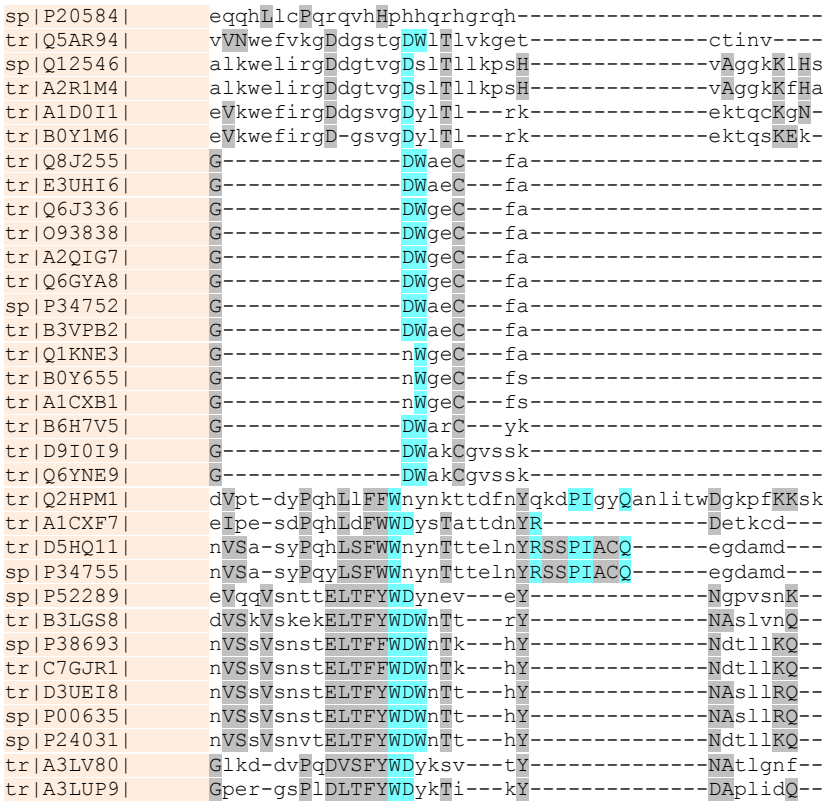

Figure 2.1: Multiple sequence alignment of 33 selected representatives of the HAP super family of fungal and yeast apases. Abbreviation of the enzyme sources are defined in Table 2.1 and represented in the phylogram in Figure 2.2. Numbers indicate the position of each presented segment within the corresponding sequence. Sequences are grouped together as discussed in the text. Similar residues are coloured as the most conserved one (according to BLOSUM62). Average BLOSUM62 score: Max: 3.0 Mid: 1.5 Low: 0.5

2.3.2 Phylogeny relationship with similar proteins from different organisms

The phylogeny map was constructed to show how related or divergent apase proteins were from each other, using alignment in Figure 2.1. These complete sequenced genomes–based on sequence homologies, assign similar proteins from organisms to clusters of orthologious groups. It became apparent that taxonomic grouping of these apase gene was not so important. Proteins were grouped according to their physiological function such as pH optima and substrate metabolism. Group A comprised of proteins such as *Aph* gene in *Aspergillus niger*, optimal at pH 6.0 and *phytB* gene from *Aspergillus niger* pH (5.0). Group B consisted of yeasts apases (encoded by *PHO5* and also *PHO3* gene in *Saccharomyces cerevisiae*) and also 'secreted acid phosphatase.' Group C of *Aspergillus niger*, pH 2.5 (PhyA) (Figure 2.2).

Within five initial selected sequences, the sequence alignment revealed that *PHO3*, *PHO5* and secreted acid phosphatases had a strong homology to each other, thus these enzymes were grouped together as group B (Figure 2.2). The secreted apases comprised of soil and plant pathogen fungi such as *Botryotinia fuckeliana.* This group also aligned well with *PHO3*. All these proteins are controlled by phosphorus concentration in the medium and are secreted glycoproteins.

Due to poor homology displayed by apase genes, few sequences were selected for alignment in order to design degenerate primers (Figure 2.3). Strong homology was found in the active sites and HD residues. These regions were used to design the primers (Figure 2.3).

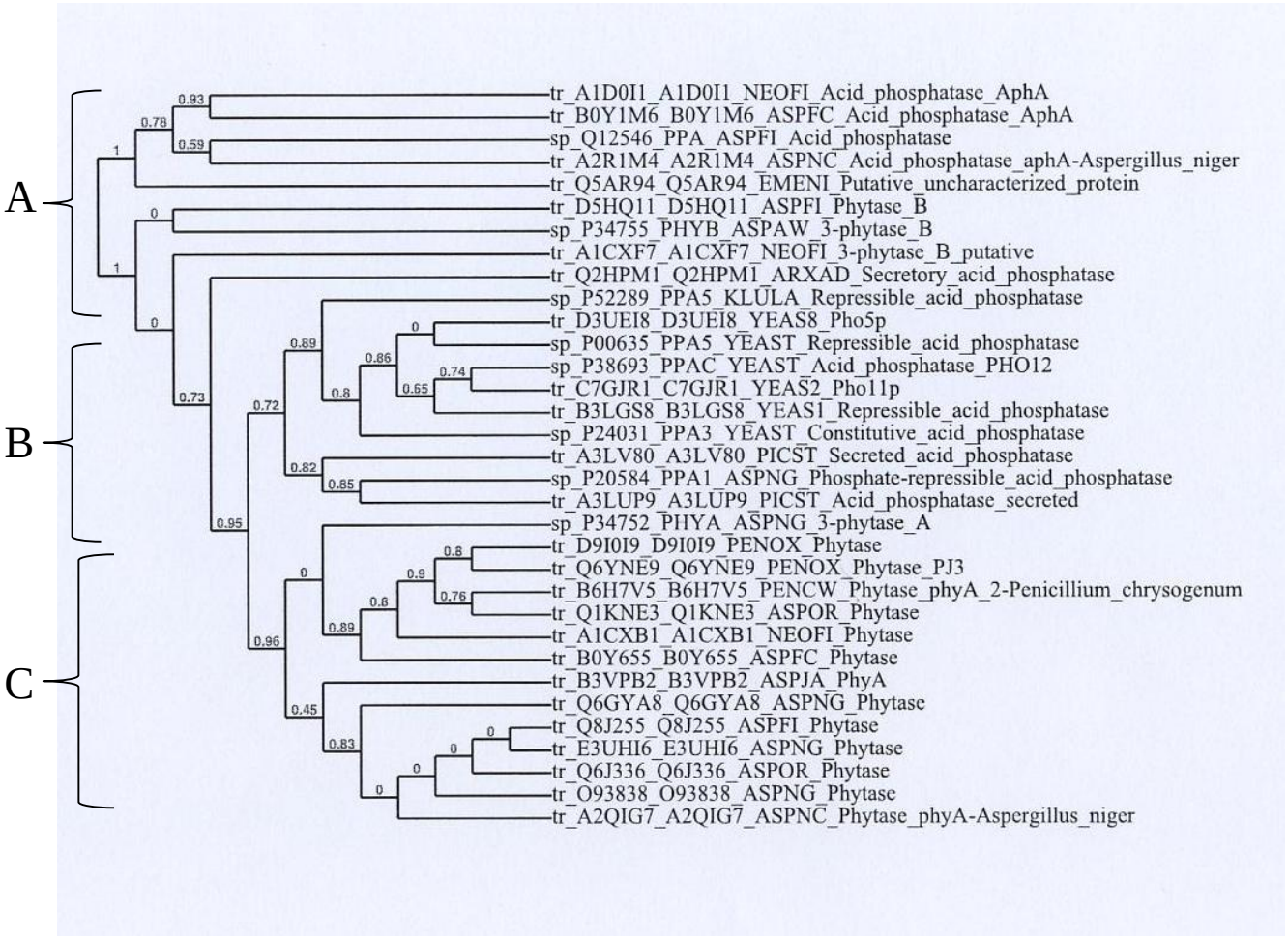

Figure 2.2: A phylogenic tree (N-J with branched length) demonstrate the order of evolutionary events in three groups of apases used in this study. All taxonomic entities are part of Actonomytes, comprising of 33 protein sequences.(A) indicate the secreted apase known as aph, encoding gene expressed at pH 2.5 (*A. niger*, *phyB*); (B) secreted apases (including yeast apases); (C) apase proteins encoding pH 5.0 (*A. niger*, *phyB*). The tree was constructed by PhyML 3.0 (www.phylogeny.Fr). Most nodes are on the same path, thus they are related to each other. The scale bar represents 30% of the estimated sequence divergence.

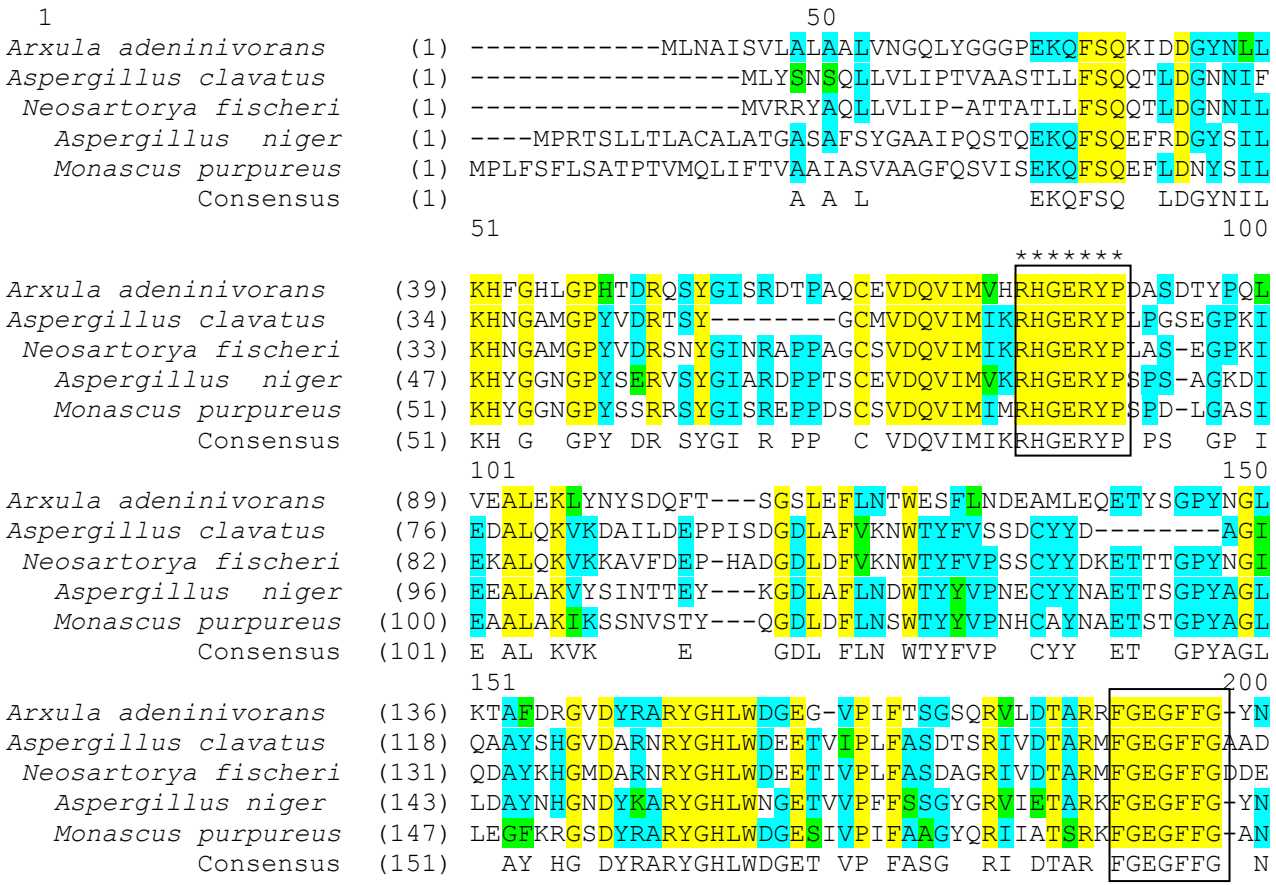

Figure 2.3: Multiple sequence alignment of apase genes from different fungal and yeast organisms used to design degenerate primers for *Cladonia portentosa*.

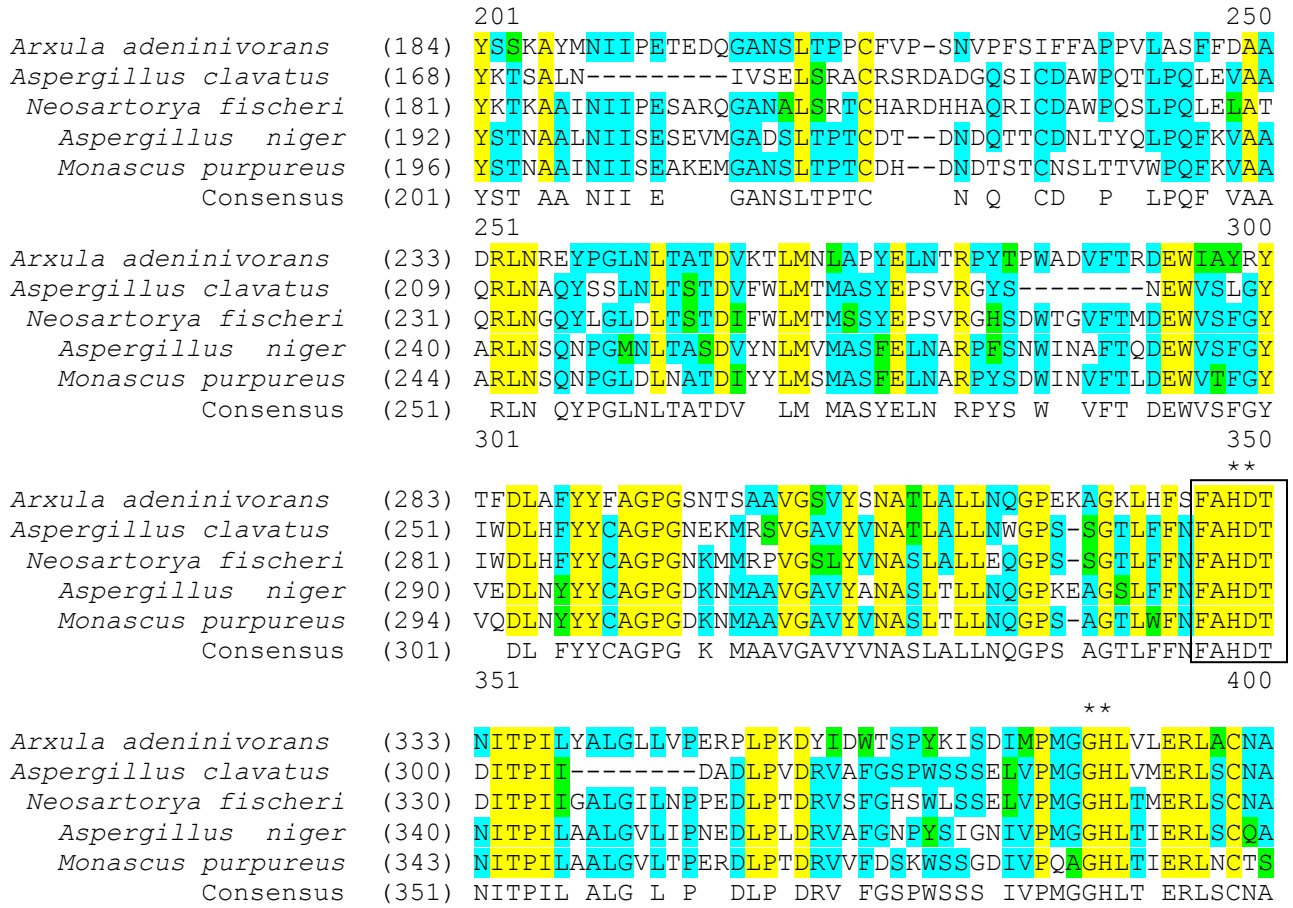

Figure 2.3 (Continued) Multiple sequence alignment of apase genes from different fungal and yeast organisms used to design degenerate primers for *Cladonia portentosa*

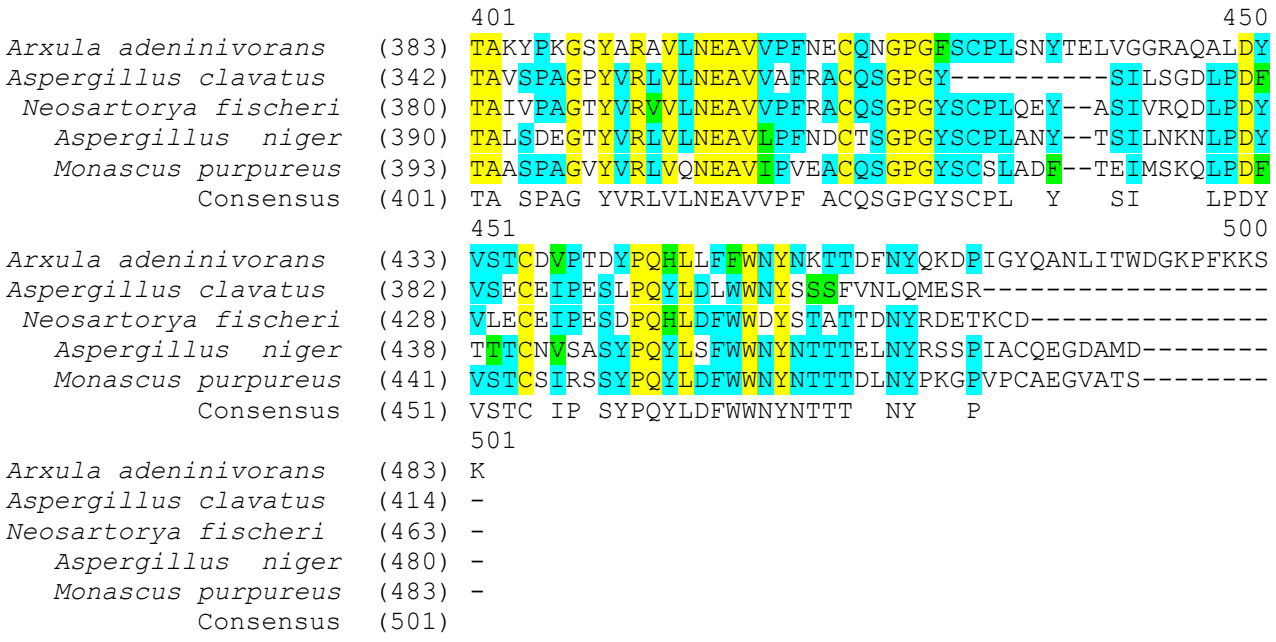

Figure 2.3: (Continued) Multiple sequence alignment of apase genes from different fungal and yeast organisms used to design degenerate primers for *Cladonia portentosa*. Numbers indicate the position of each presented segment within the corresponding sequence. Sequences are grouped together as discussed in the text according to physiological function. Sequence similarities were graded from light green (low similarity) to yellow (highest similarity). Yellow indicates identical residues, green and blue indicate similar residues and a white letter (no colour) are regions of non-homology. Functionally important residues involved in the catalytic mechanism are marked with squares. The figure was constructed using Vector NTI (Invitrogen). Gaps are indicated by dashes. The serine and arginine residues forming the catalytic sites (phosphorylation site) and phosphate binding site of fungal apases are indicated by *

2.3.3 DNA extraction

DNA yields were obtained from *Cladonia portentosa* using both commercial extractions kits as well as traditional methods. Higher concentrations of DNA were extracted by the phenol protocol (Table 2.4), compared to CTAB extraction and commercial kits. Although, CTAB extraction and commercial kits gave a much lower yield than phenol extraction, the DNA quality was better, since the pellet was white, indicating the absence of phenols or other pigments. The quality of DNA was further verified on agarose gel (Figures 2.4-2.5). Higher DNA quantity was extracted by traditional methods than with commercial kits (Figure 2.4-2.5). The DNA extracted by the three techniques was intact and no degradation was observed (Figures 2.4-2.5).

Table 2.4: Comparison of DNA extracted from *Cladonia portentosa* by by commercial kits and traditional protocols. The concentration of DNA was measured using a NanoDrop ND-1000 spectrophotometer.

Organism	**Phenol extraction (ng/µl)**	**CTAB (ng/µl)**	**Commercial protocol (ng/µl)**
Aspergillus niger	625	200	100
Neosartorya fischeri	500	180	90
Cladonia portentosa	40	25	20

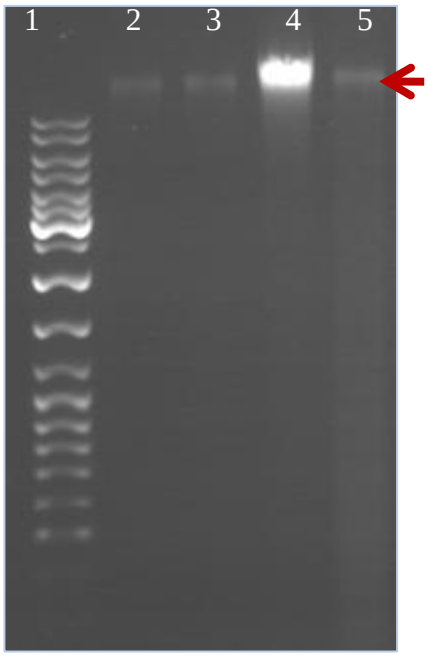

Figure 2.4: Extraction of genomic DNA from *Cladonia portentosa* using the CTAB protocol. DNA markers are indicated on the side of the gel (Lane 1). Genomic DNA extraction from *Cladonia portentosa* (25 ng/ µl) where 0.5 µl (lane 2), 1.0 µl (lane 3) and 5.0 µl (lane 3) were loaded to each well, respectively. DNA fragments were separated on 1.0% agarose gel and stained with ethidium bromide and viewed under UV light.

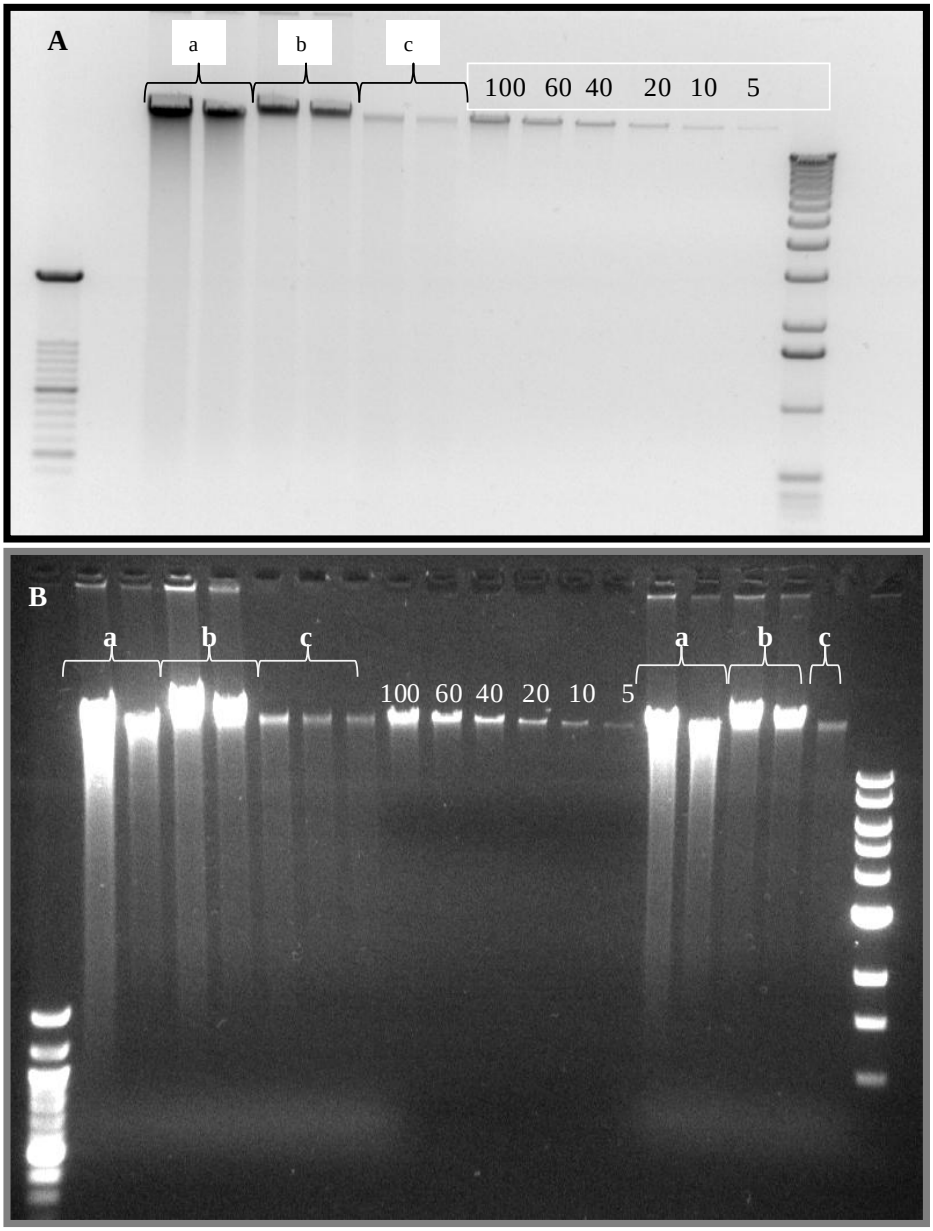

Figure 2.5: (A-B) Extraction of genomic DNA from *Aspergillus niger*, *Neosartorya fischeri* and *Cladonia portentosa*. The DNA was loaded in the following manner: 1.0 or 0.5 µl of genomic DNA mixed with a final volume of 12 µl loading buffer. (A) Genomic DNA extracted from *A. niger* (lanes a, 1.0 and 0.5 µl), *N. fischeri* (lanes b, 1.0 and 0.5 µl) and *C. portentosa* (lanes c, 1.0 and 0.5 µl) using a commercial kit. (B) Genomic DNA extracted from *A. niger* (lanes a, 1.0 and 0.5 µl), *N. fischeri* (lanes b, 1.0 and 0.5 µl) and *C. portentosa* (lanes c, 1.0 and 0.5 µl) using a phenol-based protocol. Lambda DNA markers were used to visually quantify DNA concentration ranging from 100, 60, 40, 20 10 and 5 (ng/ µl). DNA were separated on 1.0% agarose gel, prepared using TBE buffer. After electrophoresis, the gel was stained with ethidium bromide and viewed under UV light. DNA markers are indicated on the side of the gel.

2.3.4 Optimization of the PCR parameters

The first set of degenerate primers used in this experiment came from alignment that showed high homology with yeast apases, group B. However, no PCR product was amplified using the DNA template from *Cladonia portentosa*. The PCR was optimized with different concentrations of primers using the DNA template from the yeast *Pichia pastoris.* A PCR product of 600 bp was obtained (Figure 2.6).

Figure 2.6: Optimizing degenerate primers using genomic DNA from *Pichia pastoris*. Degenerate primers were designed from yeast sequences. Different concentrations of primers were used and with a genomic DNA at a concentration of 50 ng. Expected PCR fragment of 600 bp was amplified in all of most treatments. PCR products were separated on 1.0% agarose gel, prepared using TBE buffer and the gel was stained with ethidium bromide. DNA markers are indicated on each side of the gel.

Other sets of degenerate primers designed from apase sequences from group A and group C. were also used. The sequences of these organisms were included in the alignments used to design degenerate primers. Several PCR parameters were optimized as indicated in Table 2.3. Initially, less stringent conditions were used and non-specific bands were obtained in both positive controls (Figure 2.7). Expected PCR fragments of 649 bp and 954 bp were obtained in both positive controls. PCR stringencies were increased and 52 °C was optimal, using 40 or 80 pmol (Figure 2.8). Similar conditions were applied to *C. portentosa*. No bands were observed besides low molecular weights less than 100 bp (Figure 2.9).

Other factors that might influence efficient PCR amplification such as annealing temperature, $MgCl_2$ concentration, primer concentration and DNA template concentration were studied. The genomic DNA template used ranged from 5.0 ng, 10 ng, 50 ng and 100 ng. Products were amplified in both positive controls using 5.0 and 50 ng as a DNA template. Using a gradient PCR, 52 ºC using 40 pmol primer concentration and 50 ng of genomic DNA was established to be optimal for *A. niger* (Figure 2.8A). No products were observed at a concentration of 80 pmol when 50 ng and 52 ºC was used. No PCR products were amplified using genomic DNA from *C. portentosa* irrespective of DNA template concentration. In all cases, expected PCR products were amplified but only from the positive control (Figure 2.9).

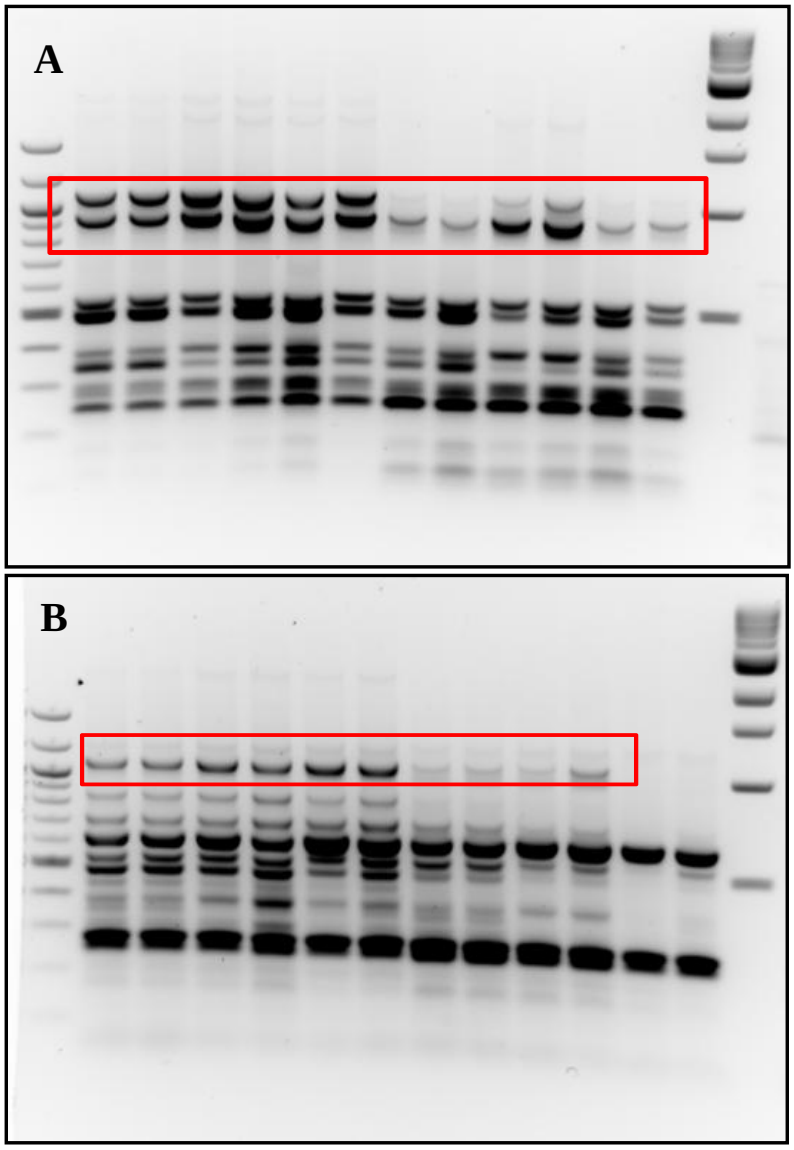

Figure 2.7: (A-B) Typical PCR profile using low stringency parameters (temperature, primer concentration and DNA template concentration) using (A) *A. niger* and (B) *N. fischeri DNA* template. DNA fragments were separated on 1.0% agarose gel, prepared using TBE buffer. Ten microliters of amplified PCR product was loaded into the gel and after electrophoresis, the gel was stained with ethidium bromide. DNA markers are indicated on each side of the gel.

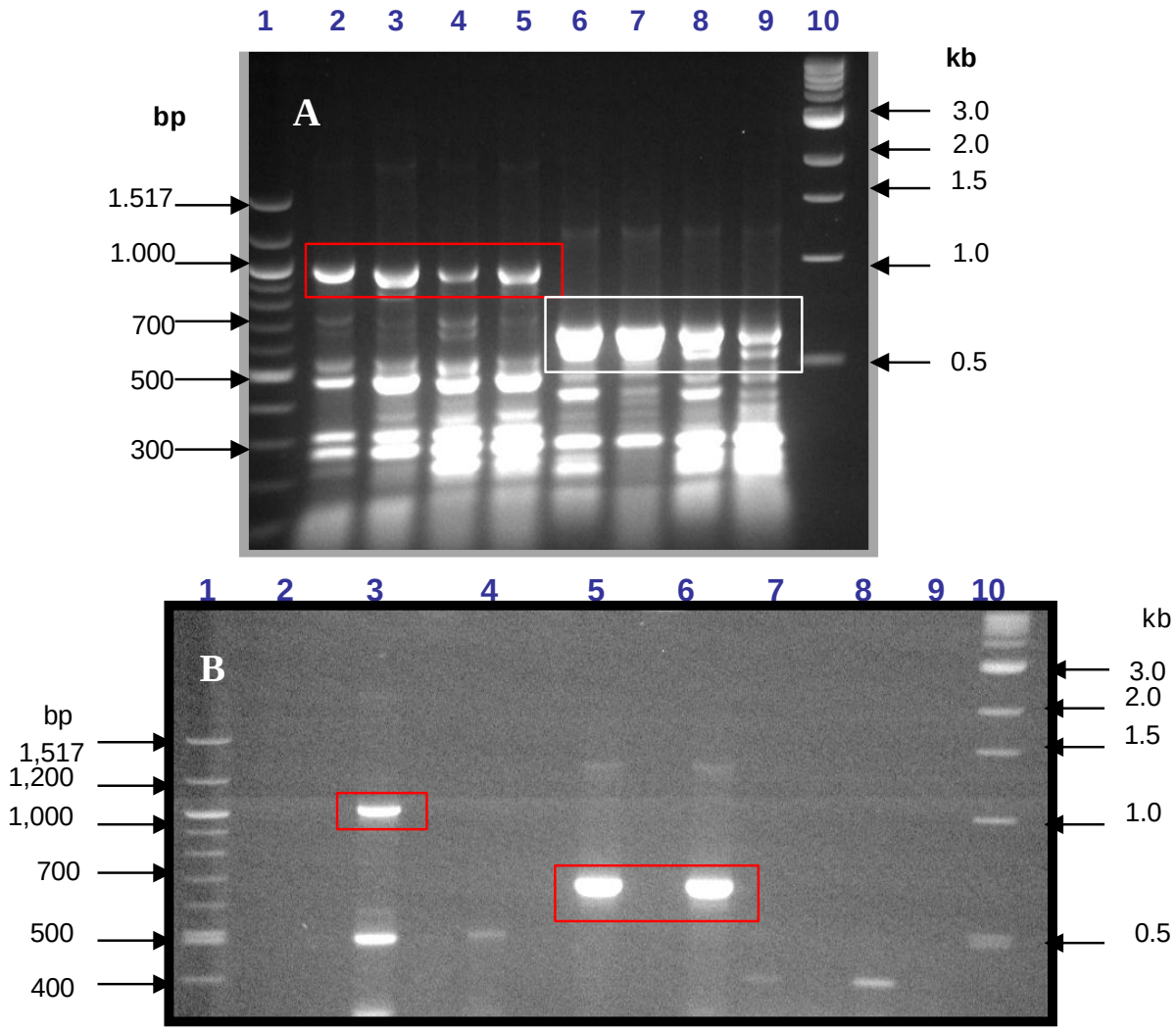

Figure 2.8: (A-B) Typical PCR profile using high stringency parameters (temperature, primer concentration and DNA template concentration) using *A. niger* (A) and *N. fischeri* (B) *DNA* template. DNA fragments were separated on 1.0% agarose gel, prepared using TBE buffer. Ten microliters of amplified PCR product were loaded into the gel and after electrophoresis, the gel was stained by ethidium bromide. DNA markers are indicated on both sides of the gel.

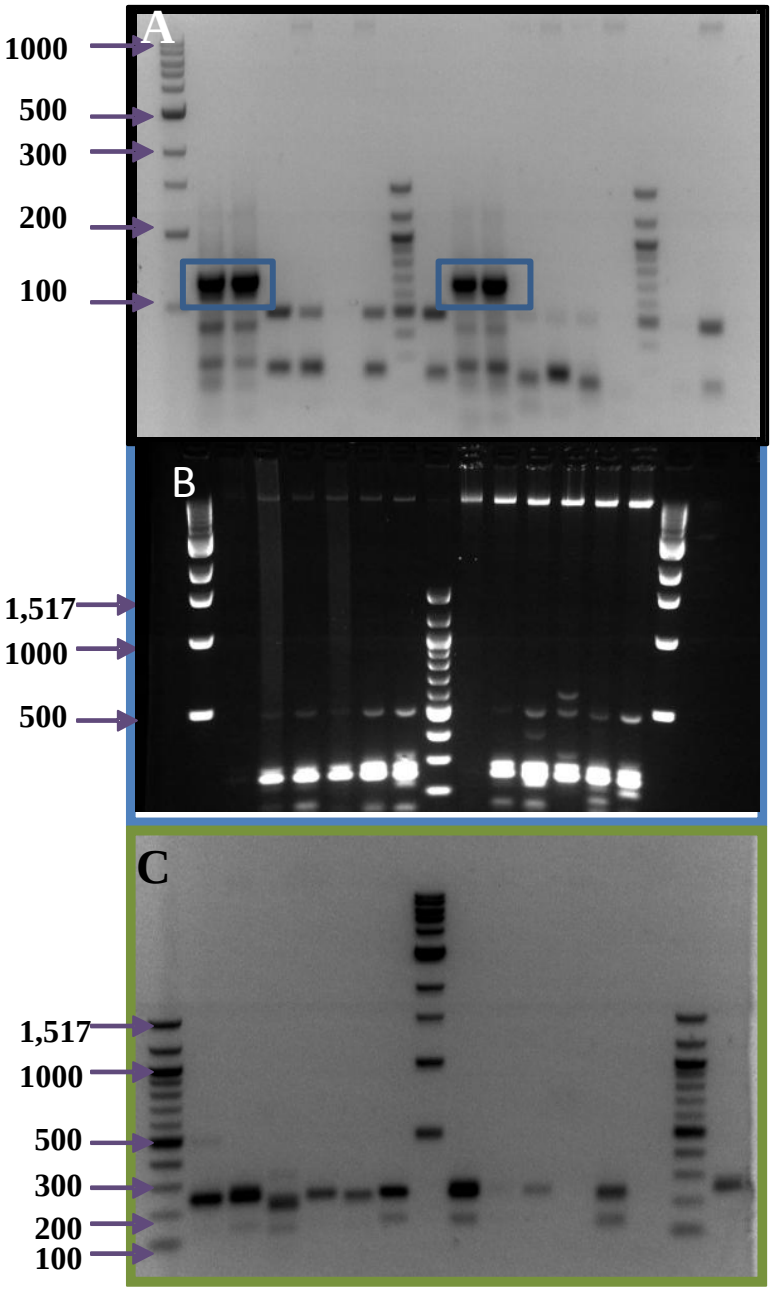

Figure 2.9: (A-C) Typical PCR profile using low stringency parameters (temperature, primer concentration, $MgCl_2$ and DNA template concentration) using a *Cladonia portentosa* DNA template. (A) Optimization using a combination of different primer concentration, 5.0 vs. 50 ng DNA template and different temperatures. Expected PCR products were not amplified but in most cases, primers present as low molecular weight bands were obsevered after ethidium bromide staining such as those indicated by the box. (B) $MgCl_2$ concentration optimization. (C) Different primer concentrations. PCR products were separated on 1.0% agarose gel, prepared using TBE buffer. Ten microliters of amplified PCR products were loaded into the gel and after electrophoresis, the gel was stained by ethidium bromide. DNA markers are indicated on each side of the gel.

2.4 DISCUSSION

2.4.1 Typical motifs in the sequences of apases and challenges in primer design

The isolation of apase gene from *C. portentosa* was conducted in order to study the apase enzyme found in this lichen. Isolation of an unknown sequence related to known sequences is a powerful method for investigating biological function **(ROSE *et al.*, 1998)**. Distance and relationship were interpreted either in phylogenetic lineage or in physiological terms. Analysis of data provided by the phylogeny map revealed that taxonomic groupings of apase genes was not so important, since many fungi produced several types/species of apases that are not related. For instance *Aspergillus niger*, produces several apase enzymes such as metallo-apase, pH 2.5 optimum, pH 5.0 and 6.0 (Table 2.1). Three apase species have been reported in *Saccharomyces cerevisiae*, which are induced by phosphorus starvation **(BAJWA *et al.*, 1984; ELLIOTT *et al.*, 1986)** (Table 2.1). In general, all proteins that are controlled by phosphorus concentration in the medium have high AA similarities (Figure 2.1, Table 2.1). For instance *PHO3*, *PHO5* and *PHO12* from *Saccharomyces cerevisiae*, *Aspergilus niger* (including both PhyA and PhyB and other secreted apase such as *Botrytinia fuckelinia*) have a higher homology and are closely related. All metallo-acid phosphatase, known as purple acid phosphates gave a poor homology when aligned with other non-metallo apases (Table 2.1). Closely related apases such as "secreted apases" from pathogenic and soil fungi such as *Botrytis* and *Monascus purpureus* also aligned well with *PHO3* and *PHO5*, thus all these apase were pooled together to design degenerate primers (Figure 2.3).

To demonstrate the capacity of PCR to amplify apase gene fragments, efforts were made in designing primers based on selected protein sequences from three groups of acid phosphatases such as that demonstrated by Figure 2.3. PCR primers were made based on protein sequences of known fungal and yeast acid phosphatases **(BAJWA *et al.*, 1984; ELLIOTT *et al.*, 1986; PIDDINGTON *et al.*, 1993; VAN HARTINGSVELDT *et al.*, 1993; EHRLICH *et al.*, 1993)**. Known apase sequences were aligned to identify conserved regions in order to design degenerate primers. In general, poor homology was obtained from sequence alignments due to the diverse nature of apase proteins (Figure 2.1). Conserved residues were mainly around conserved sites 'RHGXRXP', R and HD motifs (Figures 2.1 and 2.3). Low sequence homology

was one of several factors that made designing of PCR primers using these sites/sequences difficult. These factors include low homology, the presence of unfavourable amino acids such as serine within the conserved sequences, the absence of continuous conserved amino acids and the shortness of the conserved sites, 6 AA or less (Figure 2.3).

2.4.2 DNA extraction and PCR optimization

The data indicate that optimum DNA yield can be obtained from *Cladonia portentosa* using commercial extractions kits as well as conventional methods (Figures 2.4-2.5). In this study, the quantity of DNA isolated by commercial kits were generally lower compared to the traditional methods, although higher yield have been reported by **GRIFFITHS *et al*. (2006)** using a commercial kit. Low DNA yield obtained in using commercial kits have been attributed to the fact that some DNA molecules failed to bind to silica particles and were lost during washing or could not be eluted because of irreversible bonds **(BOOM *et al*., 1990)**. Traditional DNA protocols used in this study were labour-intensive. However, data indicated that these two methods were highly efficient in extracting high yields of genomic DNA in *Cladonia portentosa* and other fungi used in this study. Higher DNA yield increases the chances of detecting rare species **(WINTER *et al*., 1980)**.

Since the aim of the PCR was to clone the gene that was virtually unknown, low temperature stringencies were initially used in the PCR (42º to 52 ºC). The annealing temperature 42 ºC was low enough to guarantee efficient annealing of the primer to the target, but high enough to minimize non-specific binding (Figure 2.7). Later, the annealing temperature was increased to 54-60 ºC. Distinct bands were obtained from the positive controls but none were obtained from *C. portentosa* (Figure 2.8). Failure to amplify the apase gene by PCR using *C. portentosa* genomic DNA was intriguing and perplexing. Several attempts to optimise varying PCR parameters were conducted.

Visual assessment of DNA quality showed it was sufficient for PCR irrespective of the extraction method (Figures 2.4-2.5). Expected PCR products were amplified in both positive controls (*A. niger* and *N. fischeri)* using the reported extraction protocols. Most conventional

methods use toxic and hazardous reagents such as phenol and chloroform to separate cellular debris from the DNA, thus, the possible contamination of isolated nucleic acid by these toxic chemical cannot be ruled out **(GRIFFITHS *et al*., 2006; NIU *et al*., 2008)**. In addition, the use of high salt concentration buffers and proteinase K treatments of DEAE-cellulose to obtain a higher purity of DNA in several non-toxic extraction methods have been linked to compromised PCR activities **(ALJANABI and MARTINEZ, 1997; DE LA CRUZ *et al*., 1997; SHARMA *et al*., 2000; BULDEWO and JAUFEERARLLY-FAKIM, 2002; ANGELES *et al*., 2005)**. In addition, the lack of PCR product has been linked with contaminants such as tannins, polysaccharides and pigments that can inhibit the annealing of DNA or the enzymatic activity of restriction endonucleases **(PANDEY *et al*., 1996; ROGSTAD, 2003)**.

PCR amplification was further conducted using varying concentrations of genomic DNA extracted by three techniques (to eliminate all the possible inhibitors of lichen DNA). Studies by **WANG *et al*. (1989**) showed that high template concentration occurs as a result of the PCR amplifications, phenomena known "as a substrate saturation of enzyme", or product inhibition of enzyme, incomplete strand separation and product strand re-annealing can be limiting factors for efficient amplification **(WANG *et al*., 1989)**. No PCR products were obtained using genomic DNA of *Cladonia portentosa* irrespective of the DNA extraction method. The successful amplification of PCR products from positive control organisms revealed that the DNA isolated using three methods were suitable for PCR application.

Other variables that could influence the efficiency of the PCR amplification were studied: These parameters included the concentrations of dNTPS, $MgCl_2$, primers, Taq polymerase and the PCR cycle profile **(WANG *et al*., 1989)**. A difference in primer efficiency is a difficult parameter to regulate for quantitative analyses **(WANG *et al*., 1989)**. The degenerate primers were designed using sequences of close relatives of *C. portentosa*, mainly from the Ascomycete family. To improve specificity and minimize the degeneration, codon usage tables were consulted for base preference in different organisms (**INNIS and GELFAND, 1990)**. Inosine was used which pairs with all bases. In terms of primer concentration, 40 and 80 pmol were sufficient to amplify PCR products in both control organisms (Figures 2.7 and 2.8). Recommended primer concentration ranging from 1.0 µM to 3.0 µM were used since many of

the primers in the degenerate mixture are not specific to the target **(SHYAMALA and AMES, 1993)**. In either case, mismatches in oligonucleotide annealing are typically limiting **(RUBIN and LEVY, 1996)**.

Different polymerase enzymes were tried (Taq polymerase, Faststart Taq polymerase and Phusion Taq polymerase), since several studies show that the choice of enzyme can affect yield (**INNIS and GELFAND, 1990)**. FastStart Taq polymerase became the enzyme of choice in this study. The highest length of the expected fragment in this study was limited to 1500 bp. It has been shown that the amount of amplified DNA fragment in a given sample has a prevailing influence on the amplification efficiency **(LARZUL *et al*., 1990)**.

Magnesium concentration was also optimized. The concentration ($MgCl_2$) of 2.5 µM was optimal but no success was obtained for *C. portentosa* (Figure 2.9). Magnesium affects several aspect of PCR including DNA polymerase activity, which can affect yield, primer annealing, and specificity **(INNIS and GELFAND, 1990**). Higher concentrations of free magnesium can result in greater yield, but can also increase non-specific amplification and reduce fidelity **(ECKERT and KUNKEL 1990; WILLIAMS, 1989)**. Since PCR products were amplify from positive control organisms, it was concluded that failure to amplify the apase gene from *C. portentosa* was not the result of the reagents or genomic DNA.

In order to avoid time-consuming trial-and-error testing using degenerate primers, attempts to purify the apase were made. It was anticipated that the partial sequence of the purified enzymes would provide a corresponding apase gene **(KEROVUO *et al*., 1998; CHO *et al*., 2005)**.

3. PURIFICATION AND BIOPHYSICAL CHARACTERIZATION OF SECRETED ACID PHOSPHATASE FROM THE LICHEN *CLADONIA PORTENTOSA*

3.1 GENERAL INTRODUCTION

Acid phosphatase (apase) enzymes have been purified from several organisms such as bacteria, fungi and plants. Secreted apases have been characterized in many fungi such as *Aspergillus*, *Penicillium, Fusarium* and *Neurospora* species **(YOSHIDA and TAMIYA, 1971; NAHAS *et al.*, 1982; YOSHIDA *et al.*, 1989a; HAAS *et al.*, 1991; PASAMONTES *et al.*, 1997b; NOZAWA *et al.*, 1998)**.

Most apases display a high heterogeneity with regards to pH optima, molecular weight, p*I* and subunit structure/s. Several classes can be distinguished on the basis of pH optima (2.5-7.0), molecular size (high versus low weight acid phosphatases) and substrate preference such as phytic acid or protein **(ULLAH and GIBSON, 1987; VINCENT *et al.*, 1992)**. The family of high molecular weight (HMW) apases can be distinguished from other phosphatases by having a subunit molecular mass more than 40 kDa **(OSTANIN *et al.*, 1992)**. Non-specific phosphatases catalyze the hydrolysis of almost any phosphate ester, whereas protein phosphatases prefer phosphoproteins or phosphopeptides as substrates **(VINCENT *et al.*, 1992)**. Apase pH optima vary extensively amongst different filamentous fungi. For instance apase found in *Aspergillus fumigatus* is active at pH 3.0 to 7.0, with the optimum activity occurring between pH 4.0 and 6.0 **(BERNARD *et al.*, 2002)**. On the other hand, *Aspergillus niger* have several apases which display varying pH optima; such as 2.5, 5.0 and 6.0 **(ULLAH *et al.*, 1994)**. Most fungal apases are high molecular weight glycoproteins, for instances apase from *Aspergillus niger* has an apparent native molecular mass of 269 kDa with a glycosylated subunit of approximately 65 kDa and an unglycosylated form of 50.8 kDa. It forms a tetramer in solution **(KOSTREWA *et al.*, 1999)**.

Several challenges have been reported concerning the purification of apases due to their abilities to hydrolyze a wide range of phosphate esters. The problem is to separate acid phosphatase from other phosphatase species such as phytases. Although classic phytases prefer phytic acid as a substrate **(ULLAH *et al.*, 1994),** some have been reported to have dual properties **(SHIEH *et al.*, 1969)**.

The importance of secreted acid phosphatase is more recognised in plant-fungus symbiotic (mycorrhizal) relationships, as their production by soil microorganisms increases the amount of phosphate available to plants **(YADAV and TARAFDAR, 2003)**, contributing significantly to the nutrient dynamics of most ecological niches where phosphorus is deficient **(MOLLA *et al.*, 1984; JAYACHANDRAN *et al.*, 1992; TURNER *et al.*, 2001)**.

While apase from many free-living filamentous fungi have been purified and well-characterized, little is known about acid phosphatase from lichen species. The lichen, *Cladonia portentosa* (Dufour) Coem, grows in habitats that are usually very low in nitrogen and phosphorus. This study may have relevance in illustrating the role of lichen apase in phosphorus acquisition in such nutrient-poor ecosystems. Attempts to purify the enzymes, study biophysical properties and the protein sequence of secreted acid phosphatase from *Cladonia portentosa* were made with the aim of obtaining information regarding its biochemical and molecular properties; and to allow comparison with already characterized secreted acid phosphatases from other fungal sources existing in the NCBI database.

This study is divided into three sections, which address three broad but related concepts: the optimization of apase extraction (Section 3.2); purification of the apase enzyme (Section 3.3) and sequencing of the apase protein (Section 3.4).

3.2 OPTIMIZATION OF PROTEIN EXTRACTION

3.2.1 Introduction

Preliminary studies demonstrated that acid phosphatase could be easily detected in intact *C. portentosa* without disrupting the lichen. The enzyme could be released by gently shaking using 20 mM CASC buffer and addition of either 0.5 M NaCl, Triton 100 or 0.1% Tween 20. Pertaining to a large volume initially used, the proteins were concentrated by freeze-drying. Enzyme assay and electrophoretic analysis revealed that the proteins released by this method were relative low, since they could not be detected by Coomassie staining. It would appear that apase activity was reduced by the freeze-drying process. Silver staining revealed that several proteins were present in leachates in levels below 5.0 ng. For the acid phosphate protein to be sequenced, a higher yield of the protein is required. The mechanisms by which the apase enzyme is bound to the cell wall were not understood when this study was conducted. Major concerns were the use of disruptive extraction methods that would release cytosolic contaminants, or contamination with proteins of different subcellular compartments, since it was assumed that the apase was localized on the cell wall and cell membrane.

The fungal wall structure is highly dynamic, composed of chitin, glucans and other polymers, and there is evidence of extensive cross-linking between these components. The cell walls of most fungi consist of five major components (1→3)-β-glucan, (1-6) β-glucan, (1-3) α-glucan, chitin and glycoproteins (**ZONNEVELD, 1971; HEARN and SIETSMA, 1992; CHAFFIN *et al.*, 1998; MOUYNA *et al.*, 2002)**. Electron microscopy studies by **HONEGGER and BARTINICKI-GARCIA (1991)** confirmed that the cell wall of three lichen species *Cladonia caespiticia*, *Cladonia macrophylla* and *Physcia stellaris* is similar to the walls of non-lichenized Euscamycete fungi (they all belong in the same chitin-β-1,3-glucan category **(HONEGGER and BARTINICKI-GARCIA, 1991)**. The cell walls of all three mycobionts are made of polysaccharides, proteins and lipids, with glucose being the most abundant polysaccharide, however, monomeric, mannose, galactose and glucosamine were also detected (**HONEGGER and BARTNICKI-GARCIA, 1991)**. Similar observations were made in free-living fungi such as *Candida albicans* and *Aspergillus* spp. – such as *A. nidulans* and *A. fumagatus* **(ZONNEVELD, 1971; HEARN and SIETSMA, 1992; CHAFFIN *et al.*, 1998; MOUYNA *et al.*, 2002)**. Cellular disruption of fungi is a crucial step in obtaining maximum amounts of

soluble cellular contents with maximum biological activity and with minimum denaturation, proteolysis and oxidation **(WONG *et al*., 2008)**. The fungal cell wall is extremely difficult to disrupt because of its complex nature **(WONG *et al*., 2008)**. Since low protein yields were obtained in the preliminary extraction study, effective methods of solubilizing proteins with preserved biological activities were explored. This section outlines the optimization of solubilization methods for the isolation of apase in lichen *Cladonia portentosa*. Different biological detergents listed in Table 3.1 were compared. In addition, the release of apase by cell wall hydrolases was also studied (Table 3.2).

Biological detergents are a class of molecules whose unique properties enable manipulation (disruption or formation) of hydrophobic-hydrophylic interactions among molecules in biological samples **(OSBORN, 1975; HELENIUS *et al*., 1979; LICHTENBERG *et al*., 1983)**. In biological research, detergents are used to lyse cells (release soluble proteins), solubilize membrane proteins and lipids, control protein crystallization, prevent non-specific binding affinity purification, immunoassay and as additives in electrophoresis.

Biological detergents are advantageous since they are water-soluble, consequently, detergent molecules allow the dispersion of water-insoluble hydrophobic compounds into aqueous media, including the extraction and solubilization of membrane proteins. However, detergents can be denaturing (e.g. SDS and ethyl trimethyl-ammonium bromide) or non-denaturing with respect to protein structure **(LICHTENBERG *et al*., 1983)**. These detergents totally disrupt membranes and denature proteins by breaking the protein-protein interaction. Non-denaturing detergents can be divided into non-ionic detergents such as TritonX-100, bile salt such as cholate and zwitterionic detergents such as CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate) **(TAVARES and SELLSTEDT, 2000)**.

Cell wall proteins can be divided into two groups: proteins that are solubilized by ionic detergents or chaotropic agents, and those that are solubilized by degradation with hydrolases such as ß-glucanases or chitinase **(ELORZA *et al*., 1985; KAPTEYN *et al*., 1997)**. The cell hydrolase enzymes are able to cleave the glucan and chitin. Thus, it was anticipated that the use of these enzymes could release high levels of apase presumed to be localized in the cell wall

and cell membrane. This study attempted to release cell wall phosphatase using different detergents and cell wall hydrolase enzymes described in Tables 3.1 and 3.2.

Table 3.1: The following detergents were chosen for their stated properties.

Detergent(mg)/agents	Main feature	Advantages
Digitonin (0.1%)	Water-solubilizes lipids	Solubilizing membrane proteins, proteins, precipitating cholesterol and permeabilizing cell membranes
CHAPS (0.5M)	Zwitterionic	Offer combined properties of ionic and non ionic detergents
NDSB (0.6 %)	Non-detergent	Non-detergent
Water pH7.0	none	-
Citric acid-tri-citrate buffer (20 mM, pH 2.5)	none	-

Table 3.2: Cell wall hydrolase enzymes used in this study.

Enzyme	Main feature /enzyme composition	Manufacturer
Glucanex®	Contains β-glucanase, cellulase protease and chitinase activities	Sigma, UK
Caylase	Contains cellulolytic and pectolytic activities	Societe Cayla, Toulouse France
Novozyme	Glucanase, cellulase, protease and chitinase activities	Novozyme, Dernmark

3.2.2 Materials and Methods

3.2.2.1 Chemicals

A fast protein liquid chromatography system (FPLC) equipped with OS_2 software and chromatography materials including high-substituted ion exchange column with DEAE column or Superdex (10/300 GL gel filtration column) was sourced from GE Healthcare Amersham Biosciences (UK). Dextran blue 2000 was sourced from Amersham Biosciences (UK). Ampholytes 3-10 and IEF standard markers were obtained from Biorad, Germany. N-Glycosidase F (PNGase F) enzyme was purchased from New England Biolabs (UK). BCA kit was purchased from Novagen (Germany). Pre-stained native, unstained protein standard,

already-cast native PAGE and SDS-PAGE polyacrylamide gel were sourced from Invitrogen. Silver staining (The SilverQuest™) and SimplyBlue™ Safe Stain staining were bought from Invitrogen. CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate), ammonium persulfate (APS), TEMED (N,N,N,N'N'-Tetramethylethylenediamine), Fast Garnet GBC Salt, Dithiothreitol (DTT), pyrophosphate, adenosine triphosphate (ATP), adenosine monophosphate (AMP), para-nitrophenyl phosphate (pNNP), phospho-L-serine, phospho-L-threonine, phospho-L-tyrosine, ethylenedinitrilotetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), polyoxyethylenesorbitan monolaurate (Tween 80), acrylamide were all purchased from Sigma (UK). Poly-(vinylidene difluoride) membranes (0.22-0.45 µm pore size) and Viva spin ultracentrifuge were obtained from Millipore (Germany). Protease cocktail inhibitors were bought from Sigma.

3.2.2.2 Lichen collection and preparation

Fresh material of *Cladonia portentosa* (Dufour) Coem was collected in various sites in the UK: Peak District National Park, Derbyshire, England and Thurso, Scotland. After collection, the samples were air dried under low temperature (15±2 ºC) on the desk and then stored at -20 °C until used. Prior to experimentation, intact lichen thalli were rehydrated overnight in water-saturated air (over water in a desiccator) at 10 °C, and then saturated by spraying lightly with deionized water. This was to allow the lichen to become hydrated without cell rupture and leakage of solutes which can occur if uptake of water in a desiccated state is too rapid **(HOGAN *et al.*, 2010a)**. Hydrated thalli were then cleaned to remove debris. Glassware used in this study was washed with distilled water using phosphorus-free soap and acid (<10 % HCL), and thoroughly rinsed with distilled water.

3.2.2.3 Acid phosphatase assay/s

Acid phosphatase assay A

The routine acid phosphatase activity was determined using p-nitrophenyl phosphate (p-NPP) as substrate, using a method described by **TURNER *et al.*, (2001)** and optimized by **HOGAN *et al.*, (2010a)**. Briefly, samples of intact *C. portentosa* were added to 2.9 ml of 0.02 M citric

acid-trisodium citrate (CASC) buffered assay medium made up in simulated rainfall containing major ions representative of UK precipitation (20 mM $MgSO_4.7H_2O$, 8.0 mM $CaCl_2.2H_2O$, 150 mM NaCl, 15 mM NH_4NO_3, 5.0 mM KNO_3) **(HAYMAN *et al*., 2004)** and the pH adjusted to 2.5, the optimum value for apase activity in *C. portentosa* **(HOGAN *et al*., 2010a)**. Assays were initiated by the addition of 0.1 µl pNPP to yield a final concentration of 3.0 mM, a concentration found previously to saturate apase activity in *C. portentosa* **(HOGAN *et al*., 2010a)**. Samples were then placed in a shaking water bath at 37 ºC for 20 min in the dark after which the reaction was terminated by transferring 2.5 ml assay medium into 0.25 ml termination solution (1.1 M NaOH, 27.5 mM EDTA, 0.55M K_2HPO_4) and the absorbance measured at 405 nm using a NanoDrop ND-1000 spectrophotometer. Thalli were then blotted dry, oven dried for 24 h at 80 ºC and weighed. Enzyme activity was expressed as mmol substrate hydrolyzed g^{-1} dry mass h^{-1} using p-nitrophenol (pNP) to calibrate the assay.

In the case of ground samples, the routine assay was modified slightly. The reaction was performed at 37 °C by adding 100 µl of enzyme solution to 2.8 ml of 100 mM CASC buffer and 100 µl 500 µM p-NPP. The reaction was stopped by the addition of 0.25 ml 1M NaOH to the 2.5 ml solution as described above. One enzyme unit was defined as the amount of enzyme which releases 1 µmol of p-NP per min. The amount of inorganic phosphate released was calculated using an acid phosphatase from wheat (Sigma) as a standard.

Acid phosphatase assay B

For substrates other than pNPP or 4NPP, the method of **AMES (1966)** was used to measure the amount of Pi released by other phosphorylated substrates such as phytic acid, ß-naphthyl phosphate, D-glucose-6-phosphate and ß-glycerophosphate (ß-GLOP). Ascorbic–molybdate solution [2% (m/v) $C_8H_8O_8$ 0.42% (m/v), $(NH_4)6Mo_7O_{24}.4H_2O$), 1N H_2SO_4] was prepared as follows: ammonium molybdate (0.42 g) and conc H_2SO_4 (2.86 ml) were dissolved in 80 ml of dH_2O, transferred in a 100 ml volumetric flask and the volume adjusted to 100 ml with distilled water. This solution is stable at room temperature. The needed volume of ascorbic molybdate solution was made fresh in 2% ascorbic acid (the reducing agent) just before use **(AMES, 1966)**. The reaction was measured in 1.0 ml reaction mixtures containing 800 µl of assay buffer

(100 mM CSTC, pH 2.5), 100 µl of substrate and 100 µl enzyme. Incubation was carried out at 37 °C for 60 min. The reactions were stopped by the addition of 20 µl 50% (w/v) CASC. Blanks were prepared in separate tubes by either replacing the enzyme solution with aliquots of buffer and assaying as normal or stopping the reaction at time zero with 20 µl CASC (50%).

After termination of the enzyme activity, 200 µl of the reaction was mixed with 800 µl of ascorbic–molybdate solution (2% ascorbic acid, added just before use) **(AMES, 1966)**. The tubes were incubated for 20 min at 45 °C **(AMES, 1966)**. The inorganic phosphate was measured by monitoring absorbance at 820 nm. The assays were performed in triplicate and controls were run for background amounts of Pi. One unit of acid phosphatase activity catalyzes the liberation of 1 µmol of inorganic phosphate per min. Generation of an inorganic phosphate calibration curve was obtained using a phosphate standard solution which was prepared by making a 1.0 mM stock solution of KH_2PO_4 in distilled water. This was diluted to prepare a range of phosphate standard concentrations ranging from 200 to 1000 nmol/ml phosphate. The phosphorus calibration curve was performed concurrently with assay B.

3.2.2.4 Determination of protein concentration

Protein concentration was determined by a BCA kit. The test was conducted as per the manufacture's instructions. A standard protein solution consisted of Bovine Serum Albumin (BSA) stock solution (1.0 mg/ml) in distilled water. Samples were transferred to clean cuvettes and measured within 10 min at 562 nm.

3.2.2.5 Solubilization of proteins by biological detergents and cell wall hydrolases

The experiment was designed as shown in Table 3.3. The starting material was similar in all treatments, 3.0 g for ground samples and 1.0 g for intact lichens.

Table 3.3: Eluting phosphatase with detergents and hydrolase enzymes. Intact vs. mechanical extraction. N=3.

TREATMENTS	CONCENTRATION	SAMPLES	
		Intact thallus	Ground
Buffer	20 mM	3 reps	3 reps
Digitonin (w/v)	0.1%	3 reps	3 reps
NDSB 201	1M	3 reps	3 reps
CHAPS	1M	3 reps	3 reps
Glucanex	20 mg/g weight	3 reps	3 reps
Caylase	20 mg/g weight	3 reps	3 reps
Novozyme	20 mg/g weight	3 reps	3 reps

To measure the release of apase in the intact lichen samples, 1.0 g of lichen material of *Cladonia portentosa* was added to 15 ml detergents (Table 3.3). Materials were shaken gently for 1 h at 50 rpm. A protoplast isolation protocol described by **DEBETS and BOS (1986)** was adapted for isolation of apase using a hydrolase enzyme. To get whole homogenate, the intact lichen was ground in liquid nitrogen into a fine powder using a mortar and pestle. The ground samples were weighed and 3.0 g of fresh weight was put into a 50 ml flask. Cold MME buffer (1.2 M $MgSO_4.7H_2O$, 20 mM MES in 200 ml), 0.26 g (of either enzymes) and 0.08 g BSA were added to each flask. The BSA was added in order to protect the protoplast from fungal /Novozyme protease activity. The treatments were all conducted in three replicates. Reactions were incubated for 1.5-2 h while gently shaken at 30 rpm. To aid visual examination, a small sample (10 µl) was diluted with an equal volume of water (10 µl) and observed under microscopy.

To remove all the cell debris, a purification column made of a 10 ml syringe barrel with pre-autoclaved polyallomer wool was used. After the mixture had eluted, it was rinsed with NM (1.0 M NaCl, 20 mM MES buffer). The mixture was mixed gently and spun at 2500 rpm, 4 ºC for 10 min. The supernatant was removed and stored at -70 ºC until use.

3.2.2.6 Optimizing protein concentration

Protein samples obtained in this study required to be concentrated before subjecting them to further purification or SDS-PAGE analysis. Several methods were used to concentrate the proteins: sucrose, PEG, ammonium sulphate precipitation and Viva tube concentrator (Millipore, Germany).

After protein extraction, the clear fractions were divided and 100 ml of crude extract was used to determine an efficient protocol to concentrate the proteins before purification. In the case of freeze-drying treatment, 500 ml bottomless flasks were used, where the liquid was gradually frozen with liquid nitrogen by tilting the flask of liquid nitrogen, until all the liquid had turned to ice. Samples were freeze-dried overnight if the volume of 100 ml was used.

The dialysis tube was used to concentrate proteins by either PEG or glucose. The dialysis tube was initially boiled for 10 min and fractions were added to the dialysis bags. After sealing the samples, the bags were placed in a container consisting of dry glucose granules. For PEG treatment, dialysis bags were place in the liquid that consisted of 100 % (w/v) PEG solution. Solid ammonium sulphate $(NH_4)_2SO_4$ was added to the enzyme preparation where 40% (w/v) (saturated) was added to the solution and gently stirred for 10 min and centrifuged (12 000x g, 4 ^oC, 30 min).

For Viva spin columns, tubes came in 10 ml and 5.0 ml sizes. The protein samples were placed in the sample container and the Viva tubes were centrifuged at 1000x g at 4 ^oC. Once the sample had been sufficiently concentrated (5-10 fold, 2-6 h), both flow through and retentate were assayed for apase activity and protein concentration as described in Section 3.2.2.4 to assess the solute recovery.

3.2.3 Results

3.2.3.1 Solubilizing apase by hydrolase enzymes versus biological detergents

Preliminary studies indicated that acid phosphatase in *C. portentosa* is a cell membrane or cell wall protein. In order for the protein to be sequenced, a high protein yield was required. This

study attempted to release cell wall phosphatase using different detergents and cell wall enzymes. When the lichen was left intact, digitonin was more effective in releasing high levels of acid phosphatase, 3-fold higher compared to other treatments (Figure 3.1A). The buffer treatment was higher on ground samples than other treatments, 60 µmol product/g fresh weight compared to 50 and 40 µmol product/g obtained in digitonin and CHAPS, respectively (Figure 3.1B). Higher levels of acid phosphatase were obtained from the ground samples compared to the intact samples (Figure 3.1C). Although digitonin was effective in the intact lichen, it was not so effective in the ground samples. The efficiency of the detergent correlated with denaturing properties. The activity of the fraction was important in the purification of the protein inorder to determine the enzyme kinetic accurately.

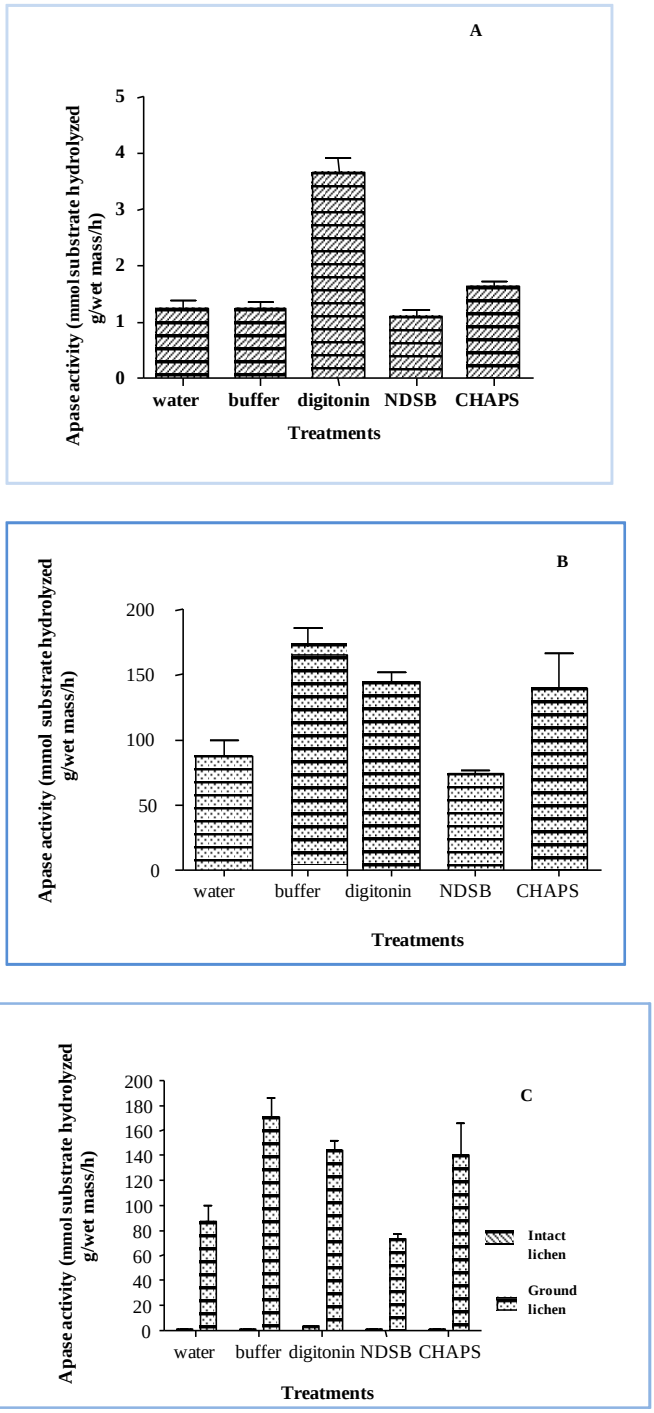

Figure 3.1: (A-C) Comparison of different treatments for the release of acid phosphatase in *Cladonia portentosa* lichen. (A) Intact thallus, (B) Ground samples and (C) Comparison between intact and ground samples). Bars indicate standard error (n=3).

3.2.3.2 Comparison of cell wall hydrolytic enzymes in the release of apases

In both ground and intact lichen material, Novozyme released high levels of apase (Figures 3.2A and B). The levels of apase in the ground samples were higher than the intact samples (Figure 3.2C). However, the presence of BSA interfered with further protein purification. After multiple centrifugation Viva tubes were clogged due to higher protein content.

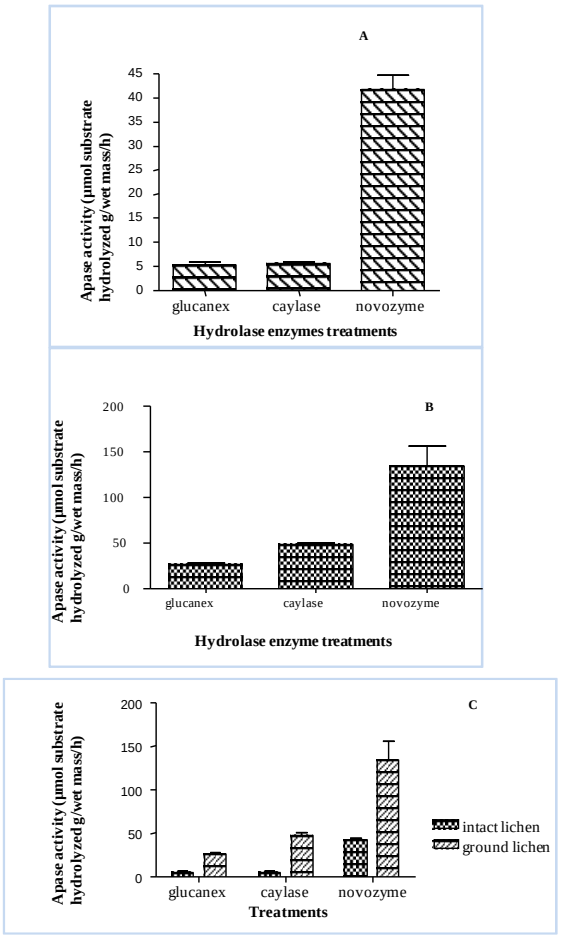

Figure 3.2: (A-C) Comparison of three cell wall hydrolase enzymes in releasing acid phosphatase from *Cladonia portentosa* lichen material. (A) intact thallus and (B) ground thallus of *Cladonia portentosa.* (C) intact vs. ground samples. Bars indicate standard error (n=3).

3.2.3.3 Comparison of different concentration methods

Active fractions of acid phosphatase were concentrated by several methods, including ammonium sulphate, freeze drying, sucrose, PEG and using Viva spin columns. Freeze drying reduced enzyme activity, especially if the sample was allowed to dry completely. High levels of acid phosphatase (±<85% and 82%, respectively) were obtained in the Viva spin columns and with the sucrose method (Table 3.4). Sucrose was also found to be effective in the long term preservation of the enzyme. However, it was very viscous and needed to be reverse-dialysed. The Viva tube was found to be sufficiently mild to retain enzyme activity and offered some advantage over other chemical precipitation methods. In addition, the Viva spin column also serve as a purification devise and thus was the best treatment.

Table 3.4: A summary of different techniques used in this study to concentrate the apase enzyme from *C. portentosa*.

Treatments	**% Enzyme activity after treatment**	**Observed advantage**	**Observed disadvantage**
Ammonium sulfate	60%	The remaining pellet was relative clean, beside the salt.	It lowered the activity
Freeze drying	25%	none	Cannot be controlled. Require the use of a bigger cointainer
PEG	60%	Relative low volumes were obtained.	Lowers activity and the recovered fractions are dirty.
Sucrose	82%	Preserved enzyme activity, effective for long term storage.	Took a long time. The concentrated samples were very viscous. It could not be loaded onto Viva spin columns. It required reverse-dialysis.
Viva spin	85-100%	It cleaned the proteins, it served as a purification device since proteins were eluted according to size. Fractions were relatively clean.	The membrane can be fragile and gets clogged easily. Higher detergent concentration can cause the membrane to break. Not recommended if used in conjuntion with reducing agents.

The polypeptide profiles of proteins extracted by detergents and hydrolase enzymes were also analyzed electrophoretically using SDS-PAGE. In general, leachates (protein extracted from intact lichen) gave a very low protein yield (Figure 3.3.A). Proteins were not detected by Coomassie staining but only by silver staining. The protein concentrations were in the region of 5.0 ng/ml (Figure 3.3.A). Apase activity was reduced drastically by freeze drying. This was supported by electrophoretic analysis where higher molecular weight proteins could not be detected by Coomasie staining (Figure 3.3. B). Further concentration by Viva tubes retained all the proteins that were above 50 kDa. However, higher molecular proteins were not detected after staining (expected in the region indicated by a rectangular box).

Cell wall hydrolase enzymes released high levels of proteins in general (Figure 3.3. C). After concentration by Viva tubes, sucrose and PEG, various bands could be visualized (Lanes 2-4, respectively). Extraction by digitonin, 20 mM CASC buffer and CHAPS were effective. After protein concentration several proteins bands could be visualized in all three treatments (Figure 3.3D). The regions where apase bands were expected is highlighted by rectangular box (Figure 3.3D). Proteins extracted by detergents were further concentrated by sucrose. Bands of various sizes were clearly visible, including high molecular weight proteins. Note the region where apase band/s were detected is highlighted by a brackect (Figure 3.3D).

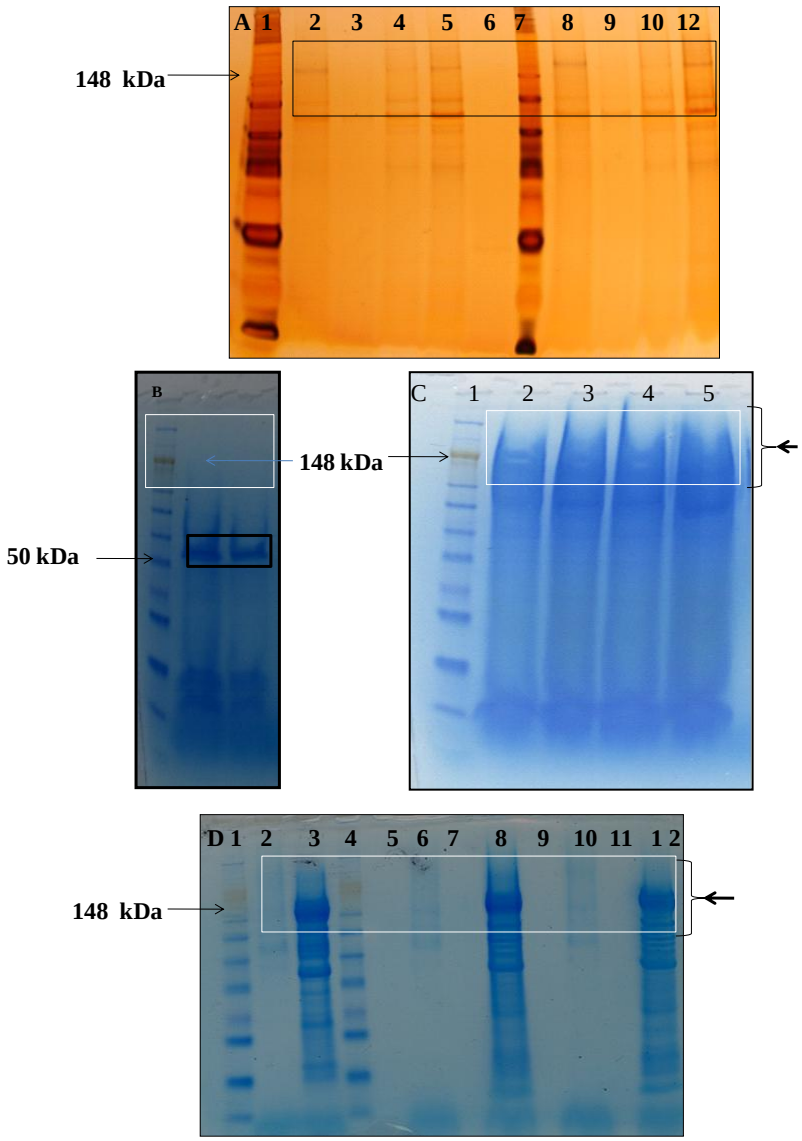

Figure 3.3: (A-D) Polypeptide profile of proteins extracted/solubilized by cell wall hydrolases and biological detergents and concentrated by various methods as mentioned in Table 3.4. (A) leachate extraction and visualized by silver staining. (B) Protein concentrated by freeze-drying. Note the presence of a few bands and the absence of higher molecular weight proteins indicated by the white rectangular block. (C) Proteins extracted by cell wall hydrolase enzymes. (D) Proteins extracted by chemical/biological detergents. Lane 1: represents the protein standard, lane 2: extraction by digitonin before protein concentration, lane 3: extracted by digitonin and concentrated by sucrose, lane 6: extraction by 20 mM CASC buffer, lane 8: extracted by 20 mM CASC buffer and concentrated by sucrose, lane 10: extracted by CHAPS before protein concentration and lane 12: extracted by CHAPS and concentrated by sucrose. Note the region where apase band/s were detected is highlighted by a bracket.

3.3 PARTIAL PURIFICATION OF ACID PHOSPHATASE

3.3.1 Introduction

Proteins comprise an extremely heterogeneous class of biological macromolecules. They are often unstable when not in their native environments, which can vary considerably among cell compartments and extracellular fluids **(PIERCE BIOTECHNOLOGY, 2005)**. If certain buffer conditions are not maintained, extracted proteins may not function properly or remain soluble. Proteins can lose activity as a result of proteolysis, aggregation and sub-optimal buffer conditions. Several classes of proteases (e.g. aspartic, cysteine, serine and metallo-proteases) are known to degrade proteins intended for purification **(PIERCE BIOTECHNOLOGY, 2005)**. The use of protease inhibitors is recommended **(WALKER, 1994)**. The addition of EDTA and PMSF also inhibits many proteases, however, care must be taken, since these compounds can interfere with protein yield or assay of active enzyme.

3.3.1.1 Purification by high pressured liquid chromatography (HPLC)

Proteins and other macromolecules can be separated based on their molecular weight (MW) by using a cross-linked porous gel. The degree of retardation of a particle is related to the molecular weight and shape. The smaller molecules will take a more convoluted path through the column and elute later while bigger molecules will be eluted first, since they are usually too big to go through the gel matrix. Since they are excluded, the distance they have to travel is reduced. There are three types of supports for gel filtration: dextran (e.g. Sephadex), polyacrylamide (e.g. Sephacryl) and agarose (e.g. Sepharose) **(JANSON and HEDMAN, 1982)**.

In ion-exchange chromatography, the mobile phase is a buffered electrolyte and the stationary phase is solid which contains covalently linked anion or cation groups **(JANSON and HEDMAN, 1982)**. Separations depend mainly on differences in the net ionic charges of the solutes at specific pH values, however, the separations depends also on the extent to which hydrophobic and hydrophilic moieties of the biomolecules interact with the hydrophobic or hydrophilic ion-exchange resins. Cation-exchangers are negatively-charged (acidic) resins

which can bind cations. Anion-exchangers are positively charged (basic) nitrogenous resins which bind anions (**JANSON and HEDMAN, 1982)**.

3.3.1.2 Polyacrylamide gel electrophoresis

SDS-Polyacrylamide gel electrophoresis is a common technique for analyzing mixtures of proteins. In this technique, proteins are reacted with the anion detergent, sodium dodecylsulfate (SDS or sodium lauryl sulphate), to form negatively charged complexes **(WALKER, 1994)**. The proteins are generally denatured and solubilized by their binding to SDS, and their complexes form a prolate ellipsoid or rod of length roughly proportionate to the protein's molecular weight, thus proteins of either acid or basic isoelectric point (p*I)* forms negatively charged complexes that can be separated on the basis of differences in charges and sizes by electrophoresis through a sieve-like matrix of polyacrylamide gel **(WALKER, 1994)**.

SDS-PAGE is the commonly used gel electrophoretic system for analyzing proteins. However, it should be stressed that this method separates denatured protein. Native PAGE is generally used to analyze non-denatured proteins, chiefly to identify a protein in the gel by its biological activity (for example, enzyme activity, receptor binding and antibody binding on such occasions when it is necessary to use a non-denaturing systems native PAGE) **(WALKER, 1994)**. When purifying an enzyme a single major band would suggest a pure enzyme. However, this band could still be a contaminant; or the enzyme is present as a weaker (even-non-staining) band on the same gel. Enzyme activity is a confirmation that the major band had enzyme activity and also that the band corresponds to the enzyme in question.

The use of stacking gel employs the principles of isotachophoresis, which effectively concentrates samples from large volumes into small zones, that leads to better separation of different species. The system is set up by making a stacking gel on top of the separating gel, which is of a different pH. When an electric field is applied, ions move towards the electrodes. However, due to the pH prevailing in the stacking gel, the proteins-SDS complexes have motilities intermediate between the Cl^- ions (present throughout the system) and glycenate ions

(present in the reservoir buffer). The system of buffers used in the gel system is as described by **LAEMMLI (1971)**.

3.3.1.3 Isoelectric Focusing (IEF)

Isoelectric focusing is an electrophoretic method for the separation of proteins, according to their p*I*, in an stabilized pH gradient **(WALKER, 1994)**. The method involves casting a layer of support media (usually a polycralymide gel but agarose gel can also be used) containing a mixture of carrier ampholytes (low molecular weight synthetic polyamino-polycarboxylic acids) **(WALKER, 1994)**.

When an electric field is applied across such a gel, the carrier ampholytes arrange themselves in order of increasing p*I* from the anode to the cathode. Each carrier ampholyte maintains a local pH corresponding to its p*I* and thus a uniform pH gradient is created across the gel. If a protein sample is applied to the surface of the gel, it will also migrate under the influence of the electric field until it reaches the region of the gradient where the pH corresponds to its isoelectric point. At this pH, the protein will have no net charge and therefore become stationary.

3.3.1.4 The release of oligosaccharides from glycoproteins

The release of oligosaccharides from glycoproteins is perfomed from proteins; firstly, to allow further studies on the core protein and secondly, to elucidate the structure of the oligosacharide moieties present **(DAVIES *et al*., 1994)**.

3.3.2 Methods

3.3.2.1 Ion-exchange chromatography

Using the optimized extraction protocol described in Section 3.2, a large scale extraction was conducted, where samples were filtered with cotton muslin cloth to remove large particles. In this study, cocktails of protease inhibitors (Roche) and phenylmethylsulfonyl fluoride (PMSF) were used to inhibit the protease enzyme. The protease inhibitors were added in the form of

tablets following the manufacture's instruction. After 30 min centrifugation (14, 000x g), the clear supernatant was filtered through a 0.22 µM disc (Millipore, Germany). The filtrate was concentrated by ultra-filtration Viva spin column (Millipore) (50 000 molecular cut-off membrane).

The enzyme fraction was purified using a DEAE column, connected to an AKTA HPLC. Equilibration of the DEAE column was carried out with 5 ml column volumes of binding buffer A (20 mM Tris-HCl, pH 8.0). Absorbance at 280 nm was monitored at a flow rate of 1 ml/min. The filtered and concentrated samples were applied to the DEAE column using a 200 µl sample loop. Elution was undertaken by using a stepwise gradient of buffer B (20 mM Tris HCl and 1.0 M NaCl, pH 8.0). Non-bound protein fractions collected during the washing step as well as fractions eluted by the increased salt concentrations were collected and further analyzed.

3.3.2.2 Gel-filtration chromatography

The concentrated fraction was injected in an AKTA purifier HPLC, Superdex column (10/300 GL, Amersham Biosciences), with the elution buffer. The elution buffer normally used contains 50 mM sodium phosphate, 150 mM NaCl, 0.2 mM Na_2EDTA and 1.0 mM sodium azide (pH 7.2). To avoid any interference of phosphorus, the elution buffer only consisted of 150 mM NaCl (pH 7.2). Absorbance at 280 nm was monitored at a flow rate of 0.5 ml/min.

The molecular mass of the purified enzyme was estimated by calibration with the standard protein, thyroglobin (670 kDa), IgG (150 kDa), Ovalbumin 44 (kDa), Myoglobin (17 kDa) and vitamin B_{12} (13.5 kDa) (Bio-Rad). Fractions with high activity were pooled and concentrated using a Viva tube (Millipore) with a molecular cut-off of 50 000. The eluted fractions were assayed for apase activity using p-NPP as a substrate by assay A. Native M_r was calculated by plotting the K_d (partition co-efficient) against log M_r using the protein standards. The void volume was estimated by dextran blue 2000.

3.3.2.3 Electrophoretic analysis of apase enzyme

In this study, electrophoresis was carried out using Native PAGE and SDS-PAGE which were commercially sourced (Invitrogen) and also manually prepared (Appendix A, Table A1). The Native-PAGE was mainly used to detect the active enzyme. SDS-PAGE electrophoresis was used to assess the protein composition of fractions from purification steps and to estimate the molecular masses of apase and its subunits.

3.3.2.4 Detection of enzyme activity by non-denaturing PAGE

Unless otherwise stated 5-20% polyacrylamide gradient gels with 3% stacking gel were used or 7% gels with 3% stacking gel were used. SDS-PAGE was performed as described by **LAEMMLI (1971)** using the electrophoresis apparatus from Invitrogen and manually-made gels according to Appendix A, Table A1. For preparation of native gels, SDS was omitted.

Non-denaturing (native) one-dimensional PAGE was carried out using 8% NativePAGE™ Novex® polyacrylamide gel (Invitrogen) or made as described in Appendix A, Table A1. To detect apase activity, the gels were equilibrated for 30 min at 37 °C in 100 ml of 100 mM sodium acetate buffer (pH 4.8). One ml of 1.0 M $MgCl_2$, and 100 mg Fast Garnet GBC Salt (Sigma) were added. The gel was immersed in the mixture and 3.0 ml substrate (1.0% ß-naphthyl acid phosphate in 50% (v/v) acetone) was added. Incubation times varied from 5 min to 5 h, at a temperature of 37 °C.

A plot of relative mobility versus log (molecular mass) was constructed with native unstained protein standard (Invitrogen). The standard consisted of IgM Hexamer (1236 kDa), IgM pentamer (1048 kDa), Apoferritin band (1), Apoferritin band 2 (480 kDa), B-phycoerythrin (242 kDa), Lactate Dehydrogenase (146 kDa), Bovine Serum (66), Soybean Trypsin Inhibitor (20). Pre-stained protein standards were Myosin (250 kDa), phosphorylase (148 kDa), BSA (98 kDa), Glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anyhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa), aprotinin (6 kDa) and insulin (4 kDa).

3.3.2.5 Estimation of molecular weight by SDS-PAGE

Denaturing one-dimensional SDS-PAGE was performed using either NuPAGE® Novex® Bis-Tris precast gels (Invitrogen) or manually prepared (7-9%) SDS-PAGE gels which were stained as described by **LAEMMLI (1971)**. For estimation of molecular weight mixtures of pre-stained standards (Invitrogen) were used: Myosin (250); phosphorylase (148); BSA (98); Glutamic dehydrogenase (64); Alcohol dehydrogenase (50); carbonic anhydrase (36); myoglobin red (22); lysozyme (16); aprotinin (6) and insulin B chain (4). The separated protein bands were stained with SimplyBlue™ SafeStain (Invitrogen) or the SilverQuest™ Staining Kit (Invitrogen).

3.3.2.6 Estimation of Isoelectric point

Isoelectric focusing was performed with polyacrylamide gel (30% acrylamide/0.8% Bis-acrylamide), with 3% Ampholytes 3-10 (Bio-Rad) and 2% (w/v) CHAPS. Ten percent ammonium persulfate (APS) (60 µl) and 1% TEMED (6 µl) and the volume was made to 10 ml. The cathode buffer (upper chamber) consisted of 20 mM NaOH (0.4 g in 500 ml). The anode buffer (lower chamber) consisted of 10 mM phosphoric acid (0.7 ml in 1L). The loading buffer IEF (2.5x) consisted of 5% CHAPS and 5% ampholytes. The electrophoresis was conducted at 1000 V for 1 h 30 min, followed by 250 V for 1 h and lastly at 500 V for 30 min. All the experiments were conducted in a cold room (10 °C).

After IEF, the gel was rinsed with distilled water. After the addition of an IEF marker protein standard to confirm the protein p*I,* the pH values were measured by cutting the gel slab into 0.5 cm segments. The slices were incubated for at least 4 h in dH_20 (0.5 ml) and the pH were measured. The rest of the gel was equilibrated in sodium acetate buffer and stained for apase activity as already described (Section 3.3.2.4). Alternatively, the gel was stained with Coomassie stain. The p*I* of acid phosphatase was determined by plotting the standard curve versus p*I* versus distance of standard markers (Bio-Rad) from the anode. The standard proteins (p*I* in parentheses) consisted of the following proteins at a concentration of 3.6 mg/ml: Phycocyanin Blue (3 bands) p*I* 4.45, 4.65,4.75, ß-Lactoglobulin B p*I* 5.1, Bovine carbonic p*I* 6.0, anhydrase, Human carbonic p*I* 6.5, Equine myoglobin (2 bands) p*I* 6.8, 7.0, Human

hemoglobin A p*I* 7.1, Human hemoglobin C p*I* 7.5 , Lentil lectin 3 p*I* 7.8, 8.0, 8.20 and Cytochrome c p*I* 9.6

3.3.2.7 Deglycosylation

N-Glycosidase F

Deglycosylation of acid phosphatase was done using N-Glycosidase F (PNGase F). The glycoprotein of apase was deglycosylated using typical reactions guidelines according to the manufacturer's instructions (New England Biolabs, Beverly, MA). The deglycosylated proteins were analyzed in a 9 % SDS-PAGE as described in Section 3.3.2.3. The gel was stained with silver.

Carbohydrate staining

The detection of the carbohydrate moiety of glycoproteins was performed by the periodic acid-Schiff reagent (PAS). The method is based on the oxidation of hexose vicinal 1,2-diol groups to aldehydes using periodate with subsequent staining by Schiff base **(JAY *et al.*, 1990)**. After electrophoresis, the gel was soaked with 200 ml of fixative (50% methanol) solution for 16 h at 25 °C. The fixative was changed once and the gel was left for 60 min with gentle agitation to remove SDS. The gel was rinsed in three changes of dH_2O for 20 min each. The gel was then replaced with 2% (m/v) periodic acid. After two brief washes with 200 ml dH_2O for 2 min, the gel was immersed in Schiff's Reagent until it turned magenta. After metabisulfite reduction, the gel was rinsed with several changes of dH_2O until the water remained clear, indicating complete removal of unreacted pararosanile.

3.3.2.8 Enzyme thermal stability and determination of optimal pH

The thermostability of acid phosphatase was determined at different temperatures (0, 15, 37, 60, 80 and 100 °C). These studies were carried using pNPP as substrate by assays A described in Section 3.2.2.3

The acid phosphatase activity was determined at different pH's (2.2; 2.5, 3.6, 4.8, 5.6, 6.6 and 7.6) using citric acid and 100 mM glycine-NaOH (8.6, 9.6 and 10.6) using assay A as mentioned in Section 3.2.2.3.

3.3.2.9 Enzyme modulators and inhibitors

Using standard assay A, enzyme modulators and inhibitors were carried-out with various divalent metal cations such as: $MgCl_2$, $MnCl_2$, $CaCl_2$, $ZnCl_2$, $CuNO_3$, $CuSO_4$, and EDTA. Known inhibitors of apase such as sodium fluoride, vanadate, molybdate, and tartrate were tested. The standard assay A was used and enzyme activity in the presence of metal cations or EDTA was expressed relative to the control.

3.3.2.10 Kinetics properties

The K_M and V_{max} values were obtained from Lineweaver-Burk (**LINEWEAVER and BURK, 1934)**) plot using various phosphatase substrates, determined using 0.4 µM to 0.5 mM concentration ranges. Substrates specificity was determined by a standard assay A and for other phosphorylated substrates such as α-naphthyl phosphate, β-glycerophosphate (GLOP), D-glucose-6-phosphate, ADP and AMP, assay B was used. The apases activity was determined at pH 2.5 measured at 37 °C. All kinetics parameters are the means of duplicate determination performed on two separate preparations of the purified enzyme and they are reproducible to within +10% SE.

3.3.3 Results

3.3.3.1 Chromatographic steps

An acid phosphatase from *Cladonia portentosa* was purified from the thallus using a two step procedure involving ultracentrifugation by Viva tubes and by a series of chromatography steps including anion–exchange and gel filtration chromatography. The concentrated fraction was

loaded onto a DEAE column and bound protein was eluted with a linear gradient of 1.0 M NaCl in an anion buffer (20 mM Tris, pH 8.0) at a flow rate of 1 ml/min.

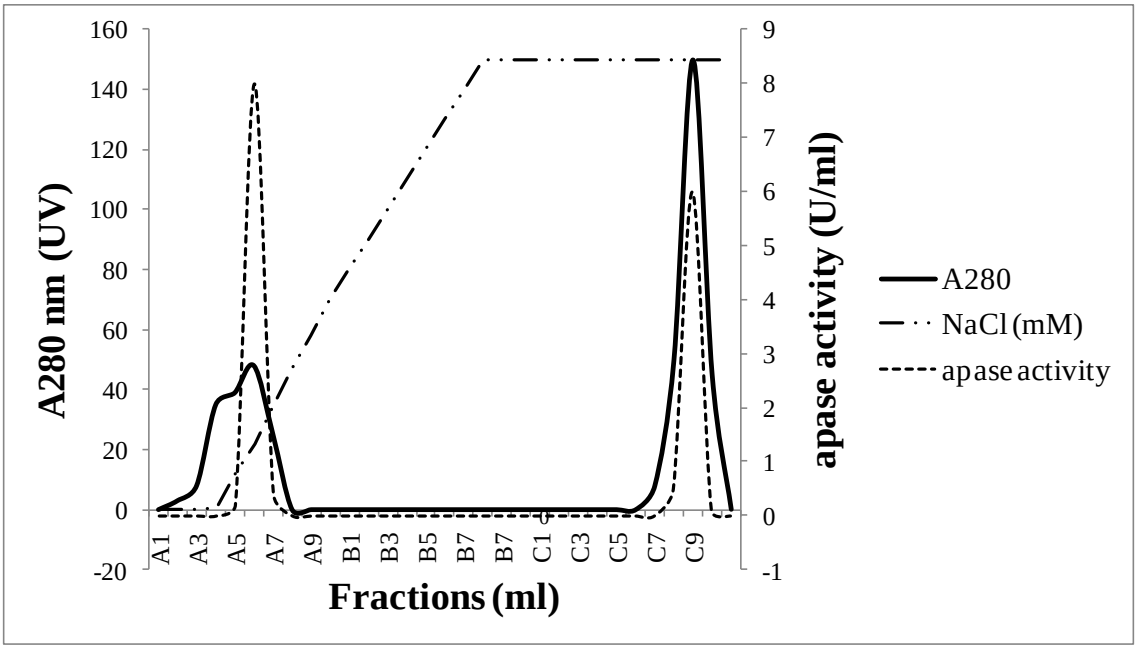

Figure 3.4: Anion-exchange chromatography of lichen *Cladonia portentosa* fraction on DEAE cellulose column (1X5cm). Binding buffer: Tris-HCl (20 mM) (pH 8); elution buffer: 20 mM Tris HCl and 1M NaCl (pH 8), flow rate: 1 ml/min. Bound protein was eluted with a linear gradient of 1 M NaCl. NaCl gradient (green line) was monitored by a conductivity meter. Solid line = protein absorbance monitored at 280 nm. Each fraction was assayed for acid phosphatase activity at 405 nm using pNNP as a substrate.

Two peaks of acid phosphatase were eluted by DEAE column chromatography (Figure 3.4). When proteins were further purified by gel filtration, very little or no protein was retrieved. Only one acid phosphatase peak was detected. All subsequent separations were then carried out using gel filtration only (Figure 3.5). Fractions were collected, pooled and concentrated by Viva tubes.

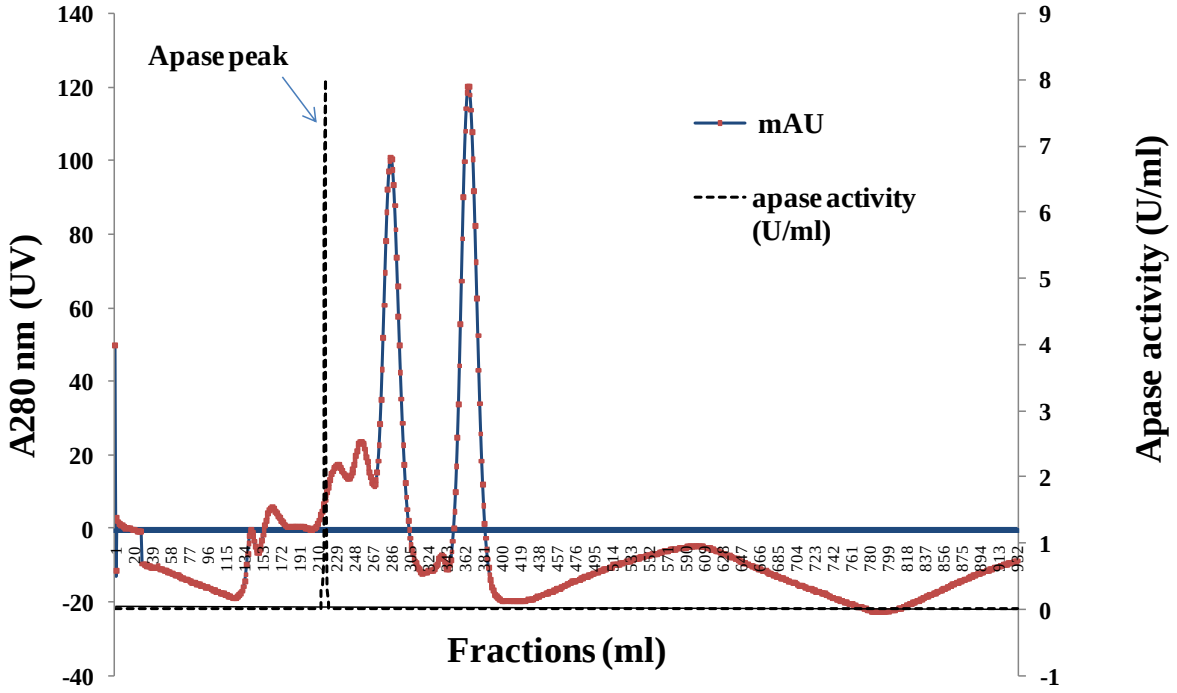

Figure 3.5: Elution profile by size exclusion gel chromatography of fractions of *Cladonia portentosa*. Fractions of *C. portentosa* were eluted by NaCl at a flow rate of 0.5 ml/min. One peak enzyme was obtained from gel chromatography. The proteins were monitored by UV at 280 nm (solid line). Each fraction was assayed for acid phosphatase activity at 405 nm using pNNP as substrate. Enzyme activity was expressed as units/ml.

3.3.3.2 Purification Scheme

A major difficulty encountered in the purification of apase was that of low yield. Preliminary studies confirmed that acid phosphatase activity was not degraded by proteases over a 24 h period and also during the extraction process. Lowest protein yield was obtained when ion exchange chromatography was used. To eliminate further protein loss this purification procedure was ignored. The experimental procedure using Viva tubes and then gel filtration permitted the partial purification of apase from *C. portentosa*. A summary of the purification scheme of acid phosphatase from *C. portentosa* is summarized in Table 3.5. The enzyme was purified 45-fold to a final pNNP hydrolyzing specific activity of 167 units/mg and an overall recovery of 18% (Table 3.5).

Table 3.5: Purification of extracellular apase isolated from the thallus of *Cladonia portentosa*. Acid phosphatase activity was assayed by NanoDrop ND-1000 spectrophotometer with pNNP as a substrate. Activity is expressed as absorbance at 405 nm, which is proportional to the amount of phosphate liberated. Protein content was estimated by BCA reagents (Novagen).

Purification step	**Total protein (mg)**	**Total activity U**	**Specific activity U/mg**	**Purification factor**	**Yield (%)**
Crude Extract	251.39	858	7.5	1	100
Ultrafiltration (Viva spin column)	162.72	638	52.56	12	92
Gel filtration	1.82	96.3	167	45	18

3.3.3.3 Electrophoretic analysis of apase enzyme

In order to assess the degree of purity of the enzyme preparations following the purification procedure summarized in Table 3.6, fractions of recovered proteins at various steps were subjected to electrophoresis under denaturing conditions. Results obtained are presented in Figure 3.6. Several proteins were observed before purification (Figure 3.6A). Silver staining revealed one band (Figure 3.6B, lane 2) and several bands above Mr 50 000 were observed after concentration by Viva spin (Mr cut-off 50 000) (Figure 3.6, lane 4-6).

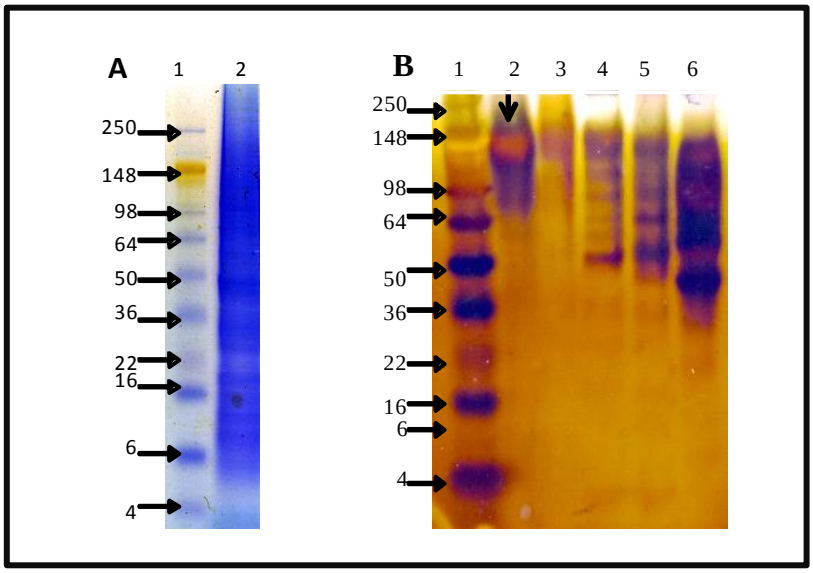

Figure 3.6: (A-B) Verification of purification steps by electrophoretic techniques. (A) Protein extraction before concentrating by Viva spin column, visualized on SDS-PAGE and stained with Coomasie stain. Lane 1 represent protein markers and lane 2 represents an unpurified concentrated fraction. (B) Silver staining of concentrated and purified fractions. Lane 1: proteins markers, lane 2: purified fraction after concentration by Viva tube. Lane 3: purified fraction before concentration by Viva tube. Lanes 4-5: different preparations of proteins prepared by Viva spin column with maximum cut-off of 50 000. Lane 6: proteins prepared concentrated by Viva spin column with maximum cut-off of 30 000.

Acid phosphatase activity was visualized by staining the native PAGE gel with Fast Garnet dye, using α-naphthyl phosphate (1.0 mM) as the substrate in 100 mM sodium acetate (pH 4.8) (Figure 3.7A-C). However, the absence of SDS in the gel, resulted in big staining smears of undistinguishable sizes (Figure 3.7B). To reduce the smearing on the gels, the enzyme activity was detected in the presence of SDS but the enzyme was not denatured. Development of reddish bands, on both purified enzyme (Figure 3.7C, lane 4) and wheat acid phosphatase (lane 8) which was used as a positive control, confirmed the presence of acid phosphatase. When the enzymes were denatured by heating for 10 min at 100°C, no activity was detected (Figure 3.7C, lanes 3 and 7).

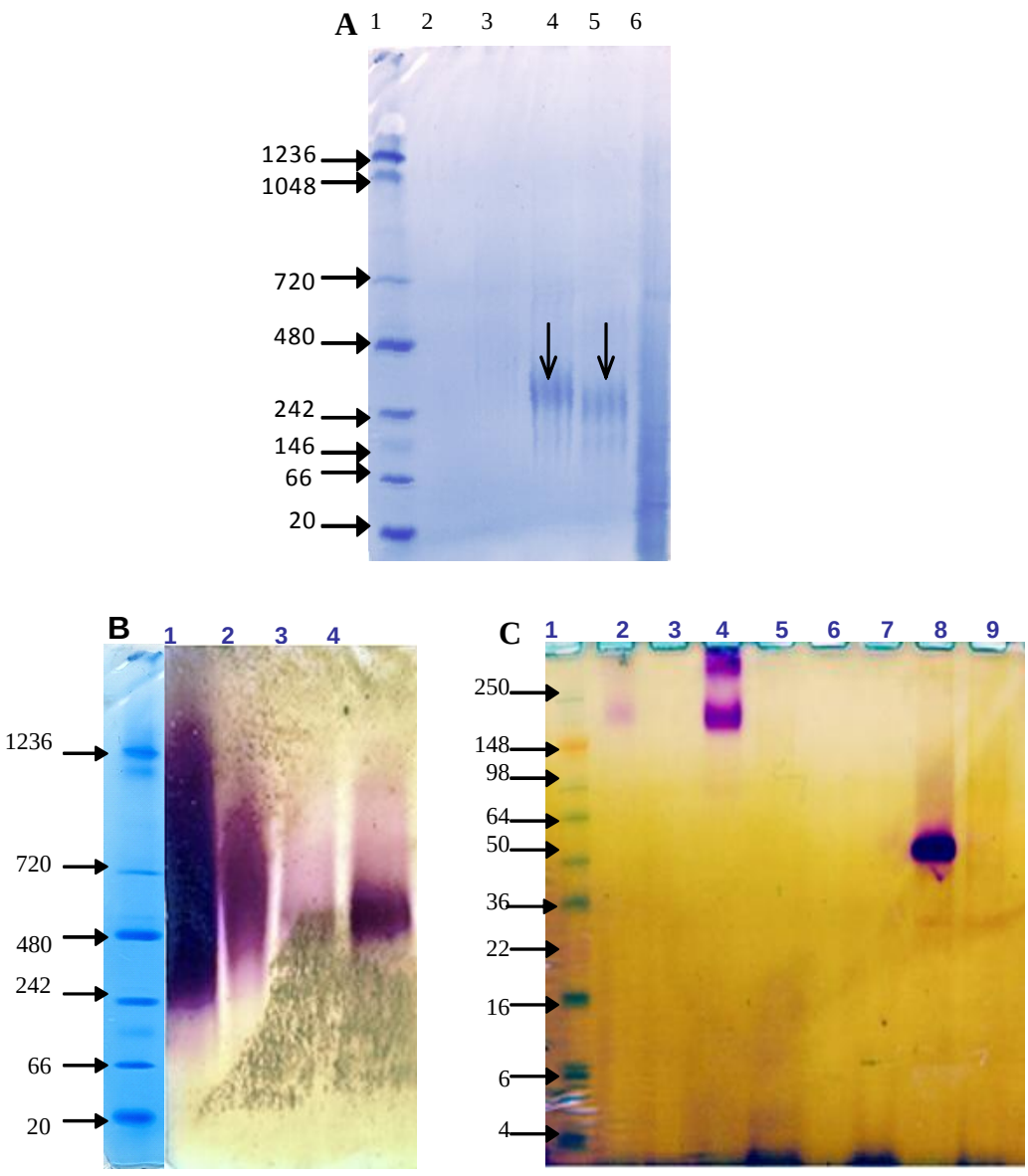

Figure 3.7: (A-C) Detection of enzyme activity using Native-PAGE gel. (A) Final acid phosphatase preparation from *C. portentosa* on the native-PAGE, stained with Coomassie blue. (B) Activity stain on the native PAGE gel stained with Fast Garnet dye, using α-naphthyl phosphate (1mM) as the substrate in sodium acetate buffer (100 mM, pH 4.8). (C) To reduce the smearing on the gels, the enzyme activity was detected in the presence of SDS but the enzyme was not denatured. Development of reddish bands, on both purified enzyme (lane 4) and wheat acid phosphatase (lane 8), which was used as a positive control, confirmed the presence of acid phosphatase. When the enzymes were denatured by heating for 10 min at 100 °C, no activity was detected (lanes 3 and 7).

Denaturing SDS-PAGE was used to estimate the molecular size of the protein. Electrophoresis of acid phosphatase on 7% PAGE gel (Fig 3.8A) indicated that the protein had mobility equivalent to a molecular weight of approximately 148 kDa. In the presence of reducing agents (mercaptoethanol or DTT), two bands of approximately 116. 25 and 37 kDa (respectively) were visualized by activity staining (Figure 3.8B, lanes 2 and 5).

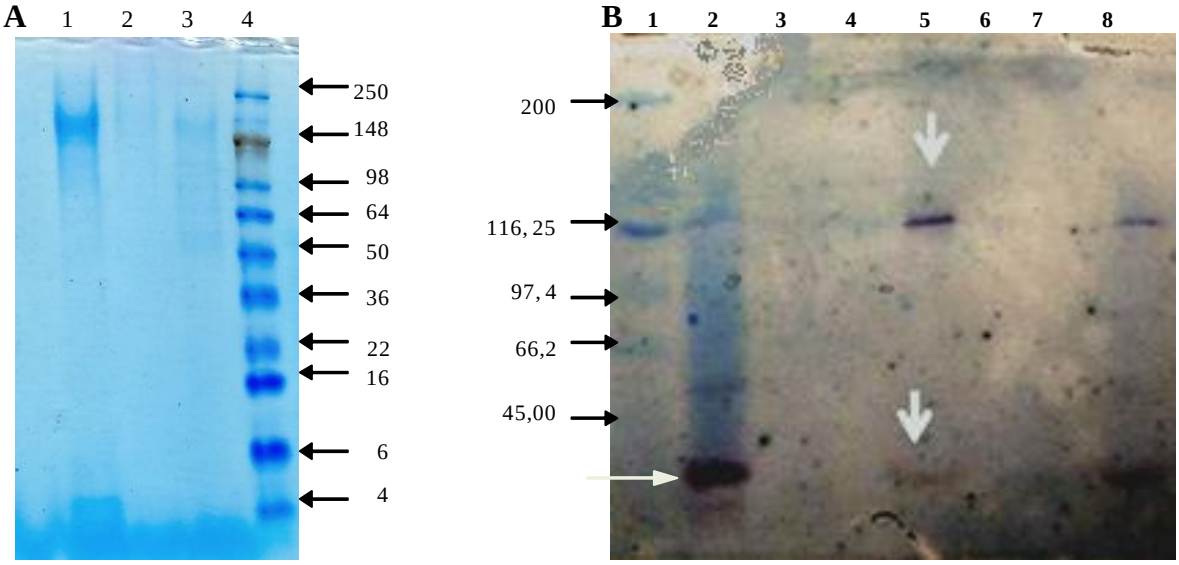

Figure 3.8: (A-B) Detection of enzyme subunit by SDS-PAGE. (A) Denatured SDS-PAGE gel stained with Coomassie blue revealed one band. (B) SDS-PAGE in the presence of reducing agent-mercaptoethanol revealed two bands. The activity was detected with Fast Garnet dye, using α-naphthyl phosphate (1.0 mM) as the substrate in 100 mM sodium acetate buffer, pH 4.8. Reddish bands confirmed the presence of acid phosphatase as they can be seen in both unpurified protein (lane 2) and purified samples lanes 5 and 8. The activity is indicated by the arrow.

3.3.3.4 Determining the pI of secreted apase

The isoelectric point (p*I*) of apase was estimated by native-PAGE. The p*I* of purified protein was estimated to be 6.4 under given experimental conditions (Figure 3.9). The activity was detected by enzyme activity using a fluoregenic substrate as explained section 3.3.2.4.

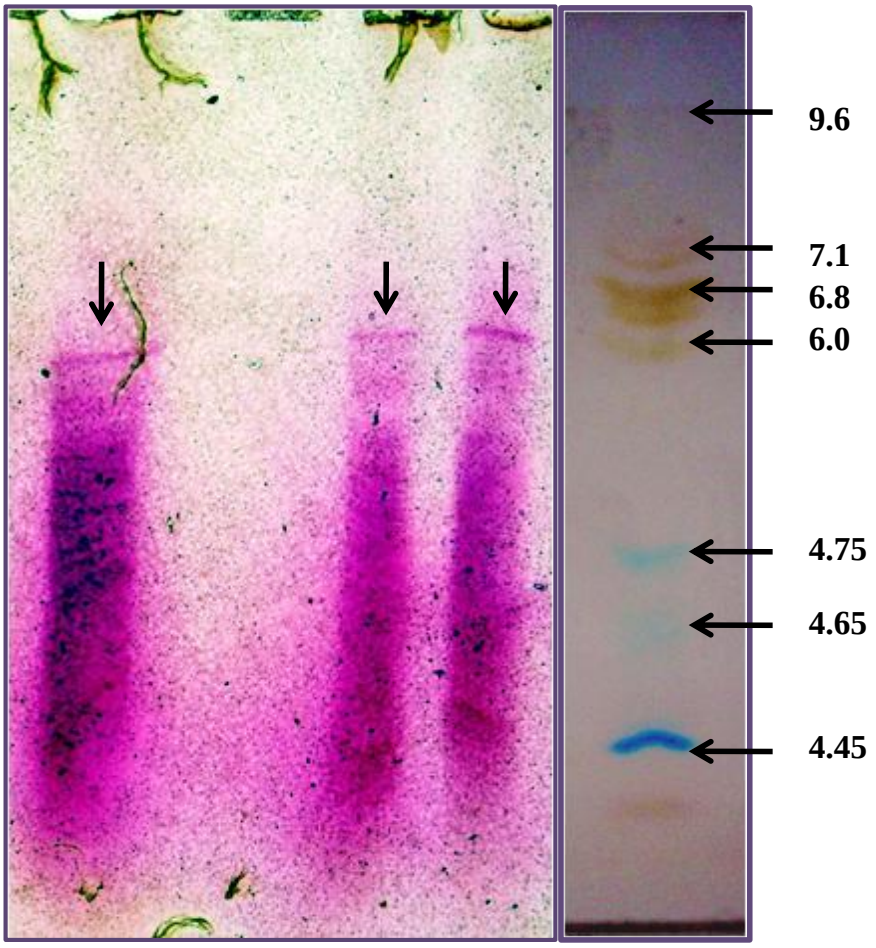

Figure 3.9: The isoelectric point (p*I*) of apase estimated by native-PAGE. The activity was detected with Fast Garnet dye, using α-naphthyl phosphate (1.0 mM) as the substrate in 100 mM sodium acetate buffer, pH 4.8. The p*I* of acid phosphatase was determined by plotting the standard curve versus p*I* versus distance of standard markers (Bio-Rad) from the anode. The standard proteins consisted of the following proteins at a concentration of 3.6 mg/ml: Phycocyanin Blue (3 bands) p*I* 4.45, 4.65,4.75, ß-Lactoglobulin B p*I* 5.1, Bovine carbonic p*I* 6.0, anhydrase, Human carbonic p*I* 6.5, Equine myoglobin (2 bands) p*I* 6.8, 7.0, Human hemoglobin A p*I* 7.1, Human hemoglobin C p*I* 7.5 , Lentil lectin 3 p*I* 7.8, 8.0, 8.20 and Cytochrome c p*I* 9.6

3.3.3.5 Effect of pH and temperature on apase activity

The enzyme showed a fairly narrow pH activity profile with a maximum occurring at 2.5 when pNNP was hydrolyzed (Figure 3.10). The purified enzyme hydrolyzed pNNP optimally between pH 2.5 and 4.8 (Figure 3.10). No evidence of alkaline phosphatase was detected. Activity decreased dramatically above pH 5.0.

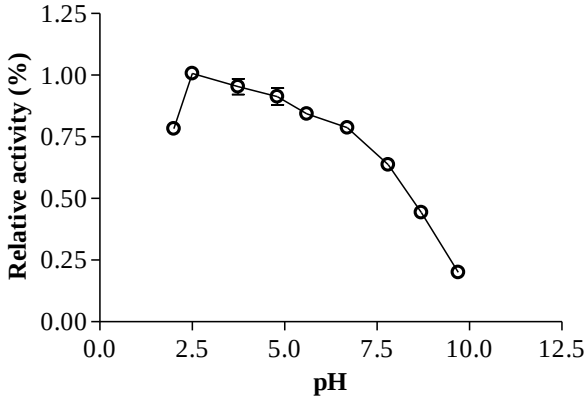

Figure 3.10: The effect of pH on the activity of acid phosphatase from *Cladonia portentosa*. The hydrolysis of pNNP was determined over a multiple pH range (1.8 to 11), expressed as relative percentage (U/ml). The temperature was maintained constant at 37 °C. Values are the means of triplicate samples (n=3).

The activity of the isolated enzyme was measured over a range of temperatures (0, 15, 37, 60 and 100 °C). The thermostability of acid phosphatase was tested by incubation for 20 min intervals at 37 °C, using the standard activity assay (Section 3.2.2.3). The results indicate that acid phosphatase is optimal at 65 °C. The activity was reduced above 65 °C (Figure 3.11).

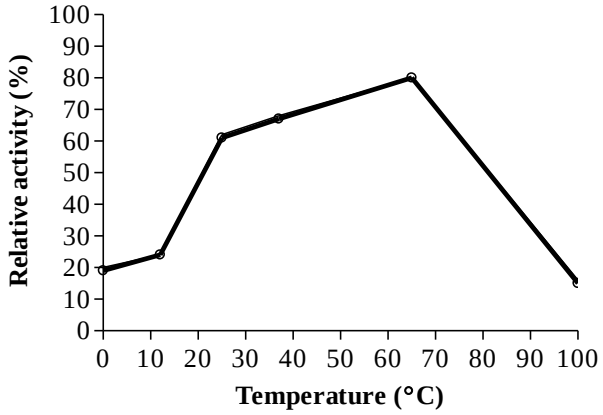

Figure 3.11: The effect of temperature on the activity of acid phosphatase from *Cladonia portentosa*. The activity was monitored with 500 µM pNNP as a substrate, expressed as relative percentage (U/ml) Values are the means of triplicate samples for each temperature tested (n=3).

3.3.3.6 Effect of bivalent metal cations

Acid phosphatase inhibitors were assayed for their effect on the enzyme activity measured by the hydrolysis of pNNP. The behaviour of the enzyme in the presence of inhibitors is summarized in Figure 3.12. Sodium fluoride, sodium molybdate and sodium vanadate were the strongest inhibitors, producing 71, 65 and 62 % (respectively) activity reduction at a concentration of 2 mM. Sodium tartrate reduced the activity by 59 % at 2 mM concentration (Figure 3.12).

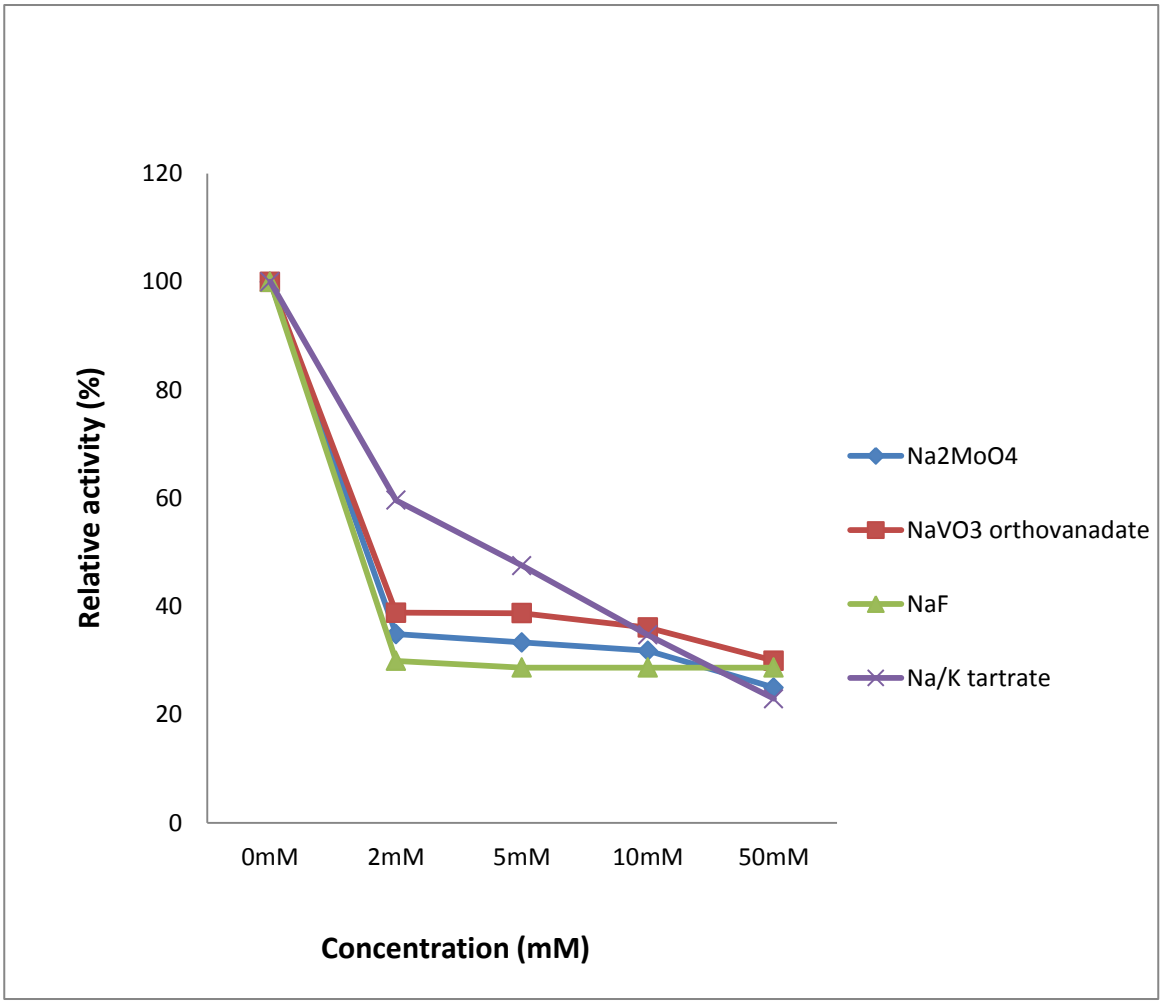

Figure 3.12: Effect of inhibitors on acid phosphatase. pNNP was used as substrate and absorption measured 37 °C at 405 nm, expressed as relative percentage (U/ml).

Different compounds were assayed for their effect on the activity of acid phosphatase using pNNP as a substrate at 37 °C (405 nm). *C. portentosa* was not activated in the presence of 2.0

mM $MgCl_2$ or $CaCl_2$. The presence of 2.0 mM EDTA did not affect the activity of acid phosphatase (Table 3.6). Other compounds had an inhibitory effect above a concentration of 2.0 mM (Table 3.6). *C. portentosa* apase was inhibited by 2.0 mM $CuSO_4$, 2.0 mM $ZnCl_2$, which caused 67% and 89% inhibition of apase activity respectively.

Table 3.6: The effect of various compounds on acid phosphatase activity of *Cladonia portentosa*.

Treatment	Concentration (mM)	Relative activity (%) (U/ml)
Control	0	100
$CaCl_2$	2	91
$CuSO_4$	2	34
$Cu(NO_3)_2$	2	75
EDTA	2	100
$MgCl_2$	2	82
$MnCl_2$	2	85
$ZnCl_2$	2	94
$PbNO_3$	2	87

3.3.3.7 The release of oligosaccharides from glycoproteins

The possible glycoprotein nature of acid phosphatase was investigated by treating the purified enzyme with N-Glycosidase F (PNGase F, which is 36,000 Da). After this treatment, the proteins were electrophoresed on SDS-PAGE. SDS-PAGE of the PNGase F-treated acid phosphatase showed a decrease in size compared to untreated sample (Figure 3.13, lanes 4 vs. 5). The mobility of the PNGase F treated sample was observed to have changed corresponding to 70 kDa. This constitutes a decrease in size for the acid phosphatase band. No enzyme activity was detected afterwards.

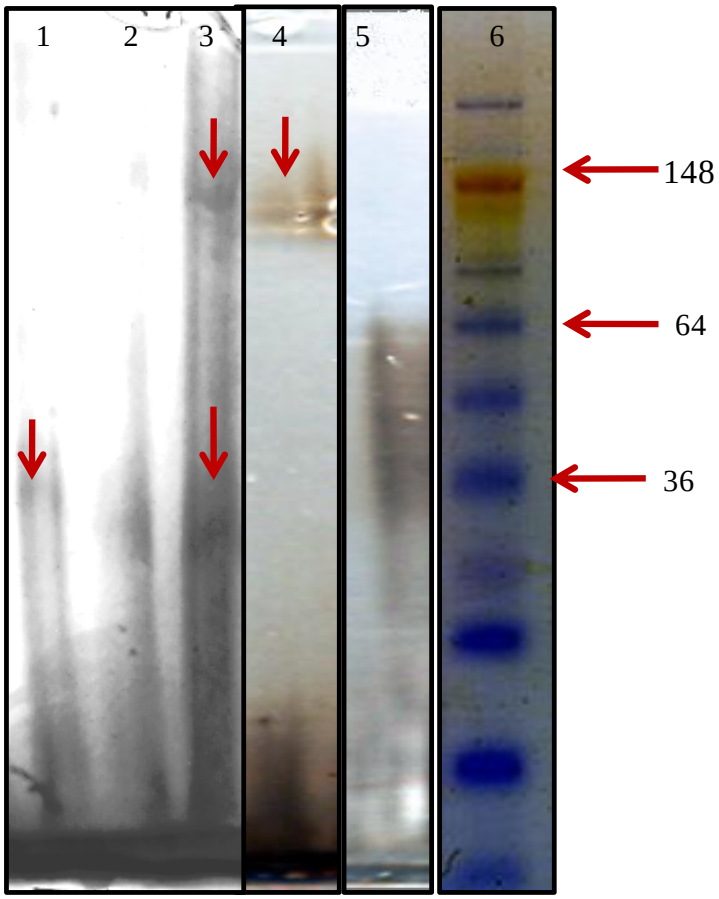

Figure 3.13: Deglycosylation of apase, using N-Glycosidase F (PNGase F) on SDS-PAGE and visualized by silver staining. Lane 1: PNGase F, 2: PNGase F, 3: purified apase and PNGase F, 4: purified apase, 5: deglycosylated apase. Note the size of the apase band before deglycosylation (lane 4). Deglycosylation resulted in band size estimated to be 70 kDa.

3.3.3.8 Kinetic parameters

Substrates specificity was tested with various phosphorylated substrates under standard conditions (assay A and B), using a concentration of 2.5 mM for each substrate (Table 3.7). The acid phosphatase from *Cladonia portentosa* hydrolyzes different phosphate esters, with pNPP, α-naphthyl phosphate and ß-glycerophosphate being amongst the best substrates for this enzyme (Table 3.7). Apase from *Cladonia portentosa* show a strong affinity towards phytic acid, with K_M of 31.2±0.25 µM (Figure 3.14). The purified enzyme was also competitively inhibited with inorganic phosphate with a K_M of 31 µM (Figure 3.14).

Table 3.7: Substrate specificity relative to pNNP of acid phosphatase from *Cladonia portentosa*.

Substrate (2.5 mM)	**Relative rate of hydrolysis (%)**
Para-nitrophenyl phosphate (pNNP)	100
4- nitrophenyl phosphate (4NNP	100
α-naphthyl phosphate	100
ß-glycerophosphate (ß-GLOP)	100
Phospho (enol) pyruvate trisodium	87
D-glucose-6-phosphate	85
Inosine-5-diphosphate (I5DP)	76
ATP	74

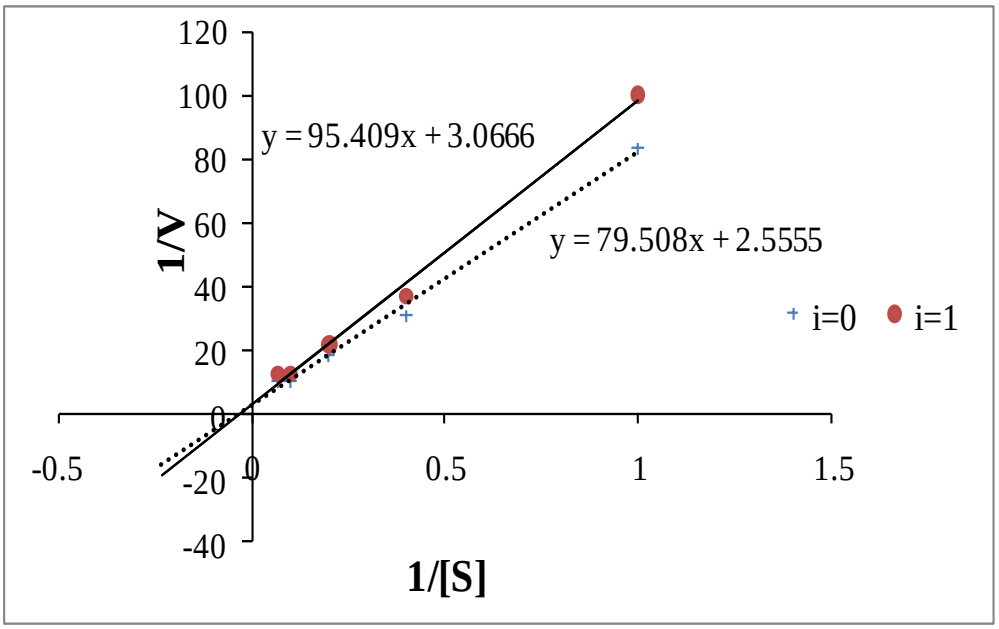

Figure 3.14: (A-B) Double reciprocal plot for determining the V_{max} and K_M values of acid phosphatase against phytic acid at 37 °C for 60 min. To determine the effect of inorganic phosphate, 1.0 mM was used as inhibitor [i].

3.4 PROTEIN SEQUENCING BY MALDI-TOF MS/MS

3.4.1 Introduction

Protein sequencing provides information about the amino acids that make up a protein. Matrix-assisted laser derption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has become a powerful and widespread analytical tool in all fields of the life sciences. It is particulary suitable for the analysis of all kinds of biomolecules including proteins, nucleic acids and carbohydrates associated with proteins **(BONK and HUMENY, 2001).**

3.4.2 Procedure

One-dimensional SDS-PAGE was performed using a 9% running and 4% stacking gel **(LAEMMLI, 1971)**. After electrophoresis, enzyme bands were visualized by SimplyBlue™ Safe Stain staining (Invitrogen). The bands of approximately148 kDa were excised from the gel slab and sequenced at Nottingham Medical School, using peptide mass fingerprinting. Peptides were generated by trypsin and purified by C18 reverse-phase high performance chromatography and analyzed. The molecular mass/charge ratio (m/z) and MS-MS fragmentation patterns were determined for individual peptides using the Mascot (Matrix Science, London, UK) program.

Protein identification was performed by searching extracted peak lists generated with one defined set of filter parameters against the NCBI database using the Mascot (Matrix Science, London, UK), Expasy and UniProt Knowledgebase (Swiss-Prot + TrEMBL) search engines. A comparison matrix was conducted by Blosum or PAM30. Sequence similarities were established using FASTS, MS Blast, and expasy search engines to include proteins found in UniProtKB/TrEMBL **(SHEVCHENKO *et al*., 2001; MACKEY *et al*., 2002)**. The matching sequences were aligned using ClustalW, and later re-arrange by alignX (Vector NTI, Invitrogen). To verify that the protein was secreted, SignalP was used to predict the result (**BENDTSEN *et al*., 2004)**. Phylogenetic relationships of *Cladonia portentosa* apase with other apases were analyzed by multiple sequence alignment using the ClustalW program **(THOMPSON *et al*., 1994; 1997)**. The bootstrap value was calculated and a phylogenetic tree was built using the Vector NTI program (Invitrogen).

3.4.3 Results

The amino acid composition and identification of the purified enzyme was achieved by mass spectrometry (MALDI-TOF). Two main problems were encountered during sequencing: - low protein yield and lack of homogeneity in the enzyme sample. The purified enzyme gave a single band on SDS-PAGE gel (Figures 3.6B and 3.8A), but sequencing data revealed multiple proteins that were co-eluted with acid phosphatase (Table 3.8). Interesting peptides ranging from stress response proteins, hydrolases (carboxylases and proteinases) with most peptide sequences showing a strong homology to fungal enzymes. However, all data not related to the enzyme of interest, apase were not further analyzed. Peptides that showed a close match to acid phosphatase were selected and analyzed in details (Figure 3.15). The alignments, conducted by ClustalW and re-arranged by Vector NTI clearly demonstrated that apase from *Cladonia portentosa* is homologous to many fungal apases (Figure 3.16).

One of the peptides generated was MLAETNPA (peptide 9, Table 3.8). The fingerprint of this peptide fragmentation data at that region of the raw data was poor. The peptide was then assigned to the Swiss-Prot, MSDB and NCBInr protein sequence database. The hit came with histidine acid phosphatase with homology (69 %) to *A. terre*us accession number (Q0CVC9 (Figure 3.16A). The E score of 4 was obtained for this peptide and the match was not significant.

Table 3.8: Amino acid sequences from the purified acid phosphatase from *Cladonia portentosa* (not shown=#, shown*). Amino acid sequences from the purified acid phosphatase from *Cladonia portentosa* (not shown=#, shown*). Data analysis is based from SDS-gel excised from 3 bands corresponding to 55, 60 and 148 kDa.

Peptide no.	**Amino acid**	**Protein**	**Estimated size**	**Accession number**	**Match**
1	EVAQLLSQSLATEAR	Stress response protein Rds1	148	Q5B2D8	*Aspergillus clavatus*
2	DGLTGLQSTPR	Putative uncharacterized protein	148	Q5B7K5	*Aspergillus nidulans*
3	TLPLGDSFP	Unknown	-	-	-
4	VLPQVLEATNR	Carboxypeptidase 5	148	Q5VJG7	*Sartorya fumigata*
5	LLNNANLNR	Unknown	-	-	-
6	VYGPLLEMFR	Unknown	-	-	-
7	GAELLVPSPK	Putative uncharacterized protein	-	-	-
8	LAWQNFPTL	Putative uncharacterized protein		Q5B2D8	*Emericella nidulans*
9	MLAETNPA*	Phytase	148		*Apsergillus terreus*
10	FLAETNPAPFGHP*	Histidine acid phophatase/phytase	148	Q0CZR1	*Aspergillus terreus*
11	AVGLGYVEELLAR*	Phytase 3	148		*Aspergillus terreus*
12	LVDGEEYGEVR	Putative uncharacterized protein	148	Q0U704	*Phaeosphaeria nodorum*
13	SNELFTANLGSAR	Aspartic proteinases	55		
14	GSNPFDLLGDVFLK	Aspartic proteinases /aspartic endopeptidase	55	Q9HF87	*Botryotinia fuckeliana*
15	AQGLGYVQEVLAR#	Acid phosphatase	148		*Aspergillus terreus*
16	FGESGVWGFLNHLDSR	Stress response gene			*Aspergillus nidulans*
17	DALPVELD	Unknown			
18	SLYALFDQGNTR	Aspartic proteinases	55kd		
19	TFTQLAPMEAPR	Unknown	60		
20	LTSTTHTVNTDQR	Galactose oxidases	60	Q4WH00	*Aspergillus fumagatus*

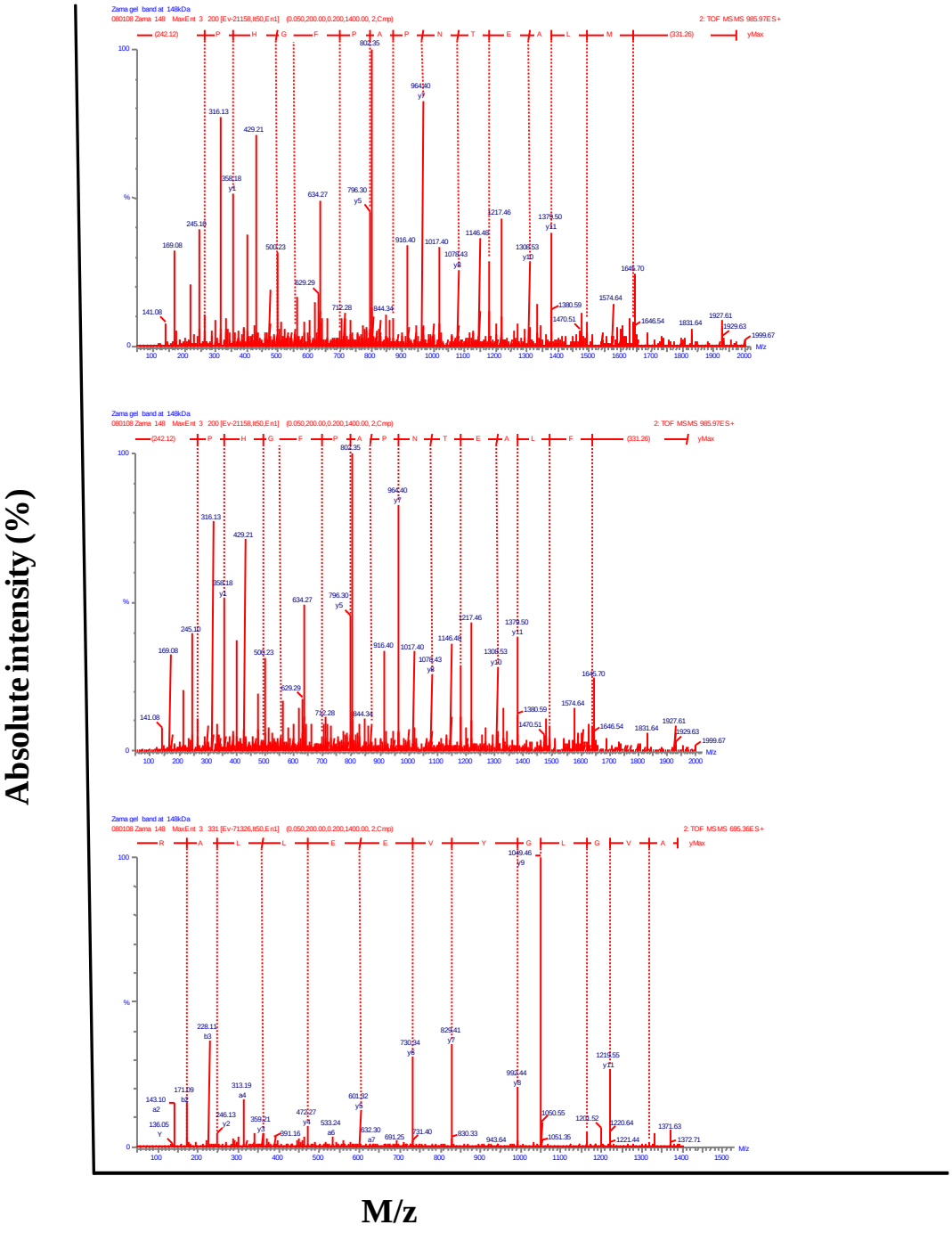

Figure 3.15: (A-C) MALDI-TOF peptide mass fingerprinting of acid phosphatase from *Cladonia portentosa*. A sample of the enzyme was resolved by SDS-PAGE and then subjected to in-gel digestion by modified trypsin. MALDI-TOF mass spectra were acquired in positive ionisation mode. The MASCOT database search retrieved peptide sequences: FLAETNPAPFGHG* and AVGLGYVEELLAR**

A) Peptide 9

Peptide 9	***Cladonia portentosa***	**mLAETNPA**	**8**
Q0CVC9	*Aspergillus terreus*	mLAETNPA	565
Q0CZR1	*Aspergillus terreus*	FLAqTNPA	540
A2R685	*Aspergillus niger*	FLAqTNPA	547

B) Peptide 10

Q0CVC9	*Aspergillus terreus*	mLAETNPAPFGq	565
Peptide10	***Cladonia portentosa***	**FLAETNPAPFGh**	**12**
Q0CZR1	*Aspergillus terreus*	FLAqTNPAPFGN	540
A2R685	*Aspergillus niger*	FLAqTNPAPFGN	547

C) Peptide 11

B2W4A5-1	*Pyrenophora tritici-repentis*	AiGIGYVeEvLAR	557
Q0U9E0	*Phaeosphaeria sodorum*	AiGVGYVqEvLAR	509
B2WJV4	*Pyrenophora tritici-repentis*	tQGVGYVNELLAR	473
Q0V0X4-1	*Phaeosphaeria sodorum*	tQGVGYVNELLAR	1001
Q5GGT6-1	*Neourospora crassa*	tQGVGYVNELLAR	596
Q7S9V5	*Neourospora crassa*	tQGVGYVNELLAR	493
B0DV43-1	*Laccaria bicolour*	vQGVGYVNELLAR	504
Q96VH9-1	*Peniophora lycii*	vQGVGYVNELLAR	473
Peptide11	***Cladonia portentosa***	**AvGlGYVeELLAR**	**13**
Q0CZR1	*Aspergillus terreus*	AvGVGYVeELvAR	540
A6SFM5-1	*Botryotina fuckeliana*	AvGIGYVqEvvAR	469
B8PES5	*Postia placenta*	AQGIGYVqELvAR	546
A6RPE1	*Botryotina fuckeliana*	AQGlGYVqELLAR	507
A7EBV4	*Sclerotinia sclerotiorum*	AQGIGYVqELLAR	527

D) Peptide 15

Q7S9V5	*Neurospora crassa*	tQGVGYVNELLAR	596
Q5GGT6	*Neurospora crassa*	tQGVGYVNELLAR	497
B2WJV4	*Pyrenophora tritici*	tQGVGYVNELLAR	473
Q96VH9	*Peniophora lycii*	vQGVGYVNELLAR	439
B0DV43	*Laccaria bicolor*	vQGVGYVNELLAR	504
B6GVW5	*Penicillium chrysogenum*	AQGIGYVeEvaAR	508
B2WJV4	*Pyrenophora tritici*	AiGIGYVeEvLAR	557
A6SFM5	*Botryotinia fuckeliana*	AvGIGYVqEvvAR	468
Q0U9E0	*Phaeosphaeria nodorum*	AiGVGYVqEvLAR	509
B8PES5	*Postia placenta*	AQGIGYVqELvAR	546
A7EBV4	*Whetzelinia sclerotiorum*	AQGIGYVqELLAR	527
Peptide15	***Cladonia portentosa***	**AQGlGYVqEvLAR**	**13**
A6RPE1	*Botryotinia fuckeliana*	AQGlGYVqELLAR	506

Figure 3.16: (A-D) Multiple alignment of peptides obtained from sequencing of acid phosphatase from *C. portentosa*. The peptides (underlined) were searched using the expasy search engine to include proteins found in the UniProtKB/TrEMBL. Pam30 was used as comparing matrix in combination with gap alignment. The matching sequences were aligned using alignX and later rearranged using Vector NTI (Invitrogen). The position of the amino acid within each database entry is shown. The alignment of amino acid sequences of acid phosphatase from various sources is indicated. The accession number (Acc no.) and name of the species from which the acid phosphatase gene was cloned or purified is shown. The length indicates the total size of the protein (in amino acids- as stated in the database entry). The different colours indicate area of homology. Identical amino acids are shaded in blue, similar amino acids are in gray and no colour=no homology.

To get a 'convincing result,' the sequencing was repeated and a longer peptide (10) (Figure 3.16B) FLAETNPAPFGHP was obtained (Figure 3.16). Interestingly, this peptide was similar to peptide 9. However, in the first peptide, the initial M/F alternate calls were considered valid only if methionine was oxidised. Methionine oxide and phe (F) are the same mass but a 'normal' methionine would not fit the data, however, the oxidation of methionine is quite common. What made this more difficult was that both peptides can act homologous to *Aspergillus terreus* and both these sequences FLAETNPAPFGHP and MLAETNPAP belong to histidine acid phosphatase, which codes for two different proteins in the same organism (accession number QOCZR1 and QOCV9, respectively) (Table 3.9). The E score of 0.76 suggests that peptide 10 was homologous to histidine acid phosphatase from *A. terreus* (75%), followed by phytase from *A. niger* (74%) (Table 3.9).

Table 3.9: Identified FLAETNPAPFGHG peptide 10 from MALDI-TOF MS/MS analysis were searched against the NCBI database using expasy website. The peptide was queried by blastp in the UniProt Knowledgebase (Swiss-Prot + TrEMBL). Comparison matrix was conducted by PAM30 using a gap alignment.

Accession number	Organism	Gene name	Score E-value	%
Q0CZR1	*Aspergillus terreus*	Histidine acid phosphatase	0.76	75
A2R685	*Aspergillus niger*	Phytase A	0.76	73
Q0CVC9	*Aspergillus terreus*	Acid phosphatase	1.0	74

A strong signal yielding a peptide sequence of AVGLGYVEELLAR (peptide 11) was generated (Figure 3.16C). The peptide identity was searched using expasy database, and blasted with known proteins in the NCBI data base. Comparison with the sequence data base using a gap alignment analysis indicated that the hypothetical protein had the highest homology (75 % identity) to a histidine acid phosphatase protein (GenBank accession number Q0CZR1) from *A. terreus* (Table 3.10). This protein also shares 73 % homology to *Botryotinia fuckeliana* apase protein (GenBank accession number A6RPE1) and shares 71 % identity to *Pyrenophora tritici-repentis* phytase gene (GenBank accession number B2WEA5). The E value score indicates that this peptide is closest to *A. terreus* (Table 3.10). A significant E value score of 0.06 was obtained.

Table 3.10: Identified AVGLGYVEELLAR peptide from MALDI-TOF MS/MS analysis was searched against the NCBI database using the expasy website: The peptide was queried by blastp in the UniProt Knowledgebase (Swiss-Prot + TrEMBL). Comparison matrix was conducted by PAM30 using a gap alignment. Histidine acid phosphatase =HAP.

Accession number	Organism	Gene name	Score E-value	%
Q0CZR1	*Aspergillus terreus*	HAP	0.06	75
A6RPE1	*Botryotinia fuckeliana*	Acid phosphatase	0.11	73
B2W4A5	*Pyrenophora tritici*	Phytase A	0.19	71
A7EBV4	*Sclerotinia sclerotorum*	HAP	1.1	65
Q0U9E0	*Phaeosphaeria nodorum*	Acid phosphatase	2.0	63
Q0U9E0	*Postia placenta*	Phytase A	3.7	61
Q96VH9	*Peniophora lycii*	Phytase A	3.7	61
Q7S9V5	*Neurospora crassa*	Phytase A	3.7	61
Q5GGT6	*Neurospora crassa*	Phytase B	3.7	61
B2WJV4	*Pyrenophora tritici- repentis*	3-phytase B	3.7	61
B0DV43	*Laccaria bicolor*	Acid phosphatase	43.7	60

There was a significant homology between the peptide 15 ‘AQGLGYVQEVLAR’ of acid phosphatase and histidine acid phosphatase protein from *Botryotinia fuckeliana* (GenBank accession number A6RPE1 and *Sclerotinia sclerotiorum* (A7EBV4) (Figure 3.16D). The E value score was 0.003, indicating the most significant hit (Table 3.11). This protein also shares 77 % identity to *Sclerotinia sclerotiorum* apase protein (GenBank accession number A7EBV4) and shares 71 % identity *to Phaeosphaeria nodorum* gene (GenBank accession number Q0U9E0). Other proteins were homologous to these peptides but the E scores were above 1, thus, were considered not significant (Table 3.11).

Interestingly, this peptide under expasy matched *Botryotinia fuckelinia*. In BLAST output, this peptide was translated as a hypothetical protein called a phytase, with the closest match *Sclerotinia.* This peptide suggests that the whole protein is also probably an acid phosphatase, but does not seem to be in related with peptide 10 and 11 but probably related to the second apase which was present in low quantity in some samples, which was only detected under ion-exchange chromatography.

Table 3.11: The AQGLGYVQEVLAR peptide was searched using the expasy search engine to include proteins found in the UniProtKB/TrEMBL. Comparison matrix was conducted by PAM30 using a gap alignment.

Accession number	Organism	Gene name	Score	E-value	%
A6RPE1	*Botryotinia fuckeliana*	HAP	0.003		85
A7EBV4	*Sclerotinia sclerotiorum*	Acid phosphatase	0.033		77
Q0U9E0	*Phaeosphaeria nodorum*	Acid phosphatase	0.19		71
B8PES5	*Postia placenta*	Phytase A	0.47		68
B6GVW5	*Penicillium chrysogenum*	Phytase	0.85		66
B2W4A5	*Pyrenophora tritici*	Phytase A	1.1		65
A6SFM5	*Botryotinia fuckeliana*	Histidine acid phosphatase	2.0		63
Q96VH9	*Peniophora lycii*	Phytase A	6.6		59
Q7S9V5	*Neurospora crassa*	Phytase A	6.6		59
Q5GGT6	*Neurospora crassa*	Phytase B	6.6		59
B2WJV4	*Pyrenophora tritici-repentis*	3-Phytase B	6.6		59
B0DV43	*Laccaria bicolor*	Acid phosphatase	6.6		59

3.5 GENERAL DISCUSSION

3.5.1 Protein solubilization

Cell wall fractionation of fungi is particularly difficult due to the thick cell wall composed of chitin, glucans and other polymers. The cell walls of most fungi consist of five major components (1→3)-*β*-glucan, (1-6) *β*-glucan, (1-3) *α*-glucan, chitin and glycoproteins. Two methods are often described in the literature for fungal cell wall fractionation. The first method is based on the formation of protoplasts by enzymatic digestion of the cell wall and collecting the supernatant as the cell wall. The second method consists of physical cell disruption (e.g. sonication) and recovering cell wall fraction as a pellet after high speed centrifugation. Both methods are labour intensive, and cytoplasmic contamination is unavoidable **(MERCHANTE *et al*., 1995)**.

High levels of apase were detected using hydrolase enzyme especially Novozyme (Figure 3.2). Overall results demonstrated that apase in *C. portentosa* is localized in the cell wall or/and cell membrane. Although this technique was successful, it was found to be labour intensive and cytoplasmic contamination could not be ruled out **(MERCHANTE *et al*., 1995)**. High levels of apase were release by digitonin treatment without resorting to disruptive extraction method (Figure 3.1A-C). However, when the digitonin was used on ground samples, the activity of apase was reduced compared to that of CASC buffer treatment (Figure 3.1B). It was apparent that after proteins extraction, the detergent needed to be removed for subsequent application of extracted proteins. Different biological detergents are commonly used and most have been reported efficient for initial proteins extraction **(LEMIEUX *et al.*, 2003; NIEGOWSKI *et al*., 2006)**. However, some compartments of cell membrane show resistance towards certain detergents **(SCHUCK *et al*., 2003)**. Even though many water-soluble proteins are functional in detergent-solubized form, membrane proteins are often modified and inactivated by detergent solubilization as a result of native lipid-interactions having been disrupted (**NIEGOWSKI *et al*., 2006)**. Thus, the choice of the right detergents is indeed important for membrane stability (**NIEGOWSKI *et al*., 2006**) and enzyme activity. In this experiment, CASC buffer treatment was efficient in extracting proteins and furthermore, it did not interfer with the apase activity. The efficiency of the buffer might have been result of the wide range of salts present in the CASC buffer mixture. In *Arabdopsis thaliana*, several salts and chelating agents were

compared for their ability to extract cell wall proteins enzyme, and calcium chloride was efficient, since 60% of the protein were released by this treatment **(BOUDART *et al.*, 2005)**. Overall, this experiment demonstrated that proteins in *Cladonia portentosa* can be extracted efficiently by biological detergents such as digitonin and CHAPS especially if the enzyme activity is not important. Such information maybe important when protein stability in necessary such as the case for X-ray crystallography and protein mining studies.

3.5.2 Apase purification and physiochemical characterization

Results from ion-exchange chromatography revealed two acid phosphatase peaks (Figure 3.4). Unfortunately, the apase were isolated in relatively low yields. In order to address this problem, protease inhibitors were added to protein samples with the intent to inhibit the suspected enzymatic degradation by proteases. The yields did not improve. The low protein yield is reported to be a problem often encountered with lichen proteins **(PRINTZEN *et al.*, 2003)**. Thus, the nature of the second protein remains to be characterized. When two techniques were used in succession, enzyme activity was reduced dramatically. After several attempts, gel filtration was the best choice for purification. The prevalent apase from *Cladonia portentosa* was purified 45-fold to near electrophoretic homogenetic and final pNPP-hydrolyzing specific unity (167 units/mg) (Table 3.5). The purified enzyme was estimated by non-denaturing PAGE to be approximately 250 kDa (Figure 3.7A). When a product was denatured, a molecular size of 148 kDa was obtained (Figure 3.8 A).

Adding the reducing agents to the purified enzyme, and analysis by SDS-PAGE followed by activity staining, revealed two protein staining bands with molecular masses of approximately 116,25 and 36 kDa, respectively (Figure 3.8B). The activity of the enzyme was not destroyed by the addition of the reducing agents. These data suggest that the native enzyme could exist as a heterodimer. Dissociation of the enzyme into subunits was obtained only after treatment with reducing agents (either 2-mercaptoethano or DTT) indicating that disulfide bridges are included in association of the subunits of this enzyme **(HOGG, 2003)**. Five disulfide bonds were predicted to exist in *Aspergillus* spp. **(ULLAH and MULLANEY, 1996)**. Using the site-

directed mutagenesis **RODRIGUEZ *et al*. (2000)** demonstrated that disulfide bonds were important for catalytic activity and conformation of acid phosphatase enzymes.

Considerable heterogeneity with regards to apase subunit structure, varying from monomeric to hetero-dimer and tetramer forms have been reported on several acid phosphatases from filamentous fungi. In *Penicillium funiculosun* the phosphatase had a 76 kDa heterodimer composed of 51 and 26 kDa subunits, when quantified on SDS-PAGE **(YOSHIDA *et al*., 1989a**). It was found that acid phosphatase from *Schizosaccharomyces pombe* exists as a dimer-tetramer dissociating mono-equilibriating system with a dimer of 180 kDa **(DIBENEDETTO and TELLER, 1981)**. In constrast, apase in *Candida albicans* was reported to be a monomer with Mr 131 kDa **(ODDS and HIERHOLZER, 1973)**. *Aspergillus niger* pH 2.5 apase has an apparent native molecular mass of 269 kDa and it also forms a tetramer in solution **(KOSTREWA *et al*., 1999)**.

Taken together, the eletrophoretic patterns of purified enzyme (native-PAGE and SDS-PAGE), fit the profile of high molecular weight acid phosphatase and thus this enzyme can be assigned to this class **(ARNOLD *et al*., 1988; VINCENT *et al*., 1992)**. The estimated molecular weight by SDS-PAGE (148 kDa) was, however, higher than that observed in other filamentous fungi of the Ascomycete family. The diffusing nature of this enzyme was attributed to glycosylation **(WANNET *et al*., 2000; MULLANEY *et al*., 2000)**. It has been estimated that about a third of all proteins that enter secretory pathways in eukaryotic cells may be N-glycosylated and so tens of thousands of glycoprotein variants may coexist in eukaryotic cells **(WALSH *et al*., 2005)**.

Thus, the discrepancy between the two estimations may be due to an overestimation of molecular mass by SDS-PAGE which is common in the context of glycoprotein **(DECEDUE *et al*., 1984)**. The variation in the extent of glycosylation of a single gene product may be associated with the regulation or targeting proteins to fulfill distinct roles **(WEBER and PITT, 1997b)**.

In addition, the sequencing data show that the apparent strong homologies to existing proteins relate to translated products of much lower expected MW's, suggesting that the protein/s

purified in this study were perhaps significantly modified (e.g. glycosylation). Again leading to an indistinct band pattern, or that they have become complexed in such a way that normal SDS-PAGE did not dissociate them to their natural apparent MW's.

PAS staining did not reveal a clear staining of glycoprotein, although the band was stained margenta after periodic stain. The colour was lost after subsequent washing steps. The removal of bound carbohydrates by endo-F collapsed the major apase into proteins of lower molecular weight (Figure 3.13). Electrophoretic pattern by SDS-PAGE analysis of N-glycosidas F-treated enzyme revealed ~78 kDa reduction in apparent molecular mass after deglycosylation. Reduction of apase in size after deglycosylation to approximated 70 kDa under denatured conditions was consistent with glycosylation reported in secreted protein in most filamentous fungi. In *Aspergillus caespitosus*, the purified enzymes were glycoproteins showing 63.0 and 58.3 % of carbohydrate content respectively **(GUIMARÃES *et al*., 2004)**. The molecular weight of the glycosylated pH 2.5 acid phosphatase produced by a *A. niger* ALKO243 is about 66 kDa and the endo-F treated deglycosylation is around 47 kDa **(MIETTINEN-OINONEN *et al*., 1997)**. *Aspergillus niger* pH 2.5 apase has an apparent native molecular mass of 269 kDa with a glycosylated subunit of approximately 65 kDa and an unglycosylated form of 50.8 kDa **(KOSTREWA *et al*., 1999)**.

Interestingly, the purified enzyme from *C. portentosa* was stable at room temperature even after several days and was not degraded by proteases, probably due to the high amount of attached sugar. The stability of the purified enzyme has long been attributed to the glycosylic nature of the protein, a phenomenon observed in several glycosylated proteins **(ULLAH, 1993)**. A high degree of glycosylation is presumed to protect enzymes from attack by proteases **(BERKA *et al*., 1991; KUBICEK *et al*., 1993; KLIONSKY *et al*., 1990)**. **ULLAH (1993)** suggested that glyco-conjugates present in acid phosphatase may even prevent the protease from degrading the peptide bonds. The results further revealed that deglycosylation resulted in a significant loss of acid phosphate activity, susceptibility to proteolytic degradation and denaturing at higher temperatures **(ULLAH, 1993)**. Similar results were noted by **HAN and LEI, (1999)** in apase and fungal xylanase **(VAN DE VYVER *et al*., 2004)**. When xylanase was exposed to proteases or rumen fluid *in vitro*, the enzyme demonstrated stability, it was obvious that glycosylation

enhances stability, thus, contributing towards the stability of exogenous enzymes **(VAN DE VYVER *et al*., 2004)**.

The p*I* of purified protein in *Cladonia portentosa* was estimated to be 6.4 under the given experimental conditions (Figure 3.9). Most fungal acid phosphatase displayed acid p*I* such as that of *A. niger* NRRL3135 which was reported to be 4.0 **(HA *et al*., 1999)** and in *A. ficuum*, the unglycosylated protein had a p*I* of 4.97 (**ULLAH, 1998**). The phytases produced by *A. niger* (NRRL 3135) PhyA and PhyB were secretory glycoprotein with p*I* 4.5 and 4.9 respectively (**ULLAH, 1998**). In *Bacillus subtillis* a p*I* of 6.5 was obtained **(KEROVUO *et al*., 1998)**.

3.5.3 Apase stabilility and pH

Temperature and pH stability were measured by incubating the purified apase in 100 mM CASC buffer at various temperatures and pHs and measuring residual activity after 20 min with p-nitrophenoly phosphatase at 37 °C. The optimum activity was at pH 2.5 as already established by **HOGAN *et al*. (2010a)**. The pH optima of the purified *C. portentosa* is in agreement with activity profiles of other apases isolated from filamentous fungi **(ULLAH, 1998; MITCHELL *et al*., 1997)**, even though this pH optima was narrower than that observed for other fungal acid phosphatases. Apase from *Aspergillus oryzae* had pH optima of 4.5, 5.0 and 5.7 and no activity detected above pH 7.0 **(FUJITA *et al*., 2003)**, the apase of *Aspergillus fumigates* was active at pH 3.0 to 7.0 with an optimum occurring between pH 4.0 to 6.0 **(BERNARD *et al*., 2002)**. In this study, no alkaline phosphatase was detected. This was surprising since most fungi from the Ascomycetes have been reported to possess both alkaline and acid phosphatases, whereas Basidiomycetestes usually possess only acid phosphatases **(EYES *et al*., 1990)**.

3.5.4 Enzyme kinetics

The presence of 2.0 mM EDTA did not affect the activity of acid phosphatase. Thus, this enzyme does not require metal ion cofactors (Table 3.6) **(WANNET *et al.*, 2000)**. Conversely, other compounds had an inhibitory affect above a concentration of 2.0 mM (Table 3.6) as demonstrated by $CuSO_4$, and $ZnCl_2$. Overall, the behaviour of purified apase fitted the the

profile of most acid phosphatases, it is well known that acid phosphatases in general are not stimulated by metallic ions **(WANNET *et al.*, 2000; KNEIPP *et al.*, 2003; GUIMARÃES *et al.*, 2004)**.

The behaviour of the enzyme under inhibitors is summarized in Figure 3.12. The purified apase was inhibited by tartrate (2 mM). Therefore the enzyme appeared to be tartrate-sensitive. The inhibition by tartrate is a common characteristic of HMW apase **(ARNOLD *et al.*, 1988)**. Using pNNP as substrate, overall results revealed that apase from *C. portentosa* was further inhibited by sodium vanadate as well as sodium molybdate and sodium fluoride which have been reported to be a potent inhibitors of apases **(WANNET *et al.*, 2000; KNEIP *et al.*, 2003)**.

The purified apase from *Cladonia portentosa* showed broad substrates specificity on a range of phosphate and phosphorylated substrates (Table 3.7). The acid phosphatase from *Cladonia portentosa* is non-specific enzyme as it hydrolyzes different phosphate esters regardless of the chemical nature of the leaving group. This was the case of pNPP, 4-NPP, α- naphthyl phosphate and ß-glycerophosphate which were the best substrates for this enzyme, suggesting that these were preferred substrates for this enzyme.

In all the substrate tested, the rate of enzymatic hydrolysis of apase from lichen *Cladonia portentosa* followed a classical Michaelis-Menten kinetics, i.e. the liberation of Pi is dependent on the substrate concentration used **(VATS and BANERJEE, 2004)**. The apparent K_M of the apase for phytic acid calculated from the Lineweaver-Burk plot was 31.2±0.25 µM. This K_M show a high affinity for phytic acid, higher than K_M values of 18.7 µM reported for *Aspergillus niger* (**NAGASHIMA *et al.*, 1999)** but lower than 40 µM reported for *Aspergillus ficuum* (**ULLAH, 1988)**. Like many fungal acid phosphatases and phytases, the purified *C. portentosa* enzyme exhibited a broad substrate specificity, including phytic acid, thus, the enzyme qualified as a phytase enzyme. In general phytase are specific for phytic acid even though some have an ability to metabolize a broad spectrum of phosphate substrates, furthermore the substrate specificity may vary due to differences in molecular molecular characteristics **(VATS and BANERJEE, 2004)**. The natural substrates for these enzymes is not known **(AZEKE *et al.*, 2010)**. It is presummed that these enzymes are released into growth media or extracellulary

where they are involved in phosphate/phosphorus mobilization from phytic and other phosphate /phosphorus containing compounds **(AZEKE *et al*., 2010)**.

Inorganic phosphate at 1.0 mM caused product inhibition (competitive inhibition of phytate hydrolysis) of apase in *Cladonia portentosa* as observed in other fungi **(ULLAH and GIBSON, 1987; GREINER *et al*., 1993; KONIETZNY *et al*., 1995**). However, in *Aspergillus ficuum*, the substrate inhibition could be observed at phytate concentration higher than 1.2 mM **(ULLAH, 1988)**.

3.5.5 Apase sequencing

In order to obtain basic structural information with respect to apase, sequencing using the MALDI TOFF/TOF instrument was performed. The MS/MS analyses resulted in 20 peptides sequences (Table 3.8). Raw spectral data of these sequenced peptides are shown for the peptides that showed homology with apase (Figure 3.16). It should be noted that the peptides presented here are not yet available in the mentioned database.

Longer peptides, FLAETNPAPFGHP, AVGLGYVEELLAR and AQGLGYVQEVLAR generated by MS/MS ion search gave successful results with statistically reliable matches found between the obtained peptide fragment masses and database protein sequences (Tables 3.9-3.11). The search conducted by BLAST matched peptides 10, 11 and 15 with several hypothetical acid phosphatases and phytases found in several fungi, in both the Ascomycete and Basidiomycetes families (Figure 3.16). In expasy expectation scores of 0.05 (indicating strong homology) were obtained with acid phosphatase putative protein from *A. terreus*. This finding confirmed the purified enzyme as a histidine acid phosphate, since it matched the AA from fungi sequences that contained a conserved histidine residue which is phosphorylated during the reaction **(MITCHELL *et al*., 1997; RIGDEN *et al*., 2002)**. Both the FLAETNPAPFGH and AVGLGYVEELLAR segments matched regions of 5' and 3' of these genes, respectively. Homologous sequences to these peptide sequences possess the active site sequences ’RHGXRXP’ which is the hallmark of most acid phosphatase and phytase enzymes **(ULLAH *et***

***al*., 1991; KOSTREWA *et al*., 1997; RIGDEN *et al*., 2002; RIGDEN, 2008)**. As can be deduced from the alignment, it appears plausible that the two peptides belong to one gene.

Interestingly, peptide 15 also belonged to the histidine apase but the sequence was different from that obtained from peptides 10 and 11. This peptide AQGLGYVQEVLAR shares high homology to histidine acid phosphate from *Botryotinia fuckeliana*, with 85% similarity and an E score of 0.003. Most remarkable was that it also shares 77, 71 and 68% similarity with acid phosphatase and Phytase-A found in *Sclerotinia sclerotiorum*, *Phaeosphaeria nodorum* and *Postia placenta*, respectively. These results suggest the existence of the second apase, even though it was not detected in a suitable quantity by ion exchange chromatography.

Existence of isoforms/multiple apases is common for most fungal acid phosphatases, which tend to be secreted under certain physiological and developmental stages. Two distinct acid phosphatase isozymes were characterized in *Dicystelium discoideum* (**BERNETT and DIMOND, 1986)**. These enzymes were later shown to occur at different developmental stages **(BERNETT and DIMOND, 1986)**. Three apase isozymes were isolated in secreted *Pholiota nameko*, one expressed under phosphate deficient conditions **(JOH *et al*., 1996; YAZAKI *et al*., 1997)**. Similar results have been reported with apases from *Saccharomyces cerevisiae* and *Aspergillus niger*, where certain isozyme apase enzymes are induced under phosphorus starvation (**TOH-E *et al*., 1976; BOSTIAN *et al*., 1983)**. The occurrence of multiple isozymes is considered to be due to either epigenetic modification molecules or the corresponding multiple genes **(YAZAKI *et al*., 1997)**. The isozymes with different electrical mobility are coded by a single structural gene and occur by the removal of an amino-terminal arginine residue **(YAZAKI *et al*., 1997)**. On the other hand, several apase isozymes of fungi have been shown to be coded respectively by different genes **(TOH-E *et al*., 1976; BOSTIAN *et al*., 1983)**. The lichen in this study was collected in various locations, where the previous physiological state or developmental stage of the lichen was not taken into consideration. It is possible that the second apase band was expressed under certain environment or stressful conditions that were not taken into consideration.

Interestingly, apase purified in this study was co-purified with other high molecular proteins including stress proteins and proteinases (Table 3.8), indicating that the lichen was under some form of stress. These proteins were not resolved by inclusion of SDS in the gel, suggesting protein-protein interaction or a synergistic relationship amongst these proteins, possibly to fulfill their biological function/s. Like apases, many proteinases are hydrolases as well, thus, considering that apases are responsible for providing inorganic phosphate (Pi) to the cell by means of cell recycling and exploitable extracellular resources **(ALTOMARE *et al.*, 1999)**, it may be possible, these enzymes work in synergy for efficient acquisition of Pi. Furthermore, in recently years, the role of proteinases have been shown to be more than breaking peptide bonds in protein but they also play a pivotal role in defence **(TEN HAVE *et al.*, 2010)**, implying that apase may also play a role in lichen defence (**JAKOBEK and LINDGREN, 2002)** or stress-related conditions. Further work in this area is needed, since this area was not a major focus of the study.

4. CYTOCHEMICAL LOCALIZATION AND REGULATION OF ACID PHOSPHATASE BY CULTURE CONDITIONS IN MYCOBIONT *CLADONIA PORTENTOSA*

4.1 GENERAL INTRODUCTION

Acid phosphatases are important hydrolytic enzymes, which play a pivotal role in the nutritive acquisition of phosphorus. The regulation of repressible acid phosphatase (Apase: orthophosphoric-monoester phosphohydrolase [acid optimum], EC 3.1.3.2) in many fungi is negatively controlled by inorganic phosphate (Pi) levels present in the growth medium (**SHIEH *et al*., 1969; YOSHIDA and TAMIYA, 1971; HAN *et al*., 1994)**. The concentration of inorganic phosphate is not the only factor reported to induce the regulation of acid phosphatase in filamentous fungi; pH of culture medium, age and the carbon source are some of the physiological factors known to induce apase **(SHIEH and WARE, 1968; TOH-E *et al*., 1978; OSHIMA, 1982; CADDICK *et al*., 1986b; ELLIOT *et al*., 1986; MORALES *et al*., 2000)**.

Apases have been localized in different fungi using a classic Gomori stain with various substrates, such as para-nitrophenyl phosphate (pNNP), β-glycerophosphate (β-GLOB) and α-naphthyl phosphate **(GOMORI, 1950; GARRISON and ARNOLD, 1983; ARNOLD *et al*., 1988; CHEREPOVA and SPASOVA, 1996; SPASOVA and GALABOVA, 1998; WEBER and PITT, 1997a)**. Recently, the use of fluorescent substrates, which were originally used for alkaline phosphatases have been employed in many apase studies. One of the substrates often used in co-labelling with antibodies is Vector blue III (Vector Laboratories, USA).

ELF-97 [2-(5-chloro-2-phosphoryloxyphenyl)-6-chloro-4-(3H) quinazolinone (CPPCQ)] (Molecular Probes, E-6601, Invitrogen), has been used to detect phosphatases in minute quantities **(GONZÁLEZ-GIL *et al*., 1998)**. This fluorogenic substrate has been used for localization of phosphatase activity in marine phytoplankton **(GONZÁLEZ-GIL *et al*., 1998)** and mycorrhizal fungi **(VAN AARLE *et al*., 2001; 2005; ALVAREZ *et al*., 2004; 2005)**. The

substrate is normally slightly fluorescent in the blue range. However, once its phosphate is enzymatically removed, the substrate forms a crystalline precipitate, which fluoresces bright green, indicating the site of activity **(HUANG *et al*., 1993; LARISON *et al*., 1995)**. Hence the process is called enzyme-labelled fluorescence (ELF).

Endocytosis is important in understanding secreted proteins especially those involved in nutritive acquisition, membrane receptors and ion exchange **(PEÑALVA *et al*., 2005)**. The membrane–selective FM4-64 belongs to a class of amphiphilic styryl dyes **(BETZ *et al*., 1992; 1996)**. This dye fluoresces significantly only when in a hydrophobic environment (e.g. a lipid rich membrane). FM4-64 has been used extensively in labelling animal cells (neuron labelling) and plant cells **(BETZ *et al*., 1996; COCHILLA *et al*., 1999; EMANS *et al*., 2002; BOLTE *et al*., 2004)**. More recently, FM4-64 is increasingly being used when studying vesicle trafficking and organelle organization in fungal cells **(VIDA and EMR, 1995; HOFFMANN and MENDGEN, 1998; FISCHER-PARTON *et al*., 2000; READ and HICKEY, 2001; ATKINSON *et al*., 2002; HICKEY *et al*., 2002)**. The use of FM4-64 to stain internal membranes was reported by **COLE *et al*. (1998)** in the slow growing mycorrhizal fungus *Pisolithus tinctorius*. **ATKINSON *et al*. (2002)** used FM4-64 to image endocytosis during conidal germination in *Magnaporthe grisea.* FM4-64 was also used for the labelling of the apical vesicular cluster in *Aspergillus nidulans* and for studying the ontogeny of the Spitzenköper in germlings in *Neurospora crassa* **(ARAUJO-PALOMARES *et al*., 2007)**.

The dye is believed to be unable to cross membranes because of its amphiphilic nature and is thought to enter the cell primarily by endocytosis; vesicles invaginated from the plasma membrane **(BETZ *et al*., 1992; READ and HICKEY, 2001)**. After internalization, the dye is distributed to different organelle membranes, perhaps via the vesicle trafficking network and thus components of secretory pathways can be labelled **(BETZ *et al*., 1996; BELANGER and QUATRANO, 2000; FISCHER–PARTON *et al*., 2000; READY and HICKEY, 2001)**.

Preliminary studies conducted by **HOGAN *et al*. (2010a and b)** demonstrated that acid phosphatase in the lichen *Cladonia portentosa* (Dufour) Coem is exclusively associated with the fungal component and the enzyme is located in the cell membrane. The aim/s of this

experiment was to illustrate by cytochemical techniques, the location of apase in the fungal mycelium and demonstrate the effect of inorganic phosphate on acid phosphatase of the mycobiont (*Cladonia portentosa*). Furthermore the relationship between Pi (KH_2PO_4) metabolism and abiotic stress on acid phosphatase were analyzed by examining the effect of oxidative stress on acid phosphatase induced by menadione. In addition, the effect of culturing on media at different pH were examined.

To address the objectives stated above, part of the study focused on two major sections: the influence of culturing conditions on the biosynthesis of apase using fungal cultures of *Cladonia portentosa* and cytochemical localization of apase in mycobiont and alga cultures.

4.2 MATERIAL AND METHODS

4.2.1 Chemicals

The substrate ELF-97 [2-(5-chloro-2-phosphoryloxyphenyl)-6-chloro-4-(3H)] quinazolinone (CPPCQ)], Hoechst dye and FM4-64 [4-(p-diethylaminophenyl-hexatrienyl) pyridinium dibromide] were purchased from Invitrogen. The inhibitors sodium flouride (NaF) and levimisole were bought from Sigma. The colorimetric substrates [*para*-nitrolyphenyl phosphate (pNNP), β-glycerophosphate (β-GLOB), phytic acid and naphthyl phosphate] and all the chemicals used for the preparation of buffers and fixing protocols were purchased from Sigma (St Louis, MO). The BCA kit was bought from Novagen (Beeston, UK). Vector blue III was sourced from Vector Laboratories, CA.

4.2.2 Isolation of axenic photobiont and mycobionts

The photobiont component that is present in *Cladonia portentosa* is *Trebouxia*. *Trebouxia* was isolated by the method described by **YOSHIMURA *et al*. (1993)**. Samples of dry thalli (0.5 g) were rinsed in distilled water to remove superficial contaminants. Samples were then macerated in a mortar with 10 ml distilled water. Homogenates were filtered through six layers of cheese cloth and filtrates were centrifuged at 100x g for 10 min. Supernatants were discarded, pellets were re-suspended in 8.0 ml 0.25 M sucrose and then, 40 ml of these suspensions were gently overlaid on top of 5.0 ml of 80% (w/v) potassium iodide (KI) in a centrifuge tube which was centrifuged at 2000x g for 45 s. Algal cells and hyphal fragments were found in a broad layer in the sucrose solution above the KI layer, whereas large fragments of non-disrupted thalli sedimented. The layer contained algal cells and hyphal fragments was recovered with a micropipette and placed on a 5.0 ml KI solution. Two ml of 10 mM phosphate buffer were added and centrifuged at 800x g for 90 s. Algal cells formed an interphase between phosphate buffer and sucrose, whereas small fragments of fungal hyphal fragments formed the pellets. The interphase containing algal cells was recovered with a micropipette, deposited on 5.0 ml KI and 3.0 ml 10 mM phosphate buffer were added and centrifuged at 1000x g for 3 min. This step was repeated twice to obtain algal cells with non-detectable fungal contamination. Alternatively,

algal cells were also scraped directly from thalli that were pre-hydrated. The scraped algals cells were then incubated on Bold Basal Medium (BBM) **(DEASON and BOLD, 1960; BISCHOFF and BOLD, 1963)** as described by **KRANNER *et al.* (2002)** consisting of the following: $NaNO_3$ (250 mg), KH_2PO_4 (175 mg), K_2HPO_4 (75 mg), $MgSO_4.7H_2O$ (75 mg), $CaCl_2.2H_2O$ (25 mg), NaCl (25 mg), EDTA (50 mg) KOH (31 mg) $FeSO_4.7H_2O$ (4.98 mg), H_3BO_3 (11.42 mg), $ZnSO_4.7H_2O$ (8.82 mg), $MnCl_2.7H_2O$ (1.44 mg), MoO_3 (0.71 mg), Cu $SO_4.5H_2O$ (1.57 mg) and $Co(NO_3)_2.6H_2O$ (0.49 mg) and made up to 1 L. The visible algal colonies were later transferred to a molten BB Medium, and dispersed in the gradient by titling the plate. Attempts to obtain a pure culture was achieve by sub-culturing frequently and contaminants were eliminated by Tween (0.2%) and 5 drops of sodium hypochlorite (JIK®) per 10 ml. The cultures were also examined under a light microscopy for contaminants.

After establishing sufficient inoculum, the axenic algal cells were inoculated (500 µl) in 125 ml Erlenmeyer Flasks consisting of BB Medium. The Pi concentration was either 0.05 mM Pi or 10 mM (K_2HPO_4). Cultures were allowed to grow for 15 days, at 18 ºC. Cultures were kept stationary under irradiances of 130 µmol photons $m^{-2}s^{-1}$, and a 14:10 light dark (L:D) cycle. The cultures were aerated with 20 L h^{-1} compressed air (in the light period enriched with 1.5% CO_2) through sterile cotton filters. At the end of the incubation period, the algae were used as assay material, using the acid phosphatase assay.

The axenic mycobiont cultures were isolated and maintained in the medium as described in Chapter 2, Section 2.6. To increase biomass of the mycelium, it was grown for 3 months in the liquid medium using the culture medium described above. After about 3 months, cultures were transferred to nutrient medium (Appendix C, Table C1).

4.2.3 The effect of Pi on apase

To achieve severe phosphorus (P) deficiency conditions, mycelium were grown in nutrient medium (Appendix C, Table C1), containing KH_2PO_4 as an inorganic phosphorus (Pi) supplement. The concentrations of Pi were: 0.05, 1.0, 3.0, 10 and 100 mM.

After establishing sufficient inoculum, the axenic algal cells were inoculated (500 µl) in 125 ml Erlenmeyer Flasks consisting of BB Medium. The Pi concentration was either 0.05 mM Pi or 10 mM (K_2HPO_4). Cultures were allowed to grow for 15 days, at 18 ºC. They were kept stationary and bubbled with air enriched with 0.5% carbon dioxide. At the end of the incubation period, the algae were used as assay material, using assay A (Chapter 3, Section 3.2.2.3).

In addition, fungal cultures were incubated at 18 °C, in the stationary medium for 4 months. Each experiment was run in triplicate and repeated at least twice; the standard deviation of analysis was <10% from the average. The mycelia were measured for total acid phosphatase activity. A portion of the samples was pooled for total RNA extraction.

Both axenic cultures of mycobionts and photobionts were collected by centrifugation. The cultures were rinsed twice with sterile distilled water to remove traces of phosphate. The samples were ground with acid-washed sand in a mortar. Ice-cold 50 mM CASC buffer ± 0.1% Triton was used to extract the enzyme. The extract obtained was centrifuged at 12, 000 rpm for 20 min to settle the fungal debris. The culture medium was maintained to test for secreted proteins. Proteins were measured by BCA kit. Clear supernatant was used to detect acid phosphatase activity using a colorimetric substrate pNPP (p-nitrophenyl phosphate) using assay A (Chapter 3, Section 3.2.2.3). Control samples were de-activated by boiling and then used as blanks. The absorbance of these samples were subtracted when calculating the phosphatase activity. The mass of mycelium was dried in the oven at 80 °C until a constant weight was obtained.

4.2.4 The effect of media pH on apase biosynthesis

Under already described growth conditions (Section 4.2.2), the effect of different media pH was investigated when the mycelium was grown with 0.05 mM vs. 10 mM Pi at four different pH values [2.5, 4.8, 6.0 and 8.0]. The mycelium was left in culture for two months. The apase enzyme was detected using pNPP.

4.2.5 The dual effect of Pi starvation and Menadione

The mycelium of *C. portentosa* grows extremely slowly. In order, to measure the optimal concentration for menadione, the mycelium was incubated on solid medium with various concentrations of menadione (2.0, 10 and 50 µM). The concentration that kills or cause browning in the mycelium within two weeks was noted as unsuitable for culturing. The aim was to select a concentration that would cause stress to the mycelium without causing visible damage to the cells. After establishing an optimal dose that did not kill the mycelium a concentration of 2.0 and 10 µM were selected as low and high treatments, respectively. The coupled effects of menadione versus Pi starvation were studied and the experiment was designed as indicated in Table 4.1.

Table 4.1: Experimental design on dual treatments of Pi versus menadione. N=5

Treatments	Replicates (n)
Low Pi-Low-Menadione, [LP-LM (0.05 mM Pi*2.0 µM Menadione)]	5
Low Pi-High-Menadione, [LP-LM (0.05 mM Pi*10 µM Menadione)]	5
High Pi-Low-Menadione, [LP-LM (10 mM Pi*2.0 µM Menadione)]	5
High Pi-High-Menadione, [LP-LM (10 mM Pi*10 µM Menadione)]	5
0.05 mM Pi (Pi)- control	5
10 mM Pi (Pi)- control	5

Controls were maintained under both -Pi (0.05 mM Pi) and high +Pi (10 mM Pi) without any chemicals added. Five replicates were used for each treatment. Samples were measured for acid phosphatase activities. Total protein was quantified by the BAC method, already mentioned in Chapter 3, Section 3.2.2.4.

4.2.6 Statisticaly analysis

All experiments were performed thrice with three replicas. Data were calculated by Graph Pad for graphical representation and SPSS version 9.0 programs. Data of specific growth rate and apase activities among different treatments and levels was firstly analyzed by the ANOVA test at $P<0.05$ and then the significance of differences between treatments were analyzed by using Duncan's new multiple range test.

4.2.7 Cytochemical localization of acid

The localization of apase was examined at the ultrastructural level using a combination of transmission electron, fluorescent and confocal microscopy.

4.2.7.1 Fixation

The cytochemical procedure for detection of apase was adapted from **MIYAYAMA *et al.* (1975)**. Cytochemical reaction was carried out in 1.5 ml micro-centrifuge tubes unless otherwise stated. Lichen samples, mycelia and intact algae were suspended in fixative solution and fixation was carried out for 16 h at 4 °C. The fixed samples were washed in three changes of buffer B (Appendix B), followed by three changes with ice-cold buffer C (Appendix B). The samples were pre-incubated in buffer C (Appendix B), containing 2.0 mM Pb $(NO_3)_2$] at 37 °C for 10 min and then incubated at the same temperature for 60 min in buffer C containing 2.0 mM $Pb(NO_3)_2$ and either pNPP (5.0 mM) or β-glycerophosphate (5.0 mM). Control samples were incubated and treated under the same conditions either in the absence of substrate or in a medium containing both substrate and NaF (5.0 mM). After incubation the samples were rinsed in three changes of buffer B (Appendix B) and re-fixed for 60 min in 2% (v/v) glutaraldehyde in buffer B. Cells were washed again in three changes of buffer B.

4.2.7.2 Electron microscopic apase cytochemistry

Fixed samples were post-fixed in ice-cold buffer B containing 2% osmium tetroxide in 0.05 M buffer for 2 to 4 h. The samples were then rinsed three times with 0.05 M Cacodylate buffer (2 x 30 min), followed by three rinses with distilled water. The samples were dehydrated in a graded series of ethanol washes (10%, 30%, 50%, 70%). The samples were finally dehydrated with 3 (x10 min) final washes of 100 % ethanol.

The samples were then embedded in propylene oxide (2 x 30 mins) followed by 25 % Epon: 75% propylene oxide and DMP (1 drop for every ml Epon)– for 2 h with tubes with the caps on. After this the samples were embedded with 50% Epon: 50% propylene oxide + DMP for 2 h with caps on. Lastly, the samples were embedded in 75% Epon: 25% propylene oxide + DMP

overnight with caps off. Finally, samples were embedded in 100 % resin and heated in an oven at 70 ºC for 48 h. Ultrathin sections were cut on an ultracut-E-microtome with a diamond knife, transferred onto 200 mesh copper grids and stained for 10 min with lead citrate. Some samples were post-stained with 2% (/v) aqueous uranyl acetate for 15 min at room temperature and stained with lead citrate (10 min). The samples were washed twice with distilled water. Micrographs were taken on a Joel 100Cx transmission electron microscope operating at 80 KV.

4.2.7.3 ELF-97 substrate

The cytochemical procedure for the localization of apase was adapted from the protocol of **ALVAREZ *et al*. (2004)** and **VAN AARLE *et al.* (2001 and 2005)**. The mycelium was removed from culture medium by centrifugation and rinsed with distilled water. Pieces of mycelium were separated using scalpels and transferred to microcentrifuge tubes (1.5 ml Eppendorf tubes). The samples (intact lichen and mycelia) were washed with 0.1 M CASC buffer and fixed in 3.7 % (m/v) paraformaldehyde (PFA) (grade 1: Sigma Co.) in 0.1 M CASC buffer (100 mM, Tri-sodium Citric Acid buffer, 200 mM sucrose, pH 2.5). PFA stock solution (23.13 ml) was added to the CASC buffer (76.87 ml). The solution was made up just before use. The specimens were rinsed three times. Just before use, the ELF-97 substrate was diluted with 0.1 M CASC buffer and then filtered (0.2 µm) to eliminate possible precipitation of the substrate. The ELF-97 substrate was either diluted 20 or 50 times for cultures incubated for 30 min or overnight cultures, respectively. After filtration, 5.0 µl of this diluted ELF solution was added to the washed cells.

The reaction was measured after 30 s and 15 min at room temperature and after 24 h at 4 °C. After the reaction had taken place, the cells were rinsed with three changes of phosphate buffered saline [PBS, pH 7.4 (8.0 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO4, 137 mM NaCl, 2.7 mM KCl, 1.0 mM $CaCl_2.2H_2O$ and 0.5 mM $MgCl_2.6H_2$)]. The solution was made to 1.0 L with distilled water. The solution was filtered and stored at 4 °C. The pH was determined by making fresh buffers of either, 100 mM CASC, (pH 2.5 or 4.8) or 100 mM sodium acetate buffer, pH 4.8. The pH of the original buffer [pH8.0] in the ELF-97 kit was used to detect alkaline phosphatase not acid phosphatase as recommended by **ALVAREZ *et al.* (2005)**. For negative

controls, samples were treated as above except that the ELF-97 substrate was omitted. Inhibitors of phosphatase, NaF and levimisole, were used to verify the results at a concentration of 5.0 mM. The samples were incubated with the inhibitors first before adding the ELF-97 substrate.

4.2.7.4 Vector Blue III

The samples were fixed for 10-30 min as already described in Section 4.2.7.3. The working solution of Vector blue III was prepared just before use by adding 2 drops of reagent to 5.0 ml of 100 mM CSTSA pH 2.5, pH 4.8 [following manufacturer's instruction, Vector Laboratories, CA]. The samples were incubated with substrate at room temperature in the dark until suitable staining developed, which was within 20-30 min. The samples were rinsed in two changes of buffer and followed by distilled water and counterstained with the second stain FM4-64.

The samples were mounted and viewed under the microscope. Vector blue III fluorescence was excited using lasers at 488 nm or 568 nm, and the emission was detected using a long pass filter designed for Cy-5 using a LSM 780 confocal scanning microscopy (Zeiss, Göttingen, Germany).

4.2.7.5 Co-staining with other fluorescent dyes

Where the ELF-97 or vector blue assay were used in combination with other dyes such as FM4-64, Hoechst and propidium iodide, labelling with the acid phosphatase substrate was performed first. Prior to staining, the mycelia was first fixed for 10-30 min and then labelled with substrate (ELF-97 overnight or 20 min for vector blue III). Following incubation, samples were washed at least three times with PBS buffer to avoid background noise. The cells were then co-stained with either FM4-64, Hoechst stain or propidium iodide followed by thoroughly rinsing as described above.

The stock solution for Hoechst was 1.0 mg/ml, where the working solution was prepared as follows: 1.0 µl was added in 4.0 ml PBS. To stain the mycelia, 10 µl of the working solution

was added to 500 µl of PBS buffer. The cells were stained for 5 min and rinsed three times with 500 µl PBS buffer. The stock solution of propidium iodide was prepared in PBS buffer at 500 µg/ml. The solution was maintained at 4 °C and protected from light. The working solution in PBS buffer was 1.0 µg/ml. FM4-64 stock solution was 1.0 mg/ml in distilled water. The working solution was diluted ten times in PBS buffer. The final working concentration was maintained between 1.0-1.5 µM.

4.2.7.6 Fluorescence microscopy

The labeled samples were blotted on tissue paper and mounted on a slide using mounting medium provided with the ELF-97 kit. The samples were observed under UV light using an Olympus AX 70 fluorescent microscope for epi-fluorescence and with a high-pressure Hg lamp. An analYSIS program (DHB, Germany) was used to take images.

4.2.7.7 Localization of apase on fungal hyphae using inverted confocal microscopy

After washing, fungal hyphae were mounted to a slide using the mounting medium and the samples were observed directly with a confocal microscope (LSM 780, Zeiss, Göttingen, Germany) equipped with argon and neon lasers. Vector blue III yields a stable, strong fluorescent reaction with an excitation peak around 500 nm and a large stokes to emission peak at 680 nm. The reaction product was excited using lasers at 488 nm or 568 nm, and the emission was detected using a long pass filter designed for Cy-5 using a LSM 780 confocal scanning microscope (Zeiss, Göttingen, Germany).

When the samples were stained with two fluorophores, controls devoid of fluorophores were used to optimize the settings on the confocal microscope. The natural autofluorescence and fluorescence by the substrate (vector blue III) were read in one channel and given the same pseudo colour green. To differentiate the autofluorescence from the vector blue III, controls were measured first to establish their maximum excitation. The microscope settings were adjusted until no further background or autofluorescence was measured.

4.2.7.8 Detecting colocalization in dual labelling

The colocalization was determined using ImageJ Analysis program (National Institute of Health, USA). During the initial experiment using the confocal microscope, the intensity of the red channel and the green channel were kept the same to avoid skewed results. Thus, we assumed that the starting value or ratios of red and green pixels were the same and there was no bleeding of colour. The fluorochromes were excited using 405, 633 laser lines. For colocalization analyses, the channels masking techniques were used in conjunction with automatic threshold to calculate colocalization statistic in several samples. The percentage of one channel volume colocalized with another channel volume in the channel mask with selected intensity. Pearson correlation coefficient in the colocalized volume was calculated and the automatic threshold feature based on an algorithm developed by **COSTES *et al*. (2002 and 2004**). The same level of threshold was applied to each data set as described by **LI *et al*. (2004)**. The ICCB tools mainly use statistics to assess the relationship between fluorescence intensities **(BOLTE and CORDELIÈRES, 2006)**. This is mostly done using correlation coefficients that measure the strength of the linear relationship between two variables, i.e. the grey values of fluorescence intensity pixels of green and red image pairs **(BOLTE and CORDELIÈRES, 2006)**.

4.3 RESULTS

4.3.1 Isolation of axenic cultures

Microbial contamination was a major problem in the isolation of axenic cultures. The isolation of algal cells by a protoplast protocol resulted in extensive bacterial growth. Scraping the algae direct from the lichen was a better isolation method in this study (Figure 4.1A-C).

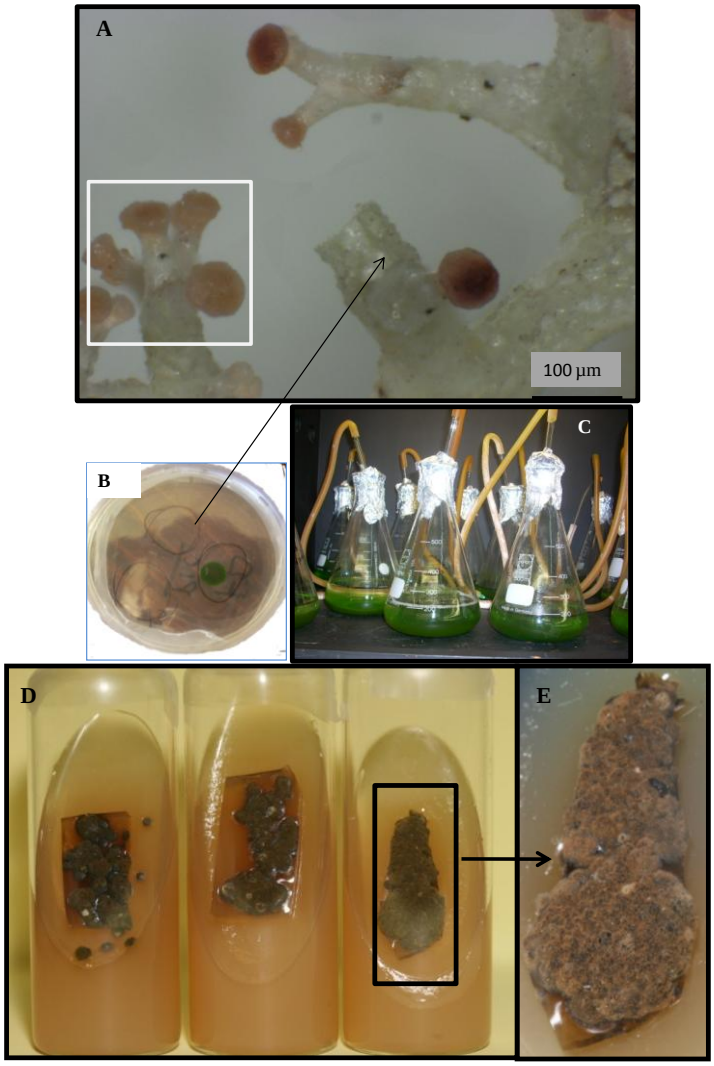

Figure 4.1: (A-E) Axenic cultures of *Cladonia portentosa*. (A) Intact lichen which was used as a starting material to isolate both mycobiont and photobiont. Fertile portion of the lichen, podetia (reddish-brown) is indicated in a square. (B) An algal colony isolated by scraping algal cells directly from lichen thallus. (C) Cultures of algae were grown on BB medium at 20 ºC. (D) Mycobiont stock cultures were maintained on the slant (malt extract, yeast extract and agar). (E) Magnification of the zone in D, indicated by a box.

In algal cultures, contamination was reduced by sub-culturing the colonies onto the molten agar, where the algal cells were buried slowly by tilting the plate. Thus, surface sterilization (0.5% Tween and 2.0% sodium hypochloride) could be applied without damaging the colonies. This technique was efficient, resulted in axenic agal colonies, which were further used for larger-scale experiments using liquid cultures (Figure 4.1 C). Fungal spores isolated from lichens were detected after ~3 months in culture from solid media (Figure 4.1D-E). To increase the mycelium biomass, liquid medium was also used. Mycelium cultures were also maintained on slants for long term storage (Figure 4.1D).

4.3.2 The effect of different Pi concentrations on apase biosynthesis

The biosynthesis of apase under different concentrations of inorganic phosphate (Pi) is shown in Table 4.2. There was a significant difference amongst treatments ($P<0.05$), however, no significant difference was observed at 3, 10 and 100 mM treatments (Table 4.2). Decreasing amounts of available Pi tended towards increasing phosphatase activity as shown by 3.0 mM Pi treatment (Table 4.2). On contrary, at high Pi (100 mM) the apase activity was reduced. The lowest Pi concentration (0.05 mM) reduced the mycelium growth, thus, resulting in the lowest dry weight when compared to other treatments (Table 4.2). High dry weight was obtained at 10 mM (Table 4.2).

Table 4.2: Apase activity from *Cladonia portentosa* cultured on different concentrations of Pi [0.05, 1.0, 3.0, 10 and 100 mM] at 18 °C for 4 months. Dry weight of mycelia were measured in g per 100 ml medium. N=3, SD is represented. Means with letters in common are not significantly different ($P<0.05$).

Pi [mM]	Mycelium (#apase activity g/dry weight)	Dry mass
0.05	1270.56±501.44 b	0.0497±0.002d
1.0	1963.27±53.17 ab	0.094±0.001b
3.0	3023.31±266.81 a	0.0957±0.002b
10	2806.73±388.86 a	0.105±0.0011a
100	2430.91±109.41 a	0.077±0.0019c

(#One unit represents the activity required to degrade 1 mol p-PNNP in 1 min.

4.3.3 The effect of menadione versus Pi starvation on apase biosynthesis

This study was conducted over a period of 2 months. The mycelium was treated under different dual treatments of menadione [low or high (2.0 µM or 10 µM, respectively) and either low (0.05 mM or high (10 mM) Pi], at a pH 2.5. Comparative studies were made with the controls which were maintained at 0.05 mM or 10 mM Pi without any menadione. The results for this experiment were not clear. A significant differences ($P<0.05$) was observed on different treatments. An increase on apase activity was obtained at low Pi and low menadione (LPi-LM) compared to other treatments (Figure 4.2). Low apase was detected in the control treatment (LPi) where a significant increase in apase activity was detected in the control treatment (10 mM, HPi) (Figure 4.2).

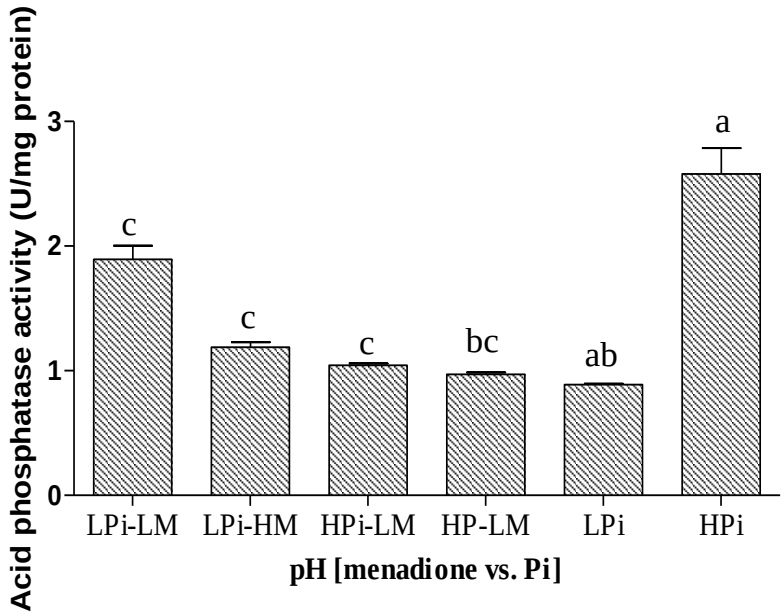

Figure 4.2: Acid phosphatase activity (units/mg protein) in the mycelia of *Cladonia portentosa* in a dual treatment of menadione vs. Pi-deficient or Pi-enriched medium. Each treatments consisted of 5 replicates (n=5). The vertical bars represent standard error. Means with letters in common are not significantly different ($P<0.05$). Low Pi and low menadione (LPi-LM; 0.05 mM Pi and 2.0 µM menadione), low Pi and high menadione (LPi-HM= 0.05mM Pi and 10 µM), High Pi and low menadione (HPi-LM; 10 mM and 2.0 µM menadione), LPi (low Pi; 0.05 mM Pi) and high Pi (10 mM Pi).

4.3.4 The effect of pH on apase

The effect of media pH on apase activity was compared using Pi-sufficient (Pi) versus P-defficient (PC) conditions. The mycelium was grown at four pH values (2.5, 4.8, 6.0 and 8.0) and the activity of apase was compared. Alkaline pH 8.0, was detrimental to the mycelium, as the cultures became necrotic, no further growth was observed, thus these data were not included in the analysis. Interesting, high apase were detected at pH 4.8 (-Pi) and pH 6.0 (-Pi and +PC). Statistical evidence show that there was no significant difference between pH 4.8 and pH 6.0. (Figure 4.3).

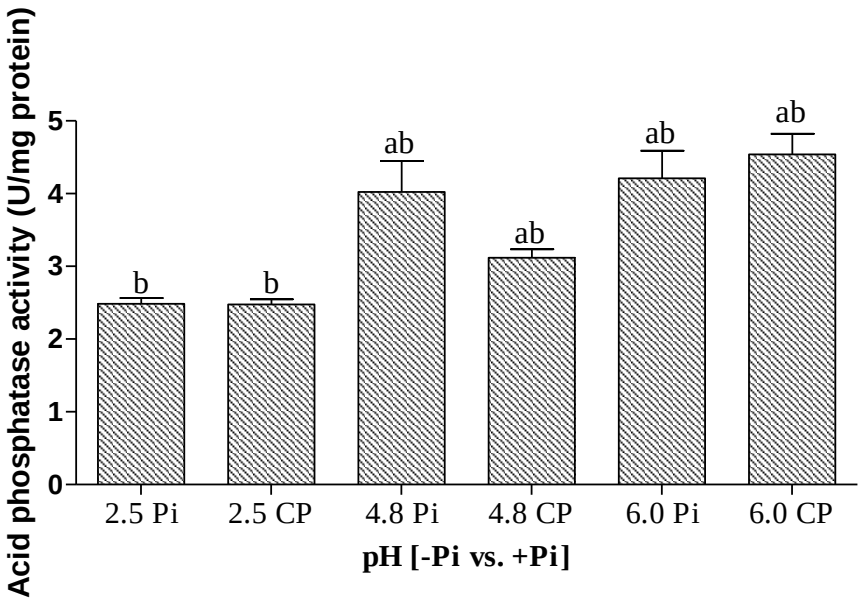

Figure 4.3: Acid phosphatase activity (units/mg protein) in mycelia of *Cladonia portentosa* under Pi-deficiency (Pi) and Pi-enriched (PC) medium. Each treatments consisted of 5 replicates. Means with letters in common are not significant different ($P<0.05$).

4.3.5 Localization of apase using TEM/ EDX

Localization of apase in the mycobiont of *Cladonia portentosa* was studied by cytochemical localization using lead as the capture agent for the inorganic phosphate released from p-nitrophenyl phosphate. Other substrates such as β-GLOB, naphthyl phosphate and phytic acid. were also used. The apase were measured in intact lichen, axenic mycobiont and algal cultures from *Cladonia portentosa.* When the samples were viewed under a light microscope they

appeared efficiently stained, however, the resolution of the light microscope was insufficient for ultra-structure localization of the enzyme (Figure 4.4A).

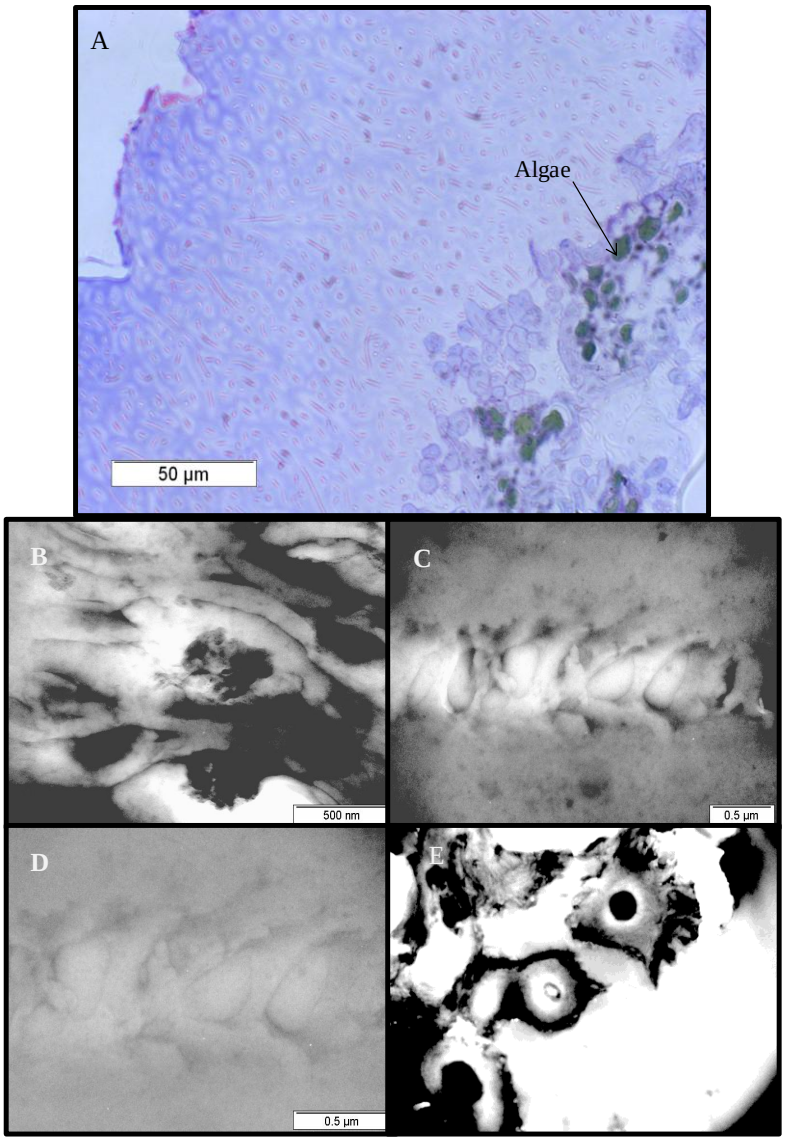

Figure 4.4: (A-E) Cytochemical detection of apase in the lichen *Cladonia portentosa* by transmission electron microscopy (TEM). (A) A cross section of intact lichen thallus viewed by light microscopy. The green spot represents algal cells. (B) Ultra-thin intact lichen sections viewed under TEM. (C) Ultra-thin section of intact lichen, devoid of apase substrate, viewed under TEM. The material was incubated in medium containing 1.0 mM β-GLOB substrate and 2.0 mM lead in 0.1 M sodium acetate buffer (pH 4.8). Lichen thalli incubated with 2.0 mM Pb with no substrate. (D) Ultra thin section of intact lichen, with β-GLOB substrate, viewed under TEM. Lichen thalli incubated with 2.0 mM Pb with no substrate. (E) Ultra thin cross section of mycobiont, viewed under TEM.

The samples were observed under TEM. Several problems were encountered with the samples. When they were subjected to high temperature under the TEM, they were torn and disintegrated. Several attempts were made to stabilize the samples by improving fixation and embedding routine using different pHs (Figure 4.4A-D).

To pin point the specific location of apase (lead), ultra-thin sections of samples were observed by transmission electron microscopy (TEM) (Figure 4.4B-E). No distinct organelle could be differentiated. Micrographs shown in Figure 4.4B and Figure 4.4C did not reveal any intense lead deposits. No major difference was observed in the controls devoid of substrates (Figure 4.4D). Mycobiont cultures were not successful under TEM, as the resin melted when the temperature was increased (Figure 4.4E).

The samples were further viewed by SEM-EDX. EDX analysis determines the element composition of the various micro-structural components (Figure 4.5-4.6; Table 4.3). Since lead was used as a capture agent, i.e. the phosphate released by enzyme would occur as insoluble precipitation, at the site of location of the enzyme, the lead deposits in the tissues could be compared. High deposits of lead on the tissue/organelle indicates the location of the enzyme. Low levels of lead were detected in the structure observed using EDX in the unidentified organelles surrounded by lipids (Figure 4.5A-B). Small organelles which appeared to be rich in lipids were abundant in starved treatments (Figure 4.5B-C). Comparison of the scanned elements indicated that higher lead levels were obtained in the two scanned areas compared to the control (3.34 and 2.34 compared to the control 1.19.)(Figure 4.6, Table 4.3). However, these structures which are surrounded by lipids were not identified (Figure 4.5).

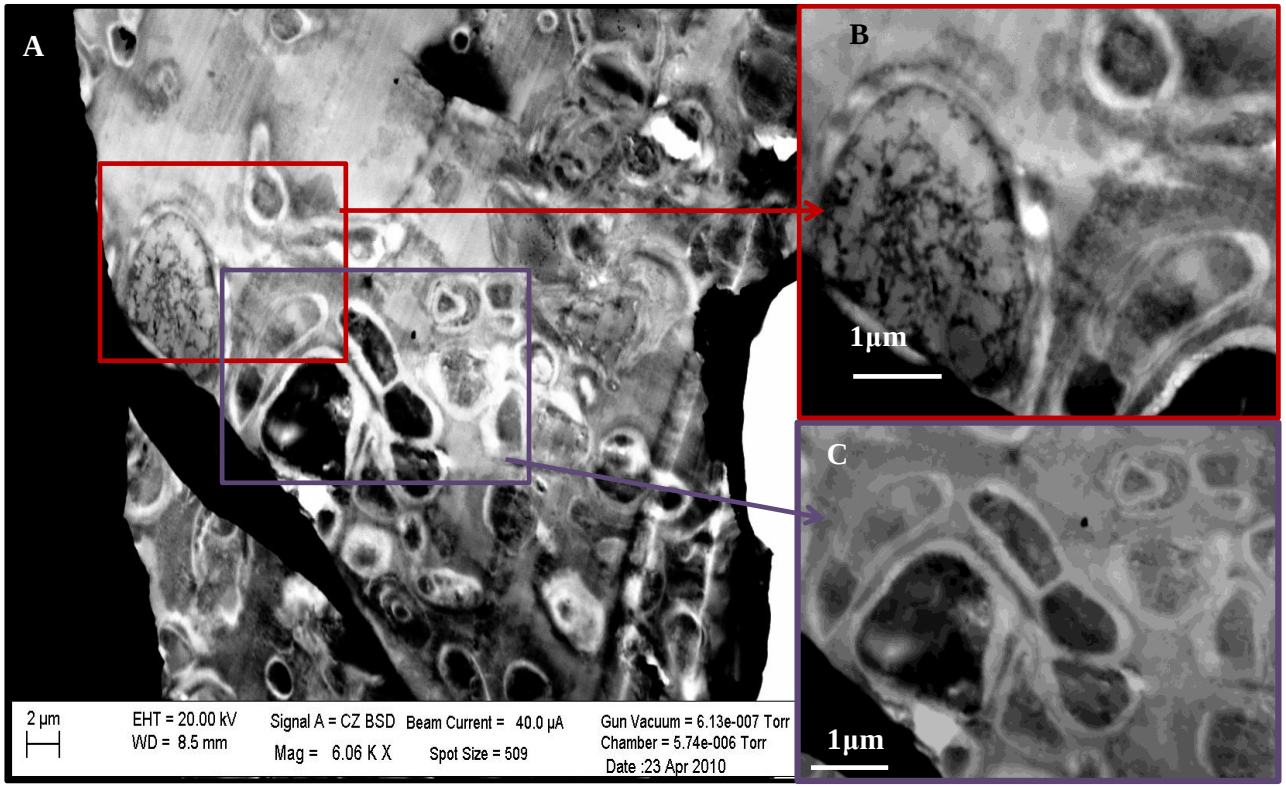

Figure 4.5: (A-C) Ultra structural localization of apase in mycobiont of *Cladonia portentosa* by scanning electron microscopy with energy dispersive X-ray analysis (SEM-EDX). (A) A cross section of mycobiont viewed with EDX. (B) Magnification of the zone in A indicated by a red square. (C) Magnification of the zone in A indicated by purple rectangular block. Bar = 1 µm in A, B, and C. Mag = 4.93 KX SPOT SIZE =445µM.

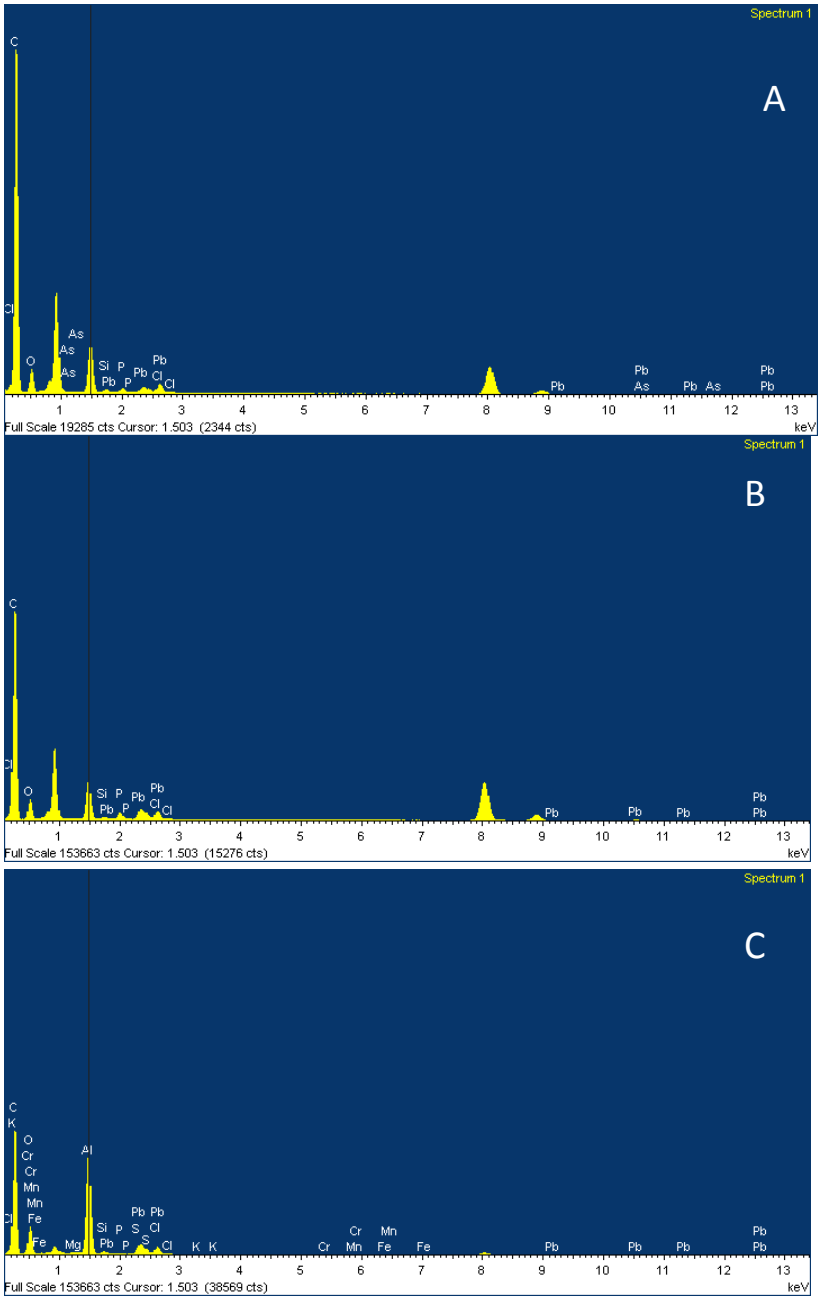

Figure 4.6: (A-C) EDX spectra of different metal elements (yellow). (A) Area 1, (B) area 2 and Control with relatively no lead (Pb).

Table 4.3: Summary of analysis by EDX. Mycobionts were grown in different concentrations of Pi (0.05 vs. 10 mM). The cells were fixed in 2% glutaraldehyde as explained in Section 4.2.2. They were then cut into thin sections by ultra microtome. This Table corresponds with the scan obtained in Figures 4.5-4.6

control			**Area 1**			**Area 2**		
Element	**Weight %**	**Atomic%**	**Element**	**Weight%**	**Atomic%**	**Element**	**Weight %**	**Atomic %**
C K	80.97	86.33	C K	76.69	84.15	C K	68.54	78.35
O K	16.32	13.06	O K	18.03	14.86	O K	19.82	17.01
Si K	0.26	0.12	Si K	0.28	0.13	Si K	0.19	0.09
P K	0.25	0.10	P K	0.67	0.29	P K	0.07	0.03
Cl K	0.75	0.27	Cl K	0.99	0.37	Cl K	0.79	0.31
Pb M	1.19	0.07	Pb M	3.34	0.21	Pb M	2.38	0.16
Totals	100.00		Totals	100.00		100.00		100.00

4.3.6 Fluorescent microscopy

In the absence of the ELF-97 substrate, intact lichen composed of close-netted hyphae appeared blue under UV light while algae cells appeared red (Figure 4.7A). The ELF-97 appeared as green fluorescent granules on the hyphal cells (intact lichen) detected with CASC buffer, pH 2.5 (Figure 4.7B). Localization of the apase in the lichen thallus indicated that the enzyme was exclusively associated with the fungal component as there was a high precipitation of ELF-97 granules in the thallus (Figure 4.7B). When the axenic mycobiont cultures were incubated with ELF-97, the untreated mycelium appeared blue under UV light (Figure 4.7D), whereas the treated samples displayed high precipitation of ELF-97 granules which were neon-green (Figure 4.7E). No apase was detected in the algae hence no ELF-97 fluorescence signals were observed (Figure 4.7C and F). The algae appeared greenish under bright field light (Figure 4.7C).

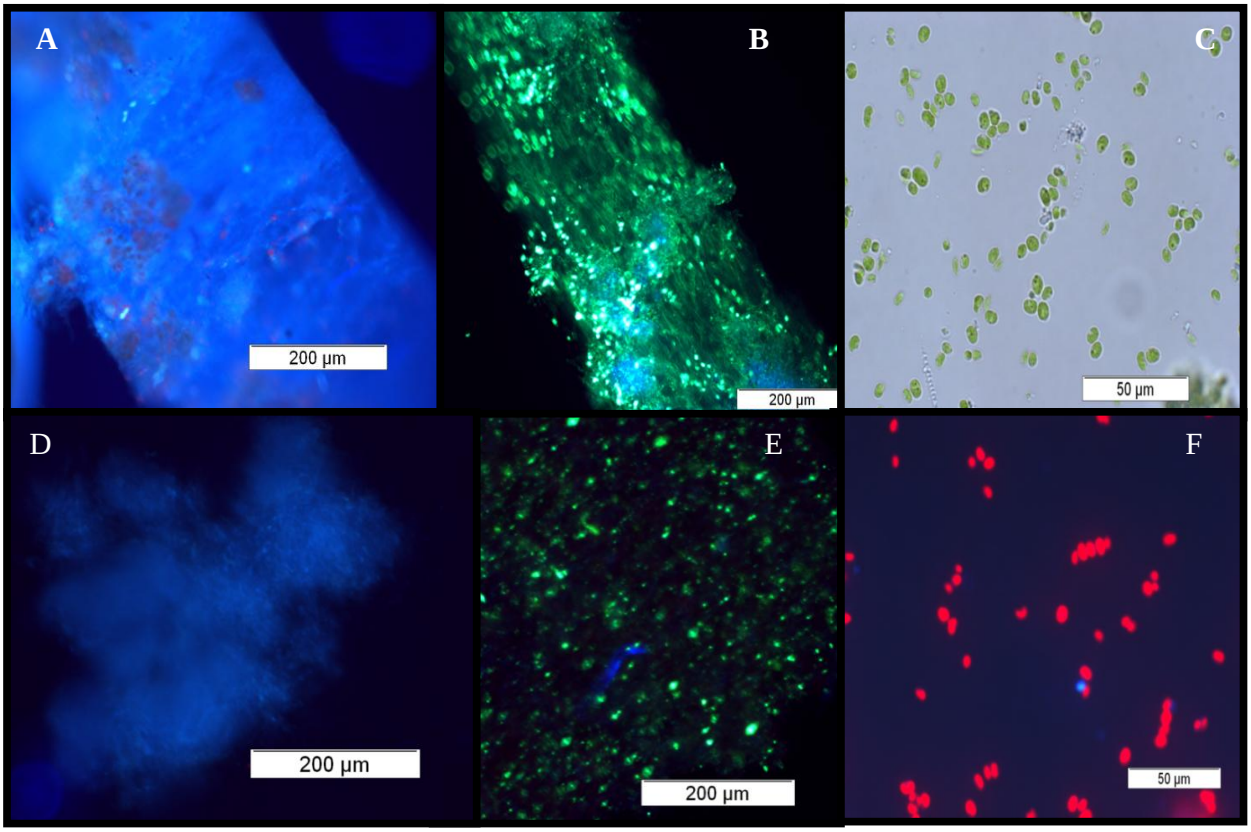

Figure 4.7: (A-E) Lichen thallus visualized using fluorescence microscope. Controls were included to differentiate between natural autofluorescence (blue) and ELF-97 (green) visualized under UV light excitation. (A) Lichen thallus viewed under UV light, (ELF-97 substrate omitted). (B) Lichen thallus with ELF-97 substrate. (C) Bright field image of algal cells (*Trebouxia*). (D) Mycelium with no substrate viewed under UV light. (E) Mycelium with ELF substrate (F) Algal cells with ELF-97, viewed under UV light.

The mycelium grown under different concentrations of Pi (0.05, 1.0, 3.0,10 and 100 mM) were incubated with ELF-97 substrate, using 100 mM CASC buffer, pH 2.5. It must be noted that no direct quantification was made amongst treatments, but the microscopy settings were similar. This was done by mounting a small patch of mycelium consisting of all three treatments on the same slide. Treatments were not mixed. A strong fluorescence was detected in the axenic mycelium at the lowest concentrations (0.05, 1.0 and 3.0 mM) (Figure 4.8A-D). At the lowest concentration, it was observed that the bigger clusters of mycelium did not fluoresce but it appeared blue as did the untreated control (Figure 4.8A-B). At 10 mM Pi, a strong fluorescent signal was detected compared to 100 mM Pi, where ELF granules appeared very sparse under the microscopy settings. In addition, when 10 mM NaF was used as an inhibitor, no apases were

detected, whereas a strong fluorescence was detected in the presence of 5.0 mM levimisole. These results confirm that the phosphatase activity observed was due to an acid phosphatase rather than an alkaline phosphatase.

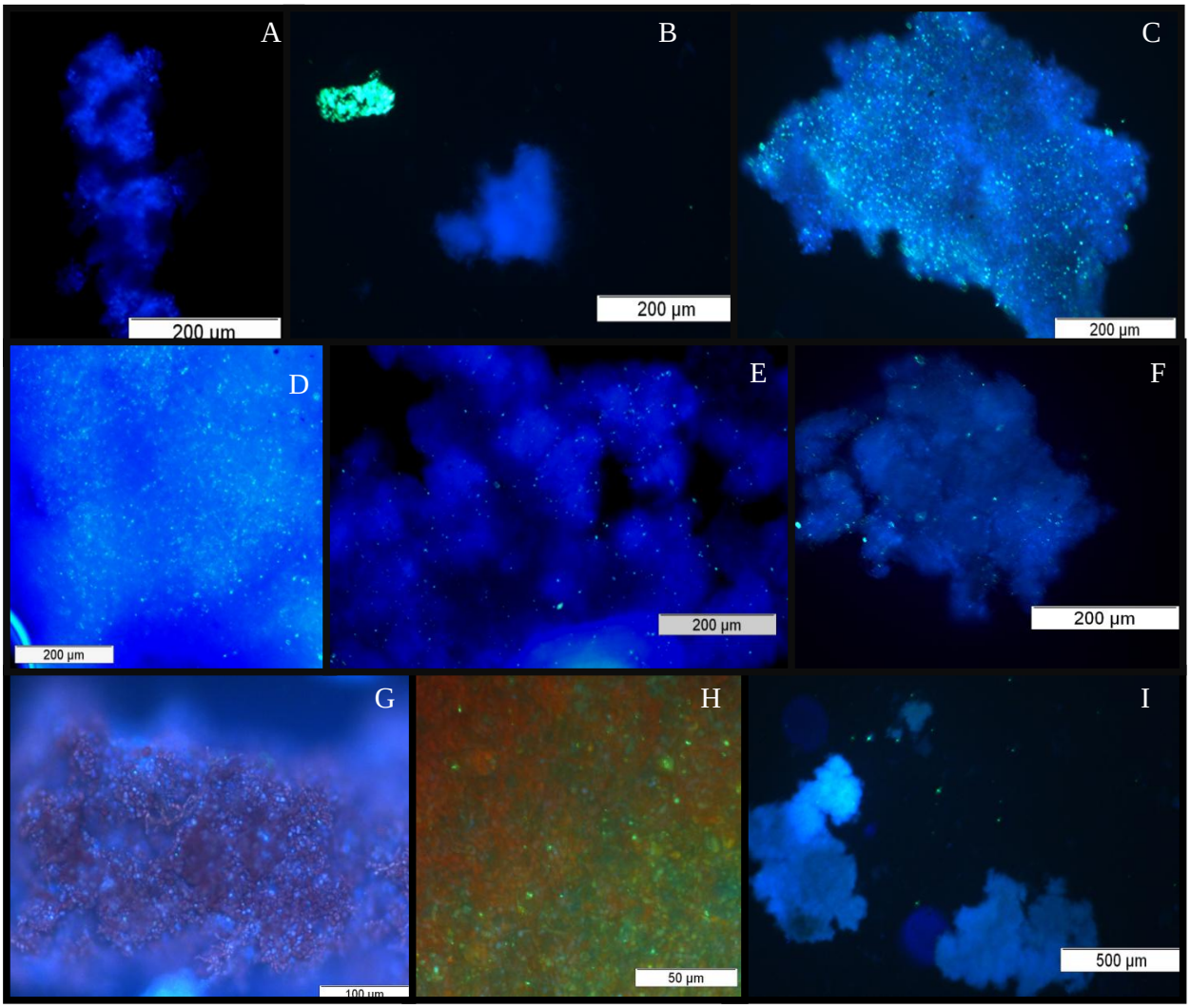

Figure 4.8: (A-F) Axenic mycelium grown under different concentration of Pi (0.05, 1.0, 3.0, 10 and 100 mM KH_2PO_4) incubated with ELF-97 viewed by fluorescence microscopy. (A) A control mycelium, devoid of ELF-97 substrate. (B) Fungi grown under 0.05 mM Pi, with smaller patch displaying strong intensity of ELF-97 signals. (C-D). Mycelium grown at 1.0 and 3.0 mM treatments displaying almost similar reactions to ELF-97. (E) Mycelium grown under 10 mM Pi displaying granules of ELF-97 substrates. (F) Mycelium grown at 100 mM with ELF-97 signals appearing as sparse granules. (G) Mycelium (0.05 mM) co-labelled with ELF-97 and propidium iodide. (H) Mycelium (0.05 mM) co-labelled with ELF-97 and acridine orange. (I) Mycelium (100 mM) co-labelled with ELF-97 and Propidium iodide.

To ascertain the viability of the mycelium, ELF-97 was co-stained with either propidium iodide or acridine orange dye (usually use to measure apoptosis). Big clusters of mycelium grown under Pi-starved conditions, stained red (dead) with only a few patches remaining blue, indicating viability when propidium iodide was used (Figure 4.8G). The mycelium grown at 1.0, 3.0, 10 and 100 mM Pi also partially stained red but the bigger clusters remained blue. It was obvious that the mycelia were at different stages of growth. The fluorescence of ELF-97 could be detected after co-labelling as indicated by green precipitation (H-I). In addition, a similar reaction was observed with acridine orange, where dead cells were stained orange and live cells were blue (Figure 4.8H).

To further illustrate the localization of the enzyme, higher magnification was used in connection with co-staining with Hoechst dye and FM4-64. A strong fluorescence was observed but no defined structures could be observed using fluorescence microscopy. Furthermore, no nucleus differentiation was observed after staining with Hoechst dye but the mycelium appeared blue. When the mycelium was stained with FM4-64, the red colour was intensified when viewed under NIBA but no membrane organelles were observed.

The mycelia were observed to be at different stages of development in different treatments. When viewed under UV light, mycelium grown at 100 mM Pi, displayed sparse ELF-97 granules compared to 10 mM Pi where the fluorescence was stronger (Figure 4.9A and B). Using bright field settings (100x), it was observed the cells at 100 mM Pi were growing vigorously and were dividing (Figure 4.9B). Compartmentalization partitioned by septa was distinct (Figure 4.9B). A mature vacuole was observed in the compartment in the cellular region delimited by septa. Apical branching (AB) to form new tips in mature hyphae were clearly distinguished (Figure 4.9B). Enlarged and globular germlings (G) could be seen at the hyphal tip (Figure 4.9B). Hyphal swelling was often observed, formed on the edge of contiguous cells, resembling appressoria (Figure 4.9B). Under UV light, a stronger ELF-97 fluorescence was observed at 10 mM Pi compared to 100 mM Pi (Figure 4.9C). Mycelia grown under 10 mM were more coiled, but germlings and apical branching were observed (Figure 4.9D). The mycelium grown at lower Pi concentrations, appeared stunted, the cells were much smaller and some parts of the mycelium were not viable (Figure 4.9E-F).

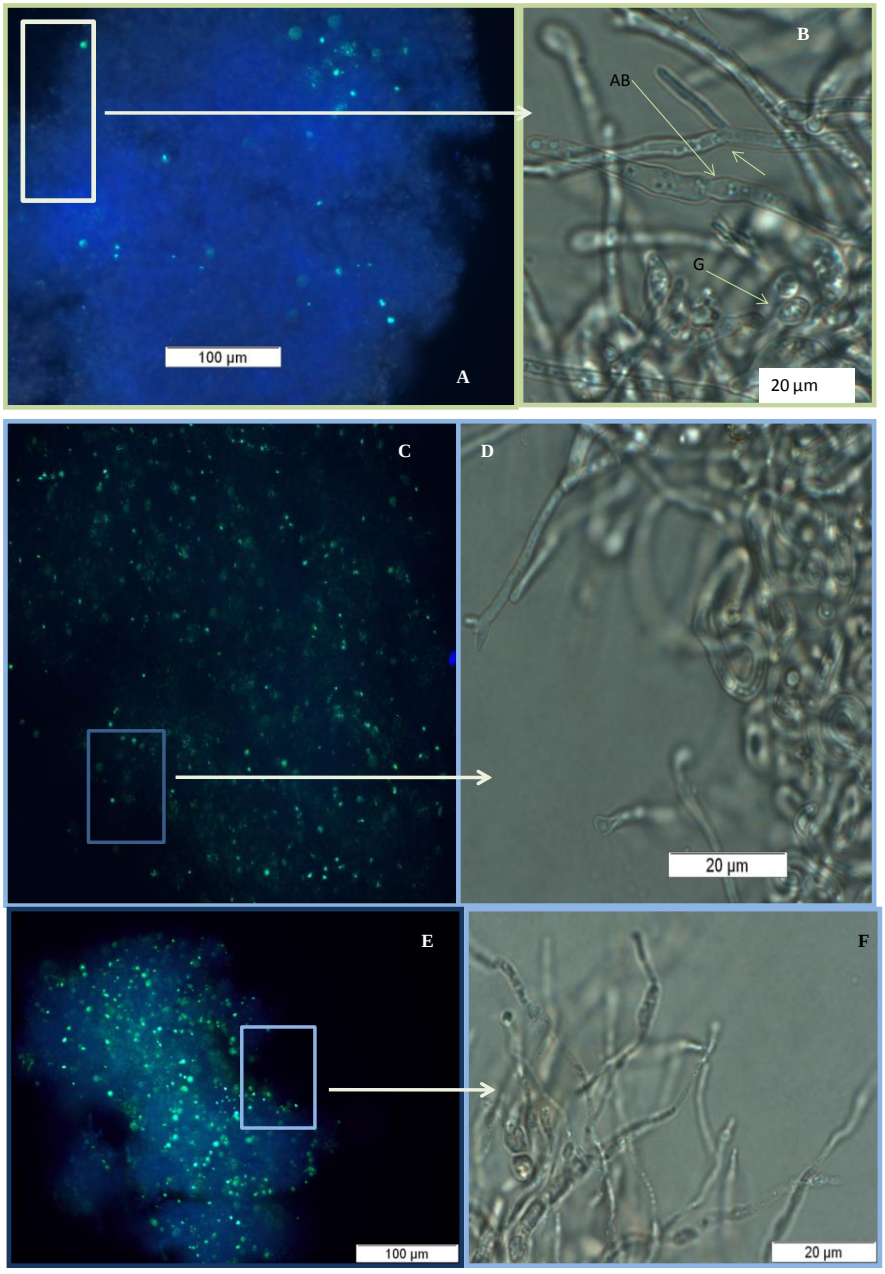

Figure 4.9: (A-F) Axenic mycelium with under different concentration of Pi (100, 10 and 0.05 mM) incubated with ELF-97 viewed with fluorescent microscope. (A) Fungi grown in 100 mM Pi with ELF-97 fluorescence appearing as sparse granules. Bar =20 µm. (B) Magnification of the zone in A, viewed under bright field, with mycelium grown at 100 mM, observed with germlings (G) and apical branching (AB). (C) Mycelium grown under 10 mM Pi, showing intense ELF-97 fluorescence, appearing as green granules. (D) Magnification of the zone in C, observed under bright field with mycelium grown at 10 mM. (E) Mycelium grown under 0.05 mM Pi, showing intense ELF-97 fluorescence, appearing as green granules. (F) Magnification of the zone in E, showing mycelium grown under low Pi, under bright field light. Bar =100 µm.

However, strong fluorescence was observed at 0.05 mM, mainly on the edge of the hyphae (Figure 4.7E). Because of the relatively low level of the resolution given by light microscopy, it was impossible to localize the reactions precisely in the mycobiont. Extending incubation time and increasing the ELF-97 did not enhance any staining pattern to allow any localization at the ultrastructural level. To gain a better understanding of the localization of the enzyme, the confocal microscope was used as an alternative.

4.3.7 Confocal microscopy

Vector blue substrate was excited using a 488 argon laser. In general, the intact lichen displayed very strong autofluorescence (Figure 4.10A-E), which interfered with substrates, since some pigments such as chlorophyll (from algae) were detected in the same wavelength range (Figure 4.10A-E) as the substrate. There were other fluorescing compounds that were exuded by the thallus (Figure 4.10E). Intact lichen was therefore excluded from this experiment, all the subsequent experiments were conducted using mycobiont cultures.

The microscopy settings were optimized by a control devoid of the substrate until all background and autofluorescence were eliminated (Figure 4.11A). The fluorescence was allocated a green pseudo colour. Strong fluorescence were detected in all three treatments viewed with microscopy after staining for 30 min (Figure 4.11B-I). In the absence of substrate, the cytoplasm looked empty (Figure 4.11A and E). At low Pi (0.05 mM), fluorescence was distributed all over the mycelium, covering the cytoplasm and cell membrane (Figure 4.11B and F). Furthermore, in Pi-starved medium, the hyphae appeared distorted with coralloid growth, hyphal swelling and short stunted branches (Figure 4.11B, F and G). At a moderate treatment (10 mM) the fluorescence was detected in cytoplasm and also in small round structure, presumed to be vacuoles (Figure 4.11 C). The presence of appresoria-like structures were often observed, which were characterized by swelling of hyphae (Figure 4.11A, C and H). This was mainly observed in older cultures. Using a younger mycelium, the fluorescence was detected in big round structures and small round structures presumed to be endosomes (Figure 4.11H). At a higher treatment (100 mM Pi), fluorescence was observed in the small and big round structures, and none were detected in the cytoplasm lumen (Figure 4.11D and I).

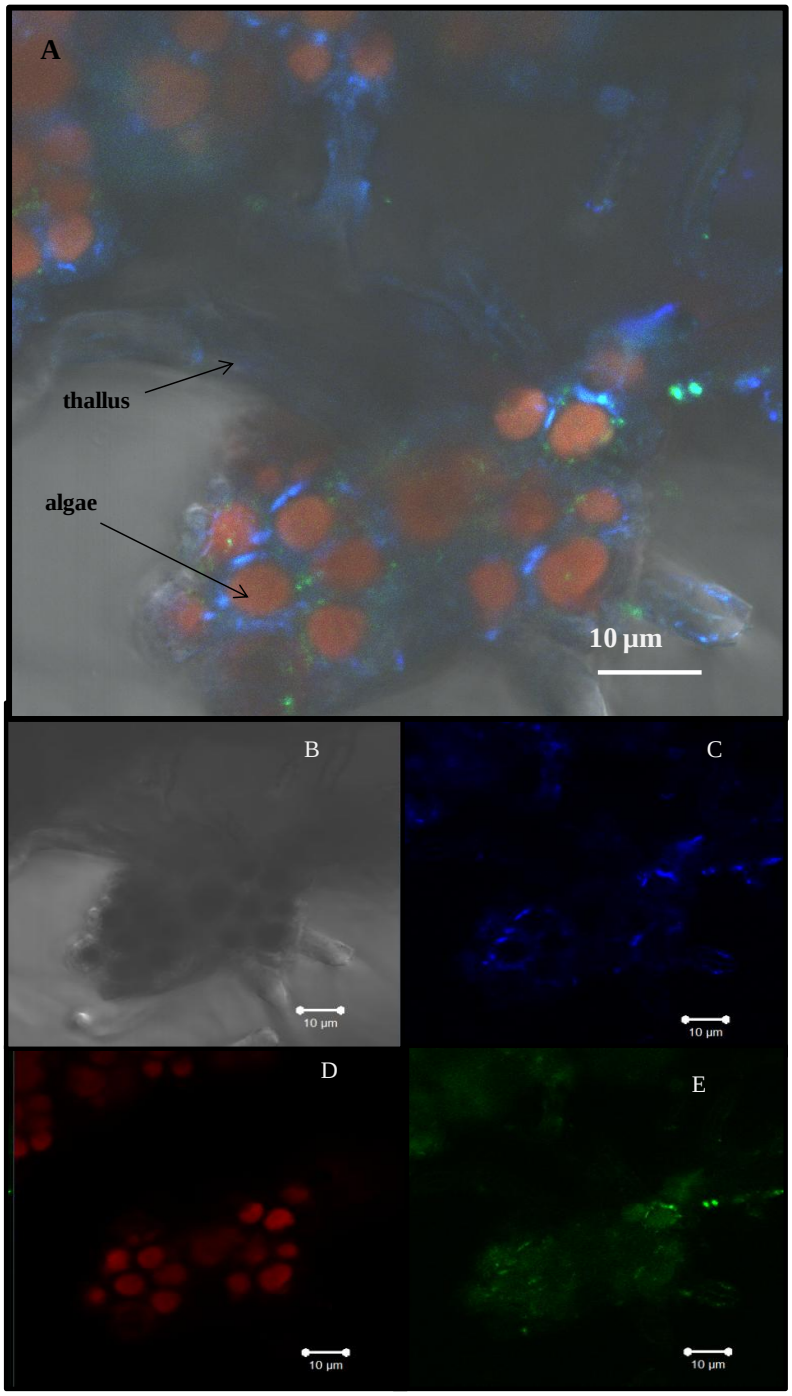

Figure 4.10: (A-E) Lichen thallus visualized using confocal microscope. The thallus was visualized without any substrate, to ascertain the extent of autofluorescence in the sample. (A) Combined image, where algae can be seen as red round spots and lichen thallus as blue. (B) DIC image visualized as grey. (C) Natural autofluorescence (blue) visualized under 488 laser excitation. (D) Algal cells as red spot (E) Unknown flourescing substance in the hyphae. Bar = 10 µM.

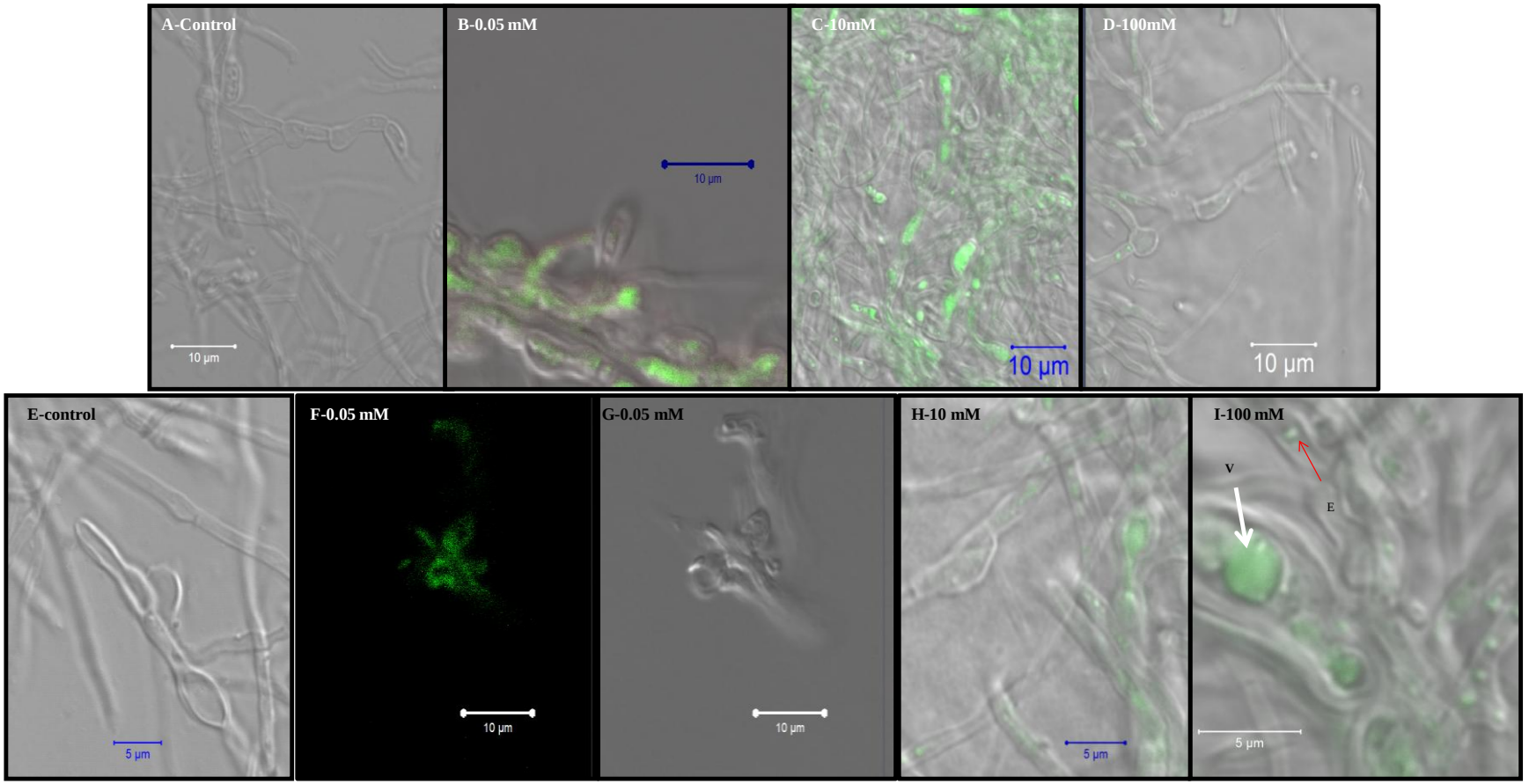

Figure 4.11: (A-I) Mycelium stained with Vector blue III substrate (VB), viewed with a confocal microscope. (A) In the control treatment (devoid of substrate), the cytoplasm appeared empty. (B) The distribution of VB could be seen in the cytoplasm, membrane and surface of the hyphae. (C) Strong fluorescence was visualized at 10 mM Pi. (D) Apase detected at 100 mM Pi treatment. (E) Control treatment devoid of substrate. (F) VB fluorescence was distributed all over the cells in older cultures with a treatment of 0.5 mM Pi. (G) DIC image of F. (H) Fluorescence detected from the cytoplasm as well as vacuole from 10 mM Pi treatment. (I) A detailed image of fluorescing organelles from the mycelium cultured under 100 mM, where apase could be seen in the vacuoles and small structures presumed to be endosomes (E) or prevacuoles (V).

To verify the nature of the organelles stained by vector blue substrate, and to rule out that this was not an artefact of fixation, the mycelium was stained only with a vital stain, FM4-64. The mycelia were analyzed by confocal microscopy after 15-30 min initial incubation with FM4-64. To differentiate the autofluorescence from FM4-64, controls were measured first to establish their maximum excitation. The natural autofluorescence was allocated a green pseudo-colour and fluorescence by FM4-64 substrate was allocated red to make a contrast. Autofluorescence (AF) appeared as a cloud of green (Figure 4.12A). FM4-64 was excited by a laser 488 nm. At low Pi (0.05 mM), poor internalisation of FM4-64 dye was observed (Figure 4.12A). The hyphal cell did not incorporate the stain but it was scattered on the surface (Figure 4.12A). The mycelium was damaged and stunted.

Using 1.0 mM Pi, strong FM4-64 fluorescence was observed, which appeared like strong red dots (Figure 4.12B). The FM4-64 was noted to stain only round punctuate organelles; no staining of the cell membrane was observed (Figure 4.12B-C). FM4-64 was seen as a strong red fluorescent spot on the mycelium which were distinct from autofluorescence displayed by mycelium in general (Figure 4.12C-F). It was noted that, at 1.0 mM Pi treatment, the mycelium looks stunted, swollen and starting to form colloid growth (Figure 4.12C). The FM4-64 staining pattern identified several red punctuate structures (red fluorescence) of different sizes which were more numerous in the Pi-starved cultures (Figure 4.12C-F). At 100 mM Pi, few fluorescing organelles were observed compared to those found at 1.0 mM (Figure 4.12G-L). Relatively large organelles were found in the higher treatment (100 mM Pi). For instance, the organelle in Figure 4.12G, identified as a mature vacuole, was located in the cytoplasm and separated by a distinct septum wall (Figure 4.12G).

Several organelles were identified in mycelium grown at 1.0 mM (Figure 4.13A-D). On closer observation, the apical region known as Spitzenköper (SPK), satellite Spitzenköper (SSPK) were observed at the edge of the hyphae (Figure 4.13D). A cluster of red punctuate structures (E), thought to be endosomes could also be seen. The bigger round clusters were identified as mature vacuoles (V) (Figure 4.13D). The staining of FM4-64 appeared to follow a hypothetical model of the organization of the vesicle trafficking network in a growing hypha based upon the pattern of FM4-64 staining (Figure 4.13E, adapted from **FISCHER-PARTON *et al.*, 2000)**.

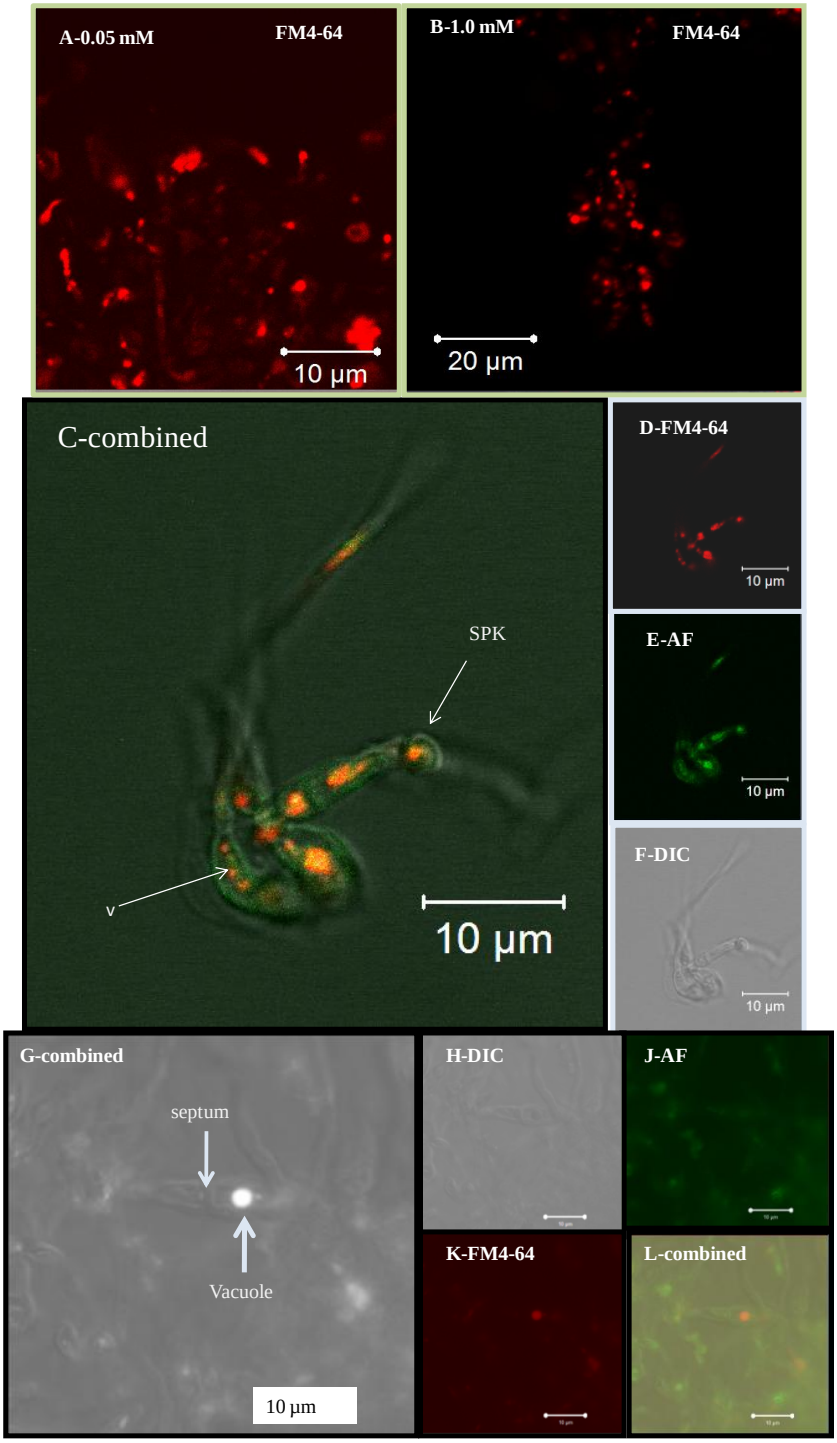

Figure 4.12: (A-L) Confocal images of the mycelium of *C. portentosa* displaying the internalization of FM4-64. (A) 0.05 mM Pi after staining with FM4-64. (B) 1.0 mM Pi after staining with FM4-64. (C) A detailed image of 1.0 mM Pi. Note numerous fluorescing organelles. (D) FM4-64. (E) Autofluorescence. (F) DIC image. (G) 100 mM Pi after staining with FM4-64. Note, the absent of small fluorescing organelles. A mature vacuole was easily distinguished (white). (H) DIC image. (J) Autofluorescence. (K) FM4-64 staining. (L) A combined image.

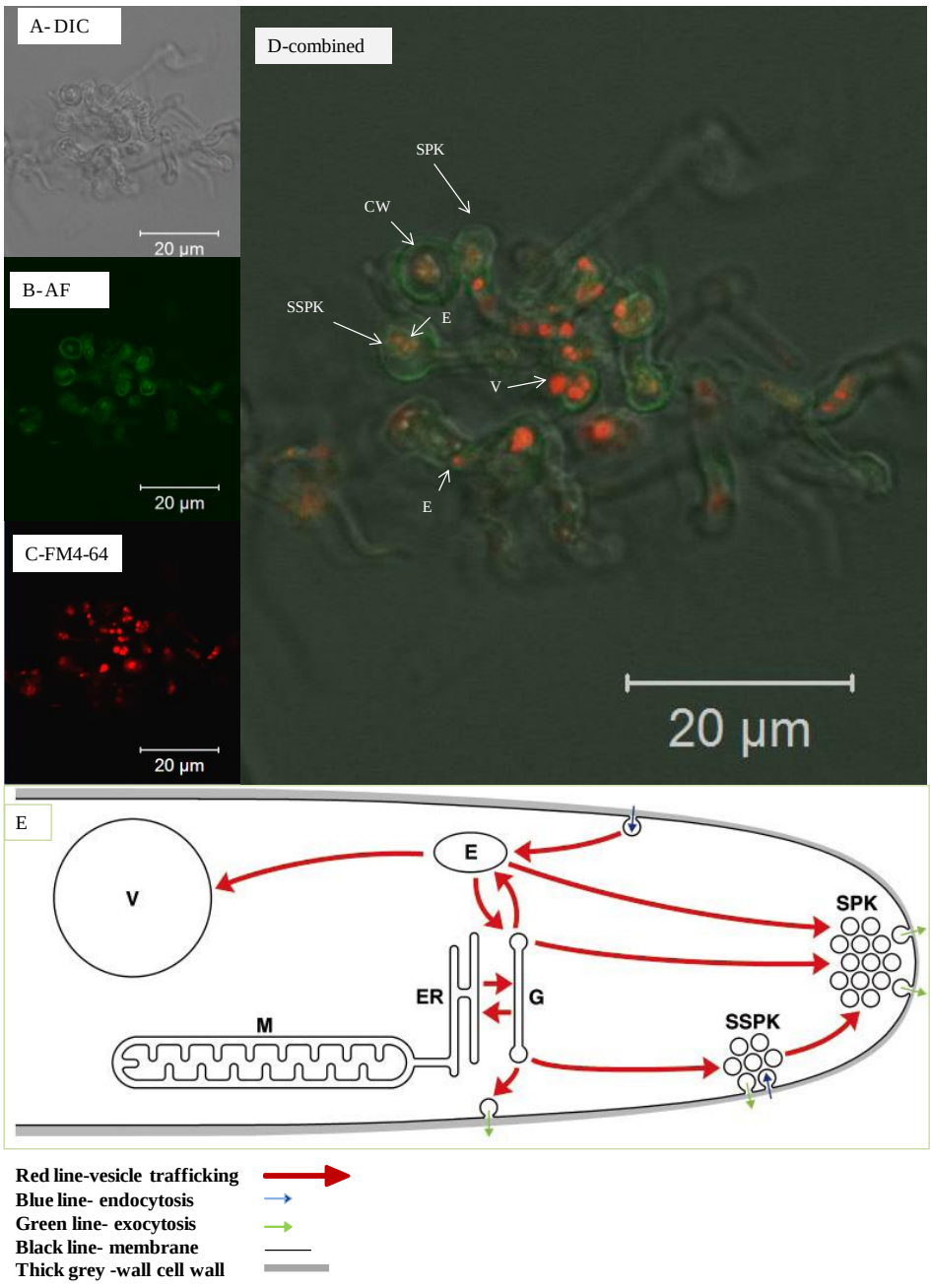

Figure 4.13: (A-D) Internalization of the FM4-64 by living hyphae grown under phosphorus starvation (1.0 mM Pi). (A) DIC represents an image under bright field light. (B) Autofluorescence (AF) seen in the background as green. (C) Fm4-64 fluorescent viewed as red dots. E = endosomes, V = vacuoles, SPK = satellite Spitzenköper and S = representing apical region known as Spitzenköper. (E) A hypothetical model of the organization of the vesicle trafficking network in a growing hypha based upon the pattern of FM4-64 staining (adapted from **FISCHER-PARTON *et al.*, 2000**).

In a second experiment, two fluorophores were used to label the mycelium. The mycelium was stained with Vector blue III first and then later co-labelled with FM4-64. The two stains were excited by two lasers, as described before and pseudo-colour green allocated to vector blue and red to FM4-64. At 100 mM Pi, colocalization of FM4-64 and vector blue was observed (Figure 4.14A-E).

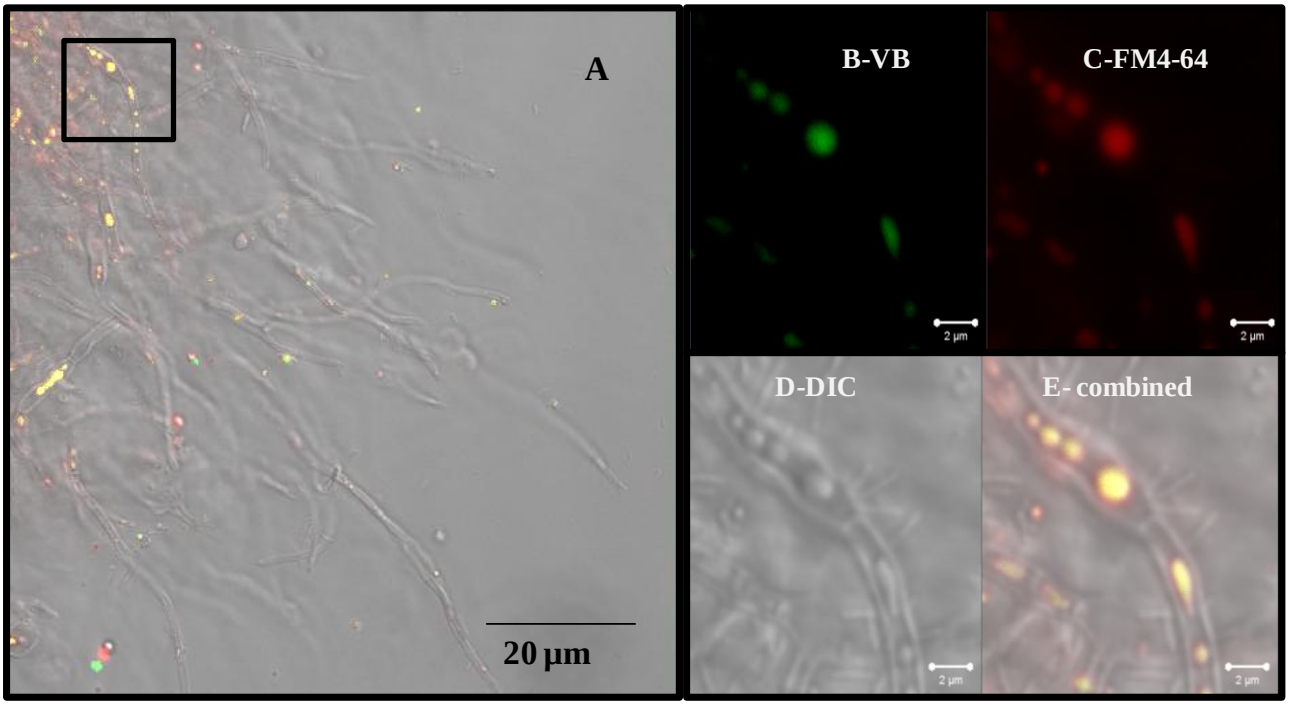

Figure 4.14: (A-E) Interactive representation of apical branching of mycelium of *C. portentosa*, showing a colocalization of VB and FM4-64 after co-labelling. The mycelium was grown at 100 mM Pi, co-stained with Vector blue and FM-4-64. (A) Combined image of the fungus (gray). (B) Vector blue. (C) FM4-64 (red). (D) DIC image. (E) Magnification of the zone in A, displaying colocalization of VB and FM4-64 (yellow). Note the enzyme is clearly represented by green pseudo colour, whereas FM4-64 is represented by red colour. Note the staining of different shaped organelles by both fluorophores. Bars = 2 µm.

At low concentration, there was a lot of background. It appeared that the fungus at this stage was dead, as no internalization was observed. Staining with vector blue revealed that the enzyme was all over the surface of the mycelium. However, the internalization of FM4-64 was not observed.

The colocalization (Figure 4.14E) was further confirmed by ImageJ Analysis where the Pearson coefficient was 0.88, indicating a very strong colocalization. Colocalization was first analyzed using isotropic structures using the **VAN STEENSEL *et al*. (1996)** method, which could distinguish between colocalization, exclusion and unrelated signals. A Pearson‘s coefficient where r=0.804 was obtained. These results revealed that the enzyme was found in the same organelles that were stained by FM4-64.

4.4 GENERAL DISCUSSION

4.4.1 The influence of Pi on the activity of apase

A concentration of 3.0 mM Pi, marginally increased the activity of apase compared to higher Pi treatment (100 mM) (Table 4.2). This confirmed that acid phosphatase in *Cladonia portentosa* is negatively regulated by the concentration of Pi as has been demonstrated in different fungi **(SHIEH *et al*., 1969; YOSHIDA and TAMIYA, 1971; HAN *et al*., 1994)**.

Even though high apase activity was detected in Pi-starved vs. P-sufficient cultures, it was not of the magnitude reported in other studies. For instance, in *Saccharomyces cerevisiae* a 1000 fold increase of apase was reported in Pi-starved cultures versus Pi-sufficient cultures **(ELORZA *et al.*, 1978)**. A three-fold increase of apase was reported by **HIDAYAT *et al*., 2005** when *Aspergillus niger* was grown as phosphate-limited cultures. In the filamentous fungus, *Humicola lutea,* a reduction of Pi from 1.0 to 0.01g/l resulted in a 5-fold increase of apase **(ALEKSIEVA and MICHEVA-VITEVA, 2000)**. Interestingly, apase activity was still detected at the highest concentration (100 mM Pi) even though it was present in low levels compared to 3.0 mM or 10 mM (Table 4.2). Constitutive acid phosphatase has been reported not to be influenced by the Pi concentration in the medium **(WEBER and PITT, 1997a)**. In *Botrytis cinerea*, Pi starvation, led to an 80-fold increase in the activity of acid phosphatase exported into the culture fluid, which was reported to be due to the secretion of a novel Pi-repressible enzyme form, in addition to low levels of a constitutive form being secreted irrespective of the Pi status of the medium **(WEBER and PITT, 1997a)**. Electrophoretic characterizations of repressible and inducible form/s of apases were not successful, probably due to insufficient protein quantity.

The level of apase synthesized was dependent on the initial concentration of inorganic phosphate in the medium (Table 4.2). The maximum activity of apase was obtained when the concentration of Pi was 3.0 mM. This suggests that 3.0 mM was the crucial concentration, which played an important role in the direction of the fungal metabolism towards enhanced apase synthesis. Beyond this concentration, competition for Pi resulted in decreasing metabolic activity of the mycelium, which was evident from overall mycelia weight (Table 4.2) **(SHIEH *et al*., 1969)**. An average dry weight of 0.096 g was obtained at 3.0 mM Pi compared to 0. 105 g

obtained at 10 mM Pi (Table 4.2). The results indicate that P is directly involved in fungal morphogenesis (**SCHWEINGRUBER and SCHWEINGRUBER, 1981)**. Increased apase activity detected at 3.0 mM Pi also indicated that the fungus was already starved of Pi, probably due to the prolonged culture conditions.

4.4.2 The influence of pH on the activity of apase

Acid phosphatase found in *Cladonia portentosa* have been shown to have an optimum pH of 2.5 **(HOGAN *et al.,* 2010a)**. Thus, high apase activity was expected in culture medium with a pH 2.5. However, the highest apase activity was detected at pH 4.8 and 6.0 (Figure 4.3). There are several reasons that might have contributed to this shift. In nature, as nutrients get depleted, the pH usually drops due to acidification since most fungi have been shown to accumulate citric acid after metabolization of carbohydrates. In the study conducted by **ASERI *et al*. (2009**), acidification of the media with time was observed in several fungal species. For instance, *Trichoderma harzianum* exhibited a marked decline in pH's of culture (7.3-2.4) after 4 weeks, followed by *Trichoderma viride* (7.3-3.0) and *Penicillium simplicissimum* (7.3-5.5) **(ASERI *et al*., 2009**). Although, the pH of culture media were not measured after the termination of the study, it is possible that the acidification in medium occurred due to the release of citric and oxalic acid. This pH shift at 6.0 and 4.8 eventually became close to those that would induce optimal synthesis of apase. At pH 2.5, it is possible that pH shift was below the critical mark where apase biosynthesis was possible, with low pH levels, probably becoming toxic to the cells; hence reducing apase activity.

Several studies have demonstrated that the biosynthesis of acid phosphatase enzymes is under pH regulation **(CADDICK *et al*., 1986b)**. It is logical to expect that most enzymes in *Cladonia portentosa* lichen would have a preference for an acidic medium, probably matching that of natural soil in order for hydrolase enzymes to carry their normal physiological function/s. Most secreted enzymes, permeases and exported metabolites, all of which must function at ambient pH are strongly influenced by pH **(NAHAS *et al*., 1982; FOSTER, 1999)**.

This biosynthesis of phosphatase enzymes under pH regulation has been shown to be useful in organisms such as *Aspergillus nidulans*, which can grow, under a wide range of pH values (2.5-9.0) **(CADDICK *et al.*, 1986b)**. Such a pH regulatory system ensures that extracellular enzymes and exported metabolites are produced under conditions of pH where they can function: for example acid phosphatase under acid conditions and alkaline phosphatase under alkaline conditions **(CADDICK *et al.*, 1986a; 1986b; DENISON, 2000)**. In *Saccharomyces cerevisiae* high apase activity was found in low Pi medium at pH 4.7 **(BOSTIAN *et al.*, 1983)**. In *Aspergillus*, maximum apase activity was observed in cultures grown at pH 2.8 and 6.3 and apase production was almost completely suppressed in a culture grown at pH 7.3 **(HIDAYAT *et al.*, 2005)**.

The pH control, ensures that gene regulation can respond appropriately to ambient pH, for example, in responses to changes in extracellular pH many organism can control factors that affect their growth including stress and virulence **(OLSON, 1993; BLANKENHORN *et al.*, 1999)**.

4.4.3 The effect of oxidative stress on apase activity

Menadione have been reported to induce a cascade of defense responses since it is a redox-cycling quinone known to cause oxidative stress. The aim of this experiment was to investigate the role of acid phosphatase under Pi starvation versus oxidative stress. In addition to their role in phosphorus acquisition it was hypothesized that acid phosphatase plays a defensive or protecting role against oxidative damage. In this study, the relationship between Pi (KH_2PO_4) metabolism and oxidative stress on acid phosphatase was analyzed.

A marginal increase in apase activity was found when the mycelium was grown under low menadione and low Pi (Figure 4.2). However, the results were puzzling and not conclusive enough. Several factors may have contributed to misleading results. Menadione caused oxidative stress, thus causing the production of ROS. Overproduction of ROS have been shown to damage cellular lipids, nucleic acids, proteins and leads to lipid peroxidation, genome instability or gene mutation, protein carbonyl formation and enzymatic inactivity resulting in

degenerative processes leading to aging **(MARTIN *et al.*, 1996; BERLETT and STADTMAN, 1997; FINKEL and HOLBROOK, 2000)**. Since many cellular organelles are surrounded with lipid membranes that get damaged due to lipid peroxidation or due to oxidative stress, a damaged membrane loses its fluidity and integrity. An increase in activity was only detected at low menadione concentration, it is possible that menadione enhanced the activity of acid phosphates in the mycelium. However, excessive oxidative stress might have markedly affected the metabolic systems and thus decreased the activity of the enzyme.

4.4.4 Cytochemical localization of acid phosphatase using Gomori stain under TEM

The cytochemical reaction described by **GOMORI (1950)** has been used in this work, employing intact lichens, mycobiont and algal cells. Several attempts were made to locate apase in samples using TEM; however, due to poor fixation this was not successful. Low levels of lead were detected in the structure observed using EDX in the unidentified organelles surrounded by lipids (Figure 4.5). Although there was a difference between the treatment and control, the results were not conclusive since other structures could not be identified. Lead has been used widely to trap the phosphate released at the enzymic site as an insoluble phosphate precipitate **(GOMORI, 1950)**. However, this technique using lead has been criticized by several authors because it may produce false positives and some enzymes are reported to be sensitive to lead **(HOEFSMIT *et al.*, 1986; KATZ *et al.*, 1988; CHAUHAN *et al.*, 1991)**. In this study it was not clear whether; the poor localization was also due to the apase in lichen being sensitive to lead. Apase enzyme isolated from lichen (Chapter 3, Section 3.2, Table 3.6) was sensitive to lead. Thus attempts to localize the enzyme by fluorescent substrates were explored.

4.4.5 Localization of acid phosphatase using fluorescent substrates

Localization of apase using the ELF-97 substrate was found to be a sensitive and simple method, corresponding with the findings described for several fungi **(VAN AARLE *et al.*, 2001)**. The lichen and fungal mycelium generally gave a blue auto-fluorescence under UV light when viewed under a fluorescence microscope whereas the reaction of ELF-97 was distinctly

bright green (Figure 4.7). Localization of the lichen thallus indicated that the enzyme was exclusively associated with the fungal component (Figure 4.7A-H). These results are in agreement with those obtained by **HOGAN *et al*. (2010a and b)**, where it was demonstrated that in lichen *Cladonia portentosa*, apase was exclusively associated with the fungus. However, direct quantification of the fluorescence substrate was not conducted, unlike in the study of **VAN AARLE *et al*. (2001)** where quantification was conducted by measuring ELF-97 fluorescent spot intensity using a scanning confocal microscope.

Although no quantification was conducted using fluorescent microscopy, under the same microscopy settings, stronger fluorescence signals were observed in Pi-starved treatments compare to Pi-sufficient cells (Figure 4.8). Similar results were also reported by **VAN AARLE *et al*. (2002; 2005)** where the presence of a high phosphorus concentration in the initial growth medium led to a decrease in ELF-97 precipitation in the hyphae compared with phosphorus-starved hyphae. The result were further confirmed by confocal microscopy studies, where under phosphorus starvation, the apase enzyme was found on the cell surface, covering the entire cytoplasm, the vacuoles and endosome structures (Figure 4.11). Filamentous fungi are known to possess apase in vacuole and lysosomes **(MALKE, 1998)**. These findings are in agreement with localization of secreted apase under Pi starvation in fungi such as *Botrytis cinerea* and *Humicola lutea*, where it has been shown to be located in the periplasmic space, vacuole, cell wall and cell membrane and secretory vesicles **(HÄNSSLER *et al*., 1975; ARNOLD and LACY, 1977; WEBER and PITT, 1997a; ALEKSIEVA *et al*., 2003**). In addition, studies by **BEEVER and BURNS (1980)** demonstrated that acid phosphatase was secreted in large quantities, especially by hyphae starved of inorganic phosphate, presumably in an effort to solubilize phosphate from sources in the environment.

In phosphorus-starved cultures, the fluorescence appeared to be diffused over the entire surface of the hyphae (Figure 4.11B, C and F). The diffusion pattern of apase localized by lead precipitation in fungus *Claviceps purpurea* was thought to be due to glycosylation **(VORÍSEK and KALACHOVÁ, 2003)**. Although similar fluorescence diffusion was observed in this study more work needs to be conducted to confirm the nature of secreted apase in *C. portentosa*, isolated from the mycobiont.

Stronger ELF-97 fluorescence was generally found in small clusters and on the edge of the hyphae; probably since this is the active region in terms of growth of new cells. It has been reported that secretory vesicles, deliver membrane, cell wall precursors and wall-building enzymes to the hyphal tip and many extracellular enzymes are released into the surrounding medium are secreted from this region **(WESSELS, 1993)**.

To ascertain the viability of the *C. portentosa* mycelium, the nucleic acids of the damaged cells (leaky) were stained with propidium iodide. This dye only enters cells with damaged membranes and substantially enhances fluorescence by binding to nucleic acid with little or no sequence preference **(HAUGLAND, 2002)**. Co-staining with ELF-97 and PI revealed that some of these clusters were not viable. Bigger patches of red were found in the Pi-starved mycelium compared to Pi-sufficient cultures. It was initially thought that the absence of Pi may have lead to death under prolonged culture periods. However, since the propidium iodide stain was taken by both high and low Pi treatments, it was clear that the death was not due to phosphorus starvation alone. It was obvious that the mycelia were at different stages of growth. Alternatively other physiological factors may have influenced the fungi but were not taken into consideration (e.g. the level of carbohydrates or amino acids in the medium). In nature, the lichen mycobionts grow very slowly, making it difficult to monitor growth.

When the mycelium was observed under bright field settings, germlings and apical branching was observed (Figure 4.9C-D). Under low Pi condition, the fungal cells were observed to be thin and not dividing as rapidly as those under Pi-sufficient condition (Figure 4.9E).

The enzyme under optimal and higher concentrations of phosphate was localized in the cytoplasm and vacuole and also in the smaller structures thought to be pre-vacuolar organelles (Figure 4.11I). Furthermore, the colocalization of VB and FM4-64 in Pi-sufficient mycelium, indicate that the same organelles were stained by both fluorophores, thus confirming the presence of acid phosphatase in these structures irrespective of the level of Pi concentration (Figure 4.14). The results in this study were supported by those found in the fungus, *Humicola lutea*, where apase under Pi-sufficient conditions was found in the cytoplasmic membrane

(ALEKSIEVA *et al*., 2003). In most fungi, apase is typically found in the vacuole and it is used as; presumably a marker enzyme for this organelle **(BORGERS *et al.*, 1991; EZAWA *et al*., 1995)**. However, it must be noted this interpretation of this result could be misleading because apase have been shown to be found in other organelles **(WEBER and PITT, 1997a)**. For instance, apase in the dimorphic fungus *Sporothrix schenckii* was localized in the vacuoles and periplasmic space using *β*-glycerophosphate **(GARRISON and ARNOLD, 1983)**. The localization of apase in the vacuole seems to suggest that this enzyme is a resident protein in these organelles since it was not intended to be secreted outside the cells **(CONESA *et al*., 2001)**.

Another attractive explanation could be that this apase is a constitutive enzyme, thus, is not affected by phosphorus concentration. The constitutive enzyme has been proposed to be secreted by alternative route- 'lower density population of vesicles' while the repressible apase is transported by 'higher density' **(SHNYREVA and EGOROV, 1990; WEBER and PITT, 1997b; BLINNIKOVA *et al*., 2002)**. The existence of an alternative secretory route has been reported to be due to post-translation modification- i.e. glycoprotein **(BLINNIKOVA *et al*., 2002)**. Glycosylation is important for enzyme secretion, especially secreted acid phosphatase **(MRŠA *et al*., 1987)**. The absence of glycosylation in *Saccharomyces cerevisiae* has been reported to result in poor secretion of extracellular apase (encoded by the *PHO5* gene) (**PRAETORIUS-IBBA *et al.*, 1997)**. Concurrent studies on enzyme purification revealed that the secreted apase in *C. portentosa* is highly glycosylated; however, more studies need to be done on the mycobiont as well to support this finding.

Staining with FM4-64 confirmed the round organelles to be vacuoles. Other smaller round structures were only stained red when FM4-64 was added. These small particulate structures were thought to be endocytic compartments, presumable endosomes/prevacuolar compartments. The staining of the endosome/vacuoles is consistent with the FM4-64 dye internalization by endocytosis observed in filamentous fungi and yeasts such as *Aspergillus nidulans*, *Botrytis cinerea*, *Magnaporthe grisea*, *Neurospora crassa*, *Phycomyces blakesleeanus*, *Sclerotinia sclerotiorum*, *Trichoderma viride* and *Aspergillus oryzae* **(RAYMOND *et al*., 1992; VIDA and EMR, 1995; PEÑALVA, 2005; SHOJI *et al*., 2006)**. In *Saccharomyces cerevisiae*, FM4-64

stained compartments such as endosomes and vacuoles **(VIDA and EMR, 1995)**. In filamentous fungi, FM4-64 is believed to be internalized by endocytosis and accumulates in endosomal organelles **(FISCHER-PARTON *et al.*, 2000)**. In *Aspergillus oryzae*, FM4-64 showed good labelling of CMAC-positive vacuoles **(SHOJI *et al.*, 2006)**. Similar observations were made in *Aspergillus nidulans*, where a class of cytoplasmic punctuate organelles which became fluorescent shortly after dye loading could move in either an apical or basal direction **(PEÑALVA, 2005)**. Although live cultures were used in the present study, no movement was observed.

Co-staining with both fluorophores revealed that the organelles where apase was present were composed of different sizes (Figure 4.8G). Recent studies have illustrated the pleomorphic nature of vacuoles **(WEBER, 2002; SHOJI *et al.*, 2006)**. These organelles form a part of vacuolar networks described as spherical, tubular and putative pre-vacuolar compartments **(WEBER, 2002)**. These acid compartments were originally implicated in intra- and intercellular transport of nutrients in the mycorrhizal fungus *Pisolithus tinctorius* **(ASHFORD *et al.*, 1999)**.

Under Pi starvation, co-staining with FM4-64 was not very successful. A closer look revealed that the mycelium was in the autophagocytosis stage or undergoing apoptosis (Figure 4.10A). Several studies have shown that a phenomenon like autophagy can occurs at a basal level in normal growing cell conditions, but most often in occurs in starving cells, for example yeast autophagy is induced by nutrient starvation, including nitrogen and carbon depletion **(YORIMITSU and KLIONSKY, 2005)**.Therefore, the internalization of FM4-64 was not successful at this condition, probably due to damaged organelles. The results were confirmed by propidium iodide staining.

FM4-64 staining using 1.0 mM treatments, revealed that the apical tips of the mycelia were swollen, distinct endosomes were labelled and Spitzenköper also clearly visible (Figure 4.13D). The dynamic behaviour of the Spitzenköper has been indicated to be intimately associated with the precise growth pattern of the hyphal apex **(GIRBARDT, 1957)**. In many fungi, this structure is predominated by secretory vesicles, making an apical vesicle cluster **(GROVE and**

BRACKER, 1970; LOPEZ-FRANCO and BRACKER, 1996). The punctuate organelles were thought to represent endosomes or lysosomes since they were at least three of them toward the tip (Figure 4.13D).

Under low Pi treatments closer observation revealed numerous punctuate structures that fluoresced red when stained by FM4-64 compared to those observed at the higher concentration (Figure 4.12C vs. G). Initially this was puzzling, but the literature seems to support the findings as not merely co-incidental. Similar observations were made by **SHOJI *et al*. (2006)**, where tubular vacuoles and clusters of small punctuate vacuoles were prominent in hyphae that were not in contact with nutrients. The authors concluded that since these hyphae have a more urgent requirement for nutrient transport, it is reasonable to speculate that tubular vacuoles develop more extensively in these hyphae and mediate transport of nutrients from hyphae that are in contact with media **(SHOJI *et al*., 2006)**.

The presence of numerous organelles, under Pi starvation further illustrate their role in terms of phosphorus acquisition, as well as the compatible relationship between a fungus and it potential phycobiont. The results support the notion that phosphate is transferred through the hyphae, and release to the algal cell over the appresoria interface **(AHMADJIAN, 1962)**, it is assumed that the vesicles are involved. This was supported by the fact that no phosphatase was detected in the algal cell even under low Pi concentration (Figure 4.5F). Channels were observed by EM in the lichen *Cornicularia normoerica* to originate within the chloroplast, cross the algal cell wall and flare to open space share with fungal and other algal cells (**WALKER, (1968)**. Interestingly, the author also noted that tubules within the channels were extensions of chloroplast vesicles **WALKER, (1968)**. In many symbiotic fungi (parasitic, and mycorrhizal fungi, haustoria have been shown to be involve in nutrients uptake/acquisition from the host (**GIOVANNETTI and CITERNESI, 1993; GIOVANNETTI *et al*., 1994; AKIYAMA *et al*., 2005**). Considering that *Cladonia portentosa* is a naturally symbiotic organism, it is plausible that it employs a vacuolar network to transport phosphorus to the algae which in turn supports its carbon requirements.

Although the mechanisms involved in the transport are still uncertain in lichens, progress in this area have emerged from other symbiotic fungi. Studies by **HARRISON (1999)** demonstrated that in the arbuscular mycorrhizal association there is a bi-directional nutrient transfer. Other studies have suggested that P is transported by Pi transporters or efflux channels **(HARRISON and VAN BUUREN, 1995)**. Molecular studies have revealed that biochemical signals and secreted proteins are involved **(KEMEN *et al.*, 2005)**. For instance **VOEGELE *et al*. (2001)** provided an important advance by characterizing a sugar transporter located at the haustorium-host interface. In mycorrhizal fungi, phosphate is assumed to be transferred through the hyphae as polyphosphate and release to the plant over the arbuscular interface **(HARRISON, 1999)**. Studies by **KEMEN *et al.* (2005)** showed that proteins are transferred from the haustorium into the plant cytoplasm for *Uromyces fabae.*

In *Pisolithus tinctorius*, tubular vacuoles were shown to contain phosphorus compounds **(ASHFORD *et al.*, 1999; HYDE *et al.*, 1999)**. Tubular vacuoles have been proposed to play a role in intracellular translocation, where transport via tubules permits the bulk flow of contents for relatively long distances without concomitant transport of the membrane **(ASHFORD, 1998; COLE *et al.*, 1998)**. Involvement of cytoplasmic streaming in the translocation of phosphate in arbuscular mycorrhizal fungi has been suggested based on calculations of the energy required for the high flux rates of phosphorus in hyphae **(TINKER, 1975; HALEY and SMITH, 1983)**. The relationship between tubular-form vacuoles and cytoplasmic streaming implies the importance of this form of transport inside hyphae **(UETAKE *et al.*, 2002)**.

The prevalence of apase in cytoplasm could be indirectly illustrating the way in which phosphurus is transported. Cytoplasmic streaming within hyphae facilitates bi-directional movement of metabolites **(EUTAKE *et al.*, 2002)**. Although cytoplasmic streaming in lichen-fungi has not been studied there seems to be no *a priori* reason why lichens should be differ in this respect from other filamentous fungi such as *Magnoporthe grisea*. Clearly, these results demonstrate indirectly how the algal component of *C. portentosa* benefits by the lichenized association.

Although the presence of apase in the cell wall is questionable, the presence of this enzyme in cell membrane and periplasmic space was clearly distinct under phosphate starvation (Figure 4.11). It has been shown that the acquisition of nutrients by a fungus is dependent on hydrolases and their transport across the plasma membrane of those hyphae involved in exploiting the substrate **(JENNINGS, 1990)**. Trans-membrane vesicular transport is a general solution among both Ascomycete and Basidomycete fungi, as many studies in several fungi and yeast have revealed the presence of similar structures across these phylogenetically distant organisms **(RODRIGUES *et al.* 2007; ALBUQUERQUE *et al.*, 2008; CASADEVALL *et al.*, 2009)**. It is sensible to imagine that in order for fungal cells to cope with low nutritional phosphate availability they will employ vesicular transportation **(CASADEVALL *et al.*, 2009)**. This study also support the current proposed route of most secreted proteins: the apase enzyme is presumed to being packed in the ER, it leaves the ER packed in the vesicles and head to the Golgi compartment, where additional modification can take place such as further glycosylation **(FISCHER-PARTON *et al.*, 2000; CONESA *et al.*, 2001)**. Finally, again packed in secretory vesicles, the enzyme is directed to the plasma membrane from where it is secreted **(FISCHER-PARTON *et al.*, 2000; CONESA *et al.*, 2001)**. These findings were further confirmed by the matching staining pattern of FM4-64, which follows a hypothetical model of the organization of the vesicle trafficking network in a growing hypha proposed by **FISCHER-PARTON *et al.* (2000)**.

It must be noted that the absence of phosphorus in Pi-deficient cultures resulted in stunted growth and the fungus became necrotic, clearly indicating that apase biosynthesis is growth-associated. However, a relationship between apase synthesis and dividing cells has not been established and this present study was not designed to make such comparisons. The slow growth of the lichen mycobiont demanded that the mycelium be maintained in culture for several months. However, in *Aspergillus flavus*, in addition to apase being present on phosphorus-sufficient cultures, apase was also detected in vegetative and conidiogenous cells **(BOJOVIĆ-CVETIĆ and VUJIČIĆ, 1982)**. Extracellulary, the enzyme was secreted into and across the walls of vegetatively hyphae growing in the medium, as well as in aerially borne conidiogenous cells where the intracellular cap was localized in vacuoles. It was also found associated with the plasma membrane where it was regionally distributed **(BOJOVIĆ-**

CVETIĆ and VUJIČIĆ, 1982). Since the morphology of *C. portentosa* thallus is not different from those observed in other filamentous fungi (e.g. *A. nidulans*), it can only be assumed that similar mechanisms observed in other fungi are present in *C. portentosa* **(CLUTTERBUCK, 1970; WOLKOW *et al.*, 1996)**. However, there are many challenges that remain to be addressed when studying lichenized fungi with slow growth being the major challenge.

5. GENE EXPRESSION PROFILING DURING PHOSPHORUS STARVATION IN THE MYCOBIONT OF *CLADONIA PORTENTOSA*

5.1 GENERAL INTRODUCTION

Phosphate starvation in microorganisms results in transcriptional activation of high-affinity Pi transporters, phosphatase genes as well as genes putatively related to other biochemical pathways **(TORRIANI-GORINI, 1994; STENZEL *et al.*, 2003)**. High affinity phosphate transporter genes, responsible for phosphate uptake, have been cloned from several fungi including AM fungus *Glomus versiforme, Saccharomyces cerevisiae* and *Neurospora crassa* **(BUN-YA *et al.*, 1991; HARRISON and VAN BUUREN, 1995; VERSAW and METZENBERG, 1995)** and plants **(KAI *et al.*, 1997; LEGGEWIE *et al.*, 1997)**.

At the molecular level, little is known about the Pi-starvation response in lichens and even less about its regulation. The objective of this study was to identify *C. portentosa* gene(s) encoding acid phosphatase, induced by phosphate starvation. One way to investigate the effect of phosphorus starvation on *C. portentosa* was to examine the effect of Pi on the gene expression profile. The abundance of particular mRNA transcripts can be used to provide an indicative measure of the level of gene expression by detecting differences in mRNA levels, thus gene(s) whose transcriptional pattern is changed by Pi starvation can be identified **(WATSON *et al.*, 1992)**.

The abundance of mRNAs in a typical eukaryotic cell can be divided into rare transcripts present at approximately 15 copies or less per cell, moderately abundant transcripts, and abundant transcripts present at over 1000 copies per cell **(SOARES *et al.*, 1994)**. The identification of rare mRNAs from a cDNA library, based on a random selection scheme, can be difficult because of their low representation. Construction of normalized cDNA libraries based on re-association kinetics has been used to significantly reduce the representation of abundant transcripts, thereby increasing the chances of obtaining the rare cDNAs **(SOARES *et***

***al*., 1994)**. However, normalization often results in a bias towards small inserts **(CARNINCI *et al*, 2000)**.

With the development of molecular technology and bioinformatics, large-scale screening for P-deficiency responsive genes has become possible. Gene expression can be measured by quantification of RNA by methods such as microarrays, Northern blotting and real-time PCR. PCR is highly sensitive and allows quantification of rare transcripts and small changes in gene expression **(PFAFFL *et al*., 2003)**.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals and enzymes

Trizol reagent was sourced from Invitrogen (Carlsbad, CA), RNeasy® (Qiagen), pGEM-T Easy Vector and JM109 competent cells from Promega, RNAse free DNase I from Qiagen and Advantage2 Taq polymerase from Clontech. TargetAmp™ 2-Round a RNA amplification kit 2.0 (Epicentre). SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) were sourced from Sigma. Isopropyl β-D-1-thiogalactopyranoside (IPTG) and diethyl pyrocarbonate (DEPC) were obtained from Fermentas.

5.2.2 Primer designing

A set of degenerate and specific primers were designed based on the short sequences obtained as outlined in Chapter 3. Amino acid sequences from *Aspergillus terreus* were selected after protein alignment indicated that this species was the closest relative to *C. portentosa*.

5.2.3 Growth of *C. portentosa* under varying phosphate regimes

Axenic mycobiont cultures were isolated as described in Chapter 2, Section 2.2.4. To achieve severe phosphorus deficiency conditions, mycelia were grown in medium containing KH_2PO_4 as the inorganic phosphorus (Pi) supplement, either at 0.05 mM KH_2PO_4 [Pi-deficient (-Pi)] or 10 mM KH_2PO_4 [Pi-sufficient (+Pi)] (Chapter 4, Section 4.2.3 and 4.2.5).

5.2.4 Extraction of total RNA and DNA from mycobiont cultures of *C. portentosa*

All isolations were prepared with water that has been treated with 0.1 % diethyl pyrocarbonate (DEPC), unless otherwise stated. Glassware was baked at 250 °C overnight to destroy RNAse activity. In this experiment, mRNA was extracted from –Pi and +Pi mycelium of *C. portentosa*. In addition, RNA was also isolated from mycelium grown in –Pi and +Pi and in the presence of 0.2 µM menadione. The mycelia were harvested by centrifuging and immediately processed for RNA isolation as described below.

Approximately, 0.1 g of mycelia were harvested from liquid medium. The mycelia were ground in Trizol Reagent. To separate the two phases, 0.2 ml of chloroform per 1.0 ml of Trizol Reagent was added. The samples were vortex vigorously for 15 sec and then incubated at room temperature for 2-3 min. The samples were then centrifuged at 12,000x g for 15 min at 4 ºC. Following centrifugation, the mixture was separated into lower red, organic phase, an interphase and a colourless upper aqueous phase. RNA remained exclusively in the aqueous phase. The upper aqueous phase was carefully transferred without disturbing the interphase into a fresh tube. The volume of the aqueous phase was measured. The volume of the aqueous phase was about 60% of the volume of Trizol Reagent used for homogenization.

RNA from the aqueous phase was mixed with 0.5 ml isopropyl alcohol per 1.0 ml of Trizol Reagent used for the initial homogenization. The samples were incubated at 15 to 30 ºC for 10 min and centrifuge at 12,000x g for 10 min at 4 ºC. The RNA precipitate, often invisible before centrifugation, formed a gel-like pellet on the side and bottom of the tube. For RNA wash, the supernatant was discarded, the RNA pellet was washed once with 75% ethanol adding at least 1.0 ml 75% ethanol per 1.0 ml of Trizol Reagent used for the initial homogenization. The samples were mixed by vortexing and centrifuged at 7,500x g for 5 min at 4 ºC. The washing step was repeated and all the ethanol leftovers were removed. The RNA was eluted with DEPC-treated Millipore water.

To remove the genomic DNA, the RNA samples were treated with RNAse free DNase (Qiagen) at 37 °C for 30 min in 20 µl reactions. The reactions was terminated by adding 1.0 µl of DNase stop solution and incubated at 65 °C for 19 min to inactivate the DNase and stored at -80 °C until used. DNA was extracted as described in Chapter 2, section 2.2.6.3 using a commercial kit.

5.2.4.1 Isolation of mRNA

The mRNA was purified from total RNA using an Oligotex Direct mRNA Kit (Qiagen). The purification was conducted as per the manufacturer's instructions. The quantity of isolated total

RNA and mRNA was examined by NanoDrop ND-1000 spectrophotometer (Thermo-Fisher Scientific, MA, USA).

5.2.4.2 Quality assessment of RNA

The quality of the RNA was also checked by the Agilent Bioanalyzer capillary electrophoresis system to assess the RNA Integrity Number which describes the RNA intactness. The reactions were set up according to the standard Lab-On-Chip protocol. Quantification of all RNA was determined using NanoDrop ND-1000 spectrophotometer.

5.2.5 Amplification of RNA using aRNA kit

To increase the yield of RNA, the total RNA was further amplified with TargetAmp™ 2-Round RNA Amplification Kit 2.0 (Epicentre). The amplification was conducted with mRNA isolated previously as described above. All the reactions were conducted on ice unless otherwise stated. The amplification involved three basic steps (i) first strand cDNA synthesis, (ii) 2nd-strand cDNA synthesis and (iii) *In vitro* transcription. The RNA amplification was conducted as per the manufacturer's instructions.

5.2.6 cDNA Synthesis for Cloning

5.2.6.1 First strand cDNA synthesis

The first strand was performed by RevertAid ^{TM} First Strand cDNA Synthesis Kit (Fermentas). After thawing, the reagents were mixed and centrifuged briefly. The reaction mixture was prepared using 0.5 ml microcentrifuge tubes. Two sets of tubes were prepared (i) using total RNA as template and (ii) using poly (A) mRNA as template. Two µl of template (poly (A) mRNA (~0.5 µg) and 1.0 µl oligo $(dT)_{18}$ primers were added to the tubes. The total volume was made to 12 µl using DEPC water. The mixtures was mixed gently and incubated for 5 min at 65 °C. Another set of reactions was prepared but in this case the template came from total RNA; which was prepared as described above, in this reaction random hexamers were used.

After incubation, the mixture was centrifuged and put back on ice, and the following reagents were added: 5X reaction buffer (4.0 µl), 1.0 µl RiboLock^{TM} RNase inhibitor, 10 mM dNTP mix (2.0 µl) and 1.0 µl RevertAid^{TM} M-MuLV Reverse Transcriptase. The total volume was made to 20 µl using DEPEC water. The reactions were centrifuged briefly.

5.2.6.2 Second strand synthesis

For the second strand cDNA synthesis, 8.0 µl of 10X reaction buffer for DNA Polymerase I was added. This was followed by RNase (1.0 µl), DNA polymerase (30 µl) made to 100 µl using DEPC water. The mixture was gently vortex and centrifuged and incubated at 15 °C for 2 h. A volume of T4 DNA polymerase (5.0 µl) was added and incubated for 5 min at 15 °C. For oligo $(dT)_{18}$ primed cDNA synthesis, the mixture was incubated for 60 min at 42 °C. As for the random hexamer primed synthesis, the mixture was incubated for 5 min at 25 °C followed by 60 min at 42 °C. Both reactions were terminated by heating at 70 °C for 5 min. Reactions were terminated by adding 5.0 µl of 0.5 M EDTA (pH 8.0).

5.2.7 PCR amplification

A standard and two-step PCR amplification were conducted using various enzymes: - Phusion Taq polymerase (Finnzymes) and Advantage2® Taq polymerase (Clontech). The PCR was conducted with cDNA product using both oligo (dT) and random hexamer primer pairs. A standard PCR was designed using the following parameters: 95 °C for 1 min, 30 cycles for 95 °C for 15 sec, 68 °C for 3 min. The two-step PCR was designed using the following parameters: 95°C for 5 min, 30 cycles for 94 °C for 0.25 sec, 72 °C for 3 min, 94 °C for 0.25, 67 °C for 3 min, 67 °C for 7 min.

PCR amplification was also conducted using specific and degenerate primers based on the short amino acid sequence from sequenced apase, Chapter 3. Cycle parameters were 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 52 °C and 1 min at 72 °C before the final 7 min at 72 °C (all at ramp rate 60 °C min^{-1}). The PCR was performed using a Gene Amp kit (Perkin-Elmer Cetus) according to the manufacturer's instructions.

5.2.7.1 PCR clean up

The bands amplified by PCR were manually excised and cleaned with Agarose GelExtract Mini Kit (5Prime). The manufacture's instructions were followed. DNA concentration was determined by NanoDrop ND-1000 spectrophotometer.

5.2.7.2 Blunt ended PCR

Thermostable DNA polymerases with proofreading activity such as Advantage2® Taq polymerase (Clontech) generated blunt-ended fragments during PCR amplification. It was necessary that the PCR fragment generated be modified using the A-tailing procedure before ligation into pGEM-T Easy Vector (Promega).

5.2.7.3 A-Tailing procedure

Purified PCR fragments generated by proofreading polymerase Advantage2® Taq polymerase was used as the starting material. A volume of 7.0 µl PCR reaction was added to a 1.5 ml tube, 1.0 µl of Taq DNA polymerase 10X reaction buffer with $MgCl_2$, 1.0 µl of dATP and 0.25 µl Taq DNA polymerase were added to make a total volume of 10 µl. The reaction was scaled-up. The reaction was incubated at 70 °*C* for 15 min.

5.2.7.4 Ligation using pGEM®T Easy vector

The ligation was conducted using pGEM®T Easy Vector (Promega). A standard ligation reaction was conducted as per the manufacturer's instructions. Briefly, a volume of 3.0 µl PCR product was used in the 10 µl final reaction volume. The reactions were as follows: 5.0 µl of 2X ligation buffer, 1.0 µl of pGEM®T Easy Vector, 3.0 µl PCR product, 1.0 µl T4 DNA ligase. The reaction was performed at either room temperature for 1 h or overnight 4 °C).

5.2.8 Transformations

The tubes containing the ligation reaction were centrifuged and 2.0 µl of each reaction were added to a sterile 1.5 ml microcentrifuge tube on ice. The competent cells (JM109 High efficiency competent cells, Promega) were thawed and gently mixed by flicking the tube. A volume of 50 µl of cells was transferred to 1.5 ml tubes for determination of transformation-efficiency. The mixture was gently flicked to mix and placed on ice for 20 min. The cells were heat-shocked for 45 sec at 42 °*C*. The tubes were immediately returned to ice for 2 min. A volume of 0.9 ml of Super Optimium Broth (SOC) medium [2.0 g tryptone, 0.5 g yeast extract, 1.0 ml 1M NaCl, 0.25 ml 1M KCl, pH 7.0 and added to a final volume of 100 ml. The solution was autoclaved and cooled to room temperature. Two ml of 2M Mg^{2+} stock and 2M glucose stock, were added by filter sterilization through a 0.2 µm filter unit (Millipore)]. The SOC medium was equilibrated to room temperature before it was added to the tubes containing cells transformed with ligation reactions. The mixture was incubated for 1.5 h at 37 °*C* with shaking at 150 rpm. Dry Luria–Bertani medium (LB) [10 g tryptone, 5.0 g yeast extract, 5.0 g NaCl, pH 7.5 and 15 g agar and volume made up to 1 liter, autoclaved at 121 °C] was allowed to cool, to approximately 55 °C and the ampicillin (125 µg/ml final concentration) was filter sterilized and a volume of 35 ml was poured into the Petri dishes. LB plates were prepared in the fume hood, where 40 µl of X-Gal (stock prepared by dissolving 400 mg X-Gal in 10 ml DMSO) and 10 µl IPTG were added and left for approx 30 min or until condensation on top of the lid was dry. A volume of 100 µl transformed cells were spread onto each agar plate, with a glass hook stick and the plates were incubated upside down at 37 °C overnight.

5.2.9 Isolation of recombinant Plasmid DNA

Single white colonies were picked using a sterile tooth pick and inoculated individually in 5 ml LB medium containing selective antiobiotics [25 µl ampicillin (100 µg/ml)]. The broths were incubated at 37 °C overnight on a shaker at 300 rpm. The culture density was measured and the bacteria cells were harvested by centrifugation at 6000x g for 15 min at 4 °C. The cells were then lysed with Qiagen Plasmid Min Kit per the manufacture's instructions. The yield was determined by a NanoDrop ND-1000 spectrophotometer (Thermo-Fisher Scientific, MA, USA).

Insertion of the PCR product of interest onto the plasmid was verified by Not1 digestion of 200 ng of recombinant plasmid and analysis by agarose gel electrophoresis.

5.2.10 DNA sequencing

Randomly-transformed plasmids were checked by mini-plasmid preparation, using the Qiagen Spin MiniPrep Kit, and the plasmids that were positive, contained the band that were sequenced. Sequencing was carried out with the ABI Prism Big Dye Cycle Sequencing Kit and the product was analyzed in an ABI Prism 3700 DNA sequencer (Applied Biosystem, Hungary). Close orthologs were assigned to each transcript, to elucidate its putative function. The EST sequences that showed high nucleotide quality were processed with the CAP3 software for contigs assembly, and the corresponding open reading frames (ORFs) were identified in the genome database and subjected to BLASTN and BLASTX search against the GENBANK database (http:www.ncbi.nlm.nih.gov).

5.3 RESULTS

5.3.1 Cloning by PCR from genomic DNA

PCR amplification was conducted using specific and degenerate primers based from the short amino acid sequenced from sequenced apase, Chapter 3. Using degenerate primers designed from short peptides generated from protein sequencing revealed an expected fragment of 1500 bp (Figure 5.1, lane 2 and 6). However, when the experiment was repeated it was clear that this was a pseudo fragment since it could be amplified with a single forward degenerate primer (Figure 5.1 B, lane 4). Attempts to clone this fragment resulted in an empty clone.

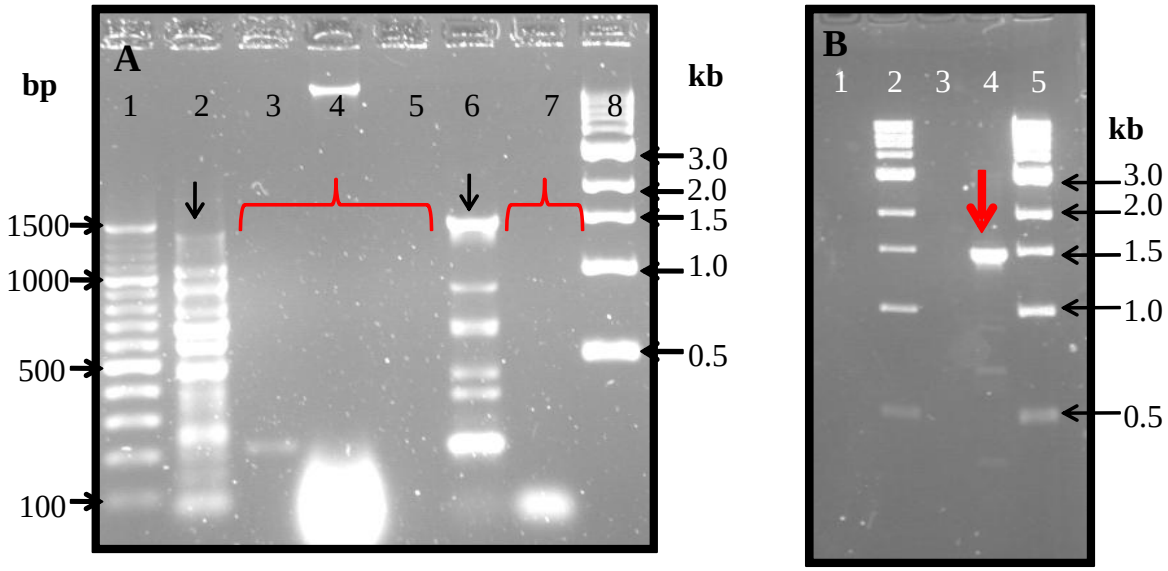

Figure 5.1: (A-B) PCR amplification of *C. portentosa* apase gene using degenerate primers. (A) PCR amplification under low stringency. Non-specific bands were obtained (lane 2 and 6). (B) PCR amplification of PCR under higher stringency. No PCR product was amplified using both primers (lane 3). A PCR product amplified using a single forward primer/s (lane 4).

5.3.2 Extraction of total RNA from mycobiont cultures of *C. portentosa*

5.3.2.1 Analysis of RNA

The quality assessment of RNA preparation extracted from *C. portentosa* was performed by NanoDrop ND-1000 spectrophotometer (Thermo-Fisher Scientific, MA, USA) (Table 5.1) and Bioanalyzer 2100 (Agilent, USA) (Figure 5.2). RNA extracted from cultures of *C. portentosa* grown under phosphorus starvation resulted in poor RNA quality and low yield (Table 5.1). Most lichen RNA lacks the common rRNA peaks; however, 18S and 28S were observed. It was observed that in most samples there were relative high abundance of low molecular weight species present (Figure 5.2). The presence of 28S and 18S RNA peaks indicated that the RNA was not degraded.

Table 5.1: Quantification of RNA isolated from +Pi sufficient vs. –Pi deficient cultures

	Pi concentration	RNA (*ng*/µl)	µg/ml
	Pi+	4.1	0.0041
	Pi-	7.3	0.0073
	Pi+	3.7	0.0037
	Pi-	1.2	0.0012
	Pi+	1.4	0.0014
	Pi-	2.0	0.002
menadione	Pi-	1.4	0.0014
menadione	Pi+	3.1	0.0031
menadione	Pi-	0.7	0.0007
menadione	Pi-	0.5	0.0005

Attempts were made to increase the yield by amplifying RNA with TargetAmp™ 2-Round a RNA amplification kit. The yield was increased to a magnitude of 20-100 fold in some samples. RNA was also extracted from cultures grown under phosphorus starved conditions and menadione. RNA samples quality was also verified on an Agilent Bioanalyzer. A profile of electropherograms of total RNA similar to the one demonstrated in Figure 5.2 was obtained for most samples.

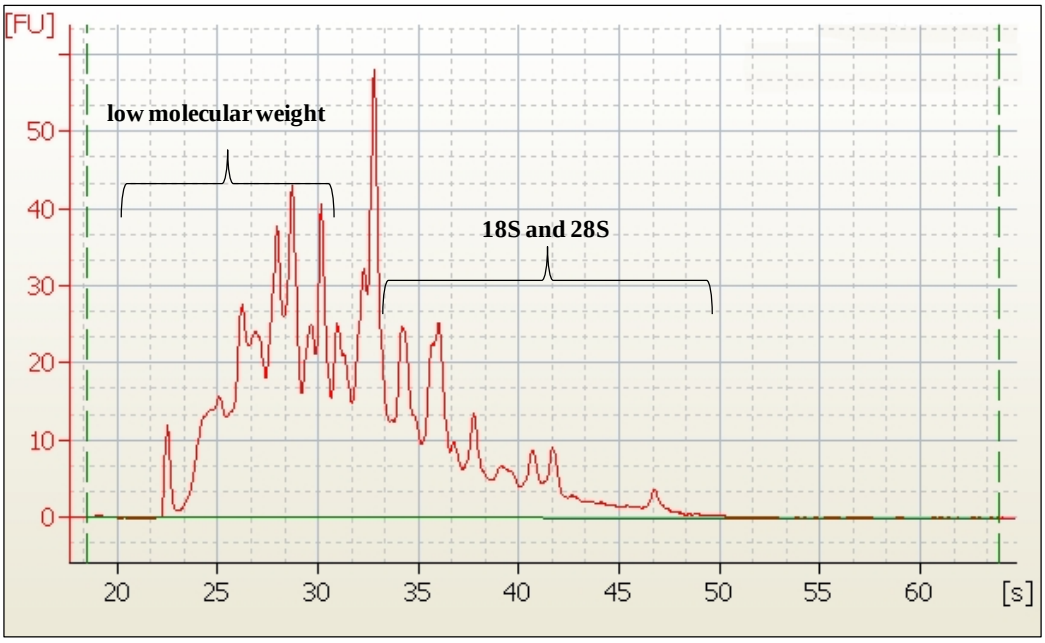

Figure 5.2: A representative of electropherograms of total RNA extracted from *C. portentosa* mycobionts. The 28S and 18S RNA bands were clearly visible. The samples were run on a Agilent Bioanalyzer.

5.3.3 Screening of transformants

The screening was done by adding antibiotic, X-gal and IPTG in already mentioned quantities. Isopropylthiogalactoside (IPTG) induces the transcription of the lacZ gene by binding with the Lac repressor protein which results in white colonies. Since transformed cells, whether they contained a recombinant or not, have antibiotic resistance and can grow on antibiotic LB-agar plates. The advantage of blue-white selection is that it can identify what cells are transformed with a recombinant plasmid when the the recombinant gives no easily detectable phenotype. Selection of recombinants is based on the fact that the insert DNA disrupts the lacZ function, making cells unable to hydrolyze lactose or X-gal, which makes these colonies. Thus it was assumed that all white colonies were transformants. However, few transformants (white colonies) were obtained in this experiment (Figure 5.3).

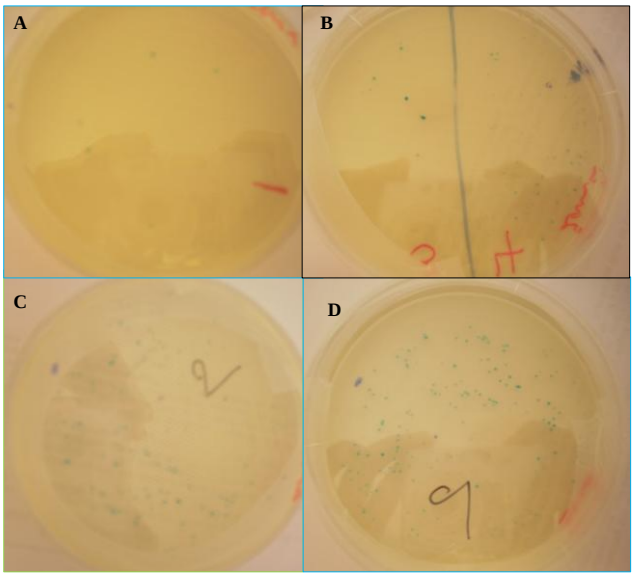

Figure 5.3: Selection using the lacZ complementation was used as a screening tool to differentiate between transformed colonies with recombinant plasmids (white) and those transformed with non-recombinant plasmids (blue).

5.3.4 Generation and identification of expressed cDNA fragments

The cDNA template was used for PCR using oligoDT and random primers. Multiple bands were obtained from PCR, where individual band was excised and cleaned as described in Section 5.2.7.1. The inserts ranged from 100 to 850 bp in size by agarose gel electrophoresis analysis (Figure 5.4).

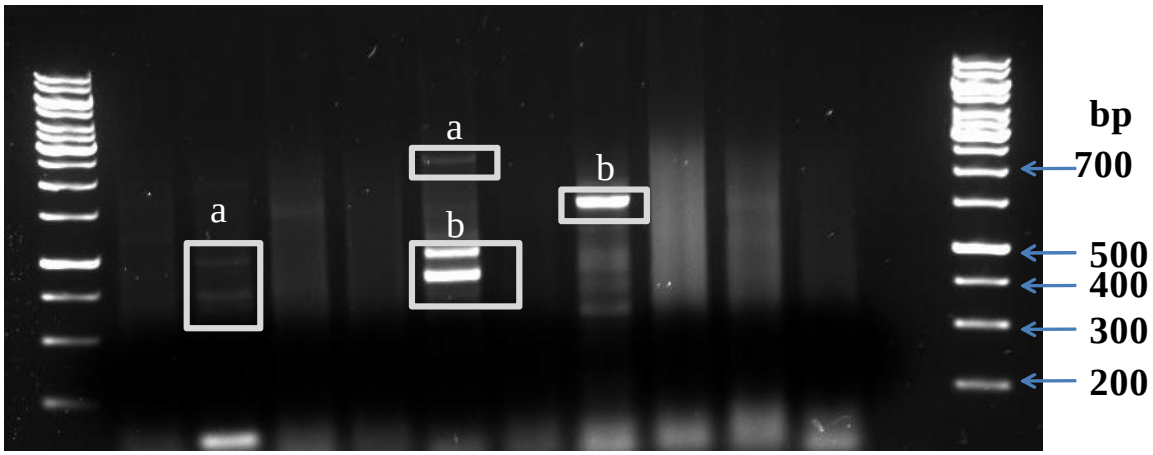

Figure 5.4: PCR amplification using oligo-DT primers. Stronger bands were selected (b) for cloning. Several bands were present but were faint and we not selected (a).

5.3.5 Cloning

Low transformation efficiency of competent *E. coli* cells was obtained. In most cases, there were one or two white colonies in the mist of blue colonies. Precautions were taken to maximize the transformation effiency such as the removal of ligase enzyme and using a sticky end protocol. The transformation efficiency remained low.

5.3.6 Sequencing and analysis

A total of 25 clones were sequenced and compared with NCBI proteins using BLASTXN. Similarity searches against public non-redundant protein databases were carried-out using BLASTX on the National Centre for Biotechnology information web server (http://ncbi.nlm.nih.gov/BLAST). These sequence similarities were judged to be significant when the expected-value (E-value) wass less than 1E-9 at the amino acid sequence level. The results are summarized in Table 5.2. No apase gene was identified. Most of the genes that were preferential expressed are stress-related and have a significant similarity to known genes, EST clones obtained from sequencing (Figure 5.5).

Table 5.2: Candidate clones from Pi starvation library from *C. portentosa*. Gene fragments preferentially expressed in the mycelia from of *C. portentosa*. After BLAST searches, the EST matched the mentioned sequences.

EST no/clone name	**Size (bp)**	**Accession no.**	**BLASTX database match**	**Protein Name**	**Similarity%**
1	628	BAC82547.1	*Penicillium chrysogenum*	ADP/ATP carrier protein (AAC)	47
2	886	EED21936	*Talaromyces stipitatus*	Eukaryotic translation initiation factor 5	42
3	919	CAD42938.2	*Taiwanofungus camphoratus*	Manganese superoxide dismutase	45
4	570	XP_385359.1	*Gibberella zeae PH-1*	Glutathione reductase	64
5	102	XP_002478082.1	*Talaromyces stipitatus* ATCC 10500	Unidentified protein	54

>EST No.1|*Cladonia portentosa* (ADP/ATP carrier protein)
TCGGAGACGAACCCGGCGCCTTTGAAGAGAGACTCGACACCCTCCTTGGCGACGATCTGGCGGGCAGCATCCAT
GGAGCTGTTGTACTTGACGGCCTCACCAGAGGTCATCATCATGCGACGGCGAACGGTGTCAAGAGGGTAAGAGG
CAACACCGGCACCGGTGGTGACGGTCCAGCCGAGCAGGAAGGAGGCGAGGAAAGAGCCCTCAAGAGGACCAAC
GAGGAGAACGGGCTTGATGGAGTCGTACATTCCGAAGTAGAGACCACGGTAGACAACAATTCCGAGAACGGAG
GGACCGAAACCACGGTAGAGACCGGCAATACCGTCGGTGGCGAGGGTCTTGCGGTAGACGTCAACGAGACCGTT
GAACTGGCGCTCACCGGTACCCTTGGAGGACTTGGCGTCGTTGGCGAGACGGGTACGGGCGTAGTCCAGGGAGT
AGACGAAGAGGAGGGAAGTGGCACCGGCAGCACCACCGGAGGCAAGGTTACCCATCATCCACTTGGCGTATCCA
TCACGGTCCTTCTTGTAGGCGAACATGGACTTGTAGGTGTCGCGGAAAGCGAAGTTCAGGGCTAGGGTACGTTGA
AAAATAGACTATCATGTCGAGTTTCGGCCTCGTTTCA

>EST No.2|*Cladonia portentosa* (eukaryotic translation initiation factor 5)
TTCGATTCGGAGACTAATCCAGCGCCGTTGGTAGAGAAATGACTAATACCCTTTCATGACTCTATTCGCGACGTT
GTTCTCGTCTAGGGCTGGTGGGCGCACATCCTCTGGAATACCGGACGACTATACGGCCACAACATNNTTTTAATC
CTGCGACTGTACAGATTTACGATGCTTTCACACATATACCACTGGATTGCGGAGCCCTCTGGAATCCTAGACGAA
CGGCGTTTTCGTATCATTGTCATGAAAAGGGCATAATCGGTTCACTTCTTTTTTGGTCATTCTCTGCATTTTCGGTT
TTTTCTTAAAGCCTTTGTTGATGTTCTTAGCAACAAAGCGCACGGTGTTACACTTGGTGGGATGGATGAACGAAA
ACCACACGATGCACATTCAGTCGGATTCTTTGACTTTTGGGCGCAGGCATATATTTTCCGCATGCTTGGGTTTCTG
CTTCTGCTGTTTACAATCCTTCTCTTCTTGTTTACGACTCTCACACAGGGAAAGAAAGGTTTCATGGCTGGTCGGG
GCGGAGATTCGGCGTCTTTGTTATTTGAGTTATGGTTTGGCAACAATCGTCGTCGAGCGAGCGAGAGGAGTCTTT
ATTGTCGGTTATACCACATGTCGGGGAAATTCTGGGACACTGGGAGTTGTTTTTGCGGNTTCGCTATTTCGAATCN
CTGGCACTTNCNTTNCTTTCNTCAANANTTCCCNCCTACNGGCCNCTGGGCANGCNGGGGCGGCCTGGAAATNTG
AAAATCCTANGAATCNGGCCCCNNAGNCACAANGGAAACTCCACNGTGGAGNAACTGNNATCAAGGCCAAAAC
TGGGAACNGGCAANGTCGGGNANGTACNNNATCCNAAAANCGAA

>EST No. 3|*Cladonia portentosa* (manganese superoxide dismutase)
ATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTC
TATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAAT
TCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAA
TTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCG
CGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCT
GCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGA
ACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTC
CGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATA
CCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCC
TTTCTCCTTCGGNANCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGGGAAGTCGTTCGCTCCA
NCTGGGCTGTGTGACNAACCCCCGTTCACCNGACGCTGCNCTTATCGGNANTATCGCNTGANCCACCGGTAAANC
NATTANCCCNTGGCACACCCTGGTACGGATACAANCNGGTTGNAGCGGGCTNAAATNTGAANGGGCTAATCGCT
ANTAAAAAAATTGATNGCCT

>EST No. 4|*Cladonia portentosa* (glutathione reductase)
CTGAGACCAATCCGGCGCCTTTTGACTGGAAGACATTCACGGACAAGCGTGCTGCATATGTCAAGCGACTGAATG
GCATCTACGAGAAGAATTTGAAGAACGACAAGGTGGAGCATCTGCATGGAACAGCAAGCTTCAAGGACCAGCAC
ACTGTAAAGGTTGTGTTGGACGACAACAGCGAGGTCGAGGTCAAGGCGAAGAAGGTGTTGATCGCTGTGGGAGG
CAAGCCCAACATTCCAGATGTTGAAGGTGCAGAGTTGGGCATTACATCCGACGGTTTCTTCGAGCTGGAGCAACA
GCCCAAGAAGGTGGCTGTTATTGGTGCTGGCTATATTGCGGTCGAATTGGCGGGCATGTTCCACCACCTGGGAAC
TGAAACCCACCTGTTCATCCGCCACGATTCGTTCCTCCGTACCTTTGACCCCATGGTCCAGGAAAAGATTGTGCA
AGAGTACGAGCGTCAAGGCATCCACATTCACAAGCAATCGTCCCAGTCCAAGGTTGAGGATATTGGCAATGGCC
AGAAGAAGCTGCACTACAAGGACTCAAAGGAGCTGGATTCGTTTCCGA

>EST No. 5|*Cladonia portentosa* (unknown)
TAGGACCGCGTCTCCGAGCTTGCCGAAGTCGAAATAACATGGAACGAGAAATCTACTTCTCCAACCAATTCCTTA
ACATTCCCAATCAACTTTTCCAAATCA

Figure 5.5: EST generated from phosphorus-starved and menadione-stressed mycelium.

5.3.7 Functional categories and analysis EST clones

One of the proteins that was up-regulated in the isolated transcripts was eukaryotic translation initiation factor 5, which was classified under RNA metabolic processes. It shows 42% similarity with *Talaromyces stipitatus*, accession no. EED21936.1, which is 1251 bp long versus that of *C. portentosa* which is 886 bp (Appendix D, Figure D1).

One transcript that was abundant was that of a protein encoding ADP/ATP (Table 5.2). This gene share 47 % similarities with *Penicillium chrysogenum* (BAC82547.1), indicated by alignments (Appendix D, Figure D2). The isolated gene was 628 bp vs. 948 bp from *P. chrysogenum.*

Transcripts that were also abundant were of the two genes that encode reactive oxygen species (ROS) and related genes such as glutathione reductase and manganese superoxide dismutase. Glutathione reductase was similar (64%) to *Gibberella zeae* PH-1 (accession XP_385359.1) (Table 5.2). The manganese superoxide dismutase (MnSD) was similar to most fungal MnSDs with *Taiwanofungus camphoratus* being the best match, sharing 45% sequence similarities. Other transcripts that were isolated were unknown since they still need to be identified. These included EST which shows high similarities (54%) with a RNA transcript clone isolated from *Talaromyces stipitatus*, which has an unknown function but exist as a putative protein.

5.4 DISCUSSION

Whilst transcriptomic analyses in yeast show that P deficiency changes hundreds of genes related to various metabolic processes **(LENBURG and O'SHEA, 1996; OSHIMA, 1997)**, there is limited information available for global metabolite changes of P-deficiency in lichens. As changes in metabolites are the ultimate 'readout' of changes in gene expression, this study attempted to profile RNA transcripts for both Pi-deficient and Pi-sufficient mycelia. The aim was to identify genes encoding acid phosphatase induced by phosphate starvation in *C. portentosa* in order to gain a better insight into the molecular mechanisms of P metabolism in this fungus. Axenic cultures of the mycobiont (*C. portentosa)* were used as starting material to construct cDNA libraries.

Under Pi starvation, it was expected that screening for the occurrence of genes encoding for acid phosphatase enzymes in mycelia would be *facile.* Major difficulties were that the total RNA yield was low even though samples did not appear to be degraded. Attempts to increase RNA yield by TargetAmp™ 2-Round RNA amplification Kit 2.0 were successful. In most cases, 20 to 100 fold increases were obtained using the kit. A cDNA library was constructed from poly (A) + RNA isolated from the mycelia cultured for 4 months in –Pi or +Pi treatments. Very low transformation rates were obtained by selection of the blue plates (ampicillin/X-Gal/IPTG). A representative cDNA library was further constructed from the young mycelium of –Pi and +Pi treated with menadione. Since the cDNA library cannot be better that the mRNA from which it is derived, it was essential to start with good quality mRNA before it was used as a template for the synthesis of cDNA.

The identities and amount of transcribed mRNA have been reported to be in a constant flux within cells **(SAMBROOK *et al.*, 1989)**. Therefore, it became apparent that the samples needed to be carefully chosen with respect to age (while the mycelium was still actively growing) and function (while the effect of Pi-starvation was significant on mycelium growth) prior to cDNA production. The apase concentration in the mycelium samples was not measured prior to this experiment. Lichen mycelium grows very slowly, with observation after 3 months showing that

the mycelia were growing under Pi starvation treatments. After four months, a difference could be seen between the two treatments (-Pi vs. +Pi). The stage with the highest production of apase remains to be identified. The concentrations used in this study, were based on the results obtained in Chapter 4 on localization of the enzyme, which revealed that under 0.05M Pi, strong fluorescent signals were obtained when compared to +Pi sufficient cultures (10 mM Pi).

When the experiment was conducted it was expected that genes would transcribe at different rates as required by the need of the hyphae. It has been reported that mRNA transcribing at lower rates, representing less than 0.5% of the total mRNA population of the cell that are classified as low abundance or rare mRNAs (<14 copies/cell) **(SAMBROOK *et al.*, 1989; FARRELL *et al.*, 2005)**. The probability of isolating cDNA clones from mRNA of this type depends on the cDNA library constructed, with a size sufficient to ensure that even rare mRNA has a good chance of being represented **(SAMBROOK *et al.*, 1989)**.

Another problem encountered in this study was that very low transformation efficiency was obtained. In some cases, there were no white colonies in the selection plates (Figure 5.3). Colonies were randomly picked and screened for inserts. To verify that the cDNA library contained cDNA synthesized from C. *portentosa*, randomly selected inserts were characterized by sequencing (ESTs). The basic scheme of an EST sequencing project relies on a cDNA library constructed from a tissue of interest under a particular condition from which randomly isolated clones are sequenced until further sequencing no longer yields an acceptable frequency of identifying novel cDNAs. The length of the homologous regions between the ESTs and the gene sequences in the databases was in some cases short which lessened the significance of the homology although the E values remained relatively high. In most cases, short sequences (<100 bp) were ignored.

Based on the BLAST results of all ESTs to the genome, most of the inserts sequenced matched the sequences deposited in the NCBI database, from other fungi. BLASTX similarity searches, revealed that 5 out of 25 showed similarities to protein-encoding genes in the Uniprot database. The BLASTX results for ESTs are demonstrated in Table 5.2. Analysis of the organisms represented by the BLASTX best hit showed that more that 70% were fungal species. Several

clones of the same gene were found in the ESTs of abundant transcripts under Pi-starvation. It has been reported that the major drawback faced in the ESTs sequencing method, "is the repeated sequencing of abundant transcripts" and, hence, the expense and effort of sequencing them **(RAY *et al*., 2004)**.

Phosphorus is not only a constituent of the key cell molecules such as ATP, nucleic acids and phospholipids, but also a pivotal regulator in many genetic processes including protein activation and amino acid synthesis **(MARSCHNER, 1995)**. Thus the limitation of phosphorus in fungi may cause changes of genetic processing such as induction of transduction, transcription factors, altered translation, some specific protein activation and modification and nucleic acid replication and repair. The genes involved in genetic information processing usually functions in transcriptional activation or repression and therefore may play an important role in the regulation of gene expression under stress **(TESFAYE *et al*., 2007)**.

A homologue to *Talaromyces stipitatus* coding for eukaryotic translation initiation factor 5 (eIF5), was amongst the ESTs isolated. It shows 42% similarity with *Talaromyces stipitatus*, and it was made up of 886 base pairs (Appendix D, Figure D1). Eukaryotic translation initiation factor 5, is a monomeric protein of 49 kDa in mammals **(RAYCHAUDHURI *et al*., 1985; CHEVESICH *et al*., 1993; DAS *et al*., 1993)** and 46 kDa in the yeast *Saccharomyces cerevisiae* **(CHAKRAVARTI and MAITRA, 1993)**. In conjunction with guanosine triphosphate (GTP) and other initiation factors, it plays an essential role in initiation of protein synthesis in eukaryotic cells **(DAS and MAITRA, 2000)**. What was the most interesting about initiation factor eIF5 was that it interacts with the 40S initiation complex to effect the hydrolysis of ribosome-bound (guanosine triphosphate) GTP. Hydrolysis of GTP causes the release of eIF2-GDP, Pi, and eIF3 from the 40S initiation complex, which is essential for the subsequent joining of the 60S ribosomal subunit to the 40S complex to form a functional 80S initiation complex (80S-mRNA-Met-tRNA$_f$) that is active in peptidyl transfer **(MAITRA *et al*., 1982; MERRICK and HERSHEY, 1996; KOZAK, 1999)**. Eukaryotic translation initiation factor 5 -dependent GTP hydrolysis has also been shown to play an important role in the selection of the AUG start codon **(HUANG *et al*., 1997)**.

The activation of autophagic-related proteins (mainly found in vacuoles and membranes) indicated the most nutrients were depleted. Macroautophagy is a nearly universal process that eukaryotic cells employ to reutilize the constituents of cytoplasm and organelles **(SUZUKI *et al*., 2001; KIM *et al*., 2002; NODA *et al*., 2002**). In most cases it has been reported under acute nutrient starvation **(TAKESHIGE *et al.*, 1992)** (major amino acids and nitrogen limitation), where increased levels of autophagy lead to the breakdown of non-vital components and release of nutrients, ensuring that vital processes can continue **(YORIMITSU and KLIONSKY, 2005)**. In response to amino acid deficiency, cells simultaneously decrease their rates of protein synthesis and increase their rates of protein degradation by the induction of macroautophaphy **(NODA *et al*., 2002)**. When autophagy is induced, double-membrane autophagosomes are produced, and these fuse with the vacuole and release single membrane autophagic bodies that are degraded by resident hydrolases and proteases **(SUZUKI *et al*., 2001; KIM *et al*., 2002; NODA *et al*., 2002)**.

Eukaryotic translation initiation factor 5 is a GTPase-activating protein (hydrolase) that binds through its C-terminus to the b-subunit of eIF2 and stimulates GTP hydrolysis by the g-subunit of eIF2 **(DAS *et al*. 2001)**. GTPases play an important role in signal transduction (transmembrane receptors), protein biosynthesis (translation at the ribosome), control and differentiation during cell division and translocation of protein through membranes. The GTPases also controls assembly of vesicle coats thus, transport of vesicles within the cell. It is suspected that when the cells become desperate for phosphate, that eIF5 is initiated to activate GTPase (which is capable of hydrolysis of γ phosphate of GTP into guanosine disphosphate (GDP)) and Pi (inorganic phosphate) which is reported to occur by the SN2 (nucleophilic substitution) in order to keep the cells alive **(IMYANITOV, 1990; SHIN *et al*., 2002)**. Since these cells are responsible for vesicle coat assembly, it is speculated that during P starvation these vesicles become numerous in order to transport nutrients to the starving cells.

In *Saccharomyces cerevisiae,* it has been observed that under nutrient deprivation conditions, double-membrane vesicles form around bulk cytoplasmic cargo destined for degradation and recycling in the vacuoles/endosomes **(KIM and KLIONSKY, 2000)**. A similar process functions to remove excess organelles under vegetative conditions in which they are no longer

needed. These speculations are in agreement with cytochemical localization of apase in vacuoles and vesicles, discussed in Chapter 4. In previous experiments (Chapter 4), numerous vesicles were detected with FM4-64 dye in Pi-starved cultures compared to Pi sufficient cultures.

One of the most abundant transcripts was a protein encoding ADP/ATP carrier (AAC). This gene share similarities with several fungi, with *Penicillium chrysogenum* (BAC82547.1) being the highest, where 47% homology was found (Appendix D, Figure D2). The isolated gene was 628 bp. AAC is a metabolite carrier **(PFANNER *et al.*, 1987)**, driven by the membrane potential, it exports newly synthesized ATP into the cytosol in a counter-exchange with ADP that is taken up into the mitochondrial matrix space for rephosphorylation. It is an integral protein of the inner mitochondrial membrane and abundant in this organelle **(KLINGENBERG, 1980)**.

The AAC gene (NCU09477, 3) which encodes an adenine nucleotide translocase is also preferentially induced at acidic pH in fungal species **(LEAL *et al.*, 2009)**. During periods of oxidative metabolism, the AAC mediates the transfer of high energy phosphate to other cellular locations of AAC. The AAC are components of the mitochondrial permeability transition pore and have a crucial role in mitochondrial mediated apoptosis and mtDNA maintenance **(PEREIRA *et al.*, 2007; KAUKONEN *et al.*, 2000; LEAL *et al.*, 2009)**. Since this protein is associated with apoptosis, it could be an indication of a physiological stage of the mycelium deprived of phosphorus in this study. It is tempting to speculate that the presence of this transcript may indicate that the mycelium was undergoing oxidative metabolism, where most nutrients, especially nitrogen were depleted and further that the mycelium was undergoing some form of apoptosis.

Interestingly, this gene was also upregulated in *Neurospora crassa* under Pi-starvation and stimulated at pH 5.4 **(LEAL *et al.*, 2007)**. This suggests that *C. portentosa* has additional metabolite functions at acidic pH like most fungi such as *Aspergillus nidulans* and *Neurospora crassa* **(FREITAS *et al.*, 2007; SILVA *et al.*, 2008)**. It is tempting to speculate that intracellular ACC might also be engaged in a coordinated cell death process. One possible role

is the mobilization of Pi from phosphorylated organic substrates. It is largely unknown in which way Pi regulates the expression of the characterized Pi-responsive genes such as RNAses (**BARIOLA *et al*., 1994)**, ACC and Pi transporters.

Transcripts that were also abundant were of the two genes that encode ROS related genes: glutathione reductase and manganese superoxide dismutase (SOD). Glutathione reductase showed a 64% similarity with *Gibberella zeae* PH-1(acc. No. XP_385359.1) (Table 5.2). The manganese superoxide dismutase (MnSD) was also similar to most fungal MnSDs with *Taiwanofungus camphorates* being the best match, sharing 45% sequence similarities. Other transcripts that were isolated were unknown since they still need to be identified. This included EST which shows high similarities (54%) with a RNA transcript clone isolated from *Talaromyces stipitatus*, which had an unknown function but exists as a putative protein in the NCBI database. Candidate genes with unknown function were highly abundant in the libraries, which might be attributed to the limited genome sequence information in the lichen genome.

It must be considered that not all the genes which change in transcription in the absence of P are involved in the phosphorus starvation response as this study was performed using a batch culture system. In such cultures, the environment is continuously changing and it is difficult to study the effects of individual physiological parameters, cell growth and metabolism or to make a reproducible comparison amongst exponential phases. Furthermore, secondary effects such as growth-rate-dependent factors tend to obscure the identification of genes really pertinent to a particular experimental treatment or physiological condition **(HAYES *et al*., 2002; LIM *et al*., 2003)**.

It was expected that some genes would be induced as a result of generalized stress response imposed by other nutrient limitation or menadione. The changes in transcription of two putative genes SOD and MnSD are more likely to be due to oxidative stress induced by menadione in the mycelium of *C. portentosa*. These two enzymes are known for their role to combat free-radical-mediated damage **(BELL and SMITH, 1994)**. SOD is required to protect aerobic organisms from the damaging and lethal effects of ROS, superoxide, which is produced in large amounts during oxidative stress. SOD provides defence by scavenging the intermediate of

oxygen reactions, by disproportionating two molecules of O_2^- to H_2O_2 and O_2 **(BELL and SMITH, 1994)**. The ROS are known to damage DNA, proteins and cellular membranes **(BELL and SMITH, 1994)**. Glutathione is a ubiquitous tripeptide of which the thiol/thiolate group of its cysteine residue is reversibly oxidized to generate oxidised glutathione, it is the most abundant intracellular redox-active sulfhydryl compound and it acts as a major cellular redox buffer **(MEISTER and ANDERSON, 1983)**.

Similar results have been reported when menadione was added to the culture medium of *Aspergillus nidulans* **(PUSZTAHELYI *et al.*, 2011)**. The logic of adding menadione to the medium was to verify if apase had an additional protecting mechanism against oxidative stress induced by menadione. Based on these data, no apase genes were detected. However, the emerging data in recent years have signified the involvement of apases in response to biotic stress **(LAWTON and LAMB, 1987)** (Chapter 4). Other environmental conditions such as deficiency of sulphur, nitrogen, thiamine and sugar affect the expression of apase genes differentially **(LINDBERG and DRUCKER, 1984; CADDICK *et al.*, 1986b; 1986c; YANG and SCHWEINGRUBER**, **1990; SCHWEINGRUBER *et al.*, 1992; RODRIGUEZ-GARCIA *et al.*, 2009)**. Furthermore, apase genes are also upregulated during saline stress and pathogen infection (**SOMSSICH and HAHLBROCK, 1998; JAKOBEK and LINDGREN, 2002**). These seem to suggest that apases are involved in the defence role in addition to their role in P acquisition.

During phosphorus starvation, many microbes have been shown to devise a series of adaptations to deal with temporary and/or spatial heterogeneities in P availability **(BUN-YA *et al.*, 1991; HARRISON and VAN BUUREN 1995; VERSAW and METZENBERG, 1995)**. These strategies include the synthesis of apase enzymes and synthesis of Pi transporter and several enzymes that provide metabolic mechanisms that use alternative forms of the limiting nutrients. However, in this study, no apase or Pi transporter transcripts were isolated due to time constrains and resources, not all transformants were screened and sequenced. It was anticipated that apase genes would be amongst the abundant transcripts isolated and sequenced. Another limitation of this study was that the expression data were acquired from old mycelia. The discrepancy in these results could be related to the duration of Pi-starvation treatments and the

age of cultures. In future, the use of younger cultures and other techniques such as qRT-PCR may shed light and give a better expression pattern. Several factors were not measured in this study they include mean cell volume, cellular RNA and protein content.

In yeasts it was demonstrated that the onset of apase derepression coincided with the time at which the mean cell volume began to decrease **(BOSTIAN *et al.*, 1983)**. This decrease in growth rate corresponded with a dramatic increase in the percentage of unbudded cells in the culture, thus derepression of apase preceded the onset of GI arrest by approximately two generation of growth **(BOSTIAN *et al.*, 1983)**. These events occurred about one generation before complete cessation of growth due to Pi starvation **(BOSTIAN *et al.*, 1983)**. It was further noted that apase derepression varied with the growth rates. In cultures, however, the above parameter held the same relationship, suggesting that it is the yield of growth versus Pi supply and not the rate that determines the point of derepression **(BOSTIAN *et al.*, 1983)**. However, this is easy to regulate in yeast and other fast-growing filamentous fungi since these organisms grow very fast. Slow growth in lichens make experimentation in the symbiont fungi particular challenging.

The transient reduction in mRNA levels can be explained by a number of mechanisms such as inhibition of transcription of changes in mRNA turn-over. This has been observed in other eukaryotic systems **(ANDERSON and LENGYEL, 1980)**. Increased transcription of phosphate-transporter genes in Pi-limiting conditions is well documented in several microorganisms **(TORRIANI-GORINI, 1994)**. *Saccharomyces cerevisiae* has both high affinity and low affinity Pi uptake systems. Transcription of high affinity phosphate (PHO84) is controlled by the availability of Pi in the medium through the action of several positive and negative regulators constituting the *pho* regulon **(YOSHIDA *et al.*, 1989b; JOHNSTON and CARLSON, 1992)**

In conclusion, although apase genes were not amongst the genes sequenced, this study illustrate that complex genes are involved in Pi starvation, since phosphorus controls many metabolic processes. Generations of a cDNA library from axenic grown mycelium treated with phosphorus provided a foundation for the identification and characterization of genes expressed

in the phosphorus treated mycelium through Expressed Sequence Tags (ESTs). Several genes were identified whose transcriptional profiles have been significantly changed by phosphorus treatment and menadione. They include genes required for signal transduction and vesicular transport, cell biosynthesis and protein metabolism and stress responses. The identification of novel genes modulated by the transcriptional factor provides new insight into the metabolic interactions between extracellular Pi and pH sensing in *C. portentosa.*

6. GENERAL CONCLUSIONS

The molecular characterization of apase in *Cladonia portentosa* presented in this study has led to a better understanding of P acquisition in this lichen. However, the challenge to provide definite answers to many of the questions posed at the beginning remained to be addressed. There were several challenges in studying these organisms such as low protein yield, slow growth rate and the lack of lichen genomic sequences in the public database.

The isolation of an apase gene from *Cladonia portentosa* using PCR was not successful. Failure to amplify the apase gene by PCR from *C. portentosa* was intriguing and perplexing, and it was concluded that the high levels of sequence diversity among phosphatases may have contributed to poor PCR amplification. Since all the PCR parameters were optimized, it could have been that the primers were dissimilar to the "unknown target" (apase gene) so as not to efficiently anneal to the original template or degenerate primers may have been too dissimilar to each other to efficiently amplify the synthesized product. PCR procedures have been employed successful in the isolation of homologous apase gene from genomes or transcriptomes **(MITCHELL *et al*., 1997; PASAMONTES *et al*., 1997a; 1997b; WYSS *et al*., 1998)**. In order to avoid time-consuming trial-and-error testing using degenerate primers, attempts to purify the apase were made. It was anticipated that the partial sequence of the purified enzymes would provide a corresponding apase gene **(CHO *et al*., 2005; KEROVUO *et al.*, 1998)**.

The purified apase protein is the first report of *Cladonia portentosa* acid phosphatase to be identified and characterized at a molecular level. The signalP showed that the enzyme possesses secretion signals. Purified apase from *Cladonia poretentosa,* like many secreted proteins that are destined for extracellular *milieu,* is a glycoproteins of 148 kDa on SDS-PAGE and has a 250 kDa size on non-denaturing gel, proving it to be a high molecular weight apase. Furthermore, kinetic properties and behavior under activactors and inhibitors, make the enzyme isolated fits the description of secreted histidine acid phosphatase (HAP). Short peptides generated after sequencing show significant homology to known acid phosphatase and phytases. Homologous sequences to this enzyme all possess the active site sequences RHGXRXP which is the hallmark for all hisitidine acid phosphatases. The present study proposes that the *Cladonia*

portentosa uses secreted apase to acquire overall efficiency of P uptake in acidic and nutrient-poor environments. Furthermore, kinetics studies revealed that apase enzyme from *Cladonia portentosa* has an ability to degrade a wide variety of substrates, including phytic acid, making it a phytase enzyme.

A second phosphatase remained to be purified. It not yet clear when it becomes expressed in this lichen. In an ecological context, the lichen *Cladonia portentosa*, may grow in P-deficient soils from spore or relatively small lichen fragment to larger mats, and it may take several months to establish itself. Consequently, it may express a different set of genes from those discussed here in order to cope with a perpetually low availability of P. Exactly what happens in between, however, remains to be elucidated.

Acid phosphatase (Apase) enzymes including phytases have broad applications in diagnostic kits, poultry feeds, biofertilizers and plant nutrition **(BRINCH-PEDERSEN *et al*., 2002; BOYCE and WALSH, 2006)**. Since phytic acid is largely indigestible by monogastric animals and it is the single most important factor hindering uptake of a range of minerals. On the large scale, knowledge contributing to phytic acid biosynthesis, deposition and degradation, combined with more information about enzyme heat stability and catalytic properties should provide the plant biotechnologist with new options for improving phosphate and mineral uptake and bioavailability **(BRINCH-PEDERSEN *et al*., 2002)**. This might benefit human and animal nutrition and the environment due to the reduced phosphate load on agricultural ecosystems and thereby alleviate eutrophication of the aquatic environment **(BOYCE and WALSH, 2006)**. Although, there are numerous numbers of phytase-producing organisms, including commercial ones from *Aspergillus niger*, the thermostable phytase with broad substrate specificity and high specific activity is still highly desirable for animal nutrition purpose and is of great commercial importance. Thus, to obtain better and alternative source of phytase, there is an ongoing interest in screening new organisms producing novel and efficient phytases with the ultimate aim to produce this enzyme at cost-effective levels and establishing the suitability for its industrial application.

In view, of the fact that most lichen grow under extreme environmental conditions, enzymes isolated in these organisms may have a potential not only for industrial but also agrobiotechnology applications **(BRINCH-PEDERSEN *et al*., 2002**; **ULLAH *et al*., 2002)**. Engineering plants to secrete phytase from their roots might also be an important strategy for mobilising phosphates reserves in the soil (**ULLAH *et al*., 2002)**. Although phytic acid is generally considered to reduce phosphate and mineral bioavailability, there is now evidence to suggest a beneficial role for phytic acid. A few studies have indicated that it is a potential antioxidant and it is has anti-neoplastic activity in the large intestine **(HARLAND and MORRIS, 1995; BRINCH-PEDERSEN *et al*., 2002)**.

This study revealed first lines of evidence regarding the localization of apase in the mycobiont *C. portentosa*. The stimulation of apase secretion by Pi-starvation and the presence of apase in the plasma membrane, vacuole and cytoplasm lends credence to the idea that under P starvation, *Cladonia portentosa* can acquire and hydrolyze P by increasing acid phosphatase. It may also be pertinent to note that, while most microorganisms can synthesize acid or alkaline phosphatase, depending on the pH of the medium, *Cladonia portentosa* can only synthesize apase (acidic environment). This may be related to the native pH of the soil where this species is found. This study further supports that phosphorus may be transported to the algal cells by cytoplasmic streaming using vacuolar networks, where phosphorus is delivered to the dependent algal cell possible through an appresorium interface and it is likely that vesicles are involve (**AHMADJIAN, 1962; WALKER, 1968)**. It represents, therefore, a starting point for further studies about the dynamic activity of this enzyme at ultrastructural level in relation to the acquisition of phosphorus under different environmental conditions.

The powerful combination of genetic and biochemical studies in *Cladonia portentosa* will lead to a more detailed and comprehensive understanding of the above questions in the near future. One of the major unsolved mechanisms is how extracellular Pi signals are detected in this lichen. Although this study, showed the changes in P starvation profile of various genes during stress induction, no apase or Pi transporters were detected, probably these were present as rare transcripts. The influenced of Pi needs to be assessed at different stages of growth, to establish the critical stage where apase enzyme induction is the highest. It is anticipated that gene

transcripts will reveal apase genes amongst other Pi transporters involved in the Pi starvation pathway. However, this may require a large scale gene isolation and less laborious techniques such as real-time PCR. The detailed study of the regulatory mechanisms of Pi-repressible gene expression could reveal how intracellular signaling network receive and process information to control Pi starvation responses in lichens. The genome sequencing of lichen fungus, such as that ongoing project at Duke University will open a new era in lichenology. It will facilitate a wide range of other approaches such as comparative genomics, genomic engineering and transcriptomics and proteomics **(OKSANEN, 2006)**.

Given its limitations, this study constitutes the first step towards understanding the molecular mechanism under P starvation in *C. portentosa*. The identification of novel genes modulated by the transcriptional factor provides new insight into the metabolic interactions between extracellular Pi, menadione and pH sensing in *C. portentosa.* The library is a resource that can be used to generate EST (Expressed Sequence tags), which can be compared with ESTs of other fungal species to assess the similarity of *C. portentosa* sequences to existing EST data and enrich the public database with novel ESTs unique to *C. portentosa.*

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APPENDIX

APPENDIX A: REAGENTS FOR PROTEIN ELECTROPHORESIS (SDS-PAGE)

Solution A: monomer solution [30% (m/v) acrylamide, 1.0 % bis-acrylamide]. Acrylamide (60 g) and bis-acrylamide (2.0 g) were dissolved in ~170 ml of dH_2O and made to 200 ml with dH_2O. The solution was stored in on amber-coloured bottle at 4 °C.

Solution B: running buffer (1.5 M Tris-HCl, pH 8.9). Trizma Base (7.28 g), TEMED (92 µl). The solution was adjusted to pH 8.9 with 1 N HCl and dH_2O added to a final 40 ml volume.

Solution C: stacking gel buffer C: 0.5 M Tris-HCl, pH 6.9 Trizma base (1.21 g), TEMED (92 µl). The pH of the solution was adjusted to 6.9 with 1.0 N HCl and the dH_2O added to a final volume of 20 ml.

Solution D: 10% (m/v) SDS. One gram of lauryl sulfate sodium salt was added to a final volume of 10 ml of dH_2O and filtered.

Solution E: initiator ammonium sulfate (APS). Sixty milligram of ammonium persulfate was added to a volume of 5 ml of dH_2O. This was prepared fresh each day.

Solution F: tank buffer [0.25 M Tris-HCl, 0.192 M glycine, 0.1% (m/v) SDS, pH 8.3]. Tris (15 g) and glycine (72 g) were dissolved and made up to 5.0 l with dH_2O. Prior to use, 5.0 ml of SDS stock (solution D) was added and made to a 500 ml volume.

Solution G: reducing treatment buffer: [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.9] Buffer C (2.5 ml), 10% SDS (4.0 ml), (Solution D), glycerol (2.0 ml) and 2 mercaptoethanol (1.0 ml) were made up to 10 ml with dH_2O.

Solution H: non-reducing treatment buffer [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, pH 6.9]. Buffer C (2.5 ml), 10% SDS (4 ml), (Solution D) and glycerol (2 ml)) were made up to 10 ml with dH_2O.

Staining stock solution [1% (m/v) Coomassie blue R-250]. Coomassie blue R-250 (2.0 g) was dissolved in a total volume of 200 ml distilled water, stirred (60 min) using a magnetic stirrer and filtered through Whatman N˚ 4 filter paper.

Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid] Methanol (500 ml) and acetic acid (100 ml) were mixed and made up to 2l with dH_2O.

Table A1: SDS-PAGE formulation for the preparation of separating and stacking gels. Separating gel final volume =20 ml. Stacking gel solution final volume 8.0 ml.

Components	**Stacking gel solution**	**Separating gel solution**				
	3%	**5%**	**7%**	**8%**	**10%**	**20%**
Deionized water (ml)	**5.52**	10.47	9.13	8.47	7.13	0.47
Solution A (ml)	**0.80**	3.33	4.67	5.33	6.67	13.33
Solution B (ml)	**-**	5.0	5.0	5.0	5.0	5.0
Solution C (ml)	**1.00**	-	-	-	-	-
Solution D (ml)	**0.08**	0.2	0.2	0.2	0.2	0.2
Solution E (ml)	**0.60**	1.0	1.0	1.0	1.0	1.0

APPENDIX B: BUFFERS AND STAINING SOLUTIONS USED IN MICROSCOPY WORK

Cacodylate stock solution*:* 1.0 M sodium cacodylate (10.7 g) was dissolve in 45 ml of distilled water, titrated to pH 7.2 with sodium hydroxide, and made up to 50 ml. For use, the stock solution was diluted to 100 mM, and the pH re-checked.

Buffer B: (100 mM sodium cacodylate pH 7.2). 200 mM sucrose. A 100 mM sodium cacodylated solution was prepared by mixing 10 ml of 1.0 M sodium cacodylate stock solution, 6.846 g sucrose and approximately 80 ml of distilled water. The pH was adjusted to pH 7.2 with dilute sodium hydroxide.

Buffer C: (20 mM sodium acetate pH 4.8 containing 200 mM sucrose).
Paraformaldehyde stock solution (16 %). Paraformaldehyde 1.6 g was dissolved in distilled water (100 ml), heated to 60 °C and cleared with minimum amount of 1.0 M NaOH. The solution was stored at -20 °C until required.

Glutaraldehyde (1%): 25 % Glutaraldehyde (1.0 ml) was diluted to 25 ml with 100 mM sodium cacodylate buffer, pH 7.2

Fixative [2% Paraformaldehyde, 1% Glutaraldehyde]. 16 % Paraformaldehyde stock (1.25 ml) and 25 % Glutaraldehyde (0.4 ml) were made up to 10 ml with buffer B. The final pH was checked and adjusted to pH 7.2 if necessary.

Epon–araldite resin. EPON812 (1 part), Araldite (CY212 (1 part), dodecyl succinic anyhydride (DDSA) (3 parts) and 2, 4, 6–trimethylmonomethyl phenol (DMP-30) (1 drop/ml).

APPENDIX C: FUNGUS CULTURE MEDIA AND SOLUTIONS

Table C1: Submerged filamentous medium was prepared as following.

Ingredient	**g/l or part /10^6**
Adonitol	8.0 g
NH_4NO_3	0.424 g
KH_2PO_4	0.44 g
$CaCl_2.2H_2O$	0.2 g
$MgSO_4.7H_2O$	0.5 g
$MnCl.4H_2O$	0.2 mg
$Fe_2(SO_4)_3$	0.2 mg
$ZnSO_4.7H_2O$	0.2 mg
$CuSO_45H_2O$	-
H_3BO_3	-
$Na_2MoO_4.2H_2O$	-
Thiamine	100 µg
Biotin	5 µg

Stock solution	**Aqueous stock solution**	**Volume of stock per litre of medium**
$CaCl_2$. $2H_2O$	5g in 250 ml	10 ml
$ZnSO_4.7H_2O$	0.1g in 500 ml	1.0 ml
$MnCl_4(2H_2O)$	0.1g in 500 ml	1.0 ml
Fe_2 $(SO_4)3$	0.05g in 250 ml	1.0 ml
$CuSO_4.5H_2O$	0.0393g in 1000 ml	0.1 ml
H_3BO_3	0.0572g in 1000 ml	0.1 ml
$Na_2MoO_4.2H_2O$	0.1262g in 500 ml	0.1 ml
Thiamine	0.5g in 500 ml	0.1 ml
Biotin	0.05g in 500 ml	0.05 ml

APPENDIX D: DNA SEQUENCES

```
Talaromyces     ATGGCTACCGTCAACGTTCGTCGGGATGTTACCGATCCCTTTTACCGTTACAAGATGGAG 60
Cladonia        -----TTCG------ATTCGGAG-------ACTAATCCAG--CGCCGTTGGTAGAG-AAA 39
                     *:*       .**** .*       ** .****.    .*****. :***  .*.

Talaromyces     AAGCTCCAAGCCAAGATCGAGGGTAAAGGTAACGGTATCAAGACCGTCGTTGTGAACTTG 120
Cladonia        TGACTAATACCCTTTCATGACTCTATTCGCGACG-----TTGTTC-TCGTCTAGGGCTGG 93
                :..**..:* **:: .: **   **:: * .***     ::*: * ****  :*..** *

Talaromyces     AACACCGTCGCCCAATCCCTTGCCCGTCCTCCGGAGTACGTTATCAAGTACTTCGGTTTC 180
Cladonia        TGGGCGCACATCCTCTGGAATACCGGACGACTATACGGCCACAACAT-NNTTTTAATCCT 152
                :. .*  :*. **:.*  .:*.** *:* :* . *  .* : *:**: .  ** ..*

Talaromyces     GAAATTGGAGCCCAGGCCAACGCAAAGCCCACTGATGACCGCTGGATCATTAACGGTGCT 240
Cladonia        GCGACTGTACAGATTTACGATGCTTT-CACACATAT-ACCACTGGATTG----CGGAGCC 206
                *..* ** * . .:  .*.* **::: *.***: ** ***.****** .    ***:**

Talaromyces     CACGATGCTCCTAAGCTTCAGGACTTGTTGGATGGTTTCATTGACAAATTCGTTCTCTGC 300
Cladonia        CTCTGGAATCCTAGACGAACGGCGTTTTCGTATCATTGTCATGAAAAGGGCATAATCG-- 264
                *:* . ..*****..* :..**. ** * * ** .**  .:***.**.  *.*:.**

Talaromyces     AAGAAGTGCAAGAACCCTGAAACCGAAGTCATCCTCAAGGACAACCGTATCACTCTTGAC 360
Cladonia        -----------GTTCACTTCTTTTTTGGTCATTCTCTG------CATTTTCGGTTTTTTC 307
                           *::*.** .::   :.***** ***:.      *. *:**. * ** :*

Talaromyces     TGCAAGGCCTGCGGCCAGCGATCTGAGGTTGATCCCCGTCTCAAGCTAAGCACTTTCATC 420
Cladonia        TTAAAG-CCTTTG---------------TTGATGTTCTTAGCAA-CAAAGCGCA------ 344
                * .*** ***  *               *****   * *. *** *:****.*:

Talaromyces     TTGAGAAAAACCCCTACCAAGGGCGGTAAAAAGGACAAGAAATCTCGTCGTGACAAGAAG 480
Cladonia        ---CGGTGTTACACTTGGTGGGATGGATGAACG---AAAACCACACGATG---------- 388
                   .*.:.::.*.**:  :.**. **::.**.*   **.*..:*:**: *

Talaromyces     AAAGAGAAAGACGAAACCAATGGAGAAAAGAATGGCAGCCCAGGAGAGAGCAATGCTTCT 540
Cladonia        -CACATTCAGTCGGATTCTTTG-----ACTTTTGG--GCGCAGGCATATATTTTCCGCAT 440
                 .* * :.**:**.*: *::**     *. ::***  ** ****..:.:. ::* *  .*

Talaromyces     GACGAAGGCGAAAATGGCGATGTCGAAATCCCAGCTGGTAGTGACGACGAGATCGTCGCT 600
Cladonia        GCTTGGGTTTCTGCTTCTGCTGTTTACAATCCTTCTCTTCTTGTTTACGACTCTCACACA 500
                *.  ..*   .:..*   *.***  *.*: **: **  *. **:  **** :   :*.*:
```

Figure D1: GAP alignment of EST transcripts isolated during phosphorus starvation (matching a protein encoding an eukaryotic translation initiation factor 5). The alignment was conducted with CLUSTAL W2 (www.ebi.ac.uk)

```
Talaromyces     GGTGCTGAGAAGATCAACATCCAGGATGAGAATGAGGAAGAGGTTCAGTGGTCCGTCGAT 660
Cladonia        G-----GGAAAGAAAGGTTTCATGGCTGG--TCGGGGCGGAGATTCGG-----CGTCTTT 548
                *     *..****:... :**.:**.**.  : *.**..***.***.*     **** :*

Talaromyces     GTCTCCGAGGAAGCCGTCAAGGCCCGTGCTAAGGACTTGCCCGACGATCTCAAGCGAACC 720
Cladonia        GTTATT-----------------TGAGTTATGGTTTGG--CAACAATCGTCGTCGAGCG 588
                ** :                    *:* **:**: * *  *.**.***  .. ***.*

Talaromyces     TTGATCTTGGAAGGCGGCGAGGATGAGGATGAAGAAGGCGGTGCTACCATCTACGATCAG 780
Cladonia        ---------------AGCGAG-AGGAGTCT-TTATTGTCGGTTATACCA--------CAT 623
                               .***** * *** .* ::.::* **** .*****        **

Talaromyces     CTCGGAAGTTGGATCATCAAGGAGGCTGAAGAGAAAGGCGGCGTCGCCAACGTCAGCGAC 840
Cladonia        GTCGG-----GGAAATTCTGGGACACTG--------GGAGTTGTT-------TTTGCGGN 663
                 ****     ***:.:**:.*** .***        **.*  **        * :***.

Talaromyces     ATCGACATCTACATGAAGGCTAAGGACCTGGGCATTGAAAACAAGCATAAGACTTTAACT 900
Cladonia        TTCG----CTATTTCGAATCNCTGGCACTTN--------------------------CN 692
                :***    *** :* .*. *..:**..** .                           *.

Talaromyces     GTGCTTGCTCAGACCATCTTTGACGAGAAGATTGTCAAACAGATTCCATCTCGTGCTGGC 960
Cladonia        TTNCTTTCN------------------------TCAANAN--TTCC--CNCCTACNGGC 723
                 *.*** *.                        **** .   ****  *.* *.*.***

Talaromyces     ATGCTCAAGAAGTTGATCACTTCCGAGCGCCATGAGAAGGCCTTCCTCGGTGGTACTGAG 1020
Cladonia        CN-------------------CTGGGCANGCNGGGGCGGCCT--------GGAAATNTG 755
                ..                   * *.**.  ..*.*..*****        **:*.*.:*

Talaromyces     CGTTTCGTAGGCAAGGACCATCCTGAGCTCATCTCTCAAGTACCGGCTATCCTTCTCGGA 1080
Cladonia        AAAATCCTANGAAT-------------CNGGCCCCNNAGNCACAANGGAAACTCCACN-- 800
                ..::** **.*.*:             *. . * *. *.. **...  *:.** *:*.

Talaromyces     TACTATCAGAACGATCTTGTTTCTGAGGATGTCCTCACCGCTTGGGGCAGCAAGGCCAGC 1140
Cladonia        ----------------------GTGGAGNAACT----------GNNATCAAGGCCAAA 826
                                     *:*** .:.**          *. * ********..

Talaromyces     AAGAAGTATGTCGATCCTTCTACCAGCCGAAAGGTTCGCAAGGCTGCTGAAAAGTTTCTC 1200
Cladonia        ACTGG----------------------GAACNG---GCAANG---------------TC 845
                *. ..                       ***..*   ****.*               **

Talaromyces     GAGTGGCTCTCGACTGCCGACAGCGATGAGAGTGAAGAAGAGTCTGAGTAA 1251
Cladonia        GGGNANGTACNNNATCCNAAAANCGAA----------------------- 872
                *.*... *.  . .* * .*.*.***:
```

Figure D1: (continued) GAP alignment of EST transcripts isolated during phosphorus starvation (matching a protein encoding an eukaryotic translation initiation factor 5). The alignment was conducted with CLUSTAL W2 (www.ebi.ac.uk).

```
Penicillium     ATGGGTTTCGGTGGTGATGTTAACCAGGCCGAGAAGTCGGTCTTCGGCATGCCCGGCTTC 60
Cladonia        -------TCG---GAGACGAACCCGGCGCCTTTGAAGAGAGACTCGACA--CCC----TC 44
                       ***   *:** *::..* . *** : .*. .*. . ***.**  ***    **

Penicillium     GTTGTCGACTTCCTGATGGGTGGTGTTTCCGCCGCTGTCTCCAAGACCGCTGCTGCCCCC 120
Cladonia        CTTGGCGACGATCTGGCGG--------------GCAGCATCCATGG----AGCTG----- 81
                 *** **** : ***. **              **:* .****:*.    :****

Penicillium     ATTGAGCGTATCAAGCTCCTGATCCAGAACCAGGATGAGATGCTCAAGCAGGGTCGTCTC 180
Cladonia        -TTGTACTTGACGG---CCT-------CACCAG----------------AGG------TC 108
                 ***:.* *.:*..   ***       .*****                ***      **

Penicillium     GACCGCAAGTACAACGGCATTGTTGACTGCTTCCGCCGTACCGCCGCCGCTGAGGGTGTT 240
Cladonia        ATCATCATG--CGACGGCG--------------------AACGGTGTC-AAGAGGGT--- 142
                .:*. **:*  *.*****.                    *.**  * * .:******

Penicillium     GTCTCCTTGTGGCGTGGTAACACCGCCAACGTCATCCGTTACTTCCCCACCCAGGCCCTG 300
Cladonia        -----------AAGAGGCAACACCGGCACCGGTG---GTGACGGTCCAGCCGAG--CAGG 186
                           ..*:** ******* **.**  .   ** **   **..** **  *. *

Penicillium     AACTTCGCTTTCCGCGACACCTACAAGTCCATGTTCGCCTACAAGAAGGACCGTGATGGA 360
Cladonia        AAGGAGGCGAGGAAAGAGCCCT-------CAAGAGGACCAACGAGGAGAACGG------- 232
                **  : ** :  ...** .***       **:*:  .**:**.**.**.** *

Penicillium     TACGCCAAGTGGATGATGGGTAACCTTGCCTCCGGTGGTGCTGCCGGTGCCACTTCCCTC 420
Cladonia        ----------GCTTGATGG--AGTCGTACATTCCGAAGT-----AGAGACCACG------ 269
                         * :******  *. * *.*.* * *:.**     .*. .****

Penicillium     CTCTTCGTCTACTCCCTGGACTACGCCCGTACCCGTCTCGCCAACGACGCCAAGTCCTCC 480
Cladonia        GTAGACAACAATTCCGAG---AACGGAGGGACCG----AAACCACG--GTAGAGACCGGC 320
                 *. :*.:*:* *** :*   :*** . * ***     ...*.***  * ..**:**  *
```

Figure D2: GAP alignment of EST transcripts isolated during phosphorus starvation (matching a protein encoding an ADP/ATP). The alignment was conducted with CLUSTAL W2 (www.ebi.ac.uk)

```
Penicillium     AAGGGTACCGGTGAGCGCCAGTTCAACGGTCTCGTTGACGTCTACCGCAAGACCCTCGCC 540
Cladonia        AA---TACCGTCGGTGGCGAG------GGTCTTGCGGTAGACGTCAACGAGACCGTTG-A 370
                **   *****  *.  ** **      ***** *  *:.*:* :*..*.***** * * .

Penicillium     ACCGACGGTATTGCCGGTCTCTACCGTGGTTTCGGTCCCTCCGTTCTCGGAATTGTTGTC 600
Cladonia        ACTGGCG--CTCACCGGTACCCTTGGAGGACTTGG---CGTCGTTGGCGAGACGGGT-AC 424
                ** *.**  .* .*****. * :  *:**: * **   *  ****  **..*  * * :*

Penicillium     TACCGTGGTCTCTACTTCGGAATGTACGACTCCATCAAGCCCGTTCTCCTCGTTGGTCCT 660
Cladonia        GGGCGTAGTC-------CAGGGAGTAG----------------------ACGAAG----- 450
                 . ***.***       *.*..:***                        :**::*

Penicillium     CTTGAGGGCTCTTTCCTCGCCTCCTTCCTGCTCGGCTGGACCGTCACCACCGGTGCCGGT 720
Cladonia        -AGGAGGGAAGT------GGCACC---------GGCAG------CACCACCGGAGGCAAG 488
                 : *****.: *      * *:**          ***:*      *********:* *..

Penicillium     GTTGCCTCTTACCCTCTTGACACCGTTCGCCGTCGCATGATGATGACCTCTGGTGAGGCC 780
Cladonia        GTTACCCATCATCCACTTGGCG----TATCCATCACGG------TCCTTCTTGT-AGGCG 537
                ***.** .* * **:****.*.    *. **.**.*.        .* *** ** ****

Penicillium     GTCAAGTACAACAGCTCCATGGATGCTGCCCGCCAGATCGTCGCCAAGGAGGGTGTCAAG 840
Cladonia        AACATG-------------------------GACTTGTAGGTGTCGCGGAAAGCG--AAG 570
                .:**:*                        *.*: .*.*  * *..***..* *  ***

Penicillium     TCTCTCTTCAAGGGTGCCGGTGCTAACATCCTCCGTGGTGTTGCCGGTGCTGGTGTCCTG 900
Cladonia        ------TTCAGGG---------CTAGGGTACGTTGAAAAATAGAC--------TATCATG 607
                      ****.**         ***. .*.*   *:..:.*:*.*        *.**.**

Penicillium     TCCATCTACGACAAGGCCCAGATGCTCCTCCTCGGAAAGAAGTTCTAA 948
Cladonia        TCGAGTTTCGG------------------CCTCG-------TTTCA-- 628
                ** *  *:**.                  *****        ***:
```

Figure D2: (continued) GAP alignment of EST transcripts isolated during phosphorus starvation (matching a protein encoding an ADP/ATP). The alignment was conducted with CLUSTAL W2 (www.ebi.ac.uk)..uk).