Microalgae Harvesting Via Co-Culture With Filamentous Fungus

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DEDICATION

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ABSTRACT

Microalgae harvesting is a labor- and energy-intensive process. For instance, classical harvesting technologies such as chemical addition and mechanical separation are economically prohibiting for biofuel production. Newer approaches to harvest microalgae have been developed in order to decrease costs. Among these new methods, fungal co-pelletization seems to be a promising technology. By co-culturing filamentous fungi with microalgae, it is possible to form pellets, which can easily be separated. In this study, different parameters for the cultivation of filamentous fungus (*Aspergillus niger*) and microalgae (*Chlorella vulgaris*) to efficiently form cell pellets were evaluated under heterotrophic and phototrophic conditions, including organic carbon source (glucose, glycerol and sodium acetate) concentration, pH, initial concentration of fungal spores, initial concentration of microalgal cells, concentration of ionic strength (Calcium and Magnesium) and concentration of salinity (NaCl). In addition, zeta-potential measurements were carried out in order to get a better understanding of the mechanism of attraction.

We have found that 2 g/L of glucose, a fungi to microalgae ratio of 1:300, and uncontrolled pH (around 7) are the best culturing conditions for co-pelletization. Under these conditions, it was possible to achieve a high harvesting performance (>90%). In addition, it was observed that most pellets formed in the co-culture were spherical with an average diameter of 3.5 mm and in concentrations of about 5 pellets per mL of culture media. Under phototrophic conditions, co-pelletization required the addition of glucose as organic carbon source to sustain the growth of fungi and to allow the harvesting of microalgae. Zeta-potential measurements indicated that (i) both microalgae and fungi have low zeta-potential values regardless of the pH on the bulk (i.e. <-10 mV) (ii) fungi can have a positive electric charge at low pH (ie. pH=3). These values suggest that it might be possible that the degree of repulsion and dispersion between these organisms is low which facilitates the attraction between them.

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CHAPTER 1
BACKGROUND

1.1. Renewable Energy

An increasing energy demand and a potential fossil fuels depletion have become major concerns for people around the world. Furthermore, climatic changes and global warming possibly caused by the emission of greenhouse gases have become additional contemporary problems that have to be solved (Borowitzka, 1999; Miao & Wu, 2006; Chisti, 2007). Therefore, renewable energy sources, such as solar, biomass, geothermal, wind and hydropower, are being used as alternative sources of energy. Biomass is a renewable, biodegradable, and carbon dioxide (CO_2) neutral energy source with plenty of resources as, for instance, agricultural residue and waste, forestry waste, municipal solid and industrial waste, terrestrial crops, and aquatic plants (Tsukahara & Sawayama, 2005).

Because of its low cultivation and production costs, bio-diesel, which is generated from oil produced by terrestrial crops, is currently a widely used alternative for transportation fuels. However, limited land availability confines the amount of biofuel that can be produced. Unlike traditional oilseed crops, microalgae show great potential due to their potential applications for industrial CO_2 removal, their higher photosynthetic efficiency, their higher biomass production, their faster growth, their production of a great variety of metabolites, and their capability to grow both in ponds and in fermentation units and thus not requiring precious farmlands and forests (Yang, Hua, & Shimizu, 2000; Miao & Wu, 2006).

1.2. Microalgae

1.2.1. What are microalgae?

Microalgae are microscopic unicellular organisms that have the same characteristics as higher plants. They have the capability to convert solar energy into chemical energy through photosynthesis. As photosynthetic microorganisms, microalgae are able to use sunlight and assimilate carbon dioxide from the air under natural growth conditions (Wang, Li, Wu, & Lan, 2008). Compared to other plant systems, microalgae can grow exponentially under variable environmental conditions (Wang, Li, Wu, & Lan, 2008; Benemann, 1997; Zeiler, Heacox, Toon, Kadam, & Brown, 1995). In addition to sunlight and CO_2, nutrients, trace metals and water are also needed for microalgae to grow. In short, the reaction in microalgae cultivation which produces biomass can be formulated as follows (Mosojidek, Torzillo, & Erik, 2008):

$$CO_2 + H_2O + \text{nutrients} + \text{light energy} \text{ -----> } \text{biomass} + O_2$$

1.2.2. Microalgal cultivation systems

In general, there are several factors to be considered before the cultivation system is chosen, including the microalgae strain, the biological needs of microalgae, the nutrient availability, the climate conditions, the availability of sunlight, water and carbon dioxide and the desired final product (Mosojidek, Torzillo, & Erik, 2008; Borowitzka, 1999).

There are two main microalgal cultivation systems: (1) open-air systems or open systems and (2) closed systems (Borowitzka, 1999). Open systems are designed for a large-scale algal biomass production with a lower cost because of its lower energy input requirement (Rodolf, et al., 2008). However, the main drawback of open systems is that the culture can be contaminated with other microalgae species or protozoa (Pulz &

Scheibenbogen, 1998). To solve this problem, closed cultivation systems were developed and applied because of their ability to reduce the risk of contamination for sensitive strains of microalgae (Chisti, 2007).

There are various types of closed systems that are operated for microalgae cultivation, including flat-plate, tubular and column photobioreactors. In general, photobioreactors are designed with a transparent vessel exposed to light, in which the microalgal cells are circulating with nutrients and CO_2. The earliest form of photobioreactor, the flat-plate photobioreactor is considered suitable for mass cultures of microalgae because of its low accumulation of dissolved oxygen and its high efficiency of photosynthesis compared to the tubular photobioreactor (Richmond, 2000). Tubular photobioreactors, however, are widely used for outdoor mass cultures because of their large surface area exposed to sunlight (Pulz & Scheibenbogen, 1998). Unlike flat-plate and tubular photobioreactors, column photobioreactors are capable to efficiently mix cultures and to control the growth conditions of microalgae (Eriksen, 2008).

It has been reported that the use of photobioreactors could have higher biomass production rates in comparison with open cultivation systems because they have a more accurate process control. However, in terms of the operational costs, the costs of photobioreactors are higher than those of the open systems (Carvalho, Meireles, & Malcata, 2006).

1.2.3. Growth conditions of microalgae

In general, there are three modes of microalgae growth, including under phototrophic (autotrophic photosynthesis), heterotrophic or mixotrophic conditions. Under phototrophic conditions, the growth of microalgae primarily depends on sunlight

and carbon dioxide as energy and carbon source. Under heterotrophic growth conditions, instead of harvesting light and assimilating carbon dioxide from air, microalgae use organic carbon substrates such as glucose, acetate and glycerol as their energy source. It was observed that the production of microalgal biomass as well as the lipid content in the microalgal cells under heterotrophic conditions were much higher than those under phototrophic growth conditions (Miao & Wu, 2006; Liang, Sarkany, & Cui, 2009). Because of the higher cell density achieved under these conditions, the microalgae harvesting costs are lower compared to phototrophic conditions. Mixotrophic growth is a combination of phototrophic and heterotrophic conditions, where some microalgae strains have the capability to combine autotrophic photosynthesis and the heterotrophic assimilation of organic compounds, either simultaneously or sequentially.

1.2.4. Harvesting methods of microalgae

The most difficult step in microalgae cultivation and utilization is the harvesting of microalgae. Since microalgae cells have specific characteristics such as a low density (typically in the range of 0.3-5 g/L) and a small size (typically in the range of 2-20 μm), they are technically challenging to be harvested. A number of harvesting techniques have been applied, such as flocculation and ultrasonic aggregation, flotation, gravity and centrifugation sedimentation, and filtration, as well as a combination of the above. A brief discussion of the most common harvesting methods is included below.

Flocculation

It is widely known that most microalgae have negatively charged cell surfaces. This charge is caused by the ionization of iongenic functional groups on the microalgal cell walls and also by the adsorption of ions from the culture medium. Algal species, the

ionic strength of medium, pH and other environmental conditions have a great impact on the development of this property (Ives, 1959; Golueke & Oswald, 1965; Shelef, Sukenik, & Green, 1984). This negative charge of the cell surface is important for microalgae growth, especially for preventing the natural aggregation of suspended cells (Grima, Belarbi, Fernandez, Medina, & Chisti, 2003).

The surface charge of microalgae cells is also considered as an important property for harvesting microalgae. Neutralizing and reducing microalgal surface charge by applying positively charged electrodes and cationic polymers is commonly done in order to flocculate the microalgal biomass. Some multivalent salts such as ferric chloride ($FeCl_3$), aluminum sulfate ($Al_2(SO_4)_3$, alum) and ferric sulfate ($Fe_2(SO_4)_3$) have been widely used as flocculants and coagulants (Koopman & Lincoln, 1983; Grima, Belarbi, Fernandez, Medina, & Chisti, 2003). Some studies also reported that the flocculation of microalgal biomass could be carried out by adjusting the pH and by simple electrolysis (Wu, et al., 2012; Poelman, De Pauw, & Jeurissen, 1997).

Since this harvesting method is producing toxic waste and is relatively expensive because of the cost of flocculants, inexpensive and nontoxic flocculants should be considered for future flocculation processes. In addition, increased efficiency and the use of a lower concentration of flocculants are also very important in order to reduce the harvesting cost (Grima, Belarbi, Fernandez, Medina, & Chisti, 2003).

Flotation

The flotation method is a gravity separation process in which the microalgal cells attach to air or gas bubbles and accumulate as float, which can be skimmed off. For some microalgal strains, natural float at the surface of the water occurs when their lipid content

increases. In addition, in most cases flocculants are also applied for effective flotation (Edzwald, 1993).

There are various flotation processes based on the method of bubble production: dissolved air flotation (DAF), electrolytic flotation, and dispersed air flotation (Shelef, Sukenik, & Green, 1984). DAF is a process where small bubbles are generated (10 to 100 μm), which is widely used for wastewater treatment rather than for microalgal biomass production (Christenson & Sims , 2011). In electrolytic flotation, gas bubbles are formed through electrolysis. Because of the use of electricity, this method is very energy-intensive. For dispersed air flotation, either foam flotation, large bubbles (1 mm) moving through porous media or froth flotation,, a combination of agitation and air injection, are applied (Shelef, Sukenik, & Green, 1984).

If small bubbles are required, the energy usage for the flotation processes will be very high, which inevitably results in high operational costs and therefore a required high investment.. The costs can be even greater when the costs of flocculants are included (Mohn, 1998). Moreover, there is very limited information and evidence showing that flotation methods are technically or economically feasible (Brennan & Owende, 2010).

Centrifugation

In the centrifugal separation process, a much greater force replaces gravity as the force dividing separation (Mohn, 1998). The force applied in this process may be from 4,000 to 14,000 times gravitational force. Various centrifugal methods have been applied for microalgae separation, such as tubular centrifuge, multi-chamber centrifuges, imperforate basket centrifuge, decanter, solid retaining disc centrifuge, nozzle type centrifuge, solid ejecting type disc centrifuge, and hydro-cyclone (Shelef, Sukenik, &

Green, 1984). It was reported that microalgal pastes with higher solid contents (above 15%) were harvested through centrifugation methods than through other methods (Sim, Goh, & Becker , 1988). Moreover, some studies revealed that microalgal recovery could be very high (>90%) when higher centrifugation speeds were applied (Heasman, Deamar, O'Connor, Sushames, & Foulkes, 2000).

Even though centrifugation is a very efficient cell harvesting method for microalgae, it is obvious that the process is energy-intensive. High capital and operational costs, and potentially higher maintenance requirements are the main disadvantages of this process (Mohn, 1998; Grima, Belarbi, Fernandez, Medina, & Chisti, 2003; Bosma, van Spronsen, Tramper, & Wijffels, 2003; Shen, Yuan, Pei, Wu, & Mao, 2009).

Sedimentation

Gravity sedimentation is a common harvesting method for algae biomass in wastewater treatment. This method is based on Stoke's Law (Eq 1.1), which determines the characteristics of suspended solid by density, radius, and sedimentation velocity of algae cells (Schenk, et al., 2008). Stoke's Law can only be used for non-flocculated particles and is not applicable for flocculated particles because of the complicated structure of the flocs (Shelef, Sukenik, & Green, 1984).

$$v = \frac{2}{9} r^2 g \frac{\rho_p - \rho_f}{\eta} \qquad (1.1)$$

Where:

v	particle settling velocity [m/s]
r	cell radius [m]
g	gravitational acceleration (9.81) [m/s^2]
ρ_p	mass density particle [kg/m^3]
ρ_f	mass density fluid [kg/m^3]
η	dynamic viscosity [N s/m^2]

Even though this method is considered as a low-cost, simple technique because there are no extra forces other than gravity, the method is only suitable for large microalgae (>70 μm) such as *Spirulina* (Munoz & Guieysse, 2006). Therefore, for microalgae with a smaller size, chemical flocculation is needed for gravity sedimentation in order to enable the harvesting of algae (Shelef, Sukenik, & Green, 1984).

Filtration

There are some types of filtration systems that have been used for microalgae harvesting, such as filter presses, vibrating screens, micro-strains, belt filters and vacuum drums (Milledge & Heaven, 2012). Membrane pore size is the most important component that is used to classify filtration systems. Filtration systems can be classified as macro-filtration (pore size of >10 μm), micro-filtration (pore size of 0.1-10 μm), ultra-filtration (pore size of 0.02-2 μm) and reverse osmosis (pore size of <0.001 μm). In most cases, pressure is needed to force the liquid through the membrane and thus accelerate the water removal (Jaouen, Vandanjon, & Quemeneur, 1999).

Macro-filtration is the most appropriate method for larger microalgae (>70 μm) such as *Coelastrum* and *Spirulina*. Micro-filtration and ultra-filtration are widely used for the recovery of smaller microalgae cells (<30μm). Ultra-filtration is suitable for fragile cells because it requires low trans-membrane pressure and low cross-flow velocity conditions. Even so, ultra-filtration has not generally been used because operating and maintenance costs are high (Borowitzka, 1999, Rossignol, Vandanjon, Jaouen, & Quemeneur, 1999; Brennan & Owende, 2010; Milledge & Heaven, 2012).

Membrane filtration can be more cost-effective for low broth volumes (<2 m^3). However, in large-scale production (>20 m^3), this method will be less effective because

of the costs of the membrane and the energy needed (Grima, Belarbi, Fernandez, Medina, & Chisti, 2003). It was also found that for large-scale and high-density algae cultivation, cell packing could be a significant problem with this method. Although stirring and shaking can offer a solution to this problem, a lot of energy is needed for this process, especially for a large amount of liquid (Morris & Yentsch, 1972).

1.3. Filamentous fungi

1.3.1. Morphology of filamentous fungi

Filamentous fungi can have various morphologies in submerged cultures, depending on the organism and on the operated culture conditions (Gibbs, Seviour, & Schmid, 2000). Morphologies include: dispersed hyphae, microscopic aggregates, loose hyphal aggregates which are commonly referred to as clumps and denser spherical aggregates, commonly referred to as pellets (Cox, Paul, & Thomas, 1998).

Filamentous organisms have the common feature hyphae, which is a polarized pattern in the form of filaments. These hyphal elements originate from the outgrowth of single cells or spores as multinucleate tubes containing cytoplast, which moves within a hypha toward the hyphal tip. The hyphal tip is the only place where the hypha grows. Out of the main hyphae, new tips are formed which produce branches, resulting in a network of hyphae, which is termed mycelia (Znidarsic & Pavko, 2001, Zmak, Podgornik, Podgornik, & Koloini, 2006,). Mycelial growth can be divided into microscopic and macroscopic morphology (Krull, et al., 2010; Krull, et al., 2013).

Microscopic morphology

The growth of fungi will begin with a single spore or several spores. Without supplying nutrients necessary for activation, the spores will remain inactive. By

absorbing much water in the early phase of the germination process, the spores extend in an isotropic manner (Osherov & May, 2001). In the early phase of cultivation, the microscopic morphology of filamentous fungi can be described by two important parameters, namely the total hyphal length L [μm], which is obtained by summing up all hyphae in a mycelium, and the number of tips n [-](Metz & Kossen, 1977; Trinci, 1974). These parameters can be determined through digital image analysis.

Kinetically, the exponential growth of mycelia can be described by a specific length growth rate μ [h^{-1}] (Grimm, Kelly, Hengstler, Gobel, Krull, & Hempel, 2004).

$$\frac{dL}{dt} = \mu \cdot L \tag{1.2}$$

In general, mycelial growth is composed of an increase in the length of each tip and an increase of the number of tips produced by branching. With q_{tip} [μm h^{-1}] being a constant tip growth rate of n tip, the total hyphal length growth can be determined (Grimm, Kelly, Hengstler, Gobel, Krull, & Hempel, 2004).

$$\frac{dL}{dt} = q_{tip} \cdot n \tag{1.3}$$

It was found that the blanching process relied on mycelial length and has been regarded as a branching constant k_{bran} [$\mu m^{-1} h^{-1}$] (Grimm, Kelly, Hengstler, Gobel, Krull, & Hempel, 2004).

$$\frac{dn}{dt} = k_{bran} \cdot L \tag{1.4}$$

An essential relationship between tip growth and hyphal branching is described as hyphal growth unit (HGU), which is the average length of each tip in a mycelium (Caldwell and Trinci 1973; Grimm, Kelly, Hengstler, Gobel, Krull, & Hempel, 2004).

$$HGU = \frac{L}{n} \tag{1.5}$$

HGU increases during the first stage of mycelial growth until a steady state is reached. Consequently, the amount of hyphal tips starts increasing exponentially (Bergter, 1978; Prosser & Trinci, 1979).

Macroscopic morphology

The final micromorphology of filamentous fungi is determined by several factors such as the properties of the strain, and the applied cultivation conditions (e.g. inoculum properties, mechanical stress, temperature, medium composition and pH) (Papagianni , 22). The morphological processes and other physic-chemical environmental treatments result in the apparent macro-morphology that is known by the size and the structure of the growth form (Krull, et al., 2013).

In terms of pellet formation, there are two ways for filamentous fungi to form pellets, namely non-coagulative formation and coagulative formation. For the non-coagulating type, pellets are formed out of one spore. This type of pellet formation has been reported for some *actinomycetes* from the genus *Streptomyces* (Vecht-Lifshitz, Magdassi, & Braun, 1990 ; Znidarsic & Pavko, 2001) and for fungi belonging to *Rhizopus spp.* and *Mucor spp* (Metz & Kossen, 1977). In coagulating pellet formation, the spores usually agregate in the early stage of cultivation and pellets are formed out of these agregates. This type of pellet formation can include *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae* and the basidiomycete fungus *Phanerochaete chrysosporium* (Metz & Kossen, 1977; Grimm, Kelly, Hengstler, Gobel, Krull, & Hempel, 2004; Znidarsic & Pavko, 2001).

Aspergillus niger, a common filamentous fungus that has been studied for a long time, gives a clear image of the microscopic morphology that is related to the formation of the pellets (Grimm, Kelly, Hengstler, Gobel, Krull, & Hempel, 2004). After inoculation, the agregation of conidia begins immediately and keeps steady during the first stage of cultivation. In this stage, pH values strongly affect the process. A second aggregation step is triggered by the germination process and the hyphal growth of germ tubes, which increase the hyphal surface area to which conidia can attach. In the final growth stage, pellet formation begins after the second aggregation (Krull, et al., 2010; Grimm, Kelly, Hengstler, Gobel, Krull, & Hempel, 2004).

1.3.2. Pellet

Depending on the operating conditions, some filamentous fungi may form pellets when they are grown submerged. Numerous studies on pellets have already been carried out. It was found that the structure of pellets could vary significantly from a very loose and irregular form to a compact and spherical form (Metz & Kossen, 1977; Liao, Liu, & Chen, 2007). Based on their structure, pellets can be classified into three groups: a) fluffy, loose pellets, where the center of the pellets is compact and the outer zone is much looser, b) Compact smooth pellets, where the whole pellet is compact and the outside of the pellet is smooth, and c) Hollow smooth pellets, where the center of the pellet is hollow due to autolysis and the outside is smooth (Metz & Kossen, 1977).

It has been observed that the structure and formation of pellets is determined by many different factors. Microbiological factors including genetics, cell wall composition, inoculum size, growth rate, nutrition, and the C-N ratio and physicochemical factors including share forces, surface active agents, pH, temperature, Ca^{2}+ ion, ionic strength,

and suspended solids have been found to impact the structure and formation of pellets (Braun & Vecht-Lifshitz, 1991). However, many studies revealed that the structural properties and the pelletization process are very strongly dependent on cultivation conditions (Metz & Kossen, 1977; Zmak, Podgornik, Podgornik, & Koloini, 2006).

1.4. Pelletization of filamentous fungi on harvesting microalgae cells

Even though pelletization of filamentous organisms is more widely used in the fungal fermentation process, recent studies revealed that a pelletization of filamentous fungi could be applied to harvest microalgal cells. Zhang and Hu's study showed that out of 12 filamentous fungi cultured together with microalgae, 7 fungi formed green-colored pellets instead of milky white-colored pellets. This indicated that some microalgal cells attached to or were entrapped in the pellets (Zhang & Bo, 2012). Moreover, another study carried out by Zhou found out that the filamentous fungus *Aspergillus orizae*, which was isolated from municipal wastewater sludge, could be used to assist in the harvesting process of microalgal cells (Zhou, et al., 2013).

1.5. Surface charge as an approach for the co-pelletization process

The concentration of counter-ion close to the surface of a particle is affected by the development of a charge at the particle surface. There are two layers surrounding the particles, namely the 'stern layer' and the 'diffuse layer'. The stern layer is a dense layer with a strong boundary where other counter-ions are difficult to access. The diffuse layer is a layer where ions can move freely along with the particle because there is an unreal boundary within this layer. These two layers create a system of the charge both in the particle surface and in the solution around the particle called the electrical double layer (Salgın , Salgin, & Bahadir, 2012; Vandamme, Foubert, & Muylaert, 2013).

The outer boundary of the diffuse layer consists of a zero charge where the concentrations of co-ions and counter-ions are equal. At the end of the diffuse layer, there is a boundary called the slipping plane where all ions do not move and stay where they are. At this boundary, there is a potential that is commonly used to evaluate the surface charge of a particle called the zeta potential (ζ) (Salgın , Salgin, & Bahadir, 2012; Cai, Frant, Bossert, Hildebrand, Liefeith, & Jandt, 2006).

Estimation of the particle surface charge by the zeta potential measurement is considerably easy because it can be determined by measuring the mobility of charged particles in an electrical field. Because of its importance in characterizing the particle surface properties, the zeta potential is widely used for a number of applications such as for the characterization of material properties in pharmaceuticals, for water treatment and separation and for the purification process ((Vandamme, Foubert, & Muylaert, 2013; Sze, Erickson, Ren, & Li, 2003).

Based on the DVLO (Derjaguin, Landau, Verwey and Overbeek) theory, the interaction between colloidal charged particles can be explained. This theory describes the interaction between two actions; (i) electrostatic repulsion and (ii) Van der Waals forces in a model of colloidal interaction (Gregory, 2006). To evaluate the stability of colloids in the systems, zeta potential is applied as a key parameter. The repulsion between particles is said to be strong when the zeta potential values are more then ±25 mV. In contrast, when the zeta potential values are close to zero (isoelectric), particles will attract each other because of the Van der Waals forces. At this condition, aggregation of particles will occur.(Vandamme, Foubert, & Muylaert, 2013).

According to Shelef, Sukenik, & Green (1984), the surface charge and the cell size and density are the main factors for the stability of microalgae suspension in the culture. In terms of surface charge, it was reported that at neutral pH, microalgae reveal a slightly negative charge because of the presence of proton-active carboxylic, phosphoric, phosphodiester, hydroxyl and amine functional groups (Grima, Belarbi, Fernandez, Medina, & Chisti, 2003; Hadjoudja, Deluchat, & Baudu, 2010). Typical zeta potential values of microalgae are within the range of -10 to -35 mV (Henderson, Parsons, & Jefferso, 2008).

1.6. Perspectives and Objectives

Since microalgae cells have specific characteristics such as a low density (typically in the range of 0.3-5 g/L, which is similar to water) and a small size (typically in the range of 2-20 μm), they are technically challenging to be harvested. Several methods have been applied to harvest microalgae cells such as flocculation, flotation, centrifugal sedimentation, and filtration. However, all of these approaches have limitations for the effective, cost-efficient production of biofuel (Shelef, Sukenik and Green 1984). A new method for harvesting microalgae cells assisted by filamentous fungi has been applied in this research. Several strains of filamentous fungi were selected and applied to microalgae cells in order to know their capability for co-pelletization (Zhang and Hu, 2012). However, in order to optimize the co-pelletization process, the optimum conditions for fungi-microalgae co-cultures under heterotrophic and phototrophic conditions must be considered. Moreover, to fully grasp the co-pelletization process, a deeper understanding of the mechanism of co-pelletization and the interaction between fungi and microalgae is needed.

The aim of this master thesis is to understand and optimize the conditions for the co-culturing of microalgae with filamentous fungi. This study can be a good foundation to develop a novel method to harvest microalgae through the pelletization of filamentous fungi by investigating the best conditions for this co-culture as well as the cause of co-pelletization. The specific objectives of this work are:

1. To quantify the effects of key factors on co-pelletization.
2. To optimize the co-culturing process.
3. To evaluate the harvest performance of co-pelletization.
4. To evaluate the morphology of pellets.
5. To gain a better understanding of the attraction mechanisms between fungi and microalgae during co-pelletization.

CHAPTER 2
MATERIALS AND METHODS

2.1. Microalgae strain

The cells of *C. vulgaris* (UTEX 2714) were purchased from the culture collection of algae of The University of Texas (UTEX, Texas, USA). The fresh microalgae cells were inoculated into a 250 mL flask filled with 100 ml of autotrophic culture medium and were stirred by a shaking incubator (150 rpm) at 25^{o}C in the presence of light (continuous light). To provide sufficient light for microalgae growth, four fluorescent lights were placed outside the flask. After the microalgae cells achieved a concentration of 8.50E+09, the broth was transferred to a 4 L flask filled with 3 L autotrophic culture medium. This was stirred with a magnetic stirrer (200 rpm) at a temperature of 24^{o}C (Figure 2.1)...The inoculation was performed inside a biological safety cabinet (Model 1823 S/N 13321-131, Forma Scientific, Inc.) to avoid contamination as well as to maintain an axenic environment.

Fresh cultural medium was routinely added (every 2 weeks) into the flask to maintain the active cell growth. This microalgae culture was used as a mother broth to provide algae seeds for the co-culture experiments. The health of the microalgae cells was routinely checked by observing the culture under a light microscope (National DC5-163 digital) using 40x magnitude and by sub-culturing them on Petri dishes, which contained approximately 20 mL autotrophic culture medium with 2% agar.

2.2. Fungus strain

Filamentous fungus *Aspergillus niger* (Ted S-OSU) was used for this study. The fungal spores were purchased from ATCC (biological resource center) and were stored

with 25% of glycerol at -71^{0}C. To activate the fungal cells, the spores were cultivated at 27^{o}C for 7 days on a Petri dish, which contained approximately 20 ml of 12 g/L of potato dextrose, 15 g/L of glucose and 2% agar. After the fungal spores were grown in the medium, 10 ml of sterilized distilled water was applied to harvest the spores (Figure 2.2). The number of the spores in the spore solution was then counted under a light microscope (National DC5-163 digital) using 40x magnitude and the solution was then applied as inoculum for the co-culture experiments.

Figure 2. 1. Mother culture of microalgae *Chlorella vulgaris*.

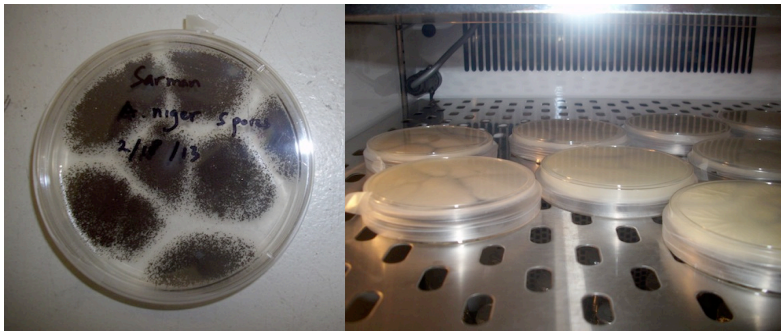

Figure 2. 2. Spores of fungus *Aspergillus niger*.

2.3. Culture medium

The mother culture medium was prepared according to Gladue and Maxey (1994) and is shown in Table 2.1. Autotrophic growth of microalgae was achieved using this medium without an organic carbon source. The trace metal solution (A_5) was added to the

medium in order to provide micronutrients for algal growth. The composition of A_5 is shown in Table 2.2 (Gladue & Maxey, 1994).

Table 2. 1. Autotrophic growth medium for *Chlorella vulgaris.*

Components	Final Concentration (g/L)
KNO_3	1
KH_2PO_4	0.075
K_2HPO_4	0.1
$MgSO_{4\cdot2}H_2O$	0.5
$Ca(NO_3)_{2\cdot4}H_2O$	0.0625
$FeSO_{4\cdot7}H_2O$	0.01
Yeast extract	0.5
Trace metal solution (A_5)	1 ml/L

Table 2. 2. The trace metal solution (A_5)

Components	Concentration (mg/L)
H_3BO	2.86
$Na_2Mo_{4\cdot2}H_2O$	0.39
$ZnSO_{4\cdot7}H_2O$	0.22
$MnCl_{2\cdot4}H_2O$	1.81
$CuSO_{4\cdot5}H_2O$	0.079
$Cu(NO_3)_{2\cdot6}H_2O$	0.049

2.4. Condition optimization for co-pelletization process

Different experiments were designed to attempt the optimization of the co-pelletization process. Different parameters such as glucose concentration, initial fungi to algae ratio and pH were studied. Generally, the cultivations were carried out in 250 mL Erlenmeyer with a working volume of 100 mL of solution. Flasks were shaken at 150 rpm, 27^{o}C for 3 days without the presence of light. Controls were also prepared with only fungi and only microalgae cultures. After 3 days of cultivation, flasks were evaluated qualitatively and quantitatively, i.e. pallet size and morphology through visual observation (Figure 2.3), the percentage of microalgae removal from the supernatant (attached to pellets), pH and cell concentrations.

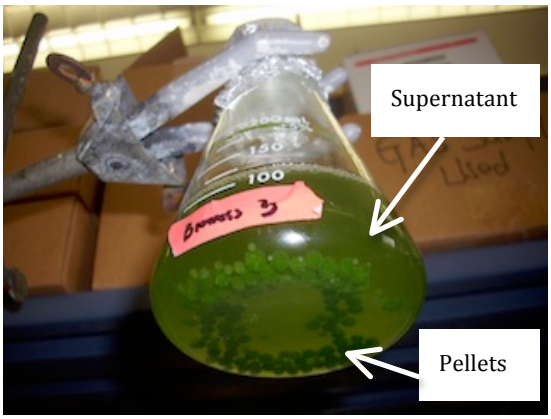

Figure 2. 3. Pellets and supernatant in the co-culture medium.

2.4.1. The effects of carbon sources on the co-pelletization process

Glucose as a carbon source

Since there was no light present under these conditions, organic carbon was the main source of both carbon and energy to support the microalgal and fungal growth. To study the effects of carbon on co-pelletization, co-culture experiments were prepared at five different concentrations of glucose (1, 2, 4, 6 and 10 g/L). No pH adjustment was applied under these conditions. The inoculum size of microalgae (2.55E+09 cells/L) and the inoculum size of fungi (8.5E+06 spores/L) were applied to this co-culture. After 3 days of cultivation, the pellets were harvested and further analyzed. The glucose concentration that corresponded to the highest removal of microalgae was applied to the next experiments. The pure culture of fungus as well as that of microalgae was used as a control and as a comparison with the co-culture. The summary of the co-culture conditions is shown in Table 2.3.

Table 2. 3. Co-culture conditions at different initial concentrations of glucose.

Shaker speed	150 rpm
Temperature	27^{o}C
Initial medium pH	6.8
Inoculum concentration of fungal spores	8.50E+06 spores/L
Inoculum concentration of microalgal cells	2.55E+09 cells/L
Total volume of the co-culture	100 mL
Cultivation time	3 days
Initial glucose concentration	1, 2, 4, 6, 10 g/L

Acetate and glycerol as carbon sources

Other carbon sources such as sodium acetate and glycerol were also applied to the co-culture to observe the effects of different carbon sources on the co-pelletization process. The optimal concentration of glucose was applied to a concentration of both sodium acetate and glycerol in this experiment. The pure culture of fungus as well as that of microalgae was used as a control and as a comparison with the co-culture. The co-culture conditions used in this experiment were the same as in Table 2.3, except for the carbon source applied.

2.4.2. The effects of light on the co-pelletization process

Under phototrophic conditions, microalgae use light and inorganic carbon (e.g., carbon dioxide) instead of organic carbon as an energy source through photosynthesis. To know the effects of light on co-pelletization and to optimize co-pelletization under phototrophic conditions, three different co-culture conditions were employed in this experiment: i) a co-culture without yeast extract and without glucose ii) a co-culture with yeast extract but without glucose and iii) a co- culture with yeast extract and with glucose. Yeast extract was used as an organic nitrogen source, while glucose served as an organic carbon source. The summary of the co-culture conditions applied is shown in Table 2.4.

Table 2. 4. Co-culture conditions in phototrophic cultivation.

Shaker speed	150 rpm
Temperature	27^{o}C
Initial medium pH	6.8
Light source	4 fluorescent lights
Inoculum concentration of fungal spores	8.50E+06 spores/L
Inoculum concentration of microalgal cells	2.55E+09 cells/L
Total volume of the co-culture	100 mL
Cultivation time	3 days
Independent variable:	
Test 1	(-) yeast extract, (-) glucose
Test 2	(+) yeast extract, (-) glucose
Test 3	(+) yeast extract, (+) glucose

Note: (-) means ***without***
(+) means ***with***
0.5 g/L of yeast extract
2 g/L of glucose

2.4.3. The effects of pH on the co-pelletization process

One of the factors that play a crucial role in a co-culture system is the pH value of the growth media (Znidarsic & Pavko, 2001). In order to know the effects of pH on co-pelletization, different initial pH values (5, 6, 7, 8, and 9) were applied to the growth media in this study. These initial pH values were kept constant during the whole cultivation by adjusting the pH every 12 hours using very diluted NaOH and HCl solutions. The summary of the co-culture conditions is shown in Table 2.5.

Table 2. 5. Co-culture conditions at different pH value.

Shaker speed	150 rpm
Temperature	27^{o}C
Glucose concentration	2 g/L
Inoculum concentration of fungal spores	8.50E+06 spores/L
Inoculum concentration of microalgal cells	2.55E+09 cells/L
Total volume of the co-culture	100 mL
Cultivation time	3 days
Co-culture medium pH	5, 6, 7, 8 and 9

2.4.4. The effects of the initial fungal spores concentration on the co-pelletization process

To observe the effects of the fungal inoculum concentration on co-pelletization, different initial concentrations of fungal spores were inoculated with a constant concentration of microalgae inoculum (2.55E+09 cells/L). The inoculum concentration of fungal spores was adjusted based on the ratio of fungus to microalgae of 1:50, 1:100, 1:300, 1:600, and 1:1000. The summary of the co-culture conditions is shown in Table 2.6.

Table 2. 6. Co-culture conditions at different initial fungal spores concentrations.

Shaker speed	150 rpm
Temperature	27^{o}C
Initial medium pH	6.8
Glucose concentration	2 g/L
Total volume of the co-culture	100 mL
Cultivation time	3 days
Initial concentration of microalgal cells	2.55E+09 cells/L
Initial concentration fungal spores (fungus : microalgae ratio)	5.10E+07 (1:50) 2.55E+07 (1:100) 8.50E+06 (1:300) 4.25E+06 (1:600) 2.50E+06 (1:1000)

2.4.5. The effects of the initial microalgal cell concentration on the co-pelletization process

The objective of this experiment was to study the effects of the initial microalgal cell concentration on co-pelletization. Different initial concentrations of microalgal cells were co-cultured with the same fungal inoculum (8.50E+06 spores/L). Table 2.7 shows the summary of the co-culture conditions applied.

Table 2. 7. Co-culture conditions at different initial microalgal cells concentrations.

Shaker speed	150 rpm
Temperature	27°C
Initial medium pH	6.8
Glucose concentration	2 g/L
Total volume of the co-culture	100 mL
Cultivation time	3 days
Initial concentration of fungal spores	8.50E+06 spores/L
Initial concentration of microalgal cells	4.25E+08
	2.55E+09
	3.83E_09
	4.46E+09
	8.50E+09

2.4.6. The effects of ionic strength (Ca^{2+} and Mg^{2+}) on the co- pelletization process

Calcium (Ca^{2+}) and magnesium (Mg^{2+}) are ionic compounds that carry a positive charge. Since microalgae cells have a negative charge, calcium and magnesium are commonly applied to assist auto-flocculation at a high pH. In addition, it was reported that calcium and magnesium salt could be used to induce the flocculation efficiency of microalgae (Shelef, Sukenik, & Green, 1984; Xia S. , et al., 2008).

To identify the effects of ionic strength on co-pelletization, different concentrations of Ca^{2+} and Mg^{2+} were used in this study. Table 2.8 shows the concentration of Ca^{2+} and Mg_{2+} applied. The summary of the co-culture conditions is shown in Table 2.9.

Table 2. 8. Different concentrations of Ca^{2+} and Mg^{2+}.

Test	Concentration of Ca^{2+} (g/L)	Concentration of Mg^{2+} (g/L)
1	0	0
2	0.005	0.025
3	0.01	0.05
4	0.03	0.15
5	0.1	0.5

Table 2. 9. Co-culture conditions at different concentrations of Ca^{2+} and Mg^{2+}.

Shaker speed	150 rpm
Temperature	27°C
Initial medium pH	6.8
Glucose concentration	2 g/L
Inoculum concentration of fungal spores	8.50E+06 spores/L
Inoculum concentration of microalgal cells	2.55E+09 cells/L
Total volume of the co-culture	100 mL
Cultivation time	3 days
Independent variable:	
Concentration of Ca^{2+}	0, 0.005, 0.01, 0.03 and 0.1 g/L
Concentration of Mg^{2+}	0, 0.025, 0.05, 0.15 and 0.5 g/L

2.4.7. The effects of salinity (NaCl) on the co-pelletization process

Sodium (Na^{+}) is one of the main components of seawater salinity. This test assessed the possibility of applying filamentous fungi to marine microalgae. Sodium chloride (NaCl) was applied to the growth medium to observe the effect of salinity on co-pelletization. The NaCl concentration (1, 5, 10, 20, and 30 g/L) was adjusted based on the composition of seawater salinity. The summary of the co-culture conditions applied is shown in Table 2.10.

Table 2. 10. Co-culture conditions at different concentrations of NaCl.

Shaker speed	150 rpm
Temperature	27°C
Initial medium pH	6.8
Glucose concentration	2 g/L
Inoculum concentration of fungal spores	8.50E+06 spores/L
Inoculum concentration of microalgal cells	2.55E+09 cells/L
Total volume of the co-culture	100 mL
Cultivation time	3 days
Initial concentration of NaCl	1,5, 10, 20 and 30 g/L

2.4.8. The role of surface charges on the co-pelletization process

The effects of pH on the surface charges of microalgae and fungus

The objective of this experiment was to study the effects of pH on the surface charges of fungus and microalgae. The summary of the co-culture conditions applied is shown in Table 2.11.

Table 2. 11. Co-culture conditions at different pH for zeta potential measurement

Shaker speed	150 rpm
Temperature	27^{o}C
Glucose concentration	2 g/L
Inoculum concentration of fungal spores	8.50E+06 spores/L
Inoculum concentration of microalgal cells	2.55E+09 cells/L
Total volume of the co-culture	100 mL
Cultivation time	7 days
Medium pH	3, 5, 7, 10

The effects of ionic stength (Ca^{2+} and Mg^{2+}) on the surface charges of microalgae and fungus

The objective of this experiment was to study the effects of ionic strength using Ca^{2+} and Mg^{2+} on the surface charges of fungus and microalgae. The summary of the co-culture condition applied is shown in Table 2.12.

Table 2. 12. Co-culture conditions at different concentrations of Ca^{2+} and Mg2+ for zeta potential measurement.

Shaker speed	150 rpm
Temperature	27^{o}C
Initial pH	6.8
Glucose concentration	2 g/L
Inoculum concentration of fungal spores	8.50E+06 spores/L
Inoculum concentration of microalgal cells	2.55E+09 cells/L
Total volume of the co-culture	100 mL
Cultivation time	3 days
Independent variable:	
Initial concentration of Ca^{2+}	0, 0.005, 0.01, 0.03 and 0.1 g/L
Initial concentration of Mg^{2+}	0, 0.025, 0.05, 0.15 and 0.5 g/L

2.5. Harvesting process

After 3 days of cultivation, the co-culture flasks were taken from the shaking incubator and the final pH of the co-culture was measured. Pictures of the co-culture in the flask as well as of the pellets were taken to evaluate the co-pelletization through visual observation. After taking the pictures, the separation process of pellets from the supernatant was then carried out by sieving the co-culture. Some pellets were taken for further analysis and the rest was put on the aluminum foil-weighing dish to be dried in the oven at 105^{o}C (Figure 2.4). The wet and dry weights of the pellets were recorded to determine the wet to dry weight ratio. In the same way, the supernatant was analyzed to determine its concentrations of both microalgae and fungi.

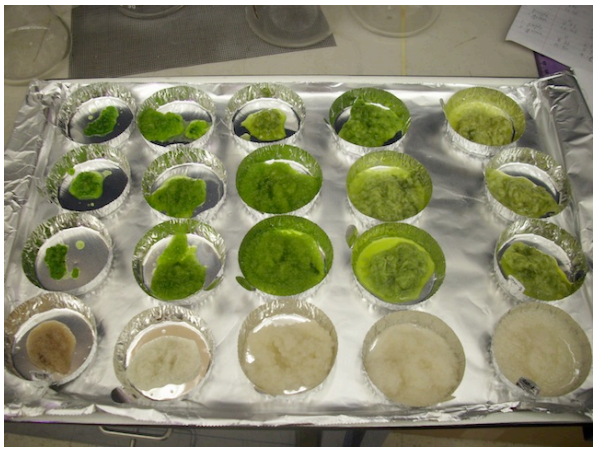

Figure 2. 4. Pellet biomass on the aluminum foil-weighing dish

2.6. Analysis

2.6.1. Morphology of pellets (form, number and size of pellets)

After the co-culture flasks were taken out of the shaker, the pellets were separated from the supernatant using a 35-mesh sieve (pore size of 0.5 mm). The pores of the sieve were small enough to separate the pellets from the algae in the supernatant. The pellets

were then put in the Petri dish and photographs of the pellets were taken using a digital camera (8 Megapixel, BenQ, USA) (Fig 2.5). Pellet count and size were calculated from the photographs through the use of a computer program (ImageJ, National Institutes of Health, USA).

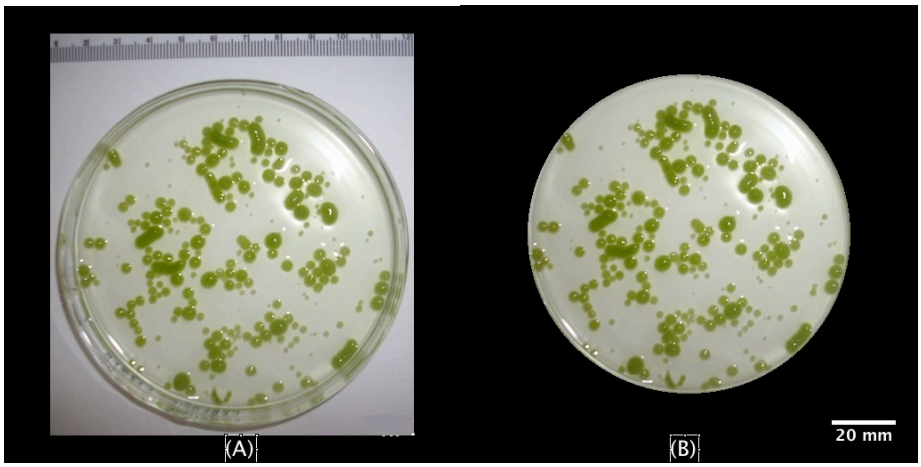

Figure 2. 5. (A) Picture of pellets taken by digital camera, (B) Picture of pellets edited using *imageJ* software.

2.6.2. Glucose utilization

Because glucose was the main carbon source under heterotrophic growth conditions, it was considered important to determine the utilization of glucose by fungi and microalgae. 3 mL of medium from each co-culture flask was taken after 12, 24, 36, 48, and 72 hours of cultivation as samples for the analysis of glucose concentration. The Dinitrosalicyclic Colorimetric Method (DNS method) was applied in this study to determine the presence of free carbonyl group (C=O).

Two reagent solutions were prepared for the DNS test. The first was a DNS reagent solution and the second one was a Rochelle salt reagent solution. For the DNS reagent, 10 g of NaOH was dissolved in 900 mL of water. After the NaOH was completely dissolved, 10 g of 3.5-dinitrosacyclic (DNS) and 2 g of phenol were added to

900 mL of NaOH solution to which distilled water was then added to complete a 1 L solution. 500 mg of sodium sulfate was then added to this 1 L DNS reagent solution. For the Rochelle salt reagent, 40% of Rochelle salt was prepared by dissolving 40 g of Rochelle salt or potassium sodium tartrate ($KNaC_4H_4O_{6\cdot4}H2O$) in 100 mL of water. After the co-culture samples were filtered through syringe filtration, the supernatant was used for the analysis. The samples and standards were prepared by adding 3 mL of DNS reagent solution to 1.5 mL of the sample in a screw-cap glass tube. The mixture was heated in a boiling water bath for 10 minutes. After the tube was taken out from the boiling water bath, 1 mL of Rochelle salt reagent solution was added and the new mixture was boiled for another 5 minutes (Fig 2.6). The absorbance was measured using a UV/Visible spectrophotometer at a wavelength of 575 nm after the sample was cooled down to room temperature. Glucose concentrations were determined using the linear regression of the standard curve (Miller, 1959).

Figure 2. 6. DNS essay experimental set up

2.6.3. pH value

For every experiment, the initial and final pH was measured and recorded using a pH meter (Oakton, SN 153400, Malaysia). The initial pH was measured before the samples were autoclaved, while the final pH was recorded at the end of cultivation (day 3). The same pH meter was used to measure the pH value of all samples.

2.6.4. Harvest efficiency (microalgae removal)

The percentage of microalgae attached to pellets was expressed by the percentage of microalgae removal (harvest efficiency).

$$\text{Harvest efficiency (\%)} = \frac{\text{microalgal cells attached to pellets}}{\text{total microalgal cells}} \times 100\% \qquad (3.1)$$

Where:
Total microalgae cells = microalgae attached to pellets + microalgae in supernatant.

All cell concentrations were expressed in mg of dry weight.

2.6.5. Dry cell weight of microalgae

The cell concentration of microalgae was determined by measuring the dry weight of algal biomass. The suspended microalgae in the co-culture medium were transferred to 50 mL centrifuge tubes and were centrifuged at 7000 rpm for 7 minutes. The biomass settled at the bottom of the tube was washed twice with distilled water to remove all impurities. The supernatant was discarded and the biomass was placed on an aluminum foil weighing dish in order to determine the initial weight. The biomass was then dried in the oven at 105^{o}C to constant weight. The final weight was then recorded. The dry weight of algae biomass was determined using equation 3.2.

$$\text{Dry weight of microalgal biomass (mg/L)} = \frac{\text{final weight - initial weight}}{\text{sample volume}} \qquad (3.2)$$

Where:
Initial weight: the weight of tube (mg)
Final weight: the weight of tube and algal biomass (mg)

2.6.6. Cell numbers

The cell concentration of microalgae was also determined by counting its cell numbers using a 0.1 mm deep Neubauer improved haemocytometer (Hausser Scientific, USA). 50 µL of supernatant was taken from the co-culture medium and put under light microscope for counting. If the sample was too concentrated, dilution was applied to make easier to count the cells. The concentration of microalgae was calculated using equation 3.3 (Guillard & Sieracki, 2005).

$$\text{Microalagal cell concentration (cells/L)} = n \times 5 \times 10^6 \times DF \qquad (3.3)$$

Where:
n : Cell number
DF : Dilution factor
5 : number of small squares

2.6.7. Chlorophyll analysis

Chlorophyll analysis was used as an indirect method to estimate the microalgae concentration in both supernatant and pellets. This method was applied based on the chlorophyll analysis of microalgae carried out by Becker (1995).

For the supernatant part, 20 mL of supernatant was removed from the co-culture flask and centrifuged at 7000 rpm for 7 minutes. After centrifugation, the liquid was discarded and the solid part (biomass) settled down at the bottom of the tube was used for chlorophyll analysis. 5 mL of 90% methanol (as a solvent) was then mixed with the biomass and homogenized using a vortex for 30 seconds. By using a syringe filter (pore

size of 0.45 μm), the solvent was separated from the biomass in the mixture; and was used to measure the absorbance using a spectrophotometer with 650 nm and 665 nm of wavelength. Equation 3.4, 3.5 and 3.6 were used to calculate the concentrations of *chlorophyll a, b and a+b* respectively.

For the pellet size, approximately 200 mg of pellets was put into a test tube and was grinded using a glass stick to break down the pellets and to release the microalgae cells that were attached to the pellets. 5 mL of 90% methanol (as a solvent) was mixed with the broken pellets and was vortexed for 30 seconds. The next steps were the same as in the above supernatant part.

$$\text{Chloropyll a} = (16.5 \times A_{665}) - (8.3 \times A_{650}) \quad (3.4)$$

$$\text{Chloropyll b} = (33.8 \times A_{650}) - (12.5 \times A_{665}) \quad (3.5)$$

$$\text{Chloropyll a+b} = (4.0 \times A_{665}) - (25.5 \times A_{650}) \quad (3.6)$$

2.6.8. Surface Charge (Zeta potential)

The surface charges of fungi and microalgae were determined based on the zeta potential measurement. A zeta potential analyzer (ZetaPALS, Brookhaven, USA) was used to measure the mobility and zeta potential of the cells. Because the size of the cells was the important factor for the zeta potential test, the size of both algae and fungal cells must be considered. Since the microalgae had small cell sizes (5-50μm), the measurement could be applied directly without reducing the cell size any further. For the pellets, breaking down to a smaller size was required. Because the pellets were so compact, a high speed blender was used to set apart the hyphae.

CHAPTER 3
RESULTS AND DISCUSSION
OPTIMIZING CO-CULTURE CONDITIONS OF MICROALGAE AND FILAMENTOUS FUNGI

Microalgal cells are relatively stable and homogeneous when suspended in the cultivation broth. Because of this characteristic, it was difficult for microalgal cells to naturally aggregate or settle down in order to harvest their cell biomass. After 3 days of co-culturing microalgal cells (*C. vulgaris*) with fungal spores (*A. niger*) in a 250 mL flask containing 100 mL of heterotrophic growth medium in a shaking incubator at 170C and 150 rpm, fungal spores in the co-culture media were transformed into pellets. All pellets are sphere-shaped, compact, and relatively homogeneous in size. Compared with the pellets that were not co-cultured with microalgae, the pellets in the co-culture were green instead of white or grey (Fig. 3.1). The green color of the pellets in the co-culture was due to microalgal cells entrapped in or attached to the pellets during co-cultivation. Co-culturing filamentous fungus (*A. niger*) with microalgal cells (*C. vulgaris*) assisted in the harvesting of these microalgal cells by attaching the latter to the pellets formed by the filamentous fungus. This technique provided an easy way to harvest microalgal cells through a simple filtration with sieves because of the size of the pellets (2-5 mm). For the following discussion, the process of pellet formation in the co-culture system is referred to as co-pelletization.

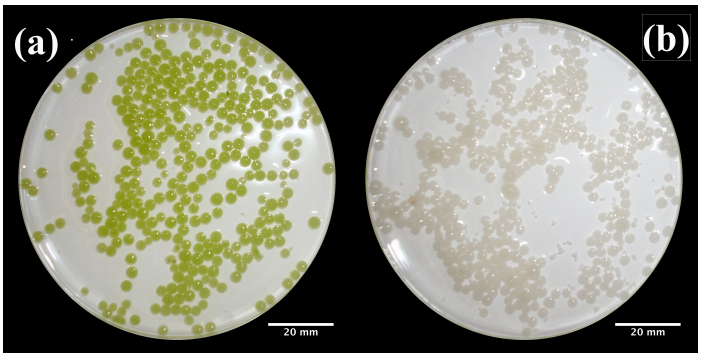

Figure 3. 1. Images of pellets from *A.niger* spores (a) with microalgae (b) Fungal culture without microalgae.

The image of the pellets showed that the microalgal cells were entrapped in and attached to the hyphae of the filamentous fungus *A niger* (Fig 3.2).

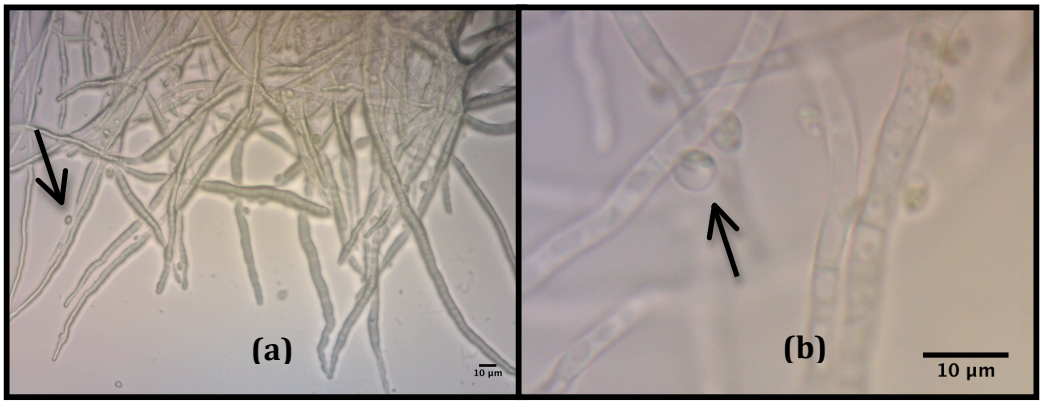

Figure 3. 2. Light microscopy images of fungus-microalgae pellets. (a) Overview of the surface of filamentous hyphae of *A. niger* and *C. vulgaris* (b) Magnification of image (a). Arrows indicate *C. vulgaris* cells.

There are two main factors that might contribute to the co-pelletization process, i.e. the hydrophobic proteins produced by filamentous fungi and the different surface charges of fungi and microalgae (Zhang & Bo, 2012; Zhou, et al., 2013). The hydrophobic proteins on the mycelial surface of some filamentous fungi have been observed and it was found that these hydrophobic proteins could help the hyphae to attach to a solid surface so that cell pelletization/granulation/aggregation could occur (Feofilova, 2010). This can be helpful for the co-pelletization of fungi and microalgae.

Surface charges, as additional cause of co-pelletization, also play an important role in cell attraction. The mechanism of co-pelletization will be discussed more in Chapter 4.

In this chapter, the impact on the co-pelletization process of certain co-culture conditions such as carbon source, medium pH, initial concentration of fungal spores, initial concentration of microalgal cells, ionic strength and salinity is discussed. The specific objectives of this work were to quantify and evaluate the effects of key factors on co-pelletization in order to optimize the co-culturing process.

3.1. The effects of carbon sources on the co-pelletization process

Glucose as a carbon source

Experiments with different concentrations of glucose as the only carbon source showed that the harvest efficiency of microalgae was higher (>80%) for all concentrations of glucose except for 1 g/L, which was about 35% (Fig. 3.3). For glucose concentrations of 4, 6 and 10 g/L, even though the percent removal of microalgae from the co-culture medium was higher (> 80%), the percentage of microalgal cells out of pellets was relatively lower (< 16%) (Fig 3.4). A possible explanation could be that the pH dropped to an acidic condition (around pH of 4) when the concentration of glucose was higher, and as such inhibited the growth of microalgae (Table 3.1).

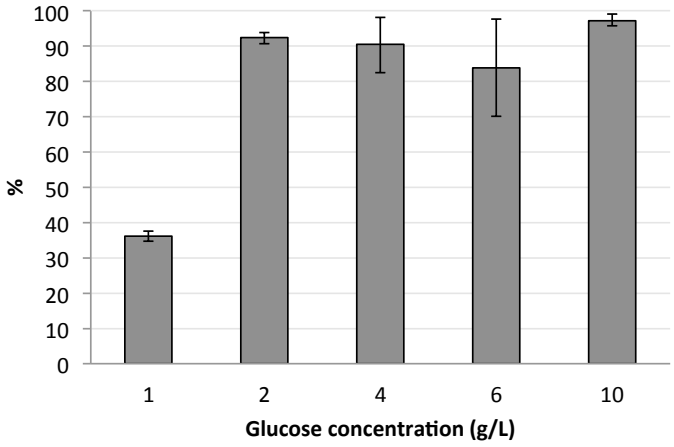

Figure 3. 3. Harvest efficiency at different glucose concentrations under heterotrophic conditions.

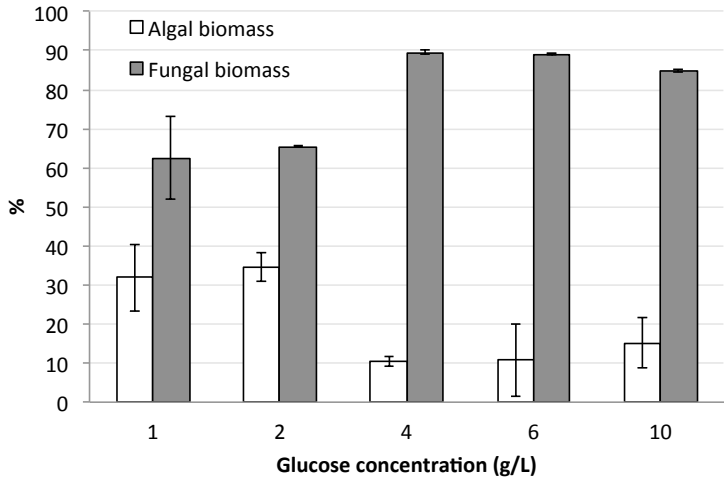

Figure 3. 4. Percentage of microalgal and fungal biomass out of pellet at different glucose concentrations under heterotrophic conditions.

Table 3.1 shows that the pure fungal culture had a much lower pH with higher glucose concentrations. This suggested that the filamentous fungus *A.niger* was possibly responsible for the acidic condition in the co-culture media. A potential explanation for this is that, during its growth, *A.niger* oxidized glucose to gluconic acid and 2-ketogluconic acid (Gottlieb, 1963). Under these conditions, the growth of microalgae is inhibited and most microalgal cells cannot survive (Lustigman, Lee, & Khalil, 1995).

Table 3. 1. pH of the co-culture after 3 days cultivation at different glucose concentrations.

Glucose concentrations (g/L)	Average pH (co-culture)	Average pH (only fungus)	Average pH (only microalgae)
1	7.1±0.02	7.25	7.35
2	7.1±0.15	6.85	7.55
4	5.6±0.94	4.54	7.60
6	4.1±0.13	4.51	7.50
10	4.0±0.17	4.33	6.86

Under heterotrophic conditions, both fungi and microalgae need an organic carbon source to sustain their growth. Competition between fungus *A.niger* and microalgae *C.vulgaris* for the glucose in the co-culture cannot be avoided since both metabolize the glucose for their growth. Fig 3.5 shows the glucose utilization profile of both fungus and microalgae during cultivation. The glucose consumption profiles in the co-culture and in the pure culture of both fungus and microalgae do not seem to be different, indicating that in the co-culture system, fungus and microalgae competed for glucose.

As shown in Fig 3.4, it also appeared that at higher glucose concentrations, the concentration of fungal biomass in the co-culture was higher. It can be confirmed by the results of the glucose consumption profile that in the co-culture system, the utilization of glucose by the fungus was faster than that by the microalgae when the amount of glucose was abundant, possibly because of the metabolism of fungi allowing them to utilize the glucose more effectively (Nielsen, 1992). It was observed that when the concentration of glucose increased up to 10 g/L, the productivity of the fungus (790 mgL^{-1} day^{-1}) was around 7 times higher than the productivity of the microalgae (115 mgL^{-1} day^{-1}).

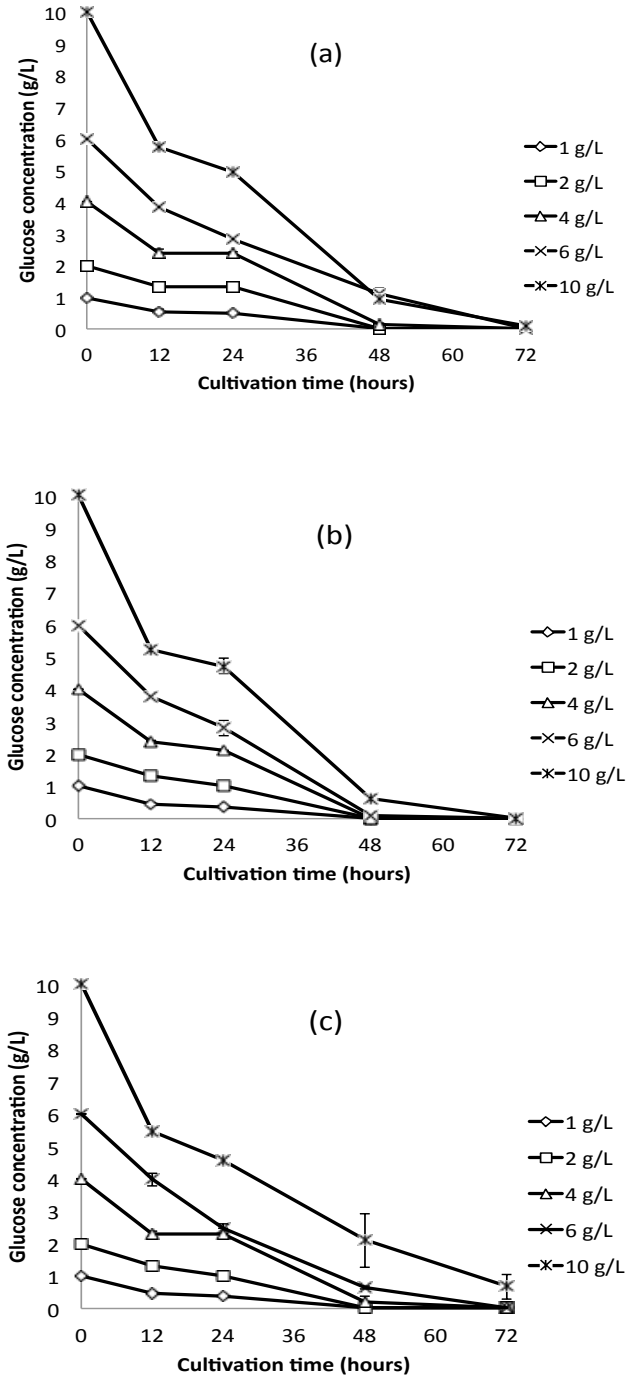

Figure 3. 5. Glucose concentration profile vs. cultivation time (a) Only fungus (b) Only microalgae (c) Co-culture (fungus and microalgae).

This can also be confirmed by the total biomass concentration of fungus and microalgae. With higher concentrations of glucose, the fungal biomass concentration in the co-culture was still the same as that in the pure culture (only fungi) (Fig 3.6), whereas

the microalgal biomass in the co-culture was much lower as compared to the biomass produced in the pure culture (Fig 3.7).

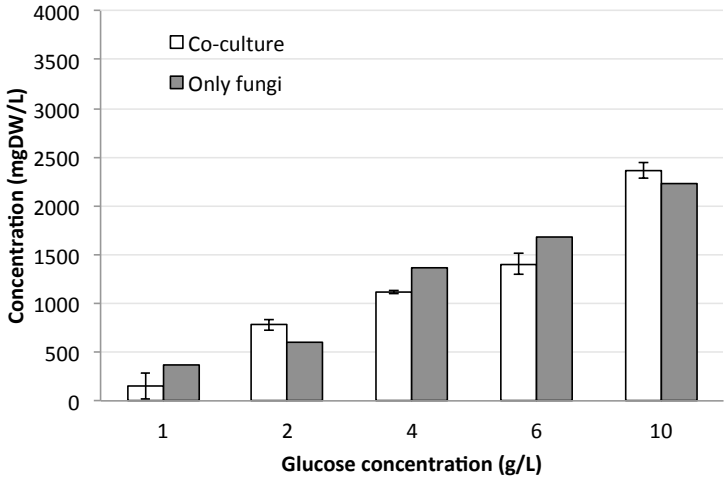

Figure 3. 6. Concentration of total fungal biomass in the co-culture and the pure culture (only fungi) at different glucose concentrations under heterotrophic conditions.

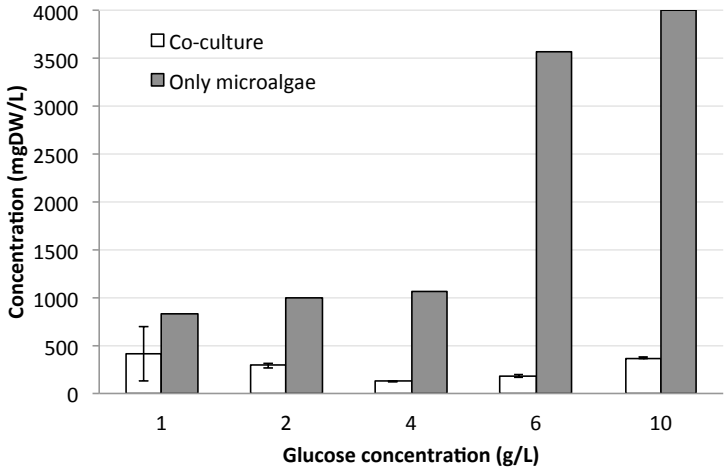

Figure 3. 7. Concentration of total microalgal biomass in the co-culture and the pure culture (only microalgae) at different glucose concentrations under heterotrophic conditions.

Based on these results, a glucose concentration of 2 g/L was identified as the optimum concentration for co-pelletization,. With 2 g/L of glucose, the harvest efficiency of microalgae can reach up to more than 90% without decreasing the pH to acidic conditions. This concentration was applied in the subsequent experiments.

When comparing the final concentration of microalgae at 2 g/L of glucose with the initial concentration in the co-culture, it was observed that the final concentration of microalgae was around 300 times higher than the initial concentration with a biomass productivity around 90 mg/L day. In the pure culture, however, the final concentration of microalgae was around 1000 times higher than the initial one, with a biomass productivity around 320 mg/L day. Results from other studies showed that the maximum biomass productivity of *C.vulgaris* under heterotropic conditions with 1% of glucose was 151 mg/Lday (Liang, Sarkany, & Cui, 2009).

In general, all pellets formed in the co-culture at different glucose concentrations are spherical with a green color. As shown in Table 3.2, with 2 g/L of glucose, the number of pellets in the co-culture was 5±1.5 pellets/mL with an average diameter of 3.3±0.3 mm. It also showed that the higher the concentration of glucose, the larger the pellet size.

Table 3. 2. Number and diameter of pellets in the co-culture and the pure culture (only fungi) at different glucose concentrations under heterotrophic conditions.

		Glucose concentration (g/L)				
		1	2	4	6	10
Co-culture	# of pellets/mL	2±0.2	5±1.5	6±0.7	7±0.7	7±0.8
	Average diameter (mm)	3±0.1	3.3±0.3	3.4±0.2	3.6±0.8	3.8±0.4
Only fungi	# of pellets/mL	5	6	7	7	7
	Average diameter (mm)	4	3.2	3.3	3.6	3.6

Sodium acetate and glycerol as carbon sources

Acetate is usually chosen for the preparation of synthetic wastewater while glycerol is a by-product of biodiesel production. Therefore, these two compounds are good carbon source models from waste streams.

The parameters used for this experiment were similar to those in the previous section (see Table 3.1). The selected concentration of both acetate and glycerol was 2 g/L, which is the optimum glucose concentration. The image of fungus-microalgae pellets from day-1 to day-3 of cultivation is shown in Figure 3.8.

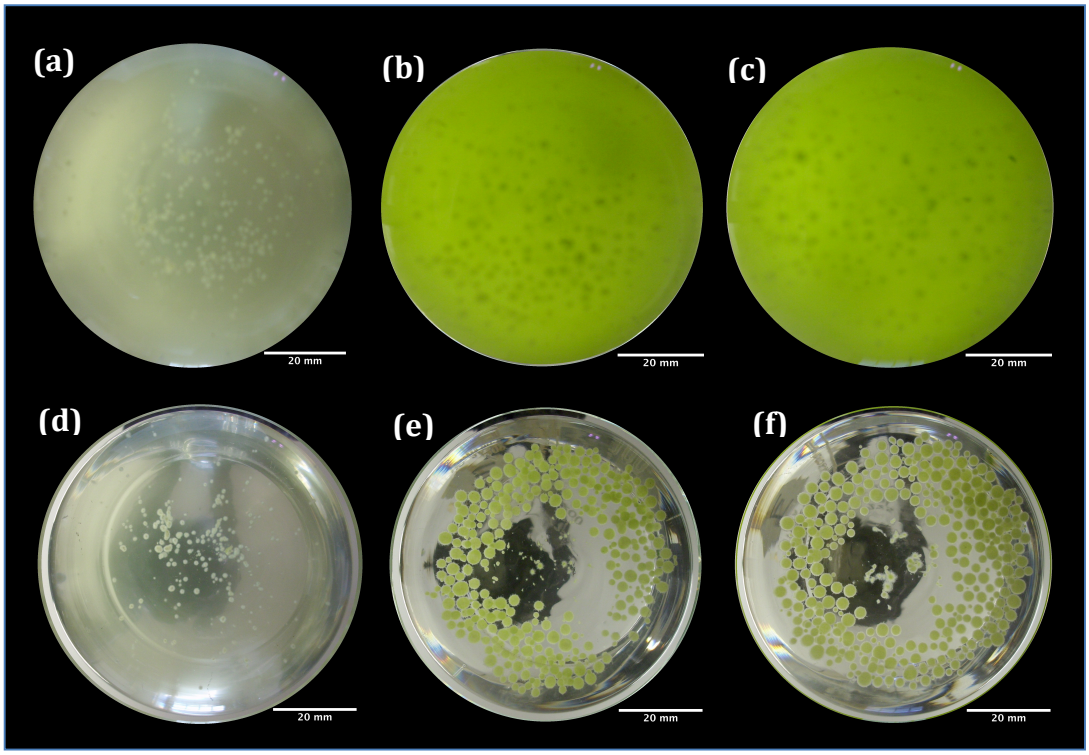

Figure 3. 8. The Image of fungus-microalgae pellets in the co-culture during the cultivation (image from the bottom side of the co-culture flasks). Co-culture with sodium acetate as a carbon source at (a) day 1, (b) day 2, (c) day 3. Co-culture with glycerol as a carbon source at (d) day 1, (e) day 2 and (f) day 3.

Overall, pellets were formed better in the co-culture with glycerol as carbon source than with acetate. When using glycerol, the harvest efficiency of microalgae in the co-culture was around 95% while the percentage of microalgal cells entrapped in the pellets was clearly lower (around 10% of total pellets) (Fig 3.9). This was probably because microalgae were not fully utilizing the glycerol for their growth. Consequently, the fungus predominantly used the glycerol.

In the co-culture with sodium acetate as carbon source, it seemed that the microalgal cells dominated the co-culture. As a consequence, the harvest efficiency was slightly lower compared to the glycerol co-culture even though the biomass concentration of microalgae from pellets was higher (Fig 3.9). This could be the result of the higher amount of microalgal cells entrapped in pellets caused by the faster growth of microalgae in the sodium acetate medium as compared to the co-culture medium with glycerol as carbon source.

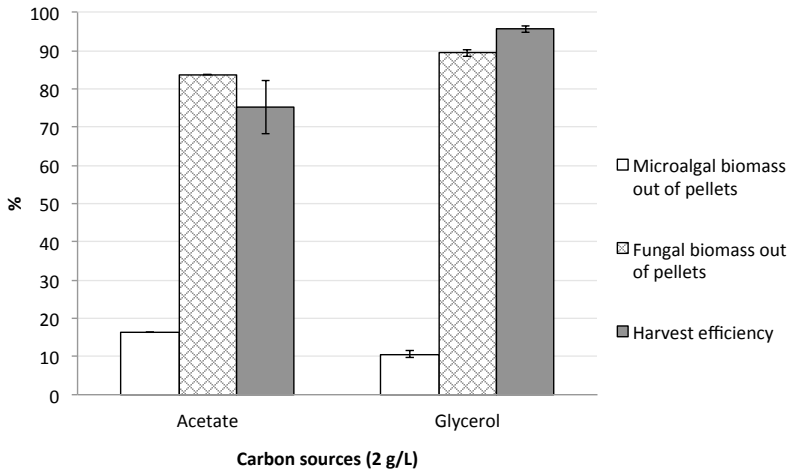

Figure 3. 9. Harvest efficiency and biomass concentrations of fungus and microalgae out of pellets from the co-culture with glycerol and sodium acetate as carbon sources under heterotrophic condition.

3.2. The effects of light on the co-pelletization process under phototrophic conditions

In this experiment, fungus and microalgae were co-cultured under three different conditions and were cultivated in the presence of light. Within 3 days of cultivation, it was observed that no pellets were formed in the co-culture without both yeast extract and glucose, while co-pelletization did occur in the co-culture medium containing yeast extract and/or glucose (Fig 3.10). The results suggested that the presence of yeast extract and glucose in the co-culture medium positively affected the co-pelletization.

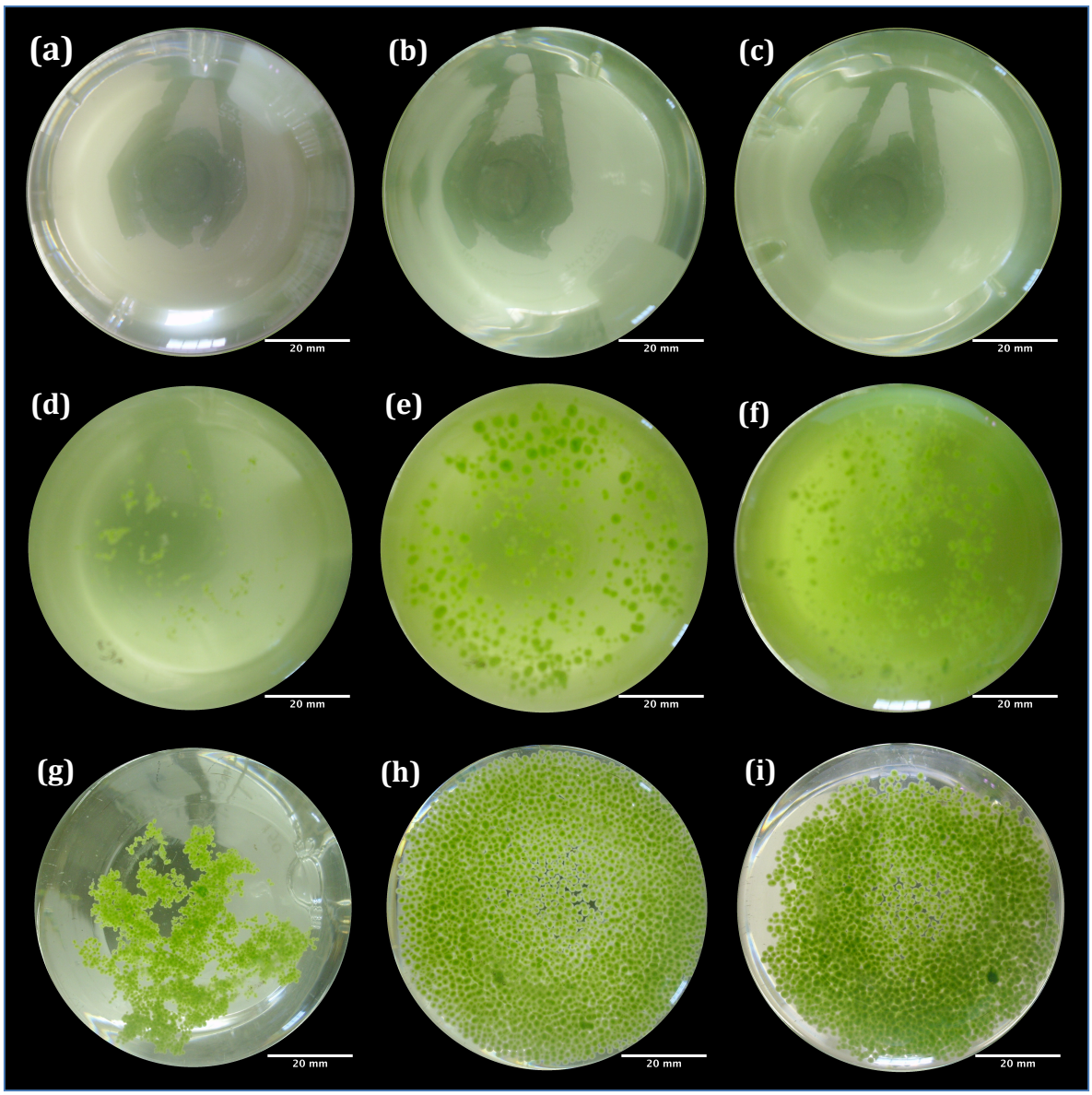

Figure 3. 10. The image of fungus-microalgae pellets in the co-culture during the cultivation under phototrophic condition (image from the bottom side of the co-culture flasks). Co-culture without both yeast extract and glucose at (a) day 1, (b) day 2, (c) day 3. Co-culture with 0.5g/L of yeast extract, without glucose at (d) day 1, (e) day 2 and (f) day 3. Co-culture with 0.5g/L of yeast extract and 2 g/L of glucose at (g) day 1, (h) day 2 and (i) day 3

The growth of microalgae was really slow and the fungal spores were not germinated. It was obvious that co-pelletization fully depended on the presence of an organic carbon and nitrogen source. In their experiments on polyunsaturated fatty acid production by the fungus *Mortierella alpine*, Bajpai & Bajpai (1992) reported that the cell growth of fungus can be promoted by the carbon source and by the supplementation

of yeast extract as an organic nitrogen. Moreover, it was also indicated that nitrogen and carbon sources affected the micelial morphology of fungus (Bajpai & Bajpai, 1993; Park, Koike, Higashiyama, Fujikawa, & Okabe, 1999; Koike, Cai, Higashiyama, Fujikawa, & Park , 2011).

An addition of 0.5 g/L of yeast extract could induce the growth of fungal spores. This signifies that the fungus can use yeast extract as an energy source in order to germinate the spores to form pellets. Even so, in terms of harvest efficiency, pellets formed in the yeast extract medium could not harvest the microalgal cells as much as pellets formed in the co-culture medium with both yeast extract and glucose (Fig 3.11 and Fig 3.12). With a glucose addition of 2g/L, the harvest efficiency increased 5-fold from the co-culture without glucose and 9-fold from the co-culture without both yeast extract and glucose.

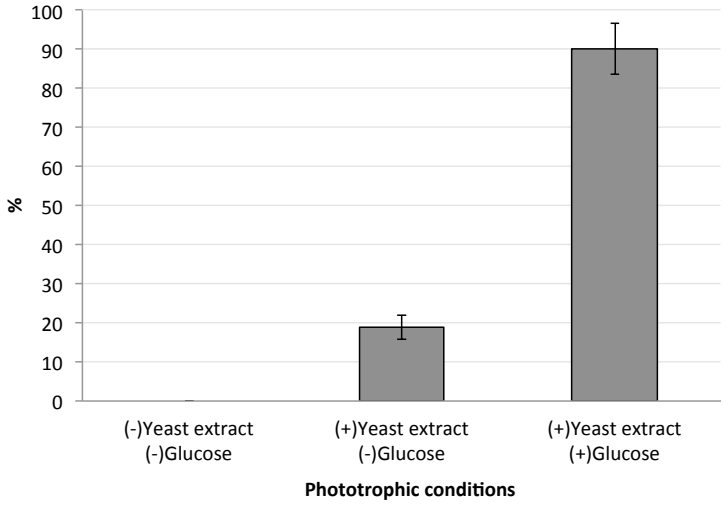

Figure 3. 11. Harvest efficiency at different phototrophic conditions.

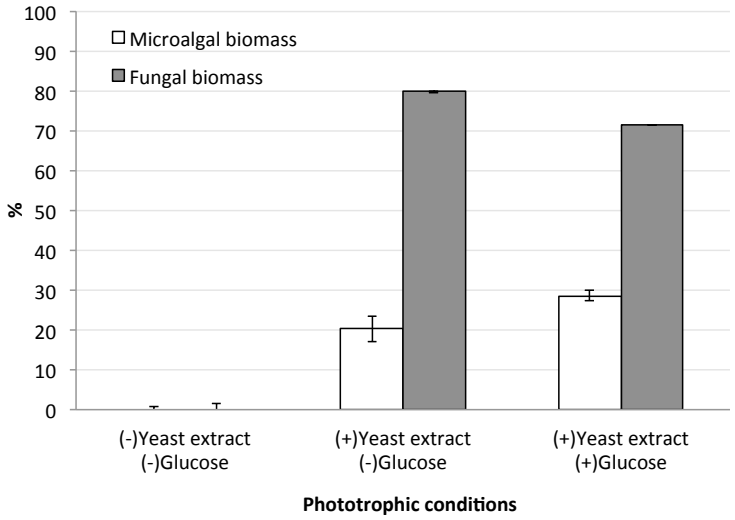

Figure 3. 12. Percentage of microalgal and fungal biomass out of pellets under different phototrophic conditions.

It was found that the concentration of total fungal biomass during the co-culture with glucose was almost 10 times higher than the co-culture without glucose (Fig 3.13). In the same way, during the co-culture experiments, the concentration of microalgal biomass with glucose was 3 times and 10 times higher with glucose than without glucose and without both yeast extract and glucose, respectively (Fig 3.14).

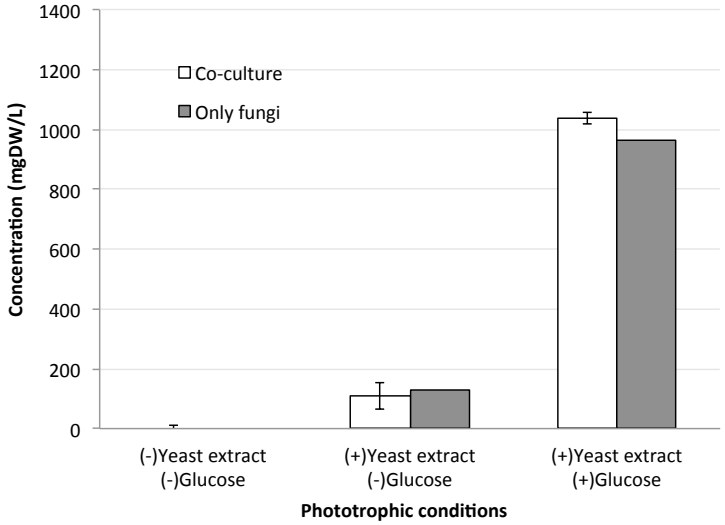

Figure 3. 13. Concentration of total fungal biomass in the co-culture and the pure culture (only fungi) at different phototrophic conditions.

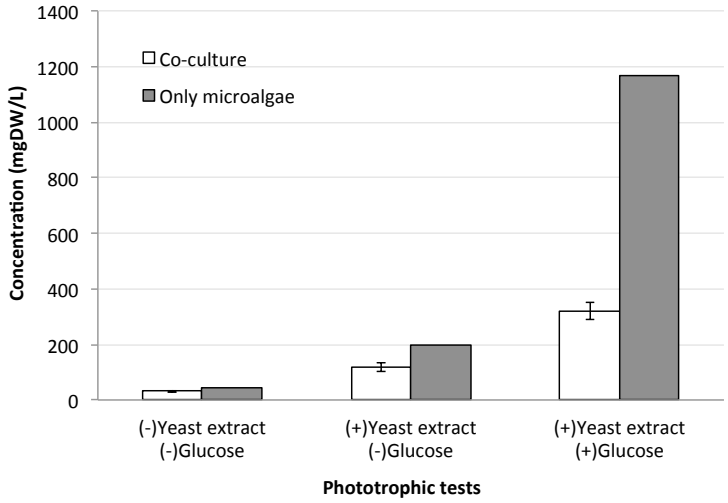

Figure 3. 14. Concentration of total microalgal biomass in the co-culture and the pure culture (only microalgae) at phototrophic conditions.

The results suggested that co-pelletization would not occur in phototrophic cultivation without the addition of a carbon source. Therefore, to identify the optimal glucose concentration under phototrophic conditions, different concentrations of glucose from 0 to 1 g/L were tested. These concentrations were chosen based on the optimal concentration of glucose used under heterotrophic conditions, which was 2 g/L.

Initial glucose concentration on the co-pelletization process under phototrophic condition.

To know the optimum concentration of glucose under phototrophic conditions, different glucose concentrations were applied (0, 0.1, 0.5, 1 g/L). The co-culture medium conditions applied in this experiment were the same as in the previous test (see Table 2.4).

After 3 days of cultivation under phototrophic conditions, it was observed that the co-culture medium for all concentrations of glucose had a green color. The highest harvest efficiency obtained from this test was 67%, which was at 1 g/L of glucose. There

was no apparent difference between the co-culture without glucose and the co-culture with an initial glucose concentration of 0.1 g/L in terms of harvest efficiency (Fig 3.15).

Fig 3.16 shows the harvest efficiency at 24, 48 and 72 hours of cultivation. It was found that the harvest efficiency was higher for all glucose concentrations compared to the one without glucose. These results indicated that the presence of organic carbon was important to optimize the harvest efficiency. In addition, the harvest efficiency could be affected by the presence of light. It has been reported that *C. vulgaris* could grow better in mixotrophic conditions (with organic carbon substrate and light) due to its capability to use both organic carbon and light for their growth (Liang, Sarkany, & Cui, 2009). Consequently, the concentration of microalgae cells suspended in the co-culture medium was higher compared to microalgae cells harvested by fungus.

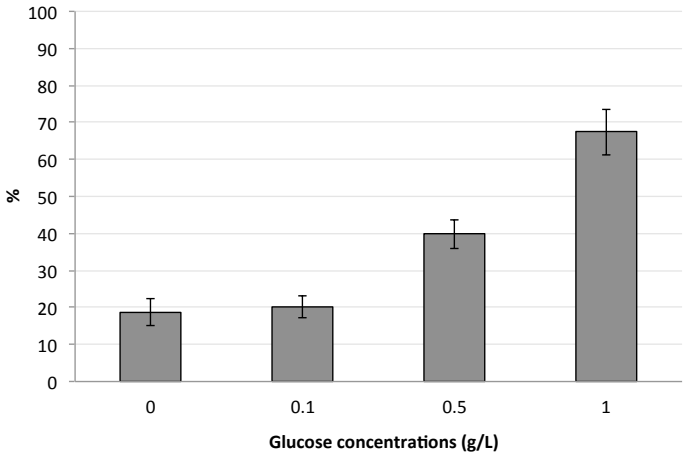

Figure 3. 15. Harvest efficiency at different concentrations of glucose under phototrophic conditions.

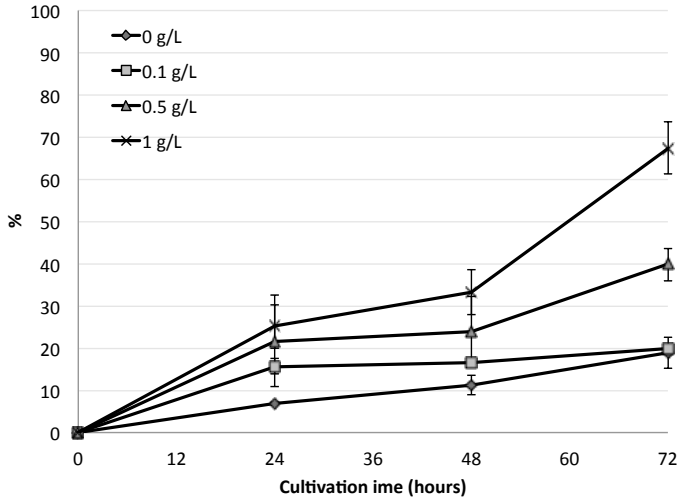

Figure 3. 16. Harvest efficiency during cultivation at different concentrations of glucose under phototrophic conditions.

As shown in Fig 3.17, it was also found that the highest concentration of microalgae cells attached to pellets was at 1 g/L of glucose. Moreover, it was observed that the percentage of microalgae cell pellets at an initial glucose concentration of 0.1 g/L was not different when compared to the co-culture without glucose. This was probably because this concentration was not sufficiently high to support the growth of fungus.

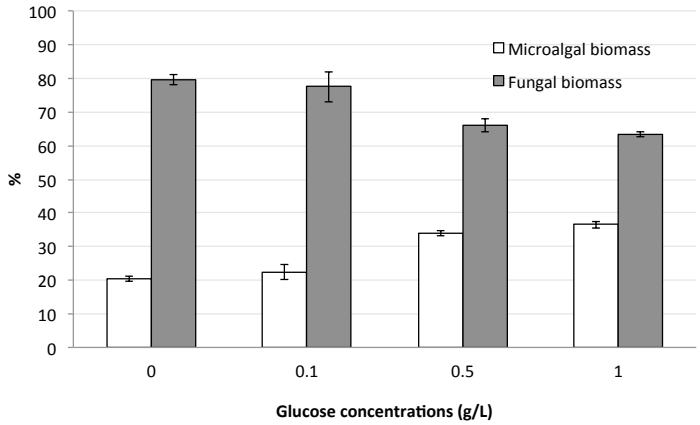

Figure 3. 17. Percentage of microalgal and fungal biomass out of pellets at different concentrations of glucose under phototrophic conditions.

It was clear that the concentration of total fungal biomass as well as the concentration of total microalgal biomass apparently increased at 1 g/L of glucose. A glucose concentration of 0.1 g/L did not clearly impact the increase neither of total fungal biomass nor of total microalgal biomass. However, an addition of 0.1 g/L of glucose in the pure culture of microalgae (only microalgae) did have a positive effect on microalgae growth (Fig 3.18 and Fig 3.19). This indicated that with lower glucose concentration, the utilization of the carbon source was quicker by fungus in the co-culture.

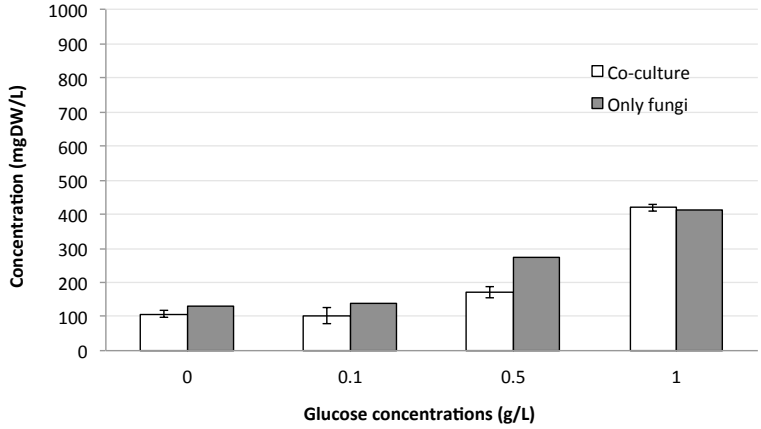

Figure 3. 18. Concentration of total fungal biomass in the co-culture and the pure culture (only fungi) at different concentrations of glucose under phototrophic conditions.

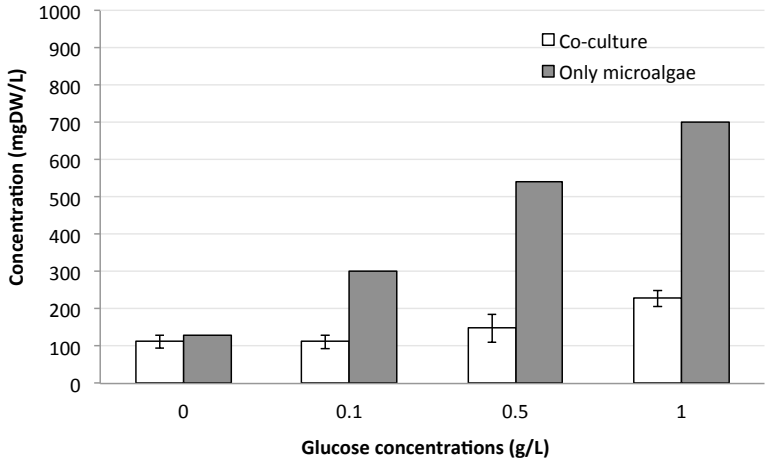

Figure 3. 19. Concentration of total microalgal biomass in the co-culture and the pure culture (only microalgae) at different concentrations of glucose under phototrophic conditions.

3.3. The effects of pH on co-pelletization

After 3 days of cultivation under different pH values (5,6,7,8 and 9), the co-culture media for all pH values were clear, except for a pH value of 9, indicating that most of the microalgal cells attached to pellets within 3 days of cultivation. The harvest efficiency for all pH values was higher than 80%, except for the pH of 9 which was around 60% (Fig 3.20).

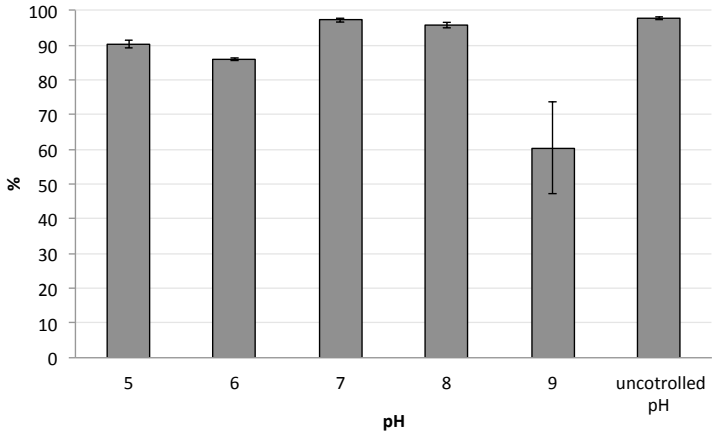

Figure 3. 20. Harvest efficiency at different pH values under heterotrophic conditions.

It was observed that when pH was higher, the percentage of microalgal biomass out of pellets was also higher (up to 70% for a pH of 9), whereas the fungal biomass decreased (Fig 3.21). This could be due to the alkaline conditions which result in a slower growth of fungus in comparison with the growth of microalgae. These results confirm earlier results from experiments carried out by Grimm, Kelly, Vo¨lkerding, Krull, & Hempel (2005), which reported that the biomass growth of fungus *A. niger* proceeded at higher rates for lower pH values.

It was reported by some studies that, even though the growth and morphology development of filamentous fungi was strain-dependent, the pH of the medium played a significant role (Krull, et al., 2013). Moreover, some studies revealed that the pH value of the medium had a decisive influence on spore coagulation (Znidarsic & Pavko, 2001). It was also reported that the morphogenesis of *A. niger* cultivation can be controlled effectively by adjusting the pH. For instance, the bio-pellet of *A. niger* formed at pH 5.5 after a cultivation period of 32 hours (Krull, et al., 2010). Zhou, et al. (2013) reported that for a pH of 9, filamentous fungi strains isolated from municipal wastewater sludge shifted from a pH of around 7 (as an initial pH) to a pH of around 5 (as a final pH) after 5 days of cultivation in typical soy broth.

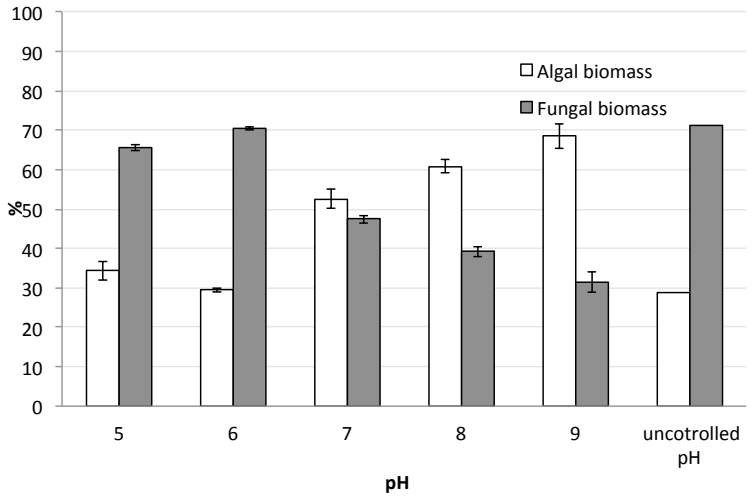

Figure 3. 21. Percentage of microalgal and fungal biomass out of pellet at different pH under heterotrophic conditions.

The results also showed that there was no clear difference between the co-culture with controlled pH at 7 and 8 and the tests with uncontrolled pH in terms of harvest efficiency (>90%). Therefore, to keep operational costs low, a co-culture with uncontrolled pH will be considered.

Fig 3.22 showed the comparison between co-culture and pure culture in terms of the concentration of total fungal biomass. It can be observed that the concentration of total fungal biomass in both the co-culture and the pure culture was the same for all pH values. However, the concentration of microalgae in the pure culture was about double than that of the co-culture (Fig 3.23). The slower growth of microalgal cells in the co-culture could be due to the limited carbon source available because of competition for glucose with the fungus.

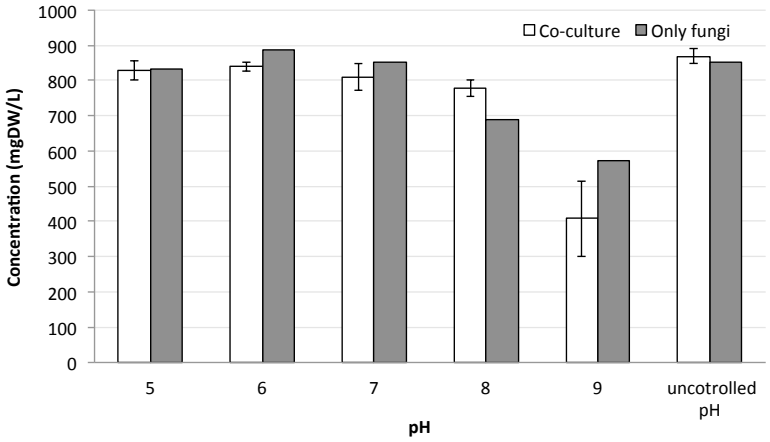

Figure 3. 22. Concentration of total fungal biomass in the co-culture and the pure culture (only fungi) at different pH under heterotrophic conditions.

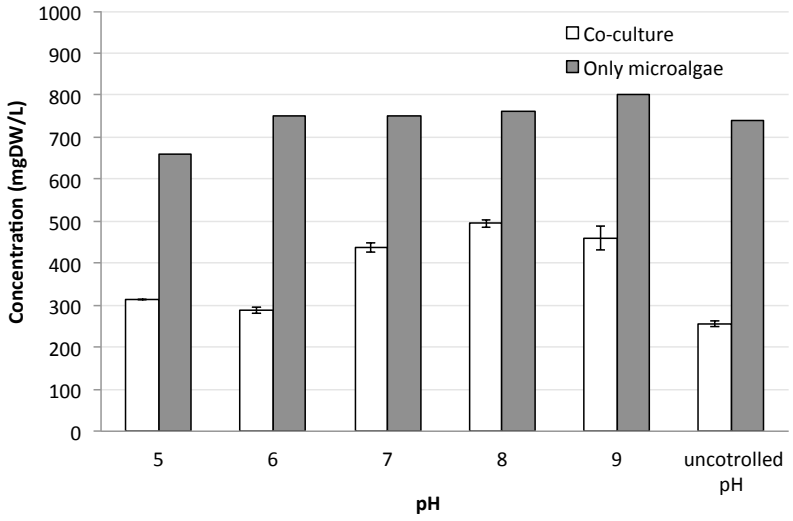

Figure 3. 23. Concentration of total microalgal biomass in the co-culture and the pure culture (only microalgae) at different pH under heterotrophic conditions.

pH values have an impact on the number and the size of pellets (Table 3.3). When the pH value increased, the number of pellets also increased (9±0.3 pellets/mL at a pH of 9), while the average diameter of the pellets decreased (2.6±0.2 mm). This indicated that, when the size of pellets was smaller, the concentration of microalgal cell pellets was higher. The surface area of pellets affecting the process of mass transfer of nutrients from outside into the pellets might be the reason of the higher concentration of microalgae in the pellets. When the pellet size is smaller, the surface area is higher and the mass transfer of substrates might be higher.

Table 3. 3. Number and diameter of pellets in the co-culture and the pure culture (only fungi) at different pH under heterotrophic conditions.

		pH				
		5	6	7	8	9
Co-culture	# of pellets/mL	3±0.1	4±0.1	5±0.3	8±0.04	9±0.3
	Average diameter (mm)	4.3±0.2	3.8±0.2	3.5±0.2	2.9±0.1	2.6±0.2
Only fungi	# of pellets/mL	5	5	5	9	9
	Average diameter (mm)	4	3.3	3.3	3.5	2.7

3.4. The effects of initial fungal spores concentration on co-pelletization

The inoculum concentration of fungal spores can affect the formation of pellets (Metz & Kossen, 1977). After 3 days of cultivation, it was found that all inoculum concentrations of fungal spores used in this test formed pellets well. Moreover, the harvest efficiency for all inoculum concentrations was almost 100%, except for the inoculum concentrations of 2.50E+06 spores/L, which corresponds to the fungus to microalgae ratio of 1:1000 (Fig 3.24). It was observed that for the co-culture with a fungus to microalgae ratio of 1:1000, the broth was clearly green which indicated that most of the microalgal cells were still suspended in the medium.

This suggested that if the concentration of fungal spores was too low, the efficiency of microalgae harvesting would decrease. Moreover, the number of pellets formed in the co-culture was relatively small (around 4 pellets/mL) (Table 3.4) as well as the percentage of fungal biomass out of pellets (Fig 3.25). This result confirms studies on fungal pelletization that reported that the inoculum concentration of fungal spores affected the size and number of pellets (Zmak, Podgornik, Podgornik, & Koloini, 2006).

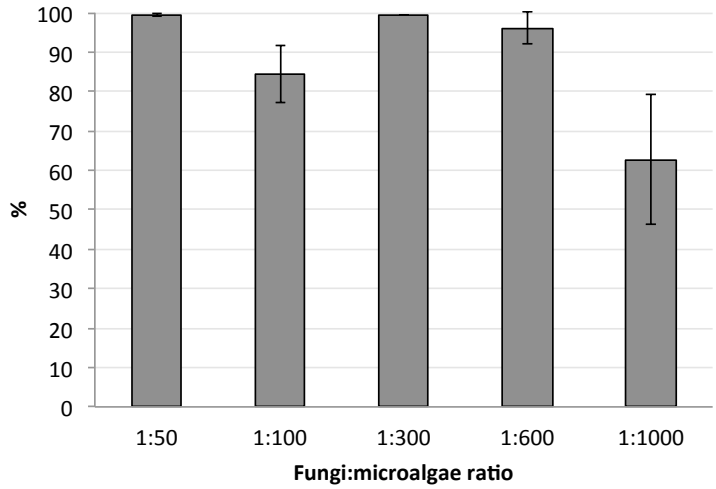

Figure 3. 24. Efficiency at different fungus:microalgae ratio under heterotrophic conditions.

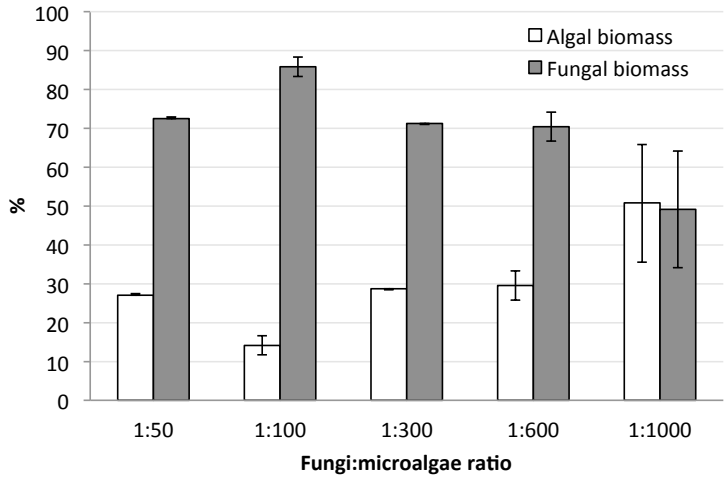

Figure 3. 25. Percentage of microalgal and fungal biomass out of pellet at different fungus to microalgae ratios under heterotrophic conditions.

Table 3. 4. Number and diameter of pellets in the co-culture and the pure culture (only fungi) at different fungus to microalgae ratios under heterotrophic conditions.

		Fungi:microalgae ratio				
		1:50	1:100	1:300	1:600	1:1000
Co-culture	# of pellets/mL	6±0.6	5±2.8	5±0.2	4±0.4	4±0.8
	Average diameter (mm)	2.6±0.4	2.9±0.4	3.4±0.2	3.6±0.9	3.6±0.6
Only fungi	# of pellets/mL	8	7	5	5	4
	Average diameter (mm)	2.9	3	3.3	3.4	3.6

It was observed that the decrease of the initial concentration of fungal spores from 4.25E+06 spores/L (ratio of 1:600) to 2.50E+06 spores/L (ratio of 1:1000) clearly decreased the concentration of total fungal biomass (Fig 3.26). However, a lower inoculum concentration of fungal spores increased the concentration of total microalgal biomass. A lower concentration of fungal spores clearly favored microalgal cells in the competition for carbon source (glucose) (Fig 3.27).

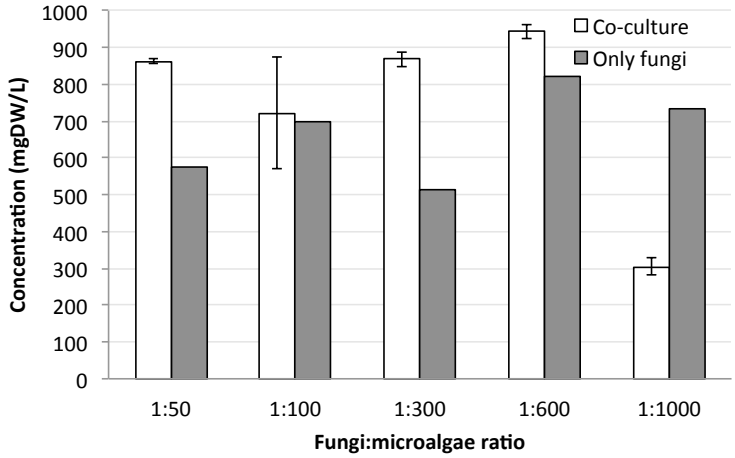

Figure 3. 26. Concentration of total fungal biomass in the co-culture and the pure culture (only fungi) at different fungus:microalgae ratio under heterotrophic conditions.

The initial spore concentration of 8.50E+06 spores/L was found to be suitable for co-pelletization in terms of harvest efficiency (> 95%) and of the total concentration of both fungal and microalgal biomass. Therefore, this initial fungal spores concentration was employed in the subsequent experiments.

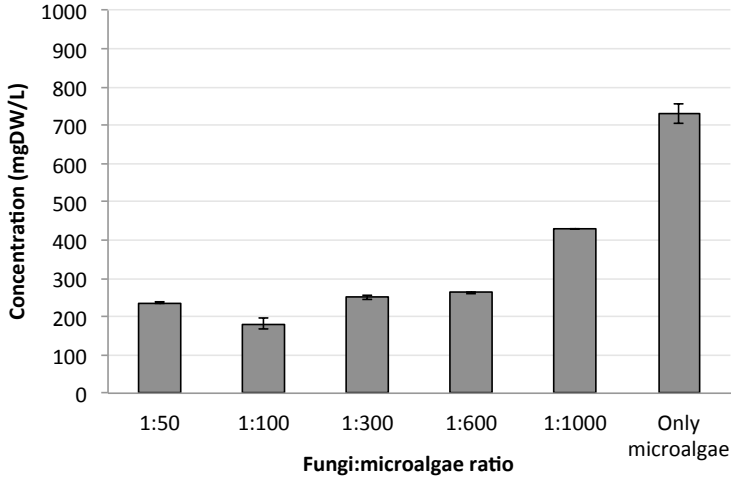

Figure 3. 27. Concentration of total microalgal biomass in the co-culture and the pure culture (only microalgae) at different fungus:microalgae ratio under heterotrophic conditions.

3.5. The effects of initial microalgal cells concentrations on co-pelletization

It was observed that the initial concentration of microalgal cells could affect the co-pelletization. After 3 days of cultivation, it was found that the medium was visually greener for the co-culture with a higher inoculum concentration of microalgal cells (3.83E+09, 4.46E+09 and 8.50E+09 cells/L). Under these conditions, the harvest efficiency obtained was 62%, 27% and 0.7%, respectively (Fig 3.28). This suggested that, if the initial concentration of microalgal cells was too high compared to the initial concentration of fungal spores, the harvest process would not be optimal.

Even though the presence of oxygen in the co-culture system was not measured in this experiment, the availability of oxygen in the co-culture could probably be a limiting factor for fungus to grow. Since there was a high concentration of microalgae cells suspended in the medium, fungus and microalgae would compete for oxygen. There was no supply of oxygen into the co-culture because the flasks were closed during cultivation.

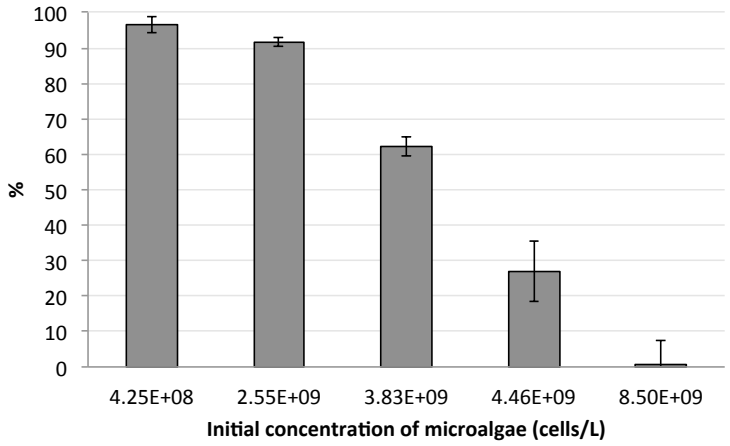

Figure 3. 28. Harvest efficiency at different initial microalgal cell concentrations under heterotrophic conditions.

As shown in Fig 3.29, the percentage of microalgae entrapped in the pellets was lower when the initial concentration of microalgal cells in this experiment was very low

(4.25E+08 cells/L) or very high (8.50E+09 cells/L). Due to the low initial concentration of microalgal cells, the latter might be easily entrapped in the fungal hyphae. However, a high initial concentration of microalgal cells (8.50E+09 cells/L) will interfere with the growth of fungus in the co-culture.

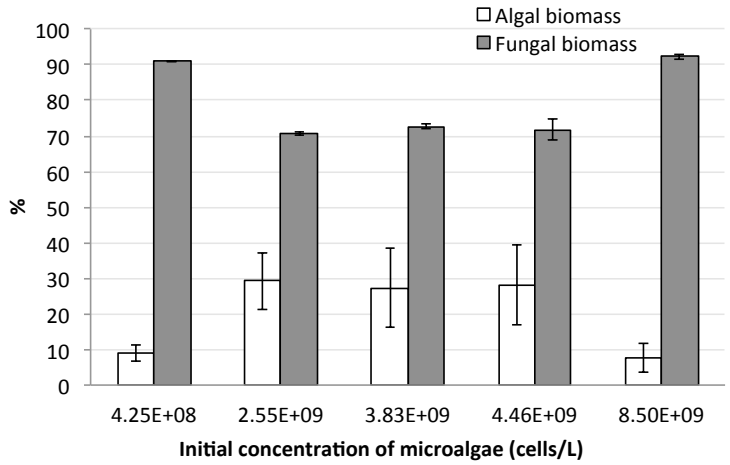

Figure 3. 29. Percentage of microalgal and fungal biomass out of pellet at different initial microalgal cell concentrations under heterotrophic conditions.

It was also observed that the initial concentration of microalgal cells in the co-culture could affect the concentration of total fungal biomass (Fig 3.30) as well as of total microalgal biomass (Fig 3.31). It was found that the concentration of total fungal biomass clearly decreased when the initial microalgal concentration of 8.50E+09 cells/L was applied. The number and the size of pellets shown in Table 3.5 also confirmed this result. The number and size of pellets drastically decreased when a great initial concentration of microalgal cells was applied.

It was observed that, even though the initial concentration of fungus as well as of microalgae was applied based on the same ratio of fungus to microalgae (ratio of 1:1000), the number and diameter of pellets formed were different. For lower initial concentrations of fungus with a constant initial concentration of microalgae (fungus to

microalgae ratio of 1:1000), the number and the diameter of pellets were larger (4±0.8 pellets/mL and 3.6±06 mm, respectively) than the number and the diameter of pellets for higher initial concentrations of microalgae with a constant initial concentration of fungus (fungus to microalgae ratio of 1:1000), i.e. 1±0.4 pellets/mL and 2.5±0.1 mm, respectively. This suggested that the concentration of microalgae applied to the co-culture affects the number and the diameter of pellets.

Table 3. 5. Number and diameter of pellets in the co-culture and the pure culture (only fungi) at different initial microalgal cell concentrations under heterotrophic conditions.

		Initial concentration of microalgal cells (cells/L)				
		4.25E+08	2.55E+09	3.83E+09	4.46E+09	8.50E+09
Co-culture	# of pellets/mL	5±1.1	4±0.5	4±0.4	3±0.4	1±0.4
	Average diameter (mm)	3.4±0.1	3.3±0.1	3.3±0.3	3.0±0.8	2.5±0.1
Only fungus	# of pellets/mL			5±1.2		
	Average diameter (mm)			3.5±2.1		

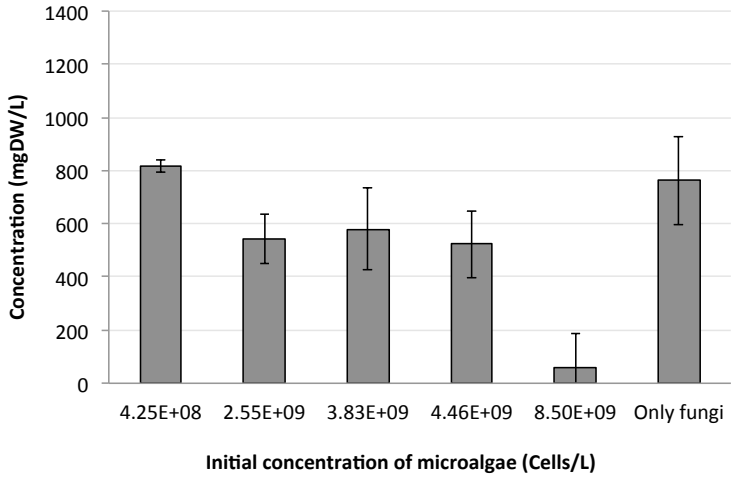

Figure 3. 30. Concentration of total fungal biomass in the co-culture and the pure culture (only fungi) at different initial microalgal cell concentrations under heterotrophic conditions.

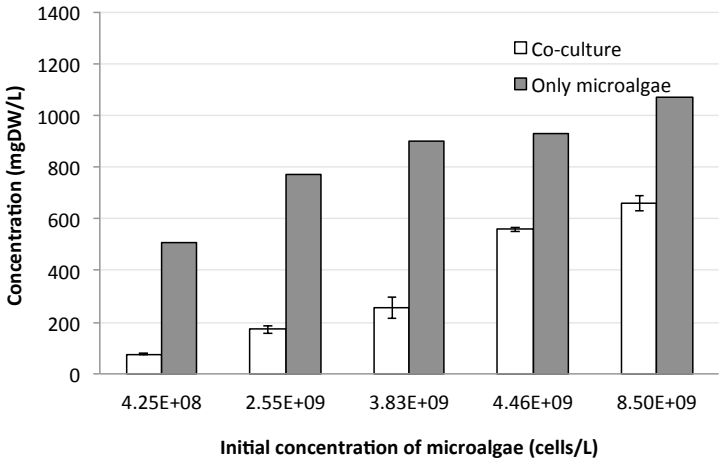

Figure 3. 31. Concentration of total microalgal biomass in the co-culture and the pure culture (only microalgae) at different initial microalgal cell concentrations under heterotrophic conditions.

From this study, the initial microalgal cell concentration of 2.55E+09 cells/L seemed to be the best concentration for co-pelletization. A previous co-pelletization study carried out by Zhang & Hu (2012) did not quantify the inoculum concentration of microalgae cells applied to the co-culture, but instead used 20 mL of microalgae broth for all experiments. Another study on fungus-microalgae pelletization carried out by Zhou, et al. (2013) also did not mention the initial concentration of microalgae used in the co-culture.

It is important to note that the initial concentration of microalgae could affect the efficiency of microalgae harvesting as well as the morphology of the pellets. By applying this concentration, a harvest efficiency of >90% was obtained without decreasing the total concentration of both fungal and microalgal biomass. Hence, this initial microalgal cell concentration was used in the subsequent experiments.

3.6. The effects of Ca^{2+} and Mg^{2+} on the co-pelletization process

The effects of Ca^{2+}

After 3 days of cultivation, it was observed that all co-culture samples were clear, which indicated that all microalgal cells were harvested by the fungus. Fig 3.32 shows that all tests at different concentrations of Ca^{2+} obtained more than 90% of harvest efficiency. It was observed that there was no clear difference between the co-culture with and the one without Ca^{2+} in terms of harvesting efficiency.

Since there was no clear difference in harvest efficiency for all concentrations of Ca^{2+} after 3 days of incubation, harvest efficiency was evaluated for shorter cultivation times (i.e. 24, 48 and 72 hours). As shown in Fig 3.33, harvest efficiency reached about 40% within 24 hours for most of the samples. After 48 hours, the harvest efficiency varied for each concentration of Ca^{2+}. It is important to note that the harvest efficiency for the test without Ca^{2+} was as high as for the one with Ca^{2+} (> 90%).

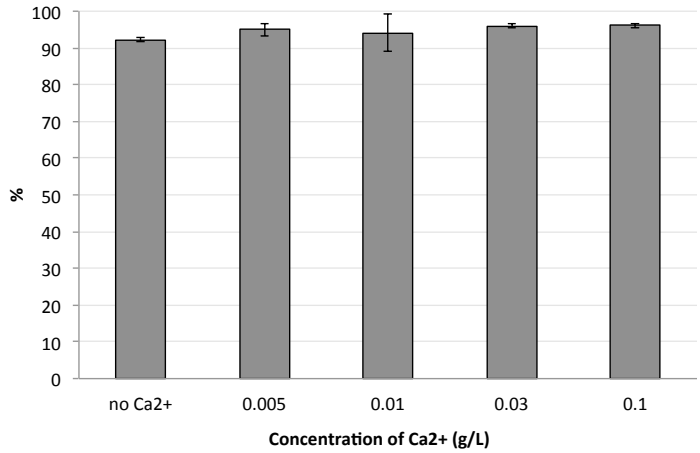

Figure 3. 32. Harvest efficiency at different concentrations of Ca^{2+} under heterotrophic conditions.

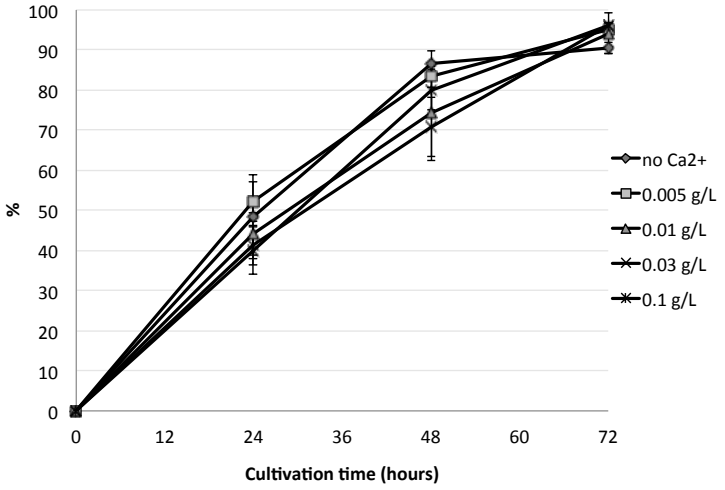

Figure 3. 33. Harvest efficiency during the cultivation at different concentrations of Ca^{2+} under heterotrophic conditions.

As shown in Fig 3.34, the percentage of microalgal cells in the pellets increased with higher concentrations of Ca^{2+} (0.1 g/L) even though there was no clear difference between a concentration of 0.01 and 0.03 g/L. This means that the presence of Ca^{2+} can be used to assist the attraction of microalgal cells to the fungal hyphae. This was probably caused by the ionic strength of the medium since calcium has bivalent cations. It was reported that calcium could be used to induce the flocculation of microalgae at high pH values because of its bivalent cations (Vandamme, Foubert, & Muylaert, 2013).

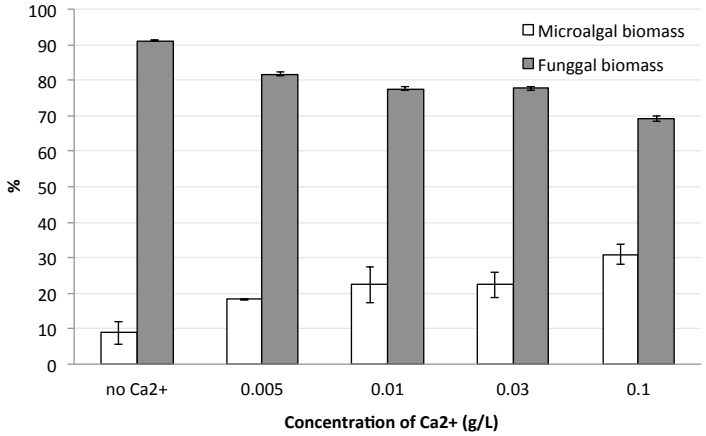

Figure 3. 34. Percentage of microalgal and fungal biomass out of pellet at different concentrations of Ca^{2+} under heterotrophic conditions.

In terms of the concentration of total fungal biomass, there was no clear difference between the different concentrations of Ca^{2+} applied to the co-culture (Fig 3.35), even though it was observed that the higher the concentration of Ca^{2+} applied, the more pellets were formed with a smaller size. It is probably because the fungus did not fully utilize Ca^{2+} for their growth. However, it seems that the presence of Ca^{2+} can affect the morphology of the fungus. In previous experiments, it was also observed that with an addition of Ca^{2+}, the number of pellets was higher (reached 10±0.9 pellets /mL) but they had a smaller diameter (2.1±0.1 mm) (Table 3.6). Fig 3.36 shows that the higher the concentration of Ca^{2+}, the higher the concentration of microalgae in the pure culture.

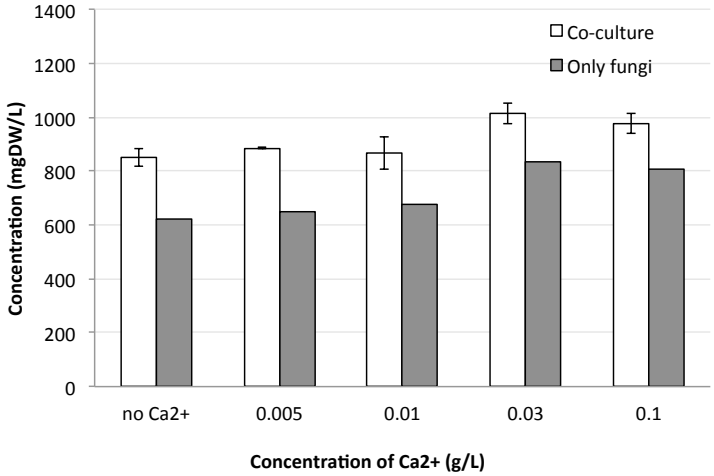

Figure 3. 35. Concentration of total fungal biomass in the co-culture and the pure culture (only fungi) at different concentrations of Ca^{2+} under heterotrophic conditions.

Table 3. 6. Number and diameter of pellets in the co-culture and the pure culture (only fungi) at different concentrations of Ca^{2+} under heterotrophic conditions.

		Concentration of Ca2+ (g/L)				
		0	0.005	0.01	0.03	0.1
Co-culture	# of pellets/mL	4±1.4	5±0.9	5±0.7	7±1.7	10±0.9
	Average diameter (mm)	3.4±0.2	3.2±0.4	3±0.1	2.4±0.1	2.1±0.1
Only fungus	# of pellets/mL	6	8	9	10	11
	Average diameter (mm)	3.5	3.3	3.1	2.3	2.2

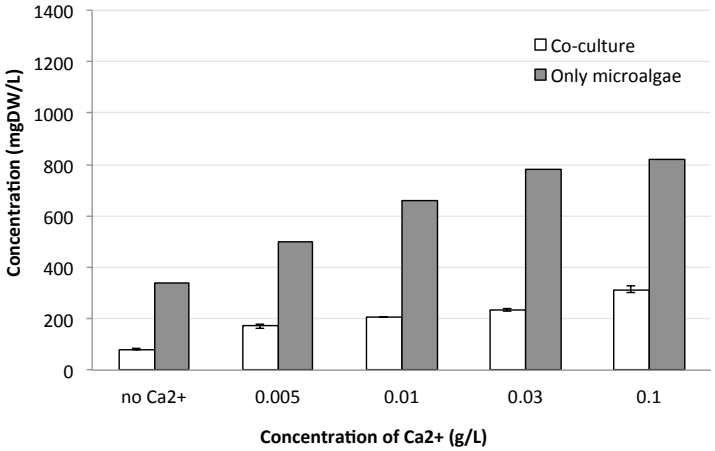

Figure 3. 36. Concentration of total microalgal biomass in the co-culture and the pure culture (only microalgae) at different concentrations of Ca^{2+} under heterotrophic conditions.

The effects of Mg^{2+}

The observation was carried out within and after 3 days of cultivation. It was found that the co-culture medium for all concentrations of Mg^{2+} was visibly clear, which indicated that all microalgal cells were harvested by the fungus. A harvest efficiency of >90% was reached by all concentrations of Mg^{2+}, including the co-culture without Mg^{2+} (Fig 3.37). It was shown that there was no clear difference between the co-culture with and the one without Mg^{2+} in terms of harvest efficiency.

Moreover, the harvest efficiency during cultivation (24, 48 and 72 hours) was monitored to determine the percentage of microalgae removal at different concentrations of Mg^{2+}. As shown in Fig 3.38, the harvest efficiency reached about 30% within 24 hours. After 48 hours, a harvest efficiency of 90% was reached for all concentrations. This result revealed that almost all microalgal cells had been harvested within 2 days. Therefore, one could conclude that it was possible to decrease the time of cultivation for co-pelletization.

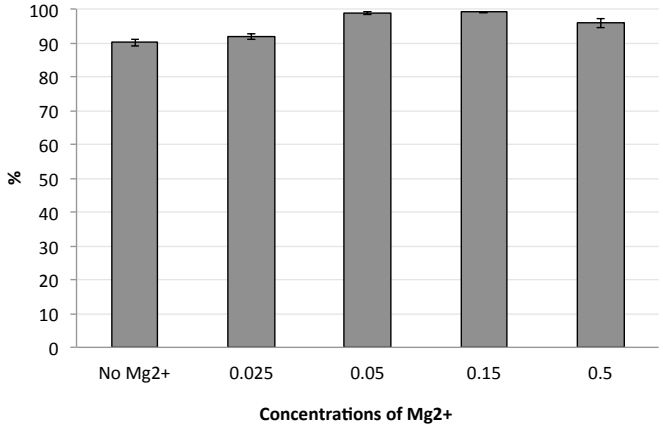

Figure 3. 37. Harvest efficiency at different concentrations of Mg^{2+} under heterotrophic conditions.

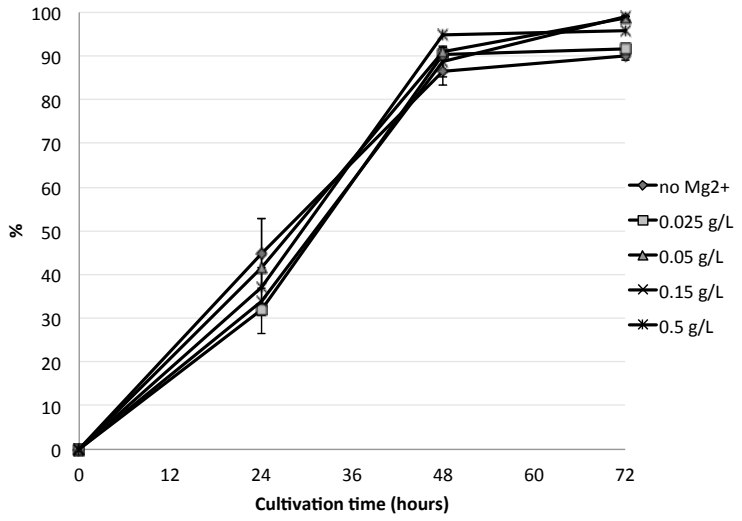

Figure 3. 38. Harvest efficiency during cultivation at different concentrations of Mg^{2+} under heterotrophic conditions.

Based on Fig 3.39, it was found that, when the concentration of Mg^{2+} increased, the concentration of microalgal cells in the pellets also increased. This suggested that the presence of Mg^{2+} could also be used to assist the attraction of microalgal cells to the fungal hyphae. A previous study by Vandamme, Foubert, & Muylaert (2013) reported that the flocculation of the microalgae *Chlorella* could be induced by the precipitation of magnesium.

In terms of the concentration of total fungal biomass, there was no clear difference between the different concentrations of Mg^{2+} applied to the co-culture (Fig 3.40), even though it was observed that the higher the concentration of Mg^{2+}, the higher the pellet number (7±1.4 pellets/mL) and the smaller the pellet size (3.8±0.3 mm) (Table 3.7). The number of pellets was fewer as compared with the Ca^{2+} tests (10±0.9 pellets/mL), but they had a larger diameter than in the Ca^{2+} tests (2.1±0.1 mm). Moreover, it was found that the presence of Mg^{2+} could induce the growth of microalgae.

Fig 3.41 shows that the concentration of total microalgal cells increased when the Mg^{2+} concentration was higher.

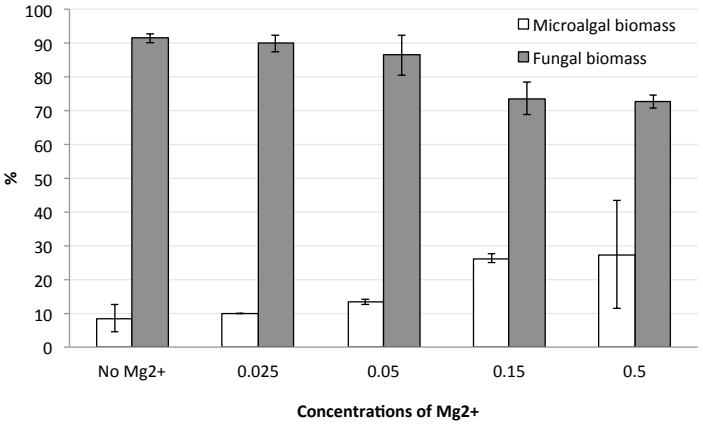

Figure 3. 39. Percentage of microalgal and fungal biomass out of pellets at different concentrations of Mg^{2+} under heterotrophic conditions.

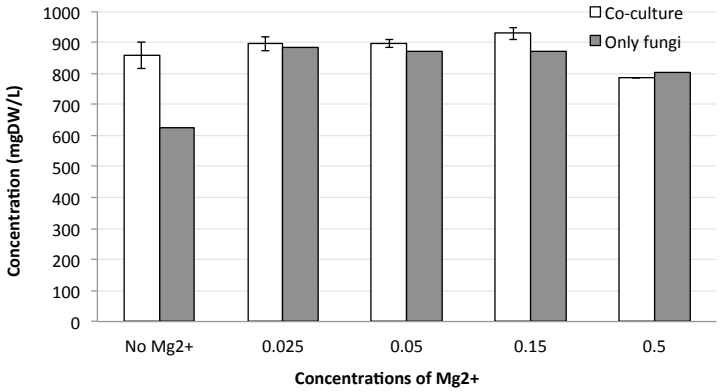

Figure 3. 40. Concentration of total fungal biomass in the co-culture and the pure culture (only fungi) at different concentrations of Mg^{2+} under heterotrophic conditions.

Table 3. 7. Number and diameter of pellets in the co-culture and the pure culture (only fungi) at different concentrations of Mg^{2+} under heterotrophic conditions.

		Concentration of Mg2+ (g/L)				
		0	0.025	0.05	0.15	0.5
Co-culture	# of pellets/mL	4±1.4	5±0.2	6±0.6	7±2.8	7±1.4
	Average diameter (mm)	3.4±0.2	4.6±0.3	4.4±0.2	4±0.3	3.8±0.3
Only fungus	# of pellets/mL	4	5	7	7	7
	Average diameter (mm)	3.5	4.7	4.1	3.9	3.8

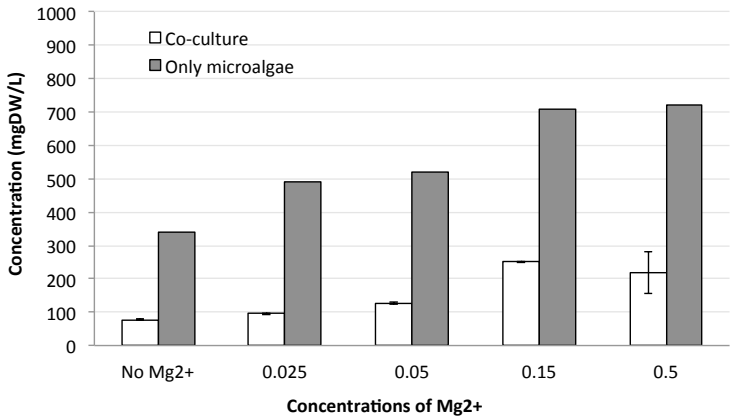

Figure 3. 41. Concentration of total microalgal biomass in the co-culture and the pure culture (only microalgae) at different concentrations of Mg^{2+} under heterotrophic conditions.

3.7. The effects of NaCl concentration on the co-pelletization process

After 3 days of cultivation, more than 80% of the harvest efficiency was obtained for all concentrations of NaCl (Fig 3.42). The highest efficiency of microalgae harvesting was at 30 g/L (almost 100%). However, in terms of the percentage of microalgal cells in the pellets, the higher the concentration of NaCl applied to the co-culture medium, the lower the percentage of microalgal cells in the pellets (Fig 3.43)..

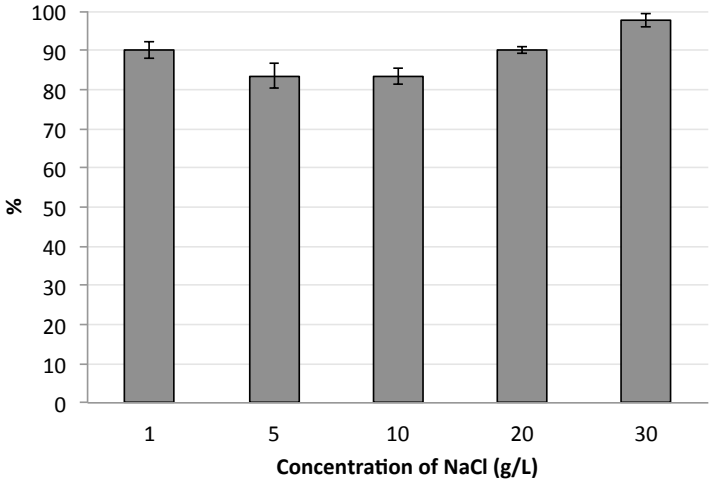

Figure 3. 42. Harvest efficiency at different concentrations of NaCl under heterotrophic conditions.

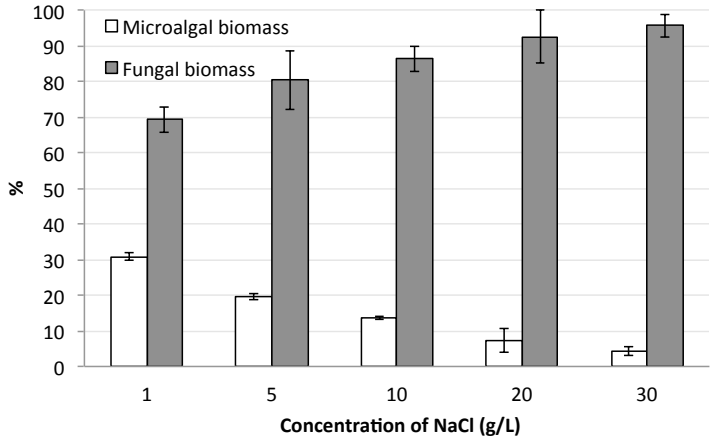

Figure 3. 43. Concentration of microalgal and fungal biomass out of pellets at different concentrations of NaCl under heterotrophic conditions.

The fungus *A.niger* survived and even grew well in higher concentrations of NaCl. This was confirmed by the increase of the total fungal biomass concentration when the initial salinity concentration was higher (up to 30 g/L) (Fig 3.44). However, the growth of microalgae *C. vulgaris* was inhibited at salinity concentrations of 20 g/L and 30 g/L (Fig 3.45). A previous study on the oil accumulation of microalgae *C. vulgaris* under salinity conditions also reported that the growth of *C. vulgaris* was inhibited at 35 g/L of NaCl (Heredia-Arroyo, Wei, Ruan, & Hu, 2011). This possibly occurred because *C. vulgaris* is a fresh water microalgae, which has its habitat in fresh water.

Interestingly, at the highest concentration of salinity (30 g/L of NaCl), which is the same concentration as marine water, the fungus was able to grow and form pellets (2.9±0.1 mm in diameter). The diameter and the size of the pellets in these tests were about the same as in the Ca^{2+} tests. The number and the size of the pellets for different salinity concentrations are shown in Table 3.8.

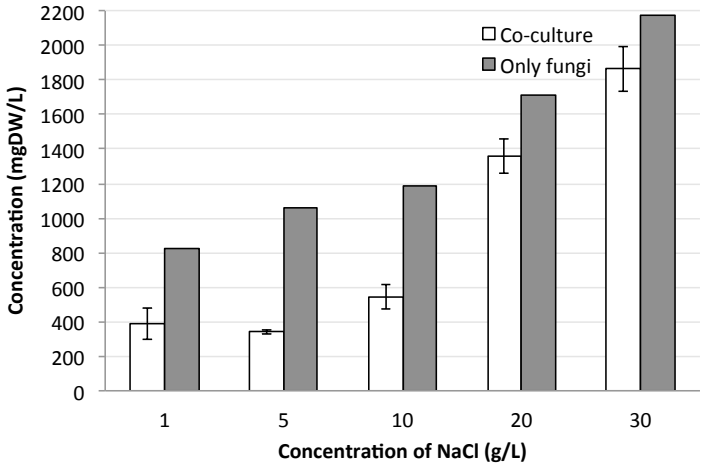

Figure 3. 44. Concentration of total fungal biomass in the co-culture and the pure culture (only fungi) at different concentrations of NaCl under heterotrophic conditions.

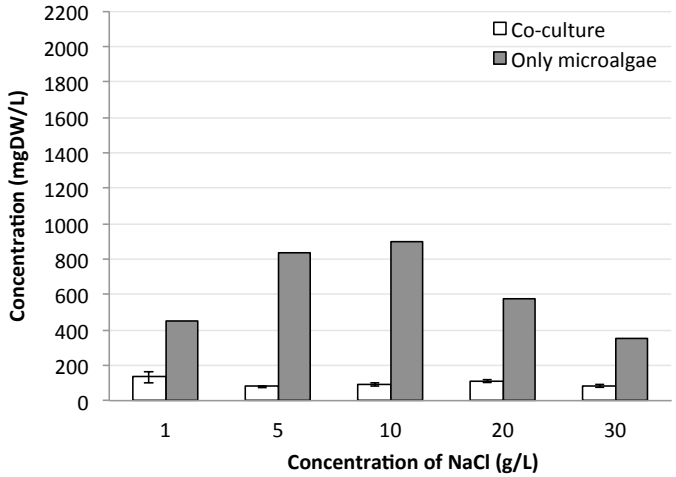

Figure 3. 45. Concentration of total microalgal biomass in the co-culture and the pure culture (only microalgae) at different concentrations of NaCl under heterotrophic conditions.

Table 3. 8. Number and diameter of pellets in the co-culture and the pure culture (only fungi) at different concentrations of NaCl under heterotrophic conditions.

		NaCl concentration (g/L)				
		1	5	10	20	30
Co-culture	# of pellets/mL	5±0.9	5±0.5	6±0.9	8±2.4	11±1.9
	Average diameter (mm)	3.8±0.3	3.7±0.3	3.5±0.2	3.5±0.6	2.9±0.1
Only fungus	# of pellets/mL	7	8	7	11	12
	Average diameter (mm)	3.9	3.7	3.7	2.9	2.7

It was reported that flocculation was not effective for marine microalgae harvesting because high salinity inhibited the flocculation process. It was also found that 5 g/L of salinity or lower was the best level for effective flocculation (Bilanovic , Shelef , & Sukenik , 1988; Sukenik , Bilanovic , & Shelef , 1988). However, the ability of fungal spores to grow in the highest salinity concentration suggested that the fungus could possibly be applied to marine microalgae for co-pelletization.

In summary, the optimization of the co-culture conditions in the co-pelletization process was discussed in this chapter. The effects on co-pelletization of certain key factors, including carbon source concentrations, pH values, initial concentrations of fungal spores, initial concentrations of microalgal spores, concentrations of ionic strength and salinity were presented. It was found that the optimum conditions for co-pelletization were 2 g/L of glucose, 8.50E+06 spores/L and 2.55E+09 spores/L for inoculum concentrations of fungal spores and microalgal cells, respectively, which corresponds to a fungus to microalgae ratio of 1:300, and uncontrolled pH (around pH of 7). It was also found that organic carbon and nitrogen played an important role in the co-pelletization process under phototrophic conditions. In addition, it was observed that Ca^{2+} and Mg^{2+} affected the percentage of microalgae in the pellet. However, without Ca^{2+} and Mg^{2+}, the harvest efficiency was still high (> 90%). Finally, it was observed that the fungus could survive and form pellets in a high salinity level (30 g/L of NaCl). This suggested that the fungus could possibly be applied to marine microalgae for co-pelletization.

CHAPTER 4
RESULTS AND DISCUSSION
UNDERSTANDING THE MECHANISM OF MICROALGAE-FUNGI ATTRACTION ON CO-PELLETIZATION

The mechanisms of co-pelletization are still not fully understood. The different surface charges of fungi and microalgae could be the main reason for the attraction between fungus and microalgae. It is widely known that microalgae carry a negative charge in their cells (Henderson, Parsons, & Jefferso, 2008). Because of this characteristic, flocculation is broadly employed to harvest microalgae by applying a positively charged chemical compound. Because of the opposite charges, the microalgal cells can easily be harvested through attraction.

Based on the idea above, it was suspected that under some conditions in the co-culture, the filamentous fungus *Aspergillus niger* could carry a positive charge in its hyphae so that attraction with microalgal cells occurred due to their opposite charges. In order to support the hypothesis that the surface charges played an important role in the co-pelletization, zeta potential measurements were carried out for the fungal pellets and the microalgal cells under two different conditions that were believed to have a significant effect on the surface charge, namely pH and ionic strength. Four different pH values were applied to the medium (i.e. 3, 5, 7 and 10) in this study. In addition, calcium (Ca^{2+}) and magnesium (Mg^{2+}) were used to apply ionic strength conditions because of their positive charge.

4.1. The effects of pH on the surface charges of microalgae and fungus

In order to determine the effects of pH on the surface charge of microalgae and fungus, four different pH values were applied to the culture medium of microalgae as

well as to that of the fungus. In this experiment, a cultivation time of 7 days was applied and analysis was carried out daily.

The zeta potential of microalgae during the cultivation period at different pH values is shown in Fig 4.1. It was observed that the zeta potential value decreased down to negative values (around -20 mV) when the pH increased (up to pH 10), However, the charge of the microalgae was closer to neutral (-3 mV) when the medium pH reached 3. During the 7 days of cultivation, it was found that the charge of microalgae at each pH value was constant. Even though the charge seemed to be more negative after day-3 of cultivation, the charge came back to the initial value after day-6 of cultivation.

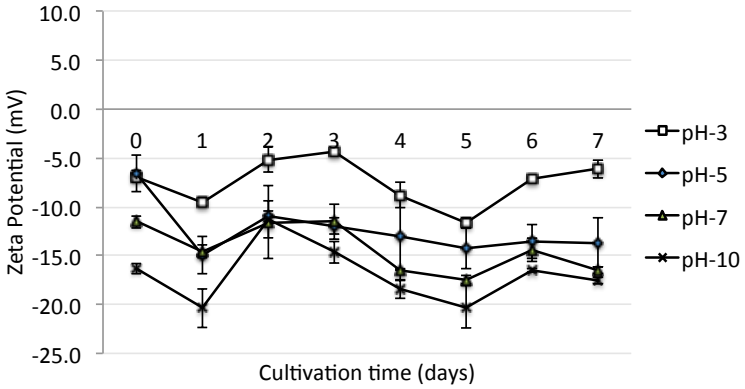

Figure 4. 1. Zeta potential of microalgae (*C. vulgaris*) at different pH.

These results confirm other studies that reported that the surface charge of microalgae was more negative at a high pH (Lavoie & de la Noue, 1982; Vandamme , Foubert , Fraeye , Meesschaert & Muylaert , 2012). Moreover, Vandamme, Foubert, & Muylaert (2013), explained in their review paper that microalgal cell suspensions are stabilized by the surface charge of the cells. This surface charge is mostly caused by the presence of carboxylic (-COOH) and amine ($-NH_2$) groups on the cell surface. Therefore,

when pH is above 4-5, the carboxylic groups were dissociated and the microalgae cells received a negative charge.

From the tests with the filamentous fungus *A. niger*, it was determined that a positive charge was reached when the medium pH was 3 (Fig 4.2). It was also observed that, even under acidic conditions, the fungal spores (day-1) were negatively charged. This is in agreement with the results of Wargenau et al. (2011). In this study, the origin of the electrostatic surface charge of *A. niger* spores under acidic conditions was determined. It was found that the spores were negatively charged even at a lower pH (pH of 2) (Wargenau, Fleibner, Bolten, Rohde, Kampen, & Kwade, 2011).

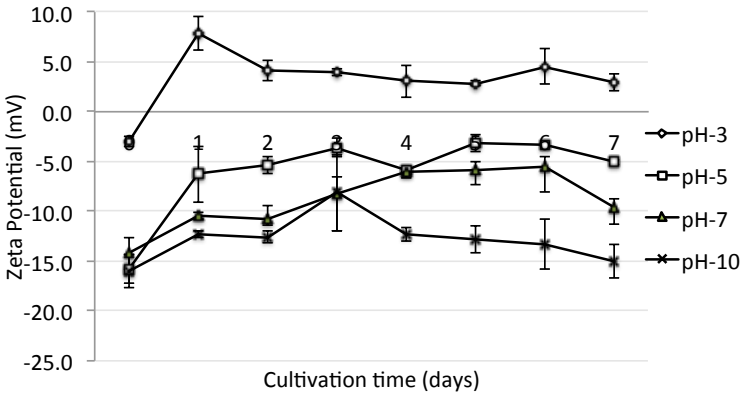

Figure 4. 2. Zeta potential of fungus (*A. niger*) at different pH.

It was also observed that the zeta potential value of the fungus tended to be more negative (< -10 mV) when the medium pH increased up to pH 10. The surface charge of the fungus was consistent for each pH value during the cultivation time. These results suggested that in order for the fungal pellet to gain a positive charge, lower pH values must be applied. Fig 4.3 compares the zeta potential of fungus and microalgae at different pH values.

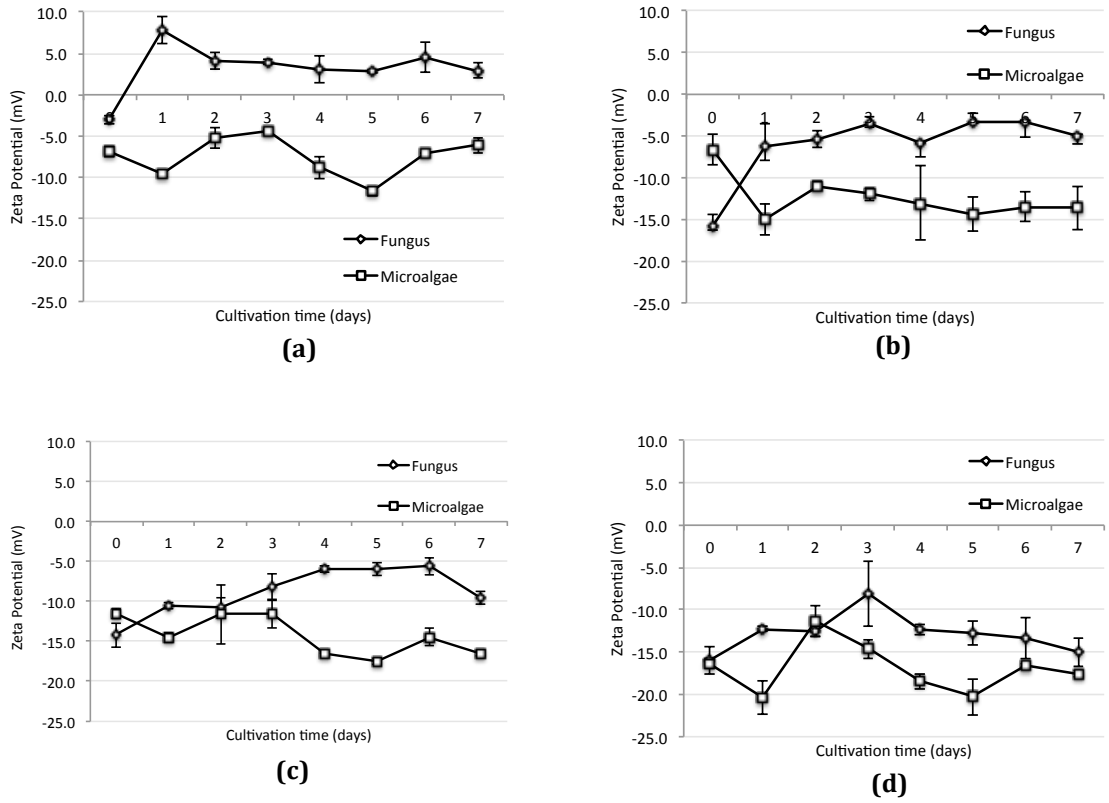

Figure 4. 3. Zeta potential of fungus and microalgae at different medium pH: (a) 3, (b) 5, (c) 7 and (d) 10

It was found that, for all pH values, negative zeta potential values for both fungus and microalgae were measured with the exception for fungus at pH 3. Even though both were negatively charged, the zeta potential of microalgae was more negative than that of fungus at all pH values. It was reported that, at pH values above 5.5, the cell walls of most microorganisms are negatively charged, tending to cause separation of the aggregating cells by electrostatic repulsion (Braun & Vecht-Lifshitz, 1991). This report supported these results, showing that the higher the pH value, the more negative the zeta potential value.

Based on the results from the previous chapter, the optimum co-pelletization occurs at uncontrolled pH. Fig 4.4 shows that even at uncontrolled medium pH, the surface charges of both fungus and microalgae were negative.

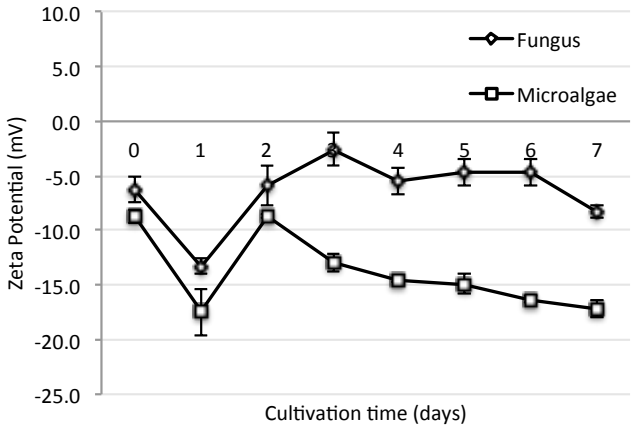

Figure 4. 4. Zeta potential of fungus and microalgae at the uncontrolled pH

From these results, it was observed that only acidic conditions (pH of 3) would shift the fungal hyphae charge to be positive. This suggested that under these conditions, the attraction between fungus and microalgae as well as co-pelletization could occur. However, it is not possible to co-culture microalgae with fungus under acidic conditions because, as discussed in the previous chapter, this would inhibit the growth of microalgae.

Interestingly, even at normal medium pH (pH of 7) with both the cell surfaces of microalgae and of fungus negatively charged, the co-pelletization process could still be observed. This suggested that the surface charges of fungal and of microalgal cells affected by the medium pH were not the main factors in the co-pelletization process.

Another potential cause of co-pelletization related to surface charge is the internal pH of fungus. It was discussed in the previous chapter that as a part of its metabolism,

fungus produced acid when glucose was utilized. It was reported that during its growth, *A.niger* oxidized glucose to gluconic acid and 2-ketogluconic acid (Gottlieb, 1963). Therefore, it was possible that, even though the medium pH was in a normal condition, the cell wall of the fungus was in an acidic condition. As a consequence, the fungal cells were negatively charged and as such the interaction between fungal and microalgal cells occurred.

4.2. The effects of Ca^{2+} on the surface charges of microalgae and fungus

It was observed that at all concentrations of Ca^{2+}, the zeta potential of both microalgae and fungus was negatively charged. In this experiment, it was clear that the surface charge of the microalgal cells was more negative (up to -23 mV) than that of the fungal cells (up to -15 mV) (Fig 4.5 and Fig 4.6).

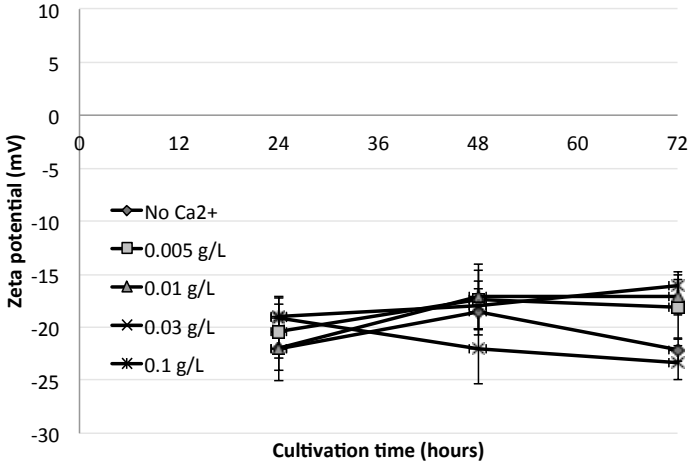

Figure 4. 5. Zeta potential of microalgae (*C. vulgaris*) at different concentrations of Ca^{2+}.

At uncontrolled medium pH, it was found that the surface charge of microalgal cells was more negative (<-20 mV) when the higher concentration of Ca^{2+} was applied (0.1 g/L), while the surface charge of fungal cells was less negative (> -5 mV). It was found that even though both fungal and microalgal cells were negatively charged, adding

Ca^{2+} to the media resulted in more differing zeta potential values between fungus and microalgae (Fig 4.7).

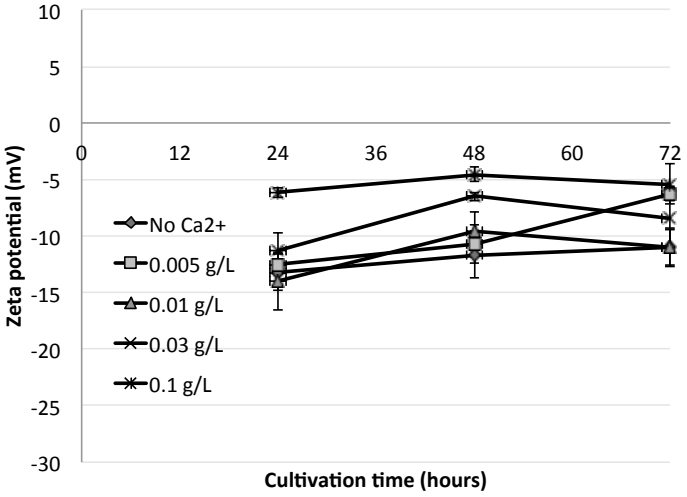

Figure 4. 6. Zeta potential of fungus (*A. niger*) at different concentration of Ca^{2+}.

Calcium was widely used in the flocculation process because of its bivalent cations, especially at high pH (Shelef, Sukenik, & Green, 1984). Vandamme et al (2012) reported that at a high pH (pH 0f 11), calcium precipitated before and after flocculation. In their results, however, it was reported that calcium at concentrations ranging between 0.001 g/L and 0.1 g/L did not induce flocculation even though it precipitated.

Contrary to the latter report, it was found in this experiment that co-pelletization can be induced by the presence of calcium, as was discussed in the previous chapter. A potential explanation for this is probably because the surface charge of the fungal cells tended to be positive due to the addition of calcium. The surface charge tests showed that the higher the concentration of calcium, the lower the negative charge of the fungal cells.

However, since the fungal and the microalgal cells had no opposing surface charges, it was suggested that the surface charge was not the main factor in the co-

pelletization process. The internal pH of fungal cells could be the cause of co-pelletization.

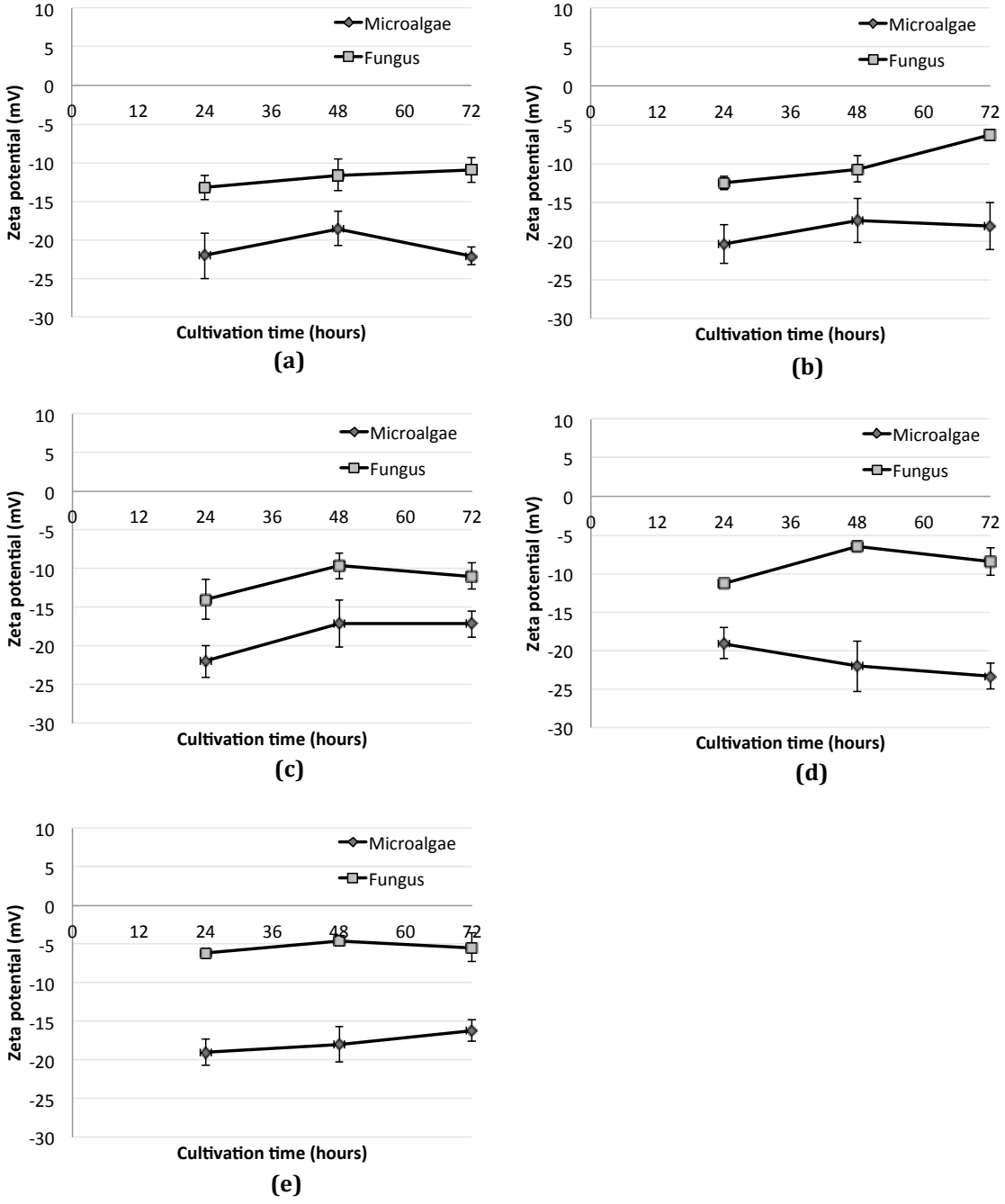

Figure 4. 7. Zeta potential of fungus and microalgae at different initial concentration of Ca^{2+}: (a) 0 g/L (without $Ca2^{+}$), (b) 0.005 g/L, (c) 0.01 g/L, (d) 0.03 g/L and (e) 0.1 g/L

4.3. The effects of Mg^{2+} on the surface charges of microalgae and fungus

It was observed that the zeta potential of both microalgae and fungus was negatively charged at all concentrations of Mg^{2+}. It was also shown that the surface charges of both fungal and microalgal cells were less negative (> -10 mV) when a higher concentration of magnesium was applied (0.5 g/L) (Fig 4.8 and Fig 4.9).

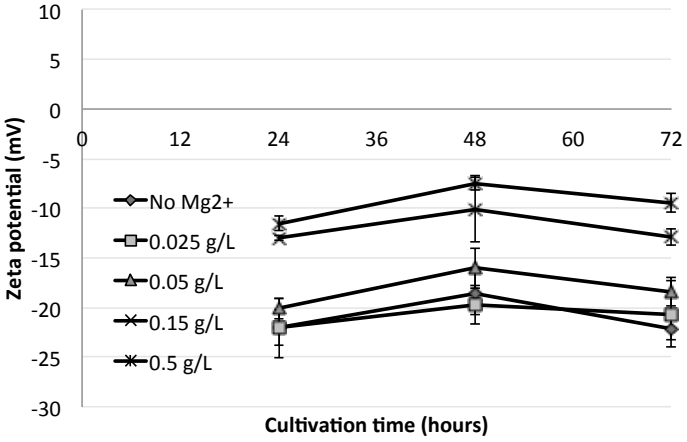

Figure 4. 8. Zeta potential of microalgae (*C. vulgaris*) at different concentration of Mg^{2+}.

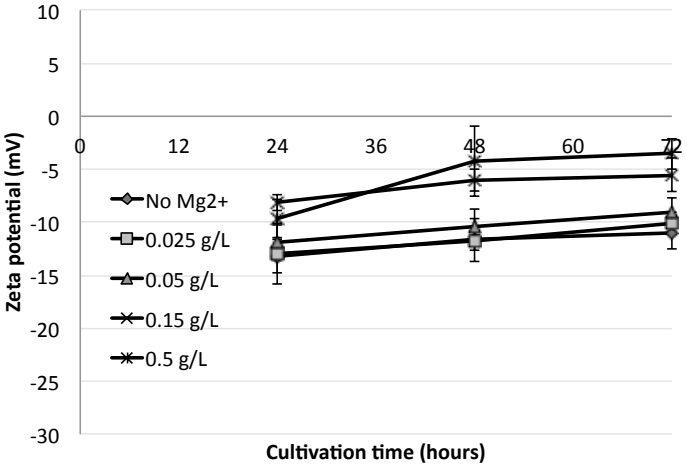

Figure 4. 9. Zeta potential of fungus (*A. niger*) at different concentration of Mg^{2+}.

At uncontrolled medium pH, it was found that by adding magnesium, the zeta potential of fungus and of microalgae were closer in value (-9 and -12 mV, respectively) (Fig 4.10), even though the surface charges of both fungal and microalgal cells were still negative.

It has been reported that the flocculation of *Chlorella* microalgae could be induced by magnesium precipitation (Vandamme , Foubert , Fraeye , Meesschaert , & Muylaert , 2012). The presence of magnesium (0.004 g/L or higher) played an important role in microalgae flocculation due to its bivalent cations, especially at a high pH (pH of 11) (Vandamme , Foubert , Fraeye , Meesschaert , & Muylaert , 2012). The same results were also reported by Leentvaar & Rebhun (1982) who applied magnesium-coagulation-flocculation (ranging between 0.005-0.044 g/L of magnesium) in wastewater treatment at a high pH (pH of 11.5)

In agreement with the report above, it was found in this experiment that co-pelletization can also be induced by the presence of magnesium, as discussed in Chapter 3. However, it was contrary to the results of the surface charge tests. It was observed that the surface charge of microalgal cells was less negative (>-12 mV) with a higher concentration of magnesium (0.5 g/L) Moreover, the presence of magnesium does not seem to affect the surface charge of the fungal cells. Even though the negative zeta potential value (>-5 mV) of fungal cells was lower for a higher concentration of magnesium ((0.5 g/L), there was no clear difference in overall surface charges at all concentrations. Therefore, it was evident that the surface charge could not be a main factor in the co-pelletization process. This suggests that there might be another

mechanism for fungal-microalgal cell attraction such a surface charge due to the internal pH of fungal pH or hydrophobicity of fungal cells.

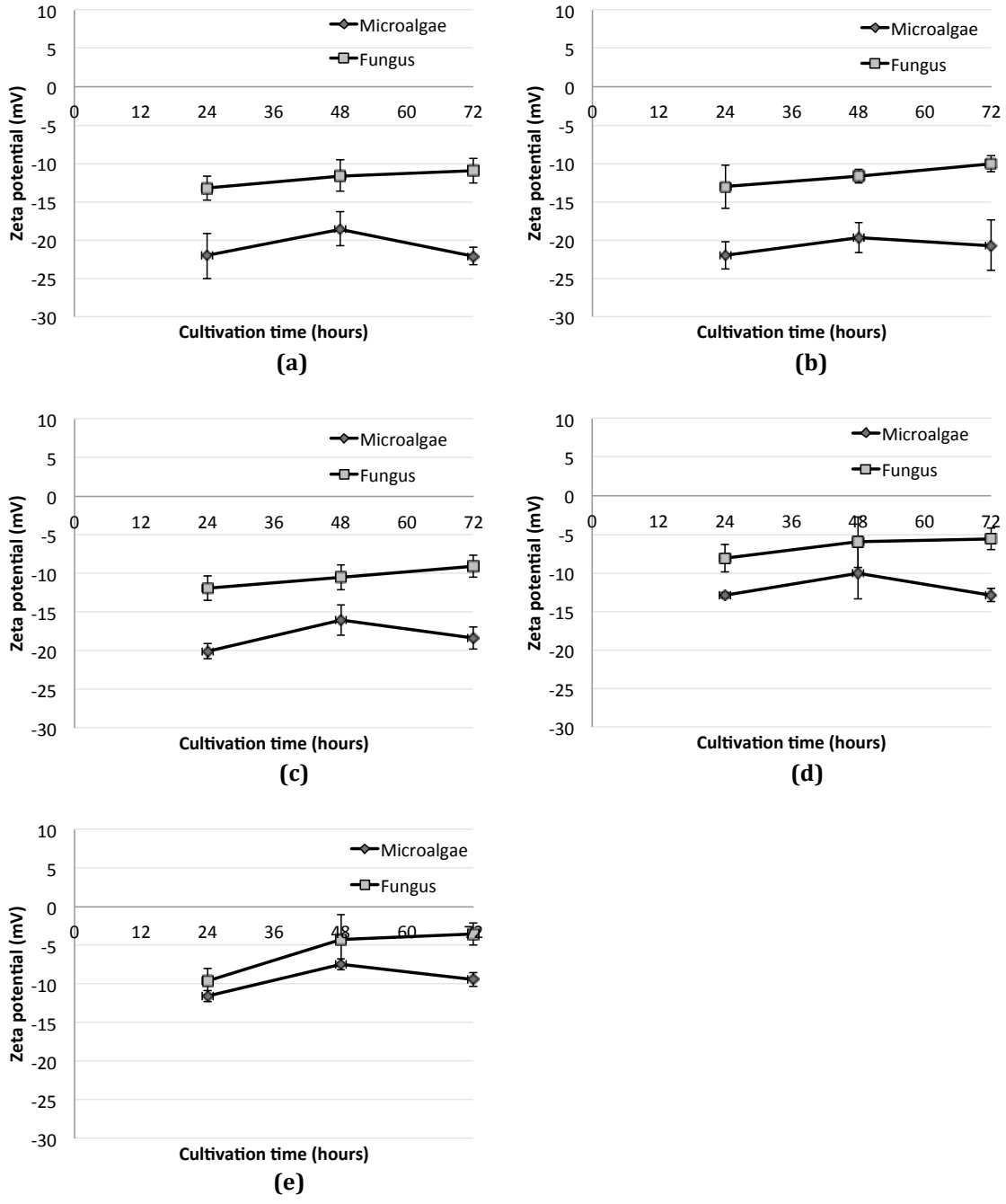

Figure 4. 10. Zeta potential of fungus and microalgae in different initial concentration of Mg^{2+}: (a) 0 g/L (without Mg^{2+}), (b) 0.025 g/L, (c) 0.05 g/L, (d) 0.15 g/L and (e) 0.5 g/L

4.4. The effects of Ca^{2+} and Mg^{2+} on the surface charges of microalgae and fungus under acidic conditions (pH of 3)

Based on the fact that the surface charge of fungal biomass was positive at pH 3, the effects of calcium and magnesium on the surface charges of microalgae and fungus under acidic conditions were tested. From this experiment, it was observed that, overall, the zeta potential values of both fungal and microalgal cells were negatively charged.

Fig 4.11 shows that the addition of both calcium and magnesium changed the zeta potential value of microalgal cells under acidic conditions. The surface charge was more negative (-10 mV) when adding calcium and even more negative (-13 mV) when adding magnesium compared to the culture medium without either calcium or magnesium. For the fungal cells, it was observed that the zeta potential value was positive after 3 days of cultivation (> +0.5 mV). In the first two days of cultivation, the zeta potential value was negative for medium with calcium and magnesium (< -0.1 mV) (Fig 4.12).

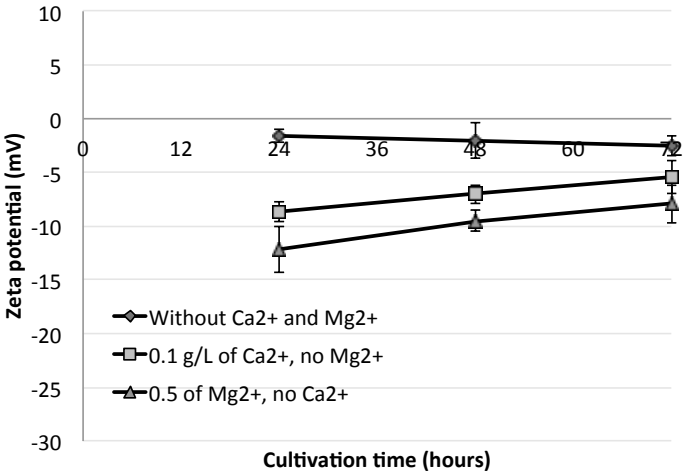

Figure 4. 11. Zeta potential of microalgae (*C. vulgaris*) without and with Ca2+ and Mg2+ at pH 3.

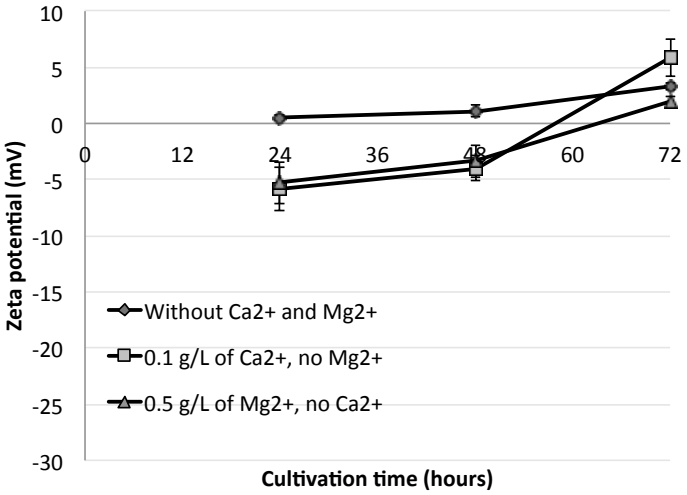

Figure 4. 12. Zeta potential of fungus (*A. niger*) without and with Ca2+ and Mg2+ at pH of 3.

These results suggest that the addition of calcium and magnesium to the medium under acidic conditions did not positively affect the surface charge of fungal cells. Compared with the previous tests with pH 3, the addition of calcium and magnesium shifted the surface charge of fungal cells to a negative value in the first two days of cultivation. In this test, the concentration of calcium and magnesium was 10 times higher than in the previous test (see Fig 4.2).

It was reported that calcium and magnesium (0.004 g/L) could be very effective for microalgae flocculation when they are applied at a high pH (pH of 11) (Vandamme , Foubert , Fraeye , Meesschaert & Muylaert , 2012). Therefore, applying calcium and magnesium under acidic conditions would probably not give an optimum result for co-pelletization. From this experiment, it can be seen that the presence of calcium and magnesium under acidic conditions could not effectively impact the surface charges of fungal and microalgal cells.

In this chapter, the hypothesis that the surface charges of fungal and microalgal cells affect the co-pelletization process was tested. pH values and ionic strength were used as important parameters to determine the surface charge through zeta potential measurement. It was determined that the surface charges of fungal and microalgal cells do not seem to be the main factor in the co-pelletization process. There must be other possible mechanisms for fungal-microalgal cells attraction, such as the surface charge affected by internal pH of fungal cells and the hydrophobicity of fungus.

CHAPTER 5
SUMMARY, CONCLUSIONS, AND FUTURE WORKS

The optimization of the co-culture conditions for the co-pelletization process and the co-pelletization mechanism of cell attraction due to the surface charge were studied and discussed in this master thesis. In this study, several key factors were tested in order to optimize the co-culture conditions for co-pelletization. The key factors include carbon source concentrations, pH values, initial concentrations of fungal spores, initial concentrations of microalgal spores, concentrations of ionic strength and salinity. In order to quantify the effects of each factor, certain parameters were used, including harvest efficiency (%), the concentration of microalgal biomass (mg DW/L), the concentration of fungal biomass (mg DW/L), and the morphology of pellets (size and diameter).

For the experiments on co-pelletization mechanisms, the surface charges of fungus and of microalgae were determined by zeta potential measurement. pH values and ionic strength as the main factors of surface charge were considered. In order to determine the effects of pH on the surface charge, three different pH values were employed (i.e. 3, 5, 7 and 10). For the ionic strength, the effects of calcium (Ca^{2+}) and magnesium (Mg^{2+}) were evaluated.

After presenting and discussing the results in this master thesis, there are certain conclusions that can be drawn:

1. The optimum conditions for co-pelletization were uncontrolled pH (a pH of around 7), 2 g/L of glucose, 8.50E+06 spores/L and 2.55E+09 spores/L for an inoculum concentration of fungal spores and microalgal cells, respectively, which

corresponds to a fungus to microalgae ratio of 1:300. Under these conditions, it was possible to achieve high harvesting performances (>90%).

2. In heterotrophic condition, glycerol and acetate can be used as carbon sources for co-pelletization. It was found that glycerol was better that acetate in terms of harvesting efficiency. However, comparing with glucose, it was found that glucose was the best carbon source for co-pelletization because of higher harvest efficiency (>90%), higher percentage of microalgal cells in the pellets (35%) and higher concentration of total fungal and microalgal cells (782 mg DW/L and 294 mg DW/L, respectively)
3. Although without Ca^{2+} and Mg^{2+}, the harvest efficiency was still high (> 90%), the presence of Ca^{2+} and Mg^{2+} could accelerate the co-pelletization process.
4. Co-pelletization can occur at a high level of salinity (30 g/L of NaCl).
5. All tests on the surface charge showed that both fungal and microalgal cells have negative charges, except at a pH of 3. It seems that the surface charge is not the main factor of fungal-microalgal cell attraction.

Fungus-microalgae co-pelletization is a promising technology for microalgae harvesting that can be used as an alternative to decrease the capital and operational costs. It was revealed that by applying the optimum conditions found in this study, the optimum harvest efficiency and biomass production out of co-pelletization could be obtained. However, some future research needs to be considered in order to make this method more economically attractive and viable:

1. Scale-up the co-pelletization process using the optimum conditions found in this study.

2. Other causes of fungal-microalgal cell attraction in co-pelletization mechanisms besides the surface charge should be explored. For instance, hydrophobicity of fungal cells or fungal cell compositions. By gaining a fundamental understanding of co-pelletization, the latter could effectively be applied in many areas.
3. Fungus *A. niger* could be applied for harvesting marine microalgae due to its ability to survive in high levels of salinity (30 g/L of NaCl).

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