ASSISTED FLOCCULATION OF *CHLORELLA SOROKINIANA* BY CO-CULTURE WITH FILAMENTOUS FUNGI

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor Philosophiae in the Department of Biodiversity and Conservation Biology, University of the Western Cape.

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November 2015

ABSTRACT

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Biofuel production from microalgae is currently not economically competitive with fossil fuels due to high operational costs. A sustainable system needs to be developed which considers cultivation, harvesting and conversion to fuels as a single loop. The harvesting step has been identified as a major bottleneck within the biofuel production process, contributing to a significant proportion of the operational cost (20-30%). Chemical flocculation is a more affordable alternative to centrifugation and filtration. Chemical flocculants however negatively impact the quality of biomass and conversion efficiency to biofuel by increasing biomass ash content. Bioflocculation with biopolymers or microbes have a minimal impact on the quality of biomass. In this study, the interaction between the filamentous fungus *Isaria fumosorosea* and the microalgae *C. sorokiniana* is investigated. Under strict autotrophic conditions at pH 7-8, co-culture of microalgae (2-20 µm) with fungal blastospores resulted in the

development of large pellets (1-2 mm) which may be easily harvested by sedimentation or filtration at 95% harvesting efficiency. Fungal assisted bioflocculation was compared to other harvesting methods with respect to cost and impact on the hydrothermal conversion process. Low cost carbon sources, including waste hydrothermal nutrients, minimal sugar concentrations and algal exudate may reduce fungal cultivation costs. Waste products, such as organic carbon, N, P, CO_2 and trace metals can be recycled and used for algae and fungal cultivation, closing the loop to make the system sustainable.

November 2015

KEYWORDS

Chlorella sorokiniana

Isaria fumorosea

Microalgae

Filamentous fungi

Lichen

Co-culture

Harvesting

Dewatering

Bioflocculation

Hydrothermal gasification

DECLARATION

I declare that **ASSISTED FLOCCULATION OF *CHLORELLA SOROKINIANA* BY CO-CULTURE WITH FILAMENTOUS FUNGI** is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

November 2015

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Supervisor: Dr. Rolene Bauer

Acknowledgements

I would like to acknowledge my financial supporters, The Swiss Government Scholarship, the Swiss State Secretariat for Education and Research in the framework of COST Action CM0903 (Project C11.0048), the Swiss South African Joint Research Programme (Project SMCJRP06) (2012-2014) and the National Research Foundation of South Africa, for their aid (2014-2015).

I would like to acknowledge my supervisor, Dr. Rolene Bauer, for her motivation, academic input and supervision, and for providing me with these opportunities. I would like to thank Dr. Jean-Paul Schwitzguébel and Prof Christoff Holliger for their supervision at the EPFL. I would like to acknowledge the members of the labs at UWC and the EPFL for their instruction, advice and constructive criticism.

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Chapter 1:

Introduction

The large scale cultivation of microalgae, such as *Chlorella sorokiniana*, may address a range of global environmental challenges including bioremediation of waste water and carbon dioxide sequestration (Farrelly *et al*., 2013; Pires *et al*., 2013). Microalgae biomass serves as a feedstock for several industries such as biofuel, animal and fish feed and the production of pharmaceutical products (Spolaore *et al*., 2006). Biofuel production is predicted to potentially contribute up to 25% of the global energy requirements (Christensen *et al*., 2011; Smith *et al*., 2010). However, the process is currently not economically feasible at industrial scale (Dassey *et al*., 2013). Major challenges include (a) high costs associated with harvesting, (b) high costs of converting biomass into biofuel and (c) lack of sustainable processes for recycling recovered nutrients.

The process of biofuel production from microalgae occurs in three major steps: (1) Cultivation; (2) Harvesting and dewatering; and (3) Conversion to biofuels. In order to reduce operational costs, individual steps as well as the system as a whole need to be optimized. Current harvesting methods are estimated to contribute 20-30% of the total cost of biomass production (Molina Grima *et al*., 2003). Being the single largest contributing factor to the economic viability of

industrial cultivation, reducing the cost of harvesting has become the main focus of recent studies. Universal harvesting methods are favoured over species specific methods and include centrifugation, filtration, gravity sedimentation, flotation, co-agulation and various flocculation technologies (Danquah *et al*., 2009). Harvesting is time consuming, energetically demanding and may contribute to a high environmental impact when performed at industrial scale (Molina Grima *et al*., 2003; Barros *et al*., 2015). Centrifugation is the most effective universal method available, but also the most energy intensive and therefore unsuitable to large scale systems (Coons *et al*., 2004).

The energetic costs of biofuel production are viable if production costs are below 10% of the energy yield (US Department of Energy, 2010). Production costs have been estimated at approximately $ 470 tonne^{-1} (Chisti, 2007), although costs can vary significantly based on the operational expenses. Production and harvesting costs are determined by the cultivation system, the cultivation conditions of the specific algal strain, harvesting and dewatering method, and the biofuel conversion process (reviewed in Molina Grima *et al*., 2003; Christi, 2007; Williams and Laurens, 2010).

In order to decrease the cost of harvesting, thickening processes are used prior to centrifugation or filtration. The use of chemical flocculants, co-aggulants and or biological flocculants has been shown to reduce the costs of harvesting by concentrating the biomass prior to dewatering by centrifugation or filtration.

Recently, the use of filamentous fungi as a bioflocculation tool by co-cultivation with microalgae to form lichen-like pellet structures has been investigated (Zhou *et al*., 2012; Zhang and Hu., 2012; Zhou *et al*., 2013; Xie *et al*., 2013; Gultom *et al*., 2013; Wrede *et al*., 2014; Muradov *et al*., 2015; Mackay *et al*., 2015). Large pellets (1-2mm) are easily harvested at low cost, and may be used in conjunction with current harvesting methodologies such as filtration or sedimentation. However, a drawback of the addition of filamentous fungi is a reduction in the lipid yield of biomass. Presently, conventional solvent extraction and transesterification of lipids derived from algal biomass is the most prevalent method, and is dependent on high lipid content (20-50%) of microalgae strains (Hu *et al*., 2008; Wijffels, 2008; Sun *et al*., 2011). Hydrothermal conversion processes, which are not reliant on the lipid content of biomass, are under development. These include pyrolysis, hydrothermal liquefaction and hydrothermal gasification, all of which have yielded improved conversion efficiencies (Elliot *et al*., 2014). Microalgae biomass can be converted through hydrothermal conversion to bio-oils, bio crude and biogas (Elliot *et al*., 2014) from a wet algal slurry (15% dry weight) (Vogel, 2009). Hydrothermal conversion processes produce CH_3, CO_2, and a range of waste organic carbons, organic nitrogen, phosphates, and trace metals which can be recovered and recycled (Cherad *et al*., 2013; Bagnoud-Velasquez *et al*., 2015).

Current harvesting methods can be a single step or a combination of a dewatering and concentrating step including centrifugation, filtration and

chemical flocculation or bioflocculation (reviewed in Barros *et al*., 2015). In addition to energy, labour and material costs, some harvesting methods may reduce the quality of the biomass. Chemical flocculants, typically metal salts or metal alkali (Vandamme *et al*., 2013), contribute metal ash to the recovered biomass which reduce hydrothermal conversion efficiencies (Haiduc *et al*., 2009). Bioflocculants include biopolymers (e.g. chitin) and microbes such as bacteria, fungi or other flocculating algae strains (Barros *et al*., 2015). No metal ash is introduced when algae are co-cultured with bioflocculants; however, they are more expensive in comparison to chemical flocculants (Mackay *et al*., 2015). With respect to fungal bioflocculation, the reliance on glucose as a carbon source is not viable for large scale continuous culturing. The use of minimal medium for culturing or cheaper, sustainable alternatives to carbon and nitrogen sources could reduce costs (Muradov *et al*., 2015; Mackay *et al*., 2015). Sustainable fungal bioflocculation methods could be implemented for direct hydrothermal conversion (Muradov *et al*., 2015). Furthermore, microalgae biomass production can be tied to the bioremediation processes as microalgae can be cultivated on a broad range of urban, industrial and agricultural wastewaters (Rawat *et al*., 2011) as well as the nutrient rich aqueous phase recovered from the hydrothermal conversion of algal biomass (Bagnoud Velasquez *et al*., 2015). The Paul Scherrer Institute (Villigen, Switzerland) is developing a hydrothermal conversion process from microalgae to methane. They have provided us with samples of the nutrient rich aqueous

phase recovered after the hydrothermal liquefaction of algal biomass (Haiduc *et al*., 2009 and Bagnoud-Velasquez *et al*., 2015). This group has confirmed the viability of the samples as a nutrient source for microalgae. In this thesis, the value of the hydrothermal liquefaction (HTL) aqueous phase as a supplementary carbon source for fungal pre-cultivation is determined in comparison to other low value carbon sources, such as organic acids.

This thesis is composed of a literature review (Chapter 2) and a research section (Chapter 3). Fungal assisted bioflocculation is compared to current flocculation methods. As the economic viability of bioflocculation and quality of the biomass is a major concern, the impact on the quality of the recovered algae-fungi biomass is investigated and compared to existing chemical flocculation and bioflocculation strategies in the context of hydrothermal conversion processes.

Aims

- Review the feasibility of hydrothermal conversion of algae harvested by fungal assisted flocculation
- Optimize fungal assisted bioflocculation to operate under autotrophic conditions at low cost
- Assess the impact of fungal assisted bioflocculation on the quality of biomass
- Evaluate the potential for cultivation, harvesting and hydrothermal conversion to operate sustainably by maximizing the use of recovered resources

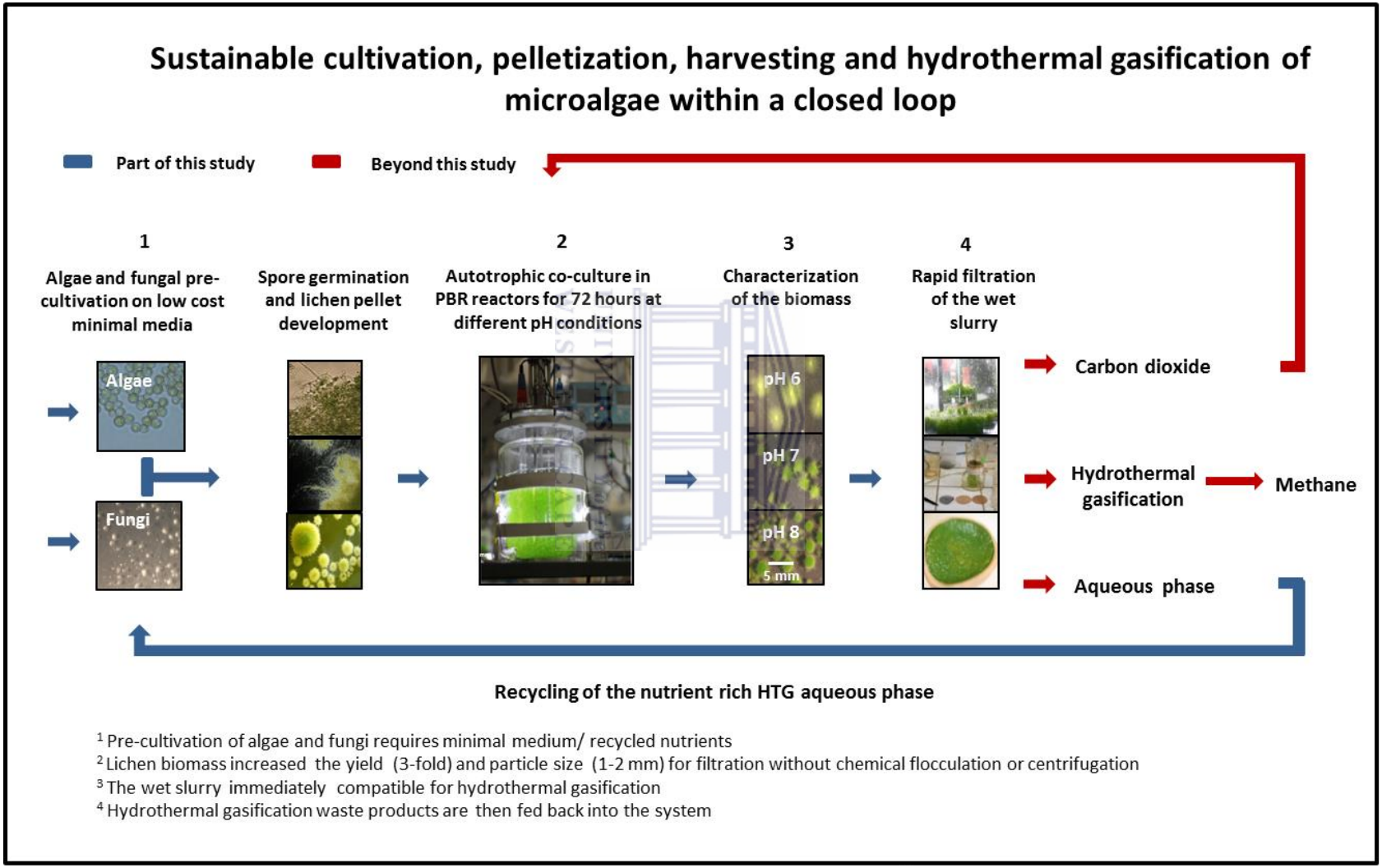

Fig 1.1: Scope of the study. Graphical abstract published in Mackay *et al.*, 2015.

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Chapter 2:

Sustainable Harvesting of Microalgae with Filamentous Fungi:

Closing the Loop by Integration with Hydrothermal Conversion

A modified version is submitted for publication to *Algae Research*, November 2015 as:

Mackay S., Bauer R. (2015) Sustainable Harvesting of Microalgae with Filamentous Fungi: Closing the Loop by Integration with Hydrothermal Conversion

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Abstract

Due to economic, energetic and environmental constraints, the production of biofuels from microalgae has not been sustainable. Harvesting of microalgae has been identified as a major bottleneck in the process chain. This paper compares harvesting technologies and highlights the use of filamentous fungi as a promising new strategy to harvest single cell microalgae (2-20 µm). In co-culture with fungi, large pellets (1-2mm) are formed which are easily separated from the culture medium. Microalgae may be immobilized by the addition of pre-cultured fungal pellets shortly before harvesting, autotrophic co-culture at

neutral pH, or mixotrophic co-culture at acidic pH. We review the algae-to-biofuel process chain from cultivation to hydrothermal conversion. By using fungal biomass as a bioflocculant, harvesting costs are decreased and biomass yield is increased. The production loop can be closed by recycling of by-products.

Keywords: Bioflocculation, Filamentous fungi, Microalgae, Harvesting, Pyrolysis, Hydrothermal liquefaction, Hydrothermal gasification

Highlights:

- Current methods for harvesting microalgae were compared to fungal bioflocculation.
- Three different harvesting strategies by fungal bioflocculation are presented.
- Algae cultivation and bioflocculation with fungi could potentially be performed within a closed loop system by coupling cultivation and harvesting with hydrothermal conversion.
- The efficiency of the cultivation and conversion process can be increased at several steps, specifically with respect to energy usage and resource recycling.

2.1 Introduction

Large scale cultivation of microalgae may address a range of environmental concerns including bioremediation of wastewater and carbon dioxide sequestration (Farrelly *et al*., 2013; Pires *et al*., 2013). Microalgal biomass is furthermore used for the production of animal and fish feed, pharmaceutical

products and biofuel (Spolaore *et al*., 2006). While biofuel production may potentially contribute up to 25% of the global energy requirements (Christensen *et al*., 2011; Smith *et al*., 2010), the process is not economically viable on industrial scale (Dassey *et al*., 2013). Major challenges include (a) sub-optimal lipid content of biomass, (b) high costs associated with harvesting and dewatering, (c) high costs of converting biomass into biofuel and (d) lack of sustainable systems for recycling nutrients.

Microalgae can be cultured on a range of industrial and agricultural wastewaters (Rawat *et al*., 2011) as well as wastewater derived from the hydrothermal conversion of algal biomass (Bagnoud *et al*., 2015). Harvesting and dewatering contribute 20-50% of the total cost of biomass production (Gudin and Therpenier, 1986; Molina Grima *et al*., 2003). Being the single largest contributing factor to the economic viability of industrial cultivation, reducing this cost has become the main focus of recent studies. Universal harvesting methods are favoured over species specific methods and include centrifugation, filtration, gravity sedimentation, flotation, co-agulation and various flocculation technologies (Danquah *et al*., 2009). Harvesting is time consuming, energetically demanding and may contribute to a high environmental impact when performed at industrial scale (Molina Grima *et al*., 2003; Barros *et al*., 2015). Centrifugation is most effective, but also most energy intensive and therefore not suitable to large scale systems. Low value products

such as biofuels do not compensate for the high energetic costs of centrifugation (Coons *et al*., 2004).

Biofuel production is considered viable if production costs (including algae cultivation and harvesting) do not exceed 10% of energy produced (US Department of Energy, 2010). Algal biomass production has been estimated to cost approximately $ 470 tonne^{-1} (Chisti, 2007) (reviewed by Williams and Laurens, 2010). Costs are determined by the algal strain, cultivation system, harvesting and dewatering method, and the biofuel conversion process. Biofuel production and economics have been extensively reviewed (Molina Grima *et al.,* 2003; Christi, 2007; Williams and Laurens, 2010).

Recent work has suggested the use of filamentous fungi as a bioflocculation tool by co-cultivation with microalgae to form lichen-like pellet structures (Gultom *et al.,* 2013; Mackay *et al.,* 2015). Pellets (1-2mm) are easily harvested and at low cost, and may be applied as a safe alternative to current harvesting methodologies. However, the addition of filamentous fungi reduces the lipid yield in biomass. Presently, the most widely used method for producing bio-oils from microalgae is through conventional solvent extraction and transesterification of lipids. This method is highly reliant on the lipid content (20-50%) of microalgae strains (Hu *et al*., 2008; Wijffels, 2008). Hydrothermal conversion processes, including pyrolysis (hydrothermal carbonization), hydrothermal liquefaction and hydrothermal gasification have shown promise due to higher conversion efficiencies as well as the generation of recyclable

resources (López *et al*., 2013; Elliot *et al*., 2014). Hydrothermal gasification is not dependent on the lipid content of biomass (Garcia Alba *et al*., 2012; López *et al*., 2013,).

This paper highlights the potential application of filamentous fungi as a bioflocculation tool to harvest microalgae. The review compares harvesting methods with respect to efficiency, economic implications and environmental considerations. The impact of methodology on biomass quality, conversion processes into biofuel, as well as the potential to recycle resources within the system, is discussed.

2.2 Microalgae biomass harvesting methods

The small size (2-20µm) and negative surface charges of microalgal cells, as well as dilute culture concentrations in open ponds (0.5-1g/L) and photobioreactors (PBRs) (>5 g/L), keep cells in suspension. Concentrating steps are required to reduce the load volumes for down-stream processes. Typically, a primary thickening step to concentrate biomass to a wet slurry (100-200 fold concentration to 2-7% total suspended solids, TSS) is followed by a dewatering step (2-10 fold concentration to 15-25% TSS) (Brennan *et al*., 2010; Uduman *et al*., 2010). The thickening step is generally costly and impacts on downstream processing strategies (Griffiths *et al*., 2011). Harvesting methods based on physical separation include centrifugation, filtration and sedimentation. Co-agulation and flocculation require the addition of chemicals or biological

flocculating agents which cause biomass aggregation for ease of harvesting by physical methods. The scale of biomass, whether produced in an open pond or photobioreactor, influences the choice of harvesting method. Other important considerations are the down-stream conversion process and product specification. Some processes require dry biomass, while others such as hydrothermal liquefaction and catalytic hydrothermal gasification facilitate wet biomass with up to 20% moisture content (Haiduc *et al*., 2009). Cost intensive harvesting methods, such as centrifugation, may be compensated by high-value products such as pharmaceuticals. Energy intensive harvesting methods are not suitable for biomass intended for low value products such as biodiesel or biogas. Reducing the cost of harvesting has indeed become the main target for sustainable production of biomass for biofuel (Uduman *et al*., 2010).

2.2.1 Thickening processes

Thickening, the primary concentrating step in algae harvesting, aims to reduce the volume of the algae slurry, which significantly reduces cost of final dewatering steps. Co-agulation and auto-flocculation by the addition of chemicals are the most commonly used thickening processes (Uduman *et al*., 2010).

2.2.1.1 Co-agulation and auto-flocculation by chemicals

The type of flocculation is defined by the nature of the flocculating agent. Co-agulation involves the addition of multivalent cationic salts, whereas auto-

flocculation involves pH manipulation with an alkali (Papazi *et al*., 2010). Both methods rely on the introduction of metal cations to neutralize negatively charged algae cell surfaces which cause small flocs of algae cells to aggregate (Papazi *et al*., 2010). The resulting increase in particle size allows for efficient capture by sedimentation and 20-100 times concentration of biomass (Vandamme *et al*., 2013). Efficiency is dependent on media pH, algal growth phase and cell surface charges (Papazi *et al*., 2010).

Co-agulation is limited to multivalent metal salts of which $FeCl_3$, $Al_2(SO_4)_3$ and $Fe_2(SO_4)_3$ have been most effective (Papazi *et al*., 2010). Although highly efficient, the introduction of toxic Al^{3+} and Fe^{3+} cations is undesirable. Auto-flocculation is therefore the preferred technology, and pH is typically raised with $Mg(OH)_2$, $Ca(OH)_2$ or NaOH. This is a relatively inexpensive harvesting method, as production cost for flocculants are low (Horiuchi *et al*., 2003; Vandamme *et al*., 2013). Flocculation efficiency with cations is dependent on pH and dosage. Rapid settling times with high auto-flocculation efficiencies (>90%) have been recorded at pH values ranging from 8.6 to 10.5 (Horiuchi *et al*., 2003; Vandamme *et al*., 2013). Flocculant dosages to cell density have been reported as linear at low density cultures (Papazi *et al*., 2010), however at high cell densities, approximately 20-fold lower dosages are effective (Schlesinger *et al*., 2012). The increased efficiency of flocculation at high cell culture densities are thought to be due to near neutral cell surface charges at late stages of growth, possibly due to a build-up of algal extracellular polysaccharides (EPS)

and proteins which interact with flocculating agents. The decrease in distance between cells and increased rate of collision require lower concentrations of alkali to neutralize and repel colliding cells. Marine species cultured in high ionic strength have required a 5 times larger dosage relative to species cultured in fresh water (Uduman *et al*., 2010). Disadvantages of auto-flocculation include the accumulation of ash in the biomass, due to the build-up of metal cations (Schenk *et al*. 2008). The re-use of culture media relies on pH neutralization and the removal of salt.

Chen *et al*. (2012) investigated the use of ammonia as an alternative flocculant to raise the pH of the medium without metal ions contamination. Flocculation was most effective for marine algae. For example, *Nannochloropsis oculata* flocculated with 99% efficiency at a dosage of 57 mmol.L^{-1} (12h, pH 10.7), while a fresh water *Chlorella sorokinina* strain flocculated at 49% efficiency with 113.3 mmol.L^{-1} (12h, pH 10). After pH adjustment with CO_2, the medium was suitable for recycling. Ammonia was metabolized by cultures as a nitrogen source and did not change the algae cell metabolic components. The specific mechanism of action remains to be elucidated.

2.2.1.2 Flocculation by cationic polymers

Naturally derived cationic polymers such as chitosan, polyacrylamides, starch and cellulose are effective flocculants for various fresh water microalgae, including *Chlorella* species (reviewed in Barros *et al*., 2015). However, high

salinity of the marine environment can be inhibitory since polymers shrink to smaller dimensions, failing in bridging cells. Chitosan, an abundant waste product from fishing industries, has been used with success to floculate fresh and seawater microalgae without introducing toxins (Show *et al*., 2014) or contaminating biomass with metal ash (Schenk *et al*. 2008). However, it is expensive and energy intensive to refine, making it only viable for high value microalgal products. Effective chitosan flocculation and filtration (8µm) are furthermore dependent on acidic pH conditions (Xu *et al*., 2013).

2.2.1.3 Bioflocculation by microbial co-culture

Bioflocculation of microalgae involves co-culture with other microorganisms and relies on the interaction between bacterial, fungal and algal secreted extracellular polysaccharides (EPS). Although relatively cost effective, co-culture may introduce biological contaminants which are undesirable for high value products such as pharmaceuticals or animal feed (Vandamme *et al*., 2013). Bacterial bioflocculants are typically applied for waste water bioremediation (Lee *et al*., 2010). Bioflocculation of microalgal cells (2-20µm) with bacteria result in a mixed EPS network with increase particle size (148-305 µm) and therefore settling time (Lee *et al*., 2013). Although the addition of chemicals can be avoided, lipid content may be significantly reduced through dilution as significant amounts of bacterial biomass are required.

Fungal strains, including yeast (Díaz-Santos *et al*., 2014) and filamentous fungi (Rajab Aljuboori *et al*., 2015), have been shown to produce bioflocculants with different degrees of efficiency. Bioflocculation by the addition of other microalgal strains has been successful on small scale (Salim *et al*., 2011) with harvesting efficiencies comparable to the sediment efficacies of chemical co-agulation (Papazi *et al*. 2010).

2.2.1.4 Gravity sedimentation

Gravity sedimentation is a time-consuming albeit energetically viable harvesting method for microalgae species with large cells (Rawat *et al*., 2011). Algae tend to settle naturally, at about 0.1-2.6 $cm.h^{-1}$, depending on cell size and density. This method is not viable for bacterial-sized microalgae such as *Chlorella* due to the slow rate of sedimentation which can result in lipid metabolism (Christenson *et al*., 2011).

2.2.1.5 Flotation

Microalgae can be captured by bubbling gas from the base of the reactor or pond (Rubio *et al*., 2002; Hanotu *et al*., 2012). Hydrophobic cell surfaces attach to the bubbles and migrate to the surface. Flotation is commonly applied in waste water treatment; however, efficient harvesting relies on supplementation with chemical flocculants (Hanotu *et al*., 2012).

2.2.1.6 Flocculation by electrical current

Due to the hydrophobic and charged nature of microalgae cell surfaces, cells in an electric field migrate in the direction of the current without the need for flocculating agents (Uduman *et al*., 2010; Zenouzi *et al*., 2013). Electrical currents can be directed to the surface (electro-flotation), opposite electrodes (electrophoresis) or towards the base (elctro-floccualtion). Sacrificial electrodes release metal cations by dissolution from the anodes into the culture, causing aggregation in a similar fashion to cationic chemical co-agulation (Uduman *et al*., 2010). Metal ion toxicity and ash contribution are, however, significantly lower (Zenouzi *et al*., 2013). Non-sacrificial electrodes (anaphoresis) cause migration and aggregation of negatively charged algae towards the anode (Uduman *et al*., 2010). Harvesting by electrical current is a relatively rapid process; however electrolysis time and energy consumption is directly proportional to culture density. Energy efficiency can be optimized by operating at acidic to neutral pH, or at higher temperature due to an increase in electrical conductivity (Gao *et al*., 2010).

2.2.1.7 Flocculation by ultrasonic radiation

Flocculation by ultrasonic radiation relies on pressure created by standing waves (Coons *et al*., 2014). Algae aggregate at the standing nodes when pressured by forward and reverse ultrasonic radiation. Ultrasonic harvesting technologies are promising as biomass quality is not impacted. Studies have

only been performed at very small scale (Bosma *et al*., 2003) and significant improvements in energy costs (reviewed by Coons *et al*., 2014) are required for this option to become viable.

2.2.2 Dewatering

The dewatering step follows the initial concentrating and thickening steps and usually involves energy intensive mechanical methods which are economically viable only when working with dense algal slurries.

2.2.2.1 Micro-straining and Filtration

Micro-straining involves rotary drums covered in straining fabrics, stainless steel or polyester (Uduman *et al*., 2010). Captured algae are washed into concentrated slurry. Micro-straining is only energetically viable for large cells, such as blue green algae. Filtration of small single cell microalgae requires filters with small pore sizes and application of constant pressure (Molina Grima *et al*., 2003). Dead-end filtration permits relatively quick flow-through and is suitable for microalgal cells larger than 70µm. Tangential flow is appropriate for smaller cells as it is less sensitive to membrane fouling (Rawat *et al*., 2011; Molina Grima *et al*., 2003). Efficient harvesting by filtration relies on prior flocculation (Show *et al*., 2014). Flocculation significantly improves costs of filtration as large particles allow for the use of filters with larger pore size which increase the sub-critical flux limit and the maximum flow rate before membrane fouling occurs (Rossi *et al*., 2008). Small cells can be harvested by

membrane filtration without prior flocculation, however, small pore size reduces flow rate, thereby increasing the cost of harvesting and maintenance (Show and Lee, 2014). Similar to tangential flow filtration, membrane filters require maintenance as biopolymers and biomass build-up restrict flow. Membrane filtration is not useful at industrial scale due to high cost of membranes and maintenance (Molina Grima *et al*., 2003).

2.2.2.2 Centrifugation

Centrifugation has traditionally been the favoured method for small scale operations as it is universally efficient for all algal strains. However, centrifugation is limited by very high equipment costs, intensive energy requirements, and low turnover for large scale operations (Dassey *et al*., 2013). Centrifugation, as with filtration, is an effective dewatering step if preceded with a harvesting technique that reduces the volume of algae cultures (Show *et al*., 2014).

2.3 Fungal assisted flocculation of microalgae

Fungal assisted flocculation of microalgae through co-culture with filamentous fungi relies on the co-agulation of algae cells on the hyphae of fungal pellets (Mackay *et al*., 2015). Although this mechanism of harvesting and dewatering is in some ways similar to other flocculation and co-agulation methods, there are significant differences in the economic and environmental impact as well as the quality of the harvested biomass. The mechanism of action is not well

understood, but appears to rely on differences in surface charge between algal and fungal cells, similar to the mechanism described for polymeric flocculants such as chitin (Xu *et al*., 2011). The size and density of the particles (1-2 mm) are significantly larger than chemical flocs (<100µm) and algae-fungi co-cultivation has led to increases in total biomass yields and efficiency of waste water bioremediation (Zhou *et al*., 2012, Wrede *et al*., 2014, Muradov *et al*., 2012). Mutual increase in biomass of algae and fungi, compared to yields of monocultures, suggests symbiosis (Muradov *et al*., 2015, Mackay *et al*., 2015). The efficiency of bioflocculation with the aid of filamentous fungi depends on compatibility with algal strains and the formation of stable pellets under normal culture conditions. Important factors to consider after harvesting are the influence on biomass quality and potential environmental implications.

2.3.1 *Pellet morphologies of filamentous fungi*

The growth morphology of filamentous fungi is dependent on culture conditions (Mackay *et al*., 2015). When cultured on solid media, large networks of hyphae grow outwardly and form conidiophores that produce aerial conidia. In liquid culture, filamentous fungi form branching mycelial networks which grow apically from germinating blastospores. Blastospores produced in liquid culture are formed from buds on the filamentous hyphae and are typically more resilient to stress conditions than aerial conidia.

Under submerged conditions and agitation, certain species of filamentous fungi form dense spherical pellets where intertwined hyphae aggregate to form dense networks ranging from loose irregular hyphal aggregates, often referred to as clumps, to denser more spherical pellets (Cox *et al*., 1998). Pellet formation originates either from the germination of a single spore referred to as non-co-agulative pellet formation, or through the germination and aggregation of multiple spores referred to as co-agulative formation (Znidarsic *et al*., 2001).

Pellet morphology varies depending on strain, culture conditions and pellet maturity (Gibbs *et al*., 2002). A pellet clump is typically small with a compact nucleus, expanding outwardly to form loose mycelial “arms” on the periphery which give pellets irregular morphologies. Compact smooth pellets are typically spherical and have dense morphologies with a regular periphery. The morphology of pellets are strain specific and influenced by genetic, chemical, mechanical and physical factors (Gultom *et al*., 2013). The quality and number of pellets formed are dictated by inoculum size, agitation rate, temperature and pH. Strong agitation and large spore inoculums increase the number of pellets formed but decrease the size of pellets. Culture medium with high C:N ratios favour smooth compact dense pellets. Low C:N ratios or nutrient limitation yield smaller irregular pellet clumps, and favour increased blastospore production (Cliquet and Jackson, 2005). Through maintenance of the pH and

nutrient conditions, the quality and quantity of pellets can be reproducibly cultured (Mackay *et al*., 2015).

2.3.2 Harvesting of microalgae by pelletization with filamentous fungi

The ultimate objective is to design a system where the fungus is a universal flocculant for different algae strains, has a low impact on the quality of the biomass and environment, and is compatible to all bioreactors, pond systems and down-stream processing operations. The cost of culture media and the timeframe dictate the viability of harvesting.

Table 2.1 summarizes the co-culture of a range of fungal and algal strains by three different strategies: (1) Inoculation of fungal spores into growing algae cultures and co-culture under mixotrophic conditions at neutral to acidic pH. (2) Addition of fungal pellet biomass to algae cultures prior to harvesting. (3) Inoculation of small pellets of fungal biomass, pre-cultured on cheap carbon sources, into growing algae cultures and co-culture under autotrophic conditions at neutral pH.

2.3.2.1 Co-cultivation of fungi and microalgae under mixotrophic conditions

The co-culture of algae and fungi by the addition of fungal spores (strategy 1, Fig 2.1a) requires an adequate carbon source and neutral to acidic pH for spore

germination. Several studies have followed this approach and are discussed below.

Zhang and Hu (2012) induced pellet formation by adding *Aspergillus niger* conidia to *Chlorella vulgaris* cultures. Co-culture at acidic pH resulted in algae-fungi pellet formation and three-fold increase in total biomass compared to algae monoculture control. The number of free cells in suspension after harvesting was however high, corresponding to ~63% harvesting efficiency. Similarly, co-culture of *C. vulgaris* with an oleaginous filamentous fungus, *Cunninghamella echinulata* has resulted in up to three-fold increase in total biomass (Xie *et al*., 2014). Glucose metabolism in the absence of pH regulation has resulted in a drop in media pH and flocculation of nearly 99% of cells. Zhou *et al*. (2012 and 2013) have shown than pH is indeed the key factor affecting formation of algae-fungi pellets. *C. vulgaris* was co-cultured with an environmental isolate of *Aspergillus oryzae*. An increase in spore inoculum and glucose concentration (up to 20g/L) resulted in a decrease in culture pH. Highest biomass yield was achieved at acidic pH, and algae-fungi pellet formation relied on pH 4.5 to 7.

Gultom *et al.* (2014) investigated the impact of light, media composition and initial concentration of fungal spores and microalgal cells on pellet formation. Co-cultivation of *C. vulgaris* and *Aspergillus niger* was not viable under strict

Table 2.1: Summary of the bioflocculation efficiencies of algae-fungi co-cultures

Reference	Fungus	Algae	FW/MW	Cultivation conditions/ Fungus Preculture	Algae/Co-culture	Stratergy	Trophic level of the co-culture	Culture scale	Optimal pH range	Time frame	Capture efficiency
Zhou *et al* ., 2012	*Aspergillus sp.*	*Chlorella vulgaris*	FW	PDB	BG-11 + 20g/L glc	1	M	Flask	4-6.7	54 h	100%
Zhang *et al* ., 2012	*Aspergillus niger*	*Chlorella vulgaris*	FW	PDB	Basal + YE + 2g/L glc	1	M	Flask	4	72 h	60%
Zhou *et a* ., 2013	*Aspergillus oryzae*	*Chlorella vulgaris*	FW	TSB	BG-11 + 10g/L glc	1	M	Flask	5	72 h	93%
Xie *et al* ., 2013	*Cunninghamella echinulata*	*Chlorella vulgaris*	FW	PDB	TAP	1	M	Flask	3.5-5	48 h	99%
Talukder *et al* ., 2014	*Aspergillus nomius*	*Nannochloropsis sp.*	MW	PPM + cooking oil	F/2	2	M	Flask	6.0-6.5	3 h	97%
		Chlorella vulgaris	FW	PPM + cooking oil	BAR	2	M	Flask	7	4h	95%
		Dunaliella teriolecta	MW	PPM + cooking oil	ATCC 1174DA	2	M	Flask	6.0-6.5	9h	93%
Prajapati *et al* ., 2014	*Aspergillus lentus*	*Croococus sp.*	FW	BG 11 + 10g/L glc N + P	BG 11 + 10g/L glc	1	M	Flask	6.5-9.2	24 h	100%
Wrede *et al* ., 2014	*Asperguillus fumigatus*	*4 algal strains*	FW	FGB + (2% glc or 1% TWS)	*N/A (differs per strain)*	2	A	Flask	*N/A*	24 h	< 90%
		7 algal strains	M	FGB + (2% glc or 1% TWS)	*N/A (differs per strain)*	2	A	Flask	*N/A*	24 h	< 90%
Muradov *et al* ., 2015	*Apergillus fumigatus*	*Chlorella protothecoides*	FW	FGB + (2% glc or 1% TWS)	*N/A* + 3% glc	2	M/A	Flask	*N/A*	24 h	< 90%
		Tetraselmis suecica	MW	FGB + (2% glc or 1% TWS)	F/2	2	A	Flask	*N/A*	24 h	< 90%
Gultam *et al* 2014	*Asperguillus niger*	*Chlorella vulgaris*	FW	Basal + YE + 2g/L glc	Basal + YE + 2g/L glc	1	M	Flask	7.1	*N/A*	95%
Al-Hothaly *et al* ., 2015	*Asperguillus fumigatus*	*Botryococcus Braunii*	FW	FGB + 2% glc	BG-11	2	A	500-L	*N/A*	12 h	98%
Mackay *et al* ., 2015	*Isaria fumorosea*	*Chlorella sorokinina*	FW	2g/L glc + 3N BBM	3N BBM	3	A	2L PBR	pH 7-8	48 h	97%

Key:
A = Autotrophic co-culture
M = Mixotrophic co-culture
PBR - photo bioreactor
FW/MW = algae freshwater/marine species
NA = available/reported
(m:m) = mass:mass
(v:v) = volume:volume
(f:a) = fungal spore: algae cell

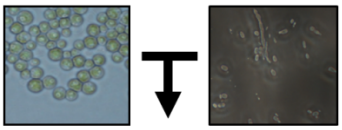
Media:
BBM = Bolds basal medium
BG 11 = Blue Green Medium
F/2 = Guillards f/2
FGB = Fungal growth broth
glc = glucose
PDB = Potato dextrose broth
PPM =Polypeptone medium
TAP = Tris acetate phosphate
TSB = tryptic soy broth
TWS = Acid treated wheat straw
YE = Yeast extract

Strategy 1: Mixotrophic co-cultivation of algae and fungal spores

Pre-cultivation

Algae culture

Spore inoculum



Co-cultivation

Harvesting

Harvesting by sedimentation and filtration

Description

- Prepared fungal spores are added to algae cultures.
- Spore cultures are prepared from glucose rich media (PDB 20g/L) for increased germination and viability.
- Spores germinate into pellets which capture algae cells over a 48-72 h period .
- Spore to algae inoculums (1:600) are effective at harvesting > 90% (Gultom *et al*., 2014)
- Spore germination requires a carbon source (2g/L glucose and a pH of 4-7 (Gultom *et al*., 2014).

Advantages

- Spores may be produced in large quantity and stored.
- Co-cultivation with glucose increases lipid yield.

Disadvantages

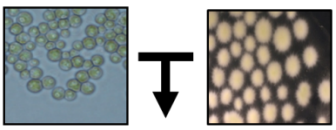
- Spore production requires glucose for viable spore production.
- Dependence on glucose for co-cultivation is costly.
- The use of glucose for co-cultivation/harvesting may introduce contamination.
- Algae cultivation <pH 7 limits autotrophic growth.

Strategy 2: Autotrophic co-cultivation of algae and fungal pellets

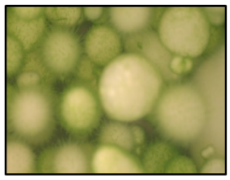
Pre-cultivation

Algae culture

Pellet inoculum



Co-cultivation



Harvesting

Harvesting by sedimentation and filtration

Description

- Prepared fungal pellets are added to algae cultures.
- Pellet cultures are prepared from glucose rich media (PDB 20g/L) for increased germination and viability.
- Mature pellets capture algae cells over a period of 24-48 h.
- Pellet to algae inoculums (1:1 m/m) are effective at harvesting > 90% (Muradov *et al*., 2015)
- Spore germination requires a carbon source (2g/L glucose and a pH of 4-7 (Gultom *et al*., 2014).

Advantages

- Pre-cultivation of pellets in glucose rich media increase the lipid yield.
- Pellet production still viable with cheaper carbon sources (1% TWS).
- Co-cultivation does not require glucose as pellets are already developed.
- Pellet development can be maintained at a specific pH.

Disadvantages

- Requires a large quantity of pellet biomass to efficiently harvest algae.
- Low algae:fungi ratio in the final pellet composition

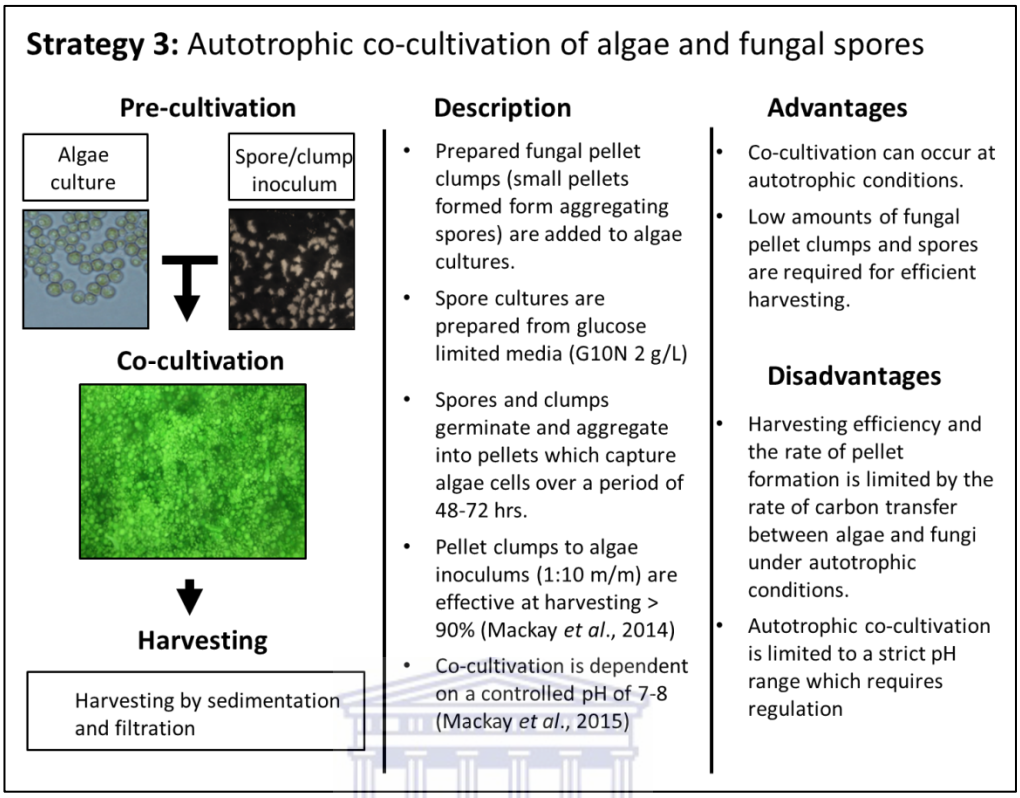


Fig. 2.1 a) Stratergy 1. Mixotrophic co-ccultivation of algae and fungal spores. Algae seed culture is innoculated with fungal spores and co-cultured at low pH under mixotrophic conditions to form algae-fungi pellets. **b)** Strategy 2: Autotrophic co-cultivation of algae and fungal pellets. Autotrophic or mixotrophic co-cultivation of algae and fungal pellets to form algae-fungi pellets. Fungal pellets are pre-cultured prior to co-cultivation. **c)** Stratergy 3: Autotrophic co-cultivation of algae and fungal pellet clumps. Pellet clump and spore biomass is precultured with limited carbon. Low ratios of pellt clump and spore biomass to algae are required to harvest algae supplied with CO_2 at pH 7-8.

photoautotrophic conditions and supplementation with an organic carbon sources was required to induced spore germination and pellet formation. The influence of different carbon sources, i.e. glucose, glycerol and sodium acetate, were compared. Co-culture with 2 g L^{-1} glucose was sufficient to harvest >90% of

cells from suspension, compared to 35% at concentrations below 1 g L^{-1}. Although co-culture resulted in a significant increase in total biomass (~66%), the algal contribution in biomass was only 30%. Media containing glycerol and acetate were able to produce pellets but at significantly lower yield. Since glycerol favoured fungal growth, the algal component of biomass harvested (>95% efficiency) was only 10%. Acetate yielded an algal composition of 16%, albeit lower harvesting efficiency (75%). The use of fungal spores as an inoculum for co-culture is clearly highly reliant on the quality of the carbon source. Harvesting efficiency was furthermore dependent on spore to algae ratio. In the presence of 2 g L^{-1} glucose, an inoculum ratio of 1:50 resulted in >95% harvesting efficiency. The lowest ratio (1:1000) tested, resulted in ~62% harvesting efficiency.

Prajapati *et al*., (2014) selected an *Aspergillus lentus* strain that was able to form algae-fungi pellets at acidic to neutral pH conditions (5-7). Fungal spores were unable to germinate under autotrophic conditions. Germination and growth relied on the addition of large concentrations of glucose. Close to 100% harvesting efficiency was achieved within 24 h after inoculation with fungal spores and a 10 g.L^{-1} glucose supplement, compared to 52 h at 5 g.L^{-1} glucose. This strategy has been successful at small scale, but clearly requires significant concentrations of glucose. Glucose may potentially be replaced with cheaper carbon sources such as wastewater. However, neutral pH needs to be maintained to favour spore germination and growth. High concentrations of

carbon increase the risk of microbial contamination, limiting this approach to closed systems such as PBRs.

2.3.2.2 Addition of fungal pellets to microalgae cultures prior to harvesting

The second bioflocculation strategy (Fig 2.1b.) is not pH sensitive (Muradov *et al*., 2015) and involves mass pre-culture of fungal pellets which are added as bioflocculant to microalgae cultures before harvesting. Studies have been primarily conducted at laboratory scale and extensive techno-economic analysis is required to make this technology economically feasible at industrial scale (Muradov *et al*., 2015).

Taluker *et al*. (2014) have reported on the use of pre-cultured fungal "cakes". Fungal "cakes" are large amorphous aggregates prepared without agitation as opposed to fungal pellets formed by agitation in submerged cultures. "Cakes" have been cultivated on various growth media such as glucose, peptone and inexpensive waste cooking oils and could immobilize freshwater and marine microalgae at acidic pH (<6.5). However, large volumes of fungal to algal biomass, up to 4:1, were required for immobilization.

Wrede *et al*. (2014) have screened an *Asperguillus fumigatus* isolate against a variety of marine, fresh water, autotrophic, and heterotrophic microalgae of different size. The authors observed increased yields in biomass production, lipid production and waste-water bioremediation during co-culture. Fungal pellets

were pre-cultured in a basal fungal growth broth supplemented with either 1% glucose or 1 % acid treated wheat straw (TWS). Pellets were added to algal cultures before harvesting. Over a 24h period, *A. fumigatus* pre-cultured in the presence of glucose flocculated all algae strains with 50-95% efficiency, compared to 50-65% when pre-cultured with TWS supplement. Flocculation efficiency remained unchanged after 48 h.

Al-Hothaly *et al*. (2015) investigated large scale harvesting of *Botryococcus braunii*, a lipid rich planktonic microalgae, with *A. fumigatus* as bioflocculant. Fungal biomass was cultured on potato dextrose broth (20g/L glucose), while *B. braunii* was cultured autotrophically in 500L tanks. The optimal volume to volume ratio of fungi to algae (1:40) resulted in the capture of 98% of biomass within 12 h. Up to 3g.L^{-1} algae-fungi biomass was recovered which was equivalent to the biomass yield from algal monocultures.

Muradov *et al*. (2015) pre-cultured a lipid rich *A. fumigatus* isolate to be used as bioflocculant for microalgae *Chlorella protothecoides*. Fungal spores were cultivated in basal medium containing 1% TWS or in Potato Dextrose Broth (PDB) containing 20g.L^{-1} glucose. Inhibitory compounds in TWS suppressed fungal growth which justified dilution to 1%. *A. fumigatus* biomass contained 2.8% lipid when grown in TWS, compared to 11.5% when grown in PDB after 48 hr. The lipid content of *C. protothecoides* monoculture corresponded to 28.2% of the biomass after 48 h when grown mixotrophically (3% glucose). Algae co-cultivation with fungi pre-cultured on TWS or PBD yielded biomass with lipid

composition of 12.35% and 21.35%, respectively, highlighting the importance of carbon source quality. However, total lipid yield (mg/L) in algae-fungi biomass pellets produced from both media was almost double than the additive lipid content of respective algal and fungal monocultures. Lipid yields correlated with increase in biomass. The synergistic effects of co-cultivation on biomass production and lipid yields have been reported in numerous studies but remain unexplained.

Large amounts of fungal biomass are required for efficient flocculation of algal cells, which may be costly if high quality carbon sources are used for fungal pre-culture. Muradov *et al*. (2015) reported a required 1:1 ratio of pellet to algae biomass (m/m) for effective harvesting for to algae biomass. Low cost, sustainable alternatives (e.g. TWS) may significantly reduce costs, albeit at the expense of total lipid yield. Key areas of research required to make this technology economically feasible at a larger scale include (a) reduction of the cost of growth nutrients through the application of alternative sources of carbon, nitrogen and phosphorus, and (b) establishment of efficient and cheap downstream processing technologies for oil extraction from fungal-algal cells and conversion to fuels (Muradov *et al*. 2015).

2.3.2.3 *Pre-cultivation of fungal biomass for autotrophic co-cultivation with microalgae*

The third strategy (Fig 2.1c) involves inoculation of actively growing algae cultures with a relatively small fungal starter, pre-cultured in e.g. waste water or a low concentration of glucose (Mackay *et al*., 2015). The starter consisted of a mixture of small pellet clumps and spores and resulted in efficient bioflocculation when co-cultured autotrophicly at constant pH of 7-8. Flocculation and biomass production relied on the symbiotic relationship of the lichen co-culture.

Mackay *et al*., (2015) prepared fungal pre-cultures of *Isaria fumorosea* in nutrient poor medium containing NH_4 and 0.2% glucose. Low nitrogen and carbon content stimulated spore production and resulted in the development of fine pellets (<0.5mm) which increased the surface area of fungal biomass. The fungal pre-cultures were inoculated into PBRs containing actively growing *Chlorella sorokiniana* cultures. Spore germination and development of co-cultures were encouraged under autotrophic conditions at constant pH of 7-8 and with the addition of CO_2. Algae-fungi pellets (1-2 mm) formed within 48 h, resulting in almost complete clearance of growth media (>95%). Close to 300% increase in biomass compared to algae monoculture were obtained and flocs consisted of 44% algae cells. Protein and carbohydrate content did not vary significantly between algal monocultures and co-cultures. The fatty acid profile shifted, displaying an increase in linoleic acid (18:2) in lichen pellets.

Although co-cultivation relies on carbon produced from photosynthesis and the development of spores to pellets, the time frame to harvest was comparable to strategies 1 (Gultam *et al*., 2014) and 2 (Muradov *et al*., 2015). In contrast to other strategies, minimal carbon was required for pre-culture of fungi, fungal inoculum was small and no supplemental carbon was required during co-culturing.

2.3.3 Yeast as bioflocculants

Díaz-Santos *et al*. (2014) investigated the potential of self-flocculating yeast strains to flocculate microalgae. *Saccharomyces bayanus* var*. uvarum* was shown to induce flocculation when paired with *Chlamydomonas reinhardtii*. The addition of an equal mass of anaerobically cultured yeast to *C. reinhardtii* cultures induced flocculation of ~90% of cells within 6 h. Aerobic cultured yeast resulted in <70% efficiency within the same timeframe. Similar results were observed for a marine microalgae *Picochlorum* sp. but at lower flocculation efficiencies. Although flocculation efficiencies are comparable to filamentous fungi, the reliance on a large inoculum is not cost effective on large scale.

Flocculation of algal biomass was due to the presence of lectins, yeast-derived flocculation proteins, rather than simple differences in zeta potential of cell surfaces (Díaz-Santos *et al*., 2014). Lectins are proteins which reversibly and non-enzymatically bind specific carbohydrates, such as flocculins and agglutinins (Sze and Tzi, 2011). Flocculation could also be induced by the addition of protein

extracted from a yeast culture (Díaz-Santos *et al*., 2014). Flocculation of *C. reinhardtii* has been optimal at a dose of 1 mg yeast protein to 10 mL of microalgal culture (OD_{660} = 1) causing 75% sedimentation in 90 min. Production of lectins may present a universal bioflocculation mechanism for algae with similar cell wall structures and warrants further investigation.

2.3.4 Impact of the carbon source on fungal biomass quality

The quality of the carbon source used for pre-culture of fungal biomass is significant. Low value carbon sources, such as TWS, reduced fungal biomass yield and lipid content, relative to glucose rich media (Wrede *et al*., 2014; Muradov *et al*., 2014). Mackay *et al*., (2015) cultured fungal biomass on various organic acids, such as acetate, butyrate, propionate, oxalate and succinate, which resulted in lower yields in comparison to glucose, sucrose and glycerol. Carbon recycled from hydrothermal gasification wastewaters produced similar results. Cultivation of fungal pellets on acetate produced lower yields, spore counts and smaller irregular shaped pellets in comparison to glucose. However, low-nutrient solutions with limited glucose favoured the formation of smaller pellets with increased surface area, resulting in increased spore production relative to media supplemented with high concentrations of glucose and peptone (Cliquet and Jackson, 2005; Mackay *et al*., 2015).

2.3.5 Bioremediation of wastewaters using algae-fungi pellets

Several studies have looked at the bio-remediation properties of biomass produced from algae-fungi co-cultures. Different types of diluted wastewaters such as municipal stream sludge (Zhou *et al*., 2012) and swine waste water (Zhou *et al*., 2012; Wrede *et al*., 2014; Muradov *et al*., 2015) have been used. Co-culture of algae-fungi pellets in swine waste water resulted in increased biomass yield and lipid content compared to algae monocultures, while COD, NH_4^+-N and PO_4^{-3}-P content in wastewater was significantly reduced. The co-culture of fungal spores with algae did not result in the formation of flocs due to the alkaline environment of waste water which causes pellet instability (Zhou *et al*., 2012). This may be overcome by a pre-culture step of spores in glucose rich media.

2.3.6 The mechanism of fungal-algal interaction

The co-culture of algae and fungi under autotrophic conditions with concomitant increase in biomass fits the definition of lichen. Lichen is currently classified based on phenotype since the molecular signalling mechanisms of lichen formation are unknown (Arnold *et al*. 2009, James *et al*. 2006, Schoch *et al*. 2009). Lichen, often growing terrestrially on rocks and trees, form symbiotic relationships that may consist of several species including algae, fungi and bacteria. The symbiotes' exchange is mutually beneficial, with carbon from photosynthesis being exchanged for a suitable terrestrial environment created by the fungus. A typical indication for symbiosis is an increase in biomass for

respective symbiotes. One mycobiont may interact with multiple photobionts and vice versa. True lichen interaction can be observed by modifications in gene expression profiles in response to changes in extracellular carbon metabolism.

Joneson (2011) investigated gene expression profiles of a model lichen consisting of photobiont *Asterochloris* sp. and mycobiont *Cladonia grayi*. The mycobiont displayed most adaptations in gene expression. The majority of changes were related to carbon partitioning, e.g. the up-regulation of 3 secreted lipases and a 15-fold increase in the expression of a hydrophobin Het-6 protein (Glass and Kaneko, 2003). Up-regulation of hydrophobins suggests that the association between hyphae and algae cells may be due to specific protein-binding interactions as opposed to solely cell surface charges (Joneson, 2011). The most significant change in the algae was the expression of a chitinase-like protein assumed to be involved in chitin recognition and binding.

In co-culture, algae and fungi bioflocculation is driven by differences in cell surface charges similar to chemical flocculation (Uduman *et al*., 2010b). Cell surface charges (zeta potential) are determined by the pH of the culturing media as well as the age and density of cultures. At neutral to acidic pH, algae cell surfaces tend to be negatively charged due to de-protonation of carboxylic-, phosphoric-, phosphodiester-, hydroxyl- and amine groups displayed on the cell surface (Hadjoudja *et al*., 2010). The zeta potential for microalgae is typically negative (-10 to -35 mV) (Rajab, 2007; Henderson *et al*., 2008), while the surface charge of fungi has been reported as positive, e.g. +46 mv for *A. flavus* (Rajab,

2007). The charge difference between cell surfaces could cause attraction by van der Waals forces.

Several recent studies have used *Aspergillus*, a genus known for its saprophytic activity, as the fungal partner. Significant increases in biomass when co-cultured have been reported (Zhou *et al*., 2012; Zhang and Hu, 2013; Zhou *et al*., 2013; Wrede *et al*., 2012; Muradov *et al*., 2013). The mechanism of carbon transfer from algae to fungi has been attributed to cellulase activity (Xie *et al*., 2013). Bekkett *et al*. (2013) have reported several Peltigeralean lichen which produce cellulases and oxidio-reductases, although the activity appears to be saprophytic under stressed conditions rather than a mechanism of carbon transfer. The co-culture of algae and fungi in glucose rich media would suppress fungal cellulase activity (Tengborg *et al*., 2001), and no cellulase and laccase activities have been detected in other studies (Joneson, 2011; Mackay *et al*., 2015). The presence of cellulase would furthermore cause rapid algal cell death in the absence of glucose. Cell wall remodelling during co-culture (Hom and Murry, 2014), as seen by reduces size of cell wall and surrounding cell sheath (Muradov *et al*., 2015), is a more likely cause for carbon transfer. A transcriptomic study of model lichen showed specific up-regulation and down-regulation of different metabolic pathways, specifically, lipid and carbohydrate metabolism, in both algae and fungi during different stages of lichen development (Joneson, 2011). Changes in carbohydrate metabolism did not include cellulase activity. Although many fungi possess cellulolytic machinery, it is more likely that the transfer of carbon occurs

directly in the shared extracellular matrix between the algae and fungi. The extracellular matrix and cell sheaths surrounding the algal cell walls consist of many carbohydrate compounds such as sucrose (Watenabe *et al*., 2006) and lipids released during cell division (Joneson, 2011). All previous reports have indicated that algal-fungal pellets remain stable after formation without supplementary carbon.

Understanding the algae-fungi mechanisms of recognition and interaction may result in the development of more efficient strains for harvesting, either by selection through broader screening, or genetic modification. Certain bacteria have been shown to improve bioflocculation, remediate wastewater and produce algal growth promoting substances (Watenabe *et al*., 2005). Some wastewaters are not viable for algal growth, containing inhibitory compounds such as propionate, a common component of wastewater, which inhibits the growth of *C. sorokiniana.* Co-culture with specific bacteria capable of metabolizing inhibitory compounds may be a solution (Imase *et al*., 2008). Microbial consortia consisting of synthetically arranged organisms including fungi, algae and bacteria may be useful in future, tailored for specific conditions (Mee *et al*., 2012).

2.4 Hydrothermal conversion of algae feedstock

Hydrothermal processing of wet feedstock such as algae slurry bypasses time and energy intensive drying steps after harvesting (Elliot *et al*., 2011). High

temperature pyrolysis of biomass takes place within an ionic aqueous environment at high pressure. Hydrothermal processing (reviewed in Elliot *et al*., 2011 and 2015) is sub-categorized according to operating conditions: (1) Hydrothermal carbonization takes place below 520 K and produces hydro-char (a composite of carbohydrate and protein) and a lipid fraction which is recovered and processed to bio-oil. (2) Hydrothermal liquefaction (HTL) occurs between 520 and 647 K and produces liquid bio-crude. (3) Hydrothermal gasification (CHG) takes place above 647 K and requires a catalyst to produce fuel gas. The chemical processes and products for microalgae of each conversion method are reviewed in López *et al*. (2013).

Direct pyrolysis (hydrothermal carbonization) requires only moderate temperatures to convert lipid rich biomass to bio-oil. HTL produces bio-crude which differs in composition from bio-oils. Bio-crude has a dense viscosity and is relatively deoxygenated, containing a higher energy density. Bio-oil and bio-crude require purification by catalytic hydro-treatment prior to refinement into petrochemicals (Elliot, 2007; Jones *et al*., 2014).

HTL is not dependent on a specific catalyst, however, the presence of alkali (Na_2CO_3) in the aqueous medium helps to drive base-catalysed condensation reactions which favour aromatic oil formation (reviewed in López *et al*., 2013). Only CHG requires a specific catalyst, such as Ru/C (Haiduc *et al*., 2009), to drive efficient conversion. The major advantage of CHG is greater conversion efficiency (Brown *et al*., 2010).

2.4.1 Direct pyrolysis vs. conventional transesterification

Direct pyrolysis and transesterification technologies are favourable for feedstock high in lipid content. Biomass generated from algae-fungi co-cultures have only been processed to fuels using direct pyrolysis (Muradov *et al*., 2015, Al-Hothay *et al*., 2015). No significant differences were observed in the production of bio-oil, bio-gas and bio-char between algae-fungi pellets and the respective mono-cultures (Muradov *et al*., 2015). Pyrolyisis converted approximately 50-55% of algae-fungi biomass to bio-oil, 6-9% to biogas and 30-38% to solids. The bio-oil component contained various low molecular weight aromatic carbon and nitrogen compounds which could be removed by hydrodesulfurization and hydrodenitrification steps. Long chain saturated alkanes (dodecane through to octadecanes), including monounsaturated alkanes in the C14-C20 range, have also been recovered. Bio-oil produced from algae monocultures contained longer straight chain alkanes C13-C17 relative to bio-oil from fungal biomass. Bio-solids obtain after pyrolysis consisted of bio-char and mineral ashes. Bio-char can be used as agricultural fertilizer or for the production of activated carbon (Azargohar *et al*., 2006).

The lipid yield by conventional extraction of algal biomass for transesterification varies depending on algal species, the extraction method (Ranjith Kumar *et al*., 2015) and organic solvents used (Li *et al*., 2014). Conventional extraction requires dry biomass, mechanical or chemical cell disruption and expensive solvents, associated with high chemical and energy demands (Elliot *et al*., 2014).

Other costs include separation of contaminating inconvertible lipids and chlorophyll from the neutral lipids.

2.4.2 Hydrothermal liquefaction

Hydrothermal liquefaction (HTL) is capable of processing most types of solid and liquid biomass feedstock as it is suited for processing under aqueous conditions. Microalgal biomass are ideal for continuous liquefaction due to small size of biomass particles and low cell density (Elliot *et al*., 2014). HTL of biomass to bio-crude has higher conversion efficiency relative to pyrolysis since lipids, carbohydrates and proteins are processed. An aqueous phase rich in inorganic salts, nitrogen and phosphates may be recovered and reused for algal cultivation. The remaining carbon in the aqueous material can be further converted by catalytic hydrothermal gasification to methane to increase total process efficiency. Different HTL setups are reviewed by Elliot *et al*., (2014). Yields are highly dependent on loading quantities, biomass compositions and operating temperatures (Jazrawi *et al*. 2013; Elliott *et al*., 2013b).

2.4.3 Catalytic hydrothermal gasification

Catalytic hydrothermal gasification (CHG) of algae biomass slurry can be operated as a single step (Haiduc *et al*., 2009) or coupled to HTL (Elliott *et al*., 2013c). Typical valuable products of CHG include, methane, CO_2 and H_2 (Haiduc *et al*., 2009). The methane component may be used for the production of syngas, while CO_2 and H_2 may be recycled for use in the algae cultivation phase and

hydro-treatment process, respectively. CHG requires a single conversion step and can recover 68-74% of the processed carbon as biogas (Haiduc *et al*., 2009).

Coupled to HTL, CHG yields both bio-crude and gas products (Elliott *et al*., 2013c). CHG-HTL has been shown to have significant advantages as assessed by the National Alliance for Advanced Biofuels and Bio-products (NAABB synopsis report, 2014). Up to 85% of the carbon in algae biomass is captured in bio-crude and biogas which may respectively be converted to liquid fuels (diesel and gasoline) and syngas. The NAABB reported an estimated cost reduction of 86 % which has been contributed to the bypassing of conventional solvent extraction of lipids. The HTL aqueous phase may furthermore contain significant amounts of organic carbon, which can be directly recovered and converted by hydrothermal gasification, increasing the efficiency of the entire process (Krochta *et al*., 1984). The aqueous phase also contains the mineral component of the biomass which can be separated by precipitation and filtration (Elliott *et al*., 2012) or by a salt separator (Haiduc *et al*., 2009) as residual salts will poison expensive CHG catalysts.

2.4.4 Recycling of the aqueous phase of HTL

Nitrogen, phosphates and the mineral ash, consisting of the trace metals from the cultivation of the biomass, can be recovered from the aqueous phase of HTL and recycled (Biller *et al*., 2012; Garcia Alba *et al*., 2013; Jena *et al*., 2011; Bagnoud *et al*., 2015). Residual organic carbon are present in the aqueous phase

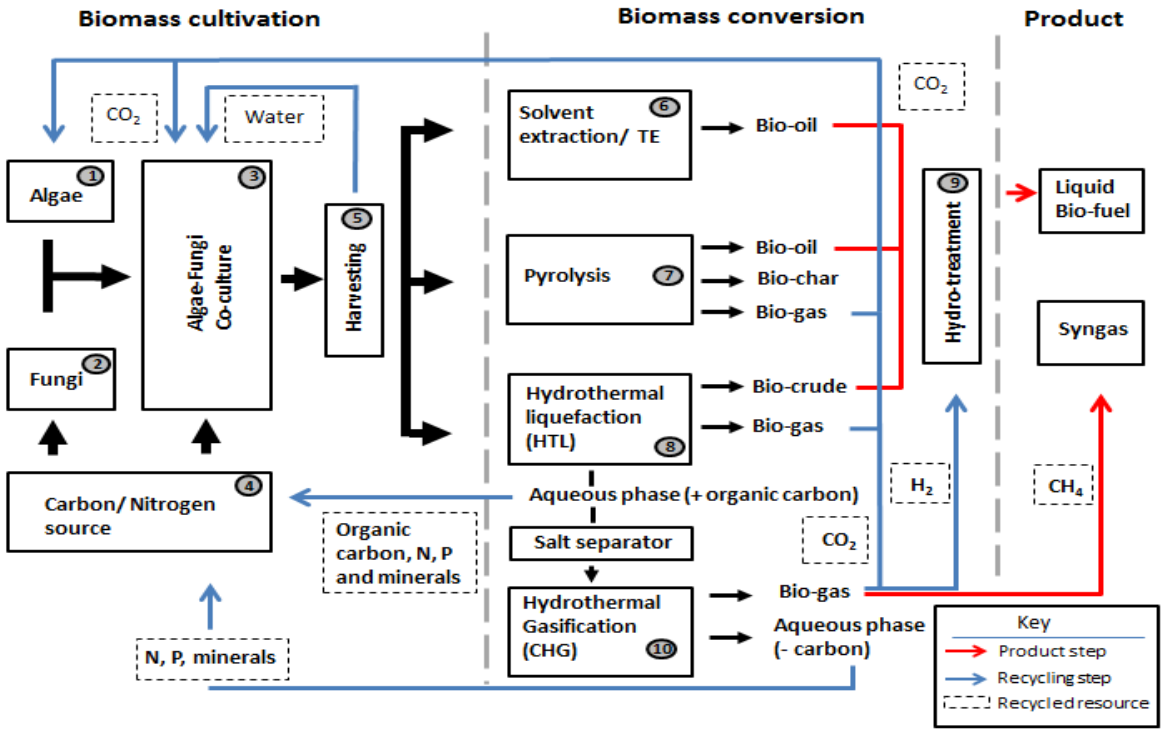


Fig 2.2: Map of the processing of algae-fungi biomass from cultivation to conversion with the respective products and by-products.

Box 2.1. Map of the processing of algae-fungi biomass from cultivation to conversion with the respective products and by-products.

Cultivation phase

Algae- fungi biomass can be cultivated by various strategies (Fig 2.1 a-c). The strategy used would dictate the limitations of the cultivation phase from points 1 to 4.

1) Algae biomass is cultivated at a large scale, either in PBRs or open ponds. The time frame of cultivation would depend on the facility (~10-21 days).

2) Fungal inoculum is cultivated in a separate reactor on-site. The addition of the inoculum depends on the strategy that is followed, either as a spore-clump pre-culture or as mature pellet biomass. Preparation of the fungal inoculum requires approximately 3 days.

3) Co-cultivation of the algae-fungi biomass. If mature pellets are added to the culture, co-culture pellet formation would take 12-48 hours depending on quantity of fungal biomass inoculated. If spore clump pre-cultures are inoculated, the co-culturing phase could take 48-72 hours if inoculated prior to the date of harvesting. Lower quantities of inoculums increase the flocculation times required but do not impact harvesting efficiency.

4) Recovered nutrients from hydrothermal conversion would be applied to algae and fungi cultivation stages. The carbon and nitrogen from the aqueous phase could be applied as supplementary nutrients to the algae pre-culture or to the fungi. Additionally, bioremediation of agricultural or urban waste waters could be used as a nutrient source.

5) The harvesting of the biomass could be performed relatively simply through various methods. As the algae-fungi co-cultures are dense, complete sedimentation is very rapid (~30 cm/ min). Alternatively, low energy screening mechanisms or filtration (0.5-1mm pore size) could be applied. Centrifugation would be unnecessary. As the biomass is wet, it is ideal for hydrothermal conversion, as it supplements the water required for the process.

Conversion phase

Biomass can be converted to biofuel pre-cursors through different processes: conventional solvent extraction, transesterification, direct pyrolysis, HTL or CHG.

6) Solvent extractions and TE are the conventional methods used. Solvent extraction is followed by transesterification. Yields of this processing option are governed by the lipid content of the biomass. As the lipid content of fungi is lower than for algae, the co-culture would have a lower lipid yield g^{-1} biomass.

7) Pyrolysis, or hydrothermal carbonization, operates at temperatures below 520K, producing solid bio-char, bio-gas and bio-oil. The marginal gas component (CO_2) can be fed back into the cultivation stages of subsequent cycles. The inconvertible bio-char consists of the protein and carbohydrate fraction. The bio-oil is extracted which can be further processed.

8) Hydrothermal liquefaction operates at 520-647 K and produces a bio-gas phase, liquid bio-crude and an aqueous phase containing low MW soluble organic carbon and inorganic nitrogen, phosphates and metal salts. The bio-gas (CO_2) can be recycled back in to the subsequent cultivation cycle while the viscous bio-crude is converted to biofuel by catalytic hydro-treatment. The aqueous phase can be fed back into the subsequent cultivation step as a nutrient source.

9) Hydro treatment is a catalyst based system which removes remaining nitrogen, sulphur and oxygen to form liquid biofuel.

10) Hydrothermal gasification is a catalyst dependent process which operates above 647 K. Hydrothermal gasification can operate as a subsequent step to HTL, or as a single step in the conversion of biomass to methane. The aqueous phase would need to be processed through a salt separator as some metals cause damage to the catalyst. The organic fraction is converted to bio-gas consisting mainly of methane which can be converted to syngas, CO_2 which can be recycled into the cultivation steps, and H_2 which can be recycled into the catalytic hydro-treatment process. The inorganic fraction contains reusable inorganic N, PO_4^{3-} and macro- and micro nutrients.

but at low concentrations (<1%) (Peterson *et al*., 2008; Matsumura *et al*., 2005). Nutrients such as nitrogen and CO_2 may be recovered and re-used for algae or fungal cultivation (Bagnoud *et al*., 2015; Mackay *et al*., 2015). Re-use of the aqueous phase necessitates dilution (200-400 fold) since the accumulation of inhibitors such as phenols and nickel may inhibit growth (Jena *et al*., 2011; Bagnoud *et al*., 2015).

Recycling can significantly reduce culturing costs, as the aqueous phase may provide up to 50% of the nitrogen demand (Cherad *et al*., 2013). However, the misbalance of metals may cause underperformance of subsequent cultures and a nutrient supplementation is recommended (Garcia Alba *et al*., 2013; Bagnoud *et al*., 2015). An overview of the algae biomass to biofuel process and the resources which can be recovered is depicted in Fig. 2.2 and Box 2.1.

2.5 Economic viability and the impact of biomass on down-stream processing

The success and viability of hydrothermal conversion of biomass is dependent on post-harvesting operational costs, pre-treatment of biomass, conversion efficiencies and wastewater recycling. Harvesting of microalgae has however been identified as the major bottleneck in the algae-to-biofuel process chain, hampering economic viability of biofuel production. The use of fungal biomass as a bioflocculant is a promising alternative to current harvesting technologies. The induction of auto-flocculation by the addition of chemicals such as $Mg(OH)_2$ and

$Ca(OH)_2$, which raise the pH and drive aggregation, is probably the most comparable method to fungal bioflocculation with respect to efficiency and infrastructure costs.

The cost of pH-induced auto-flocculation with $Ca(OH)_2$ (slaked lime) for low density cultures has been estimated at ~\$7.50 ton^{-1} algae flocculated. Due to the non-linear nature of auto-flocculation, cost of harvesting high density cultures (*Nannochloropsis*) may be as low as \$3.50 ton^{-1} (Schleiser *et al*., 2012). The use of chemical flocculants presents several disadvantages. The pH of the culture media requires neutralization and the size of the flocs are relatively small (<100 µm). Considerable quantities of the chemical flocculant is used, e.g. ~12 mg $Ca(OH)_2$ per g algae DW at pH 10.8 (Vandamme *et al*., 2012). Down-stream desalting steps are required since residual metal salts preclude the direct re-use of culture media. Metal alkali salts have a high affinity for algal organic fractions and are not easily removed during salt separation (Bagnoud *et al*., 2013). Residual alkali salts may poison catalysts during hydrothermal conversion at supercritical conditions (Waldner *et al*., 2007) or suppress methanation reactions (Gadhe and Gupta, 2005).

Biomass generated with fungal bioflocculation is more suited to hydrothermal conversion as it contains no harmful alkali salts. Bioflocculation with fungi however necessitates an on-site bio-reactor to prepare fungal pre-cultures. Compared to chemical flocculation, fungal bioflocculation is only limited by the cost of culture media. Culturing of fungi on minimal glucose or sucrose has been

estimated to cost $64 or $50 tonne $^{-1}$ algae harvested, respectively (Mackay *et al*., 2015). Filamentous fungi studies Algae-fungi co-cultures form 1-2 mm pellets, 10-20 fold larger than chemical flocs (20-100 µm), which can be harvested by simple filtration. Co-culture significantly impacts biomass quality with respect to protein, lipid and carbohydrate composition. High concentrations of proteins can result in accumulation of unwanted chemical by-products and catalyst poisons, such as phenol (Jena *et al*., 2011) and sulphates (Haiduc *et al*., 2009). No significant changes in composition were reported in algae-fungi biomass, in comparison to algae pure culture, when biomass was cultured on autotrophic medium (Mackay *et al*., 2015). Significant differences were observed in mixotrophic cultures, in particular with respect to lipid content (Muradov *et al*., 2015). The percentage fungus in harvested biomass is dependent on the carbon source and algae cultivation strategy. Autotrophic cultivation yielded biomass pellets with almost equal proportions (m:m) of fungi and algae (Mackay *et al*., 2015), whereas the fungal component increased under mixotrophic culture to more than 70% (Gultom *et al*., 2014).

2.6 Conclusions

Majority of costs in the algae-to-biofuel process chain are contributed by two key processes, biomass harvesting and conversion to fuels. Fungal bioflocculation significantly decreases costs of microalgal harvesting, while biomass is better suited for hydrothermal conversion. By integrating fungal bioflocculation with hydrothermal conversion, such as HTL and CHG, overall process efficiency can be

increased. Further reduction of costs is possible by recycling nutrients from the hydrothermal conversion processes for biomass cultivation steps, driving the system towards sustainability.

2.7 Acknowledgements

This work was supported by the South African National Research Foundation.

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Chapter 3:

Harvesting of *Chlorella sorokiniana* by co-culture with the filamentous fungus *Isaria fumosorosea*: A potential sustainable feedstock for hydrothermal gasification

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Published in Bioresource Technology as:

Mackay, S., Gomes, E.P., Holliger, C., Bauer, R., Schwitzguébel, J-P. (2015), Harvesting of Chlorella sorokiniana by co-culture with the filamentous fungus Isaria fumosorosea: A potential sustainable feedstock for hydrothermal gasification. *Bioresource technology,* 185: 353–361.

Abstract

Despite recent advances in down-stream processing, production of microalgae remains substantially limited by operational costs. Harvesting and dewatering are the most energy-intensive processing steps in their production and contribute 20-30% of total operational cost. Bioflocculation of microalgae by co-cultivation with filamentous fungi relies on the development of large structures that facilitate cost effective harvesting. A yet unknown filamentous fungus suitable for this purpose was isolated and identified as *Isaria fumosorosea*.

Blastospores production was optimized in minimal medium and the development of pellets, possibly lichens, was followed when co-cultured with *Chlorella sorokiniana* under strict autotrophic conditions. Stable pellets (1-2 mm) formed rapidly at pH 7-8, clearing the medium of free algal cells. Biomass was harvested with large inexpensive filters, generating wet slurry suitable for hydrothermal gasification. Nutrient rich brine from the aqueous phase of hydrothermal gasification supported growth of the fungus and may increase the process sustainability.

Keywords: Microalgae, Filamentous fungi, Lichen, Harvesting, Flocculation

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Highlights:

- A new strain of *Isaria fumosorosea* growing with algae was isolated and characterized.
- The fungus was co-cultivated with *Chlorella sorokiniana* as lichen for easy harvesting.
- This approach has the potential to reduce operational cost of microalgae production.
- Co-culture of microalgae and fungi should improve the sustainability of the process.

3.1 Introduction

Large industrial scale cultivation of lipid-rich microalgae could potentially reduce our dependence on fossil fuels. However, high energy demand and associated costs of several key areas within the cultivation, harvesting and down-stream processing of algae biomass need to be addressed to achieve a sustainable process. Harvesting and dewatering of algal biomass can contribute between 20-30% of the operational costs (Molina Grima *et al*., 2003). Methods include centrifugation, filtration and flocculation with various charged polymers. Advantages and disadvantages of different methods with regard to energy and processing costs have been recently reviewed (Leite *et al*., 2013; Coons *et al*., 2014). Centrifugation is time, labour and energy intensive and therefore only viable for high value products. Chemical flocculation is a more cost effective option for low value feedstocks. However, the addition of chemicals can have detrimental impact on the quality of biomass and recovered water and nutrients, necessitating further down-stream operational costs. Non-toxic biopolymer flocculants, such as chitosan, are relatively costly as they require chemical preparation.

Microalgal biomass can be converted into energy through several mechanisms, producing biodiesel, bioethanol or methane. Traditionally, fatty acids are extracted from lipid rich microalgae and converted to biodiesel through transesterification. This system remains relatively unsustainable due to costs involved (Hu *et al*., 2008). Hydrothermal gasification (HTG) has potential as a

sustainable option for the generation of methane or hydrogen. It can be operated as a closed loop system capable of recycling by-products and nutrients (Haiduc *et al*., 2009; Bagnoud-Velasquez *et al*., 2015). Recent advances in catalyst-based HTG allow for superior thermal efficiency (>70%) relative to microbial anaerobic digestion (25-35%). Major advantages of HTG are rapid reaction time and the capacity to process w*et al*gal slurry (15% dry mass), avoiding an energy and time consuming drying step (Vogel, 2009).

Co-culture of microalgae, mostly *Chlorella vulgaris* strains, with filamentous fungi to achieve assisted bioflocculation has been recently reported (Zhang and Hu, 2012; Zhou *et al*., 2012; Xie *et al*., 2013; Zhou *et al*., 2013) and reviewed by Gultom and Hu (2013). Large structures (1-5mm) are formed upon the addition of fungal spores and a carbon supplement to microalgae cultures. The germinating filamentous fungi have been shown to capture free microalgal cells in suspension in acidic to neutral pH conditions.

The present work is part of the SunCHem project aiming to develop an integrated process for the hydrothermal production of methane from microalgae (Haiduc *et al*., 2009; Bagnoud-Velasquez *et al*., 2015). It reports on the isolation and characterization of a yet unknown filamentous fungus and its association when co-cultured with *Chlorella sorokiniana.* As the microalgae are cultivated under strict autotrophic conditions and the fungi rely on the microalgae to grow without any addition of external sugar, then the algal-fungal pellet functioned as lichen, in which both partners co-existed in a symbiotic structure (Zhou *et al*.,

2012; Gultom and Hu, 2013). Co-cultivation and the rate of clarification in photobioreactors (PBRs) were thus investigated under autotrophic conditions and tightly regulated pH values optimal for industrial applications and hydrothermal gasification. Biomass quality was characterized with respect to biochemical composition and filtration efficiency. The potential for cost effective fungal blastospores production was furthermore investigated using minimal medium and wastewater recycled from the aqueous phase of HTG that may allow for sustainable cultivation, harvesting and recycling of nutrients within a single system.

3.2 Materials and Methods

3.2.1 Cultivation media

3.2.1.1 Microalgae cultivation medium

Modified Bold's Basal Medium (MBBM): Bold's recipe for green algae cultivation (Bischoff and Bold, 1963) was modified to include 0.47 mM NH_4Cl, 0.17 mM $CaCl_2$, 0.3 mM $MgSO_4$, 0.43 mM K_2HPO4, 1.29 mM KH_2PO_4, 0.43 mM NaCl and 6 ml/L trace elements from a stock solution (per L) of 0.75 mg Na_2EDTA, 97 mg $FeCl_3.6H_2O$, 41 mg $MnCl_2.4H_2O$, 5.0 mg $ZnCl_2.6H_2O$, 2.0 mg $CoCl_2.6H_2O$ and 4.0 mg $Na_2Mo_4.2H_2O$.

3.2.1.2 Fungal cultivation media

Sabouraud (SB) medium: 200 mM glucose, 10 g/L peptone, pH 6.0; G10N: MBBM supplemented with 10 mM glucose, pH 6.0; A10N: MBBM supplemented with 10 mM acetate, pH 6.0.

3.2.2 Isolation and identification of the mycobiont

A pellet-forming community was isolated as a contaminant from a laboratory microalgal culture. The mycobiont was cultured axenically on SB solid medium with the addition of chloramphenicol (100 µg/ml), ampicillin (100 µg/ml), and kanamycin (50 µg/ml). DNA was isolated from mycelia using a modified cetyl trimethylammonium bromide (CTAB) extraction method (Zhou *et al*., 1996). A sample of 6 mg freshly grown mycelium was incubated at 37°C for 30 min in an extraction buffer (100 mM TRIS, pH 8.0, 100 mM EDTA, 100 mM Na_2HPO_4, 1.5 M NaCl, 1% CTAB, 20 mg/ml Proteinase K), followed by 2 hours at 65°C after the addition of 1.5% SDS. Genomic DNA was purified by two consecutive extractions in phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1) for 10 min at 13,000 x g. DNA was precipitated with 0.6 vol. isopropanol and centrifuged after 30 min at room temperature (RT). The DNA pellet was washed once with 70% ethanol, air dried, resuspended in 10 mM TRIS (pH 8.0). The full 18S rRNA gene (2323 bp) was amplified using universal fungal primers NS1 5’ - GTAGTCATATGCTTGTCTC -3’ and ITS4 5’-TCCTCCGCTTATTGATATGC-3’ (White *et al*., 1990) with KAPA Hi-Fi TAQ (Promega). Internal primers were used

to sequence the full length of the amplicon. Phylogenetic analysis was conducted using MEGA software v5.1 (Tamura *et al*., 2011).

3.2.3 Screening of microalgae for co-cultivation with the fungus

Scenedesmus vacuolatus SAG211-8B, Chlorella vulgaris CCAP 211/12 and Chlorella sorokiniana CCAP 211/8k were cultivated in MBBM with 34 µg/ml chloramphenicol. Starter cultures were prepared by growing cells to an OD_{600} of ~1.0, and diluted to OD_{600} 0.5 with a final glucose concentration of 10 mM. Blastospores were inoculated to a final concentration of ~$1x10^5$/L. The pH and pellet formation were monitored over a period of 3 days.

3.2.4 Photo bioreactor cultivation

Small scale continuous stirred tank photobioreactors (PBRs) (Applicon Biotechnology, Netherlands) were used to grow *C. sorokiniana* CCAP 211/8k in pure culture and in co-culture with the mycobiont, *Isaria fumosorosea* gr. IHEM 26293. The batch culture medium consisted of MBBM supplemented with 0.1 ml/L polypropylene glycol as an antifoam agent and 34 µg/ml chloramphenicol. A light source (3 x 10 LEDs strips; 24 V LedFlex 07 High Performance, Barthelme, Nürnberg, Germany) provided a PAR value of 145 µmol photons $m^{-2}s^{-1}$, measured in the centre of the reactor when filled with medium. Air was supplemented by sparging at 1L air L^{-1} min^{-1} (Lambda mass flow meter). The PBR was stirred with two radial stirrers on a single rod at 75 rpm (Stirr controller P100 AbI 1032 Applikon Biotechnology, Netherlands). An automated pH relay system (Endress &

Hauser) regulated the pH within a narrow range by the addition of 1M NaOH and 1M HCl (Masterflex pump drive). All PBR experiments were carried out at 22°C and pH was strictly regulated at 6.0, 7.0 or 8.0. Strict autotrophic growth was supported by supplying CO_2 to cultures grown at all three pH conditions. Mixotrophic growth was only evaluated at pH 6.0 by the addition of 10 mM acetate.

Starter cultures of *C. sorokiniana* CCAP 211/8k were prepared in duplicate in 500 ml Erlenmeyer flasks containing 200 ml MBBM supplemented with 5 mM acetate and cultured for three days until late exponential phase. Monocultures of *C. sorokiniana* were prepared by transferring two 200 ml starter cultures to sterile PBRs filled to a final volume of 2 L with MBBM (OD_{660} = 0.4-0.6) (Biospectrometer, Eppendorf). Fungal starter cultures were prepared by inoculating 1 ml blastospores (~$1x10^6$) into 100 ml G10N followed by incubation at 25°C at 125 rpm to yield a cloudy mixture of small germinating pellet clumps and blastospores after three days. PBR co-cultures were initiated by 30 ml (15 mg DW/L) of fungal clump/spore culture per 2L medium (a final blastospores concentration of ~$4x10^6$/ml).

3.2.5 Characterization of the biomass

3.2.5.1 Evaluation of pellet development

PBR mono- and co-cultures were evaluated over a period of 72 h and sampled at 24 h intervals. Growth rate and clarification were measured as OD_{660} by

spectrophotometry (Biospectrometer, Eppendorf). Culture OD_{660} was correlated against cell count (R^2= 0.974) and dry weight (R^2 = 0.987) by standard curve. Pellets were photographed using a Nikon Eclipse E800 microscope and Nikon DS-2M CCD camera, linked with the DS-U2 connection, and analysed with NIS Element BR v3.0 software.

Culture supernatants were filtered by 0.22 µm syringe (Millipore) and analysed by HPLC to screen for organic acids and sugars released by algal mono- and co-cultures. Extracellular organic carbon (EOC) was screened by refractive index using an Aminex® HPX-87H column (#125-0140). The injection volume was set to 20 µl, 0.005 M H_2SO_4 was used as the mobile phase at a 0.6 ml min^{-1} flow rate, and the temperature of the column and mobile phase was kept constant at 65°C.

3.2.5.2 Evaluation of biomass composition and yield

After 72 h of cultivation, each 2L PBR culture was vigorously mixed and divided into two fractions. Biomass from a 1L fraction was collected by centrifugation (20 min at 16,000 x g at 4°C) for composition analysis. Pellets were washed twice with ddH_2O and lyophilized. The remainder was used for dry weight analysis and filtration studies. Aliquots were dispensed to equal volumes of 50 ml and passed through three copper mesh filters (63, 125 and 250 µm) in triplicate by vacuum filtration at 5 PSI for 10 seconds. Biomass was separated into filtered and filtrate fractions, dispensed into glass HPLC receptacles and dried for 3 days at 105°C. In control experiments, aliquots were not passed through the filters and were used

as reference for biomass determination. Controls were used to calculate the total biomass (g/L DW) of the reactor. The dry weight of the filtrate was expressed as a percentage relative to the unfiltered control.

3.2.5.3 Chlorophyll composition

Chlorophyll was extracted by dissolving 2 mg of lyophilized biomass in 1.9 ml methanol. Samples were incubated for 24 h at room temperature on a horizontal shaker. Chlorophyll content was measured by spectrophotometry (A_{665}) and expressed as µg chlorophyll per mg DW (MacKinney, 1941).

3.2.5.4 Protein, lipid and carbohydrate determination

Total protein was extracted from 5 mg lyophilized biomass, freeze fractured by grinding in liquid nitrogen, suspended in 2 ml 0.5 M NaOH, and incubated for 2 h at 80^{o}C (Rausch, 1981). Total protein was determined using the Bio-Rad DC Protein assay.

Carbohydrate content was analysed using a modified version of the phenol-sulphuric acid assay by Masuko *et al*. (2005). Lyophilized biomass samples (2 mg) were resuspended in 280 µl H_2O and a mixture of 600 µl concentrated H_2SO_4 and 120 µl phenol was added. The sample was incubated for 5 min at 90^{o}C, cooled to room temperature and turbidity measured at OD_{490} nm. Glucose was used as standard.

Lipids were extracted according to Laurens *et al*. (2012) specified for microalgae. Samples of 5 mg lyophilized biomass were extracted with C13:0Me as the recovery standard and C19:0Me as internal standard. The fatty acid profile and relative percentage composition was determined using FAME and BAME libraries on a Thermo Scientific Trace 13 10 GC-MS with ISQ MS ZB-5MS column.

3.2.6 Characterization of the mycobiont

3.2.6.1 Growth on defined media

While maintained in ½ SB medium, blastospores inoculum was prepared in G10N medium. Blastospores were harvested by centrifugation (9,000 x g for 10 min, 4^{o}C) after 3-5 days of incubation, washed, and resuspended in ddH_2O and 0.02% Tween 80 prior to inoculation. Growth was initiated by ~1x10^5 blastospores per ml in 100 ml flasks and germinated under fluorescent light with constant shaking at 120 rpm. Total biomass and blastospore production of the mycobiont were evaluated in ½ SB, G10N and A10N. Blastospores were counted every 24 h under microscope using a Neubauer cell counter. The pellet biomass was washed 3 times in ddH_2O and dried at 105°C for 3 days. All experiments were performed twice with triplicate samples.

Growth of the fungal mycobiont was evaluated on a range of substrates and at different temperatures using a modified microtitre plate method described by Meletiadis *et al*. (2001). A volume of 100 µl blastospores (2x10^5/ml) was inoculated into each well of flat bottom plates (Sterilin) containing 100 µl of the

respective media. Growth was evaluated in the presence of chloramphenicol (34 µg/ml), ampicillin (100 µg/ml) and kanamycin (50 µg/ml) to eliminate bacterial contamination. Mycelial growth was measured as an increase in biomass at OD_{405} nm (Granade *et al*., 1985) in a flat bottom 96 well plates (Biotek reader). Growth kinetic assays were performed at 28°C and measured over 72 h at 12 h intervals without shaking. All plates were sealed with light cycler clear PCR sealing film pierced with a needle to allow air transfer and reduce condensation. Condensation was removed by placing the underside of a pre-warmed heating block on the surface of the film for a few seconds. All plate assays were repeated twice with 6-8 replicates per sample.

Growth of the mycobiont was also followed on several organic acids typically associated with the aqueous phase from hydrothermal wastewater (Ogbonna *et al*., 2000) namely acetate, butyrate, propionate, succinate and oxalate. Growth on sugars such as sucrose and glucose as well as glycerol was also evaluated. All substrates were added to 2xMBBM (pH 6.3) to a final concentration of 2 mM. A total volume of 100 µl was loaded per microtitre plate well, each containing 100 µl of the blastospore culture. A control well containing 100 µl ddH_2O with 100 µl of spore inoculum was included for each experiment to account for the influence of residual glucose from the spore culture. This value was subtracted with the blank from their respective time points.

3.2.6.2 *Growth on the aqueous phase of hydrothermal gasification*

Samples were obtained from three different aqueous phases collected after hydrothermal gasification (HTG) of biomass produced by the microalgae *Phaeodactylum tricornutum* (Bagnoud-Velasquez *et al*., 2015). Samples contained high concentrations of nitrate, ammonium and phosphate (Bagnoud-Velasquez *et al*., 2015), various organic acids, as well as phenol, ethanol and glycerol (Bagnoud-Velasquez, personal communication). Growth of the mycobiont on HTG samples diluted with water (1:19 and 1:79) was evaluated in microtitre plates.

3.2.6.3 *Growth on recycled cultivation medium*

Supernatants were collected by centrifugation from algae mono-cultures grown for 72 h in PBRs (pH 7 and 8). Samples were filter-sterilized, diluted 1:1 and used as a substrate for microtitre plate assays to determine if the mycobiont could be cultured on extracellular carbon released by the algae.

3.2.7 Statistical analysis

All statistical analysis was performed using Graph Prism 6 software. All experiments were repeated at least twice with 3-8 replicates to determine standard deviation. Significant differences between samples were calculated using ANOVA followed by Fischer's LSD tests. Values of $P < 0.05$ were deemed as significantly different.

3.3 Results and discussion

3.3.1 Isolation and phylogenetic analysis of the mycobiont

The mycobiont was isolated from a pellet-forming community that developed spontaneously in a *C. sorokiniana* culture. The isolate was identified by molecular characterization of a 2323 bp amplicon (supplementary data) encompassing the 18S, ITS1, 5.8S and ITS2 rRNA regions (Fig. 3.1).

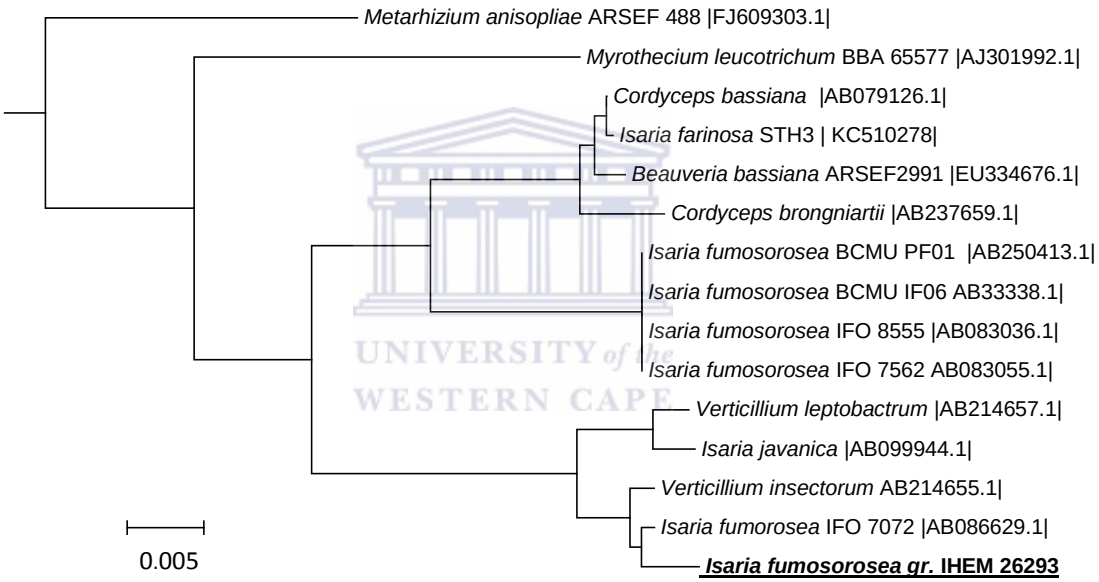

Figure 3.1: Phylogenetic analysis of the mycobiont isolate IHEM 26293. The full 18S rRNA gene sequence (2183 bp) included the 18S, ITS1, 5.8S and ITS2. The phylogenetic tree was constructed using the neighbour-joining method, rooted to *Metarhizium anisolia,* which showed close sequence similarity with several well characterized entomopathogens.

Phylogenetic analysis of the mycobiont showed high sequence identity to four *Verticillium* and *Isaria* isolates from plant root environments: 99.0% with *Isaria fumosorosea* IFO 7072 (formerly *Paeciliomyces fumosoroseus*), 99.0% with *Verticillium insectorum,* 98.0% with *V. leptobactrum* and 98% with *I. javanica*. The isolate was deposited at the BCCM and designated *Isaria fumosorosea* IHEM 26293 as it was more similar in sequence and morphological descriptions (blastospore, hyphae colour and pellet formation) to *I. fumosorosea*.

The genus *Isaria* is highly polyphyletic and difficult to distinguish from the anamorphic morphologies of *Verticillium* (Pacioni *et al*., 2007). Many *I. fumosorosea* strains are entomopathogens and have been well studied as an environmentally safe biological control of various insect pests (Zimmerman, 2008). The possible entomopathogenicity of the strain IHEM 26293 was not tested in the present study.

3.3.2 Microalgal strain specificity

The specificity of the isolated mycobiont for pellet formation was screened against three commercially important microalgae photobionts. The development of pellet structures was followed by monitoring free algae cell counts in growth medium, as well as the morphology of the pellets, over a period of 3 days and in the presence of glucose. The mycobiont (*I. fumosorosea* IHEM 26293) showed specificity only for *C. sorokiniana* CCAP 211/8k and formed a stable association. Immobilization of algae cells on fungal mycelium was not observed when co-

cultured with *S. vacuolatus SAG211-8B* or *C. vulgaris* CCAP 211/12 8k (Fig. B1, Appendix B).

3.3.3 Pelletization in photobioreactors

Pelletization was studied at pH values of importance to industrial applications. Since CO_2 solubility, and therefore algae growth, is optimal between pH 6 and 8, photobioreactors were used to strictly regulate pH and growth was followed over 72 h. The following experiments were thus carried out in PBR and initiated with a *C. sorokiniana CCAP* 211/8k inoculum corresponding to an OD_{660} of ~0.5 (0.138 g DW/L). For co-culture, blastospores were added to a final concentration of $5x10^4$ - $1x10^5$/ml.

Pellet development was monitored by measuring the density of free algal cells in the culture medium (OD_{660}), i.e. the growth rate for algal monocultures (Fig. 3.2a) and rate of clarification for co-cultures (Fig. 3.2b).

The growth rates of algae monocultures under strict autotrophic conditions at pH 6, 7 and 8 were comparable, and cell density increased steadily over a period of 72 h, reaching an OD_{660} of approximately 1.0 (Fig. 3.2a). The growth rate under mixotrophic conditions (pH 6, 10 mM acetate) was significantly higher, reaching an OD_{660} of approximately 2.0 within 48 h. When co-cultured with the mycobiont under autotrophic conditions (pH 6, 7 and 8), free algae cell density was not significantly affected over the first 24 h of cultivation (Fig. 3.2b). Over the next 24 h at pH 7 and 8, pellets captured most of the free algae from the culture

supernatant as seen by a drop in OD_{660} from 1.0 to almost 0. This was due to the immobilization of algae on the fungal mycelium and resulted in the formation of stable structures.

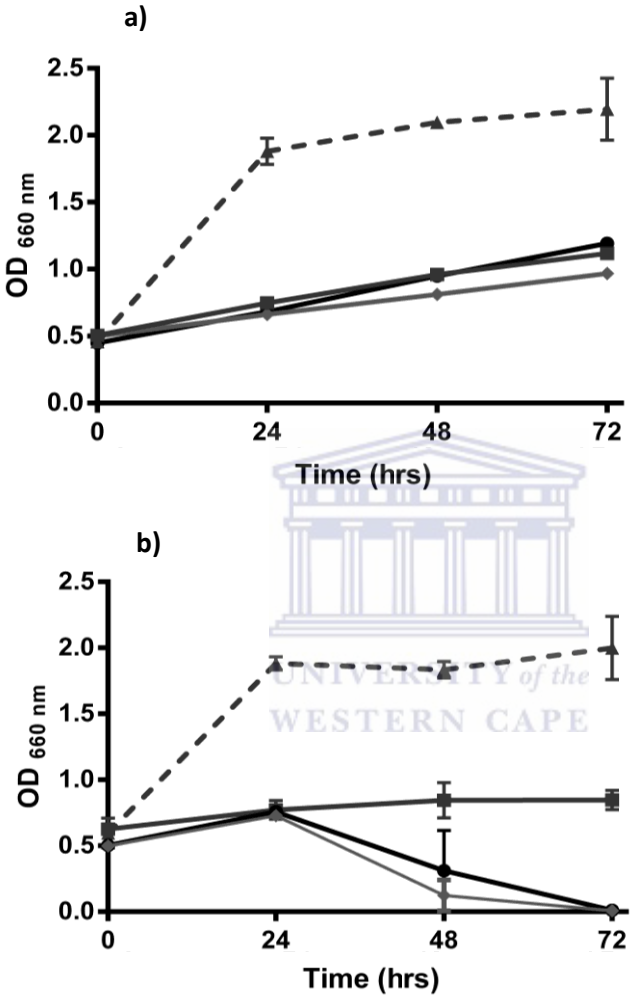

Figure 3.2: Lichen development and rate of clarification under different pH conditions. Free cells density of algae monocultures (a) and co-cultures (b) at pH 6 (▪), pH 7 (●), pH 8 (◆), and at pH 6 in a medium supplemented with 10mM acetate (▲). Values are presented as means ± SD of 2 independent experiments, each with 3-8 replicates. Two-way ANOVA followed by Fischer's LSD test were used to determine statistical significance ($P < 0.05$).

Both partners seemed to co-exist in a symbiotic structure, since cultivated under strict autotrophic conditions without the addition of any external organic carbon source. Pellets cultivated at pH 7 and 8 completely settled within 1 min when agitation was stopped (Fig B4, Appendix B).

In contrast, co-culture at pH 6 did not result in a reduction of cell density and was comparable to growth of monocultures. Similarly, mixotrophic cultivation at pH 6 (10 mM acetate) did not decrease the relative free cell density when compared to the monoculture equivalent. Pellet structures were thus not stable at pH 6 or below, even when growth was supplemented with acetate. In contrast, recent studies (Zhang and Hu, 2012; Xie *et al*., 2013; Zhou *et al*., 2013) have shown that pellet formation is limited to an acidic pH range and dependent on supplementation with glucose.

3.3.4 Biomass yield and recovery by filtration

Biomass of algal mono- and co-cultures was collected after 72 h of cultivation in PBRs. The total biomass of co-cultures was significantly more than the biomass produced by algae mono-cultures at the respective pH values, with a ~66% increase at both pH 7 and 8 (Fig. 3.3a), indicating a probable increase in CO_2 fixation and an efficient symbiotic relationship. At pH 6, the increase in biomass corresponded to 37% and 21% when cultured under autotrophic and mixotrophic conditions, respectively. The initial mycobiont inoculum (15 mg/L)

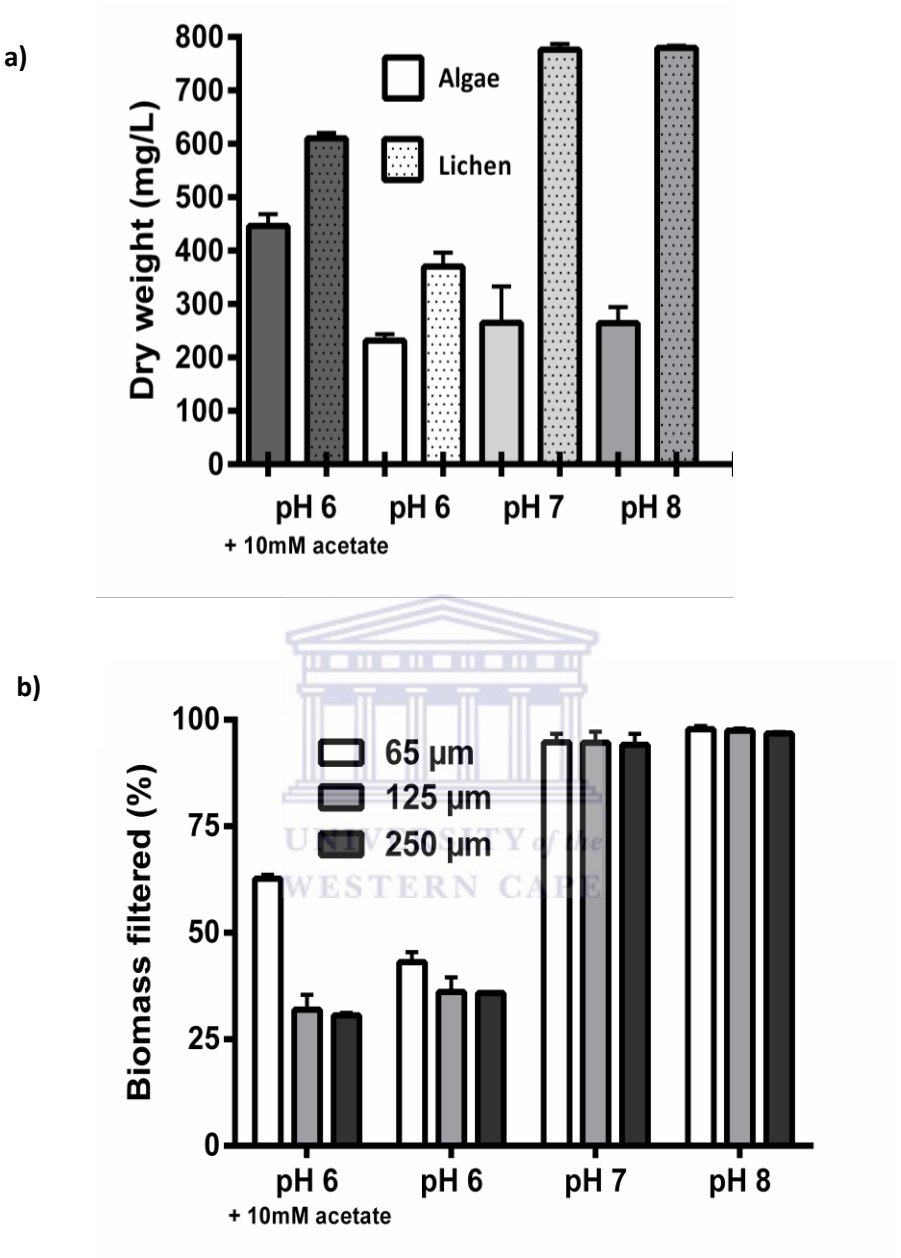

Figure 3.3: Biomass recovery by filtration. Cultures were grown for 72 h at different pH values and trophic conditions. a) Relative total biomass (mg/L DW) produced by algae monocultures and co-cultures. b) Recovered biomass after filtration using different filter pore sizes under constant pressure. Values are presented as means ± SD of 2 independent experiments, each with 3-8 replicates. Two-way ANOVA followed by Fischer's LSD test were used to determine statistical significance (P <0.05).

contributed to the equivalent of 10 % of the initial algae biomass (150 mg/L), and therefore did not account for the significant increase in biomass in co-cultures. An increase in total biomass production, probably due to a mutual stimulation of cell growth of both microalgae and filamentous fungi has already been reported, even if not fully understood (Zhang and Hu, 2012; Gultom and Hu, 2013; Xie *et al*., 2013).

Due to the small size of *C. sorokiniana* (1-10 µm), fine filtration systems are generally required for harvesting. The formation of pellets increased the size of the biomass to 1-2 mm in diameter, making easier the harvesting by filtration or sedimentation.

Large robust metal filters with pore sizes of 63, 125 and 250 µm were used to harvest pellets. Constant pressure was applied to negate capillary action. The filtration capacity was determined by the percentage of biomass that passed through the filters. Biomass retained by the filters was easily collected by rinsing. Under autotrophic conditions (pH 7 and 8), lichen biomass was harvested almost entirely (94-97%) by all three filters (Fig. 3b). Only partial filtration was achieved when co-cultures were grown at pH 6. Such a robust filtration process should reduce costs of harvesting as it is compatible with gravitational flow and/or low energy centrifugation.

3.3.5 Pellets morphology

Morphological differences in the macrostructures of pellets were scrutinized by microscopy after 72 h of cultivation. No significant morphological differences in lichen structures were observed when cultivated at pH 7 and 8 (Fig. B2, Appendix B). Lichen pellets were spherical and up to 2 mm in diameter. Microalgal cells were evenly dispersed within the pellets and decreased slightly in abundance towards the surface where newly formed mycelia were growing apically. The centres of the pellets remained compact, consisting of a network of hyphae and algal cells. Small arm-like structures protruded radially from the surface of the lichen pellets and increased in length with a decrease in pH of the growth medium. At pH 6, the arm-like structures contributed to approximately half of the diameter of the pellets. The centre remained dense with algal cells, while several arms were partially covered with algae. The initial mycobiont inoculum contained several small clump pellets (Fig. B2, Appendix B) which were significantly smaller than lichen pellets that developed after 72 h of growth. Algal cells formed strong attachments to the exposed hyphae rather than auto-flocculation with other algae. Lichen structures were stable and maintained their shape after mechanical agitation or centrifugation.

Chlorophyll was extracted and measured at OD_{665} and was proportional to the algae content of the pellet. At pH 7, pellets contained 44% of the chlorophyll relative to algal monocultures (Table 3.1). This would infer that the lichen pellets were of approximately equal composition of algae and fungi. The significant

mutual increase in total biomass of the algae-fungi co-culture and analysis of the chlorophyll content indicated that both organisms increased in total biomass suggesting a genuine mutualistic relationship existed.

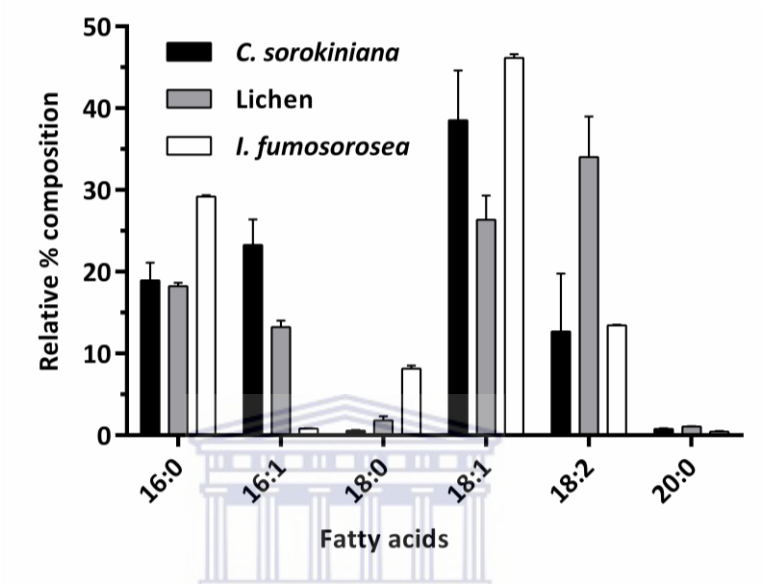

Figure 3.4: Relative fatty acid composition of the biomass produced by *C. sorokiniana, I fumosorosea* and the lichen co-culture. Values are presented as means ± SD of 2 independent experiments, each with 3-8 replicates. Two-way ANOVA followed by Fischer's LSD test were used to determine statistical significance (P <0.05).

3.3.6 Chemical composition of the biomass

The chemical composition of the biomass from algal mono- and co-cultures was evaluated to predict the impact on down-stream processing, such as recovery of useful compounds and hydrothermal gasification (HTG), with specific emphasis on total protein and carbohydrate content, as well as on relative fatty acid composition. The protein content did not vary significantly between algal

monocultures (57%) and co-cultures (56%). Carbohydrate content was not significantly greater in monocultures, comprising of 14.7% compared to 11.2% in lichen pellets (Table 3.1). In contrast, the lipid composition varied significantly between the respective algae, fungi and lichen cultures (Fig. 3.4). The fatty acid profile indicated shifting in the concentrations of several fatty acids, specifically an increase in linoleic acid (18:2) in lichen pellets.

Table 3.1: Biochemical characterization of the biomass (pH 7)

	C. sorokiniana		**Lichen**	
	µg/mg	SD	µg/mg	SD
Protein	570.90	± 51.99	559.58	± 12.64
Carbohydrate	147.94	± 10.23	112.34	± 13.58
Chlorophyll	16.59	± 1.51	7.32	± 0.56
Total DW (mg/L)	265.30	± 95.80	776.00	± 16.00

Values are presented as means ± SD of 2 independent experiments, each with 3-8 replicates. Two-way ANOVA followed by Fischer's LSD test were used to determine statistical significance (P <0.05).

3.3.7 Sustainable production of fungal spores

Cultivation of the mycobiont for blastospores production introduces additional processing steps and associated costs which may reduce sustainability. The effect of different culture conditions on blastospores production and viability was

evaluated, with the aim to reduce the economic and energetic inputs. For such a purpose, different alternative potential growth media were tested.

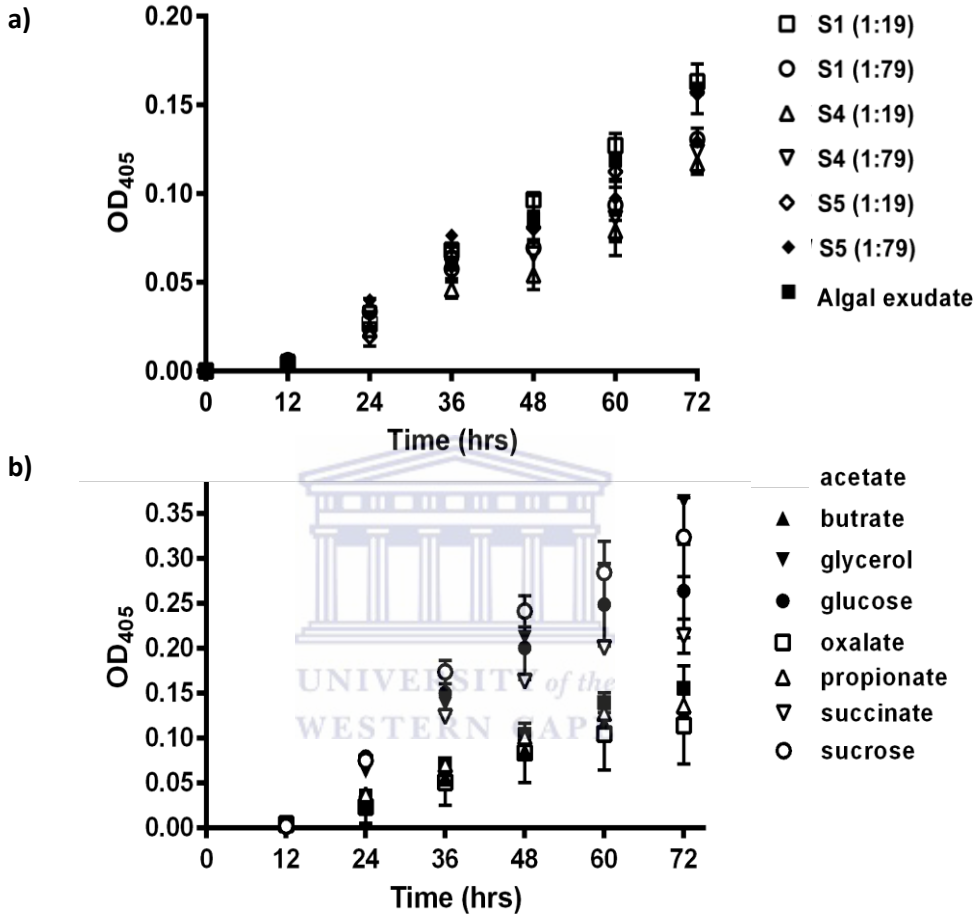

Figure 3.5: Relative growth of *I. fumosorosea* gr. IHEM 26293 on sugars, organic acids and waste water substrates. **a)** Growth on 1:19 and 1:79 dilutions of HTG aqueous phase and on recovered PBR algal culture medium; **b)** Growth on sugars, glycerol and organic acids, each given at 2 mM. Values are presented as means ± SD of 2 independent experiments, each with 3-8 replicates. Two-way ANOVA followed by Fischer's LSD test were used to determine statistical significance (P <0.05).

After 72 h of microalgae monocultures in PBR, cell-free culture supernatants were analysed by HPLC. Organic acids, mono and disaccharide sugars were not detected in the recovered PBR culture medium (data not shown). The mycobiont was however able to grow in the sterilized supernatant, reaching an OD_{405} of 0.15 over a period of 72 h (Fig. 3.5a).

New generations of blastospores (ungerminated) were detected after 72 h in lichen cultures grown at pH 7 and 8 at spores concentration of 5 ± 0.82 $x10^4$/ml and 4.91 ± 0.59 $x10^4$/ml, respectively. The re-appearance of blastospores in the PBR culture media indicated that sustainable co-cultivation should be possible over longer periods. No new blastospores were however generated at pH 6.

Hydrothermal gasification (HTG) of organic material such as algae produces a waste nutrient brine rich in minerals, nitrogen, phosphate (Bagnoud-Velesquez *et al*., 2015) and various organic compounds, without significant differences between fresh water or marine microalgae (Bagnoud-Velasquez, personal communication). The production capacity of both blastospores and mycelia, cultured on a range of alternative substrates associated with the nutrient brine in the HTG aqueous phase, were thus investigated. Samples (S1, S4 and S5) were recovered from the aqueous phase of three HTG reactions which consisted of organic acids (acetate, butyrate, propionate, tartrate, succinate, oxalate) and several other potential substrates like glycerol, ethanol and phenol, with high concentrations of nitrate, ammonium and phosphate (Bagnoud-Velasquez *et al*., 2015). Growth of the mycobiont was evident on dilute HTG samples, unidentified

algal extracellular organic carbon (Fig. 3.5a) as well as defined concentrations of organic acids as identified in the HTG nutrient brine (Fig. 3.5b). Although the reference carbon sources (glucose, sucrose and glycerol) sustained the highest growth rates and biomass yields, all tested organic acids supported growth of the mycobiont. Equivalent growth rates were noted on the 2 mM defined organic acids, i.e. acetate, propionate and butyrate (Fig. 3.5b), and the dilute HTG aqueous phase samples (Fig. 3.5a).

Growth of the mycobiont in G10N medium (pH 6.3) was optimal in the range of 25-30°C, while 32°C was lethal (Fig. 3.6a). Similar results have been reported for other *Isaria fumosorosea* strains (Zimmerman, 2008). Blastospores production and pellet biomass yield were monitored over a period of 7 days in ½ SB, G10N and A10N. Biomass yield of cultures grown in rich ½ SB media (2.95 g DW/L) was 5.2 and 8 fold, respectively, of yield in G10N (0.57 g DW/L) and A10N (0.39 g DW/L) (Fig. 3.6b). Blastospores production was measured concurrently in media supernatant (Fig. 3.6c). Germination occurred within 6-8 h, independent of media composition, and visible pellets began to form within 24 h. In contrast to the total biomass yield, subsequent generations of blastospores were produced more rapidly and at a greater concentration when cultivated in carbon limited G10N medium. New blastospores were formed between 48 and 72 h after inoculation in G10N medium compared to 72 to 96 h in ½ SB medium.

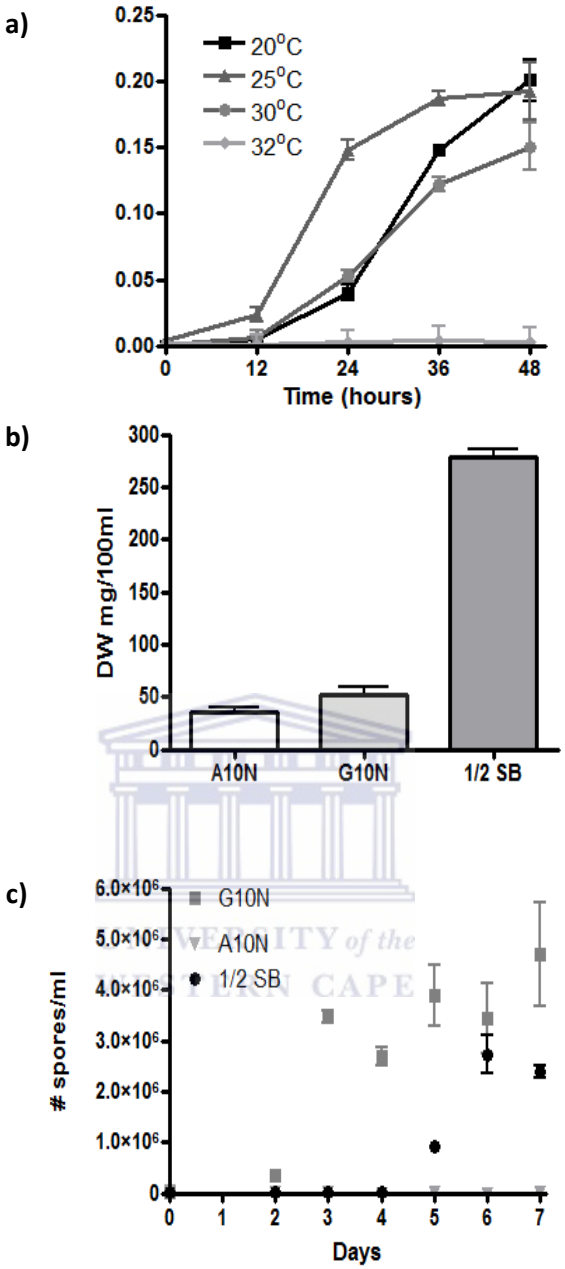

Figure 3.6. Optimal temperature, total biomass and blastospore production of the mycobiont in different media. **a)** Optimal temperature of the spore inoculum; **b)** total pellet biomass after 7 day period; **c)** blastospores production over 7 days. Values are presented as means ± SD of 2 independent experiments, each with 3-8 replicates. One-way ANOVA (b) and two-way ANOVA (a,c) followed by Fischer's LSD test were used to determine statistical significance (P <0.05).

The experiments indicated that a minimal medium (G10N) with low concentrations of glucose (10 mM) and limited nitrate was sufficient to generate viable pellet clumps and blastospores in suspension. Blastospores production in medium rich in glucose (100 mM) and peptone ($^1/_2$ SB) was significantly less, while no blastospores were produced in minimal medium supplemented with acetate (A10N). High blastospores production in G10N could be attributed to high C:N ratio and low nitrogen content. Glucose supplemented medium has been shown to significantly increase spore germination and viability, while nitrogen depletion increases blastospores yield (Jackson *et al*., 1997; Cliquet and Jackson, 2005). Optimal mass production and conditions for storage of *I. fumosorosea* blastospores have been established (Jackson *et al*., 1997).

Isaria fumosorosea IHEM 26293 blastospores germinated and aggregated at acidic-neutral pH to form small pellet clumps. Rich medium containing glucose and peptone produced spherical smooth pellets with a consistent uniformity. Minimal media with low concentration of glucose and ammonium resulted in smaller, less regular, clump-like pellets. In conclusion, blastospores production with minimal medium or wastewater supplemented with low concentrations of glucose should drastically reduce the cost of mycobiont cultivation.

3.3.8 *Surface interactions between algae and fungal hyphae*

Pellet development was optimal and highly stable at pH 7 and 8 but became less stable and immobilized fewer algal cells at pH 6. In contrast, previous studies

(Zhang and Hu, 2012; Xie *et al*., 2013; Zhou *et al*., 2013) have reported on pellets stability only under acidic growth conditions. Bioflocculation using chemical flocculants has been attributed to the interaction between the negative surface charges of algae at acidic pH and positively charged cations or polymers such as chitosan (Reviewed in Vandamme *et al*., 2013). Joneson (2011) has investigated gene expression during early lichen development. In the mycobiont, up-regulation of lipases, enzymes in carbon partitioning pathways, and hydrophobin-like proteins have been noted. Hydrophobins mediate extracellular interactions with the environment and mediate self and photobiont recognition and adhesion (Whiteford and Spanu, 2002). The most significant change in gene expression in the photobiont is the up-regulation of a chitinase-like protein, presumably a lectin involved in cell wall recognition (Joneson, 2011). Immobilization of algae to fungal cell surfaces may be due to differences in surface charges at acidic and strong alkaline pH. Stable binding above neutral pH, as observed in this study, was more likely driven by specific lectin-recognition and protein-protein interactions.

3.3.9 Carbon transfer in lichen structures

The mechanism of carbon transfer within lichen structures remains unclear. Degradation of algal cell wall components, specifically cellulose, by the mycobiont has been suggested as a mechanism for carbon transfer (Zhang and Hu, 2012; Xie *et al*., 2013; Zhou *et al*., 2013). However, cellulase and laccase activities were not detected when the mycobiont was screened on azure-blue

cellulose (Fig. B3, Appendix B). The *C. sorokiniana* sheath matrix is comprised of a mixture of sugars (mainly sucrose), sugar alcohols, proteins and divalent cations which can be hydrolyzed by associated symbionts (Watanabe *et al*., 2006). Cell-free culture supernatants collected from algal monocultures cultivated in PBRs were capable of supporting growth of the mycobiont (Fig. 3.5a), even though HPLC analysis did not detect simple sugars, organic acids or small sugar alcohols. Growth was likely supported by extracellular accumulation of algal derived polysaccharides and lipids outside the detection range of HPLC, opposed to sucrose leaking from the cell sheath matrix. Other studies have confirmed the accumulation of high concentrations of unspecified organic carbon within the culture medium of *C. sorokiniana* (Watanabe *et al*., 2005).

3.3.10 Economic viability of lichen as a microalgae harvesting tool

Organic polymers are favoured as bioflocculants as they can be easily processed down-stream without pre-treatment and are effective when used in combination with filtration. Chitosan, an organic cationic polymer derived from chitin, is an abundant sustainable resource (Renault *et al*., 2009). Chitosan flocculated *C. sorokiniana* with high efficiency (98%), but its use is restricted to acidic pH conditions. The relatively small size of chitosan-algae flocculants furthermore requires 8 µm pored filters relative to 250 µm used for lichen pellets. Bioflocculation with chitosan requires a low dosage of ~10 mg chitosan/g (DW) algae, at an estimated cost of ~$200 per metric tonne algae (DW) flocculated (Xu *et al*., 2013). Alternatively, chemical flocculants such as $Ca(OH)_2$ are fairly cost

effective. Flocculation with 12 mg/g DW is estimated at ~$18 per tonne algae (DW), but is only effective when applied at very alkaline conditions (pH 10.8) (Vandamme *et al*., 2012). The highly alkaline pH and associated ash content have a negative impact on the quality of the biomass and recycled nutrients, requiring additional treatment steps.

The cost of flocculation by co-culture with filamentous fungi is dependent on the production cost of the spore inoculum. According to the World Bank Commodity Outlooks, glucose and sucrose were priced at approximately $ 450 and 350 per tonne, respectively, in October 2014. Growth medium (G10N) supplemented with 10 mM of either sugar would cost $ 64 or $ 50, respectively, per tonne lichen (DW) harvested. Cheaper carbon sources, such as those present in wastewater, or low cost raw products, can further reduce the costs.

Due to the large sizes of the lichen pellets (~1-2 mm), harvesting can be achieved without centrifugation requiring only robust filters and gravity flow. A limitation on harvesting through co-culture with filamentous fungi is the time required for pelletization. This may be addressed by inoculating spores earlier in the culturing phase of microalgae. Continuous production of blastospores during algae cultivation should furthermore reduce costs associated with spore production in the pre-culture phase.

3.4 Conclusions

Reducing energy input and operational costs of feedstock production and processing, combined with the recycling of waste nutrients, would significantly increase the sustainability of microalgae-derived biofuels. The inoculation of *Chlorella sorokiniana* cultures with blastospores of *Isaria fumosorosea* and their co-cultivation under strict autotrophic conditions resulted in the formation of large, stable lichen pellets, reducing the costs of harvesting as well as significantly increasing biomass yield. Finally, the wet biomass slurry is compatible with hydrothermal gasification, while a potential closed loop system allows for the recycling of valuable nutrients.

Acknowledgements

This work was supported by the Swiss State Secretariat for Education and Research, in the framework of COST Action CM0903 (Project C11.0048), and the Swiss South African Joint Research Programme (Project SMCJRP06). Stephen Mackay acknowledges the Swiss Federal Commission for Scholarship for Foreign Students (academic year 2012-2013) and the South African National Research Foundation (2014). We would like to thank Dr Mariluz Bagnoud and Dr Frédéric Vogel (Paul Scherrer Institute, Villigen, Switzerland) for supplying the hydrothermal gasification aqueous phase samples, as well as Mr Jean-Pierre Kradolfer and Dr Pierre Rossi for technical assistance.

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Chapter 4:

General Discussion and Conclusions

4.1 General Discussion

The production of biofuel from microalgae is a potential alternative to current fossil fuels. The use of biofuels could significantly reduce environmental pollution and reduce CO_2 emissions that contribute to climate change. To be an effective replacement, biofuel would need to be economically viable to compete with current oil prices. Microalgae cultivation costs are relatively inexpensive, since atmospheric CO_2 and light are fixed to carbon by photosynthesis. The high costs of harvesting microalgae are however problematic for sustainable biofuel production, contributing an estimated 20-50% of the operational cost. Chemical and biological flocculation methods have the potential to reduce the cost significantly. However, improvement of the harvesting methods needs to be considered as part of the entire microalgae-to-biofuel process. The harvesting method needs to flexible and compatible within the system to optimize the entire process.

Chapter 2 considered the position of the harvesting method within the microalgae-to-biofuel process. The algae feedstock and cultivation practices as well as the harvesting method impact on downstream conversion steps. The

quality of algae biomass is largely influenced by the scale of cultivation, the use of photobioreactors (PBRs), pH regulation, CO_2 supplementation and the strain of microalgae. Strain selection is governed by cell size and the lipid, carbohydrate and protein compositions. High lipid producing strains such as *Chlorella vulgaris*, *C. sorokiniana* and *Nannochloropsis oculata* are typically favoured as they have greater bio-oil and bio-crude turnover. Bioremediation and supplementation with waste carbon and nitrogen can significantly influence biomass composition.

Conversion processes of harvested algal biomass to biofuels include conventional trans-esterification or more efficient hydrothermal conversion processes such as direct pyrolysis, hydrothermal liquefaction (HTL) and catalytic hydrothermal gasification (CHG). Transesterification systems are concerned only with the lipid fraction of the biomass while hydrothermal processes convert protein and carbohydrates in addition to lipids. Nutrients recycled from the hydrothermal conversion steps into the cultivation stages, such as water, CO_2, trace metals, organic acids and nitrogen and phosphorus can significantly increase efficiency and decrease costs.

Harvesting methods are critical for the economic viability of microalgae derived biofuels. Algae cultures are typically at low density and require dewatering prior to processing. Costs vary significantly and depend on the approach and intended use of the biomass. High value biomass requires expensive harvesting methods, such as centrifugation, which do not influence the quality of the biomass. Biofuels are considered low value products and biomass recovery requires cost

effective methods such as flocculation over energetically demanding methods. However, chemical flocculation impacts the quality of biomass by increasing the ash content which can contaminate the culture medium and limit water and nutrient recycling. Bioflocculants don't contribute to ash content but may have an undesirable impact on biomass composition. Chapter 3 focused on algae harvesting by bioflocculation with filamentous fungi. Fungal bioflocculation was shown to change the texture of the biomass, forming large algae-fungi pellets which can be easily captured by sedimentation or filtration.

In this study, a fungal assisted bioflocculation method was developed. A fungal pellet was isolated from a contaminated algae flask culture. The fungal contaminant was identified by rRNA sequencing and designated *Isaria fumosorosea IHEM 26293*. The fungus formed a pellet in suspension culture and was tightly associated to the *Chlorella sorokiniana* in the flask culture. Selectivity of the fungus for the capture of microalgae was compared with two other commercial strains, *C. vulgaris* and *Scenedesmus vacuolatus*. Neither *C. vulgaris* nor *S. vacuolatus* formed the same pellet formations with *I. fumorosea*.

Photo bioreactors (PBRs) allowed for larger biomass concentrations to be sampled under controlled pH, temperature, light and CO_2 and mixing. Constant pH and CO_2 supply facilitated autotrophic culturing conditions which are not possible in unregulated flask cultures. *C. sorokiniana* and *I. fumosorosea* were co-cultured at maintained pH values of 6, 7 or 8. Fungal pre-cultures were added to growing algae cultures in PBRs at specific ratios and cultured and sampled over a

period of 72 h. The density of the *C. sorokiniana* control cultures were compared to the densities of the free algae concentrations of the co-cultures. Algae-fungi pellets began to form within 24 hours and almost all free algae cells were captured after 48 hours at pH 7 and 8. Biomass pellets, averaging 1-2mm, were harvested (>94% efficiency) with filters with pore sizes of 250 µm.

The same experiments were repeated at pH 6 under autotrophic and mixotrophic conditions, since previous studies have reported that algae-pellet formation was limited to mixotrophic conditions at a low pH range of 4-6. Harvesting efficiency and total biomass was however significantly lower than at pH 7 or 8. Co-cultures were supplemented with 10mM acetate to create mixotrophic conditions. Algae biomass of monocultures and the algae-fungi biomass of co-cultures approximately doubled due to the addition of acetate. However, harvesting efficiency was not increased as most of the biomass consisted of free algae cells. After 72 hours in co-culture, an increase in biomass of approximately 300% was achieved at pH 7 and 8 relative to monocultures. At pH 6, increases of 137% and 121% were observed for the autotrophic and mixotrophic co-cultures, respectively.

Under magnification, pellets at pH 7 and 8 showed no differences in morphology. Algae cells were dispersed over the surfaces of the hyphae of the fungal pellets, decreasing in abundance from the centre of the pellet towards the periphery. Pellets cultivated at pH 6 showed significant morphological differences. Fungal pellets were less dense with hyphal arms extending with significantly fewer

attached algae on the surfaces of hyphae. Chlorophyll content of the algae-fungi pellets indicated that pellet composition at pH 7 comprised of 44% algae. No differences were observed in protein content (57 to 56%) or carbohydrate content (14.7 to 11.2%) between algal biomass from monocultures and co-cultures. Fatty acid profiles shifted towards a combination of fungal and algal fatty acids with an increased percentage composition of linoleic acid (18:2). No significant changes in composition occurred between the algae and co-cultured biomass, suggesting that the biomass should have similar hydrothermal conversion efficiencies.

The only significant cost of harvesting algal biomass in co-culture with fungi is the preparation of the fungal inoculum. Low cost and recycled carbon and nitrogen sources for pre-cultures could significantly minimize operational costs. Previous studies have produced the fungal inoculum either as spore or pellet cultures in glucose rich media, reviewed in Chapter 2. In order to test low cost growth media, minimal media was supplemented with either 10mM acetate (A10N) or 10mM glucose (G10N) and compared to glucose and peptone rich ½ Sabouraud (SB) media. Cultivation on A10N produced poor quality fungal biomass and no blastospores. Cultivation on G10N produced more blastospores than ½ SB, however, the biomass had formed irregular pellet clumps as opposed to dense spherical pellets formed in ½ SB. The low cost of G10N and increased blastospore concentration, induced by a low C:N ratio, favoured G10N over glucose rich media. The pellet clump biomass produced in G10N had several advantages over

biomass cultured in ½ SB. Aside from the increased blastospore count, pellet clumps were smaller, creating more nuclei and surface area for pellet co-culture development in the PBRs. Small pellet clumps have low densities and do not separate form suspended blastospores which resulted in heterogeneous biomass. All PBR co-cultures were performed with a fungal pellet clump and blastospore inoculum, prepared in G10N. The optimal ratio of fungal inoculum to algae biomass was 1:10 (m:m). The cost effectiveness of a fungal inoculum grown on G10N could potentially be improved by supplementation with carbon sources such as organic acids, or the aqueous phase recovered from HTL. A high through-put 96 well assay was modified to screen the growth kinetics of filamentous fungi using different carbon sources simultaneously. The assay was also used to screen growth of *I. fumosorosea* at a range of temperatures. Carbon sources were selected based on the composition of the recovered aqueous phase of HTL and included several organic acids, diluted HTL aqueous phase samples and the algae PBR culture exudate. Control carbon sources included glucose, sucrose and glycerol. Growth kinetics indicated that the fungus was capable on growing on organic acids and the diluted HTL aqueous phase. G10N could be supplemented with waste organic acids and the diluted HTL aqueous phase to reduce cost of the fungal pre-culture. Interestingly, *I. fumosorosea* was capable of growth on the recovered algal exudate. The exudate supported *I. fumosorosea* growth at a similar rate as 2 mM acetate or the 1:79 diluted HTL aqueous phase samples. The nature of the composition of the exudate was not identified, however, cell wall

sugars were ruled out by HPLC analysis of the supernatant. The exudate most likely consists of polysaccharides and lipids released during algae cell division. It may prove possible to prepare fungal pre-cultures in recovered algae culture exudate, avoiding almost all cost. However, the production of fungal biomass on low cost carbon sources may not produce sufficient blastospores which are required for effective harvesting.

Fungal bioflocculation may be a viable replacement for current harvesting methods, due to relatively low cost, low environmental impact, and compatibility with catalytic hydrothermal conversion. Bioflocculants such as chitosan have been estimated to cost $200 per metric ton algae harvested in contrast to chemical flocculation with $Ca(OH)_2$ which has been estimated to cost $3.5-7.5 per metric tonne^{-1} algae harvested. Fungal bioflocculation was estimated to cost ~$ 64 metric tonne^{-1} if G10N is used to pre-culture the fungal inoculum. This cost could be further reduced if the glucose was substituted with sucrose (~$ 50 metric tonne^{-1}) or supplemented with waste carbon sources such as organic acids, the recovered HTL aqueous phase or the recovered algae exudate form culture medium.

4.2 Conclusions

- An alternative strategy to fungal assisted bioflocculation of microalgae was developed by using a combination of pellet clumps and blastospores as co-culture inoculum. Compared to previous work, only 10% of fungal biomass was used as inoculum. Additionally the use of fungal culture

medium, G10N as opposed to glucose rich media, such as potato dextrose broth, reduced the cost by >90%.

- A novel fungus *I. fumosorosea* was isolated and characterized and the fungal assisted bioflocculation of *C. sorokiniana* was reported for the first time.
- PBRs were used to maintain pH and supply CO_2 to the algae-fungi co-cultures. Constant growth parameters allowed for cultivation in true autotrophic conditions. Co-cultivation of algae and fungi was optimal at pH 7-8 in contrast to the acidic pH values previously reported.
- Algae-fungi pellets (1-2 mm) was efficiently harvested at pH 7 and 8 between 48-72 h at >94% efficiency. A 300% increase in biomass was achieved of which 44% consisted of algae.
- A high-throughput 96-well plate method was optimized to monitor the growth of filamentous fungi on multiple growth media and under different growth conditions, including temperature. Growth kinetics of filamentous fungi is difficult to quantify due to heterogeneous morphology. This method can screen multiple variables accurately and reproducibly over a shorter time frame than conventional flask culture experiments.

4.3 Recommendations for future work

- **Continuous culture:** All experiments have been performed as batch cultures. Algae and fungi were co-cultured, harvested and analysed in

individual cycles. The number of blastospores in culture supernatants detected at 72 h was an adequate inoculum for subsequent algae cultures. The possibility of continuous culture should be further explored as it could significantly reduce cost of production of fungal inoculum.

- **Low cost carbon sources:** The identification of low cost alternatives to glucose may significantly reduce the cost of production of fungal inoculum. In this study, several organic acids and the diluted HTG aqueous phase were identified as alternative candidates for pellet biomass production. Other waste carbon sources or waste waters with complex carbohydrates may substitute glucose in future.

- **Investigating the potential to cultivate fungal biomass on algal exudate.** The *C. sorokiniana* exudate from PBR monocultures released an unidentified carbon source which was metabolized by the fungi as determined by plate assays. HPLC analysis of the culture medium did not detect any simple sugars and it is presumed to consist of lipids and polysaccharides. The possibility to culture fungal biomass on recycled algal exudate should be investigated as a sustainable low cost carbon source.

- **Investigating the interaction between algae and filamentous fungi:** The interaction between algae and fungi is still poorly understood. Interaction has shown to significantly increase the biomass yields of algae and fungi co-cultures. Further investigation into the mechanism of binding and transfer of carbon could result in further increases in harvesting efficiency and biomass production.

Appendix A: Supplementary information for chapter 2

Sustainable Harvesting of Microalgae with Filamentous Fungi: Closing the Loop by Integration with Hydrothermal Conversion

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Author contribution:

Stephen Mackay: Doctoral candidate

Authored and reviewed all written work.

Dr. Rolene Bauer: Principal supervisor.

Critical review of the written work

Submitted for publication to Algae Research, November 2015 (Impact factor 5.014)

Appendix B: Supplementary information for chapter 3

Harvesting of *Chlorella sorokiniana* by co-culture with the filamentous fungus *Isaria fumorosea*: A Potential Sustainable Feedstock for Hydrothermal Gasification

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Supplementary Data

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2. Figure BV1: Lichen pellet sedimentation.
3. Figure B2: Lichen morphology at different pH conditions
4. Figure B3: Screening of the mycobiont for cellulase and laccase activities
5. Graphical abstract for publication
6. 18S sequence: Isaria fumosorosea gr. IHEM 26293 (18S, ITS1, 5.8S and ITS2 rRNA regions)

Author contribution:

Stephen Mackay: Doctoral candidate

Experimental design, data analysis and author of all written work

Dr. Eduardo Gomes: Post-Doctoral fellow

Critical review of experimental design

Prof. Christof Holliger: Director of the laboratory at the EPFL

Provided feedback on experimental work, funding and access to equipment

Dr. Rolene Bauer: Principal supervisor at UWC.

Critical review of data analysis and written work

Dr. Jean-Paul Schwitzguébel: Principal supervisor in Switzerland

Critical review of data analysis and written work

Published in Bioresource Technology (Impact factor 4.494) as:

> Mackay, S., Gomes, E.P., Holliger, C., Bauer, R., Schwitzguébel, J-P. (2015), Harvesting of Chlorella sorokiniana by co-culture with the filamentous fungus Isaria fumosorosea: A potential sustainable feedstock for hydrothermal gasification. *Bioresource technology,* 185: 353–361. http://dx.doi.org/10.1016/j.biortech.2015.03.026

Bioresource technology (Elsevier) gives the right of authors to publish papers as part of a dissertation or thesis: http://www.elsevier.com/about/company-information/policies/copyright#Author%20rights

Supplementary figures

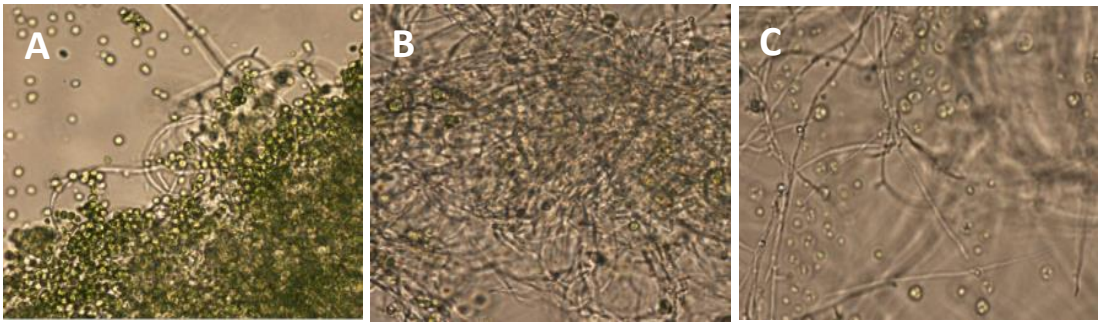

Fig. B1: Photobiont selectivity by co-culturing *I. fumosorosea* pellets with different algae strains. A) *C. sorokiniana* CCAP 211/8k. B) *C. vulgaris* CCAP 211/12 8k. C) *S. vacuolatus SAG211-8B*. Potential photobionts were screened as candidates for *I. fumosorosea* IHEM 26293 when supplemented with glucose. Only *C. sorokiniana* CCAP 211/8k formed lichen structures.

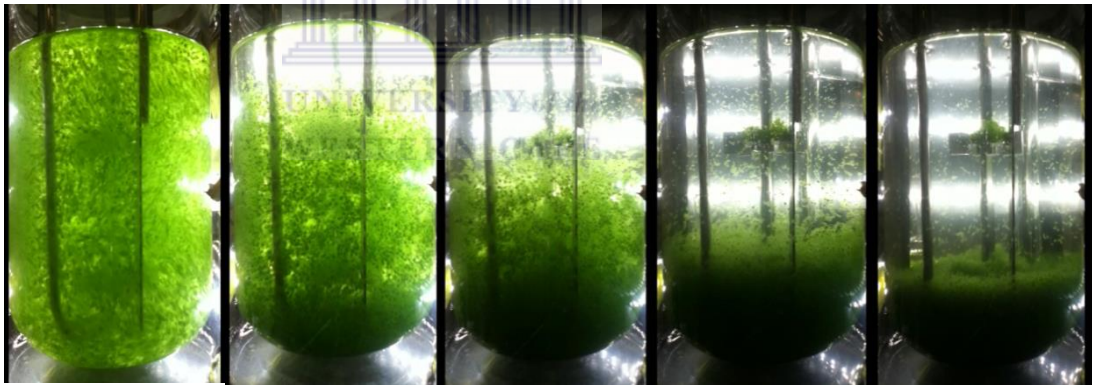

Fig. BV1: Still frame progression of lichen sedimentation. Lichen pellets in submerged culture cultivated at pH 7 after 72 hours. Algae starter cultures were inoculated with blastospores and co-cultured for 72 hours as described in the materials and methods. A video is available in supplementary data of online publication, depicting rapid sedimentation of lichen pellets within 1 min.

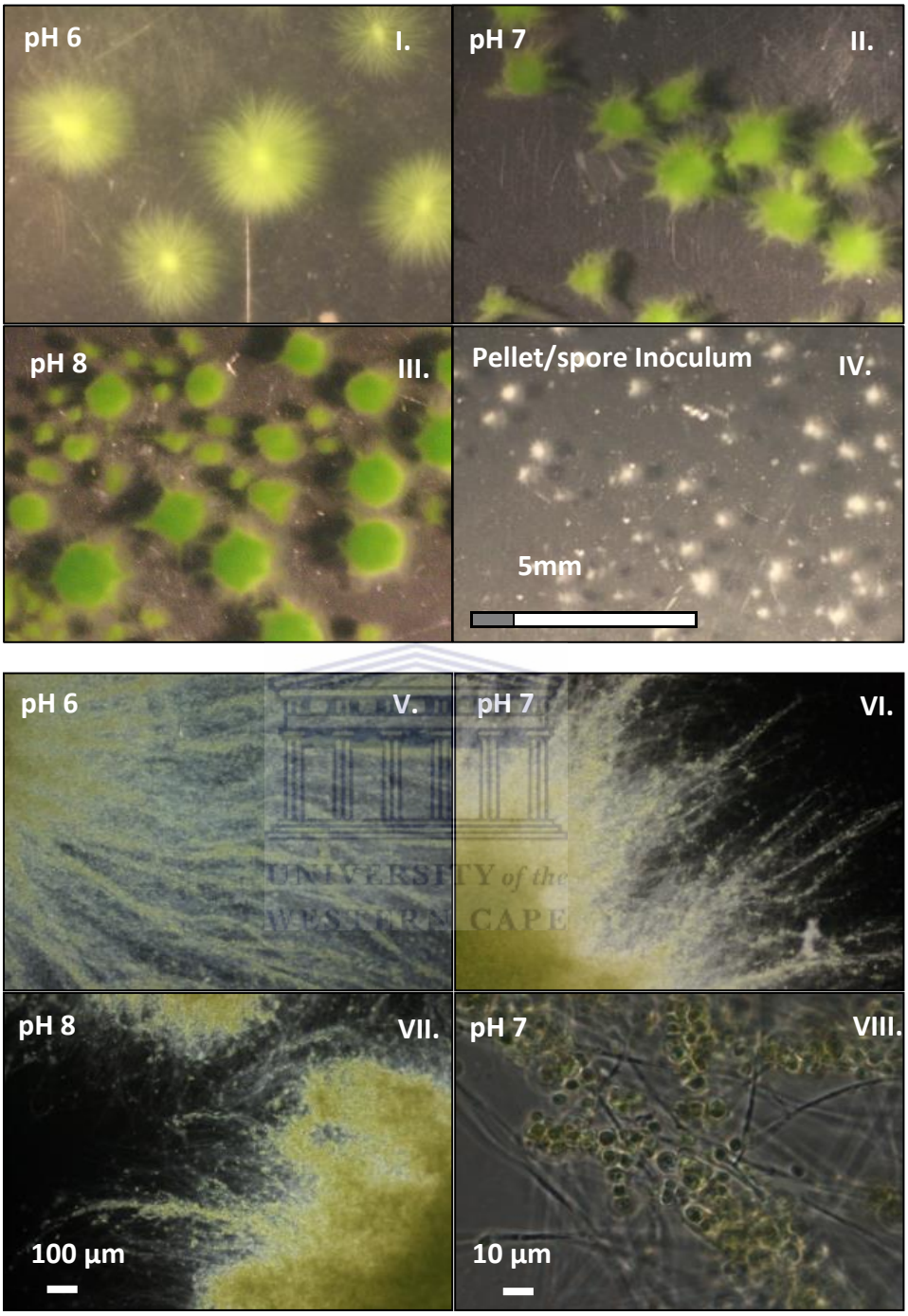

Fig. B2: Lichen morphology at different pH conditions. Pellets were harvested from PBRs after 72 hours of incubation. Stereo microscope images of pellets grown under autotrophic conditions (I-III) at different pH values (scale 1-5 mm). Pellet clumps used in the starting inoculum are presented for scale (IV). Light microscope images of lichen pellets are shown at 10- (V-VII) and 100 X magnification (VIII).

Cellulase activity

Fungi were screened for cellolytic activity with a solid phase test tube assay containing azure blue (Smith, 1977). Both base and top agar layers consisted of MBBM supplemented with 1.5% g agar, 0.1% yeast extract, and 0.5% malt extract, pH 6.8. The top layer contained an additional 0.05% azure blue. Spore cultures (50 µl) of the mycobiont *Trametes versicolor* or *Saccharomyces cerevisiae* Y187 were inoculated onto agar double layers prepared in test tubes (Smith, 1977). Experiments were performed in triplicate, and monitored up to for 4 weeks. Migration of the purple dye from the top layer into the clear lower layer indicates cellulase activity. Subsequent discoloration of the dye indicates laccase activity. Cellulase activity and laccase activity were both observed for the positive control only as expected. The *I. fumorosea* strain did not degrade either the cellulase or discolour the dye indicating the absence of cell wall degrading activity.

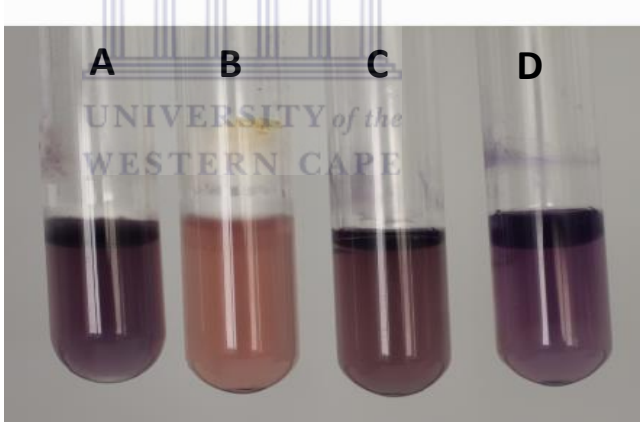

Fig B3: Screen for cellulase and laccase activity of *I. fumorosea.* Cellulase and laccase activity were screened using azure cellulase blue. Migration indicates cellulose degradation where discoloration indicates laccase activity. No activity was observed for *I.fumorosea.* A) *I. fumorosea*. B) *T. versicola* positive control (cellulase and laccase activity). C) *S. cerevisiae* negative control. D) Uninnoculated control.

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***Isaria fumosorosea* gr. IHEM 26293 (18S, ITS1, 5.8S and ITS2 rRNA regions)**

GTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGCAATTATACGGCGAAACTGCG
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Appendix C:

List of Abbreviations

BCCM	-	Belgian Coordinated Collections of Microorganisms
NAABB	-	National Alliance for Advanced Biofuels and Bioproducts
US DOE	-	United States Department of Energy
ANOVA	-	Analysis of variance
BAME	-	bacterial acid methyl esters
EPS	-	extracellular polysaccharides
FAME	-	fatty acid methyl esters
GC-MS	-	gas chromatography mass spectrometry
HPLC	-	high-performance liquid chromatography
HTG or CHG	-	catalytic hydrothermal gasification
HTL	-	hydrothermal liquefacation
LED	-	light emitting diode
PBR	-	photobioreactor

Units of measurement

°C	-	degrees Celsius
µg	-	micrograms
µl	-	microlitres
µm	-	micrometres
bp	-	base pairs (nucleotides)
DW	-	dry weight
g	-	grams
h	-	hours

L	-	litres
M	-	molar
mg	-	milligrams
min	-	minutes
ml	-	millilitres
mm	-	millimetres
mM	-	millimolar
nm	-	nanometres
OD	-	Optical density
PAR	-	photosynthetically active radiation
PSI	-	pounds per square inch
pH	-	potential hydrogen
RT	-	room temperature
SD	-	standard deviation
xg	-	gravitational force

Chemicals and media

CTAB	-	cetyl trimethylammonium bromide
DNA	-	deoxyribonucleic acid
EDTA	-	ethylenediaminetetraacetic acid
MBBM	-	modified Bold's basal medium
PDB	-	potato dextrose broth
rRNA	-	ribosomal ribonucleic acid
SB	-	Sabouraud medium
SDS	-	sodium dodecyl sulfate
TRIS	-	tris(hydroxymethyl)aminomethane
TWS	-	acid treated wheat straw

Appendix D**:**

Curriculum vitae and list of publications

Curriculum Vitae

Name: Stephen Mackay

Date and Place of Birth: 1 July 1984, Cape Town, South Africa

Nationality: South African

Education

2003-2005	Bsc (Plant Biotechnology) University of Stellenbosch, South Africa
2006	Bsc(Hons) (Plant Biotechnology), University of Stellenbosch, South Africa
2007-2010	Msc (Plant Biotechnology), University of Stellenbosch, South Africa
2010-2015	PhD (Biotechnology), University of the Western Cape, South Africa
2012-2014	Internship/PhD, École Polytechnique Fédéral de Lausanne (EPFL), Switzerland

List of Publications

Mackay, S., Pereira Gomez E., Bauer R., Holliger, C. Schwitzguebel, J.P. (2015) Harvesting of *Chlorella sorokiniana* by co-culture with the filamentous fungus *Isaria fumosorosea*: A potential sustainable feedstock for hydrothermal gasification, *Bioresource Technology*, **185** 353–361

Mackay, S., Bauer, R. (2015) Sustainable Harvesting of Microalgae with Filamentous Fungi: Closing the Loop by Integration with Hydrothermal Conversion, Submitted to *Algal research* (November 2015)

Msc Thesis

Mackay, S., Kossmann J.M., (2010) Identification of the Genes Encoding Enzymes Involved in the Synthesis of the Biopolymer Paramylon from *Euglena gracilis*

Book Chapters

Taylor, M.P., Bauer, R., Mackay, S., Tuffin, M., Cowan, D.A. Extremophiles and their application to biofuel research (Book Chapter). In: Sustainable resources and Biotechnological Implications. OV Singh, Ed; Wiley (in press, 2012)

Oral Presentations

Mackay S., Gomes E., Bauer R., Holliger C., Schwitzguébel J-P (2013) Harvesting of *Chlorella sorokiniana* by Co-culture with the Filamentous Fungi, *Isaria fumosorosea*: An application as a feedstock for hydrothermal gasification, Oral Presentation at the 4th UBIOCHEM COST ACTION meeting, Valencia, Spain.

J.-P. Schwitzguebel, S. Mackay, E. Gomes, R. Bauer and C. Holliger. Microalgae for wastewater treatment, CO2 mitigation and biofuels : dream or sustainable maid for all work? 11th International Phytotechnology Conference, Heraklion, Crete, Greece, September 30 - October 3, 2014.

List of Posters

Mackay S, Huddy R, Tuffin IM, Cowan DA, Bauer R (2011) A Single Step Method for the Isolation of Inhibitor-Free High Molecular Weight DNA. Poster presented at FEMS Microbiology conference, Zurich, Switzerland

Mackay S, Huddy R, Tuffin IM, Cowan DA, Bauer R (2011) An Improved Method for the Isolation of Inhibitor-free High Molecular Weight DNA from Environmental Samples. Poster abstract accepted for SASM 2011, Cape Town, South Africa

Mackay S., Williams W., Tuffin M., and Bauer R. (2012) Adapted Methods for the Isolation of HMW DNA from a Range of Extreme Environments. Poster presented at PASSA 2012, Qolora, South Africa.

Nyman, T., Naidoo, R., Albakosh, M., Scheepers I., Mackay, S., Bauer, R. (2012) Assesing the Bacterial Community Associated with the Brown Algae, *Splachnidium rugosum*. Poster presented at PASSA 2012, Qolora, South Africa.

Huddy R, Mackay S, Ohloff C, Smart M, Mulako I, Kirby B, Bauer R, Tuffin, IM, Cowan DA (2012). Analysis of Assembled Metagenomic Sequences. Poster presented at SASBMB 2012.

Huddy R, Mackay S, Ohloff C, Smart M, Mulako I, Kirby B, Bauer R, Tuffin, IM, Cowan DA (2011). Integrating metagenomics, high-throughput screening and next-generation sequencing technologies for novel gene discovery. Oral presentation at SASM 2011.

Mackay, S., Huddy, R.J., Tuffin I.M., Cowan, D.A., and Bauer, R. (2012) Metagenomic Discovery of Two GH9 Cellulases from a Thermal Compost Source, Poster presentation at 9th Extremophiles conference in Seville, Spain

Mackay, S., Pereira Gomes, E., Rossi, P., Schwitzguebel, J.P. and Holliger, C. (2013) Pelletization of Micro-algae by Induced Lichen Formation through Co-culture with Filamentous Fungi, Poster presented at 5th Swiss Microbial ecology Meeting, Murten, Switzerland 2013

Mackay, S., Pereira Gomez E., Bauer R., Holliger, C. Schwitzguebel, J.P. (2014) Pelletization of Micro-algae by Induced Lichen Formation through Co-culture with Filamentous Fungi, Poster presented at 28th Congress of the Phycological Society of Southern Africa, Melkbosstrand, South Africa