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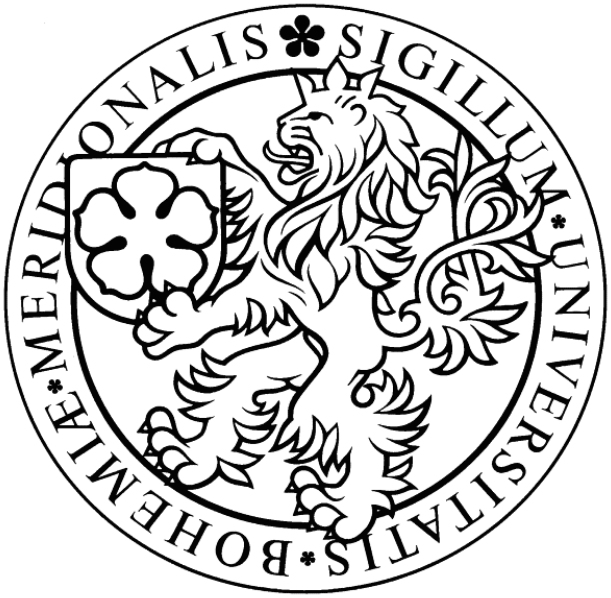

Master thesis by
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Biofuels from algae: Physiological characterization of candidate diatom species

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Annotation

The aim of this study was to isolate and extensively test promising candidate diatom species for biodiesel cultivation. Diatom strains were isolated from natural habitats in the state of Ohio (USA). These strains were tested to find optimal growing conditions and media (concentration of Nitrogen, Phosphorus, Silica, source of Nitrogen, temperature etc.).

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Abstract

Given the recent rise in gasoline prices combined with the recognition that global climate change represents a real challenge to human populations, there has been a recent resurgence in interest in developing biofuels from algae that would be a carbon-neutral source of energy. In this thesis project, a screening program for candidate algal species was undertaken, similar to the program undertaken by the Aquatic Species Program in the 1980's.

Local diatom strains were isolated from natural habitats in the state of Ohio. The fastest growing strain with high lipid content, *Fistulifera saprophila* LANGE-BERTALOT and BONIK, was identified in the process of initial screening. This strain was extensively tested to find optimal growing conditions and media. The best growth was observed in modified WC medium with urea as a source of nitrogen and half strength concentrations of nitrogen and phosphorus. A possible negative effect of urea as the nitrogen source was a decrease in lipid content in the biomass (from 28.57 % in WC medium to 18.24 %). Optimal light intensities for growth were found to be in the range of 350-450 $\mu E \cdot s^{-1} \cdot m^{-2}$. The most rapid growth was observed at ~25 °C.

Fistulifera saprophila seems to be a promising candidate species for biodiesel cultivation. It has rapid growth, high lipid content, and aggregates well (a characteristic that makes harvesting easier). It shows better growth in water both low in nitrogen and phosphorus, which provides an additional economic advantage. The next phase of this work will be the growing of this strain in outdoor ponds in Ohio, a project that has already begun.

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1. INTRODUCTION

The concept of algae cultivation as a source of biodiesel is not new. The first attempt was made during World War II (BURLEW 1953). In the 1970's the United States experienced massive oil shortages and increasing gas prices. In response, the Carter administration initiated an extensive effort to find alternative energy sources. In 1977, the Solar Energy Research Institute (SERI) was established. This federally-funded laboratory had several programs, one of which focused on production of ethanol from grasses, and production of biodiesel from microalgae. This effort was known as the Aquatic Species Program.

The goals of the Aquatic Species Program were: 1) cost benefit analysis of the technology; 2) characterization of saline groundwaters to be used for algal production; 3) isolation of high-lipid containing strains with fast growth rates; 4) optimalization of growing media and growing conditions; 5) establishment of an outdoor test facility; and 6) genetic modification of lipid-producing strains to improve yield. Late in the program a subsidiary goal was to use algal production to capture waste carbon dioxide from coal-fired power plants. Because of global stabilization and gas price decline in the 1980's, and a shifting of political party control at the national level, solar energy became unpopular in Congress, and the budget for SERI was cut dramatically by Reagan and the first Bush administration. The goal of successful biodiesel production was not reached because the project was shut down completely during the initial outdoor cultivation trials (SHEEHAN et al. 1998).

The political and economic landscape with regard to energy consumption has changed dramatically in recent years. Global climate change due to carbon dioxide

release from fossil fuel consumption has been accepted worldwide by scientists and most policy makers. Costly wars were fought in oil-rich countries (Kuwait, Iraq). Despite this effort to control the flow of oil to the United States, oil and gas prices have climbed sharply. These developments have brought attention once again to alternative energy sources. The most promising and widely studied options are carbon-neutral biodiesel and bioethanol. The energy required to produce an equal amount of energy is definitely lower for biodiesel than bioethanol (CHISTI 2007a).

Biodiesel fuels are generally fatty acid methyl esters originating from vegetable oils and animal fats. This type of fuel has been produced and used for more than 20 years, mostly in Europe. The crude oil consists of triglycerides that are converted to methyl esters in a reaction with methanol known as transesterification (CHISTI 2007b). Recently, the most-used crop plants for biodiesel production have been soybeans, palm oil, and corn in the Unites States, and rapeseed in Europe. The capability to meet worldwide fuel demand have none of these plants (CHISTI 2007b). Production of these high-in-oil crop plants requires large areas of land that are used to produce food. Thus biodiesel production from conventional crop plants is in direct conflict with food production. The promising alternative to crop plants is microalgae.

Microalgae are photosynthetic organisms that are able to convert carbon dioxide into organic matter by using sunlight as a source of energy (SAWAYAMA et al. 1995). They are more efficient converters than terrestrial plants due to their high growth rates (1-5 doublings per day), a capacity to grow in freshwater or brackish/coastal waters, and to tolerate marginal lands (such as desert) (DISMUKSEN et al. 2008). Their ability to inhabit harsh areas and still produce up to 50% of their dry weight in lipid, makes them suitable

for biofuels production (HU et al. 2008). In contrast, the biomass from terrestrial plants contains large amounts of lignin and other compounds that are needed as a support material for plants. These resistant carbohydrates are not broken down in biodiesel production and become a problematic waste product. Lignin and cellulose decrease the net oil production from terrestrial plants (GRESSEL 2008).

Algal cultivation can be done in two different ways – open culture systems or closed culture systems (bioreactors). In open culture systems the algae are usually grown in large outdoor raceway ponds or variable scale tanks (CHAUMONT 1993). This type of biomass production is fairly cheap (utilizing sun as an energy source). Compared to closed culture systems the final concentration and yield is usually lower, but is available in large amounts due to the size of the ponds. There are two types of closed culture systems. In the first type, the culture is closed in a transparent bioreactor and exposed to the sunlight. This type allows better control over the system; however, the costs are higher (BOROWITZKA 1999). In the second type algae are grown heterotrophically in closed vessels, so there is no light demand. The yield is higher; however, it is more costly and difficult to produce large amounts of biomass (WEN and CHEN 2003).

Presently, the main groups of microalgae utilized for biomass production are: cyanobacteria, diatoms and green algae. Cyanobacterial biomass is low in oil, and thus not useful for biodiesel production. Green algae have proven to produce biomass high in oil (up to 55%), but in order to achieve these lipid concentrations, they must be grown heterotrophically or at least mixotrophically (XU et al. 2006, LI et al. 2007). As mentioned earlier this type of cultivation is quite expensive and requires organic sources

that have to be taken from terrestrial crop plants, and thus it is not a tenable solution. Diatoms seem to be the only acceptable source of renewable biodiesel.

Diatoms are unicellular photosynthetic eukaryotes very abundant in all kinds of habitats. Diatoms, overall, are thought to produce as much as 25% of global primary production (SCALA and BOWLER 2001). The large diversity and ability to grow in a broad spectrum of conditions predetermine them to be used for biotechnological production. Despite their diversity in nature, only a few species have been used in biotechnology production (LEBAU and ROBERT 2003a and 2003b). To be able to grow algae (in particular, diatoms) in large scale we have to understand their growth requirements first. It has been shown that the most important factors for diatom growth are light intensity, temperature, nitrogen concentration, phosphorus concentration, silica concentration, pH, and carbon dioxide concentration (SÁNCHEZ-SAAVEDRA and VOLTOLINA 2006, KRICHNAVARUK et al. 2007).

Recently many studies have been published about diatom cultivation. In aquacultures they are usually grown in small-scale indoors and then transferred to outdoor ponds. The most important factor found increasing production was carbon dioxide (ARAÚJO and GARCIA 2005). Heterotrophic production of *Nitzschia laevis* has been successful for production of eicosapentoic acid for the pharmaceutical industry (WEN and CHEN 2000, CHEN at al. 2007), but because of the cost it can not be used in large scale for biodiesel production. Another cultivation method is closed helical or flat plate bioreactors used outdoors. Both methods have been proven to be successful (ACIÉN FERNÁNDEZ 2003, GOKSAN et al. 2003), but they have many limitations to be used for large scale cultivation. The only known way to produce large amounts of biomass with

low cost is cultivation in open race-way pond systems. This type of cultivation has been used many times in the last 50 years, but never was completely successful for production of biodiesel.

In this study, local diatom species were isolated from natural habitats in the state of Ohio. By using local species we can use the advantage of pre-adaptation to light and temperature conditions of the region, and avoid spreading potentially invasive species from other areas at the same time. Among the strains I isolated, the fastest growing strain with high lipid content was extensively tested to determine optimal nutrient, temperature and light levels. This thesis reports the results of the characterization and growth optimization of the diatom strain with greatest promise. This characterization is the first step in the process of diatom use as a source of biodiesel. Other steps, including large scale outdoor cultivation, are not the subject of this thesis, but will likely follow this work.

2 METHODS

2.1 Isolation of strains

Plankton samples were obtained during fall 2007 and summer 2008. Diatom strains were isolated from 18 ponds and rivers located in the state of Ohio (Table 1). The water chemistry analysis was done in the Old Woman Creek Reserve Water Chemistry Laboratory. Cultivation medium used for initial growth was WC Medium (GUILLARD & LORENTZEN 1972). The medium consists of the following nutrients ($mg \cdot l^{-1}$): TRIS buffer (500); $NaNO_3$ (85.01); $CaCl_2 \cdot 2H_2O$ (36.76); $MgSO_4 \cdot 7H_2O$ (36.97); $NaHCO_3$ (12.6); $Na_2SiO_3 \cdot 9H_2O$ (28.42); K_2HPO_4 (8.71); $Na_2EDTA \cdot 2H_2O$ (4.36); $FeCl_3 \cdot 6H_2O$ (3.15);

$CuSO_4 \cdot 5H_2O$ (0.01); $ZnSO_4 \cdot 7H_2O$ (0.22); $CoCl_2 \cdot 6H_2O$ (0.01); $MnCl_2 \cdot 4H_2O$ (0.18); $Na_2MoO_4 \cdot 2H_2O$ (0.006); H_3BO_3 (1). Medium was prepared in 4 l Erlenmeyer flasks with pH adjusted to 7.8-8 and autoclaved for 45 minutes at 121°C. Once the medium had cooled, vitamins were added. The vitamin solution consists of Thiamine·HCl (vitamin B_1) 0.1 $mg \cdot l^{-1}$; Biotin (vitamin H) 0.5 $\mu g \cdot l^{-1}$; Cyanocobalamin (vitamin B_{12}) 0.5 $\mu g \cdot l^{-1}$. The vitamin solution was filter-sterilized (0.2 µm mesh filter) and frozen before use.

Two methods of isolation were used. In the first case a single diatom cell was picked up with a Pasteur pipette directly from the sample using a compound microscope (100x magnification, Olympus BH2, Japan) and transferred into autoclaved cultivation tubes containing 5 ml of the WC media.

In the second case 0.7 ml of sample was transferred into autoclaved cultivation tubes containing 10 ml of the WC medium and grown for two weeks. Then, the sub-sample was spread on an agar plate and a single diatom cell was isolated with the Pasteur pipette using a stereomicroscope (Nikon SMZ-1, Japan) and transferred into 5 ml of the WC medium as in the first case.

Tubes with isolated cells were maintained on a cultivation wheel (New Brunswick Scientific, New Jersey, USA) with a continuous light regime (700-400 $\mu E \cdot s^{-1} \cdot m^{-2}$; measured with a LI-COR LI 1000, Nebraska, USA). The tubes were then checked for successfully isolated diatom strains and transferred into fresh media. The unialgal strain collection was obtained and maintained throughout the course of this study. Sub-samples of the cultures were acid cleaned, rinsed, mounted in Naphrax resin to make permanent microscope slides, and identified using optical microscopy (SGRO and JOHANSEN 1995).

Problematic taxa were examined using transmission electron microscopy (Zeiss EM-900, Germany).

Erlenmeyer flasks (125 ml) were used to test the growth rates of all successfully isolated diatom strains. Five ml of the diatom culture was added to 25 ml of the standard WC medium. The diatoms were grown for one week and then split into 6 Erlenmeyer flasks (5 ml of inoculum and 25 ml of fresh WC media). The optical density was measured daily with an Evolution 60 spectrophotometer (Thermo Scientific, Wisconsin, USA) at 750 nm. The flasks were grown under continuous illumination at ~80 $\mu E \cdot s^{-1} \cdot m^{-2}$ irradiance to obtain initial growth curves and growth rates. Based on the results from this experiment the best candidate species was chosen to be tested in detail.

The best candidate species was then grown in glass spinner flasks with continuous light (~80 $\mu E \cdot s^{-1} \cdot m^{-2}$) for five days to obtain a significant amount of biomass. This biomass was sent for oil content analysis to Eurofins Scientific Inc., Iowa, USA.

2.2 Comparison of nitrogen sources and nitrogen concentrations

The first experiments compared nitrogen sources and nitrogen concentrations. The candidate species, *F. saprophila*, was grown in small glass cultivation tubes (20 ml) on the cultivation wheel with a bright continuous light (700-400 $\mu E \cdot s^{-1} \cdot m^{-2}$) regime. Five different nitrogen sources were tested to find the best growth: sodium nitrate (the standard source in WC medium), ammonium nitrate, urea, ammonium hydroxide and ammonium bicarbonate. The nitrogen concentration from WC medium (14 $mg \cdot l^{-1}$) was used as a standard. The experimental concentrations were as follows: 7, 14, 21 and 28 $mg \cdot l^{-1}$. The media were prepared a day prior to the experiment as described earlier. The

only difference from WC medium was the nitrogen source and its concentration (overall 20 different media in a fully crossed design).

The batch culture of *F. saprophila* was transferred into fresh WC media in 3 l spinner flasks and grown with continuous light regime (~80 $\mu E \cdot s^{-1} \cdot m^{-2}$) for three days prior to each experiment to reach the exponential phase. At the start of the experiment, the biomass was harvested and washed twice with fresh WC nitrogen-free media. This culture was then used as an inoculum for the experiment.

0.1 ml of the inoculum was added to 10 ml of each medium, to obtain an initial optical density of ~0.01 at 750 nm. Each treatment was replicated three times (60 tubes altogether). The experiment was run for eight days; the optical density was checked daily at 750 nm. All tubes were harvested at day eight and ash free dry weight (AFDW in $g \cdot l^{-1}$, CHELF 1990) was obtained at the end of the experiment.

The design of the second experiment was identical to the one described above. The only difference was the duration of the test. Based upon the results from the first experiment, four days was chosen to be the optimal experimental period for the growth of *F. saprophila*.

Finally, based upon the results from first two experiments only sodium nitrate and urea were tested. The media and inoculum were prepared as described earlier. The number of replicates was increased from three to five.

2.3 Comparison of silica concentrations

In this experiment, the influence of silica concentration on the growth of *F. saprophila* was tested. The silica concentration from WC medium (2.81 $mg \cdot l^{-1}$) was used

as a standard. The experimental concentrations were 1.41 and 2.81 $mg \cdot l^{-1}$. The media (with urea as source of nitrogen) and inoculum were prepared as described above. Five replicates for each treatment were grown for four days in the same conditions as previous experiments. All tubes were then harvested AFDW in $g \cdot l^{-1}$ was obtained and analyzed.

2.4 Comparison of media with and without vitamins

In the next experiment *F. saprophila* was grown in a standard WC medium (with urea as source of nitrogen) and in the same medium in which the vitamins were omitted. Standard procedures from previous experiments were used.

2.5 Comparison of phosphate sources and nitrogen concentrations

This experiment compared phosphorus sources and concentrations. Two sources of phosphorus were used: potassium phosphate (the standard source in WC medium) and diammonium phosphate. Two phosphorus concentrations were tested: 0.775 $mg \cdot l^{-1}$ and 1.55 $mg \cdot l^{-1}$ (standard WC medium concentration).

In both tests, two nitrogen concentrations were tested. When potassium phosphate was used, all nitrogen was provided as urea at 7 $mg \cdot l^{-1}$ and 14 $mg \cdot l^{-1}$ (standard WC medium concentration). When diammonium phosphate was used, one fifth of the nitrogen was provided by this source. The remaining nitrogen (four fifths) was provided by urea. Overall, nitrogen concentrations were identical to the potassium phosphate (7 and 14 $mg \cdot l^{-1}$). Additionally, potassium in the media with diammonium phosphate was provided by potassium chloride.

Fistulifera saprophila was grown under the same conditions as the previous experiments in a fully crossed design (eight treatments altogether) for four days. All tubes were then harvested AFDW in $g \cdot l^{-1}$ was obtained and analyzed.

2.6 Comparison of nitrogen and phosphorus concentrations in large bioreactors

In the first experiment using large bioreactors, *F. saprophila* was grown in full and half strength nutrient media to confirm results from the small cultivation tubes. Six large polycarbonate tubes (17 l) were equipped with a draft tube installed centrally in the tube. The house air was provided at the bottom at a flow-rate 1 $l \cdot min^{-1}$ to allow gentle circulation of the media. Each tube was equipped with two fluorescent tubes on each side. Overall irradiance was 160-180 $\mu E \cdot s^{-1} \cdot m^{-2}$ in a 16h:8h light:dark cycle. Tubes were washed with 1.5% bleach solution and rinsed with DI water immediately prior to the experiment to eliminate algal contaminants and minimize bacterial contaminants.

Nitrogen concentration (urea as a source) in the standard WC medium was 14 $mg \cdot l^{-1}$ and phosphorus concentration (potassium phosphate as a source) was 1.55 $mg \cdot l^{-1}$. In the second medium, nitrogen concentration was 7 $mg \cdot l^{-1}$ and phosphorus concentration was 0.775 $mg \cdot l^{-1}$. Media were prepared in cleaned large carboys from sterilized stock solutions, but the DI water source was not sterile.

Inoculum of *F. saprophila* was added to the media to obtain an initial optical density of ~0.01 at 750 nm. Each treatment was run in three tubes. After four days, the experiment was terminated and analyzed.

2.7 Effect of carbon dioxide on the growth

In the last experiment the effect of carbon dioxide on the growth of *F. saprophila* was tested. The medium with half strength of nitrogen and phosphorus was prepared as described earlier. In three tubes, standard house air was provided. For the other three tubes the house air was enriched with carbon dioxide (~1%). The carbon dioxide was provided only during the light cycle of the experiment. All other conditions were maintained from the previous experiments.

At the end of the experiment the biomass was sent for oil content analysis to Eurofins Scientific Inc., Iowa, USA.

2.8 Crossed gradient of temperature and light experiment

F. saprophila was grown on a crossed gradient table to determine optimal temperature and light intensity conditions for growth. The experimental strain was grown in 125 ml Erlenmayer flasks for four days in the medium demonstrated to be optimal from previous experiments. The temperature range tested was 20°C – 35°C and the light intensity range was 350 – 850 $\mu E \cdot s^{-1} \cdot m^{-2}$ (Fig. 1). At the end of the experiment, the entire volume of the culture was harvested and the AFDW calculated as in previous cases.

2.9 Statistical analysis

All statistical analysis were done using SPSS 16.0 software (SPSS Inc., Illinois, USA). The analyses included one-way analysis of variance (ANOVA) and LSD post hoc tests for experiments in which there were more than two treatments. Independent sample

t-tests were used to analyze differences between two treatments. The rejection level used in all tests was α=0.05.

3 RESULTS

3.1 Isolation of strains

More than one hundred and fifty strains were successfully isolated from 18 natural lakes and rivers located in Ohio during fall 2007 and spring and summer 2008. Approximately 40 of them were able to grow in the collection for more than one month, with the others failing within that time. Based upon initial growth in test tubes, the ten fastest growing diatom strains were chosen for more detailed investigation. The majority of fastest growing strains (5) consisted of strains from the genus *Fistulifera*. The other genera included *Aulacosira* (2), *Stephanodiscus*, *Nitzschia* and *Cyclostephanos*.

The ten best strains were screened for growth in standard WC medium in 125 ml Erlenmeyer flasks. *Fistulifera saprophila* LANGE-BERTALOT and BONIK (Fig. 2) showed the best growth and thus was selected to be the candidate species for biodiesel production (3.6 doublings·day^{-1}). This strain was isolated on 1 January 2008 from a Muskingum River sample. The river sample was collected on October 15, 2007 by Pilný and Johansen. Results of the water chemistry analysis at the time of collecting were: conductivity 406 µS·cm^{-1}; turbidity 77.2 NTU; total alkalinity 77.93 mg·l^{-1}; NH_3-N 0.037 mg·l^{-1}; NO_3-N 10.9 mg·l^{-1}; NO_2-N 1.74 mg·l^{-1}; silicate 2.14 mg·l^{-1}; sulfate 56.02 mg·l^{-1} and soluble reactive phosphorus 99.3 µg·l^{-1}.

3.2 Comparison of nitrogen sources and nitrogen concentrations

Overall, lower concentrations of nitrogen provided better yield than higher concentrations. The AFDW means for the four nitrogen concentration levels were: 0.033 $g \cdot l^{-1}$ for half strength, 0.027 $g \cdot l^{-1}$ for full strength, 0.017 $g \cdot l^{-1}$ for double strength and 0.012 $g \cdot l^{-1}$ for triple strength (Fig. 3). Results of the effect of nitrogen source were not clear due to the difference in growth that occurred after day four. After this time period, some cultures entered senescent growth (Fig. 4), and the variance around replicate samples became high (Fig. 3). While some overall significant differences were detected in source (ANOVA $p < 0.001$, dfn = 4, dfd = 40, LSD $\alpha = 0.05$, sodium nitrate gave significantly lower final AFDW than all other sources), this experiment was not considered to be definitive given the problems in post-exponential growth variance. Due to the irregularity in growth after four days, the duration of subsequent experiments was four days.

3.3 Comparison of nitrogen sources and nitrogen concentrations II

In the second experiment the same design was used as in the previous case, with the only difference being the duration of the test (four days instead of eight). Again lower concentrations of nitrogen were found to be superior to higher concentrations. Overall, the AFDW for the four nitrogen concentration levels were: 0.056 $g \cdot l^{-1}$ for half strength, 0.057 $g \cdot l^{-1}$ for full strength, 0.038 $g \cdot l^{-1}$ for double strength and 0.040 $g \cdot l^{-1}$ for triple strength. In a comparison of different nitrogen sources (Fig. 5), urea and ammonium bicarbonate were found to be superior sources over the others (ANOVA $p < 0.001$, dfn = 4, dfd = 40) (AFDW means for ammonium bicarbonate and urea, 0.059 $g \cdot l^{-1}$ and 0.051

$g \cdot l^{-1}$, respectively). However, the data for OD showed almost exactly the same results for urea, ammonium bicarbonate and sodium nitrate. Ammonium bicarbonate has a higher cost, and requires pH adjustment of the medium. Because of these negative factors, ammonium bicarbonate was not considered to be a good nitrogen source for this technology, and consequently, sodium nitrate and urea were used in all subsequent experiments.

3.4 Comparison of urea and sodium nitrate as nitrogen sources

In a third experiment on nitrogen source utilizing only urea and sodium nitrate, the replicate number was increased from three to five to improve resolution of the results. Urea was demonstrated to be the superior source (Fig. 6). Nitrogen levels of in both tests were identical (14 mg $N \cdot l^{-1}$), the standard concentration of nitrogen from WC medium. After four days, AFDW of diatoms with urea was two fold higher than with sodium nitrate ($p < 0.001$). This experiment was run twice, with nearly identical results. The data reported are from the first test. These results led to the use of urea as the nitrogen source for subsequent tests.

3.5 Comparison of silica concentrations

After four days, AFDW of diatoms with full strength of silica was almost two fold higher than with half strength of silica ($p < 0.001$) (Fig. 7). In subsequent experiments full strength of sodium metasilicate was used.

3.6 Comparison of media with and without vitamins

The growth in media enriched with vitamins was significantly higher ($p < 0.001$), with a mean of 0.156 g AFDW·l^{-1} in contrast to 0.091 g AFDW·l^{-1} (Fig. 8). In all subsequent experiments vitamins were added to the media.

3.7 Comparison of phosphate sources

In a comparison of potassium phosphate vs. diammonium phosphate as a phosphorus source, there was a slight trend for higher AFDW within diammonium phosphate ($p = 0.010$), but the magnitude of the difference in AFDW was minimal (overall mean AFDW for potassium phosphate = 0.171 g AFDW·l^{-1} as opposed to diammonium phosphate = 0.165 g AFDW·l^{-1}) (Fig. 9). Total biomass accumulation was exactly the same for full strength and half strength phosphorus concentrations. However, biomass accumulation with lower nitrogen was significantly higher ($p < 0.001$, mean for half strength = 0.174 g AFDW·l^{-1}, mean for full strength = 0.162 g AFDW·l^{-1}).

3.8 Comparison of nitrogen and phosphorus concentrations in large bioreactors

Trends observed for various nitrogen and phosphorus concentrations in test tubes were further tested in large bioreactors with the addition of aeration with house air. After four days of cultivation the biomass was significantly higher in the media with half strength of nutrients ($p = 0.002$, with mean AFDW 0.138 g·l^{-1} for half strength and 0.103 g·l^{-1} for full strength) (Fig. 10). These results supported the trend observed in the previous tests.

3.9 Effect of carbon dioxide on the growth

Biomass accumulation was significantly higher ($p = 0.026$) when only house air was provided (mean AFDW for house air 0.129 $g \cdot l^{-1}$ as opposed to 0.118 $g \cdot l^{-1}$ for house air enriched with CO_2) (Fig. 11).

3.10 Crossed gradient of temperature and light experiment

The fastest growth was obtained in the area where the temperature reached 25-26 °C and where the light intensity was approximately 350-450 $\mu E \cdot s^{-1} \cdot m^{-2}$ (Fig. 12). These optimal conditions represented the lower light and lower temperature combinations achievable on the light gradient table.

3.11 Chemical analysis of *F. saprophila*

Biomass grown in WC medium contained 46.03% of protein, 28.57% of lipid, less than 15.4% of crude fiber and 11% of ash (Fig. 13). In contrast, biomass grown in the medium with half strength phosphorus and half strength nitrogen (urea as a source) contained 38.82% of protein, 18.24% of lipid, less than 11.86% of crude fiber and 11.86% of ash.

4 DISCUSSION

In this study forty strains were tested for high growth rate and high lipid content. The most promising strain with fastest growth rate and high lipid content was tested in detail to optimize growing conditions and growing media.

A four day growing period was found to be optimal for the growth of *Fistulifera saprophila* LANGE-BERTALOT and BONIK. After a short period in lag phase, the culture entered exponential phase, and began stationary phase in day four. The start of the stationary phase is the ideal time for harvesting biomass, as the cells shift from rapid growth with little storage to a phase of rapid lipid accumulation. Many authors have reported that four days is an optimal time period for growth of diatoms in batch cultivation (BROWN et al. 1996; MCGINNIS et al. 1997). The *F. saprophila* strain used in this study had growth comparable to the work of BARCLAY et al. (1986) and TADROS and JOHANSEN (1988).

All experiments demonstrated that optimal growth of *F. saprophila* occurs in media with a lower nitrogen concentration. From an economical point of view, this finding is very promising for mass cultivation of this strain. KRICHNAVARUK et al. (2007) reported that the diatom *Chaetoceros calcitrans* also preferred low nitrogen levels.

The inconsistent results in the first experiment were due to an eight day test period. The growth was very slow at the end of the experiment, and some tubes had obviously entered a senescent growth phase. In the second experiment the results were better overall, but the variation in the data was still high. This might be a consequence of the low number of replicates. However, the low number of replicates allowed me to test a broad spectrum of nitrogen sources and concentrations at the same time. I conclude this research design is a good tool for future studies of other candidate strains.

In a direct comparison of nitrogen sources, urea was found to be superior to sodium nitrate. This observation is in agreement with the work of TADROS and JOHANSEN (1988). These authors found that urea was the best nitrogen source for *Fistulifera*, but

surprisingly not for other diatoms tested. This might be a genus-specific trend. However, *F. saprophila* grows in a broad spectrum of conditions. It is possible that more than one species is encompassed by the current description of *F. saprophila*, as the species has been reported from high salinity waters (TADROS and JOHANSEN 1988) as well as fresh waters (LANGE-BERTALOT and BONIK 1976). The species is also polysaprobic, occurring in fairly clean waters to hypereutrophic waters. The striae range is given as 48-81 in 10 µm, an unrealistically variable range. My strain has a very narrow striae density range, 50 in 10 µm in the center, and 60 in 10 µm at the ends. The taxon needs more intensive investigation.

Fistulifera saprophila was found to grow better in standard silica concentration. This is consistent with other diatom studies (KRICHNAVARUK et al. 2007). Further increase of silica concentration did not improve growth significantly. Diatoms are very effective in consumption of silica from the environment and higher silica concentration in the medium often helps in the utilization of this source and supports faster growth.

This study proved that successful growth of *F. saprophila* requires vitamins in the medium. These findings support of the work by CROFT et al. (2006). The same trend was also observed in the work of KRICHNAVARUK et al. (2007). In their study a further increase of the vitamin concentration in the medium did not affect growth. This leads me to the conclusion, that vitamins are crucial for the growth of diatoms, although lower concentrations are sufficient for rapid growth.

From the experiment testing of nitrogen and phosphorus together in a crossed design it can be concluded that the concentration of nitrogen is more critical for the growth of *F. saprophila*. The growth in different phosphorus concentrations was identical

and thus phosphorus is not limiting for optimal growth even at varying nitrogen concentrations. This observation is consistent with many other studies (CHELF 1990; HEGARTY and VILLAREAL 1998; KRICHNAVARUK et al. 2007). The fact that the strain grows slightly better in the media with half concentration of nutrients is definitely an advantage for mass cultivation because of lower cost of production. This trend held up in other experiments, including those conducted in the large bioreactors.

The negative effect of carbon dioxide on growth is surprising. Carbon dioxide enrichment usually supports the growth of diatoms (de CASTRO ARAÚJO and GARCIA 2005; RAGHAVAN et al. 2008). In our experiment the negative effect probably was caused by too steep of a decrease in pH, which may be very important for diatom growth. Another possible explanation might be insufficient control over the carbon dioxide in the house air mixture.

The optimal light intensity range for *F. saprophila* growth found in this study is slightly higher than the range usually reported by other authors (LIANG et al. 2006; BROWN et al. 1996). The temperature of ~25°C found to be optimal for efficient growth is in agreement with the work of other authors (RAGHAVAN et al. 2008). Lower temperatures usually do not support rapid growth and higher temperatures decrease the percentage of lipid produced by diatoms (de CASTRO ARAÚJO and GARCIA 2005). However, TADROS and JOHANSEN (1988) in their experiment with saline *F. saprophila* found a temperature of 30°C produced faster growth. Their strain was isolated from the coastal waters of Florida and thus was adapted to growing in higher temperatures. Unfortunately, the poor design of the crossed gradient table did not allow me to test a finer range of temperatures and light intensity.

The biomass grown in standard WC medium had a higher lipid content (28%) compared to the biomass grown in the medium optimized in this study (18%). The protein fraction in the biomass grown in the optimal medium also slightly decreased. The other compounds (crude fiber and ash) were detected at very similar levels. If all compounds are included, 100% of the biomass is expressed as these four compounds for WC medium. In the medium with urea as a source of nitrogen and lower concentration of nutrients the sum is only 81%. Saline strains of *Fistifulifera saprophila* have been found to produce significant amounts of carbohydrate in some media (BARCLAY et al. 1986). This interesting result definitely requires further experiments.

Fistulifera saprophila seems to be a promising candidate species for production of biodiesel in mass cultivation. This species showed better growth under lower nutrient concentrations of while still producing high lipid content in the biomass. The other advantage of using *Fistulifera* is its ability to create compact aggregates. This ability allows fast sedimentation and thus easier harvesting. Several strains of the same species were intensively tested in the Aquatic Species Program at the Solar Energy Research Institute (BARCLAY et al. 1986). However, only saline strains were tested in this program, so the data are not completely comparable. The temperature optimum of saline strains was higher by 5 °C, with a maximal growth rate of 4 doublings·day^{-1}. Almost identical results were observed in the work of TADROS and JOHANSEN (1988) using several strains isolated in Aquatic Species Program. Lipid content for *Fistulifera* was definitely lower in these studies (22% and 16% respectively) compared to the 28.5 % yield in the present study.

This study is only the first step in the long process of biodiesel production. I completed isolation of local candidate species with rapid growth and high lipid production. Local species are well adapted to conditions occurring in the area of cultivation, and it is also critical to not introduce non-native taxa to an area because of the threat of invasive species. *F. saprophila* showed better growth in lower nutrient concentration which is important for the economy of the whole project. Optimal temperature and light intensity was determined and will be considered in subsequent outdoor cultivation.

Future laboratory experiments will include cultivation of *F. saprophila* in nutrient-deficient medium. It is well known that this treatment can increase the lipid concentration in the biomass. Depending on the deficient nutrient (nitrogen, phosphorus, silica) in the medium, the increase in lipid concentration can be up to 200% (BARCLAY et al. 1986; TADROS and JOHANSEN 1988; MCGINNIS et al. 1997).

The biggest challenge for outdoor mass cultivation is contamination by other organisms. Bacterial (including cyanobacterial) contamination might be treated with antibiotics specific for prokaryotes. Similar treatments can be used for amoeba or rotifer contamination. Contamination by other eukaryotic algae is very problematic, specifically green algae. They have very fast growth, and antibiotics against eukaryotic organisms can not be used. The most promising way to keep other algae out is cultivation in extreme conditions, such as high pH or high salinity (VONSHAK and RICHMOND 1985). Not all strains can tolerate these extreme conditions so media with a low content of heavy metals have been developed and successfully used specifically to grow diatoms (DEBENEST et al.

2008). These approaches will be combined in subsequent tests to reach sufficient growth of the target species without development of other microorganisms.

Other significant problems in large-scale cultivation include oxygen stress and photoinhibition. Fast-growing, dense algal cultures produce large amounts of oxygen that can became toxic to diatoms. The whole volume of medium has to be sparged with air periodically to release the oxygen (RODOLFI et al. 2008). Photoinhibition occurs when the algae are exposed to high light intensities. Possible solutions to this problem include increasing population density and providing a high amount of mixing in the cultures, or using specifically designed photobioreactors (TORZILLO et al. 2003).

In my future work I will try to find optimal conditions for increasing lipid content through use of nutrient-deficient medium (low N and low Si, most likely). The other effort in the laboratory will be to test media containing low amounts of heavy metals to evaluate their effectiveness in preventing other algal contaminants from growing in the media.

5 REFERENCES

ACIÉN FERNÁNDEZ, F.G.; HALL, D.O.; CANIZARES GUERRERO, E.; KRISHNA RAO, K. and MOLINA GRIMA, E. (2003): Outdoor production of *Phaeodactylum tricornutum* biomass in a helical reactor – Journal of Biotechnology 103 – 137-152.

ARAÚJO, S. de C. and GARCIA, V.M.T. (2005): Growth and biochemical composition of the diatom *Chaetoceros* cf. *wighamii* brightwell under different temperature, salinity and carbon dioxide levels. I. Protein, carbohydrates and lipids – Aquaculture 246 – 405-412.

BARCLAY, W.; JOHANSEN, J.R.; CHELF, P.; NAGLE, N.; ROESSLER, P. and LEMKE, P. (1986): Microalgae culture collection 1986-1987. 149 pp. Solar Research Institute. Golden.

BOROWITZKA, M.A. (1999): Commercial production of microalgae: ponds, tanks, tubes and fermenters – Journal of Biotechnology 70 – 313-321.

BROWN, M.R.; DUNSTAN, G.A.; NORWOOD, S.J. and MILLER K.A. (1996): Effects of harvest stage and light on biochemical composition of the diatom *Thalassiosira pseudonana* – Journal of Phycology 32 – 64-73.

BURLEW, J.S. (ed.) (1953): Algal culture from laboratory to pilot plant. 357 pp. Carnegie Institution of Washington. Washington DC.

CHAUMONT, D. (1993): Biotechnology of algal biomass production: a review of systems for outdoor mass culture – Journal of Applied Phycology 5 – 593-604.

CHELF, P. (1990): Environmental control of lipid and biomass production in two diatom species – Journal of Applied Phycology 2 – 121-129.

CHEN, G.; JIANG, Y. and CHEN, F. (2007): Fatty acid and lipid class composition of the eicosapentaenoic acid-producing microalga, *Nitzschia laevis* – Food Chemistry 104 – 1580-1585.

CHISTI, Y. (2007a): Biodiesel from microalgae – Biotechnology Advances 25 – 294-306.

CHISTI, Y. (2007b): Biodiesel from microalgae beats bioethanol – Trends in Biotechnology 26 – 126-131.

CROFT, M.T.; WARREN, M.J. and SMITH, A.G. (2006): Algae need their vitamins – Eukaryotic Cell 5 – 1175-1183.

de CASTRO ARAÚJO, S. and GARCIA V.M.T. (2005): Growth and biochemical composition of the diatom *Chaetoceros* cf. *wighamii* Brightwell under different temperature, salinity and carbon dioxide levels. I. Protein, carbohydrates and lipids – Aquaculture 246 – 405-412.

DEBENEST, T.; SILVESTRE, J.; COSTE, M.; DELMAS, F. and PINELLI, E. (2009): A new cell primo-culture method for freshwater benthic diatom communities – Joutnal of Applied Phycology 21 – 65-73.

DISMUKSEN, G.C.; CARRIERI, D.; BENNETTE, N.; ANANYEV, G.M.; and POSEWITZ, M.C. (2008): Aquatic phototrophs: efficient alternatives to land-based crops for biofuels – Current Opinion in Biotechnology 19 – 235-240.

GOKSAN, T.; DURMAS, Y. and GOKPINAR, S. (2003): Effects of light path lengths and initial culture density on the cultivation of *Chaetoceros muelleri* (Lemmermann, 1898) – Aquaculture 217 – 431-436.

GRESSEL, J. (2008): Transgenics are imperative for biofuel crops – Plant Science 174 – 246-263.

GUILLARD, R.R.L. AND LORENTZEN, C.J. (1972): Yellow-green algae with chlorophyllide *c*. – Journal of Phycology 8 – 10-14.

HEGARTY, S.G. and VILLAREAL, T.A. (1998): Effects of light level and N:P supply ratio on the competition between *Phaeocystis* cf. *pouchetii* (Hariot) Lagerheim (Prymnesiophyceae) and five diatom species – Journal of Experimental Marine Biology and Ecology 226 – 241-258.

HU, Q.; SOMMERFELD, M.; JARVIS, E; GHIRARDI, M.; POSEWITZ, M; SEIBERT, M. and DARZINS, A. (2008): Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances – The Plant Journal 54 – 621-639.

KRICHNAVARUK, S.; POWTONGSOOK, S. and PASAVANT, P. (2007): Enhanced productivity of *Chaetoceros calcitrans* in airlift photobioreactors – Bioresurce Technology 98 – 2123-2130.

LANGE-BERTALOT, H. and BONIK, K. (1976): Massentwicklung bisher seltener und unbekannter Diatomeen als Indikator straker Abwasserbelastung in Flüssen. Algological Studies – Archiv für Hydrobiologie Supplements 49 – 303-332.

LEBAU, T. and ROBERT, J.M. (2003a): Diatom cultivation and biotechnologically relevant products. Part I: Cultivation at various scales – Applied Microbiological Biotechnology 60 – 612-623.

LEBAU, T. and ROBERT, J.M. (2003b): Diatom cultivation and biotechnologically relevant products. Part II: Current and putative products - Applied Microbiological Biotechnology 60 – 624-632.

LI, X.; XU, H. and WU, Q. (2007): Large-scale biodiesel production from microalga *Chlorella protothecoides* through heterotrophic cultivation in bioreactors – Biotechnology and Bioengineering 98 – 764-771.

LIANG, Y.; BEARDALL, J. and HERAUD, P. (2006): Effects of nitrogen source and UV radiation on the growth chlorophyll fluorescence and fatty acid composition of *Phaeodactylum tricornutum* and *Chaetoceros muelleri* (Bacillariophyceae) – Journal of Photochemistry and Photobiology B: Biology 82 – 161-172.

MCGINNIS K.M.; DEMPSTER, T.A. and SOMMERFELD, M.R. (1997): Characterization of the growth and lipid content of the diatom *Chaetoceros muelleri* - Journal of Applied Phycology 9 – 19-24.

RAGHAVAN, G.; HARIDEVI, C.K. and GOPINATHAN, C.P. (2008): Growth and proximate composition of the *Chaetoceros calcitrans* f. *pumilus* under different temperature, salinity and carbon dioxide levels – Aquaculture Research 39 – 1053-1058.

RODOLFI, L.; ZITTELLI, G.C.; BASSI, N.; PADOVANI, G.; BIONDI, N.; BONINI, N. and TREDICI, M.R. (2008): Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor – Biotechnology and Bioengineering 102 – 100-112.

SÁNCHEZ-SAAVEDRA, M. del P. and VOLTOLINA, D. (2006): The growth rate, biomass production and composition of *Chaetoceros* sp. grown with different light sources – Aquacultural Engineering 35 – 161-165.

SAWAYAMA, S.; INOUE, S.; DOTE, Y. and YOKOYAMA, S.Y. (1995): CO_2 fixation and oil production through microlga - Energy Conversion and Management 36 – 729-731.

SCALA, S. and BOWLER, C. (2001): Molecular insights into the novel aspects of diatom biology – Cell Molecular Life Science 58 – 1666-1673.

SGRO, G.V. and JOHANSEN, J.R. (1995): Rapid bioassessment of algal periphyton in freshwater streams p. 291 – 311. In: BUTTERWORTH, F.M. et al. (eds.): Biomonitors and biomarkers as indicators of environmental change: a handbook. Plenum Press. New York.

SHEEHAN, J.; DUNAHAY, T.; BENEMANN, J. and ROESSLER, P. (1998): A look back at the U.S. Department of energy's Aquatic Species Program – Biodiesel from algae. 296 pp. National Renewable Energy Laboratory. Golden.

TADROS, M.G. and JOHANSEN, J.R. (1988): Physiological characterization of six lipid-producing diatoms from southeastern United States – Journal of Phycology 24 – 445-452.

TORZILLO, G.; PUSHPARAJ, B.; MASOJIDEK, J. and VONSHAK, A. (2003): Biological constraints in algal biotechnology – Biotechnology and Bioprocess Engineering 8 – 338-348.

VONSHAK, A. and RICHMOND, A. (1985): Problems in developing the biotechnology of algal biomass production – Plant and Soil 89 – 129-135.

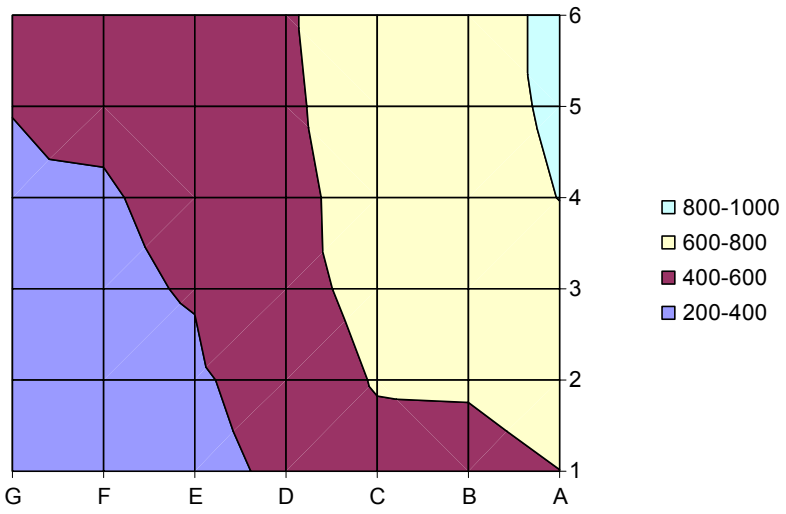
WEN, Z. and CHEN, F. (2000): Production potential of eicosapentaenoic acid by the diatom *Nitzschia laevis* – Biotechnology Letters 22 – 727-733.

WEN, Z.Y. and CHEN, F. (2003): Heterotrophic production of eicosapentaenoic acid by microalgae – Biotechnology Advances 21 – 273-294.

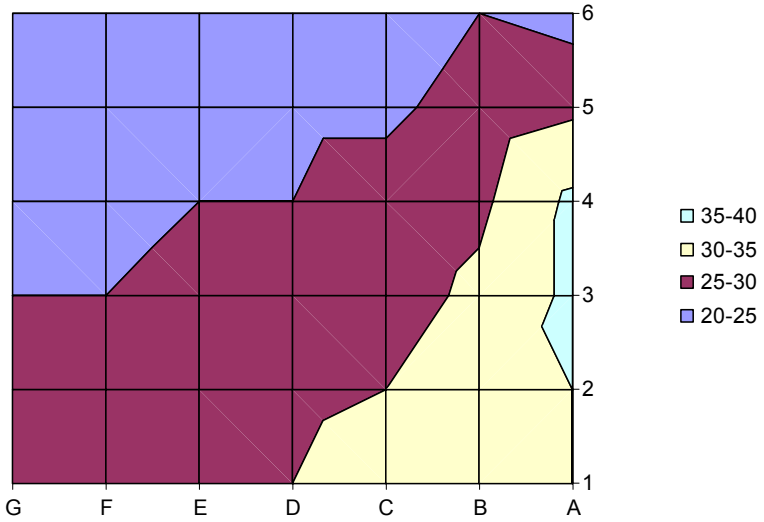
XU, H; MIAO, X. and WU, Q. (2006): High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters – Journal of Biotechnology 126 – 499-507.

Table 1. List of localities sampled in Ohio.

Site name	Date of collection
Aquilla lake	September 6, 2007
Ashtabula River at Ashtabula	September 28, 2007
Bass lake	September 6, 2007
Black River at Lorain	October 19, 2007
Chagrin River at Cleveland	September 28, 2007
Cuyahoga River at Cleveland	October 19, 2007; August 1, 2008
East Branch Reservoir	September 19, 2007
Grand River at Madison	September 28, 2007
Huron River at Huron	October 20, 2007
Lake Erie at Cleveland	October 19, 2007; August 1, 2008
Lake Erie at Magee Marsh	October 20, 2007; August 1, 2008
Magee Marsh	October 20, 2007; August 1, 2008
Maumee River at Toledo	October 20, 2007
Muskingum River at Connesville	October 15, 2007
Old Woman Creek	October 20, 2007; August 1, 2008
Portage River at Port Clinton	October 20, 2007
Punderson Lake	September 19, 2007
Vermillion River at Vermillion	October 20, 2007



a)



b)

Figure 1. Measurements of light intensity (**a** – in $\mu E \cdot s^{-1} \cdot m^{-2}$) and temperature (**b** – in °C) in the crossed gradient experiment.

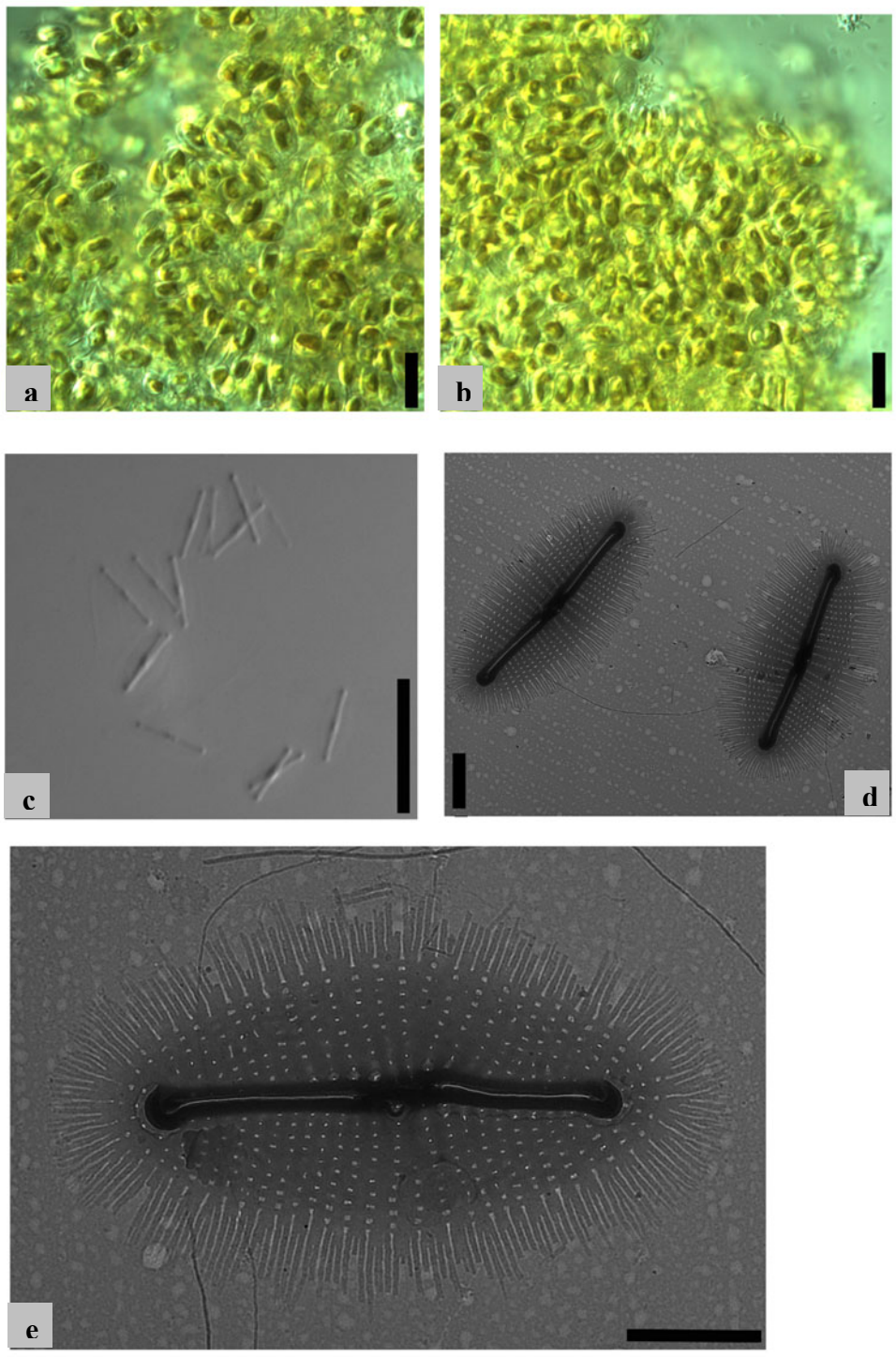


Figure 2. *Fistulifera saprophila* LANGE-BERTALOT and BONIK. Light microscopy – **a**, **b** at 630x; **c** at 1600x. TEM – **d** at 2000x, **e** at 5000x. Scalebar: a,b,c = 10 µm; d,e = 1 µm.

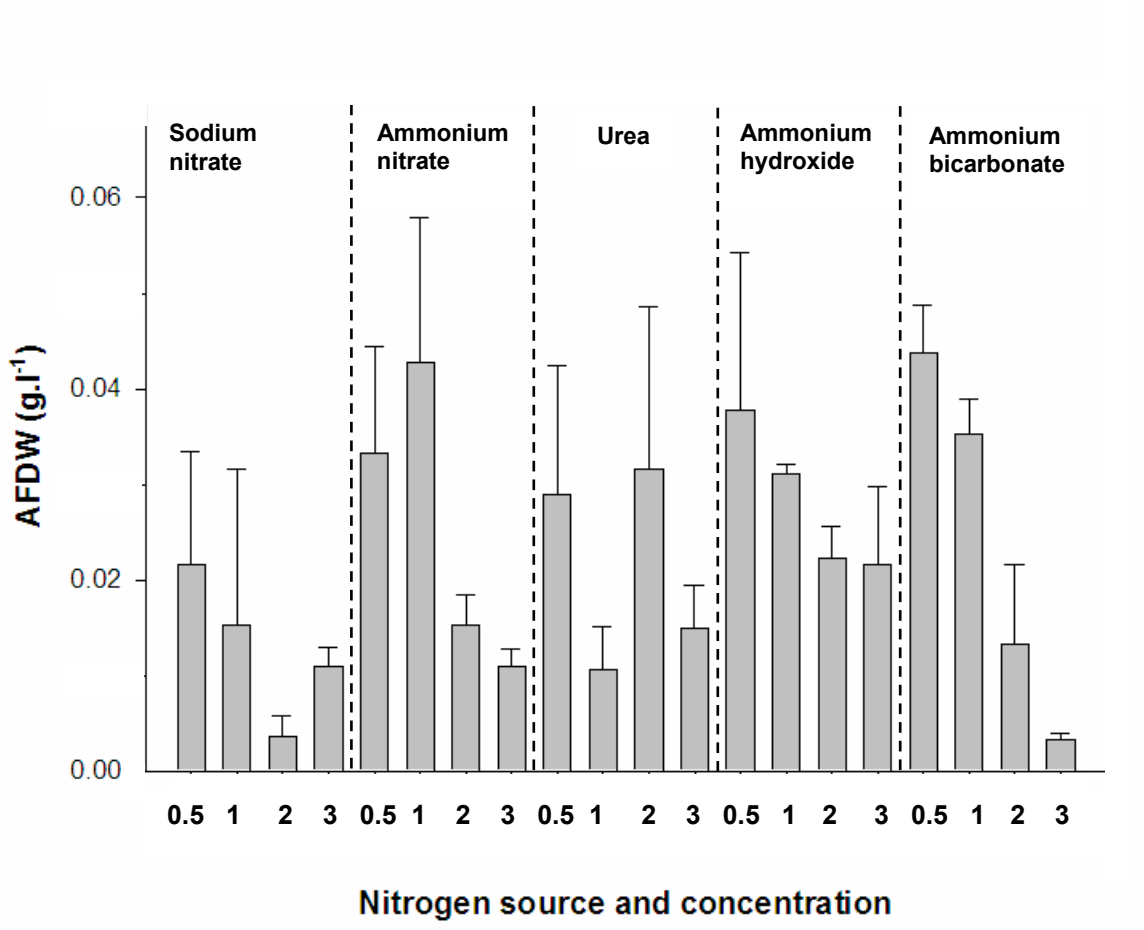


Figure 3. *F. saprophila* biomass accumulated over eight days with different nitrogen sources and concentrations. Data are means ± SD (n = 3). Concentration 0.5 = half strength, 1 = full strength, 2 = double strength and 3 = triple strength of WC medium.

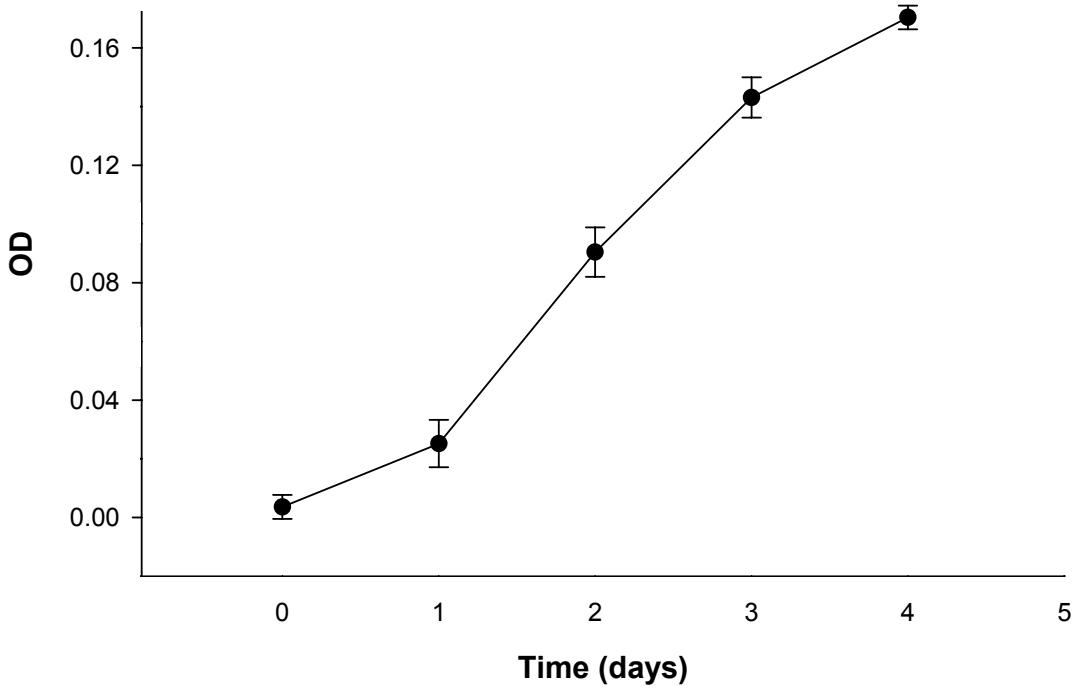


Figure 4. Typical growth curve of *F. saprophila* grown in WC medium. Data are means ± SD (n = 6).

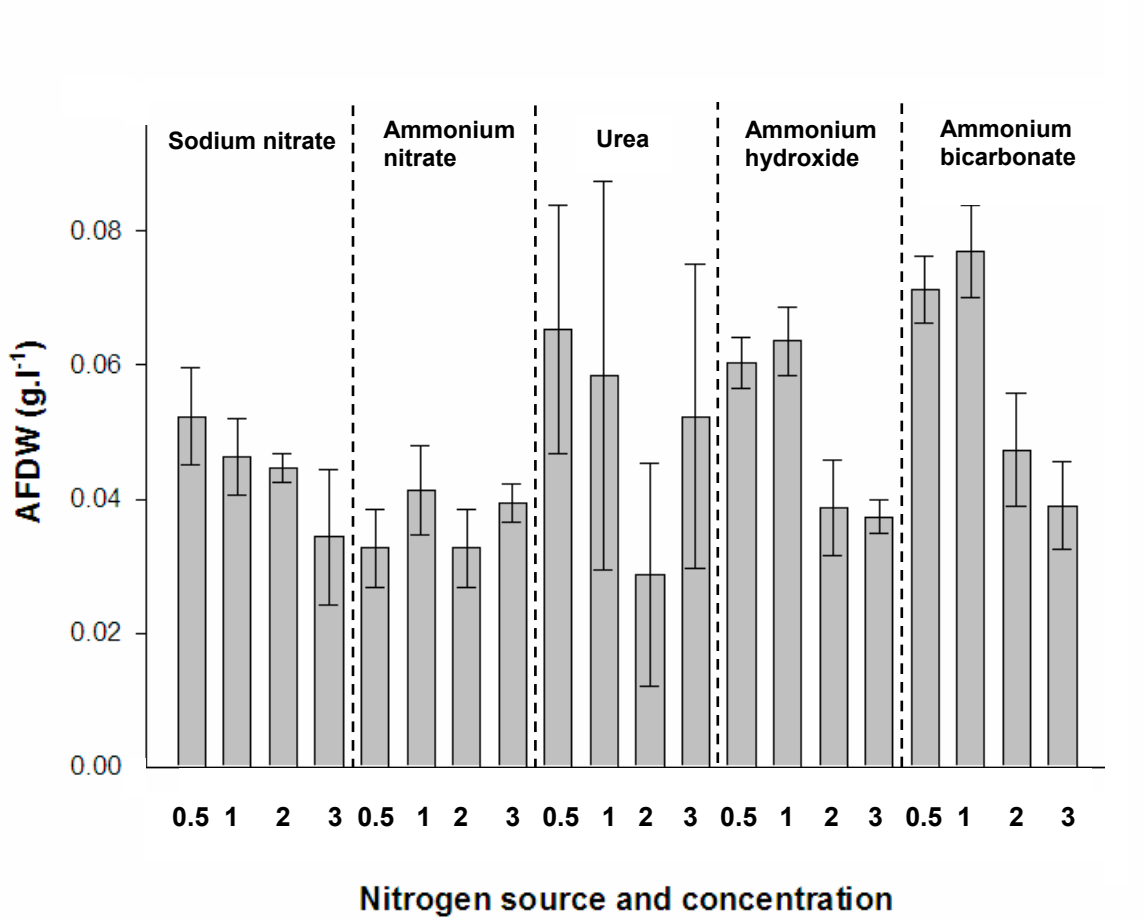


Figure 5. *F. saprophila* biomass accumulated over four days with different nitrogen sources and concentrations. Data are means ± SD (n = 3). Concentration 0.5 = half strength, 1 = full strength, 2 = double strength and 3 = triple strength of WC medium.

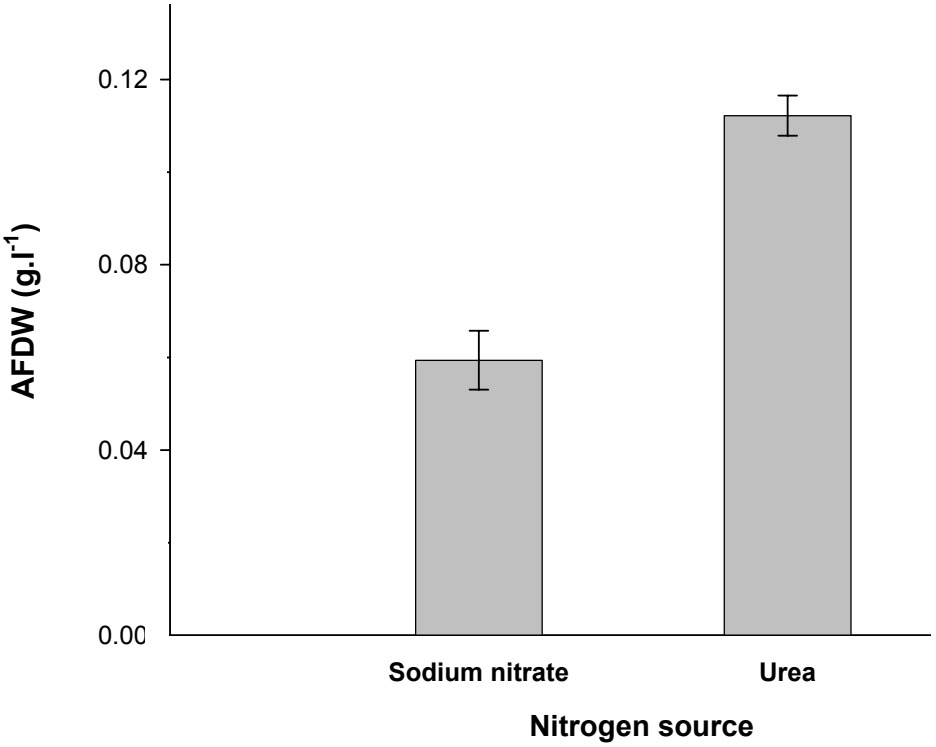


Figure 6. Comparison of *F. saprophila* biomass accumulation grown in media with sodium nitrate or urea as a nitrogen source. Data are means ± SD (n = 5).

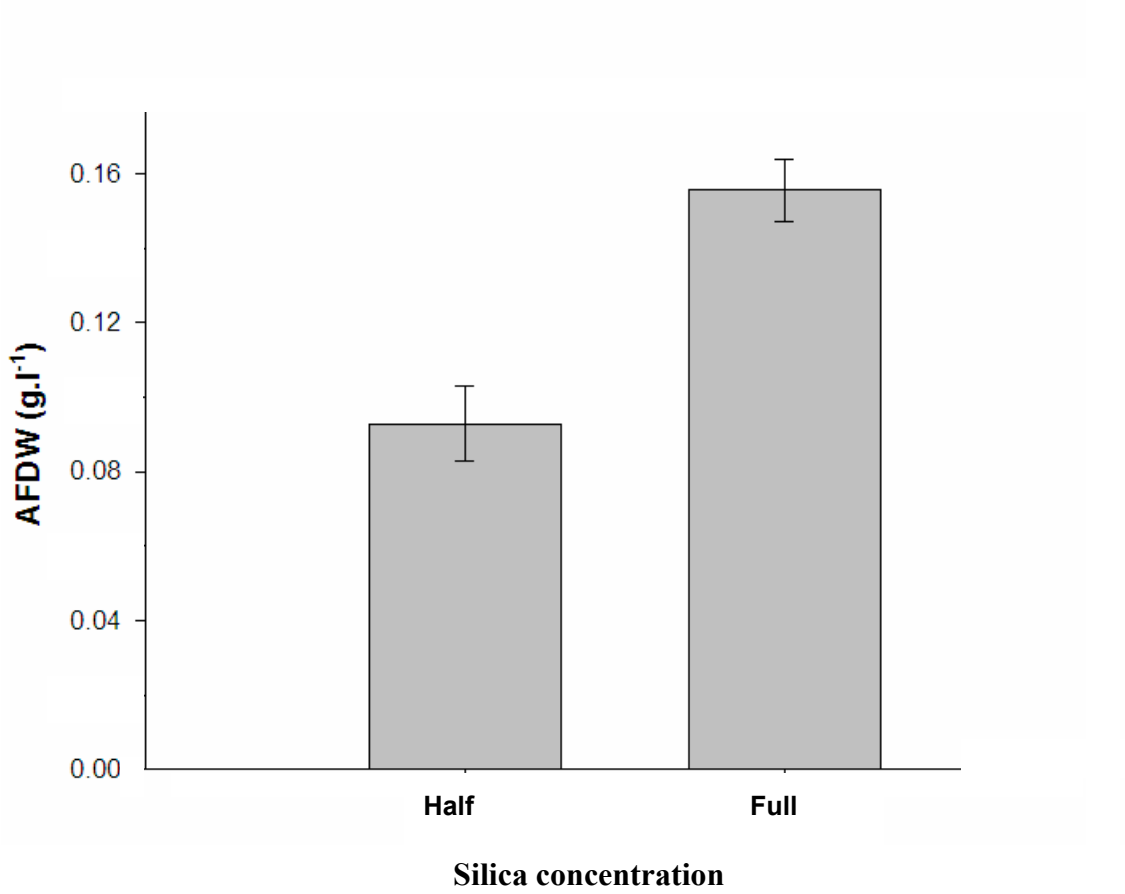


Figure 7. Comparison of *F. saprophila* biomass accumulation grown in media with half silica strength and full silica strength. Data are means ± SD (n = 5).

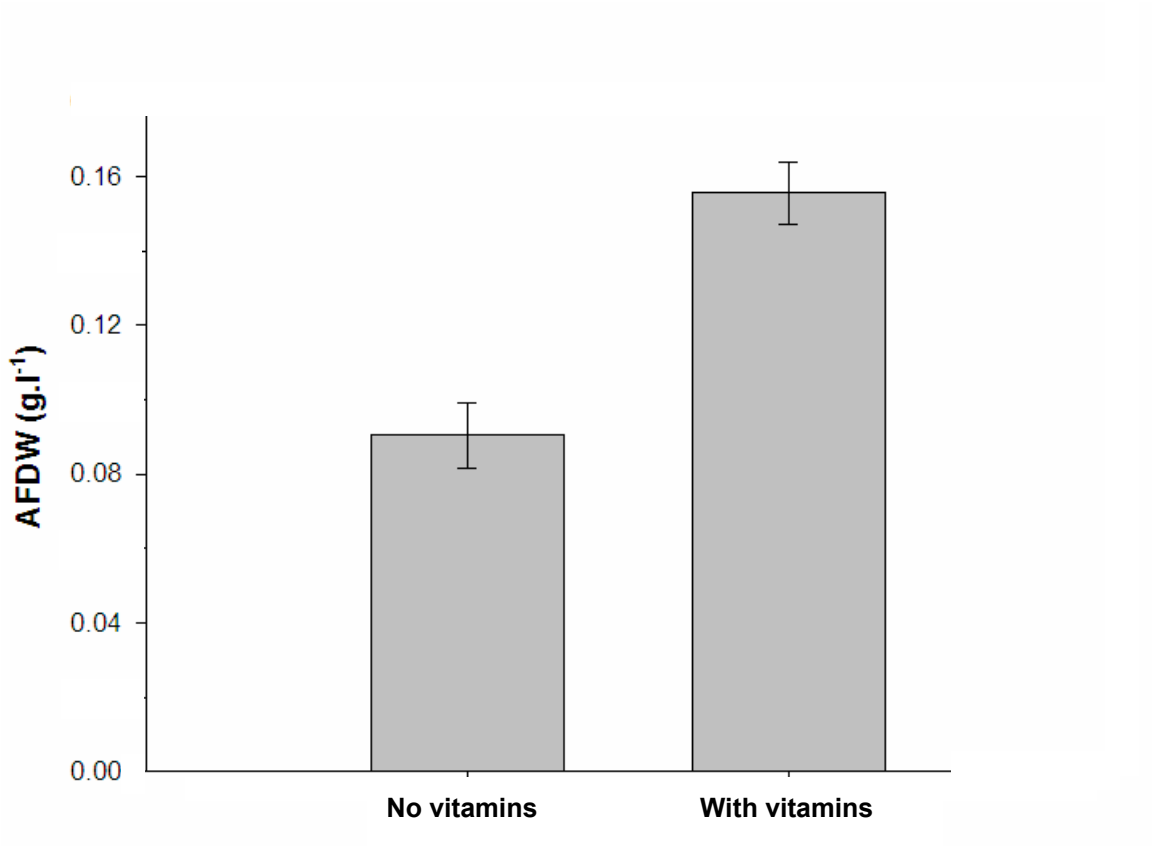


Figure 8. Comparison of *F. saprophila* biomass accumulation grown in the media with and without vitamins. Data are means ± SD (n = 5).

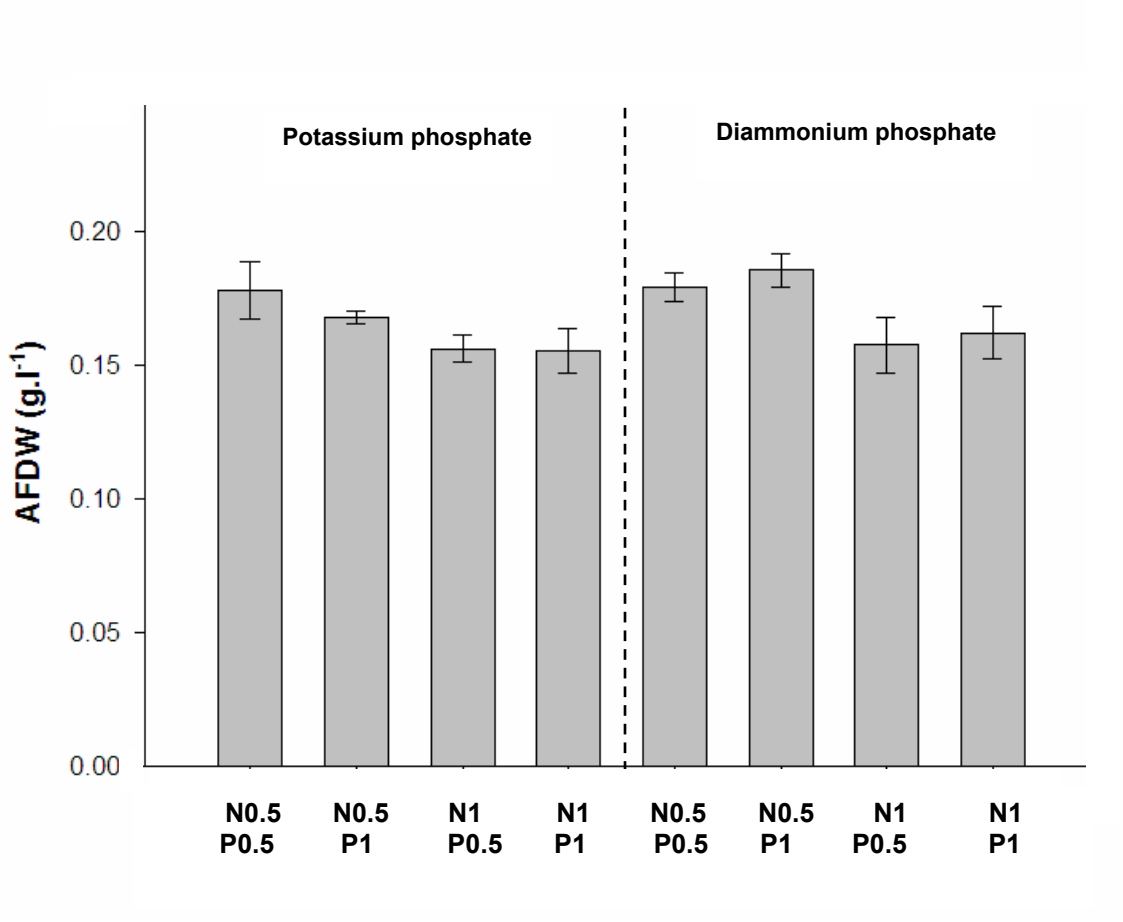


Figure 9. *F. saprophila* biomass accumulated over four days of experiment with different phosphorus sources and different nitrogen and phosphorus concentration. Data are means ± SD (n = 5). N = Nitrogen, P = Phosphorus. Concentration 0.5 = half strength, 1 = full strength of WC medium.

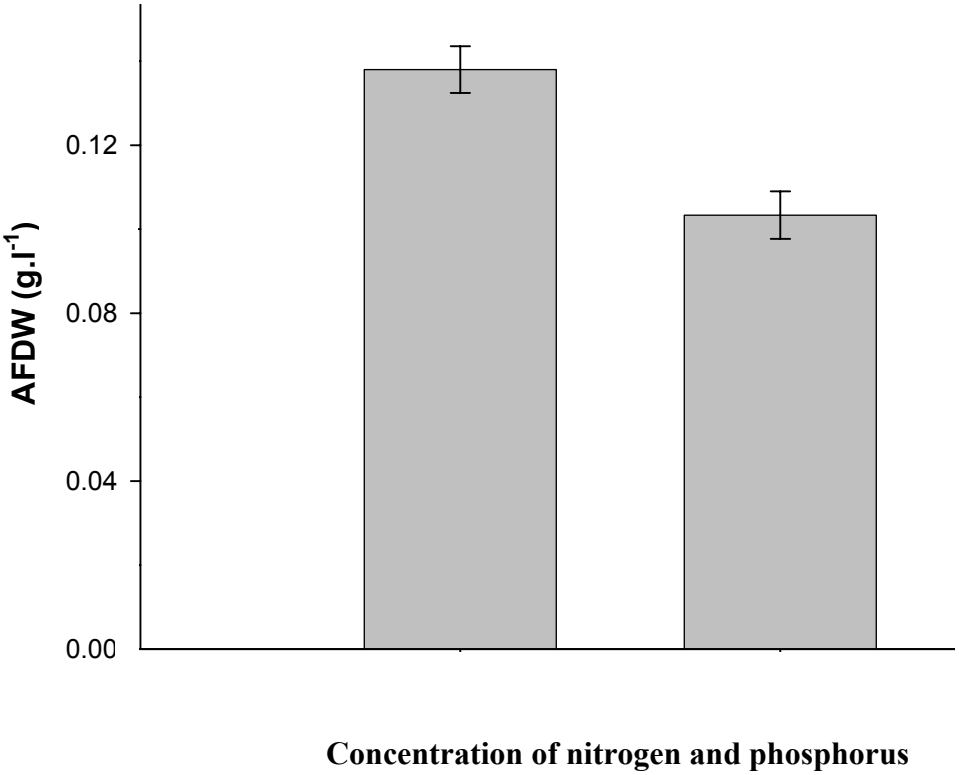


Figure 10. Comparison of *F. saprophila* biomass accumulation grown in the media with half strength of nitrogen and phosphorus and full strength of nitrogen and phosphorus in large bioreactors. Data are means ± SD (n = 3).

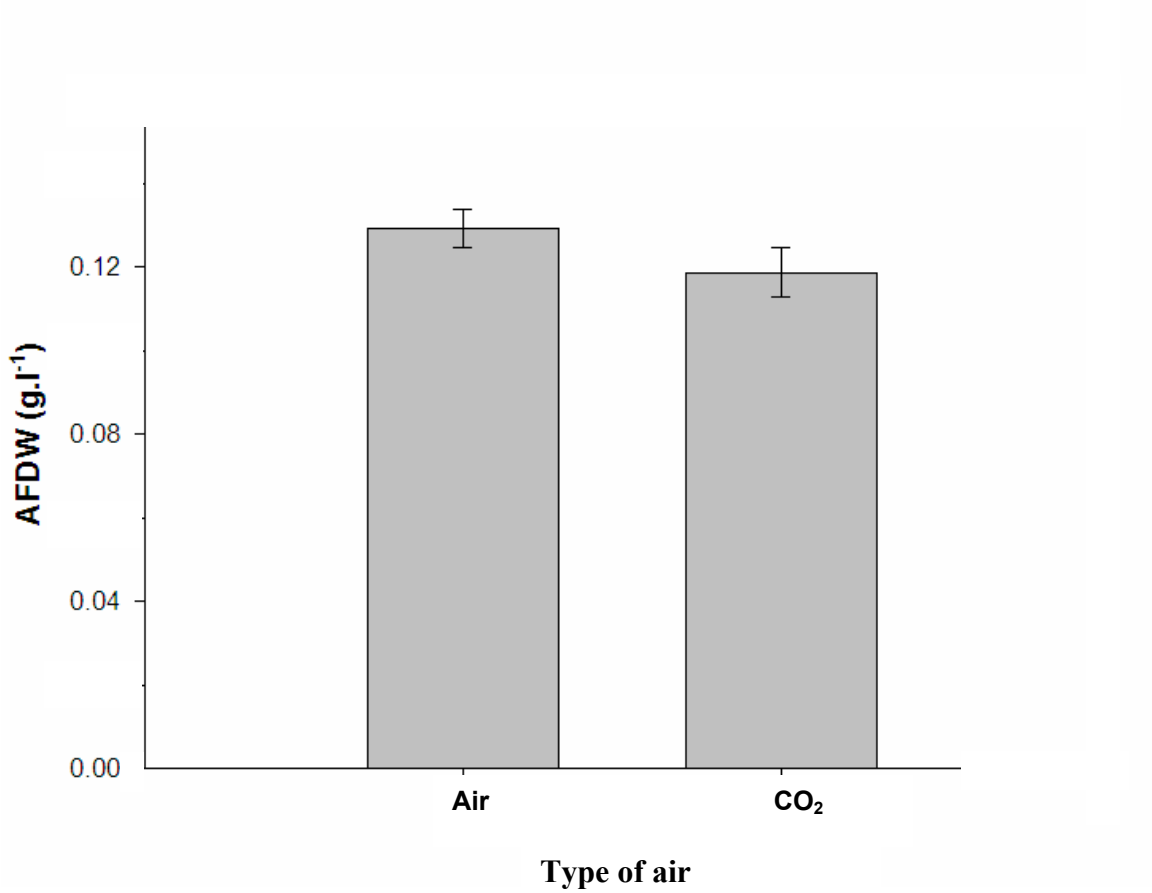


Figure 11. Comparison of *F. saprophila* biomass accumulation grown in large tubes for four days with house air and house air + carbon dioxide provided. Data are means ± SD (n = 3).

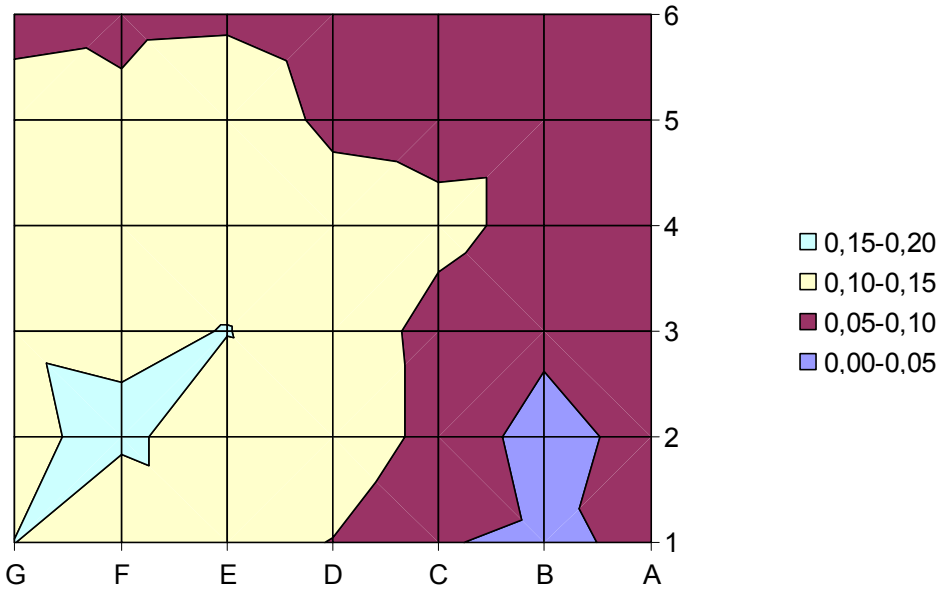


Figure 12. Comparison of the growth of *F. saprofila* in the crossed gradient of light and temperature grown for four days (expressed as g of AFDW·l^{-1}). Data are means (n = 3).

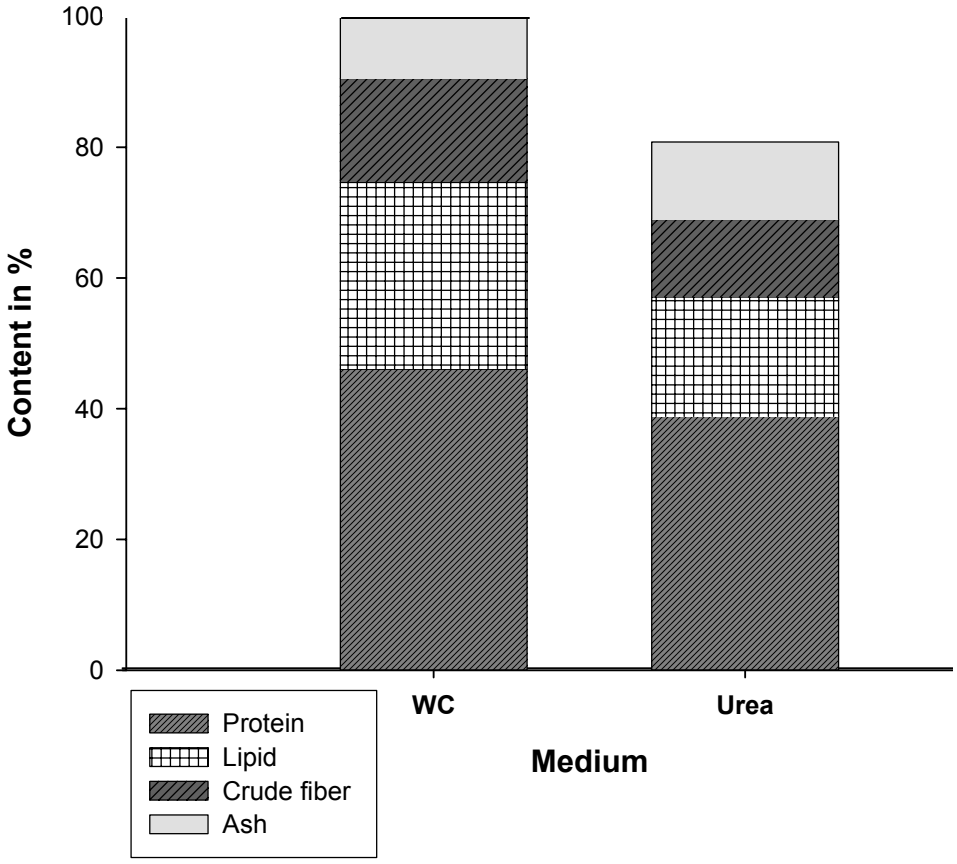


Figure 13. Comparison of *F. saprophila* biomass analysis grown in standard WC medium and medium optimized in this thesis (urea as a source of nitrogen).