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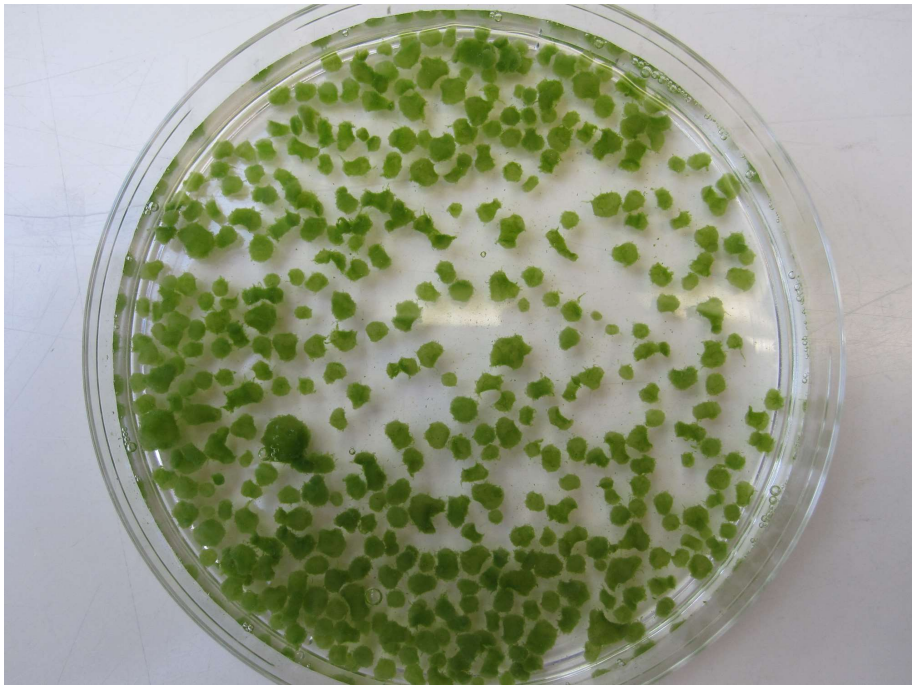

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Abstract

This work is concerned with the use of fungi to harvest microalgae. The ability of *Aspergillus niger* and *Trichoderma harzianum* to harvest cells of *Chlorella vulgaris* and *Scenedesmus* ssp. was assessed in several experiments designed to elucidate the adhesion mechanism. While *A. niger* was able to form co-pellets with both *C. vulgaris* and *Scenedesmus* ssp. by addition of spores to the algal culture, *T. harzianum* only produced filamentous mats. When KCl was added to the medium, the supernatant of the co-culture with *A. niger* and *C. vulgaris* was opaque compared to the clear supernatant of the co-culture without KCl. This implies that the adhesion of microalgal cells to the fungal hyphae is influenced by electrostatic interactions. Cells of *C. vulgaris* adhered to the surface of pre-made pellets of both fungal species but the pellets *A. niger* collected more cells than those of *T. harzianum*. The capability of fungi to harvest microalgae can be an important aspect of the transition to a biobased society as microalgae is seen as a promising renewable source of both oil and fermentables for the bioenergy industries.

Contents

1 Introduction

Microalgae has been recognized as an ideal candidate for alleviating the current situation of depleting fossil carbon resources and increasing concentrations of CO_2 in the atmosphere. One of the primary reasons why the production of microalgae is seen as promising is the possibility of utilizing several aspects of the harvested product. Together with the low requirements concerning location, microalgal production plants can be placed in areas unsuitable for food and feed production (Brennan & Owende, 2010). Oil is considered as the most promising bulk product from microalgae as it can reach 80% of the dry weight in some species and be processed into biodiesel by already developed methods. When compared to oil crops, the productivity is several orders higher which is a necessity for reaching the goal of replacing the petroleum based diesel (Chisti, 2007).

To make it economically feasible to use microalgae as a bioenergy feedstock, the costs of nutrients and carbon sources have to be minimized which can be accomplished by utilizing output streams from other processes. Nutrient rich wastewater from municipal and agricultural sources and CO_2 from industrial facilities can be used as affordable input streams (Clarens et al., 2010). Microalgae could thus be cultured autotrophically for the simultaneous assimilation of CO_2 and the prevention of nutrient rich effluents from reaching lakes, rivers and the ocean (Brennan & Owende, 2010).

The major drawback of autotrophic cultures compared to heterotrophic ones is the low cell density. However, under mixotrophic conditions it is possible to have both the carbon fixing ability of the autotrophic culture and the high biomass generation of the heterotropic culture (Gultom & Hu, 2013). But even though the culture conditions can be optimized, harvesting of the microscopic and easily dispersed algae still stands as an obstacle for a large scale production (Uduman et al., 2010).

The aim of the present study was to investigate the forces responsible for the adherence of microalgal cells to fungal pellets and how this principle can be utilized. To test the generality of the principle, *Aspergillus niger* as well as *Trichoderma harzianum* was used to harvest cells of both *Chlorella vulgaris* and *Scenedesmus* ssp.

Definitions

The terms describing the processes throughout this work are taken from the IUPAC recommendations (Slomkowsi et al., 2011). The main processes are

- Adhesion - The process of when a particle, biological or otherwise, becomes attached to a surface different from the particle itself.
- Aggregation - The process of when particles of the same type becomes attached to each other.
- Flocculation - A reversible process where particles of the same type are brought together but not attached to each other.

1.1 The behavior of a biological suspension

The stability of an aqueous suspension is dependent on the properties of both the solvent and the suspended particles. The extended DLVO (XDLVO) theory takes into account the Lewis acid-base, electrostatic and van der Waals-interactions between the surfaces of the suspended particles and the solvent to predict the stability of the suspension. In a stable suspension the particles repel each other due to an energy barrier that is insurmountable at close distances. In an unstable suspension particles tend to aggregate due to an increase in released energy as the distance between the particles approaches zero (Wu et al., 1999).

For relatively homogeneously shaped biological particles, such as *Chlorella vulgaris*, Sirmerova et al. (2013) showed that the XDLVO theory can predict the behavior regarding adhesion to surfaces such as glass and plastic based on the properties of the particle, medium and surface. However, when the suspended cells contains protrusions and irregularities in the cell wall the theory can fail to predict the adhesion behavior due to the formation polymeric bridges (van Loosdrecht & Zehnder, 1990). These bridges can span the distance of the repulsive electrostatic force and have been shown to contribute to both adhesion in bacteria (Azeredo et al., 1999) and aggregation in fungi (Gerin et al., 1993; Dynesen & Nielsen, 2003) and microalgae (Salim et al., 2014).

1.1.1 Behavior according to XDLVO theory

Algae

The cell surface of *C. vulgaris* have been classified as negatively charged down to the isoelectric point of ∼2.9 (Hadjoudja et al., 2010). The negative charge have been stated to be origin of the repellent electrostatic force inhibiting the aggregation of the algae and ensuring the stability of the suspension (Uduman et al., 2010). Henderson et al. (2008) showed that microalgae can by harvested by aggregation through the modification of the zeta potential, either by changing the pH or by introducing a positively charged coagulant. The correlation between aggregation and zeta potential fits the case of Sirmerova et al. (2013) who showed that in weak ionic medium, the electrostatic interaction is the most important factor regarding attraction.

While the surface of *C. vulgaris* is negatively charged, it is also hydrophilic which characterizes the surface as monopolar (Hadjoudja et al., 2010). Particles with these surface characteristics are readily dispersed in water as the attractive van der Waals-component of the XDLVO theory are several orders lower than the repellent electrostatic and Lewis acid-base interactions. However, when plurivalent cations are added to the medium the particles behaves completely differently and aggregate. This is attributed to a complete shift in hydrophobicity which overcomes the still repellent electrostatic interaction (Wu et al., 1999). This shift was also seen by Sirmerova et al. (2013) in high ionic strength medium where the algae formed larger colonies on hydrophobic surfaces.

Based on the findings of Wu et al. (1999), the results of Henderson et al. (2008) can be interpreted as an aggregation driven by hydrophobic rather than electrostatic interactions. Both studies used low concentrations of plurivalent cations to induce aggregation but give different explanations for the mechanism of attachment. Powell & Hill (2014) shines light on the question by showing that algal cells binding calcium were more prone to adhere to a hydrophobic surface than cells without calcium. Taken together, these studies show that plurivalent cations lowers the zeta potential of the cells and thus the repellent electrostatic forces between the cells which enables the cells to get close enough to interact hydrophobically.

Fungi

It has long been recognized that fungi can form pellets in submerged cultures (Burkholder & Sinnott, 1945). However, it is just in the recent years that the biophysical mechanism of this morphological change has been getting attention. A majority of the studies have focused on the aggregation of spores and its influence on the formation of larger pellets. For *A. niger*, it has been shown that the aggregation of spores is prevented by the electrostatic interaction and that the hydrophobic spore coat layer can not make up for the repellent force (Wargenau & Kwade, 2010). However, this does not prevent the aggregation of germinating spores, and later pellet formation, as the spore coat layer is shed during the germination (Tsukahara, 1968; Preignitz et al., 2012).

As opposed to the spores of *A. niger*, the spores of *Trichoderma* ssp. have been recognized to have a hydrophilic surface (Jin & Custis, 2010). This makes the surface similar to that of *C. vulgaris* and the spores should thus behave similarly in an aqueous suspension. According to Fontaine et al. (2010), the morphological difference between *A. niger* and *Trichoderma* ssp. continues during the germination as *Trichoderma* ssp. is unable to produce any aggregated morphology.

1.1.2 Polymeric aggregation

Algae

Although microalgae generally grows freely dispersed, there are phenotypes that spontaneously form aggregates. Both *Chlorella vulgaris* JSC-7 (Alam et al., 2014) and *Scenedesmus obliquus* AS-6-1 (Guo et al., 2013) utilizes a polysaccharide located in the cell wall to form bridges between cells, while the aggregation in *Ettlia texensis* is due to a glycoprotein (Salim et al., 2014). The purified aggregation agent from these species can be used to aggregate freely suspended cells of *C. vulgaris*. Salim et al. (2014) does not report on the molecular architecture of the glycoprotein but the polysaccharides of both *C. vulgaris* JSC-7 and *S. obliquus* AS-6-1 contains units of mannose, galactose and glucose (Guo et al., 2013; Alam et al., 2014). However, the ratio between the sugar units varies and the polysaccharide of *S. obliquus* contains rhamnose and fructose units as well which rules out identical binding mechanisms. Although

there are differences between the structures, the dosages of the purified polysaccharide compounds for removal of non-aggregating *C. vulgaris* are similar, 0.5 mg/l for *C. vulgaris* JSC-7 and 0.6 mg/l for *S. obliquus* AS-6-1. This shows that there might be a similar mechanism, possibly involving the hydroxyl and carboxyl groups as binding sites (Guo et al., 2013; Alam et al., 2014).

Fungi

Since the surface properties of the spores of *A. niger* are lost during the germination, the reason for aggregation in this species has to be sought elsewhere. This also eliminates the classical notion of Metz & Kossen (1977) that the formation of fungal pellets begins with the aggregation of spores. During the germination of both *A. niger* and *A. fumigatus*, the loss of the spore coat layer reveals the hydrophilic cell wall beneath (Tsukahara, 1968; Dague et al., 2008). In *A. fumigatus*, the polysaccharide α-1,3-glucan is a major part of the cell wall and it was shown that this compound was singularly responsible for the aggregation of hyphae and formation of pellets (Fontaine et al., 2010; Henry et al., 2012). α-1,3-glucan is also a major part of the cell wall in *A. niger* which might lead to a similar aggregation behavior (van Munster et al., 2015).

1.1.3 Fungal-assisted harvesting

Several studies have shown that it is possible to use fungi as a mean to aid the harvesting of microalgae (Zhou, Cheng, et al., 2012; Zhang & Hu, 2012; Zhou, Min, et al., 2013; Prajapati et al., 2014; Wrede et al., 2014). The studies are in agreement of the general conditions required for pellet formation between the algae and the fungi: slightly acidic conditions, addition of glucose to at least 5 g/l and agitation of the co-culture. However, using *A. lentulus*, Prajapati et al. (2014) managed to achieve pelletization irrespective of pH which shows that the process is strain-specific.

So far, the key mechanism of the adhesion of microalgal cells to the fungi have not been elucidated. Prajapati et al. (2014) reports that directly upon germination of the spores, algal cells adhere to the fungal cell wall and are mostly contained within the complete fungal-algal pellet. Similar cases are given by Zhang & Hu (2012), Zhou, Min, et al. (2013), and Wrede et al. (2014). Based on the XDLVO theory, the adherence can either be of electrostatic nature, due to van der Waals interactions or due to the similar hydrophobicity of the entities. Zhang & Hu (2012) mentions the hydrophobins of the fungal cell wall as a possible means of attachment but this requires the algal cells to be of hydrophobic nature for the interaction to be energetically favorable. It was however shown that the surface of *C. vulgaris* was clearly hydrophilic which eliminates the possibility (Sirmerova et al., 2013). Another reason to dismiss the nonpolar interaction is that if it was indeed the case, the highly hydrophobic ungerminated spores of *A. niger* would attract the algae which Zhang & Hu (2012) showed is not the case.

In the study of Rajab (2007), it was shown that the surface of the pellet of *A. flavus* was positively

charged. If this is the case for the other strains used in algal pelletization it might explain the adherence since the surface of the algae is negatively charged (Uduman et al., 2010). As was mentioned earlier, both microalgae and fungi can use specific polysaccharides for aggregation. While electrostatic interaction can either be repulsive or attractive, the formation of polymeric bridges can either overcome the repulsive force and/or strengthen the adhesion (van Loosdrecht & Zehnder, 1990).

2 Methods

2.1 Searching for α-1,3-glucan synthase homologs

Based on the fact that α-1,3-glucan was the key factor in the aggregation of hyphae in *A. fumigatus*, one of the *Ags* genes coding for α-1,3-glucan synthase in *A. niger* (ANI_1_1472184) was used to search for homologs in the database OrthoDB v8 (http://www.orthodb.org/). The species containing homologs were then compared to fungal species from previous studies of microalgal adhesion.

2.2 Pre-trials

To establish the important characteristics of the fungi for the adhesion of microalgae, *A. niger* ATCC 16888 (acquired from the American Type Culture Collection) and *T. harzianum* CBS 226.95 (acquired from CBS-KNAW Fungal Biodiversity Centre) was used throughout this study. Spores of the fungi were harvested from petri dishes using 10 ml distilled water and the spore concentration was determined using a Bürker chamber. The pre-trials were made in triplicates with 50 ml liquid in 100 ml Erlenmeyer flasks which were shaken for at least 48 hours at ∼100 rpm.

To find the right conditions for fungal pelletization, trials were performed with different liquid media. In a first test to find the minimal requirements, spores ($6 \cdot 10^8$ spores/l) were added to distilled water adjusted to either pH 4 or 7 by addition of HCl or NaOH with glucose (6 g/l) or freeze dried *C. vulgaris* (0.5 g/l) as carbon source. When no signs of germination could be seen after 115 hours, new cultures were made with fresh BG-11 medium adjusted to pH 4 or 7 and supplemented with 0.5 g/l K_2HPO_4, 0.5 g/l NH_4NO_3 and 5 g/l glucose as per Prajapati et al. (2014). This medium is henceforward called supplemented BG-11. Cultures made with supplemented BG-11 without glucose were also made for comparisons of the growth.

2.3 Microalgal adhesion

Spores of both strains ($2.37 \cdot 10^9$ spores/l) were inoculated at 2% (v/v) to cultures of *C. vulgaris* adjusted to pH 4 or 7 and supplemented with the salts according to 2.2 both with and without addition of glucose. The co-cultures were examined for co-pellets after 48 hours on a shaker set to 100 rpm.

2.4 The influence of surface charge on microalgal harvest

To examine if microalgae adhere to the fungal pellets due to electrostatic forces, an experiment was performed with varying amounts of ions. Cultures of *C. vulgaris* at a cell density of $8.75 \cdot 10^8$ cells/l and volume of 50 ml was supplemented according to 2.2 in 100 ml Erlenmeyer flasks. The pH and concentration of KCl was varied between 4 and 7 resp. 0 and 80 mM. The cultures were inoculated with spores of *A. niger* or *T. harzianum* at a volume of 2% of the final volume and concentration of $1.75 \cdot 10^9$ spores/l. The flasks were put on a shaker at 100 rpm together with uninoculated negative controls without *C. vulgaris* for 66 hours in room temperature without any additional light in the laboratory.

2.5 Algal adhesion to fungal cell wall

Guggenheim & Haller (1972) described a secreted enzyme degrading α-1,3-glucan from *T. harzianum* and this feature was used to test whether this polysaccharide is an important feature of microalgal adhesion in *A. niger*. To harvest the algae, spores of *A. niger* ($6.25 \cdot 10^8$ spores/l) were added to a supplemented culture according to 2.2 of *C. vulgaris* ($2.13 \cdot 10^9$ cells/l) and put on shaker at 100 rpm for 48 hours. As a control, spores were added to fresh supplemented BG-11 medium with glucose according to 2.2 without microalgae. For the evaluation of enzyme production, spores of *T. harzianum* were added to supplemented BG-11 medium with glucose according to 2.2 and put on shaker at 100 rpm for 96 hours.

The pellets from both cultures were removed by filtering through a 100 micron sieve. The supernatant of *T. harzianum* was centrifuged for 15 minutes at 3500 rpm in room temperature and then sterile filtered through a 0.2 µm filter to remove ungerminated spores. Pellets from both the co-cultures and the controls were added to 3 ml supernatant of *T. harzianum* in 12-well microtiter plates and incubated for 3 hours in room temperature at 100 rpm.

2.6 Algal harvest with premade pellets

Pellets of *A. niger* and *T. harzianum* were grown in supplemented BG-11 with glucose (see 2.2) adjusted to pH 4 and removed through filtering using a 100 micron sieve. The pellets were then added to cultures of *C. vulgaris* (conc. $3 \cdot 10^8$ cells/l) for harvest of the algae. Triplicates with 2 g pellets in 50 ml algal suspension were made and put on shaker on 100 rpm for 72 hours.

2.7 Co-culture with *Scencedesmus*

Cultured algae of the species *Scenedesmus* ssp. were supplemented with salts according to 2.2 and the pH was adjusted to 4. The cultures were inoculated with spores of *A. niger* and *T. harzianum* of a concentration $5 \cdot 10^8$ spores/l and put on a shaker at 100 rpm for 48 hours.

Table 1. Homologs to α-1,3-glucan synthase

Species in studies	Homolog	Fungal pellet	Co-pellet	Study
Aspergillus flavus	Yes	Yes	Yes	Zhang & Hu, 2012
Aspergillus niger	Yes	Yes	Yes	Zhang & Hu, 2012
Aspergillus oryzae	Yes	Yes	Yes[1]	Zhang & Hu, 2012; Zhou, Min, et al., 2013
Aspergillus versicolor	No	Yes	Yes	Zhang & Hu, 2012
Aspergillus lentulus	No	Yes	Yes	Prajapati et al., 2014
Aspergillus fumigatus	Yes	Yes	Yes	Wrede et al., 2014
Aerococcus viridans	No	Yes	Yes	Zhang & Hu, 2012
Phanerochaete chrysosporium	No	Yes	No	Zhang & Hu, 2012
Leucogyrophana arizonica	No	Yes	Yes	Zhang & Hu, 2012
Mucor circinelloides	No	Yes	Yes[2]	Zhang & Hu, 2012

Species used in studies for harvesting microalgae and the existence of a gene coding for α-1,3-glucan synthase in their genome. [1] = Co-pelletization only under certain conditions. [2] = Green pellets but poor harvesting.

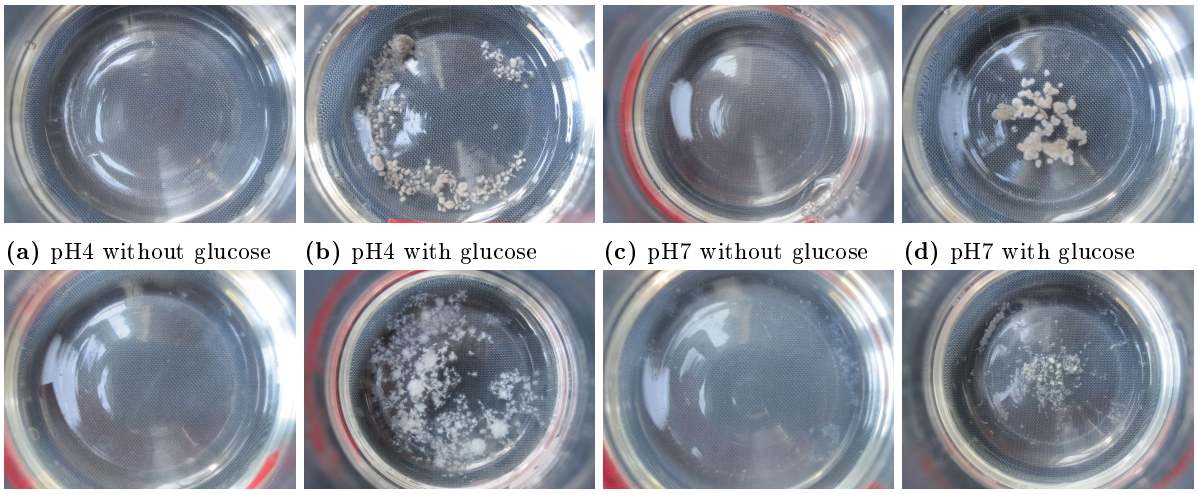
3 Results

3.1 Finding of homologs

177 hits in 96 species of fungi were found when searching for homologs to the *Ags* gene ANI_1_1472184 coding for α-1,3-glucan synthase in *A. niger*. *T. harzianum* was not one of these species. A comparison between the hits and the strains from previous studies of fungal pelletization of microalgae shows that the existence of an *Ags*-homolog is not necessary for neither fungal pelletization nor co-pelletization with algae (table 1). Among the strains belonging to *Aspergillus*, both *A. versicolor* and *A. lentulus* lacks *Ags*-homologs which points to different mechanisms within the same genus by which fungal pellets can form. And moreover, the poor co-pelletization with *A. oryzae* shows that the existence of α-1,3-glucan in the cell wall does not guarantee good microalgal adhesion.

3.2 Pre-trials

With signs of growth for both strains after 48 hours in the flasks containing supplemented BG-11 but a complete lack of biomass in the flasks containing water supplemented with glucose or freeze dried *C. vulgaris*, it was clear that neither of the strains could survive on glucose alone or utilize the algae for nutrition. When grown in BG-11, both pH and sugar had an impact on the amount of biomass produced by both of the strains. When glucose was added more biomass was produced and a low pH enhanced the fungal biomass production. Regardless of pH or glucose, there were morphological differences between the species with *A. niger* producing distinct similarly sized pellets while *T. harzianum* produced aggregates or loose pellets of varying size (figure 1).



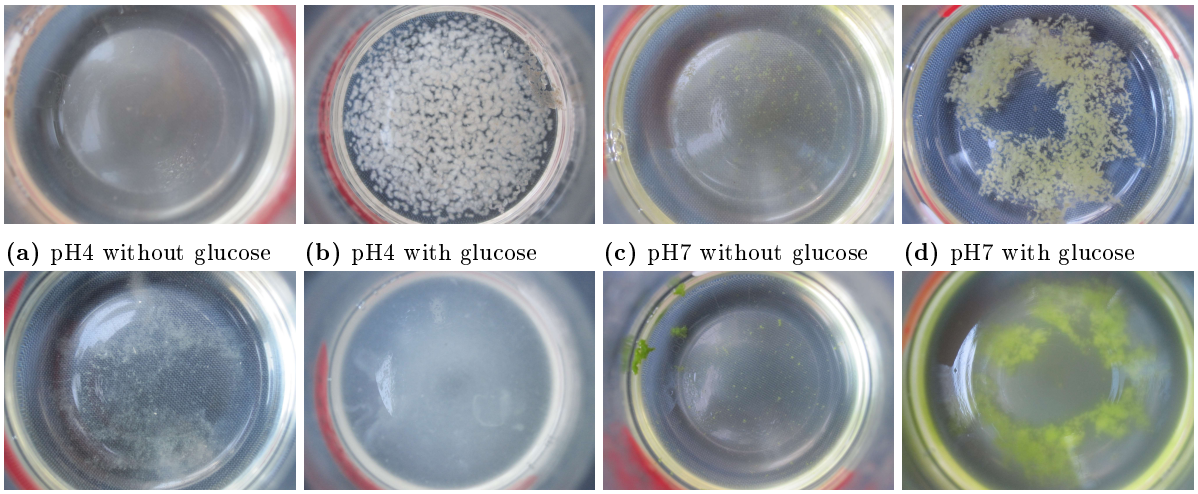
(a) pH4 without glucose **(b)** pH4 with glucose **(c)** pH7 without glucose **(d)** pH7 with glucose

(e) pH4 without glucose **(f)** pH4 with glucose **(g)** pH7 without glucose **(h)** pH7 with glucose

Figure 1. Comparison of the ability of *A. niger* and *T. harzianum* to from pellets in BG-11 medium. a-d *A. niger*, e-h *T. harzianum*.

3.3 Microalgal adhesion

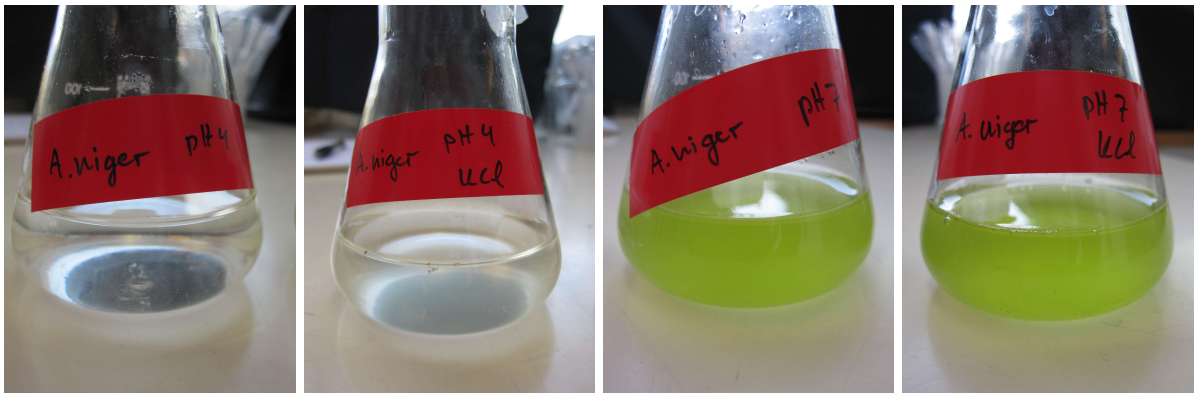
The two species performed differently with regards to pH but both were able to form more or less defined structures with the algae. While *A. niger* formed spherical pellets at both pH4 and pH7 with glucose added, the amount and color differed (fig. 2a-d). With *T. harzianum* the morphology of the cultures varied between the treatments. When glucose was added, the fungus adopted a filamentous growth at pH4 while very loose floccs were formed at pH7. Without the added carbon source, *T. harzianum* managed to produce a clear supernatant but not any defined structures, in pH4 clouds with small flocs was seen and in pH7 a few large flocs were formed (fig. 2e-h). Overall, the cultures adjusted to pH4 lost all the green color of the algae irrespective of fungal species, morphology and carbon source.



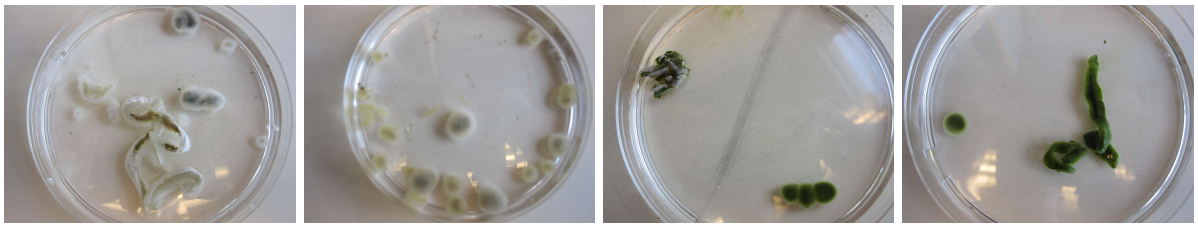
(a) pH4 without glucose **(b)** pH4 with glucose **(c)** pH7 without glucose **(d)** pH7 with glucose

(e) pH4 without glucose **(f)** pH4 with glucose **(g)** pH7 without glucose **(h)** pH7 with glucose

Figure 2. Co-pelletization of fungus and *C. vulgaris*. a-d *A. niger*, e-h *T. harzianum*.



(a) pH4 without KCl **(b)** pH4 with KCl **(c)** pH7 without KCl **(d)** pH7 with KCl



(e) pH4 without KCl **(f)** pH4 with KCl **(g)** pH7 without KCl **(h)** pH7 with KCl

Figure 3. Supernatant (a-d) and pellets (e-h) of co-cultures of *A. niger* and *C. vulgaris* with and without KCl addition.

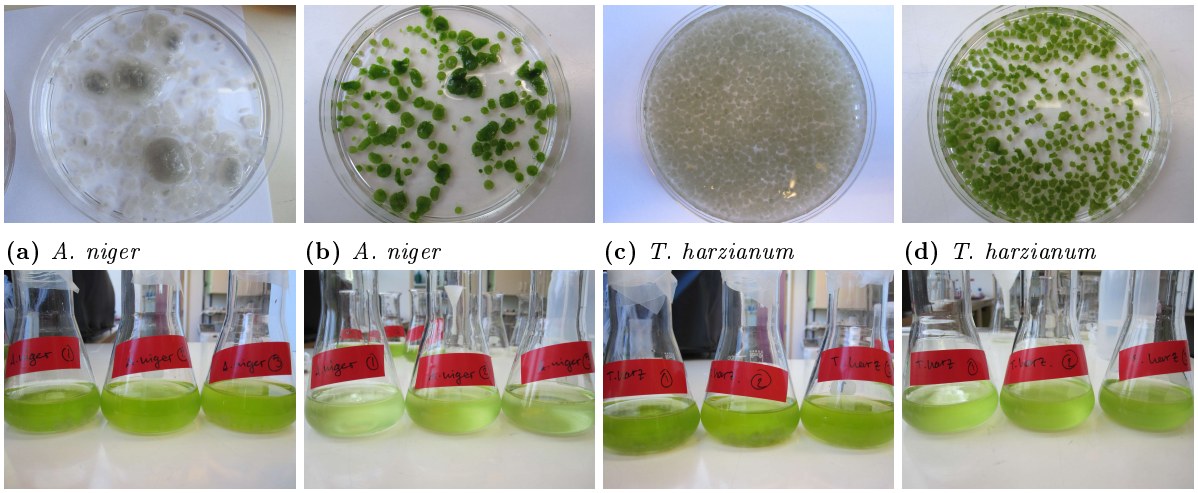
3.4 Influence of ionic strength on harvest efficiency

By visually comparing the color of the supernatant between the treatment and the species, it is cleat that the addition of KCl resulted in a lower harvest efficiency. *T. harzianum* produced large amounts of filamentous biomass when co-cultured with algae adjusted to pH4 both with and without KCl but behaved differently when cultured in pH7 (no picture). When KCl was added to cultures adjusted to pH 7, small homogeneous pellets roughly 2 mm in diameter were formed but the supernatant remained green. Without KCl loose clumps of fungus and algae formed but much of the algae remained in the supernatant.

The cultures of *A. niger* all formed fungal-algal pellets of roughly 8 mm in diameter (fig. 3e-h). The long structures seen in the pictures are the result of fungal attachment to the side of the container. These fungal structures have also gathered algae which shows that the process is not restricted to suspended pellets. The harvest efficiency was better in pH4 than in pH7 where the supernatant remained green (fig. 3a-d). In pH4 without KCl the supernatant was completely clear compared to the culture with KCl.

3.5 Algal adhesion to fungal cell wall

Following 3 hours incubation in room temperature nothing macroscopically had visually happened in terms of disintegration of the pellets or release of algae into the supernatant.



(a) *A. niger* **(b)** *A. niger* **(c)** *T. harzianum* **(d)** *T. harzianum*

(e) *A. niger* **(f)** *A. niger* **(g)** *T. harzianum* **(h)** *T. harzianum*

Figure 4. Pellets (a-d) and supernatant (e-h) of cultures of *C. vulgaris* before and after 72 hours of treatment with fungal pellets.

3.6 Algal harvest with premade pellets

Following the 72 hours, the premade pellets were all completely green from the algae (fig. 4a-d). The supernatant of both *A. niger*- and *T. harzianum*-treated cultures were green after 72 hours, either due to incomplete harvest or to the release of algae from the pellets (fig. 4e-h). Provided that all free cells in the supernatant belongs to *C. vulgaris*, table 2 shows that there is a correlation between the color of the supernatant and the number of free cells. The table also shows that although the color of the culture treated with pellets of *T. harzianum* is less green than before the treatment, the number of algal cells have increased.

Table 2. Number of free microalgal cells after pellet treatment (10^8 spores/l). n=3.

	A. niger	*T. harzianum*
Mean	1,96	3,08
Std. dev	0,62	0,52

3.7 Co-cultures with *Scenedesmus* ssp.

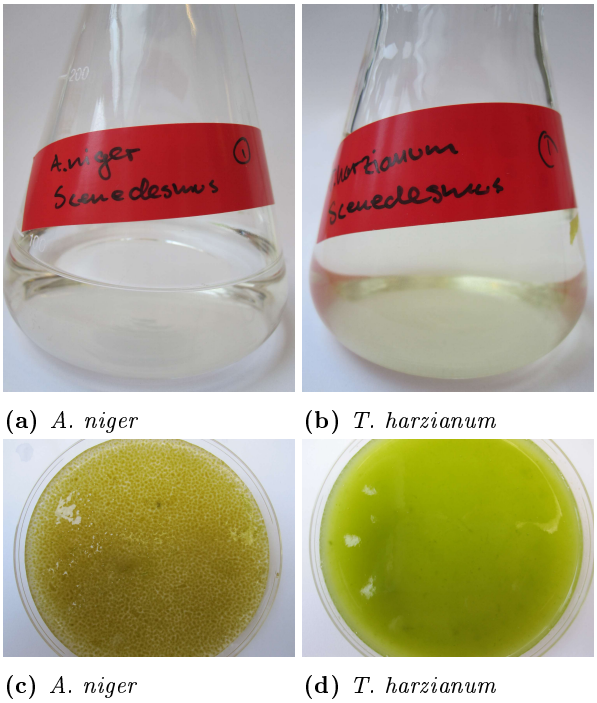
A. niger managed to completely bind all the *Scenedesmus* in two of three replicates and produced a completely clear supernatant (fig. 5a). This could not, at least in this experiment, be repeated with *C. vulgaris* where the supernatant still contained a lot of algae. The pellets produced with both microalgal species were small, about 2 mm in diameter, and homogeneous (fig. 5c).

T. harzianum produced filamentous mats with the algae attached which made the filtering process very hard and time consuming (fig. 5d). The mats formed with *Scenedesmus* were thick enough to be separated by the filter but that was not the case with *C. vulgaris*.

4 Discussion

4.1 Fungal growth requirements and morphology

The pre-trials showed that BG-11 medium supplemented with 0.5 g/l NH_4NO_3 and 0.5 g/l K_2HPO_4 as well as glucose at 5 g/l was sufficient for the spores of both *A. niger* and *T. harzianum* to germinate and produce biomass. As expected, without the carbon source the germination and biomass production severely decreased (fig 1). The difference in morphology between the species can be attributed to differences in the structure of the cell wall which makes the hyphae more or less prone to adhere to itself. α-1,3-glucan might have a similar role in *A. niger* as in *A. fumigatus* where it was pivotal to the aggregation (Henry et al., 2012). This glucan chain can perhaps interact more strongly with itself than other cell wall polysaccharides making the hyphae of *A. niger* form more dense structures than otherwise.



(a) *A. niger* **(b)** *T. harzianum*

(c) *A. niger* **(d)** *T. harzianum*

Figure 5. Separated co-cultures of the fungi and *Scenedesmus* ssp after 48 hours.

The difference in morphology between the glucose supplemented cultures of *T. harzianum* at pH 4 and 7 could be the result of a physiological aspect of the fungus in that it prefers a more acidic environment. One explanation for this could be that the glucose transporter *gtt1* is up regulated and the protein transports glucose through the cell membrane at a faster rate in acidic conditions compared to more neutral ones (Delgado-Jarana et al., 2003). The increased activity of the transporter can perhaps contribute to the higher biomass production seen in figure 1f compared to 1h.

The alternative method of microalgal harvest put forward by Wrede et al. (2014) was investigated by the addition of pre-made pellets to cultures of *C. vulgaris*. One of the advantages of this method is that the fungal pellets are produced in a separate step that can be optimized in terms of nutrition, pH and agitation. An interesting observation is the difference in morphology between the pellets of *T. harzianum* in this experiment (fig. 4c) compared to the pellets made in pre-trials (fig. 1f). While the conditions concerning medium and pH were identical, the time, volume and initial spore concentration differed. This may reflect an aspect of the pelletization mechanism in this species, that an increased spore concentration leads to a higher amount of germinating spores and therefore a higher probability of aggregation and subsequent pelletization of the hyphae.

4.2 Co-cultures and surface modifications

In the co-cultures of the fungi with *C. vulgaris*, the morphology of the resulting structure varied greatly between the fungal species. *T. harzianum* produced loose filamentous structures which contrasts both its own morphology in pure culture and the co-culture of *A. niger* and *C. vulgaris*. One possible explanation for this is that the algae induce the production of enzymes which affects the surface of the hyphae in such a way that no ordered macroscopical structure can be achieved, with or without the algae. Gómez-Mendoza et al. (2014) showed that the carbon source greatly influences the regulation of secreted enzymes in *T. harzianum* and that the difference in enzyme activity between cultures grown on glucose and a native complex substrate can be several orders. Coupled with the differences in morphology seen when pure and co-culture are compared (fig. 1f and 1h vs. fig. 2f and h), it points to a mechanism ensuring an efficient use of energy and material. By inhibiting self-aggregation through enzymatic cell wall modifications when facing a complex substrate, the fungus ensures a maximum coverage of the substrate and thus an increase in potential nutrient uptake.

The addition of KCl influenced the forces governing the adhesion of *C. vulgaris* to the hyphae of *A. niger*. When comparing fig. 3a and 3b, it is clear that KCl in the medium leads to a less transparent supernatant. According to Sirmerova et al. (2013), the potassium ions changes the zeta potential of the algae towards a more positive value and Rajab (2007) reported a positive zeta potential of the pellets of *A. flavus*. If this holds true for *A. niger* as well the decrease in transparency can be explained in terms of a repelling electrostatic force between the surfaces.

The attempt to enzymatically modify the surface of *A. niger* by treatment with the supernatant of *T. harzianum* supposedly containing mutanase (Guggenheim & Haller, 1972), did not result in any visual changes of neither fungal nor co-pellets. The reason for this can once again be sought in the differential secretion of enzymes depending on the carbon source. Gómez-Mendoza et al. (2014) found that cultures of *T. harzianum* with glucose as carbon source had minimal enzymatic activity in the supernatant and this might apply to the mutanase activity as well.

When the method of alternative microalgal harvest was investigated, pre-made pellets of both *A. niger* and *T. harzianum* collected algae to their surface. As with the case of Rajab (2007), the mechanics of this adhesion can possibly be explained by electrostatic attraction. The amount of algae still in the supernatant varied with *A. niger* showing a better harvest efficiency than *T. harzianum* (table 2), perhaps due to differences in surface charge. These numbers also shows that there was a considerable growth of the algae during the harvesting step as the amount was higher after the harvesting with *T. harzianum* than before. This shows that the harvesting process should be quick enough to counteract the growth of the algae or that the pellet load should be high enough to handle the continued growth. Wrede et al. (2014) reported that the number of free algae in the supernatant increased after 24 hours of treatment with pellets of *A. fumigatus* and explained this by growth or release of the algae. In either case, this

method allows the replacement of the fungal pellets after an optimized time span by simple sieving due to the size of the pellets. This time span may vary between fungal species and/or the combination of fungus-microalgae.

The last experiment expanded on the results of the co-culture with *C. vulgaris* and showed that *Scenedesmus* ssp. also can be harvested by a similar process. The morphological differences between *A. niger* and *T. harzianum* could once again be seen with the latter producing thick filamentous mats compared to the homogeneous co-pellets of the former. The results of the control with *C. vulgaris* varied both from the earlier co-pelletizations and the co-pelletization with *Scenedesmus* ssp. This can be explained by the fact that the culture of *C. vulgaris* were kept at 8°C for three days prior to this experiment which possibly killed or changed the physiology of the cells. If the cells died, compounds might be released into the medium and the cell wall structures might not accommodate interactions as efficiently.

5 Conclusions

This project have shown that it is possible to harvest microalgae with the help of fungi, both through co-culture pelletization and through pre-made fungal pellets. The process in not limited to single species of either fungus or microalgae but each combination requires specific conditions. In the co-culture it is of high importance that the conditions favors the germination and growth of the fungus as the amount of fungal biomass is one of the deciding factors of the amount of microalgae that can be harvested.

In the scope of this project it was not possible to produce co-pellets between *T. harzianum* and the microalgae, possibly due to enzymatic cell wall modifications of the fungus induced by the algae. *A. niger* did not have this problem and can therefore be considered easier to work with as both co-cultures and pre-made pellets can be used as harvesting methods. Based on the opacity of the supernatant when KCl was added to the medium, it can be said that electrostatic interactions to some extent influences the microalgal adhesion to the fungal hyphae.

5.1 Recommendations

To further elucidate the mechanism by which the microalgae adhere to the hyphae, experiments with pre-made pellets can be made. By using pre-made pellets instead of co-cultures it is easier to assess the number of free microalgal cells in the supernatant since the size of the cells and the ungerminated spores are roughly equal. By increasing the ionic strength of the medium, as in 2.4, the question of electrostatic interactions can be more thoroughly examined. If a similar result is obtained as in 3.4, it can be said with some certitude that electrostatic interactions does play a major role. And by adding plurivalent cations or surfactants the polar interactions can be assessed.

6 Applications

Much of the previous work concerned with fungal pelletization of microalgae have focused on the utilization of the co-pellets in treatment of wastewater (Zhou, Cheng, et al., 2012; Zhou, Min, et al., 2013; Wrede et al., 2014). This is indeed an efficient use of biological matter as nutrient filters which strips the wastewater of its carbon-, nitrogen- and phosphorous-containing compounds (Zhou, Cheng, et al., 2012). The co-pellets can then be a source of both lipids and fermentable products which can be processed into biofuels (Wrede et al., 2014). While this is an efficient recycling of waste output streams, fuel and energy are low-value end-products (Bos-Brouwers et al., 2012), which makes it important to minimize the processing costs to keep the end-products competitive.

The source of the wastewater decides how it can be utilized, while a municipal or agricultural source can be used for bioenergy purposes due to the impurities, wastewater from the food industry can be used for more value-added products. By culturing microalgae and/or fungi in 'clean' wastewater the end-product can be used as food or feed. Depending on the usage, the harvesting process will differ and the use of fungal pelletization can be used in cases where the algal purity is of secondary importance. Alternative harvesting processes, such as algal aggregation agents (Guo et al., 2013; Salim et al., 2014) or biological surfactants can instead be used when microalgal purity is the main goal.

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