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.

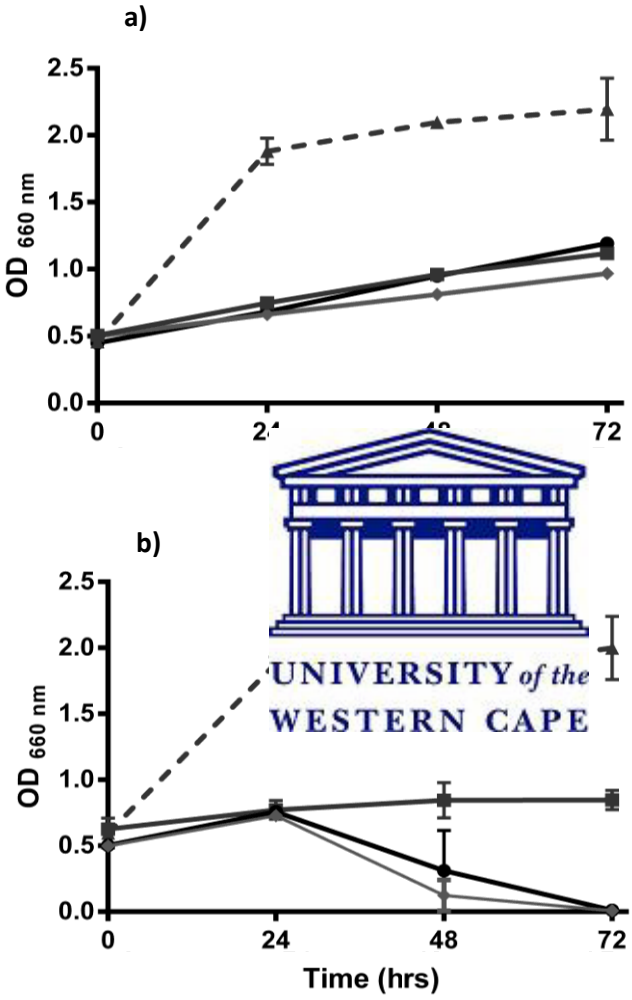

Figure 3.2: Lichen development and rate of clarification under different pH conditions. Free cells density of algae monocultures (a) and co-cultures (b) at pH 6 (▪), pH 7 (●), pH 8 (◆), and at pH 6 in a medium supplemented with 10mM acetate (▲). Values are presented as means ± SD of 2 independent experiments, 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)

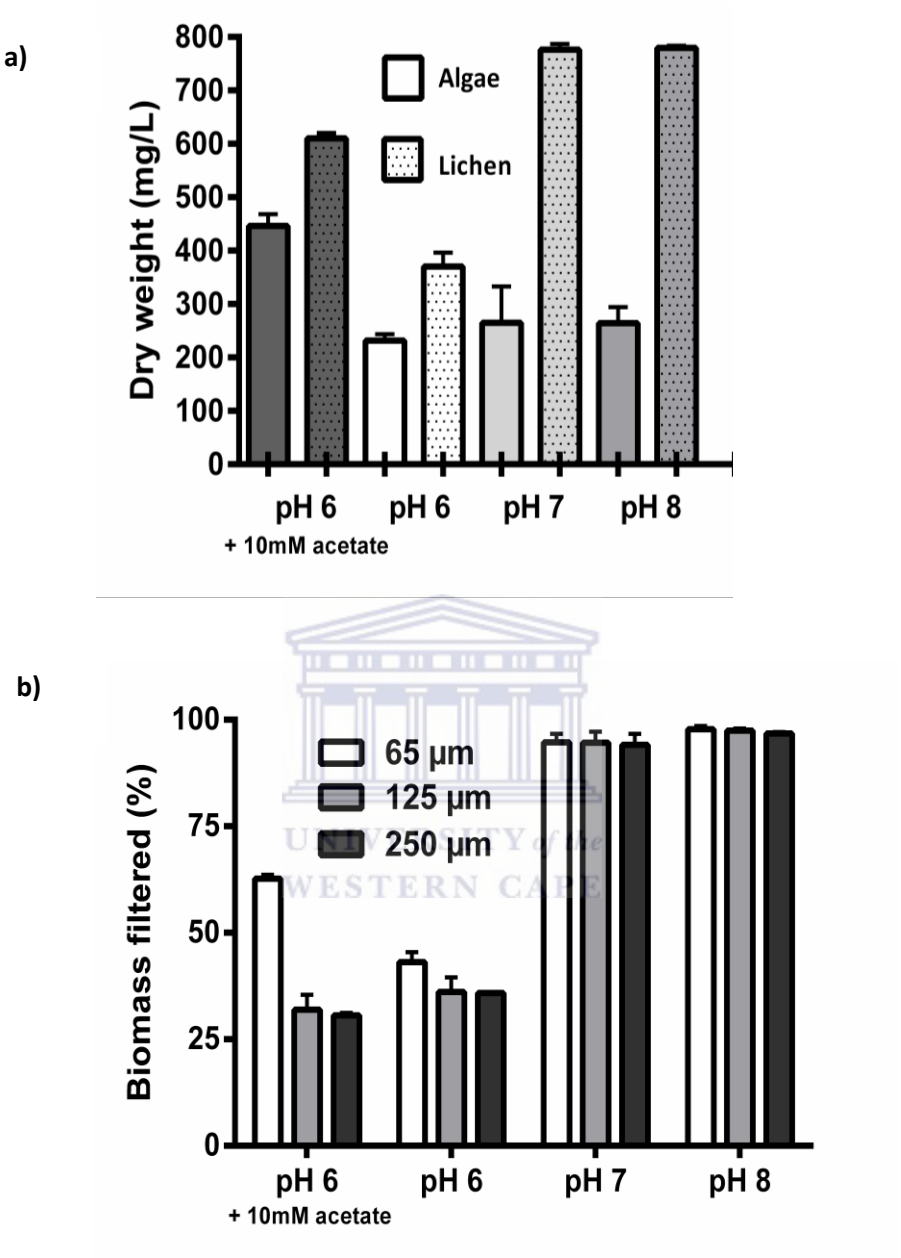

Figure 3.3: Biomass recovery by filtration. Cultures were grown for 72 h at different pH values and trophic conditions. a) Relative total biomass (mg/L DW) produced by algae monocultures and co-cultures. b) Recovered biomass after filtration using different filter pore sizes under constant pressure. 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.

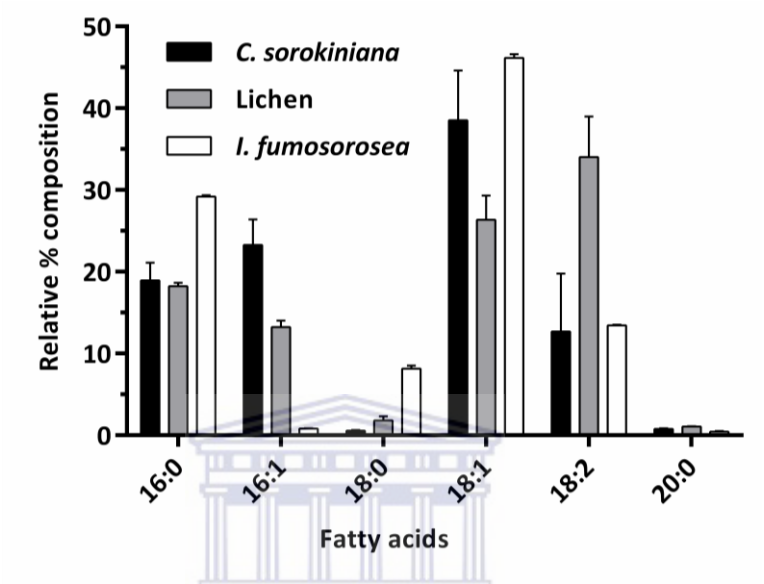

Figure 3.4: Relative fatty acid composition of the biomass produced by *C. sorokiniana, I fumosorosea* and the lichen co-culture. Values are presented as means ± SD of 2 independent experiments, each with 3-8 replicates. Two-way ANOVA followed by Fischer's LSD test were used to determine statistical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

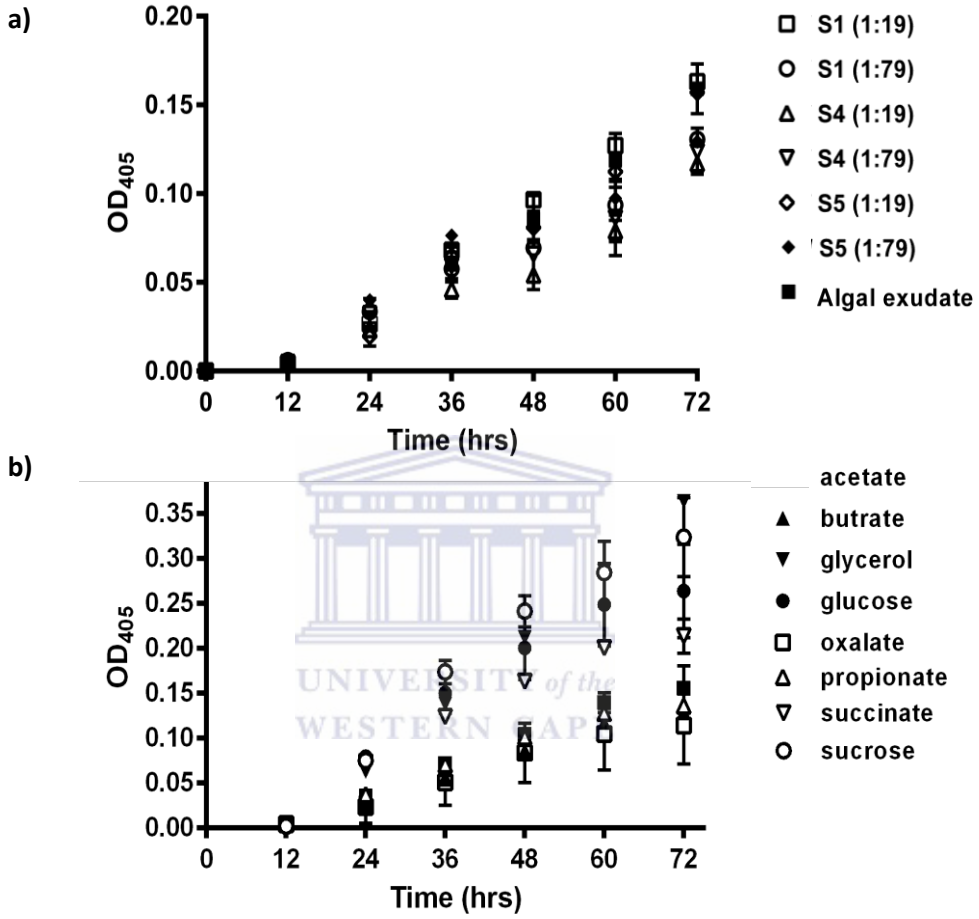

Figure 3.5: Relative growth of *I. fumosorosea* gr. IHEM 26293 on sugars, organic acids and waste water substrates. **a)** Growth on 1:19 and 1:79 dilutions of HTG aqueous phase and on recovered PBR algal culture medium; **b)** Growth on sugars, glycerol and organic acids, each given at 2 mM. Values 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.

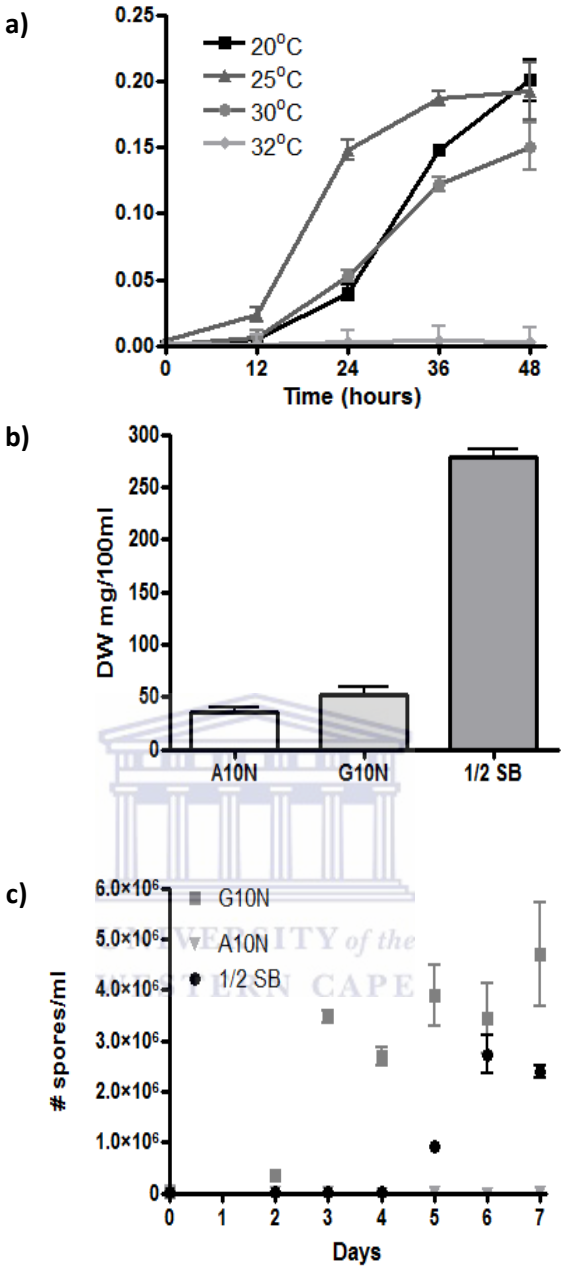

Figure 3.6. Optimal temperature, total biomass and blastospore production of the mycobiont in different media. **a)** Optimal temperature of the spore inoculum; **b)** total pellet biomass after 7 day period; **c)** blastospores production over 7 days. Values are presented as means ± SD of 2 independent 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.

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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

Mackay S.[1], Bauer R.[2]

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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

Mackay S.[1], Gomes E.[2], Holliger C.[2], Bauer R.[3], Schwitzguébel, J-P[2]

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Supplementary Data

List of contents

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

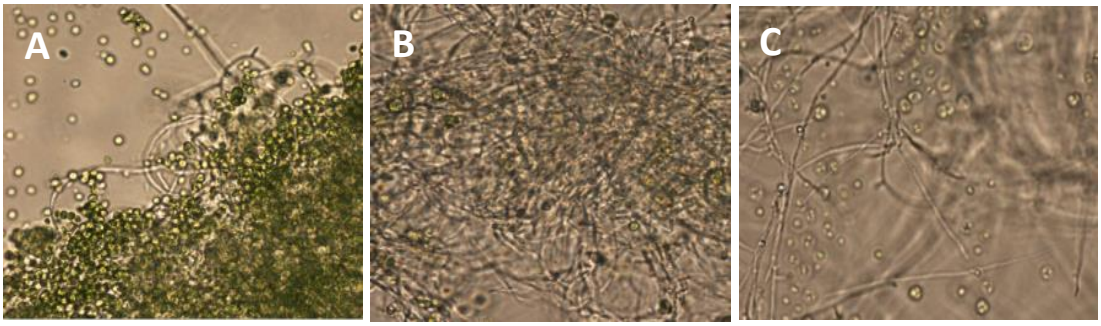

Fig. B1: Photobiont selectivity by co-culturing *I. fumosorosea* pellets with different algae strains. A) *C. sorokiniana* CCAP 211/8k. B) *C. vulgaris* CCAP 211/12 8k. C) *S. vacuolatus SAG211-8B*. Potential photobionts were screened as candidates for *I. fumosorosea* IHEM 26293 when supplemented with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

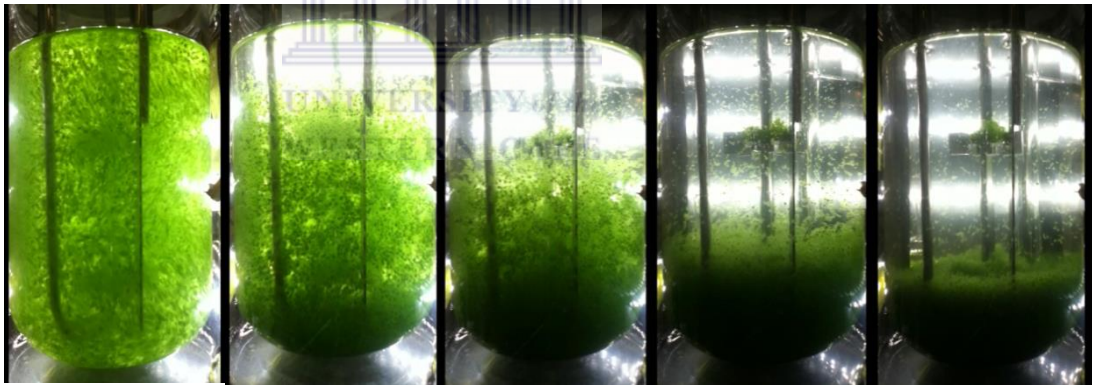

Fig. BV1: Still frame progression of lichen sedimentation. Lichen pellets in submerged culture cultivated at pH 7 after 72 hours. Algae starter cultures were inoculated with blastospores and co-cultured for 72 hours as described in the materials and methods. A video is available in supplementary data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

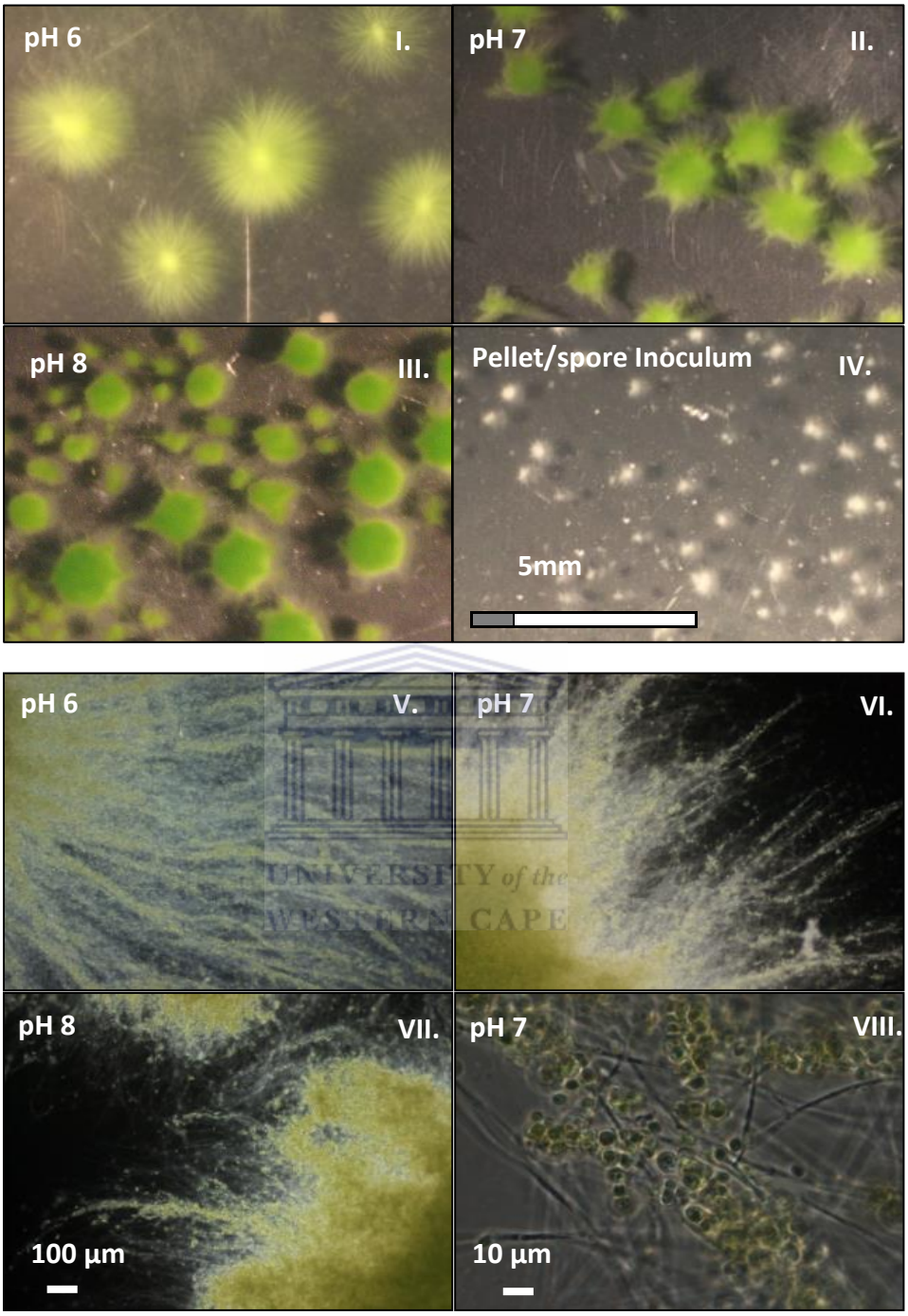

Fig. B2: Lichen morphology at different pH conditions. Pellets were harvested from PBRs after 72 hours of incubation. Stereo microscope images of pellets grown under autotrophic conditions (I-III) at different pH values (scale 1-5 mm). Pellet clumps used in the starting inoculum are presented for scale (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.

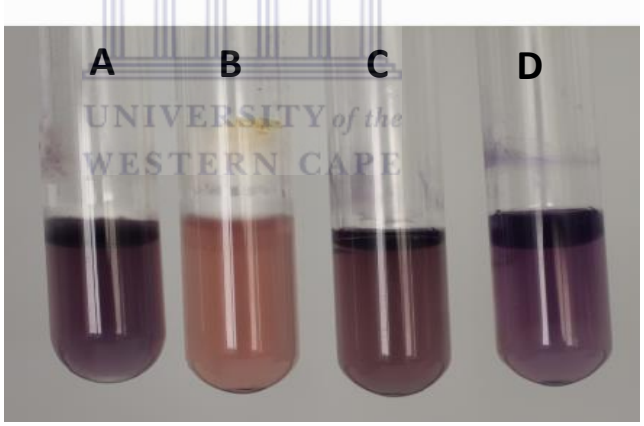

Fig B3: Screen for cellulase and laccase activity of *I. fumorosea.* Cellulase and laccase activity were screened using azure cellulase blue. Migration indicates cellulose degradation where discoloration indicates laccase activity. No activity was observed for *I.fumorosea.* A) *I. fumorosea*. B) *T. 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)

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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.

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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.

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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