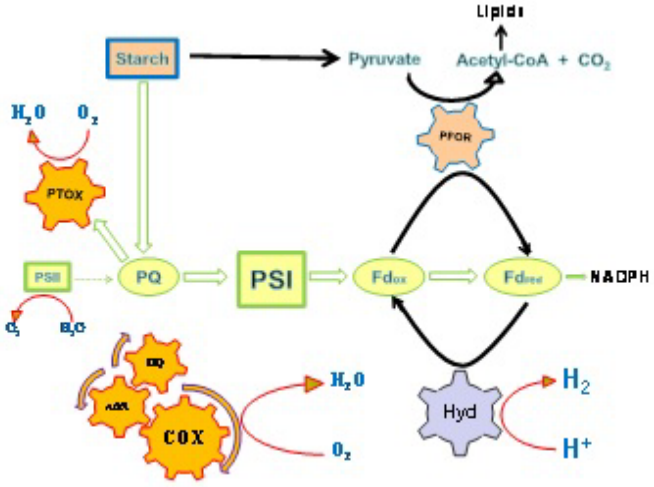
University of Crete

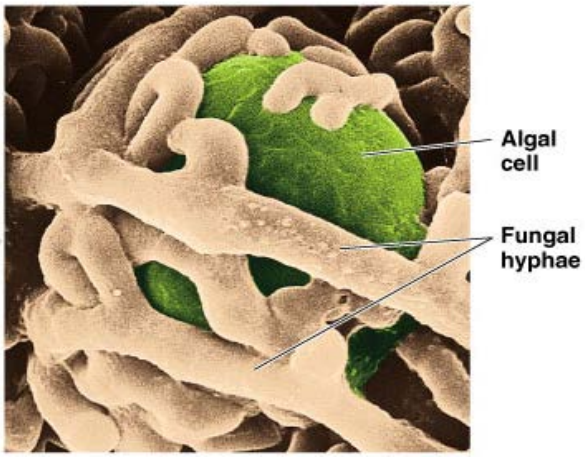
Department of Biology

Graduate Course: Plant Molecular Biology and Biotechnology

Master Thesis of Elizabeth Kastanaki

Hydrogen Production by Lichens





Supervising Professor: Prof. Kiriakos Kotzabasis

September 2015

Three-member Examination Committee

K. Kotzabasis - S. Pirintsos - E. Tsagris

ACKNOLEDGEMENTS

The completion of this master's thesis project would not have been possible without the help and support of many people. I would like to foremost thank my Professor Kiriakos Kotzabasis, for his continuous oversight and guidance throughout this thesis project. Also, many thanks to my committee members, Professor Stergios Pirintsos and Professor Efthimia Tsagris who was very helpful through the revision stage of this manuscript. I would especially like to thank Dr. Pirintsos for his relentless efforts in collecting and identifying the various lichens species required for the completion of this project. In addition, I am extremely grateful for the help and supervision of my thesis adviser Dr. Aikaterini Papazi for all of her time and energy spent assisting me throughout my experimental procedures. I owe a big thanks to all the individuals in our lab but especially to Dr. Nikos Ioannidis and Irini Gjindali.

Finally, I would like to thank my father Nikos Kastanakis for all of his sacrifices over the years and his continual financial support and tireless encouragement throughout my life. I thank my mother Marty, brother Yianni, sister Despina, aunt Lorry and my friends for all the love and support I could ever need.

THANK YOU

ABSTRACT

This master's thesis project examines the capability and the optimization of hydrogen (H2) production by lichens in a closed system. Lichens are symbionts formed by different species of fungi (mycobiont) and a photosynthesizing partner (photobiont) usually green algae or in some cases cyanobacteria. As the mycobiont provides protection from the external environment and accumulates water resources the photobiont provides chemical energy by means of photosynthesis. The photosynthetic H_2 production occurs in the green algae when they are under anoxic conditions, anoxic conditions (in a closed system) are primarily established via the respiratory O_2 consumption from the fungus but also from algae. A range of parameters affecting photosynthesis and respiration were experimentally tested in an attempt to identify the best experimental conditions for hydrogen production by the lichen *Pleurosticta acetabulum* in a closed system. These were; the concentration of glucose as an exogenously supplied carbon source, the incubation medium volume, the composition of the incubation medium, temperature and light intensity. Hydrogen production (in photobiont) and oxygen consumption (in photobiont and mycobiont) were quantified using Gas Chromatography (GC-TCD). A series of important photosynthetic, respiratory and hydrogen production proteins (PSaA, D1, COX, AOX, PTOX, PFOR and HYDA) were analyzed for their expression using western blots in order to identify and confirm the specific hydrogen production pathways under the optimal hydrogen production experimental conditions identified. The results demonstrated that lichens are able to establish anoxic conditions in a closed system mainly through O_2 consumption by the mycobiont while producing high yields of hydrogen by the photobiont utilizing three different pathways (PSII-dependent, PSII-independent and dark fermentation pathways). Under light conditions, they successfully use mainly the PSII-dependent and the PSII-independent pathways to transfer electrons to hydrogenase, while under dark conditions, lichens use the PFOR enzyme and the dark fermentative pathway to supply electrons to hydrogenase. Following the experimental testing of the lichen *Pleurosticta acetabulum,* the optimum conditions were utilized in the examination and analysis of H_2 production in a number of other lichen species which confirmed the general capability of lichens to produce large quantities of H_2 in a closed system. These benefits in conjunction with lichens' ability to survive in extreme environments constitute them as invaluable hydrogen producers, with great potential in future hydrogen production applications. A large part of the results presented in this master thesis were published in PLOS ONE [Papazi A,

Kastanaki E, Pirintsos S, Kotzabasis K (2015) Lichen Symbiosis: Nature's High Yielding Machines for Induced Hydrogen Production. PLoS ONE 10(3): e0121325. doi:10.1371/journal.pone.0121325].

ΠΕΡΙΛΗΨΗ

Με την παρούσα εργασία μελετήσαμε για πρώτη φορά τη δυνατότητα παραγωγής υδρογόνου (H_2) από λειχήνες σε κλειστό σύστημα και παράλληλα ελέγξαμε τις συνθήκες βελτιστοποίησης του. Ο λειχήνας είναι ένας συμβιωτικός οργανισμός που αποτελείται από διάφορα είδη μυκήτων και από κάποιο είδος φωτοσυνθετικού οργανισμού όπως χλωροφύκη ή σε κάποιες περιπτώσεις κυανοβακτήρια. Ο μύκητας παρέχει προστασία από το εξωτερικό περιβάλλον και διοχετεύει νερό στο χλωροφύκος το οποίο ως αντάλλαγμα σε αυτήν την συμβιωτική σχέση παρέχει χημική ενέργεια στον μύκητα μέσα από την διαδικασία της φωτοσύνθεσης. Η φωτοσυνθετική παραγωγή H_2 λαμβάνει χώρα στα χλωροφύκη όταν αυτά βρίσκονται σε ανοξικές συνθήκες, ενώ η επίτευξη των ανοξικών συνθηκών (σε ένα κλειστό σύστημα) γίνεται πρωτίστως μέσω της αναπνευστικής κατανάλωσης O_2 πρωτίστως του μύκητα αλλά και των χλωροφυκών. Πολλοί παράγοντες επηρεάζουν τη φωτοσύνθεση όπως και την αναπνοή, και ως εκ τούτου και την παραγωγή H_2. Κάποιοι από αυτούς είναι η παρουσία οργανικού άνθρακα, η κατάλληλη συγκέντρωση αλλάτων στο θρεπτικό μέσο, ο όγκος του θρεπτικού, η θερμοκρασία και η ένταση του φωτός. Για αυτόν τον λόγο πραγματοποιήθηκε σειρά πειραμάτων ελέγχοντας ξεχωριστά κάθε παράγοντα χρησιμοποιώντας το συγκεκριμένο είδος λειχήνα *Pleurosticta acetabulum.* Επισημάνθηκαν οι βέλτιστες συνθήκες παραγωγής H_2 χρησιμοποιώντας αέρια χρωματογραφία (GC-TCD) για την ποσοτικοποίηση παραγωγής υδρογόνου και κατανάλωσης οξυγόνου από τον λειχήνα και πραγματοποιήθηκε ανάλυση της έκφρασης κεντρικών πρωτεινών της φωτοσυνθετικής διαδικασίας, της αναπνοής αλλά και του ενζύμου της υδρογενάσης (PSaA, D1,COX, AOX, PTOX, PFOR, HYDA). Βάσει όλων των πειραματικών αποτελεσμάτων επιβεβαιώθηκε για πρώτη φορά η δυνατότητα υψηλής παραγωγής υδρογόνου από λειχήνες τόσο στο φως (με τη χρήση πρωτίστως του ανεξάρτητου και εξαρτημένου PSII-μονοπατιού) όσο και στο σκοτάδι (με τη χρήση του μονοπατιού της σκοτεινής ζύμωσης) και προτάθηκε ένα λειτουργικό μοντέλο του μηχανισμού παραγωγής H_2. Σε συνέχεια των πειραματικών δεδομένων με τον λειχήνα *Pleurosticta acetabulum*, χρησιμοποιήθηκαν οι βέλτιστες συνθήκες παραγωγής υδρογόνου σε μια σειρά άλλων ειδών λειχήνων που επιβεβαίωσαν τη δυνατότητα

εν γένει των λειχήνων (σε κλειστό σύστημα) να παράγουν μεγάλες ποσότητες H_2. Αυτά τα πλεονεκτήματα της συμβίωσης των λειχήνων σε συνδυασμό με την ικανότητά τους να επιβιώνουν σε ακραία περιβάλλοντα, αναδεικνύουν τους λειχήνες σε πολύτιμα βιολογικά εργοστάσια παραγωγής H2 και εμπνέουν μελλοντικές βιοτεχνολογικές εφαρμογές ακόμη και για το διάστημα. Αυτή η διατριβή είναι η πρώτη επιβεβαίωση παραγωγής υδρογόνου από λειχήνες και ένα μεγάλο μέρος αυτών των αποτελεσμάτων έχει δημοσιευθεί στο PLOS ONE [Papazi A, Kastanaki E, Pirintsos S, Kotzabasis K (2015) Lichen Symbiosis: Nature's High Yielding Machines for Induced Hydrogen Production. PLoS ONE 10(3): e0121325. doi:10.1371/journal.pone.0121325].

Contents

1. Introduction

1.1 Lichens

1.2 Lichen Anatomy and Morphology

1.3 Lichen Dispersal and Propagation-Reproduction

1.4 The Symbiosis: Mycobiont and Photobiont

1.5 The Photobiont

1.6 The Mycobiont

1.7 Uses and Applications of Lichens

1.8 Hydrogen Production Pathways

2. Materials and Methods

2.1 Organisms

2.2 Regeneration Protocol for Lichens

2.3 Experimental Procedure for Lichens

2.4 Inductive Fluorescence-Physicochemical Analyses of the Molecular Structure and Function of the Photosynthetic Apparatus

2.5 Extraction of Proteins - Quantitative Analysis of Protein-Electrophoretic Separation

2.6 Immunodetection - Western Blots of Proteins: Cytochromic Oxidase, Alternative Oxidase, Plastid Terminal Oxidase, PSaA, D1, Hydrogenase and Pyruvate Ferredoxin Oxidoreductase

2.7 Qualitative and Quantitative Analysis of Hydrogen (H_2) and Oxygen (O_2) by Gas Chromatography with Thermal Conductivity Detector (GC-TCD)

3. Results

Hydrogen Production by the Lichen *Pleurosticta acetabulum*

3.1 The Effect of Regeneration Time and Glucose Concentration on the Photosynthetic Activity of *Pleurosticta acetabulum* During Regeneration

3.2 The Effect of Glucose on Hydrogen Production

3.3 The Effect of the Culture Medium Volume on Hydrogen Production

3.4 The Effect of the Medium Composition on Hydrogen Production

3.5 The Effect of Temperature on Hydrogen Production

3.6 The Effect of Light Intensity on Hydrogen Production

3.7 Changes in Central Proteins of the Photosynthetic and Respiration Electron Transport Chains under Light and Dark Conditions

3.8 Is *Pleurosticta acetabulum* the Only Lichen Species Able to Produce Hydrogen?

4. Discussion

References

1. INTRODUCTION

1.1 Lichens

Lichens can be defined as the symbiosis between an alga and a fungus. This relationship is so interrelated that lichens are often regarded as a single organism (Bendre & Kuma, 2010). The fungal partner is known as the mycobiont (cyanophyceae or chlorophyceae) and the algal counterpart as the photobiont (green alga or cyanobacterium). The alga involved in this symbiotic relationship is unicellular. Generally, the mycobiont is an ascomycete however, in exceptional circumstances it can be a basidiomycete (Bendre & Kumar, 2010). Many times, the actual formed lichen tissue does not morphologically resemblance either of the partners. The various degrees of lichenization make it incredibly complicated to define the broad range of relationships that occur within these organisms (Nash, 2008).

The photobiont may have phenotypic morphological control over the entire morphology of the lichen due to the fact that the mycobiont is able to form discrete yet unified thalli with a green alga or cyanobacteria. Experimentally, the mycobiont only begins lichen formation in the first minutes of contact with its photobiont, up to this point it remains rather unstructured. Many species of photobionts can be found both in the lichenized state in addition to free-living unicellular organisms. However, because not many lichen alga have been definitively assigned to species as well as the undetermined systematics of many cyanobacteria and unicellular green algae, it is difficult to verify which species are able to survive in either life form. Nevertheless, most lichens express high specificity in relation to their photobiont (Nash, 2008). Lichens are able to develop in a wide range of habitats, from the tropics to polar regions, generally they are found in terrestrial environments but sometimes occur in marine intertidal zones and fresh water streams (Nash, 2008). They are defined as epiphytes, growing on the surface of other plants, and epiliths, growing on the surface of rocks. Lichens can generally be found on trees, rocks, ground surfaces and manmade surfaces. Because of the nature of their symbiosis, they can occupy habitats that if separated, as fungi or unicellular algae, would never be able to survive in (Nash, 2008). Taking advantage of the protection of lichenzation the photobionts are able to tolerate high light intensities that adversely affect them and gain water absorption due to the low water potential of the

fungus, symbiotic mechanisms such as these have enabled their expansion and proliferation in extremely harsh environments.

1.2 Lichen Anatomy and Morphology

Morphological variation in lichens is fairly diverse, varying in color from orange, yellow, red, green, gray, brown, black to size (range of length from mm to m) and growth rate. The majority of lichens are perennials, meaning they survive for a period of time longer than two years but some ephemerals (surviving a short period of time) have been identified.

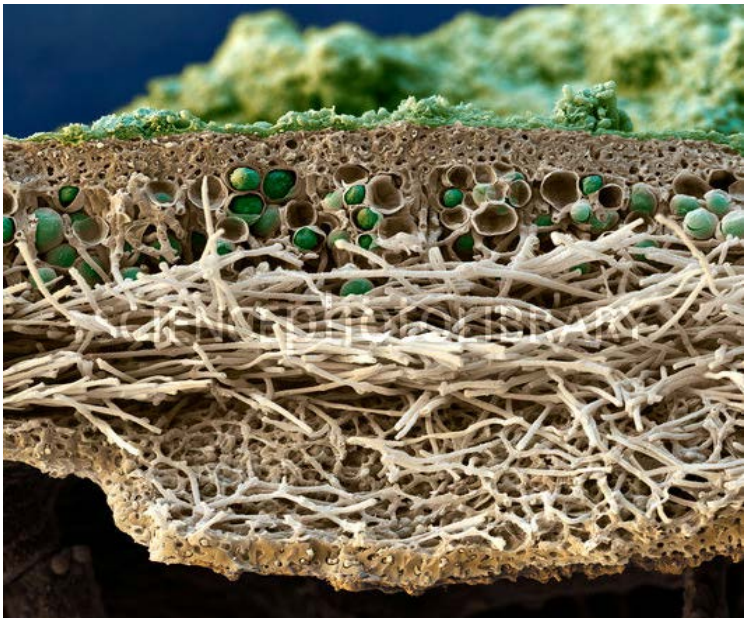


Figure 1. Scanning Electron Microscopy cross section of lichen *Parmelia sulcata.*
Source: **http://www.sciencephoto.com/media/16655/view**

Lichens are poikilohydric, which enables them to alter their water content based on their environmental surroundings in a passive way. They are also able to make use of various forms of water sources such as fog, dew and moisture from unsaturated air by taking advantage of the water potential gradient existing in the atmosphere of the lichen and its own low osmotic value within the thallus. Lichen classification

takes into consideration their growth and development, as well as their physiology (form) and the mode of attachment to their substrate. Lichens are classified in three major categories as follows: 1. Crustose or Crustaceous, 2. Foliose or Foliaceous and 3. Fruticose or Filamentous. This classification is based on the nature of the fungal component found in the lichen, the organism can be categorized as a colichen, if the fungal component is an ascomycete or as a basidiolichen if the fungal component is a basidiomycete (Bendre & Kumar, 2010).

The central characteristic of Foliose lichen anatomy is its four distinct layers. When inspected from top to bottom they can be identified as: Upper Cortex, Algal Layer, Medullary Layer and Lower Cortex (Wong, 2011). The upper cortex foliose lichens are leaf-like, have a flat surface and are only partly adhered to the substrate. Foliose thalli are either homoiomerous (gelatinous lichens) or heteromerous. In general, they have a dorsiventral organization with distinct upper and lower surfaces. In many circumstances, the thallus is separated into lobes, resulting in a range of branching levels. Foliose lichens develop a wide array of thallus sizes (Nash, 2008). Figure 1 shows the complex relationship and physical contact between the mycobiont (white/gray) and the photobiont (green spheres). *Xanthomaculina convoluta* and *Chondropsis semivirdis* are foliose lichen species which occupy deserts and semideserts. These types of lichens display hygroscopic movement, meaning that when they are in a dry state the thalli remain rolled up, revealing their lower cortices. Under such conditions the physiological activity of lichens approximates zero allowing them to survive extreme conditions, even a remarkable range of temperatures (from −196 °C to +60 °C) (Werth, 2011). After the absorption of liquid water, the thalli unroll and expose the upper surface of the thalli to the sunlight. As a result photosynthesis is considerably increased. In their dry state and inwardly rolled up, lichens can easily be transported by the wind and as soon as dewfall occurs, they unroll and expose the upper surface again (Nash, 2008). Lichens are masters of their environments by spending the harsh summer months (high light intensity and high temperatures) in a dehydrated state slowing down their metabolic needs and preserving themselves until the fall when rain and moisture are abundantly available.

The thallus lobes of Fruticose lichens are hair-like, strap-shaped or shrubby and the lobes can have a flat or cylindrical shape, usually extending out of the surface of the substrate. Some groups have dorsiventrally arranged thalli (for example, *Sphaerophorus melanocarpus and Evernia prunastri*), but

the majority have radial symmetric thalli [e.g. *Sphaerophorus globosus*, *Usnea* species and *Ramalina* species (Figure 2)].

Figure 2 Left: *Usnea barbata* Right: *Ramalina fraxinea*

Considerable variation exists in the branching pattern of lobes among different systematic groups but also within a single genus. The size of lichens differs considerably, ranging from some *Usnea* species which are able to grow several meters long, to minuscule species that are only 1 or 2 mm long. Fruticose lichens occupy a broad range of climates, from wet rainforests to desert habitats and can be found on various types of substrates (Nash, 2008). The *Usnea* genus demonstrates a peculiar beard-like type of anatomy, which has a strong central filament of periclinally arranged, conglutinated hyphae providing further support and strength along the longitudinal axis. Fruticose lichens that are well branched have a high surface to volume ratio, giving the tissue the advantage of drying and absorbing water more rapidly compared to lichens that have lower surface to volume ratios. Fruticose growth forms can be distinctly found in either very wet, humid climates, as in the case of *Usnea xanthophana* and *U. rubicunda* in temperate rain forests, or in arid climates with the continuous formation of dew and fog, as for example *Teloschistes capensis* in the Namib Desert (Nash, 2008). Foliose lichens can be recognized by the leaf-like appearance of their thallus. This category of lichens utilizes rhizens (root-like structures) to adhere themselves to the substrate, at a variety of points, on which they grow. Due to their dispersed attachment, foliose lichens can be easily removed from their substrates (Wong, 2011). Moreover, some

of the characteristics important in lichen substrate association are bark pH, water holding capacity, substrate texture, richness in nutrients and temperature (Spier et al., 2010)

1.3 Lichen Dispersal and Propagation- Reproduction

Dispersal is the movement of propagules or spores required for gene flow. This movement can be accomplished through water in the form of droplets, surface trickles, streams, and rivers. It is unlikely that flowing water plays an important role in carrying ascospores to a suitable habitat, with the exception of aquatic species. However, lichen fragments have been found in arctic drift ice and meltwater. Vegetative propagation, involving either whole thalli or its fragments as well as specialized structures (e.g. soralia or isidia), is a common method of reproduction in lichens. Transport is also facilitated by invertebrates and to a lesser extent by vertebrates (mostly through mammalian fur or bird feathers). Isidia form discrete outgrowths from the cortex and are usually constricted at their bases to assist in the detachment from the thallus by mechanical means. (Nash, 2008).

Soridia disbursement occurs via slow wind speeds and water droplets. Erratic and itinerant species have the astonishing capability to re-establish themselves independently in new locations by the detachment of major tissue parts or even complete thalli. This dispersal mechanism can result from frost, insolation, or animal interference acting on a parent body often established on an unstable substrate, common in arid areas. Lichens are also able to disperse ascospores (and indeed whole ascomata), as well as other propagules in addition to thallus fragments, while in mobility. Alternatively, vegetative reproduction through growth occurs in some foliose, squamulose, and lobate crustose lichens where the radially developing thalli begin to die off in the center of the tissue mass, leaving only an outer ring of outwardly-growing lobes (Nash, 2008). Thallus fragmentation is a result of very selective invertebrate grazing significantly benefiting the dispersal of lichens. Even though, in many instances dispersal is local, propagation along further distances can take place with the aid of flying insects. It has been found that some insect larvae-cases are in part, made up of lichen fragments. Human activity plays a central role in inadvertent introductions (for instance, lichens can be accidentally transported to new habitats on unmarked firewood, timber, stone or peat) leading in some cases to an increase in cosmopolitan taxa (Nash, 2008).

Even though adopting asexual reproductive strategies is quite advantageous, the production of many propagules with viable bionts are equipped to exploit new micro and macro environments, the potential for genetic recombination and therefore all possibility for further evolutionary development is lost. The efficient distribution of reproductive propagules does not guarantee their successful establishment and development, appropriate substrates and environmental conditions are required (Nash, 2008). Specifically, the successful germination and establishment of different types of lichen propagules depends on the characteristics of the substrate, such as texture, pH, nutrient status, micro-topography, in addition to, many other environmental factors, like humidity and temperature. Furthermore, competition between the established lichens and other organisms for space, light, and nutrients by the newly developing propagule is significant. This competition may develop by mechanical action, inhibition of gaseous exchange, reduction in light intensity and the release of toxic chemicals (Nash, 2008). Finally, symbiotic associations may be controlled by the symbiont's reproductive mode. Lichens are able to reproduce sexually as well as asexually. Specifically, mycobionts produce either sexual spores, requiring new associations with photobionts in each generation, or vegetative propagules containing fungal tissues and photobiont cells (Casano et al., 2011).

1.4 The Symbiosis: Mycobiont and Photobiont

The photobionts living within the mycobiont help form the joint thallus establishing the entire lichen morphology. This incredible symbiosis is a result of the opportunity for the green algae to take advantage of the mycobiont's ability to provide a beneficial microenvironment. In opposition to the notion that the mycobiont may have a greater advantage point in the symbiosis, here we may be able to deduce that the algae have been able to expand their habitats while maintaining the use of different bioenergetic pathways through evolution. It might be assumed that important functional links between photobionts and mycobionts, and possibly even other associates, would mean that these partners evolve simultaneously. However, this is certainly not always the case because sequencing in the Cladoniaceae has shown that the fungus and alga have evolved quite independently of each other. This can be understood in view of low specificity between some partners. Nevertheless, lichens undoubtedly represent one of the most successful forms of symbiosis in nature. They are to be found worldwide,

exploiting not only all manner of natural, usually stable, micro and macro environments, but in many cases adapting to extreme conditions, including some brought about by human disturbance (Nash, 2008). As poikilohydric organisms, lichens have an extraordinarily high stress tolerance, similar to that of the poikilohydric bryophytes. In an experiment performed by the European Space Agency, two lichen species, *Rhizocarpon geographicum* and *Xanthoria elegans*, were launched into space, and exposed to space conditions for 16 days mounted on an Earth-orbiting satellite. After returning to earth, the same specimens regained full metabolic activity within 24 hours, after having survived exposure to high-vacuum, extreme temperatures, as well as vast levels of UV and cosmic radiation, conditions lethal for bacteria and most other microorganisms (Werth, 2011).

1.5 Photobiont

Photosynthesis is the process by which organism (plants, photosynthetic bacteria and algae) absorb light energy emitted by the sun and convert it to chemical energy using it for all their metabolic and physiological needs. Close to forty genera of green algae and cyanobacteria have been documented as photobionts in various lichen species. The most common photobionts found in lichens are green algae. Cyanobacteria are of prokaryotic nature and lack chloroplasts, mitochondria, and a nucleus, all of which are found in eukaryotic algae. Metabolite transfer from the autotrophic photobiont to the heterotrophic mycobiont depends on the type of photobiont involved. The organization of green-algal photobionts is simple: only coccoid, sarcinoid or filamentous forms are known (Nash, 2008). The photobiont cell population is housed, maintained, and controlled within the fungal thallus. (Nash, 2008).

This large inconsistency in numbers suggests that many lichen fungi share a common pool of photobionts. The photobionts found in lichens include eukaryotic green algae (phylum Chlorophyta), other eukaryotic algae (phylum Heterokontophyta), as well as cyanobacteria. Most of the photobionts taking part in this symbiosis can be classified into four genera: the green algae *Trebouxia* and *Trentepohlia* and the cyanobacteria *Nostoc* and *Scytonema* (Werth, 2011). Numerous mycobionts associate with a single species, or even a single clade of photobiont. However, the same photobiont may associate with several different lichen fungi. In addition, most of the studies on population structure

have reported the presence of a single primary photobiont per thallus. In other cases, multiple algal genotypes have been found in a single thallus which may confer advantages in the lichen's ability to respond to environmental changes or to occupy diverse microenvironments. Further support for this argument is obtained by considering lichen thalli as micro ecosystems in which the fungus is the host and the photobiont(s) the primary producer(s). In nature, there are multiple examples demonstrating that positive interactions among potential competitors can sustain the stable coexistence of multiple species (Casano et al., 2011).

1.6 Mycobiont

Lichenization is a very successful nutritional strategy adopted by heterotrophic organisms like fungi in an attempt to acquire fixed carbon. The acquisition of fixed carbon from a population of minute, living algal and cyanobacterial cells, is a common and widespread method to ensure that nutritional needs can be met. One out of five fungal species is lichenized. A high percentage of lichen-forming fungi are ecologically obligate, but physiologically facultative biotrophs (organisms that obtain nutrients from a living host). In other words, they can be cultured in the aposymbiotic (''free-living'') state but in nature almost exclusively the symbiotic phenotype is found. Non-lichenized germ tubes or other free hyphae of lichen mycobionts certainly exist in natural ecosystems, but, due to their notoriously slow growth rates, they cannot be recovered with conventional isolation techniques. The majority of lichen mycobionts overgrow photobiont cells on or within the substrate and form microfilamentous, microglobose, or crustose thalli, some of which are quite inconspicuous (Nash, 2008).

The formation of sporangia has been noticed from lichenised photobionts; these sporangia may develop either into aplanospores or zoospores. As they are able to survive the gut passage of lichenivorous mites, lichen photobionts could be dispersed by these invertebrates and form free-living colonies derived from faeces. There is evidence for the existence of free-living *Trebouxia* colonies, but the free-living populations appear to be small and not persistent (Werth, 2011). Lichen have the potential for long-distance dispersal due to their tolerance to stress and the microscopic size of their spores (propagules). It has long been recognized, that lichens with *Trebouxia* photobionts are more or less pervasive whereas lichens with *Trentepohlia* photobionts (e.g. Roccellaceae) are most common in

the Mediterranean, Subtropics and Tropics (Werth, 2011). The association with a spatially structured, locally adapted photobiont pool enables lichen fungi to survive in different habitats, and to be capable of surviving under a variety of selection pressures. These examples highlight that despite the wide distributions of many lichen fungi and presumably many photobiont species, the lichen symbiosis is not an association among random partners, but is fine-tuned in space, along environmental gradients (Werth, 2011).

1.7 Uses and Applications of Lichens

The occurrence of drastic changes in our natural and managed environments in the last century has been linked to accelerated technological development threatening the integrity of many natural environments. Disasters as droughts, floods and industrial failures such as oil spills and nuclear accidents, have urged us to place great concern and value on environmental quality. Moreover, with extremely high risks posed to human health, vitality and functionality in addition to the vitality of our natural world it would be wiser and much more efficient to prevent, rather than undo destruction. In this effort, monitoring has become an invaluable tool in predicting and identifying the environmental destruction caused by the contamination of air and water with pollutants. Biomonitoring can be defined as the mapping and monitoring of pollution on selected organisms. It is applied as a monitoring technique to water quality, air pollution, heavy metal accumulation, climate change and resource management such as the effects of habitat fragmentation as well as assessing conservation practices and biodiversity levels (Nimis et al., 2002). Biomonitors are organisms utilized in the quantitative identification of pollutants (Conti & Cecchetti, 2001)

Lichens are extensively known in the field of biomonitoring in terrestrial environments and are extremely widespread in terms of habitat colonization, which ranges from deserts to arctic regions and from natural to managed environments (Conti & Cecchetti, 2001). They can be found on various types of substrates such as trees, rocks, ground surfaces and even manmade surfaces. As a result their use as biological monitors in a wide range of ecosystems and in both urban and rural settings is very effective (Nimis et al., 2002) In addition, lichens have been proven to be of value and have been used in other areas of research such as in pharmacology. The antimicrobial activity of lichens is common, both against

disease organisms and soil microbes, as for example mycorrhizal species and wood decaying fungi. More than three hundred and fifty secondary metabolites are known from lichens (Turk et al., 2003). In some cases, the sealing of the outer surface of mycobiont hyphae occurs in order to restrict and direct the movement of water and solutes in the thallus to the apoplastic continuum inside hyphal walls. This actions helps to create and maintain dry intercellular spaces for the free exchange of gases important for metabolic processes (Nash, 2008). Lichens have also been utilized by the pharmaceutical industry. More specifically, many secondary compounds secreted as defense agents against herbivores and decomposer have been used by pharmaceutical companies as antibacterial or antiviral compounds. In addition, the utilization of other secondary compounds has led to the production of perfumes or as natural dyes (Nimis et al., 2002). Furthermore, lichens take part in the weathering of rocks and the development of new soils as they can be considered very important biological weathering agents. Research has demonstrated that lichens are capable of biodeteriorating stone substrates in a rather short timescale. Information is now available to support the fact that lichens often can produce substantial biomass through weathering and support a large range of biodiversity in micro and macro organisms, helping to create intricate food webs while greatly increasing energy flow and mineral cycling near the colonized substrates (Nash, 2008).

1.8 Photosynthetic Hydrogen Production Pathways by Green Algae

It is known that oxygenic photosynthetic organisms for example, green algae under anoxic conditions are able to utilize the photosynthetic apparatus to produce hydrogen gas. In this process water oxidation and H_2 production are linked through the utilization of the photosynthetic electron flow of these organisms (Dubini & Ghirardi, 2014). It has been proven that green algae are capable of producing hydrogen under anoxic conditions through the activation of three different pathways which use the hydrogenases as catalysts (Dubini & Ghirardi, 2014). Because green algae are able to directly link water oxidation through photosynthesis to hydrogen generation by hydrogenases there is much hope in the production of ample energy from the fundamentally limitless sources, of water and sunlight (Dubini & Ghirardi, 2014). The history of hydrogen production by green algae begins with the experiments completed by Gaffron in 1939 using the green alga *Scenedesmus obliquus.* Three classes of organisms

are known to be able to produce H_2 utilizing the process of photosynthesis. These are: photosynthetic bacteria, cyanobacteria and green algae (Ghirardi et al., 1997). There are three main biosynthetic pathways that result in hydrogen production utilized by green algae under anoxic conditions which are depicted in Figure 3. More specifically: the Photosystem II-dependent (PSII), PSII-independent and Dark Fermentation pathways.

In the PSII-dependent hydrogen production pathway the photosynthetic apparatus captures light and the recovered energy is used to facilitate water splitting, resulting in the transport of electrons down the electron transport chain to produce a low-potential reductant, which can be used to reduce a hydrogenase enzyme. The major disadvantage of this process is the extreme sensitivity of the hydrogenase enzyme to the O_2 produced and released by the dissociation of water (Hallenbeck & Benemann, 2002). On the other hand, in the PSII-independet pathway, electrons are released through starch catabolism and Photosystem II is bypassed resulting in its inactivation. In this pathway of hydrogen photoproduction the metabolite accumulation serves as an intermediate step between the photosynthetic oxidation of water and hydrogen production. Therefore, the reactions of oxygen evolution and hydrogen generation are separated from each other (Melis et al., 2000). This way the problem of the sensitivity of the hydrogen evolving process is potentially circumvented by the temporal or spatial separation of oxygen evolution and hydrogen generation (Hallenbeck & Benemann, 2002).

Finally, in the Dark Fermentation pathway the oxidation of hydrogen is coupled to the reduction of carbon dioxide while the concurrent respiratory uptake of oxygen is used to generate ATP (Hallenbeck & Benemann, 2002). The majority of microbial hydrogen production is driven by the anaerobic metabolism of pyruvate, formed during the catabolism of various substrates. The breakdown of pyruvate is catalyzed by one of two enzyme systems: Pyruvate formate lyase (PFL) and pyruvate ferredoxin oxido reductase (PFOR). Thus in both these biological systems, the pyruvate generated by glycolysis is used, in the absence of oxygen, to produce acetyl CoA, from which ATP can be derived, and either formate or reduced ferredoxin, from which electrons will be transferred to hydrogenase and H_2 can be produced. The overall yields in these metabolic pathways are relatively low. This can be attributed to the fact that fermentation pathways have been optimized through evolution to generate cell biomass and not hydrogen (Hallenbeck & Benemann, 2002).

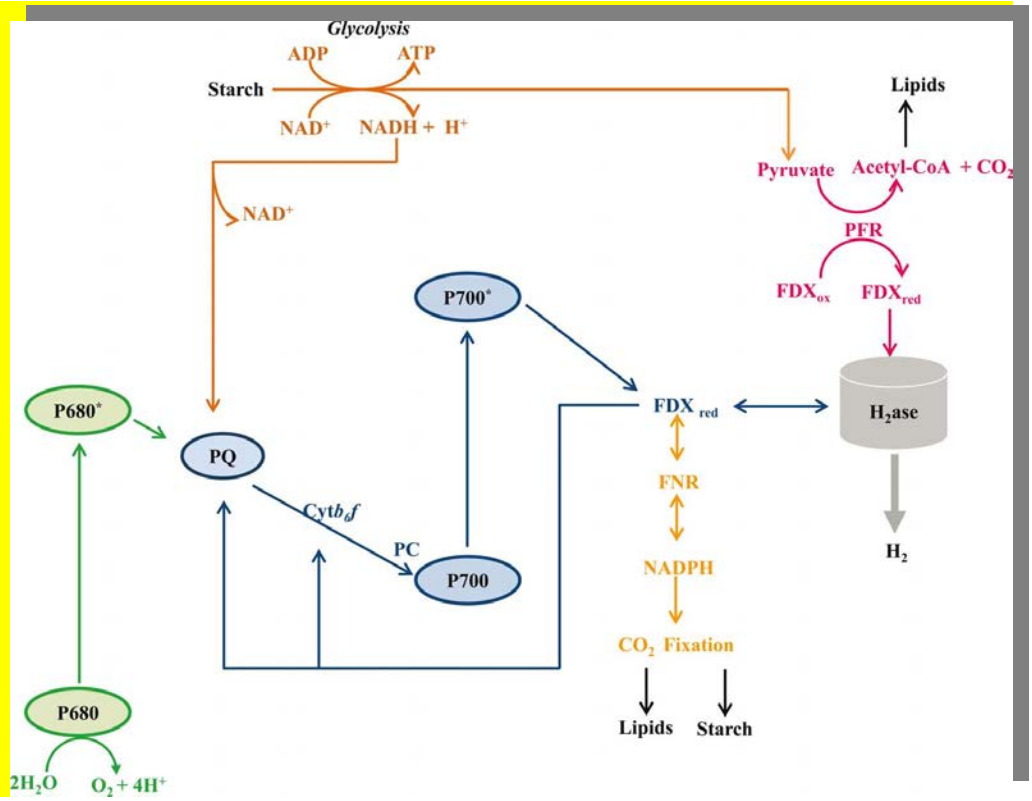


Figure 3. H_2 production pathways in green algea: PSII-dependant in Green/Blue, PSII-independent in Brown/Blue and Dark Fermentation in Brown/Pink

The most advantageous aspect of a photosynthetic hydrogen production system is the use of water as the source of a reductant (Ghirardi et al., 1997). Some of the limitations that should be addressed when working with green algae hydrogen production are: The sensitivity of its hydrogenases to oxygen, the competition for electrons that exists between the CO_2 fixation pathway and the H_2 production pathway as well as the low equilibrium pressure of H_2 release (Ghirardi et al., 1997). Some of the methods and techniques used to face these limitations are discussed below.

Continual purging algae cultures with inert gases within a closed system has been proposed as a means to eliminate the presence of O_2 and hence resolve the problem of the sensitivity of hydrogenase to oxygen. However, this method is considered expensive and impractical for the large scale production of hydrogen (Dubini & Ghirardi, 2014). As mentioned before, hydrogen production is catalyzed by the reversible hydrogenase, which is induced in green algae cells upon exposure to anaerobiosis for a short period of time. However, this activity is quickly blocked when illumination occurs due to the instant deactivation of the reversible hydrogenase by the presence of photosynthetically produced oxygen

(Melis et al., 2000). The reduction of protons by hydrogenase forms hydrogen gas, relieving the electron pressure within a cell by dissipating it. Sulfur deprivation has been proposed as a two stage model relying on the idea of sulfur playing the role of a reversible switch in the metabolic regulation of the activity of the oxygen evolving PSII complex. Hydrogen may be generated by algae as a mechanism to produce adequate amounts of the ATP required for survival under sulfur depleted anaerobic conditions. This occurs due to the deficiency of oxygen and the inactivation of PSII as a result, mitochondrial respiration and oxygenic photosynthesis are no longer viable pathways for the formation of ATP (Melis et al., 2000). Sulfur is required for the biosynthesis of cycteine and methionine. In sulfur depleted media of green algae cultures the turnover of the D1 protein, one of the two major subunits of PSII reaction center is inhibited. As a result, the repair cycle of PSII is unable to function and the corresponding oxygen evolution quickly decreases. Photodamaged D1, is not effectively repaired because of the elimination of sulfurylated amino acids. Therefore, it is apparent that endogenous substrate metabolism is a tremendously crucial factor in the production of hydrogen by green algea (Ran et al., 2009). In addition to sulfur deprivation potassium deficiency has been presented as a biochemical / bioenergetic switch for the sustained high yield production of hydrogen utilizing the photosynthetic apparatus of green algae. Potassium can partially be replaced by sodium in the majority of biochemical processes and as a result the system remains functional. However, sodium cannot replace potassium in the conversion procedure of glucose to starch. This fact significantly increases the yield of hydrogen production through the Photosystem II independent pathway, since electrons originating from the metabolism of glucose used for continuously donating electrons to the plastoquinone-pool of the photosynthetic electron chain. Additionally, PSII inactivation (and therefore the inhibition of O_2-production), the further synthesis and over activation of Photosystem I and plastidic hydrogenase, generates a sustained increase in hydrogen production, mainly through the PSII-independent pathway. The self regulation of these multistage processes in hermitically closed static systems of *Scenedesmus obliquus* cultivation, permittes the establishment of anoxic conditions and the continuous electron supply to highly activated hydrogenase, resulting in a sustained high yield hydrogen production (Papazi et al., 2014).

Another method for addressing biological hydrogen production obstacles is presented utilizing a combinational biological system where the biodegradation procedure of one *meta*-substituted dichlorophenols (*m*-dcps - chemical pollutants in industrial wastewater) is the key element for maintaining continuous and high rate H_2-production of the green alga *Scenedesmus obliquus* (Papazi et

al., 2012). In particular, it has been reported that reduced *m*-dcps, according to their redox potential, take on the role of electron donors in the photosynthetic electron flow, close to the plastoquinone pool (PQ). In parallel, they block the activity of photosystem II and the release of O_2, leading to the establishment of oxygen-depleted conditions in a closed system. Additionally, the first step of *m*-dcps biodegradation seems to be the *m*-dcps reduction that supports a continuous circuit between oxidized and reduced *m*-dcps, which continuously promotes strong electron flow to the PQ-pool, and in turn to ferredoxin. As a result, photosynthetic hydrogen production is induced strongly and continuously by the hydrogenase activation, because of the establishment of oxygen-depleted conditions. This finding allows for the possibility of green microalgae to operate as "smart bioenergetic machines" for a continuous H_2-production, through an electron bypass between the *m*-dcps biodegradation pathway and the photosynthetic procedure. The regulation of these multistage and highly evolved redox pathways leads to high yields of photosynthetic hydrogen production utilizing simple energy sources and one *meta*-substituted dichlorophenol as regulating elements (Papazi et al., 2012).

The work presented in this thesis focuses on the study of the nature and adaptability of the lichen's metabolism, specifically the bioenergetic pathways utilized by the photobiont (for H_2 production) when adapting to a closed-system environment where anoxic conditions have been established through mycobiont respiration. The novelty of our idea that lichens could be the ideal organisms for H_2 production derives from their symbiotic relationship; they consist of O_2 consumers (fungus) as well as O_2 and H_2 producers (green algae) in a common phenotype. In a closed system, the fungus consumes oxygen, establishes anoxic conditions and ensures the appropriate conditions for effective hydrogen production by it's photobiont. In other words, lichen symbiosis could be viewed as a high yield natural machine for hydrogen production. In the present work, I tested the above hypothesis by exploring the capacity of the lichen *Pleurosticta acetabulum* to produce hydrogen under various incubation conditions such as different glucose concentrations, medium factors (volume and composition), temperature levels and light intensities. In addition, to further explore the range of validity of this hypothesis, various lichen species were tested for their capability to produce hydrogen, based on the best hydrogen producing conditions outlined by *Pleurosticta acetabulum*.

2. MATERIALS AND METHODS

2.1 Organism

In the following research project the species *Pleurosticta acetabulum* was used in the majority of experiments while additional lichen species were utilized to test whether other lichen species are also able to produce H_2. Its phylogenetic taxonomy is reported below:

Kingdom: Fungi

Division: Asomycota

Class: Lecanoromycetes

Order: Lecanorales

Family: *Pleurostictaceae*

Genus: *Pleurosticta*

Species: *Pleurosticta acetabulum*

Pleurosticta acetabulum is a green algae lichen and can be described as having a dark olive-green to brown-grey (oily green when wet) thallus, closely appressed and spreading, wrinkled towards the centre, with lobe ends turned up appearing dotted with dark spots. Its lower surface is pale brown to black. There are no soredia or isidia but large apothecia with red-brown discs and in-rolled margins are common.

The species *Pleurosticta acetabulum* shown in figure 5 in its regenerated state was utilized in the testing of all the preliminary experimental parameters to determine the optimal hydrogen producing experimental conditions. The decision to use this particular species was based on its abundance in the field and the feasibility of its collection. Following, a screening of various lichen species was completed using the same experimental parameters pinpointed by the H_2 productivity conditions of *Pleurosticta acetabulum*. The protocol number for the permission to collect our lichen samples is 108436/956 and was giving by the Greek Ministry of Environment Energy and Climate Change. Additionally, thirteen different species of lichen were tested for their hydrogen producing capability, these include: *Ramalina fraxinea, Ramalina farinacea, Ramalina calicaris, Pseudevernia furfuracea, Evernia prunastri,*

Pleurosticta acetabulum, Parmelia sulcata, Parmelina pastillifera, Cladonia rangiformis, Cladonia convoluta, Anaptuchia ciliaris, Usnea barbata, Xanthoria parietina.

Pleurosticta acetabulum and *Xanthoria parietina* samples were collected from *Acer sempervirens* and *Quercus coccifera* substrates of Mountain Idi. *Cladonia convoluta* and *Cladonia rangiformis* have been collected from a Mediterranean shrubland area of Northern Greece close to the city of Kilkis. Finally, the remaining species (*Ramalina fraxinea, Ramalina farinacea, Ramalina calicaris, Pseudevernia furfuracea, Evernia prunastri, Parmelia sulcata, Parmelina pastillifera, Anaptuchia ciliaris, Usnea barbata)* have been collected from deciduous substrates (mainly Quercus trees) of Mountain Kerkini (Belles). Permission for the investigation and collection of lichen material was granted by the Greek Ministry of the Environment, Energy & Climate Change, Special Secretariat for Forests, Directorate of Aesthetic Forests, National Parks and Game Management, Athens, Greece (108436/956).

2.2 Regeneration Protocol for Lichen Species

As much of the tree bark, weeds, mosses and dirt as possible was removed from the lichen samples so that the sample dry weight (Fig. 4) was comprised of only lichen tissue. 2g (dry weight) of the lichen tissue was weighed for each sample bottle. Following, 2g of lichen tissue was placed in a piece of tool, with the edges gathered and closed with a clip so that all the pieces of lichen tissue were secured inside the tool. A large plastic container was filled with Deionised Water (DI) in which the pouch of tool with the lichen sample inside was sunk and incubated for 10 minutes. The pouch was taken out of the DI water and opened, the lichen tissue was placed on an absorbent piece of paper towel and sprayed with deionised water using a water dispenser. The lichen tissue was placed on the wet paper towel with the edges folded making a rectangle. The paper towel-packaged lichen was placed in a small plastic zip-lock bag, that was sprayed again with DI water before the bag was closed, so that there was plenty of moisture for the duration of the regeneration.

Figure 4. ***Pleurosticta acetabulum*** **dry lichen tissue**

Figure 5. ***Pleurosticta acetabulum*** **lichen tissue after regeneration**

The packaged lichen sample was then let to sit in the moistened bag at room temperature for 1 hour at 20 ^{o}C and afterwards for 20h at 5 ^{o}C. The quality of the regeneration process was tested using fluorescence induction measurements and the JIP-test (see below). Figure 5 shows the regenerated lichen tissue after the completion of the regeneration protocol.

2.3 Experimental Procedure

After regeneration the lichen sample zip-lock bags were removed from the cold room (4 ^{o}C) and a sterile environment was prepared. The sample was carefully transferred into glass 125 mL hermetically sealed bottles (Fig 6, diameter 5 cm, height 9.5 cm) with an initial volume of 10 mL sterilized medium solution (deionised water with 5 g L^{-1} glucose). The bottles were placed sideways (as to maximize exposure to light) in a temperature-controlled chamber (30 ^{o}C) at a light intensity of approximately 20 μmol m^{-2} s^{-1}. The above conditions were the usual ones. Any changes are explained in detail in the appropriate subsection of the results.

Figure 6. *Pleurosticta acetabulum* lichen tissue sealed in a 125mL bottle under incubation conditions

2.4 Inductive Fluorescence-Physicochemical Analyses of the Molecular Structure and Function of the Photosynthetic Apparatus

Only a fraction of the energy absorbed by the pigments of the photosynthetic apparatus is used in the photochemistry of photosynthesis. The excess light energy is emitted either as fluorescence or as heat. The induction of fluorescence from photosynthetic organisms was first observed by Kautsky and Hirsch in 1931 (Maxwell & Johnson, 2000). Plant fluorescence takes place in two phases, the first of which is rapid and the second slow. Today, the study of the fluorescence induction curve especially the fast phase has evolved into a valuable research tool for the study of the molecular structure and function of the photosynthetic apparatus.

Fluorescence increases when the photochemical efficiency of photosynthesis is inhibited. For example when there is no available oxidized electron acceptor at any point in the course of electron transport. When the photosynthetic mechanism (regenerated lichen tissue), after dark adaptation, is continuously illuminated the fluorescence increases from the initial level (F_o) to a maximum level (F_m) and then gradually decreases until reaching a constant level (F_S) as shown in figure 7. Measurements of the induction of fluorescence were completed using the portable Plant Efficiency Analyzer (Handy PEA Hansatech Instruments) (Fig. 8), followed by data analysis using specialized software: JIP-test (Biolyzer HP 4.0), according to the methodology of Strasser & Strasser (1995).

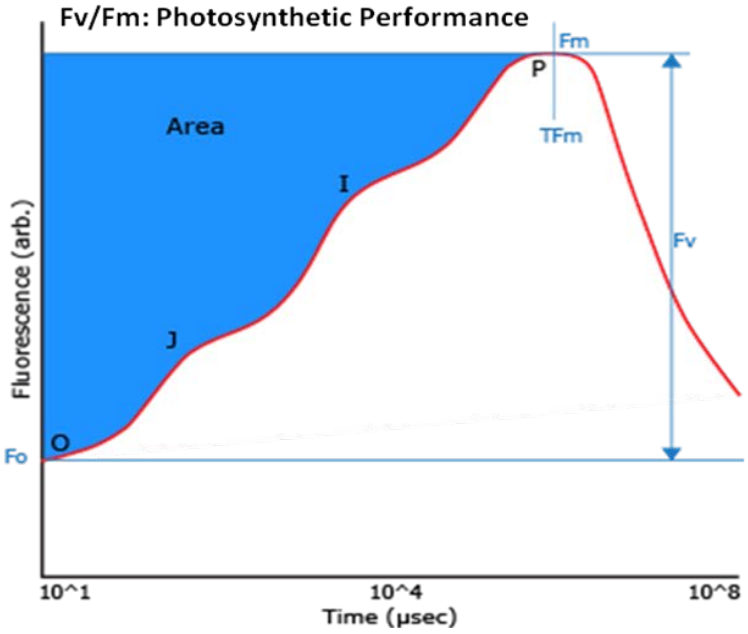


Figure 7. Typical Fluorescence Induction Curve. Showing the different stages of the curve and photosynthetic parameters Fo: Minimum Fluorescence, Fm: Maximum Fluorescence and Fv: Variable Fluorescence. Source: http://hansatech-instruments.com

All measurements were made using special clips that isolate a portion of the lichen tissue surface to be measured. An incubation time of 5 min in the dark was required to "empty" the reaction centers of electrons. The ratio F_v/F_m (Variable Fluorescence/Maximal Fluorescence) was used as an indicator of maximum photosynthetic efficiency (Strasser & Strasser, 1995) to insure the successful regeneration of our sample lichens and thus securing good quality lichen tissue for the beginning of the experiments.

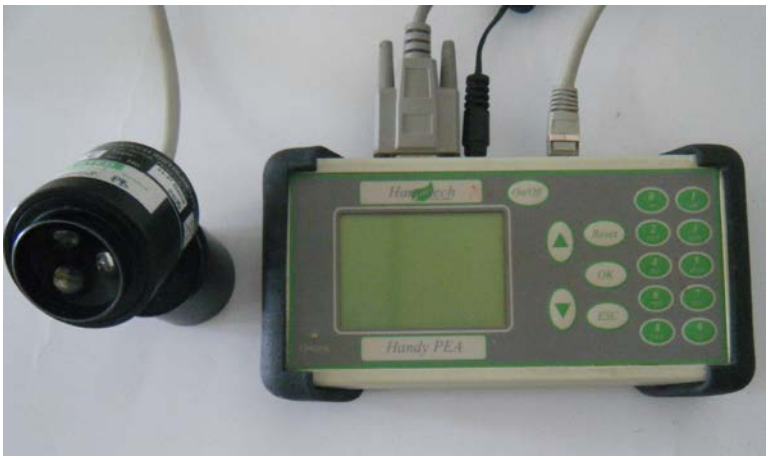


Figure 8. Handy PEA Plant Efficiency Analyzer

In addition, by applying the JIP-TEST for fluorescence values, corresponding to defined stages [O, J, I and P] we calculated individual characteristics of the photosynthetic apparatus. The ratio ABS/RC, which is associated with the functional antenna size of the Light Harvesting Complex of PSII. The ratio DI_o/RC, associated with the dissipation energy lost as heat and the ratio RC/CS_o, linked to the density of the active PSII reaction centers and PI_{ABS} expressing photosynthetic performance per amount of energy absorbed (see details in Table 1).

This method is based on measurements of the rapid variation of the fluorescence with a resolution of 10 μs in a period of 1 second. Fluorescence was measured with 12-bit resolution and the induction was done by lighting three diodes (LEDs) with a radiation intensity of 3000 μmol m^{-2} s^{-1} red light (650nm). The Handy PEA data sampling operates at a maximum frequency of 100 kHz only for the first 300 μs and then the frequency decreases.

Table 1. OJIP-Test Measurement Parameters

Abbreviation of Measurement (Variable)	Definition
F_t	Fluorescent value at time t after irradiation
$F_{50\mu s}$	Fluorescence intensity at 50 μs
F300μs	Fluorescence intensity at 300 μs
$F_J = F_{2\mu s}$	Fluorescence intensity, step J (2μs) curve OJIP
FI = F30μs	Fluorescence intensity in step I (30 μs) the curve OJIP
F_P (= F_m)	Maximum fluorescence intensity at P curve OJIP
Area	Total additional area between the curve and the straight OJIP passing from F = Fm
JIP- Test parameters	
F_o	Minimum fluorescence value, correspondding to open centers (open PSII RC t = 0)
F_m	Fluorescence maximum, corresponding to closed centers (closed PSII RCs, t = tF_m)
F_v	Variable fluorescence at time t
$F_v = F_m - F_o$	Maximum variable fluorescence value
$V_t = (F_t - F_o) (F_m - F_o)$	Relative fluorescence change at time t

$V_J = (F_J - F_o) (F_m - F_o)$	Relative fluorescence change in step J
$M_o = (\Delta V/\Delta t)_o = 4(F300\mu - F_o) /(F_m - F_o)$	Initial slope of the curve in ms V = f (t)
S_m = (Area) / $(F_m - F_o)$	Supplemental area of the OJIP curve, normalized in terms of Fv (a measure of redox cycles of QA)
$S_s = V_J / M_o$	Supplemental OJIP area of the peak corresponding only to the OJ phase (interval, the QA of the RC is reduced once)
$N = S_m / S_s = S_m M_o (1/V_J)$	The number of reduction cycles of QA in the interval
Specific energy flows (per QA reduction center)	
$ABS/RC = M_o (1/V_J)(1/\Phi_{Po})$	Size of functional Light Harvesting Complex
$TR_o / RC = M_o (1/VJ)$	Energy trapped by the reaction center (for t = 0)
$ET_o / RC = M_o (1/VJ)$ WO	Electron flux per reaction center (for t = 0)
$DI_o / RC = (ABS / RC) - (TR_o / RC)$	Dissipated energy per reaction center (for t = 0)
Quantum efficiencies or flux ratios	
$\Phi_{Po} = TR_o/ABS = [1-(F_o/F_m)]$	Max quantum yield for primary photochemistry (at t = 0)
$\Psi_o = ET_o/TR_o = 1 - V_J$	Probability that trapped exciton moves electron into the electron transport chain beyond Q
$\Phi_{Eo} = ET_o/ABS = [1-(F_o/F_m)]\Psi_o$	Quantum yield for electron transport (at t=0)
$\Phi_{Do} = 1 – \Phi_{Po} = F_o/F_m$	Quantum yield of electron diffusion
Energy flows excited by region	
ABS / CS_o	Absorbed excitation energy per area by F_o
ABS/CS_m	Absorbed excitation energy per area by Fm
$TR_o/CS_o = FP_o (ABS/CS_o)$	Caught energy per excited area of the membrane (for t = 0)
$ET_o/CS_o = (ABS/CS_o)$	Flow per electron excitation region (for t = 0)
$DI_o/CS_o = (ABS/CS_o) - (TR_o/CS_o)$	Diffused excitation energy per area (for t = 0)
Density of active reaction centers	
RC/CS_o	Density of active reaction centers
Performance indicators	
$PI_{ABS}=(RC/ABS)(\Phi_{Po}/1-\Phi_{Po})(\Psi_o/1-\Psi_o)$	Performance per energy absorbed
$PI_{CSo}=(RC/CS_o)(\Phi_{Po}/1-\Phi_{Po})(\Psi_o/1-\Psi_o)$	Performance per stimulated region (t = 0)
$PI_{CSm}=(RC/CS_m)(\Phi_{Po}/1-\Phi_{Po})(\Psi_o/1-\Psi_o)$	Performance per stimulated region ($t = tF_m$)
$SFI_{abs} = (1-\Phi_{Po})(1-\Psi_o)$	Functionality Index
Ability to Produce Work (Production Capacity)	
$DF_{ABS} = \log (PI_{ABS})$	Estimated production output per energy absorbed

2. 5 Extraction of Proteins - Quantitative Analysis of Proteins - Electrophoretic Separation

Total proteins were extracted from lichen tissue, according to a modified method of Siminis, et al.(1993). Lichen tissue was first homogenised by grinding with liquid nitrogen. Grounded tissue was incubated in an Extraction Buffer [200 mM Tris-HCl pH 8.0, 10 mM EDTA, 200 mM NaCl, 200 mM MgCl2, 0.5 mM PMSF, 5 mM DDT, 10 mM Leupeptin, 10% (v/v) glycerol, 0.25% Triton X-100] at 4 ^{o}C overnight to ensure tissue breakdown and then centrifuged for 30 min at 4^{o}C and 10.000 g. The supernatant was collected. Quantification of protein, was completed using the method of Lowry et al. (1951). Specifically, 10 ml of protein extract were precipitated by adding equal volume of 20% (v/v) TCA and the retention of the samples at 4 ° C for at least 30 min. Followed by centrifugation for 10 min at 17.000 g, the supernatant was disgarded and the precipitate redissolved by adding 100 ml of the solution A (10 g Na_2CO_3, 0.1 g K-Na Tartrate, 2 g NaOH per 500 mL water). Following, 1 ml of solution C [10 mL A and 0.2 mL B (0.5 g $CuSO_4.5H_2O$ per 100 mL water)] was added and incubated for 10 min. Then, 100 ml of the solution D containing the phenol reagent Folin Ciocalteu (Merck, Darmstadt, Germany) diluted 1:1 (v/v) with dH_2O. The samples were incubated at room temperature for 30 min and their absorbance was measured at 625 nm.

The samples were quantified utilizing their optical density, based on a reference curve constructed with bovine serum albumin (BSA). As a negative control measurements without protein samples were made. The relative concentration of proteins was confirmed by staining the gel SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) with Coomasie brilliant blue R-250. The proteins were mixed with sample buffer, in the ratio 1:6 respectively, (sample buffer: 25 mL of 20% (v/v) SDS, 13 mL Tris / HCl 1M pH 6.8, 0.24 g bromophenol blue and supplemented with glycerol up to 50 mL) to which the required amount of mercaptoethanol was added shortly before use (7 μL mercaptoethanol per mL sample buffer) (Mattoo et al., 1981). The samples were then boiled for 5 minutes and immediately placed on ice. The samples were loaded in a 10% or 13% acrylamide gel depending on the protein tested. The loading amount of each sample was determined by the protein concentration using the method of Lowry, so that all the samples were loaded with an equal amount of protein. In the beginning, electrophoresis (Fig. 9) operated at 80 mV for the stacking gel with consistency: [stacking gel 4% - 4,5 mL dH_2O, 1.85 mL upper tris (6.06 g Trisma base pH 6.8 with

concentrated HCl), 1 mL Acrylamide: Bis (58.4 g acrylamide and 1.6 g bis in 200 mL dH_2O), 26 µL ammonium persulfate 10% (v/v), 11 ML TEMED]. Followed by an increase in voltage to 120 mV for the resolving gel with consistency: [resolving gel 10% - 5.9 mL dH_2O, 3.75 mL lower tris (36.3 g Trisma base pH 8.8 with concentrated HCl), 5 mL Acrylamide: Bis (58.4 g acrylamide and 1.6 g bis in 200 mL dH_2O), 0.29 mL glycerol 50% (v/v), 64 µL ammonium persulfate 10% (v/v), 5 µL TEMED].

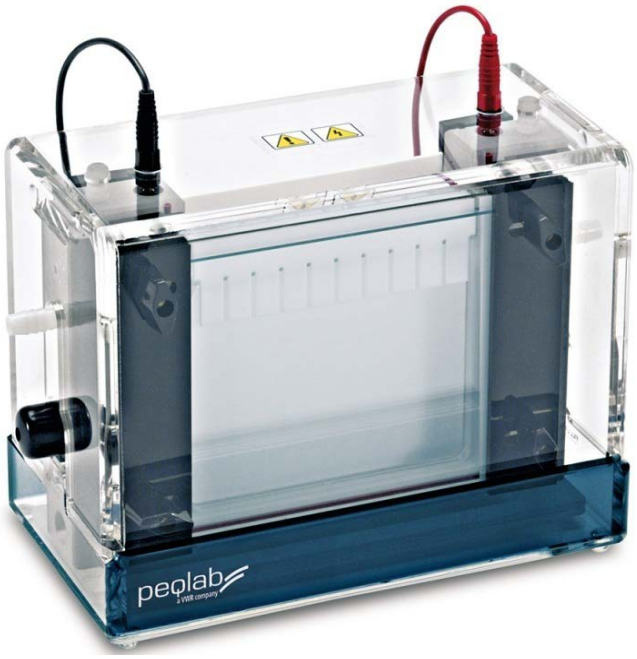


Figure 9. Electrophoresis Device.
http://www.peqlab.com/wcms/en/products/index.php?do=getArticleDetails&which=45-1614-C

After electrophoresis was completed the gel was placed in a solution of Coomasie brilliant blue R-250 (50% methanol, 10% acetic acid, 40% dH_2O and 2.5 g Coomasie brilliant blue R-250) being gently agitated at room temperature for approximately 20 min, followed by decolorization with a solution of 50% (v/v) methanol, 10% acetic acid and 40% dH_2O (v/v). The gel was then rinsed with dH_2O and photographed with a digital camera using the software Kodak Digital Science v1.9.

2.6 Immunodetection -Western Blots of proteins HydA, PsaA, D1, PFOR, PTOX, COX, AOX,

The total protein extracts were separated by 10% SDS-PAGE, as described above for the PTOX, AOX, PFOR and HydA proteins, while for the D1 protein, COX and PsaA 13% SDS-PAGE was used. After the electrophoresis, the proteins were transferred electrophoretically (western blot) to nitrocellulose membrane (Porablot, 0.45 microns, Macherey-Nagel GmbH & Co.KG.Duren). The transfer solution contained 3g Trisma base and 14.4 g of glycine per liter dH_2O. The transfer of proteins from the gel to the nitorcellulose membrane was completed at 80V for 1 h. The antibodies used were anti-protein PSaA, mounted in PSI (Agrisera), protein D1, which is mounted in the reaction center of PSII (Agrisera), hydrogenase HydA (Agrisera), the enzyme that synthesizes molecular hydrogen in the chloroplast and PFOR (Pyruvate Ferredoxin Oxidoreductase), the enzyme that is activated during Dark Fermentation and transfer electrons to Hydrogenase. As well as the respiration antibodies PTOX (Plastid Terminal Oxidase) which catalyzes the oxidation of the plastidic plastoquinone pool. The mitochondrial Cytochromic c Oxidase (COX) receives an electron from each of four cytochrome c molecules, and transfers them to one oxygen molecule, converting molecular oxygen to two molecules of water. Alternative Oxidase (AOX) an enzyme that forms a portion of the electron transport chain in mitochondria. The antibodies were used in the most appropriate dilution in each case, which was confirmed after preliminary testing of the respective antibody at various dilutions.

For the completion of the western blots the protocol of Agrisera was followed whereby the membranes initially were moistened for 10 min with PBS (8 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 0.2 g KCl, pH 7 with KOH in 1 liter dH_2O). Then the membrane was placed in a blocking solution with 4% milk powder in PBS-T for 1h (PBS solution with 0.05% Tween 20) and two 10 min washes in PBS-T and two 5 min with PBS, before incubation in the antibody for one hour at room temperature. The washing step was repeated (twice, 10 min with PBS-T and 5 min with PBS) before incubation with the anti-antibody for 1h. At this point, four 5 min washes with PBS-T and one 15 min with dH_2O were done, to avoid any interactions of the Tween with ECL (Enhanced Chemi Luminescence). The ECL solution was then added, after the slight drying of the nitrocellulose membrane that would cause intense background signaling in the development of the film, which followed. The protein bands obtained by the western blots of PSaA, HydA , D1, PTOX, COX, AOX and PFOR were quantified and expressed as a

percentage normalized to the band intensity of the control sample. The quantification of western band proteins took place utilizing the image analysis program imageJ and was expressed as % relative intensity of the control.

2.7 Qualitative and Quantitative Analysis of Hydrogen (H_2) and Oxygen (O_2) by Gas Chromatography with Thermal Conductivity Detector (GC-TCD)

For the qualitative and quantitative analysis of oxygen and hydrogen produced, Thermal Conductivity Gas Chromatography GC-TCD was used (Shimadzu GC 2010 Plus, Kyoto, Japan - Figure 10), with Argon as the carrier gas (5 bar Pressure - 23.5 ml min^{-1}). A one mL gas sample was injected in the GC-TCD via auto-sampling, where the separation of H_2 and O_2 were based on thermal conductivity of gases. The thermal conductivity of Argon is 0.0001772 W $(cmK)^{-1}$, that of Oxygen is 0.0002674 W $(cmK)^{-1}$ and Hydrogen is 0.001815 W $(cmK)^{-1}$. A capillary column of 30 m, 0.53 mm in diameter and 20 microns thick (Vici Metronics MC Poulsbo, USA) with the oven temperature at 120 ^{o}C was used for gas separation. The TCD detector temperature was 200 ^{o}C with a temperature of 180 ^{o}C at the injection entry point. The quantification of H_2 and O_2 was determined by creating a reference curve using known quantities of gas. Each experiment was repeated at least three times and each treatment included five independent samples. Standard deviations of the average values are presented on each diagram.

Figure 10. Gas Chromatography Thermal Conductivity Detector

3. RESULTS

3. 1 The effect of regeneration time and glucose concentration on the photosynthetic activity of *Pleurosticta acetabulum* during regeneration

For a lichen to be able to produce hydrogen due to the nature of the symbiotic relationship between the mycobiont and the photobiont it must be metabolically active and able to function at its highest photosynthetic efficiency. To insure this important parameter of the experimental testing, it was essential to achieve the highest possible quality of regenerated lichen tissue with the highest possible photosynthetic efficiency (performance). Therefore, initial experiments were completed testing the effect of regeneration time and glucose concentration on the photosynthetic efficiency of the lichen tissue. It was expected that an optimal range of regeneration time would be necessary for the lichen to become metabolically active after "awakening" from its dry state, so that the tissue would adapt and function in accordance to its new regenerated state. Also, a range of different glucose concentrations (0, 0.5, 1 , 2.5 and 5g/L) were used as a exogenously supplied organic carbon source to kick start the lichens metabolic processes in the regeneration stage. The JIP TEST was used to assess the potential photosynthetic efficiency of the regenerated lichen tissue. Regeneration time increments include 30min, 1h, 2h, 3h, 5h and 24h . The thirty minutes of regeneration correspond to room temperature and one hour regeneration was also completed at room temperature. The time increments from 2h-24h include a 1h incubation at room temperature before being placed in a cold room at 4 ^{o}C for the remainder of each corresponding regeneration time. Measurements of the fluorescence induction are represented in the graphs demonstrating the OJIP curves.

The results of the regeneration kinetic experiments are shown in Figures (11-14) where all data are normalized with the control time of thirty minutes and compared with the control concentration of 0g/L glucose. As demonstrated in Figure 11, all fluorescence induction curves are in extremely close proximity to or above the control curve of 30 min. There is an evident increase in the maximal fluorescence as regeneration time passes, with the exception of the 24h regeneration time mark falling slightly lower than the 5h one but still surpassing the control.

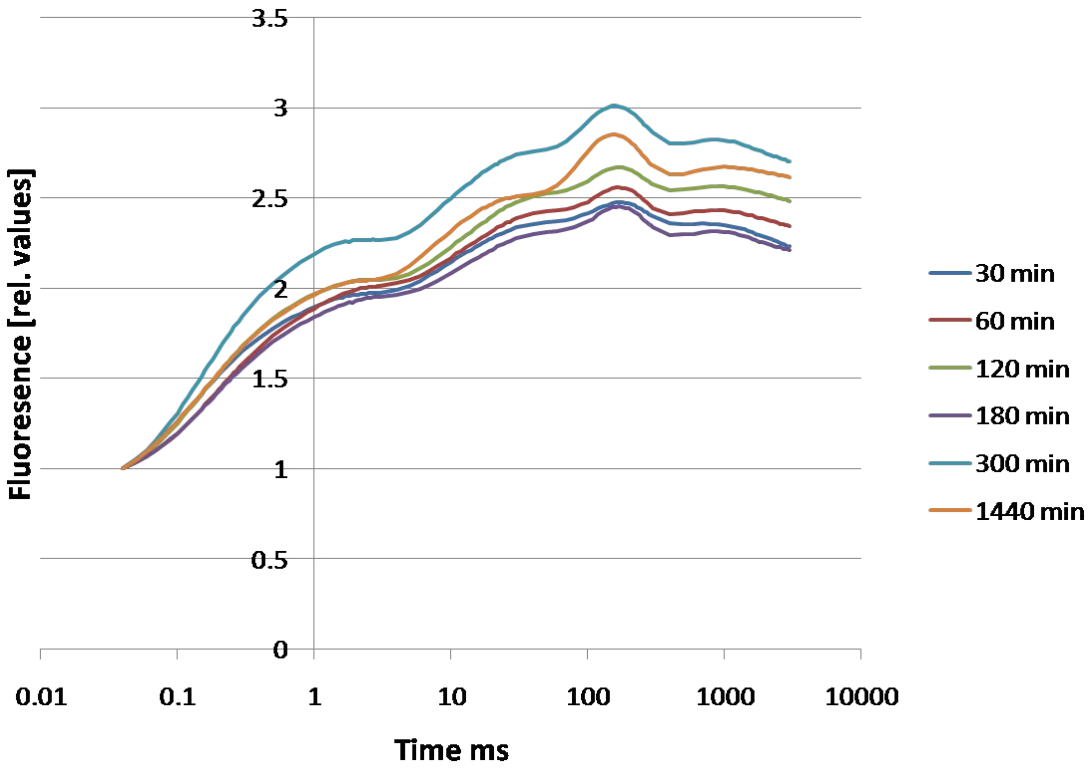


Figure 11. OJIP Fluorescence induction curve showing maximum fluorescence at various regeneration times (30', 1h, 2h, 3h, 5h, and 24h) for *Pleurosticta acetabulum* at 0g/L glucose concentration and normalized at 4 msec with the regeneration time of 30'

The parameters chosen to test the vitality and photosynthetic, activity of each lichen tissue sample can be found in the table below with a brief definition. Fv/Fm was chosen as a parameter suitable to provide evidence for the general vitality of the lichen tissue. It represents the maximum possible efficiency of Photosystem II of each dark adapted sample. However, it does not measure the real photosynthetic rate, only the potential performance of the photosynthetic light reaction. The usual range of this index for lichens is 0.60-0.76. Nevertheless, some crustose species can demonstrate lower values such as 0.50-0.60. (Nimise et al., 2002). PI $_{ABS}$ was utilized as an index for the overall photosynthetic efficiency. RC/CS_o represents the density of the active reaction centers, DI_o/RC is the dissipation energy per reaction center, ABS/RC refers to the size of the functional light harvesting complex and finally, PSI_o is the primary photochemistry.

The radar plot shown in Figures 12 represents the results of the above mentioned experiments demonstrating the photosynthetic activity of the sample in each case utilizing the main photosynthetic efficiency parameters.

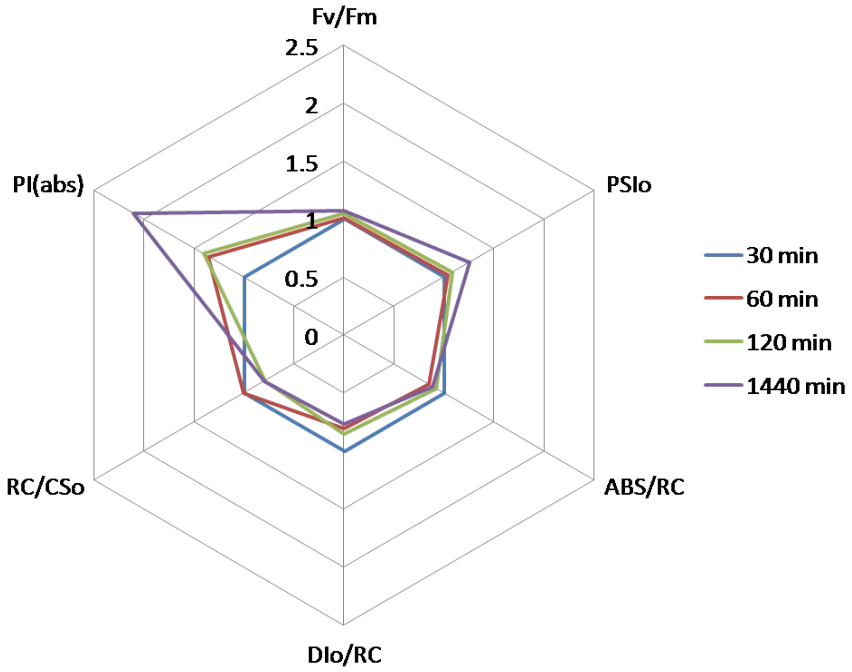


Figure 12. Radar Plot showing changes in the six photosynthetic parameters based on four different regeneration times (30', 1h, 2h, and 24h) for *Pleurosticta acetabulum* at 0g/L glucose concentration.

It is clear that in Figure 12 the PI values increase substantially as regeneration time increases. Fv/Fm also shows a slight increase with time whereas dissipation energy per reaction centre decreases, meaning less energy is wasted as heat. The size of the Functional Light Harvesting Complex and the Density of the Active Reaction Centers has decreased in comparison to the control showing no signs of a stress response. After considering both the induction curves and the radar plots of each regeneration time point, (24h) was chosen as the ideal one to optimize lichen tissue vitality and photosynthetic activity prior to the beginning of the incubation procedures.

Apart from the regeneration time another parameter examined was the addition of glucose to the regeneration DI water as a means to activate the lichens metabolic processes faster or more efficiently prior to incubation. The fluorescence induction curves of samples regenerated in different glucose concentrations after 24 hours are shown in the graph below (Fig. 13) The data are normalized using 24h and 0g/L glucose. The glucose concentration of 1g/L glucose seems to have the highest value in comparison to the control. Nevertheless, regeneration time may be a much more important factor than the addition of glucose in the regeneration stage.

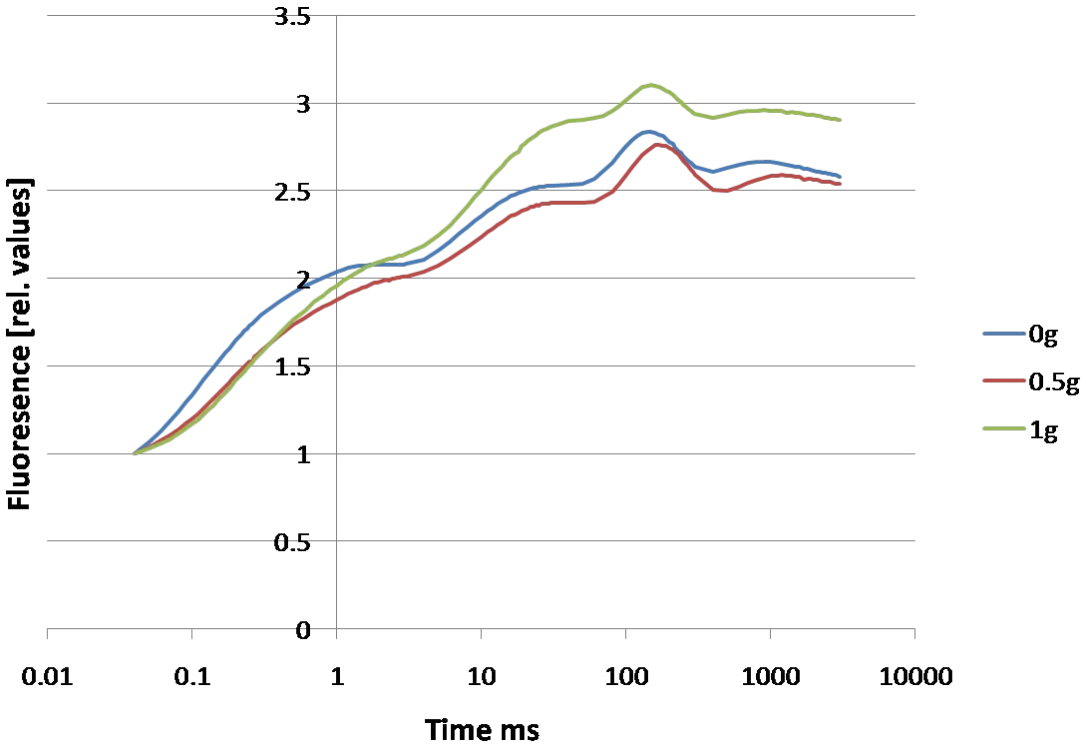


Figure 13. OJIP Fluorescence Induction Curve showing maximum fluorescence at the optimum regeneration time of (24h) for *Pleurosticta acetabulum* at various glucose concentrations (0, 0.5, 1g/L) and normalized with 24h at 0g/L glucose

The radar plot of the same samples is shown below in Figure 14. It is evident that 1g/L glucose shows the highest level of photosynthetic efficiency. Dissipation energy is lower in all glucose concentrations in comparison to the control. Fv/Fm seems to be at the same level of the control and the primary photochemistry is minimally higher that the control. The photosynthetic efficiency is increased in all

glucose concentrations but is the highest in 1g/L glucose. To further validate the role of the addition of glucose in the regeneration stage for the production of hydrogen a series of experiments were completed and are described below. The results were analyzed by gas chromatography validating or not the detection of hydrogen produced by lichens in the various glucose concentrations during the regeneration stage. Where the concern of the vitality and photosynthetic efficiency of the lichen tissue was jointly compared with the central hypothesis of the hydrogen producing capacity and optimization of *Pleurosticta acetabulum* lichen tissue samples.

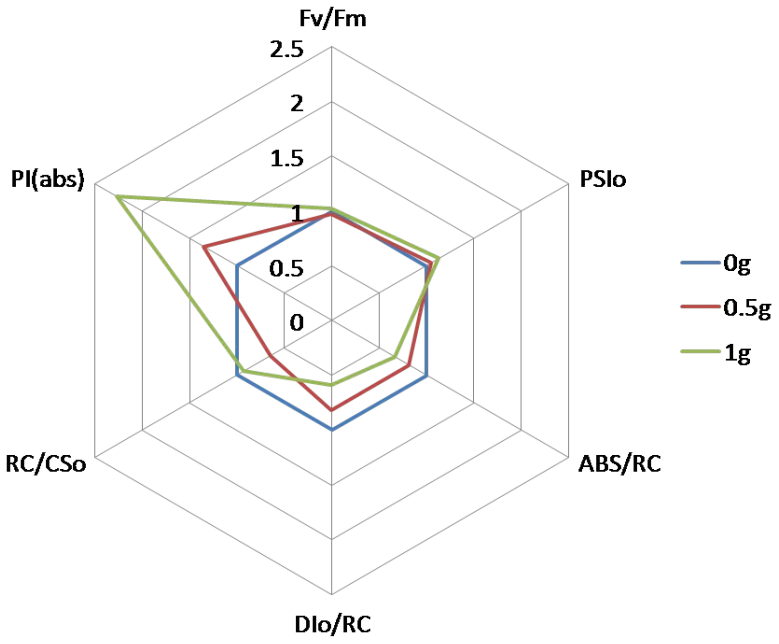


Figure 14. Radar Plot showing changes in the six Photosynthetic parameters based on the optimum regeneration time of (24h) for *Pleurosticta acetabulum* at various glucose concentrations (0, 0.5, 1g/L) and normalized with 24h at 0g/L glucose

3.2 The effect of glucose on hydrogen production

The central hypothesis tested in this work concerned the ability of lichens to produce hydrogen. In addition, if lichens could produce hydrogen then under which conditions would this hydrogen production be optimized? Many of the initial experimental parameters were chosen based on previous work in our lab regarding hydrogen production optimization by the green alga *Scenedesmus obliquus*. All lichen samples were collected in a dry state thus it was important to determine and develop an appropriate regeneration protocol that would "stimulate" the lichen's metabolic processes and allow both the mycobiont and photobiont to take part in the potential production of hydrogen. The lichen species *Pluerosticta acetabulum* was chosen as the appropriate lichen species to undergo all the preliminary experimental hydrogen production testing due to its abundant availability in the field and handling ease.

After the confirmation of this particular specie's ability to produce hydrogen under specific experimental conditions a variety of other lichen species were also tested. Previous work completed in our lab on hydrogen production by the green alga *Scenedesmus obliquus* has demonstrated that an organic carbon source is required to optimize photosynthesis (metabolic processes) and in turn may result in increased hydrogen production. The use of glucose as an exogenously supplied organic carbon source has also been confirmed to have a positive effect on the hydrogen productivity of green algae (Papazi et al., 2012; Papazi, et al., 2014; Papazi & Kotzabasis, 2013). As such, an increase in respiration (by the mycobiont) as well as an increase in hydrogen production (by the photobiont) was expected. Therefore, five different glucose concentrations (0, 0.5, 1, 2.5, 5g/L) were tested with the aim of determining the optimal concentration for enhanced hydrogen production. The Figure 15 demonstrates the hydrogen production and the corresponding oxygen consumption of 2g samples of the lichen *Pluerosticta acetabulum* following the regeneration stage and under a five day incubation period, in the above mentioned glucose concentrations. The regeneration procedure was followed as is explained in the Materials and Methods (M&M) section, utilizing deionised (DI) water. Sample incubation took place in a temperature and light intensity controlled chamber, where the temperature was set to 28-30^{o}C and

the light intensity measured as low light (LL) 20-25 μE.

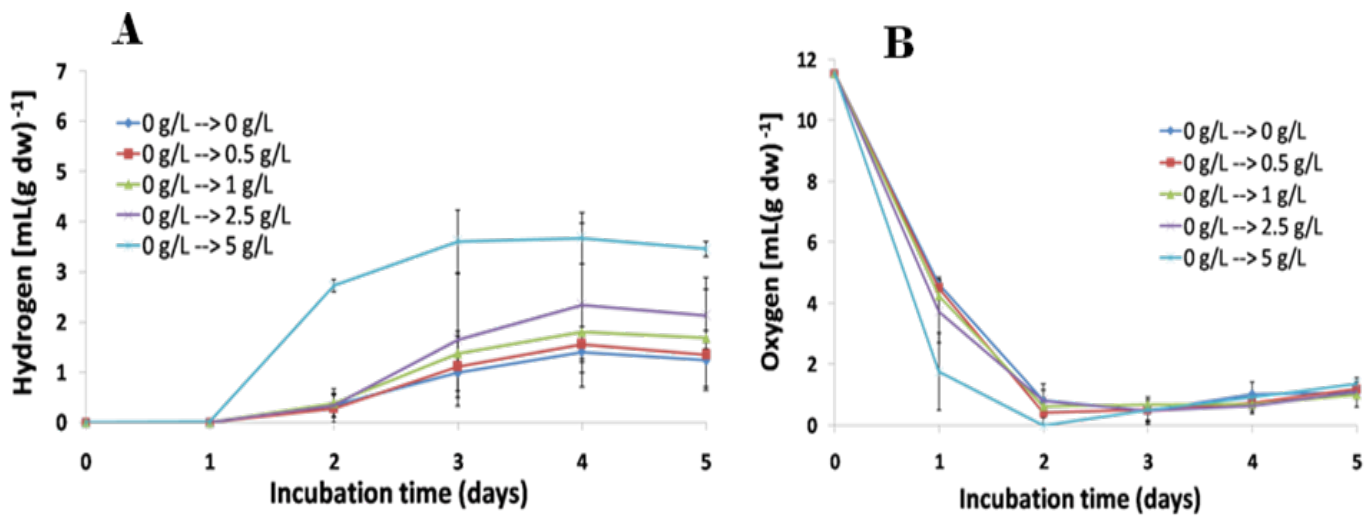


Figure 15. A. Hydrogen production and B. Oxygen consumption by the lichen *Pleurosticta acetabulum*. Regeneration in 0g/L glucose and incubation in various glucose concentrations (0, 0.5, 1, 2.5, 5g/L)

The glucose concentration of 5g/L was identified as the upper limit of the range based on previous experimental procedures which demonstrated that a higher concentration than this could result in osmotic stress. It is clear that the concentration of 5g/L glucose during the incubation stage is optimal regarding hydrogen production, resulting in a mean value of 3.7mL of H_2 per g of lichen dry weight in comparison to the control (0g/L glucose in the incubation stage) of 2.5g The samples represented in Figure 15 were regenerated in deionised water (no addition of glucose), while the medium used in the hermitically closed bottles contained glucose in the gradient concentrations previously mentioned. The increase in glucose concentration indeed resulted in the expected increase in oxygen consumption and, as a result in higher hydrogen production. Similar results appear in Figure 16, where the experimental conditions were reversed. In this case, the regeneration stage included the addition of glucose (gradient increase of glucose concentration), while the medium was devoid of it. This experimental treatment (using glucose only in the regeneration stage and not in the incubation stage) resulted in the same trend where higher glucose concentrations led to higher oxygen consumption and consequently to higher hydrogen

production. However, the actual hydrogen values were lower compared to the values in Figure 15.

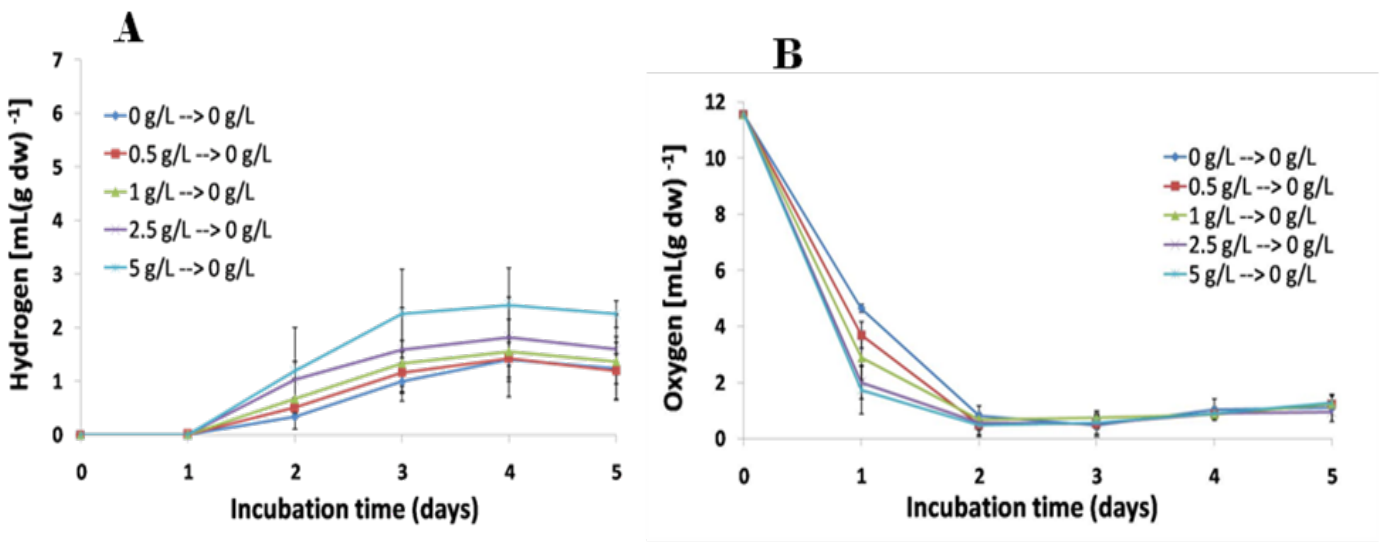


Figure 16 A. Hydrogen production and B. Oxygen consumption by the lichen *Pleurosticta acetabulum*. Regeneration in various glucose concentrations and incubation in 0g/L glucose

Specifically, the hydrogen productivity in the treatment of 5 g/L glucose only in the regeneration stage yielded 2.5 mL of H_2 per g dry weight, as compared to 3.7 mL of H_2 per g dry weight in the case of the 5 g/L glucose addition only in the medium of the incubation stage. The above results showed that in either case (the regeneration stage or in the incubation stage), the addition of glucose was beneficial for the optimization of hydrogen production. Moreover, these results demonstrated that the higher the tested glucose concentration, the higher the hydrogen production detected. Because of these observations the combined effect of using glucose in both the regeneration stage as well as in the incubation stage was experimentally tested. The results are presented in Figure 17. The influence of the addition of glucose in the regeneration stage was minuscule when glucose existed in the incubation medium in the optimum concentration of 5 g/L. Therefore, all of the following attempts for the optimization of the lichen's hydrogen production were completed in deionised water for the regeneration

stage and in 5 g/L glucose for the incubation stage.

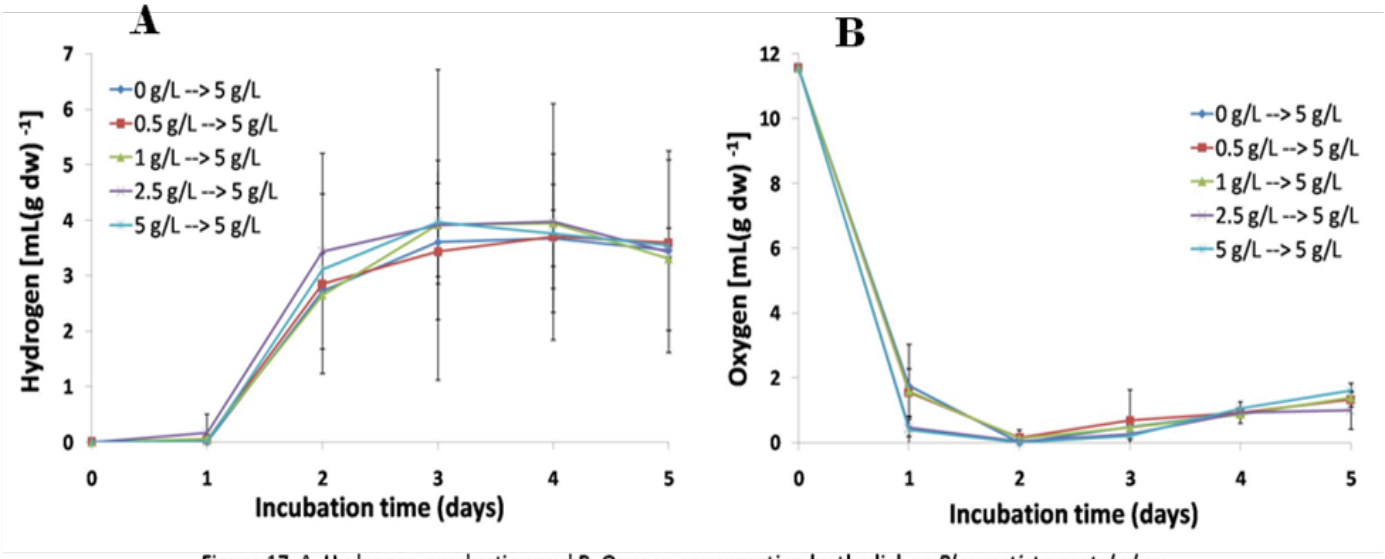


Figure 17. A. Hydrogen production and B. Oxygen consumption by the lichen *Pleurosticta acetabulum*. Regeneration in various glucose concentrations and incubation in 5g/L glucose

3.3 The effect of the culture medium volume on hydrogen production

In addition to the fact that the addition of glucose in the incubation medium of the hermitically closed bottles, increased the hydrogen yield of the lichens, the volume of the incubation medium could also indirectly play an important role in the optimization of hydrogen production by lichens. It was expected that a larger volume of the liquid incubation medium would lead to a lower initial available oxygen concentration (in the remaining air space of the 125 mL hermitically closed bottles), enabling the earlier activation of the hydrogenase enzyme.

Furthermore, an increased absorbance of glucose was anticipated, due to the improved contact of the lichen tissue within the medium that would lead to higher hydrogen production. This hypothesis was the main reason for testing the lichen's hydrogen production in several different liquid culture medium volumes (10, 25, 50 and 100 mL). The results for oxygen consumption were as expected (increase of medium volume led to the decrease of oxygen), but the hydrogen yields measured in the air space were exactly the opposite. The larger the incubation medium volume the lower the hydrogen yield measured,

as it is shown in Figure 18.

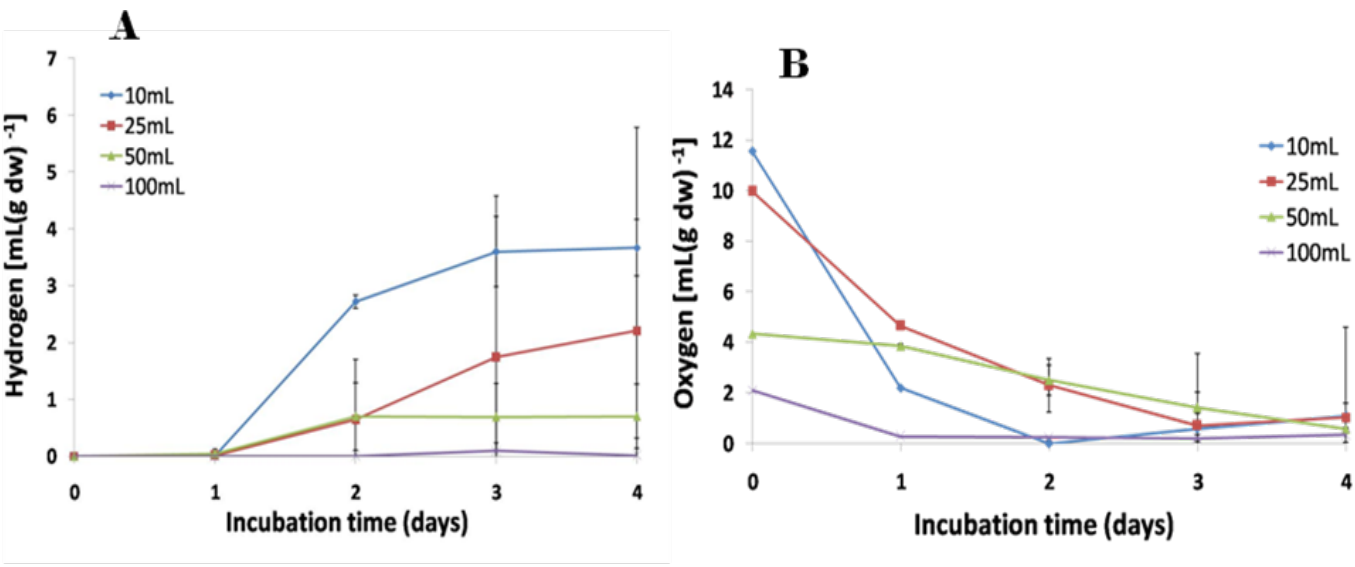


Figure 18. A. Hydrogen production and B. Oxygen consumption by the lichen *Pleurosticta acetabulum* in various incubation volumes (10, 25, 50 and 100mL)

This observation can be explained by the existence of the partial pressure of the gases in the air-liquid interface. The larger liquid volume increases the pressure of the now lesser remaining air space resulting in the confinement of much of the H_2 gas produced to the liquid-medium phase. The increased pressure of the air space did not allow the release of hydrogen gas from the liquid phase into the air space, and hence was not detected. This phenomenon is fully explained in the previous work of Papazi et al. 2014. The increased incubation medium volume resulted in earlier oxygen depleted conditions, but the partial pressures did not allow the release of the produced hydrogen from the liquid phase to the air phase in order to be detected by the gas chromatographer. During sampling, as a small volume of air from the air space was extracted from each hermitically closed bottle there was a slight air decompression. Air bubbles began to appear in the liquid phase (incubation medium) which immediately travelled to the air phase of the hermitically closed bottle, due to the induction of a new pressure balance.

In addition, previous studies on lichen tissue saturation indicate that free water is not usually present in the internal parts of the lichen thallus and that many foliose and fruticose lichens maintain their medullary space filled with air even under water-saturated conditions to help facilitate gas exchange (Souza et al., 2000). This fact could also help explain why larger medium volumes did not increase hydrogen production as there is an upper limit of saturation. In nature, lichens accumulate water in many different forms (dew, rain, high relative humidity). Similarly when used in experimental studies lichens are hydrated in relation to different protocols or methods. Lichen of different microhabitats respond differently to supersaturated conditions (high hydration levels that reduce net photosynthesis through increased resistance to diffusion). Therefore, it would be expected that lichens from different habitats develop unique mechanisms in an attempt to avoid supersaturation (Souza et al., 2000). Consequently, the volume of 10 mL of incubation medium (the one used up to this point) was chosen as the most suitable for further experimental procedures without altering the conditions of the hermitically closed bottle-systems.

3.4 The effect of the medium composition on hydrogen production

It was evident that the incubation medium would play a crucial role in the lichen's hydrogen productivity. All the experimental treatments mentioned above were completed using the well-known medium for green algae enriched with 5 g/L glucose (Bishop & Senger, 1971). This specific medium could be beneficial to green algae but not to the mycobiont or even the lichen as a whole, since they are terrestrial organisms and many are epiphytes or epiliths. Therefore, lichens may not be capable of managing the accumulated salts that are present in the incubation medium. Different concentrations of

the initially used incubation medium were tested and the results are presented in Figure 19.

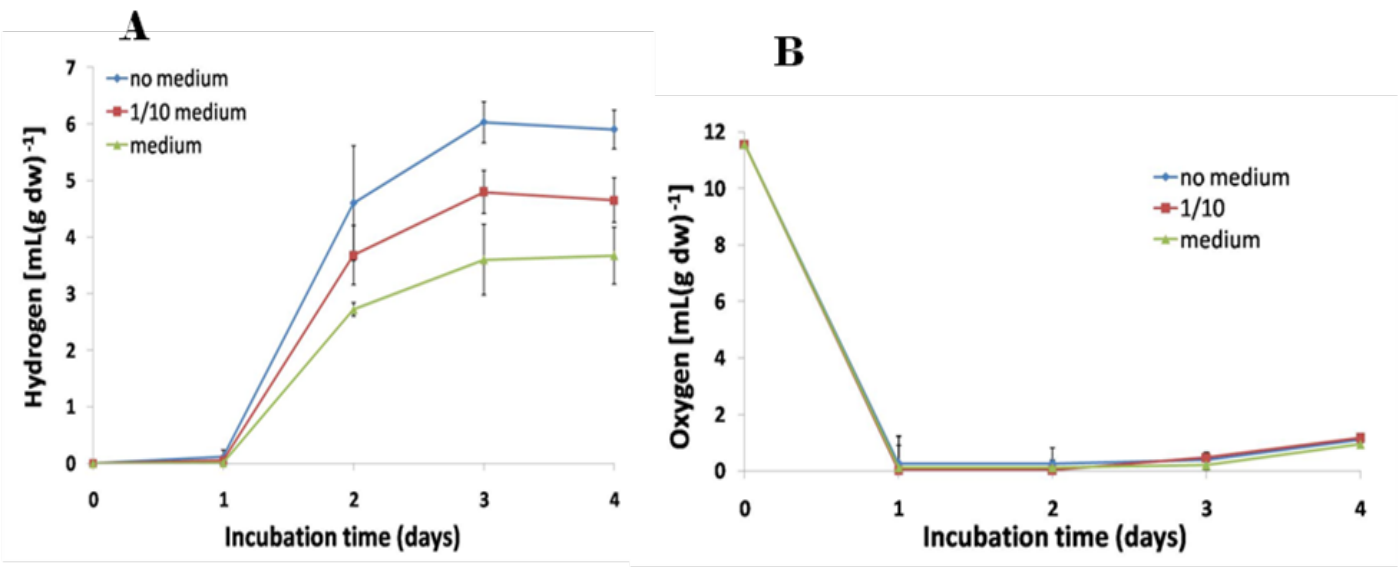


Figure 19. A. Hydrogen production and B. Oxygen consumption by the lichen *Pleurosticta acetabulum* in various incubation medium concentrations (no medium, 1/10 medium and full medium)

The most interesting observation was that every tested concentration of the incubation medium resulted in lower values of hydrogen production as compared to deionised water enriched with 5 g/L glucose. Specifically, the lower the concentration of the medium the higher the hydrogen values detected. As such, deionized water enriched with 5 g/L glucose was selected for the continuation of the experimental procedures in an attempt to maximize the lichen's capability for hydrogen generation.

3.5 The effect of temperature on hydrogen production

Temperature is an important abiotic parameter that significantly affects the metabolic rates and the activation of enzymes (Shi et al., 2013; Wang & Wan, 2008). Higher metabolic rates were expected in higher temperatures. However, the activation and rate of an enzymatic reaction usually has an

optimum temperature, where lower or higher temperatures than that could lead to reduced efficiencies.

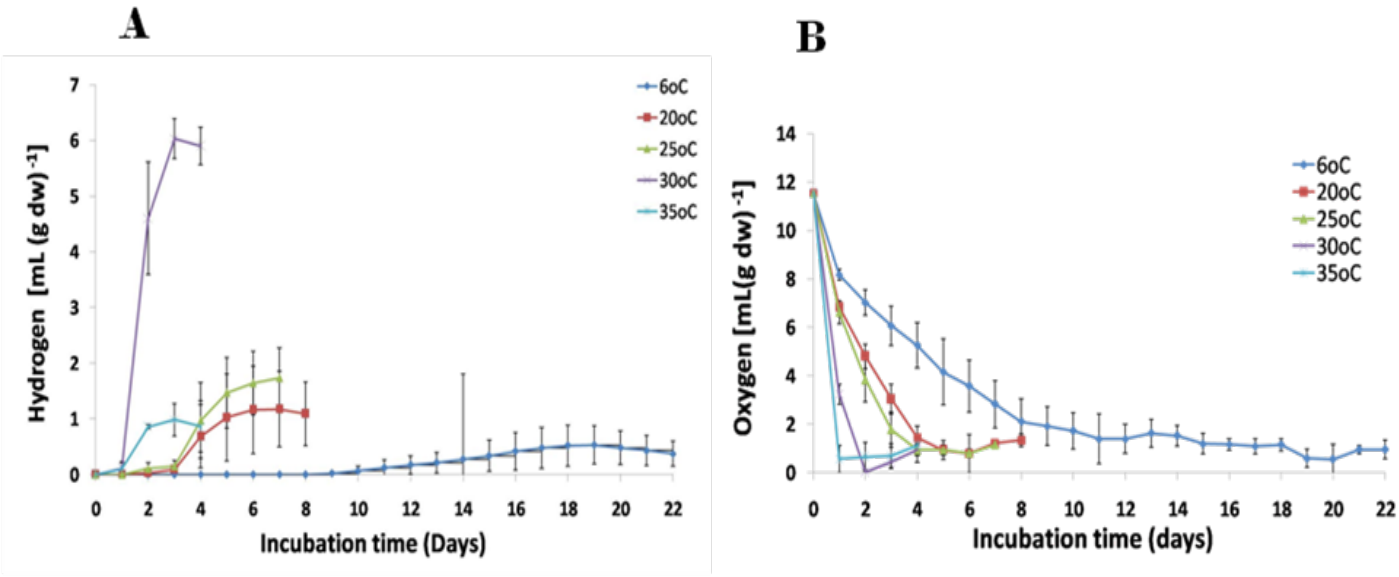


Figure 20. A. Hydrogen production and B. Oxygen consumption by the lichen *Pleurosticta acetabulum* under various incubation temperatures (6, 20, 25, 30 and 35 degrees Celsius)

The effect of incubation temperature on the hydrogen productivity of *Pleurosticta acetabulum* was examined using five different temperatures (6 ^{o}C, 20 ^{o}C, 25 ^{o}C, 30 ^{o}C and 35 ^{o}C). The temperature of 30 ^{o}C was the one used up to this point. The results regarding hydrogen production and oxygen consumption for each temperature are presented in Figure 20. As expected, the oxygen consumption was higher with the gradient increase of temperature, because of a corresponding increase in the lichen's metabolic processes. Higher temperatures led to more intensive respiration rates mainly attributed to the mycobiont component of the lichen and potentially enabling higher hydrogen production by the photobiont. The overall best hydrogen yield measured was during incubation in 30 ^{o}C. Higher temperatures (35 ^{o}C) led to an earlier detection of hydrogen, but overall lower hydrogen productivities, similar to those of 20 and 25 ^{o}C (Fig. 20). This was a result of the lichen metabolic processes put in overdrive and forced to adapt to heat induced stress which led to earlier lichen tissue breakdown. Lower temperatures (6 ^{o}C) demanded additional incubation time for the generation of hydrogen (after the 9th day) and the detected values were extremely low compared to all the other tested temperatures. This observation was the result of the lower metabolic rates of the lichen's mycobiont component. At such low temperatures the mycobiont could not respire at an effective rate to consume the undesirable oxygen which interferes with the activation of the photobiont's hydrogenase enzyme. As a consequence the

hydrogen enzyme was insufficiently activated leading to very low hydrogen yields. However, the lichen tissue remained intact for a much longer period of time, attributed to its preservation in a minimally metabolic active state. Finally, the temperature of 30 ^{o}C was found the best for increased hydrogen production and was continued throughout the following experimental procedures.

3.6 The effect of light intensity on hydrogen production

The intensity of light is a crucial parameter for photosynthetic organisms and in such for the photobiont component of the lichen. Regarding the production of hydrogen, light intensity has a great effect on oxygen production and consequently the activation of the hydrogenase enzyme.

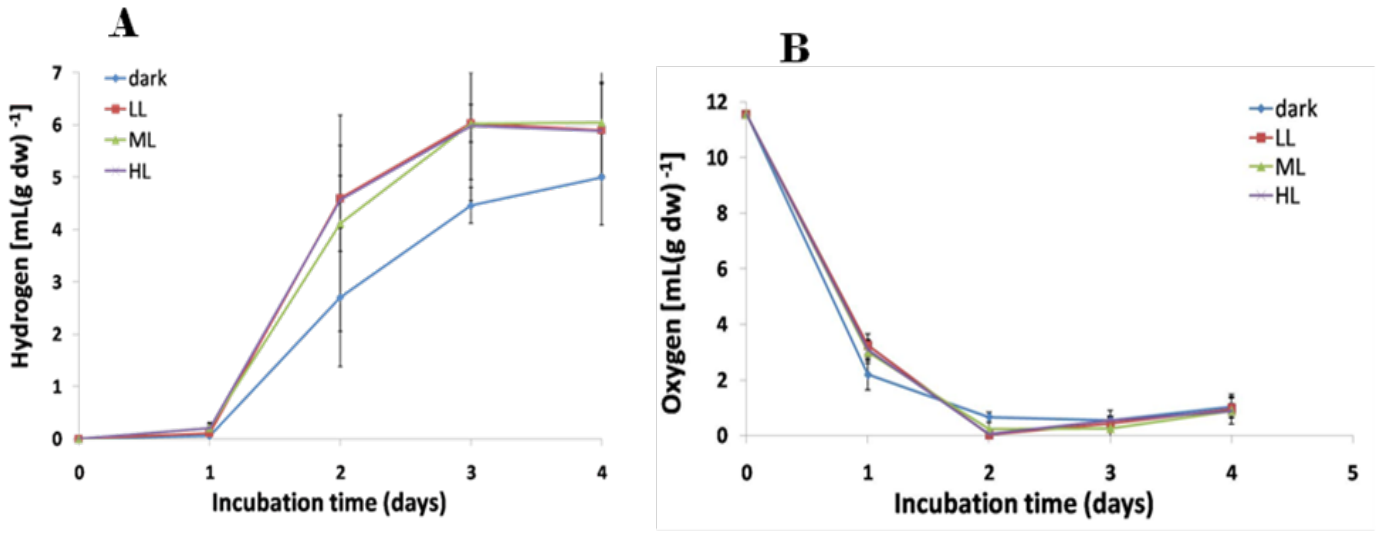


Figure 21. A. Hydrogen production and B. Oxygen consumption by the lichen *Pleurosticta acetabulum* under various incubation light intensities (Dark: 0 μE, Low Light: 20-25 μE, Medium Light :80-100 μE, High Light: 200-250 μE)

The effect of light intensity on hydrogen generation of the lichen *Pleurosticta acetabulum* was tested using four different light intensities [dark (D): 0 μE, low light (LL): 20-25 μE (the one used up to this point), medium light (ML): 80-100 μE and high light (HL): 200-250 μE]. The kinetics of hydrogen production and oxygen consumption under the above mentioned light intensities are presented in Figure 21. Two very interesting observations emerged from these results. Firstly, the hydrogen production of lichens is not exclusively a light induced process. Hydrogen was detected both in darkness (possibly

through dark fermentation) and in light conditions (PSII-dependent and/or PSII-independent pathways). Secondly, the hydrogen yield measured under the light conditions was not significantly affected by the tested intensities (LL, ML and HL), since all the hydrogen values were quite close in range. The actual hydrogen yield in the light (approximately 6 mL H_2 per g dry weight) was higher than the corresponding one measured in the dark (approximately 5 mL H_2 per g dry weight). This difference in hydrogen productivity (measured in dark and light conditions) could lead to the better understanding of the bioenergetic mechanisms that regulate hydrogen production in lichens.

3.7 Changes in central proteins of the photosynthetic and respiration electron transport chains under light and dark conditions

The expression level of central proteins of the photosynthetic and respiration electron transport chains were examined in order to thoroughly study the pathways of hydrogen production in lichens (Fig. 24, 25 ,26). There are three known respiration electron transport chains. Two of them are located in mitochondria (the cytochromic and alternative pathways) (Atkin et al., 2002; Siedow & Umbach, 1995) and the third in chloroplasts (chlororespiration) (Bennoun, 1982, 1994; Rumeau et al., 2007). The cytochromic oxidase protein (COX) (Figure 24) was chosen for the testing of the cytochromic pathway, the alternative oxidase protein (AOX) (Figure 24) for the alternative pathway and the plastid terminal oxidase protein (PTOX) (Figure 24) for chlororespiration. The cytochrome c oxidase (COX) enzyme is a large transmembrane protein complex (Complex IV) occupying the mitochondrial membrane of eukaryotes or the bacterial membrane of membrane of bacteria. It is the final enzyme in the respiratory electron transport chain and receives four electron from cytochrome c molecules transferring them to one oxygen molecule. In this way, molecular oxygen is converted into two molecules of water with the translocation of four protons across the membrane. Consequently, a difference in transmembrane proton electrochemical potential is generated which is utilized by ATP synthase to produce chemical energy (ATP).

The alternative oxidase (AOX) is an enzyme that establishes a part of the electron transport chain in mitochondria organelles of various organisms. The AOX protein is tightly bound to the inner mitochondrial membrane and provides a secondary route for the electron transfer through the chain (Siedow & Umbach, 1995). The electrons are transferred from the reduced ubiquinone to molecular

oxygen, producing water as the reduced product. Proton motive force is not produced generated during the electron flow between ubiquinol and oxygen in the alternative pathway (Siedow & Umbach, 1995). As a result, all of the free energy released during electron flow from ubiquinol through the alternative pathway is lost in the form of heat and is not used in the synthesis of ATP. Because in this alternative pathway respiration only causes the translocation of protons at NADH dehydrogenase (Complex I) it has a lower ATP yield than the full pathway (Siedow & Umbach, 1995). Even though this may seem as an energy waste the use of this pathway can help maintain the energy flow in mitochondria and come in use when electron flow through the main cytochrome pathway is restricted. The expression of the AOX gene can be influenced by different stresses as low temperatures, reactive oxygen species and pathogen infections or other factors that may reduce the electron flow through the cytochrome pathway (Siedow & Umbach, 1995).

PTOX is a chloroplast targeted quinol oxidase, a homolog to the alternative oxidase which is found in mitochondria. It is involved in the processing of carotenoids and has been proposed to play a safety valve role by preventing over-reduction of the electron transfer chain in conditions of excess light (Rumeau et al., 2007). It is known that high light intensities induce Photosystem II (PSII) damage and photoinhibition. It has also been proposed that the function of the chlororespiratory pathway may be to reduce the electron pressure on PSI electron acceptors by recycling the electrons to the Plastoquinone Pool (PQ) and ultimately to PTOX (Rumeau et al., 2007). As such, it was expected that there would be a PTOX protein abundance in high light conditions. Western blot analyses showed an increase in the quantity of all the respiration proteins after 33 hours of incubation compared to the ones analyzed immediately after the regeneration stage (Fig.22)

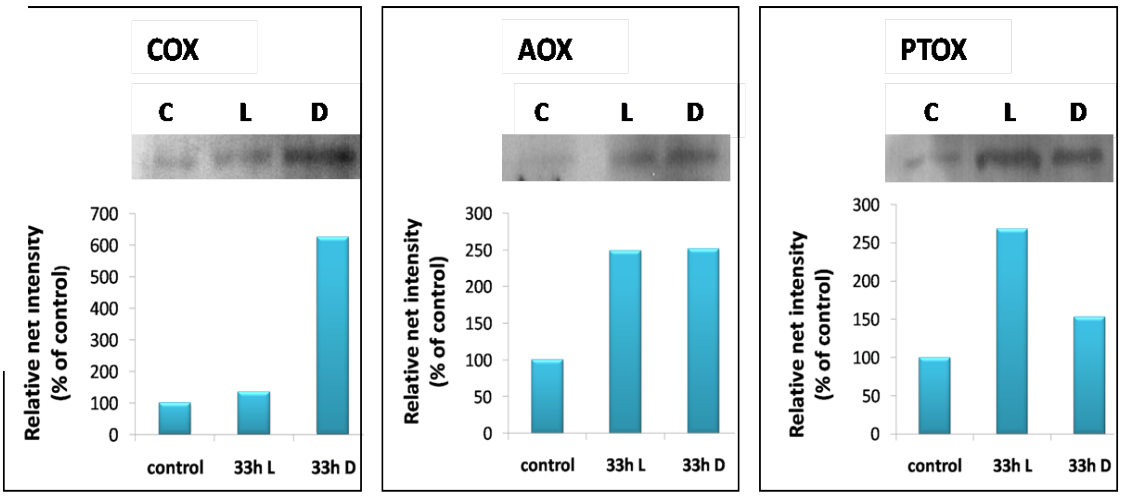


Figure 22. Western blot analysis of the respiratory proteins Cytochromic Oxidase, Alternative Oxidase and Plastid Terminal Oxidase, control (C) immediately after the regeneration stage and after 33 hours of light (L) and dark (D) incubation accordingly

As a result, all oxygen consumers (COX, AOX and PTOX) were fully activated. COX and AOX were more abundant under dark conditions, while PTOX was more abundant in the light, as it was expected. The activation of respiration O_2 consumers was substantiated by the oxygen decrease during the incubation period (approximately zero value) in the hermitically closed bottles with the lichen, measured using a gas chromatographer (Figs. 15-21) and was in absolute agreement with the protein abundance (Fig. 22). The establishment of oxygen depleted conditions was essential for the activation of hydrogenase.

In accordance to the photosynthetic electron transport chain the hydrogenase enzyme was fed with electrons through ferredoxin. The origin of these electrons could be the splitting of water (PSII-dependent pathway), the reduction of glucose (PSII-independent pathway) and dark fermentation through pyruvate ferredoxin oxidoreductase (PFOR). The examination of PSII took place through the detection of the D1 protein, as it is part of the PSII reaction center and along with D2, binds most of the cofactors and is involved in the PSII-dependent electron transport. (Figure 23), the examination of PSI by the PSaA protein a PSI-core protein (Figure 23) and the examination of dark fermentation by the abundance of the PFOR protein (Figure 24). The western blot analyses showed a decrease in the PSII core protein (Figure 23) and in parallel an increase in the PSI core protein (Figure 23). These changes were more severe under light conditions than under dark, as was expected, and in combination with the over expression of respirational oxidases (COX, AOX and PTOX) created the optimal conditions for increased hydrogen productivity. The above observations can explain the hydrogen production under light conditions by means of the induction of the PSII dependent and PSII-independent pathways.

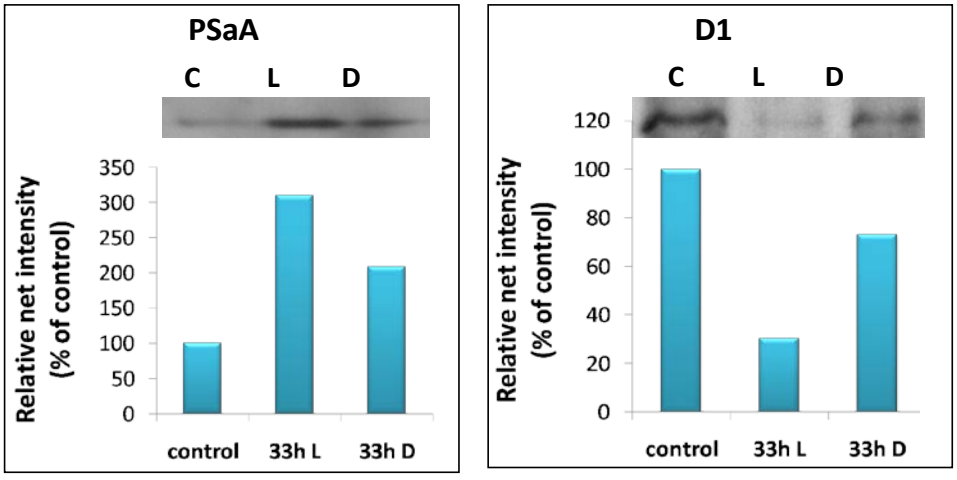


Figure 23. Western blot analysis of the photosynthetic electron transport chain proteins PSaA (PSI core protein) and D1 (PSII core protein) control (C) immediately after the regeneration stage and after 33 hours of light (L)and dark (D) incubation accordingly

In fact, the hydrogenase protein (Fig. 24) was more abundant under light as compared to dark conditions. However, after dark incubation the hydrogenase protein was more abundant than the protein measured extracted from samples immediately after the regeneration stage (control). The detection and activation of hydrogenase in the dark was a paradox result since the source of electrons and consequent transfer to hydrogenase could not come from the splitting of water (PSII dependent pathway) or the reduction of glucose (PSII independent pathway). This was the main reason for examining the abundance of the PFOR protein that is dominant in the dark fermentation pathway.

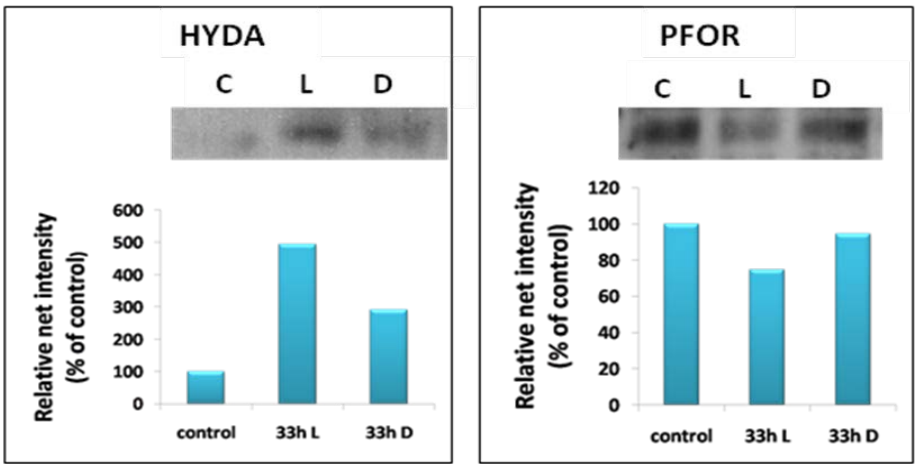


Figure 24. Western blot analysis of Dark fermentation proteins Hydrogenase and Pyruvate Ferredoxin Oxidoreductase immediately after the regeneration stage control (C) and after 33 hours of light (L) and dark (D) incubation accordingly

It was observed that PFOR (Figure 24) was the lowest under light compared to dark conditions as well as after the regeneration stage. This was mainly due to the fact that under light conditions electrons are directed to hydrogenase by the PSII dependent and PSII independent pathways, while under dark conditions, where these pathways were not able to function, the dark fermentative pathway was dominant (Fig.24).

3.8 Is *Pleurosticta acetabulum* the only lichen species able to produce hydrogen?

Pleurosticta acetabulum is a lichen species that can produce hydrogen in both light and dark conditions. Is this capability a specific characteristic of the particular lichen species or is it a general aspect of more than one lichen species? The experimental conditions for optimum hydrogen productivity of *Pleurosticta acetabulum* were chosen for testing a range of various lichen species for their ability to produce hydrogen in dark and low light conditions (20-25 μmol m^{-2} s^{-1}). In summary, these conditions were regeneration in deionised water and addition of 10 mL sterile deionised water enriched with 5 g/L glucose in hermitically closed bottles in a controlled temperature of 30^{o}C. The results are presented in Figures 25 and 26 (for light and dark conditions respectively) and support that lichens in general have the ability to produce hydrogen.

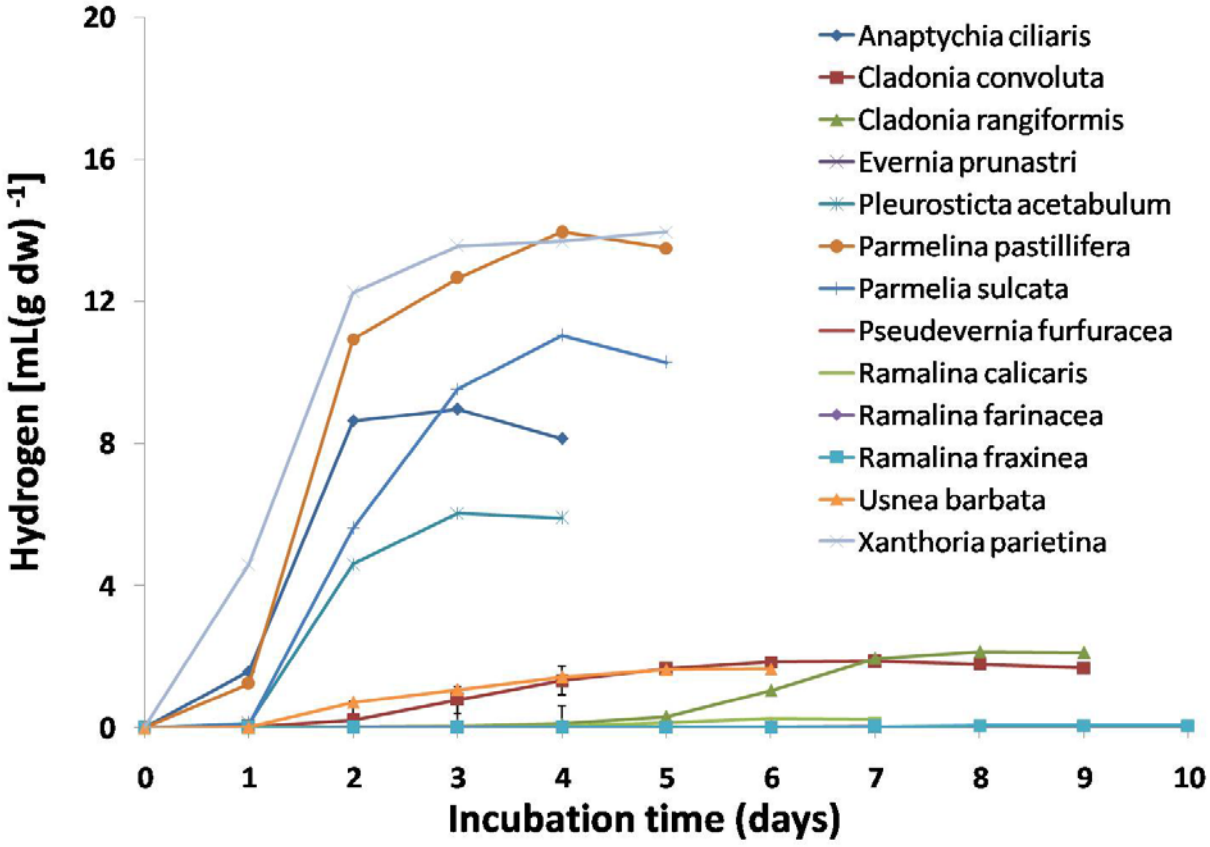


Figure 25. Hydrogen Production of various lichen species under low light (20-25 μmol m^{-2} s^{-1}) conditions

The majority of the lichen species tested produced hydrogen under dark and light conditions. A variety of incubation times were required for the induction of hydrogenase, which closely correlated with oxygen consumption. The different ratio of mycobiont to photobiont biomass in each species, their different evolutionary origin, their occurrence in various natural habitats, their unique thallus morphology or their different shape, texture and pigmentation as well as the specific species of photobiont in each symbiosis could all be some of the parameters that substantially affected the final hydrogen production under the tested experimental conditions.

Among them *Ramalina calicaris* and *Ramalina fraxinea* produced small values of hydrogen only under light conditions (approximately zero value), while *Ramalina farinacea* produced hydrogen mainly under dark and extremely low quantities under light conditions as did the species *Ramalina fraxinea* and *Ramalina calicaris*. *Evernia prunastri* was not able to produce hydrogen under the tested experimental conditions neither in the light nor in the dark. *Xanthoria parietina* seemed to be the best species concerning hydrogen production under dark conditions (approximately 16.3 mL H_2 per g of dry weight), while *Xanthoria parietina* and *Parmelina pastillifera* were those most efficient under light incubation (approximately 14 mL H_2 per grams of dry weight). *Anaptychia ciliaris, Parmelia sulcata, Parmelina pastillifera* and *Xanthoria parietina* produced higher hydrogen yields compared to *Pleurosticta acetabulum* under light conditions, while *Cladonia convoluta, Cladonia rangiformis, Usnea barbata* followed with lower values. Finally, the hydrogen production measurements of *Ramalina calicaris*, *Ramalina fraxinea and Pseudevernia furfuracea* were extremely low.

Under dark conditions, *Pseudevernia furfuracea, Anaptychia ciliaris, Parmelia sulcata, Parmelina pastillifera and Xanthoria parietina* demonstrated a higher hydrogen production compared to *Pleurosticta acetabulum*. *Cladonia rangiformis, Ramalina farinacea* and *Usnea barbata* produced hydrogen values similar to the lichen *Pleurosticta acetabulum*, while *Cladonia convoluta* comparatively produced extremely lower quantities.

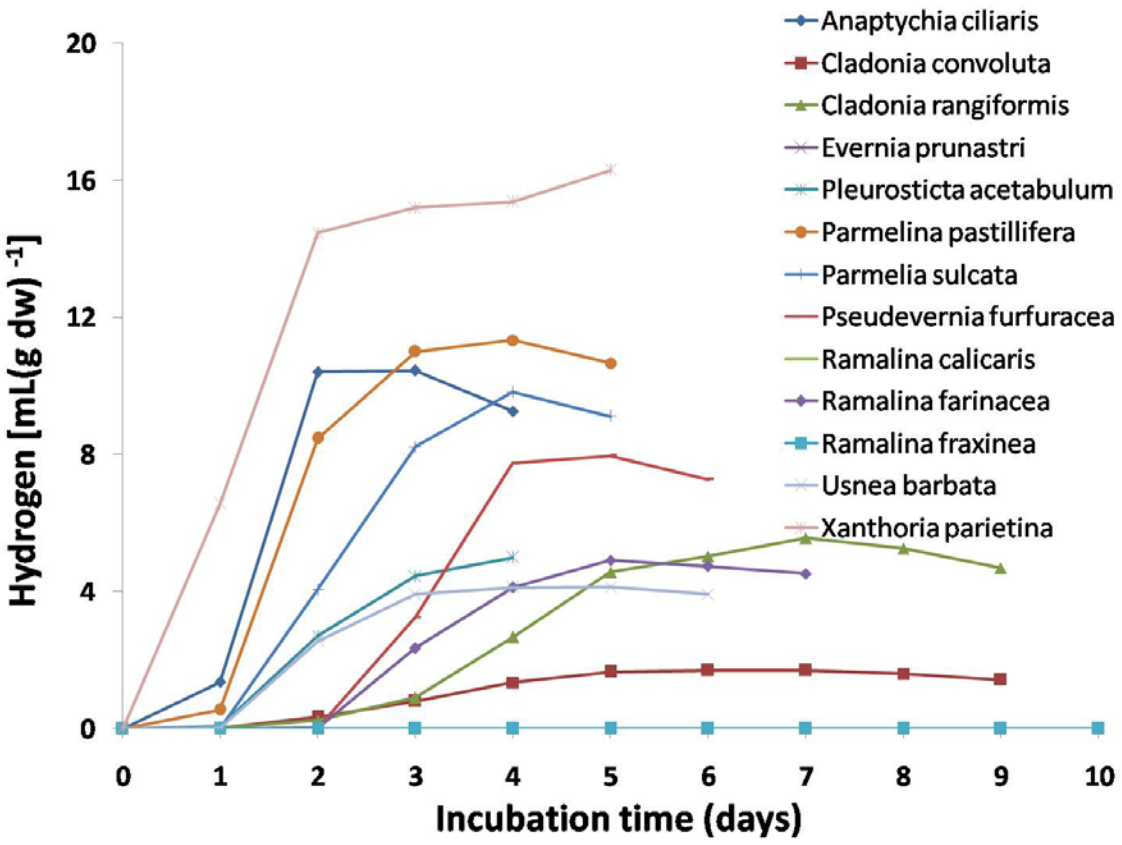


Figure 26. Hydrogen Production of various lichen species under dark conditions (0 µmol m^{-2} s^{-1})

To confirm the photobiont species existing within each of the tested lichen species an examination of the available literature was completed. Table two shows the lichen species tested with its corresponding photobiont (green algae species) identified and the representative reference from which this information was acquired. It is evident the photobiont associated with most of the lichen species used in this master's thesis project are green algae of the genus *Trebouxia*.

Table 2. Green algae as symbiotic photobionts in the studied lichen species

Lichen species	Green algae	Reference
Anaptychia ciliaris	*Trebouxia decolorans*	**[11]**
Cladonia convolute	*Asterochloris*	**[43]**
Cladonia rangiformis	*Asterochloris*	**[43]**
Evernia prunastri	*Trebouxia jamesii*	**[12]**
Pleurosticta acetabulum	*Trebouxia arboricola*	**[10,3,15]**
Parmelina pastilifera	*Trebouxia* sp	**[27]**
Parmelia sulcata	*Trebouxia impressa*	**[15]**
Pseudevernia furfuraceae	*Trebouxia* sp *Trebouxia simplex*	**[45,26]**
Ramalina calicaris	*Trebouxia jamesii*	**[12]**
Ramalina farinacea	*Trebouxia jamesii*	**[12]**
Ramalina fraxinea	*Trebouxia jamesii*	**[12]**
Usnea barbata	*Trebouxia* sp	**[35]**
Xanthoria parietina	*Trebouxia jamesii, Trebouxia arboricola*	**[11,2]**

For each of the species tested there is a separate graph, Figure 27 which represents the hydrogen production and oxygen consumption of each lichen species under light and dark conditions on its own. Most of the lichen species tested demonstrated that they were able to produce hydrogen both under dark and light conditions.

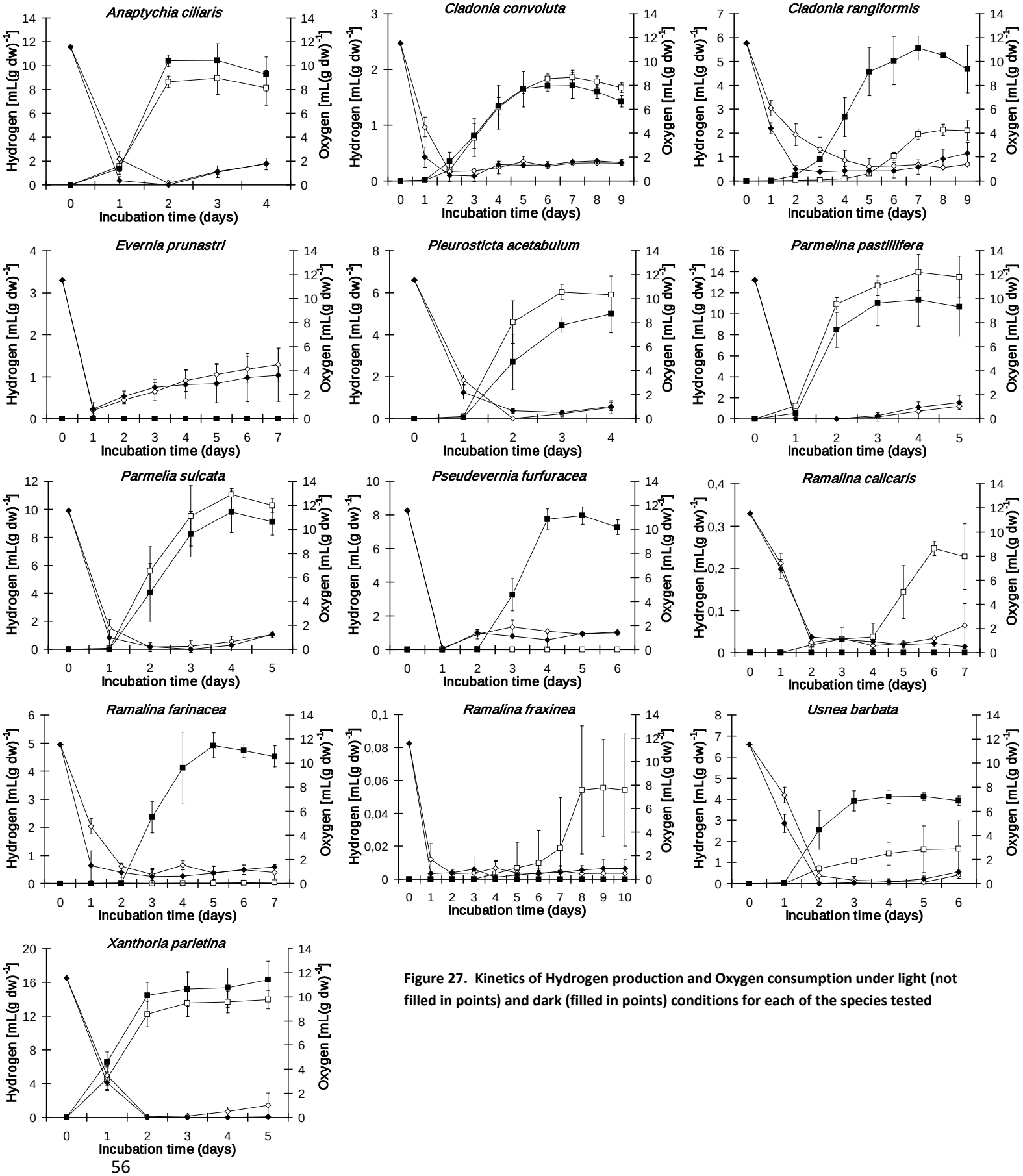


Figure 27. Kinetics of Hydrogen production and Oxygen consumption under light (not filled in points) and dark (filled in points) conditions for each of the species tested

4. DISCUSSION

This work constitutes the first report highlighting the symbiotic synthesis of lichens (mycobiont & photobiont) as nature's hydrogen biofactories. Lichens are naturally occurring organisms that under certain experimental conditions have shown to possess the capability of producing high yields of hydrogen. The nature of the symbiotic relationship between a mycobiont (oxygen consumer) and a photobiont allows the lichen in a closed system to self establish anoxic conditions that induce the synthesis of the hydrogenase enzyme and additionally to utilize three possible pathways for H_2 production in light (mainly PSII-dependent and PSII-independent pathway) and in darkness (dark fermentative pathway). This adaptability constitutes lichens' greatest advantage regarding hydrogen production. The consumption of oxygen mainly attributed to the mycobiont in a closed system creates the ideal environment for the activation of the O_2-sensitive chloroplastic hydrogenase by the photobiont. Electrons originating from the photosynthetic splitting of water are transferred through the photosynthetic electron transport chain to ferredoxin which transfers them to hydrogenase utilizing the PSII-dependent pathway (Gaffron, 1939, 1940, 1942; Gaffron & Rubin, 1942). Electrons originating from the reduction of glucose are associated with the PSII-independent pathway (Gfeller & Gibbs, 1984; Gibbs et al., 1986) and the dark fermentative pathway through pyruvate ferredoxin oxidoreductase (Hallenbeck & Benemann, 2002).

Numerous attempts to overcome the inhibition of hydrogenase by the presence of oxygen [continuous nitrogen, argon or helium flow (Terashima et al., 2010), sulfur deprivation (Melis & Happe, 2001), nitrogen deprivation (Philipps et al., 2012) or potassium deprivation (Papazi et al., 2014), addition of *meta* substituted dichlorophenols to the culture medium (Papazi et al., 2012; Papazi & Kotzabasis, 2013) or genetically modified organisms (Radakovits et al., 2010)] have all been experimentally examined. However, the elusive solution to this problem has already existed in nature for millions of years and has been identified through the biochemical mechanisms of lichens. In addition, when taking into consideration the extremely low algal density within the lichen thallus, the hydrogen yield detected is correspondingly, extremely higher compared to cultures exclusively containing green algae. It was demonstrated that the majority of the parameters that affect hydrogen production in algae also had a similar impact on the hydrogen production of the tested lichen species *Pleurosticta*

acetabulum. It has been established that glucose is an important exogenously supplied carbon source which improves hydrogen yields. Higher hydrogen productivities were detected in cases where glucose was added to the incubation stage instead of the regeneration stage. The most efficient and cost effective lichen tissue regeneration, taking hydrogen productivity into consideration, occurred after a twenty four hour incubation time in deionized water. The volume of the medium in the hermitically closed bottles also played a crucial role in hydrogen release. As the volume of the medium inside the bottles increased so did the partial pressure in the remaining air space of the bottle. Because pressure is released by a higher gradient to a lower one, the hydrogen produced in the liquid phase was not able to be released into the air space (higher pressure) to be effectively detected by the gas chromatographer. Therefore, the lower the medium volume the higher the hydrogen productivity detected. Temperature was another abiotic parameter that greatly affected the production of hydrogen by the lichen *Pleurosticta acetabulum*. However, as in all enzymatic reactions there is an ideal range of temperature for the activation of hydrogenase (Shi et al., 2013; Wang & Wan, 2008). In this case, the temperature of 30 ^{o}C was proven to be the optimal one in terms of overall hydrogen yield, even though the temperature of 35 ^{o}C resulted in earlier anoxic conditions. The composition of the medium did not significantly affect the yield of the hydrogen produced. This could be due to lichens' existence as epihytes or epithils and their inability to cope with all the different salts in the medium. As such the use of deionized water enriched with 5 g/L of glucose served to minimize unnecessary expenditures. The light intensity (low, medium or high) did not significantly impact the hydrogen generation of *Pleurosticta acetabulum*.

Nevertheless, the presence or absence of light had a significant effect on the hydrogen produced since in each case, different pathways are activated. All the specific parameters mentioned above, were confirmed with the experimental testing of the lichen species *Pleurosticta acetabulum* and utilized in the scanning of a wide range of other lichen species. These screenings support that the ability of lichens to produce hydrogen is a general

aspect of the genus or family and is not limited to only the lichen species *Pleurosticta acetabulum*. A proposed mechanism for hydrogen production under the tested experimental conditions is explained in a simplified model (Figure 28). Figure 28A refers to the time point immediately after the regeneration stage of the lichens. Figure 28B refers to the oxygen depleted conditions under light incubation conditions and Figure 28C to the oxygen depleted conditions under dark incubation.

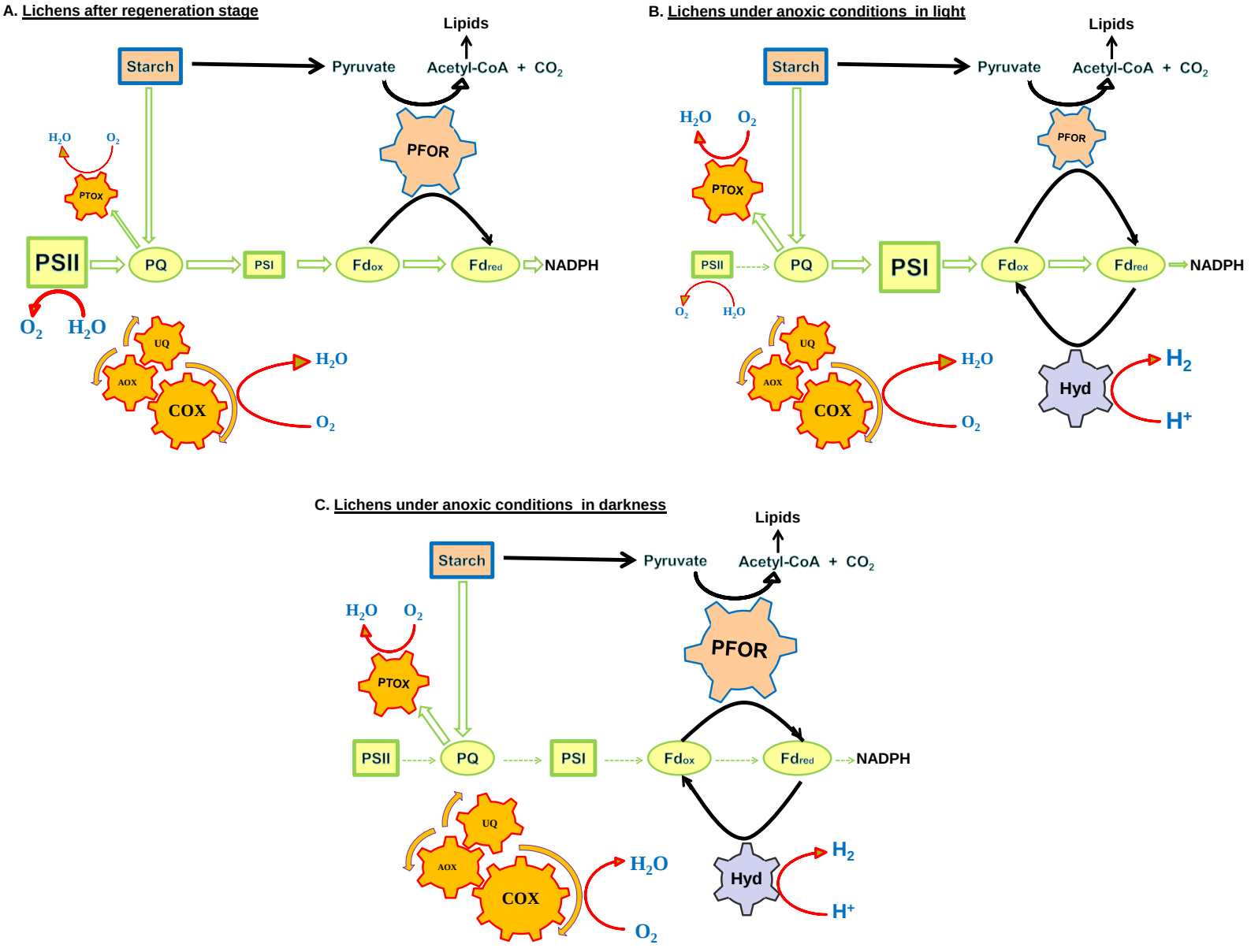


Figure 28. Proposed model of hydrogen production in *Pleurosticta acetabulum* after the regeneration stage (A), under light (B) and dark (C) conditions

In Figure 28A, where the processes immediately after the regeneration stage are presented, the PQ pool is fed with electrons mainly due to the light induced water splitting in PSII and incidentally due to the reduction of glucose (endogenously or exogenously supplied). The above electrons are transferred to ferredoxin through PSI (photosynthetic electron transport chain) or through PFOR (dark fermentation) for NADPH production, lipid and growth increase. In addition to the photosynthetic electron transport chains of photobiont, there are mechanisms for consuming oxygen and producing energy, such as the cytochromic oxidase and alternative oxidase mitochondrial pathways (in mycobiont and photobiont) as well as the chloroplastic chlororespiration (in photobiont). Under these conditions, the oxygen concentration within the closed system remained high and as a result hydrogenase activity was completely inhibited.

In Figure 28B, where the processes during light incubation are presented, as the lichens are placed in the hermitically closed bottles, anoxic conditions are established, because of the over activation of oxygen consumption through the respiration electron transport chains (in mitochondria and chloroplasts). The oxygen depleted conditions in combination with the inactivation of PSII create the optimal conditions for hydrogen generation. Specifically, PSII was deactivated and the remaining electrons were transferred from PSI to ferredoxin and then to hydrogenase (PSII-dependent pathway). The hydrogen production was further induced by the reduction of organic substrates through the PSII–independent pathway. These electrons are led to the plastoquinone pool and through PSI and ferredoxin, are transferred to hydrogenase for hydrogen production. In parallel, electrons are alternatively allocated to pyruvate and through the PFOR protein transfered to ferredoxin and then to hydrogenase. It is obvious that under light incubation the PSII-dependent and the PSII-independent pathways are more active than the dark fermentative one. In contrast, in Figure 28C which corresponds to dark incubation conditions, the dark fermentative pathway is the dominant route for feeding ferredoxin with electrons since the other two pathways are absolutely deactivated due to the absence of light. In dark conditions, oxygen consumers are more active, as was expected and hydrogen production was observed at earlier incubation times.

In conclusion, all the experiments clearly demonstrated that lichens could be nature's solution for overcoming the problem of the hydrogenase enzyme inhibition by the presence of oxygen. Lichens are able to establish anoxic conditions in a closed system mainly through O_2 consumption by the mycobiont

while producing high yields of hydrogen by the photobiont utilizing three different pathways (PSII-dependent, PSII-independent and dark fermentation pathways). They have the ability to activate the appropriate bioenergetic pathways under anaerobic conditions in order to produce hydrogen. Depending on the specific incubation conditions they can efficiently use either light induced hydrogen production or dark fermentation. These benefits in conjunction with lichens' ability to survive in extreme environments (de Vera et al., 2004) constitute them as invaluable hydrogen producers, with future applications even in space. These properties establish lichens as exceptionally important organisms in the field of biohydrogen-energy production. Further investigation could focus on the lifecycle and sustainability of this system with attempts to increase lichen lifetime as well as overall hydrogen yields. Future research on the specific metabolic pathways and molecular adaptations of lichen hydrogen production could provide major insight in ways to address the most difficult problems mentioned above in the field of biohydrogen energy production. The majority of the results presented in this master thesis were published in PLOS ONE. [Papazi A, Kastanaki E, Pirintsos S, Kotzabasis K (2015) Lichen Symbiosis: Nature's High Yielding Machines for Induced Hydrogen Production. PLoS ONE 10(3) : e0121325. doi:10.1371/journal.pone.0121325].

REFERENCES

1. Atkin, O. K., Zhang, Q., & Wiskich, J. T. (2002). Effect of temperature on rates of alternative and cytochrome pathway respiration and their relationship with the redox poise of the quinone pool. Plant Physiol., 128, 212-222.
2. Beck A, Friedl T, Rambold G (1998) Selectivity of photobiont choice in a defined lichen community: inferences from cultural and molecular studies. New Phytol 139: 709-720.
3. Beck A, Koop, HU (2001) Analysis of the photobiont population in lichens using a single-cell manipulator. Symbiosis 31: 57–67.
4. Bendre, A. Kumar, A. (2010). A Text Book of Practical Botany -1
5. Bennoun, P. (1982). Evidence for a respiratory chain in the chloroplast. Proc. Natl. Acad. Sci. U S A, 79(14), 4352-4356.
6. Bennoun, P. (1994). Chlororespiration revisited: mitochondrial-plastid interactions in Chlamydomonas. Biochim. Biophys. Acta, 1186, 59-66.
7. Bishop, N. L., & Senger, H. (1971). Preparation and photosynthetic properties of synchronous cultures of Scenedesmus. In A. San Pietro (Ed.), Methods in Enzymology (Vol. 23, pp. 130-143). New York: Academic Press.
8. Casano, L. M., del Campo, E. M., Garcia-Breijo, F. J., Reig-Arminana, J., Gasulla, F., Del Hoyo, A., Barreno, E. (2011). Two Trebouxia algae with different physiological performances are ever-present in lichen thalli of Ramalina farinacea. Coexistence versus competition? Environ Microbiol, 13(3), 806-818.
9. Conti, M. E., & Cecchetti, G. (2001). Biological monitoring: lichens as bioindicators of air pollution assessment--a review. [Review]. Environ Pollut, 114(3), 471-492.
10. Dahlkild Å, Källersjö M, Lohtander K, Tehler A (2001) Photobiont diversity in the Physciaceae (Lecanorales). Bryologist 104: 527-536
11. Dal Grande F, Alors D, Divakar PK, Bálint M, Crespo A, Schmitt I (2014) Insights into intrathalline genetic diversity of the cosmopolitan lichen symbiotic green alga Trebouxia decolorans Ahmadjian using microsatellite markers. Mol Phyl Evol 72: 54-60.
12. del Campo EM, Catala S, Gimeno J, Hoyo A, Martínez-Alberola F, Casano LM, Grube M, Barreno E (2013) The genetic structure of the cosmopolitan three-partner lichen Ramalina farinacea evidences the concerted diversification of symbionts. FEMS Microbiol Ecol 83: 310-323.
13. de Vera, J. P., Horneck, G., Rettberg, P., & Ott, S. (2004). The potential of the lichen symbiosis to cope with the extreme conditions of outer space II: germination capacity of lichen ascospores in response to simulated space conditions. Adv Space Res, 33(8), 1236-1243.

14. Dubini, A. Ghirardi, M. L. (2014). Engineering photosynthetic organisms for the production of biohydrogen. Photosynth Res, 123, 241–253.
15. Friedl T (1989) Systematik und biologie von Trebouxia (Microthamniales, Chlorophyta) als phycobiont der parmeliaceae (lichenisierte Ascomyceten). Ph.D.Thesis. Universtät Bayreuth. Available: Smithsonian Institution Libraries, Catalog Source No.: (OCoLC)ocm42464469.
16. Gaffron, H. (1939). Reduction of CO2 with H2 in green plants. Nature, 143, 204-205.
17. Gaffron, H. (1940). Carbon dioxide reduction with molecular hydrogen in green algae. Ann J Bot, 27, 273-283.
18. Gaffron, H. (1942). Reduction of Carbon Dioxide Coupled with the Oxyhydrogen Reaction in Algae. J Gen Physiol, 26(2), 241-267.
19. Gaffron, H., & Rubin, J. (1942). Fermentative and Photochemical Production of Hydrogen in Algae. J Gen Physiol, 26(2), 219-240.
20. Gfeller, R. P., & Gibbs, M. (1984). Fermentative Metabolism of Chlamydomonas reinhardtii: I. Analysis of Fermentative Products from Starch in Dark and Light. Plant Physiol., 75(1), 212-218.
21. Ghirardi, M. L., Kosourov, S., Tsygankov, A., & Seibert, M. (2000). Two-Phase Photobiological Aldal H2-Production System Proceedings of the 2000 DOE Hydrogen Program Review.
22. Ghirardi, M. L., Togasaki, R. K., & Seibert, M. (1997). Oxygen sensitivity of algal H2- production. Appl Biochem Biotechnol, 63-65, 141-151.
23. Gibbs, M., Gfeller, R. P., & Chen, C. (1986). Fermentative metabolism of Chlamydomonas reinhardii, II: Photoassimilation of acetate. Plant Physiol, 37, 649-655.
24. Govindjee .(1995).Sixty-three Years Since Kautsky: Chlorophyll Fluorescence., Aust. J. Plant. Physiol. 22, 131-160
25. Hallenbeck, P. C., & Benemann, J. R. (2002). Biological hydrogen production; fundamentals and limiting processes. Int J Hyd Energy, 27, 1185-1193.
26. Hauck M, Helms G, Friedl T (2007) Photobiont selectivity in the epiphytic lichens Hypogymnia physodes and Lecanora conizaeoides. Lichenologists 39: 195-204.
27. Honegger R (1987) Isidium formation and the development of juvenile thalli in Parmelia pastillifera (Lecanorales, lichenized Ascomycetes). Botanica helvetica 97:147-152.
28. Kosourov, S., Seibert, M., & Ghirardi, M. L. (2003). Effects of Extracellular pH on the Metabolic Pathways in Sulfur-Deprived H2-Producing Chlamydomonas reinhardtii Cultures. Plant Cell Physiol, 44(2), 146-155.

29. Lowry O, Rosebrough N, Farr A, Randall R (1951) Protein measurement with the folin phenol reagen. J Biol Chem 193: 265-275.
30. Mattoo, A.K.,Pick, U., Hoffman-Falk, H., & Edelman, M. (1981). The rapidly metabolized 32,000-dalton polypeptide of the chloroplast is the "proteinaceous shield" regulating photosystem II electron transport and mediating diuron herbicide sensitivity. Proc Natl Acad Sci U S A, 78(3), 1572-1576.
31. Melis, A., & Happe, T. (2001). Hydrogen production. Green algae as a source of energy. Plant Physiol, 127(3), 740-748.
32. Melis, A., Zhang, L., Forestier, M., Ghirardi, M. L., & Seibert, M. (2000). Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga Chlamydomonas reinhardtii. Plant Physiol, 122(1), 127-136.
33. Nash, T. H. (2008). Lichen Biology. New York Cambridge University Press (pp. 13-486).
34. Nimis, P. L., Scheidegger C., Wolseley P. A (2002). Monitoring with Lechins- Monitoring Lichens. Netherlands: Kluwer Academic Publishers.
35. Palmqvist K (2000) Carbon ecology in lichens. New Phytol 148: 11-36.
36. Papazi, A., Andronis, E., Ioannidis, N. E., Chaniotakis, N., & Kotzabasis, K. (2012). High yields of hydrogen production induced by meta-substituted dichlorophenols biodegradation from the green alga Scenedesmus obliquus. PLoS One, 7(11).
37. Papazi, A., Gjindali, A. I., Kastanaki, E., Assimakopoulos, K., Stamatakis, K., & Kotzabasis, K. (2014). Potassium deficiency, a "smart" cellular switch for sustained high yield hydrogen production by the green alga Scenedesmus obliquus. International Journal of Hydrogen Energy, 39 (34), 19452-194641-13.
38. Papazi, A., & Kotzabasis, K. (2013). "Rational" management of dichlorophenols biodegradation by the microalga Scenedesmus obliquus. PLoS One, 8(4),
39. Philipps, G., Happe, T., & Hemschemeier, A. (2012). Nitrogen deprivation results in photosynthetic hydrogen production in Chlamydomonas reinhardtii. Planta, 235(4), 729-745.
40. Radakovits, R., Jinkerson, R. E., Darzins, A., & Posewitz, M. C. (2010). Genetic engineering of algae for enhanced biofuel production. Eukaryot Cell, 9(4), 486-501.
41. Ran, C., Zhang, F., Sun, H., & Zhao, B. (2009). Effect of Culture Medium on Hydrogen Production by Sulfur-deprived Marine Green Algae Platymonas subcordiformis. Biotechnology and Bioprocess Engineering (14), 835-841.
42. Řídká T, Peksa O, Rai H, Upreti PK, Skaloud P (2014) Photobiont diversity in Indian Cladonia lichens, with special emphasis on the geographical patterns. In: Rai H, Upreti PK, editors. Terricolous lichens in India. Volume 1: Diversity patterns and distribution ecology. New York: Springer. pp. 53-71.

43. Rumeau, D., Peltier, G., & Cournac, L. (2007). Chlororespiration and cyclic electron flow around PSI during photosynthesis and plant stress response. Plant Cell Environ., 30(9), 1041-1051.
44. Scheidegger C, Schroeter B, Frey B (1995) Structural and functional processes during water vapour uptake and desiccation in selected lichens with green algal photobionts. Planta 197: 399-409.
45. Shi, X., Kim, D. H., Shin, H. S., & Jung, K. W. (2013). Effect of temperature on continuous fermentative hydrogen production from Laminaria japonica by anaerobic mixed cultures. Bioresour Technol, 144, 225-231.
46. Siedow, J. N., & Umbach, A. L. (1995). Plant Mitochondrial Electron Transfer and Molecular Biology. Plant Cell, 7(7), 821-831.
47. Siminis C, Kanellis A, Roubelakis-Angelakis K (1993) Differences in protein synthesis and peroxidase isoenzymes between recalcitrant and regenerating protoplasts. Physiol Plant 87: 263-270.
48. Souza-Egipsy, V., Valladares, F., & Ascaso, C. (2000). Water Distribution in Foliose Lichen Species: Interactions between Method of Hydration,Lichen Substances and Thallus Anatomy. Annals of Botany(86), 595-601.
49. Spier, L., van Dobben, H., van Dort, K.(2010). Is bark pH more important than tree species in determining the composition of nitrophytic or acidophytic lichen floras? Environmental Pollution(158), 3607- 3611.
50. Strasser BJ, Strasser RJ (1995) Measuring fast fluorescence transients to address environmental questions: the JIP-test. In: Mathis P, editor. Photosynthesis: from light to biosphere. Dordrecht: Kluwer Academic Press. pp. 977-980.
51. Terashima, M., Specht, M., Naumann, B., & Hippler, M. (2010). Characterizing the anaerobic response of Chlamydomonas reinhardtii by quantitative proteomics. Mol Cell Proteomics, 9(7), 1514-1532.
52. Turk, A. O., Yilmaz, M. Kivanc, M., Turk, H. (2003). The antimicrobial activity of extracts of the lichen Cetraria aculeata and its protolichesterinic acid constituent. Z Naturforsch C, 58(11-12), 850-854.
53. Wang, J., & Wan, W. (2008). Effect of temperature on fermentative hydrogen production by mixed cultures. Int J Hyd Energy, 33, 5392-5397.
54. Werth, S. (2011). Biogeography and phylogeography of lichen fungi and their photobionts Biogeography of Microscopic Organisms: Is Everything Small Everywhere? (pp. 192-207): Cambridge University Press.
55. Wong, G. (2011). Symbiosis: Mycorrhizae and Lichens, from http://www.botany.hawaii.edu/faculty/wong/BOT135/Lect26.htm