ENHANCEMENT OF BIOMASS AND LIPID PRODUCTION OF MICROALGAE IN MIXED POPULATIONS IN WASTE WATER.

A dissertation submitted in partial fulfilment of the requirement for the degree of

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CERTIFICATE

This is to certify that Ms. Neha Katyal has worked under my guidance from January 15th to May 30th, 2013 in Lahti University of Applied Sciences, Faculty of Technology, Finland. The practical laboratory work has been supervised by Ms Marika Tikka (MSc). Ms Katyal's MEng dissertation work titled by "Enhancement Of Biomass And Lipid Production Of Microalgae In Mixed Populations In Waste Water" has been done in co-operation with University of Helsinki as a part of ALGIND project. Ms Katyal's project consisted of working with algae cultivations both in laboratory scale and in photo bioreactor, analysis of waste water, lipid extraction and analysis as well as other basic laboratory work, analysis and measurements. It is my pleasure to describe her as an active and hardworking student with excellent social skills. She was always ready to learn new subjects.

I will like to wish her a successful career.

24th May, 2013

Dr Silja Kostia
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This is to certify that **Ms. Neha Katyal (Roll No. FET/BTM/1001)** D/o Mr. Katyal has worked under the guidance of **Dr. Silja Kostia, Dean, Faculty of Technology, LUAS and Dr. Sarita Sachdeva, Prof. & Head, Biotechnology, FET, MRIU** for her dissertation from January to June 2013. During this period she worked on "***Enhancement Of Biomass And Lipid Production Of Microalgae In Mixed Populations In Waste Water.*** The dissertation work enabled her to become familiar with several routinely used techniques in the area of biofuel research. Her willingness to learn and hard work coupled with sincerity has helped her to learn several techniques in short period. I am impressed with her quality of work and dedication.

I will like to wish her a successful career.

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Here is my thesis.

NEHA KATYAL

ABBREVIATIONS

Sp.	Species
PUFAs	Poly unsaturated fatty acids
FAME	Fatty acid methyl esters
CO_2	Carbon-dioxide
mgL^{-1}	Milligrams per litre
day^{-1}	Per day
FA	Fatty acid
DW	Dry weight
gL^{-1}	Grams per litre
Cells mL^{-1}	Cells per milli-litre
GA	Gravimetric Analysis

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Enhancement of biomass and lipid production of microalgae in mixed populations in waste water.

Suitability to wastewater purification and biomass and lipid production

LAHTI UNIVERSITY OF APPLIED SCIENCES
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ABSTRACT

ALGIND which is "Algae Energy Business Opportunities in India" aims to form a realistic picture for enhanced business opportunities in algal biofuel markets in India. This project aims at integrating biodiesel production with waste and wastewater treatment in processes that flexibly can be tailored and linked to local facilities and offer a way to reduce operational costs related to algal cultivation. This project is realized in co-operation between University of Helsinki (UHEL), VTT, Lahti University of applied sciences (LUAS), Aalto University and international partners are: The energy and Resources institute (TERI), G.B. Pant Univ. of agriculture and technology (GBPUAT) and Manav Rachna International University (MRIU).

This Master's thesis assesses the potential of microalgae for waste water purification and biodiesel production in single and mixed populations. The wastewater used in these experiments was pressed from mixture of new municipal organic waste and partly composted garden waste. The species that have been chosen for growth during the experiments are *Euglena gracilis, Selenastrum sp*. and *Chlorella pyrenoidosa* and their various combinations. The experiments have been carried out at lab-scale and photobioreactors. Samples were taken on a regular interval (3 times a week) for determination of growth (DW), growth rate and nutrient consumption. Microscopy was used for calculation of cell concentrations (cells mL^{-1}) in the end of the cultivation. In the laboratory scale experiments, individual population of *C.pyrenoidosa* had highest and *Selenastrum* lowest biomass concentration in the end of cultivation (1.2±0.3 and 0.7±0.1 gL^{-1} DW). Biomass concentration of *E.gracilis* culture reached 1.0 ±0.04 gL^{-1} DW. Development of biomass was used for determination of growth rate in pure and mixed cultures. The growth rates were with *C.pyrenoidosa* (0.11d^{-1}), *Selenastrum* (0.10 d^{-1}) and *E.gracilis* (0.14 d^{-1}) in laboratory cultures. The combination of *C.Pyrenoidosa* with *E.gracilis* and *C.pyrenoidosa* with *Selenastrum* showed biomass concentrations 0.7±0.1 gL^{-1} DW and 0.8±0.1 gL^{-1} DW. In the laboratory scale cultivation, the growth rates were similar for mixed populations of *C.pyrenoidosa* and *E.gracilis* and *Selenastrum* and *E.gracilis* at 0.13 d^{-1}. The population of *C.pyrenoidosa* and *Selenastrum* showed a growth rate of 0.16 d^{-1} and all the three strains together showed biomass yield of 1.0±0.1 gL^{-1} DW and growth rate of 0.2 d^{-1}. In individual populations, the cell concentrations of *C.pyrenoidosa* have been the maximum as compared to all other cultures (8.68E+06 cells mL^{-1})*E.gracilis* and *Selenastrum* a combination show survival of both the species but cell concentration of *Selenastrum* strain was lower (2.75E+05

cells mL^{-1}) than when it was cultured alone (2.29E+06 cells mL^{-1}). Also, there was presence of *C.pyrenoidosa* and *E.gracilis* together. Thus, *E.gracilis* individually and in combinations with *Selenastrum* and *C.pyrenoidosa* was selected for growth in reactors. In photobioreactors mixed cultures, *E.gracilis* survived in existence of other species better than any other species. In the end of the cultivation *E.gracilis* was the only strain which was observed under microscope but however there were differences in the growth rates and biomass yields between cultures. The growth rates of the cultures in photobioreactor experiments were: *E.gracilis* and *C.pyrenoidosa* 0.3 d^{-1}, *E.gracilis* and *Selenastrum* 0.46 d^{-1} and *E.gracilis* in in pure culture 0.37 d^{-1}. The combination of *E.gracilis* and *Selenastrum* showed biomass 0.72±0.1 gL^{-1} DW in the end of cultivation which was slightly lower than biomass of single population of *E.gracilis* which showed highest biomass (0.8±0.1 gL^{-1}). Mixed population of *C.pyrenoidosa* reached the biomass The cell concentration of *E.gracilis* in individual population and with presence of *Selenastrum* was found to be similar (1.21E+06 cells mL^{-1}). The cell concentration of *E.gracilis* was minor (8.48E+05 cells mL^{-1}) when it grew in combination with *C.pyrenoidosa* in reactors. Ammonium removal from the wastewaters in photobioreactor was most efficient. The ammonium removal was attained highest by the combination of *Selenastrum* and *E.gracilis* (99.18%) and *E.gracilis* alone followed closely by combination of *E.gracilis and C.pyrenoidosa* (93%). The nitrate removal was seen maximum by *E.gracilis* and *C.pyrenoidosa* (41.6%) followed by *E.gracilis* combination with *Selenastrum* (30.56%) and individual population (22.4%). The phosphate removal was, however, seen maximum by combination of *E.gracilis* (78.4%) followed by *E.gracilis* and *C.pyrenoidosa* (76.2%) and least by *E.gracilis* and *Selenastrum* (54.3%).

Solvent extraction (Chloroform and methanol) was used for extraction of lipids. After methylation fatty acid methyl esters (FAMEs) were analysed with GC-MS.Total lipid content was determined gravimetrically and total fatty acid content was expressed as sum of individual fatty acids (FA). The total lipid and FA contents were determined during the last four days of cultivation in the reactors. The content of polyunsaturated fatty acids (PUFAs), however varies in the cultures. They show the highest content in *E.gracilis* and *Selenastrum* (56.68±1.68% DW) and comparatively less in case of *E.gracilis* (51.47±6.06% DW) and *E.gracilis* and *C.pyrenoidosa* (51.93±4.80% DW). Some PUFAs have established health benefits but are not desirable in biodiesel raw material. The total FA content in *E.gracilis* population was maximal on the last day of cultivation (8.32±0.73 % DW) which is nearly double from day 1 (4.32±0.30% DW). *E.gracilis* and *Selenastrum* showed the highest FA content (12.14±2.27%DW) in the end of the cultivation and total FA content in mixed culture of *E.gracilis* and *C.pyrenoidosa* was 6.17±0.71%DW in the last day. The monounsaturated FA content was maximal in the culture of *E.gracilis* and *Selenastrum* (20.85±1.02%DW). The saturated FA content is seen maximally in the culture of E.*gracilis* and *C.pyrenoidosa* (44.97±0.34% DW). Results showed that biomass yield in the mixed cultures was mainly lower than pure cultures but there was enhancement in lipid production when *E.gracilis* and *Selenastrum* were cultured together. However, there are still concerns about these FAs since saturated FA chains are problematic for cold flow and polyunsaturated FA chains are problematic for oxidative stability (28).

However results indicate that cultivation in mixed populations can reach higher lipid production but fatty acid profile of *E.gracilis* is not optimal for biodiesel applications.

Key words: Microalgae, mixed cultures, nutrients, wastewater, photobioreactor,Fatty acids , FA, PUFAs,biodiesel

CONTENTS

1 INTRODUCTION

With rising populations and rapidly developing nations, the demand for natural resources and energy is always growing. Amongst the renewable energies, one of the most important energy sources in near future is biomass. Biofuel is a renewable energy source produced from biomass, which can be used as a substitute for petroleum fuels. It is being stated that algae are the only feedstock that have the potential to completely replace world's consumption of transportation fuels. They are single celled organisms that duplicate by division. The benefits of using algae biomass over traditional food crops include higher yields of oil (10-100 times) and potential of algae to grow almost anywhere. Also algae are excellent bioremediation agents - they have the potential to absorb massive amounts of CO_2 and can play an important role in sewage and wastewater treatment (1).

Lipids are one of the main components of microalgae and lipid content of algae biomass is 2–70 percent of total cell dry matter, depending on the species and growth conditions [2]. Lipids are presented in membrane components, storage products and metabolites in algae cells Algae oil can be converted to biodiesel. Unfortunately, the cost to produce biodiesel from algae is much too high to compete with petroleum-based fuels. To reduce the production cost of algae, wastewater has been discovered to be an excellent growth media and also prevent the need for freshwater. For microalga cultivation, the huge consumption of water resources and inorganic nutrients is costly (3). Addition of organic carbon, though found highly stimulatory for micro-algal growth (4, 5, 6, 7), increases the feedstock cost (8). Thus, an economically acceptable and environmentally sustainable organic carbon source for alga-based biodiesel production is currently

needed. One promising approach is to couple biodiesel production with wastewater treatment, as algae can be successfully cultivated in wastewaters (9). Cultivation of microalgae in swine wastes, dairy manure, and other animal residues has been reported by several authors (10, 11, 12, 13, and 14). Also synthetic and urban wastewater is being employed in various studies.

Most cultures of algae today are grown as monocultures. There are a number of reasons for this, but the primary reason appears to be that specific strains of algae contain the high value products that are desired for harvest. One example would be β-carotene, which is present in all algae but which only certain strains of algae contain in sufficiently large amount to justify harvest. When looking at a product like lipids, however, there are a number of relatively fast growing strains of algae which produce oil in large amounts, and these various strains of algae tend to have varying optimal growth conditions. In this situation it may be more stable to employ a mixed culture in order to regulate growth over wide ranges of environmental conditions (15).Considering this, experiments have been conducted with mixed populations of algae.

The species that have been selected for the experiments are *E.gracilis* (Fig 1), *Selenastrum sp*. (Fig 2), *C.pyrenoidosa* (Fig 3) and combinations of *E.gracilis* and *Selenastrum sp*., *Selenastrum sp*. and *C.pyrenoidosa*, *E.gracilis*, *Selenastrum sp*. and *C.pyrenoidosa, E.gracilis* and *C.pyrenoidosa.* These species have shown adaptivity towards each other in EG (complex organic medium) medium and can survive together. Waste water used for cultivation in these experiments was presswater from mixed biowaste and garden waste originating from Kujala waste management center locating in southern Finland (Fig 4). Algae strains were first cultivated in laboratory scale and most promising candidates were selected to

experiments in photobioreactors. Total lipid content and fatty acid profile was determined from last four days of cultivation. The basic work flow that has been adopted for conducting the experiments can be explained by following figure (Fig 5).

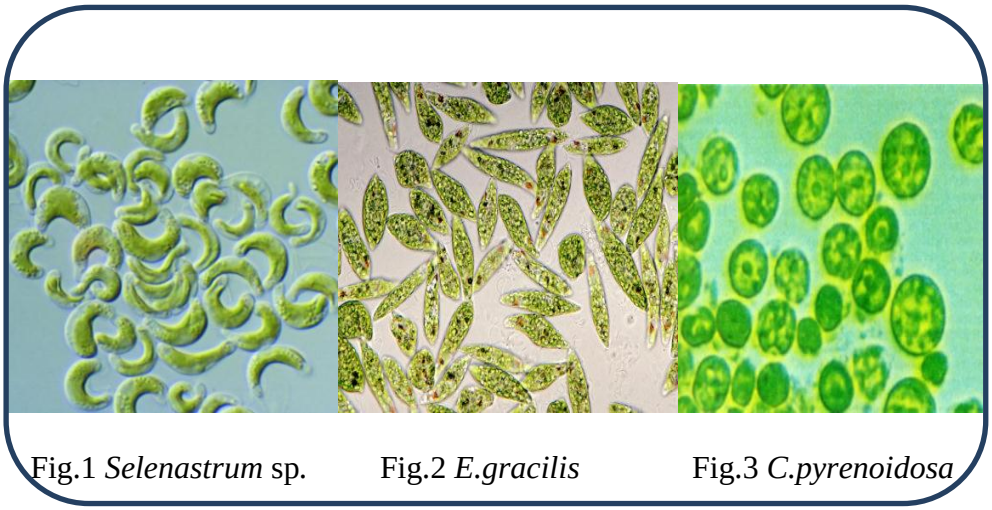

Fig.1 *Selenastrum* sp. Fig.2 *E.gracilis* Fig.3 *C.pyrenoidosa*

Images of species used in the experiments.(44)

Algae can be grown in open ponds or photo-bioreactors. Growing algae outdoors exposes them to elements that can weaken the strain and slow down production. It is also seen that when left to natural development, algae does not produce a very high yield of oil. In such cases, photo-bioreactors are implemented that would allow researchers more control over the conditions the algae are exposed to. The reactors allow the controllers to provide all the elements necessary for photosynthesis to occur (16, 17). Open and closed types of reactors usually have carbon dioxide injection to enhance productivity and some type of mixing. Both also have light penetration issues. In order to efficiently produce biodiesel from algae, strains have to be selected with a high growth rate and oil content. It is a

combination of high nutrient water, ample light, a large supply of carbon dioxide, and a high-oil content strain of algae that will provide the best bet for success.

Lipid accumulation in algae usually occurs during periods of environmental stress, culture under nutrient-deficient conditions is most often referred to. Saturated FA chains are problematic for cold flow properties and polyunsaturated fatty acid chains for oxidative stability during storage.. In order to improve fuel properties, desirable fatty acid composition offers acceptable cold flow and oxidative stability simultaneously. However, it may be noted in this connection that algal oils enriched in omega-3 polyunsaturated fatty acids (PUFAs) have been of interest in the past for physiological applications due to the recognized health benefits of these acids.

Biodiesel has usually been obtained from commodity oils such as soybean, rapeseed or palm oil with other feed stocks such as animal fats or waste cooking oils also playing a role. Recently feed stocks such as inedible oils, for example jatropha (18, 19) and pongamia (karanja) (20) have found increasing interest. These feed stocks have often been stated to be of interest because they address the supply issue and the food *versus* fuel issue, as microalgae are not the staple food for human consumptions. Besides these feed stocks, algae have found significantly increased interest as potential biodiesel feed stocks in recent years. Algal feed stocks have generated considerable discussion in the literature with numerous review or perspective articles often containing supporting or critical statements regarding the feasibility of algae-derived biodiesel being published (Table 1).

Table 1 Comparison of some sources of biodiesel [46]

Crop	Oil yield(L ha^{-1})
Corn	**172**
Soybean	**446**
Canola	**1,190**
Jatropha	**1,892**
Coconut	**2,689**
Palm	**5,950**
Microalgae$_a$	**136,900**
Microalgae$_b$	**58,700**

[a] 70% oil (by wt) in biomass
[b] 30% oil (by wt) in biomass

Fig.4 Kujala waste management Center

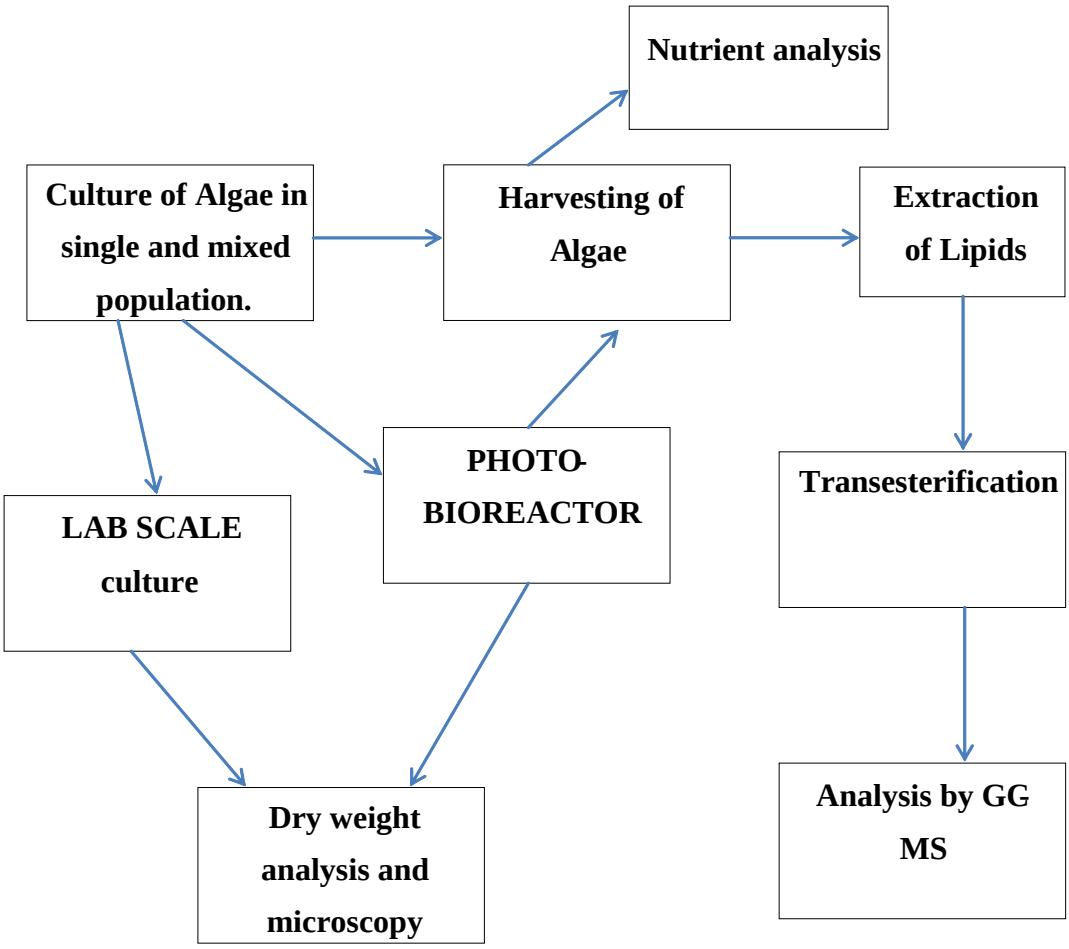

Fig.5 Work-flow adapted in the experiments.

2 LITERATURE REVIEW

Many research publications are available that have described the potential of microalage in various aspects of growth and biomass production. This section presents a review of all the articles that were relevant to this experiment.

2.1 Algae as a feed stock for biofuel production.

Many species of microalgae have been screened (*Chlorella vulgaris, Spirulina maxima, Nannochloropsis sp., Neochloris oleabundans, Scenedesmus obliquus and Dunaliella tertiolecta*) in order to choose the best one(s), in terms of quantity and quality as oil source for biofuel production. Researchers suggest that if the purpose is to produce biodiesel only from one species, *Scenedesmus obliquus* presents the most adequate fatty acid profile with low concentrations of linolenic and other polyunsaturated fatty acids. It was also found out in this particular study that the physical and fuel properties of biodiesel from micro-algal oil in general (e.g. density, viscosity, acid value, heating value, etc.), are comparable to those of fuel diesel. Also the entire process has been tried to be made as economical as possible by using biomass cake remaining after oil has been extracted , as fertilizer or feed, can undergo anaerobic fermentation to obtain biogas and/or a pyrolysis process, or to extract high value chemical compounds. It has been found that micro-algal biodiesel is technically feasible and to be economic competitive with petro-diesel, micro-algal production, harvesting and extraction must be optimized, and also there is scope in improvements to algal biology through genetic and metabolic engineering. [26]

There are many review articles that overview the process of biodiesel production with microalgae as feedstock. The methods associated with this process (e.g. lipid determination, mass culture, oil extraction) are also compared and discussed.

Neutral lipids include tri-, di- and mono-acylglycerols, waxes, among which triacylglycerols (TAGs) are frequently found to be accumulated as energy storage under various stress conditions. Although almost all types of micro algal lipids can be extracted, only TAGs are easily transesterified into biodiesel by traditional methods. The conventional method used for determination of lipid content involves a complicated lipid extraction with solvent, separation, concentration and gravimetric determination (which is time consuming and tedious). Chromatographic methods with internal standards can provide information on both fatty acid quantity and profile in a single analysis, and has been a commonly employed method in several fields. Some in situ measurement methods have been evaluated and used in determination of micro algal lipid content, including Nile Red (NR) staining, time domain nuclear magnetic resonance (TD-NMR), colorimetric quantification and others. The levels of lipid accumulation are also affected by nutrients especially nitrogen deprivation and other environmental stress conditions. Understanding biochemistry and molecular mechanisms of lipid accumulation of microalgae needs to answer two important questions, that are why do some strains accumulate more lipids than others, and what triggers lipid accumulation under stress conditions. There is a strong correlation between the activity of malic enzyme and the extent of lipid accumulation, indicating that malic enzyme plays a determining role in controlling the lipid accumulation in fungi. [30]

Recently some researches have examined the ability of E.gracilis sp. growing in wastewater ponds for biofuel production and treatment of wastewater. The algae were isolated from the sewage treatment plants and were tested for their nutrient removal capability. Compared to other algae, E.gracilis sp. showed faster growth

rates with high biomass density at elevated concentrations of ammonium nitrogen (NH4-N) and organic carbon (C). Profuse growth of these species was observed in untreated wastewaters with a mean specific growth rate (μ) of 0.28 day^{-1} and biomass productivities of 132 mg L^{-1} day^{-1}. Gas chromatography and mass spectrometry indicated the presence of high contents of palmitic, linolenic and linoleic acids (46, 23 and 22 %, respectively), adding to the biodiesel quality. Good lipid content (comprised quality fatty acids); efficient nutrient uptake and profuse biomass productivity make the E.gracilis sp. as a viable source for biofuel production in wastewaters. The investigators have found out that there were thirteen different fatty acids identified from E.gracilis sp. grown in domestic wastewaters, among which palmitic acid (C16:0), linolenic acid (C18:3) and linoleic acid (C18:2) are the abundant fatty acids. Also, the lipid profile shows a dominance of C16 (42 %) and C18 (50 %) methyl esters, emphasizing that the lipids produced from E.gracilis sp. are similar to vegetative oil and have good biofuel properties. But the concern is PUFA content of 46 % observed in this study that could lead to susceptibility to oxidation (instability) during storage, requiring a partial catalytic hydrogenation for stability and to be used as biodiesel. [36]

Table2. Advantages and limitations of lipid determination for microalgae (30)

Methods	Advantages	Limitations
Solvent extraction and gravimetric method	High accuracy and reproducibility	Time consuming and labor intensive, requirement of large amounts of biomass (10–15 mg wet weight)
Chromatographic method	Good reproducibility, a single analysis generates data of both quantity and profile of fatty acids, small samples required	Requirement of cell disruption, extraction and derivatizing of fatty acids; requirement of expensive analytical equipment
Nile Red (NR) staining	In situ measurement, high throughput, simple, rapid and efficient	Variable efficiencies in some microalgal species, accuracy can be influenced by many factors; primarily for neutral lipids staining
Time-domain nuclear magnetic resonance (TD-NMR)	In situ measurement, rapid, simple and less expensive	Accuracy is dependent on high lipid content and cellular activities; requirement of special analytical equipment
Colorimetric quantification	Rapid, simple, cheap high throughput	Not applicable to detect fatty acids with chain length of less than 12 carbons

2.2 Choosing wastewater as a source of growth medium for algae.

The wastewater discharged into the water bodies are hazardous to environment and cause various health problems in human beings. Eutrophication is one such major environmental problem caused due to the discharge of nutrient rich wastewater into the nearby water bodies. Several methods, commonly chemical treatment and conventional biological methods are used to remove nutrients (phosphorus and nitrogen) from wastewater. However, high cost and more sludge production, are the major disadvantages limiting its use and hence researchers are now focusing on microalgae for nutrient removal from wastewater as it is less expensive and results in less sludge production. Microalgae cultivated in the wastewater can be used for the biodiesel production and as feed for animals. This dual process (microalgae cultivation in effluent coupled with biodiesel

production) has several advantages such as less cost and less energy input for biodiesel production, and less greenhouse gas emission during biodiesel production. [36]

The researchers have considered algae as a potential choice for treatment of wastewater from urban, industrial or agricultural origins. Their studies indicate that cultivation of algae in wastewater can lead to the bio-treatment of wastewater along with the production of biomass that can be of commercial value. It has been suggested that the effluents from the waste show concentrations of nitrogen and phosphorus up to three times higher in magnitude as compared to the natural concentrations in lakes. It is thus necessary to treat the effluents before discharging the same into natural water bodies. Micro-algal cultures offer an elegant solution to tertiary and quaternary treatments due to the ability of microalgae to use inorganic nitrogen and phosphorus for their growth and their capacity to remove heavy metals and some toxic organic compounds. The abiotic factors have been considered to be complicated due to large variations in effluent compositions or climatic conditions whereas control of biotic factors has been considered to be easier to control. It is also possible that the production factor can modify the productivity and hence treatment efficiency. This has been done by taking an example to show the importance of operating conditions, low irradiance and use of a different culture system, in which cells are starved in the first phase and then put in contact with nutrient loaded effluent, gives a much higher productivity have been established. The possible alternatives to harvesting are also reviewed with an emphasis on immobilized systems. [22]

The use of wastewater as a nutrient source for algal growth as a biodiesel feedstock could increase the viability of this process and reduce wastewater

discharge of nitrogen and phosphorus-bearing compounds. The idea of using wastewater as a medium is because of the ability of algae to grow in nutrient-rich wastewaters, and perform nitrogen (N) and phosphorus (P) removal, thus reducing nutrient loads to receiving water bodies. It has been concluded that algal biodiesel could actually generate greenhouse gas emissions and require significantly more energy inputs than biodiesel produced from conventional corn and canola feedstocks, although the authors acknowledged that most of the environmental burdens associated with algae could be offset if wastewater were used as a nutrient source. In this study lipids were identified and quantified by gas chromatography- mass spectrometry (GC-MS). All lipid profiles were dominated by C16 and C18 methyl esters, confirming that the algal oil produced in these mixed-species bioreactors was very similar to oil seed feedstocks such as soybean. The results show that algal biomass can be cultivated in open pond reactors utilizing wastewater as a nutrient source. [27]

In order to reduce the cost of the production of microalgae for biodiesel, the feasibility of using the mixture of seawater and municipal wastewater as culture medium and CO_2 from flue gas for the cultivation of marine microalgae has been investigated. Effects of different ratios of municipal wastewater and 15% CO_2 aeration on the growth of *Nannochloropsis sp.* were examined, and lipid accumulation of microalgae was also studied under nitrogen starvation and high light. It was found that optimal growth of microalgae occurred in 50% municipal wastewater, and the growth was further significantly enhanced by aeration with 15% CO_2. [35]

2.3 Monocultures and Mixed cultures.

Many researchers have pointed to that fact that mainly monocultures are tested for their productivities of various compounds. They have conducted experiments with mixed cultures to draw a comparison between the two. It has been mentioned in the article by Kyle et.al. that in algae cultures grown primarily for lipid production, or as a stage in wastewater remediation, mixed algae cultures may mitigate environmental risk [22]. When looking at a product like lipids, however, there are a number of relatively fast growing strains of algae which produce oil in large amounts, and these various strains of algae tend to have varying optimal growth conditions. In this situation it may be more stable to employ a mixed culture in order to regulate growth over wide ranges of environmental conditions. The media chosen for this purpose has been municipal wastewater since it is one area where algae may be very economically competitive and it makes sense to postulate that in the future biodiesel production and municipal wastewater treatment with algae may be done simultaneously in the same facility and with the same algae. The sets used for experimentation have been 100% *Chlorococcum sp*., 100% *Scenedesmus sp*., 100% *Chlorella sp*., 100% *P. tricornutum* and a mixed culture with 25% of each of the inoculation volumes. Analysis of samples taken from the inoculation cultures showed that the mixed cultures were composed, by mass, of an average of 24.4% *Scenedesmus sp*., 28.2% *Chlorella sp*., 30.7% *Chlorococcum sp*., and 16.7% *P. tricornutum*. Results have been analyzed on the basis of effect of light intensity and temperature on biomass. Also results from the mixed culture growth show that under all conditions the mixed cultures were less productive than themost fit monocultures for each situation, but more productive than the least fit monocultures. *Scenedesmus sp*. is among the

most productive strains at all lighting and temperature conditions and *Chlorella sp*. is among the most productive in all lighting conditions.
Interestingly, *Chlorella sp*. is not one of the most productive strains at most temperatures. The lipid production of the mixed culture, once again, closely tracks the most productive strains. [24]

2.4 Growth of algae cultures in photo-bioreactors.

As already mentioned, economic feasibility is one of the major concerns in biofuel production. The researches have aimed to highlight some of the key biological and engineering challenges presented by the very large scale of an algae biofuels plant which need to be resolved. Different types of systems such as open, closed and hybrid systems have been viewed with their pros and cons in this review highlighting the fact that it is very important for any culture system to maintain a balance between turbulence, culture depth (length of light path) and biomass concentration for maximum light capture and optimum biomass productivities with increasing reactor size. Culture reliability and stability are essential for commercial micro-algal cultivation on a large scale and therefore careful selection of species and strains best suited to the culture system and the prevailing environmental conditions plays a crucial role. There is also mention about different methods used in lipid extraction like chloroform-methanol and soxhlet method from the algae. In the few studies with wet algal biomass the presence of water has been found to significantly reduce the yield of the fatty acid methyl esters (FAME) during lipid extraction. However, by increasing the amount of methanol, the effect of the water could be reduced but this would increase costs.

So far all of these studies have only been carried out at the laboratory scale only and no comparative economic studies between the various methods have been carried out. [23]

Many researches give an account of various types of photo-bioreactors available for mass production of algae keeping mass transfer in view as a major factor. Few articles also give prospects and limitations of various types of bioreactors namely, open pond (Fig 6), vertical column photo-bioreactor (Fig 8), Flate-plate photo-bioreactor and tubular photo-bioreactors (Fig 7). The comparison has revealed that more efforts are still required to improve photo-bioreactor technologies and know-how of algal cultures. Photo-bioreactor development is perhaps, one of the major steps that should be undertaken for efficient mass cultivation of algae. The authors suggest that large-scale outdoor photo-bioreactors should have large volume and occupy less land space. In addition, they should have transparent surfaces, high illumination surfaces, high mass transfer rates and should as well, beable to give high biomass yields. Furthermore, design and construction of any photo-bioreactor should depend on the type of strain, the target product, geographical location, as well as the overall cost of production. Apparently, while many photo-bioreactors are easily operated at laboratory scale, only few of them can be successfully scaled up to pilot scale. Scale-up of photo-bioreactors can be done by increasing the length, diameter, height or the number of compartments of the culture systems (depending on the type of photo-bioreactor). These scale-up strategies are very challenging, mainly due to difficulty in maintaining optimum light, temperature, mixing, and mass transfer in photo-bioreactors. [31]

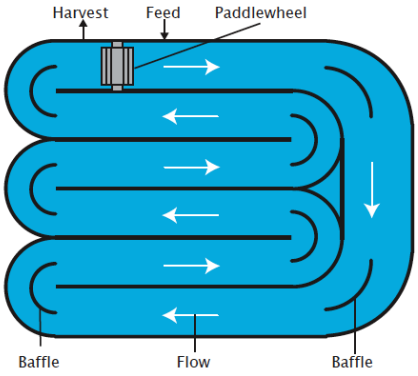

Fig.6 Raceway ponds (44)

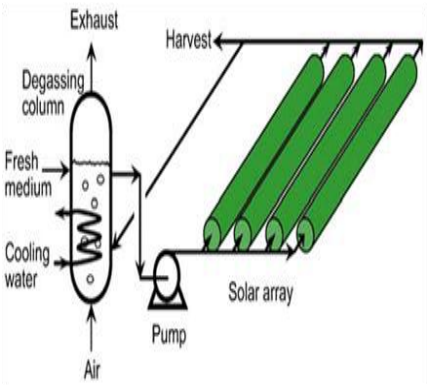

Fig.7 Tubular photo-bioreactors (44)

Fig.8 Column photo-bioreactors (44)

2.5 Lipid production from Algae.

The most important constituent of the algae species is its lipid content. Accurate quantification of lipids and their respective fuel yield is crucial for comparison of algal strains and growth conditions and for process monitoring. As an alternative to traditional solvent based lipid extraction procedures, the researchers have developed a robust whole-biomass in situ transesterification procedure for

quantification of algal lipids (as fatty acid methyl esters, FAMEs) that (a) can be carried out on a small scale (using 4–7 mg of biomass), (b) is applicable to a range of different species, (c) consists of a single-step reaction, (d) is robust over a range of different temperature and time combinations, and (e) tolerant to at least 50% water in the biomass. Unlike gravimetric lipid quantification, which can over- or underestimate the lipid content, whole biomass transesterification reflects the true potential fuel yield of algal biomass. The researchers have laid emphasis on a single step procedure based on in situ HCl/MeOH catalysis of algal biomass is applicable to small quantities of biomass. Also, there is data on the efficiency of transesterification of different catalysts at varying temperature, time and pretreatment conditions, which allowed for the statistical analysis of effects. When varying reaction conditions around 85 °C and 60 min they found no significant differences in total FAME yield, suggesting that for the strains investigated, the HCl catalyzed method has proven to be robust over a wide range of conditions. [25]

Articles compare biodiesel derived from vegetable oils and biodiesel obtained from algae in light of fuel properties. While the properties of biodiesel fuels derived from vegetable oils are well-known, the properties of biodiesel obtained from algal oils have usually not been reported. Biodiesel fuels derived from algae in many cases likely possess poor fuel properties, *i.e.*, both poor cold flow and low oxidative stability simultaneously. This observation shows that production potential alone does not suffice to judge the suitability of a feedstock for biodiesel use. Intensive study of the fuel properties has been done by the researchers to conclude that saturated FA chains are problematic for cold flow, polyunsaturated

FA chains are problematic for oxidative stability besides CN and combustion. In order to improve fuel properties, therefore, a fuel composition is desirable which is highly enriched in a FA that compromises on these properties, *i.e.*, offers acceptable cold flow and oxidative stability simultaneously.

The researchers suggest that strain selection of algae for biodiesel production would need to be guided by the aspect of favorable fuel properties imparted by an advantageous FA profile. On the other hand, it may be that algal species with more favorable FA profiles are not necessarily those with the highest productivity. Algae with the most favorable FA profiles, like all other algae, are likely sensitive to conditions (temperature, light intensity and nutrients) and minor deviations from these conditions may lead to an ultimately less favorable product. Therefore, it appears that production conditions would need to be tightly controlled in order to obtain a product with more favorable properties. [28]

Oil-accumulating microalgae have the potential to enable large-scale biodiesel production without competing for arable land or biodiverse natural landscapes. High lipid productivity of dominant, fast-growing algae is a major prerequisite for commercial production of micro algal oil-derived biodiesel. However, under optimal growth conditions, large amounts of algal biomass are produced, but with relatively low lipid contents, while species with high lipid contents are typically slow growing. Major advances in this area can be made through the induction of lipid biosynthesis, e.g., by environmental stresses. Lipids, in the form of triacylglycerides typically provide a storage function in the cell that enables microalgae to endure adverse environmental conditions. Essentially algal biomass

and triacylglycerides compete for photosynthetic assimilate and a reprogramming of physiological pathways is required to stimulate lipid biosynthesis.

There have been a wide range of studies carried out to identify and develop efficient lipid induction techniques in microalgae such as nutrients stress (e.g., nitrogen and/or phosphorus starvation), osmotic stress, radiation, pH, temperature, heavy metals and other chemicals. [35]

3 MATERIALS AND METHODS

3.1 Algae strain and cultivation conditions in laboratory scale

The microalga *C.pyrenoidosa pyrenoidosa* (UTEX 1230), *E.gracilis gracilis* (CCAP 1224/5Z) and *Selenastrum* sp. were used in the experiments. *C.pyrenoidosa* and *E.gracilis* were from culture collections. *Selenastrum* strain which is own isolate of University of Helsinki is originating from lake Iso-Ruuhijärvi located in southern Finland. EG (complex organic medium) [39] (autoclaved 120 ^{0}C, 15 min, 1 bar).was used as a base medium in pre cultivation and waste water from Kujala waste management center in experiments. All the three algae strains (*C.pyrenoidosa, E.gracilis and Selenastrum*) along with mixed populations (*C.pyrenoidosa and E.gracilis gracilis, E.gracilis and Selenastrum, C.pyrenoidosa and Selenastrum and C.pyrenoidosa, E.gracilis and Selenastrum)* were inoculated into plastic tissue culture bottles (600 mL, containing 500 mL waste water) and cultivated in growth chamber (SANYO growth cabinet MLR-350 H; 294L) at 20 ^{0}C with light: dark cycle of 16:8 (Fig 9). Intensity of light was 150 µmoL m $^{-2}$s $^{-1}$. Before starting the experiments cultures were adapted to living together two months.

Fig.9 Single and mixed cultures (in replicates) in growth chamber

3.2 Algae strain and Cultivation conditions in photobioreactor

Combinations of strains selected to the photobioreactor experiments were single strain culture of *E.gracilis*, mixed culture of *E.gracilis* and *C.pyrenoidosa* and mixed culture of *E.gracilis and Selenastrum* (Fig 10). This selection was based on the growth at laboratory scale cultivation.

Photobioreactors (volume of 200 L) were run with 180 L of 1% diluted wastewater. Fluorescence tubes (16 X 36 W Osram Lumilux 954) was used to illumination. Aeration and mixing was achieved pumping compressed air through perforated pipe in the bottom of the bioreactor (Piston pump Hailea 318,

adjustable 0-15 l/min). CO_2 feed was conducted through silicon tubes (Ø 6 mm) and aeration stone with pore size 0.5 µm at its end. Temperature was controlled at 25±2 ^{o}C. (Aquarium Systems Newatt 250 W). The pH was measured through pHT-electrode (SI-Analytics, 425 mm, gel electrode with integrated Pt1000 sensor. CO_2 feed was coupled to pH controller and feeding was switched on automaticly when pH rised to 7.

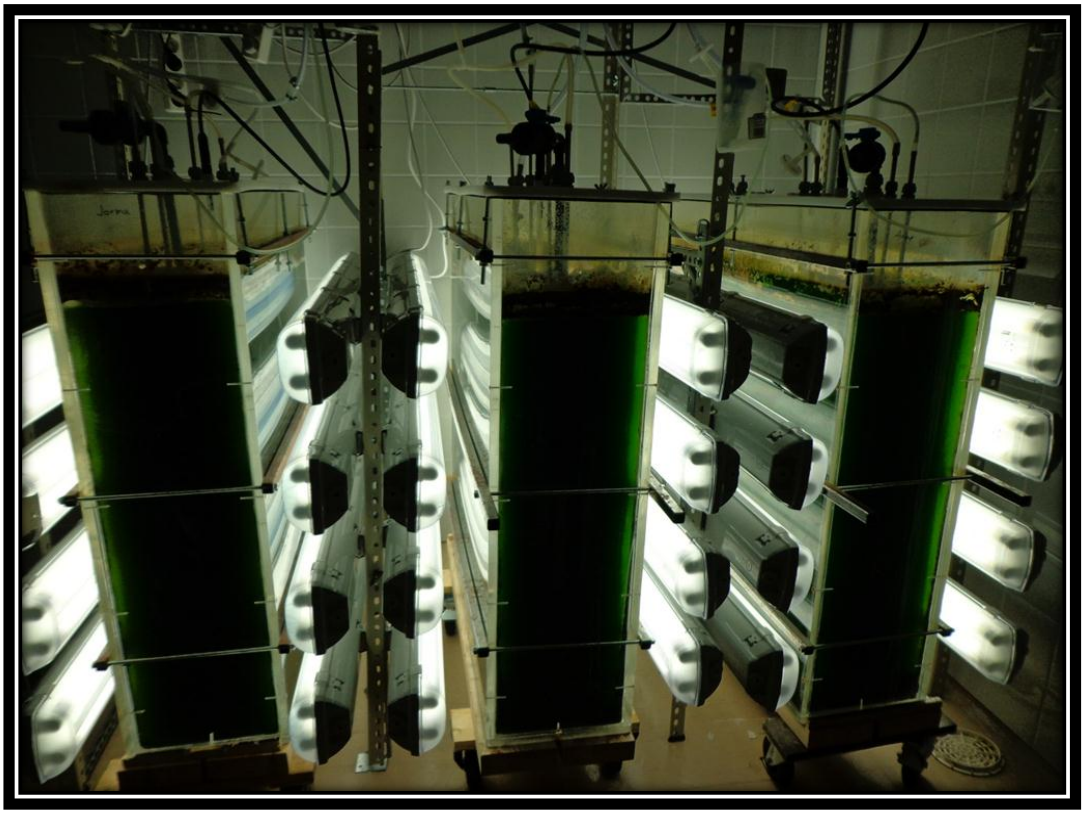

Fig.10 *E.gracilis* and *C.pyrenoidosa*, *E.gracilis* and *E.gracilis* and *Selenastrum* (from left to right) in reactors.

3.3 Wastewater collection and treatment before experiments

Wastewater was collected from Kujalan Komposti Oy, located in the Kujala waste management center. The Kujala center is a waste sorting and treatment center located in Finland in the city of Lahti. The wastewater used in these experiments

was pressed from mixture of new municipal organic waste and partly composted garden waste. The waste water was first centrifuged (Multifuge 1 S-R, Kendro laboratory products) at 4000 rpm for 20 minutes and supernatant was filtered (Whatman filters No.4,20-25µm) to remove organic as well as inorganic particles (Fig 11).. This wastewater was then autoclaved for 60 minutes at 1 bar and 121^0C. After dilution of wastewater to 1%, pH was adjusted to 7 ± 0.28 with NaOH (0.1 M). Based on nutrient concentrations (N & P) wastewater concentration 1% was used for the cultivation of algal strains. Wastewater was diluted in distilled water in laboratory scale and tap water in photobioreactor experiments.In the reactors pH was aadjusted to 7 with NaOH (0.1M).

Fig.11 Wastewater from Kujala during filtration.

3.4 Analysis of wastewater before and during cultivation

Nutrient (ammonium, nitrate, phosphate, total phosphorus and total nitrogen) concentrations, COD, and pH were determined before experiments from the original wastewater. For the determination of consumption of nutrients and COD from wastewater the supernatant was collected during the dry weight determination procedure as described later in chapter 2.5. The filters used in this procedure were pre-combusted at 450 degrees, 4h. The measurements of nutrientconcentrations and COD was done through specialized kits (HACH LANGE, Germany: LCK: 303, 339,614,138,348). Ph was measured with pH meter SCHOTT ba12272de_2_pH.

3.5 Determination of dry weight

Dry weight was used for determination of development of algal biomass during the growth (Fig 12). Dry weight was determined by filtering algae samples trough pre-combusted (450 ^{o}C, 4 h) filters (GF/C, Ø 47, Whatman). Three replicate were used in filtration. Filters were rinsed with distilled water (20 mL) after filtration of sample and dried overnight at 105 ^{o}C before weighing. (36).This analysis was done three times a week to obtain the growth and biomass yield during the cultivation. The supernatant of the samples were collected for nutrient and COD analysis. Growth rate was calculated from period of logarithmic growth using the formula

$$\boldsymbol{K' = ln(N_2/N_1)/(t_2 - t_1)}$$

Where, k is specific growth rate, N_2 is biomass in time t_2 and N_1 is biomass in the time t_1 .

Fig.12 Filters for dry weight estimation.

3.6 Strain identification and cell counting using Microscope

Samples for microscoping were taken during growth to Eppendorf tubes and stained with acidic Lugol's solution. The development of cell existence in single and mixed populations were observed and calculated under a light microscope (Lecia, DM 1000, Germany; total magnification X200). It was an important aspect to observe in mixed combinations that all the species are surviving and not superimposing each other. Lund chambers were used to observation and calculation of cells (Lund 1959). Cells were counted using a cell counter in 20 different regions of the slide. Different dilutions were used to count species in mixed populations. The formula used for the calculations is:

$$\text{Cells mL}^{-1} = N_c * A_c / N_f / V_c / \pi r^2$$

where,

N_s = counted amount of cells, A_c= Chamber area (mm^2), N_f = number of counted fields, V_c = volume of chamber, r = radius of field.

3.7 Harvesting algae for lipid analysis in photobioreactor experiments

Algae from all the three reactors were harvested in the second week of cultivation when it had attained the stationary phase. Samples for lipid and nutrient analysis and cell counting were withdrawn in plastic bottles during the growth. The harvesting was done in two parts. Approximately 200mL of algae from the different reactors was harvested in plastic tubes (50mL) and centrifuge (Multifuge 1 S-R, Kendro laboratory products) and washed with water.This biomass was then transferred to eppendorf tubes. In the end of the cultivation photobioreactors were connected to the centrifuge (Evodos 2/10) through the harvesting pipes 1100 mm (PVC, 16mm) for harvesting rest of the biomass. The biomass was stored in freezer (-70^{0}C).

3.8 Lipid extraction

The biomass collected from the reactors was freeze dried. For lipid extraction approximately 10 mg of freeze dried algae paste was weighed to test tube and 1 mL of isopropanol was added to inactivate lipolytic enzymes. Tubes were centrifuged at 2500 rpm for 1 minute, the extract was removed to the collection tube and the solvent was evaporated under the nitrogen stream. 8mL of ice cold chloroform: methanol (2:1), 1mL of water, 20µL BHT (1% in methanol) and100 µL of internal standard (final concentration in sample 3 ng µL) (Nonadecanoic acid 72332-1 G-F/analytical standard , Sigma Aldrich) was added and tubes were closed under the nitrogen stream. Samples were then sonicated on ice for (10 min), vortexed (1min) and centrifuged (3000 rpm, 3 min, 4^{0}C). The organic

bottom layer was removed to other tube using Pasteur pipette. Pipet was then flushed with approximately 3mL of ice cold chloroform and tube was closed under nitrogen stream. This procedure was repeated twice or thrice until lower phase was clear, meaning that all the lipids were extracted. The solvents were then evaporated under the nitrogen stream and 1 mL of hexane was added before closing tubes under nitrogen stream until methylation. The entire method was performed for 1-2 blanks along with the samples. (37, 38) (Fig 13)

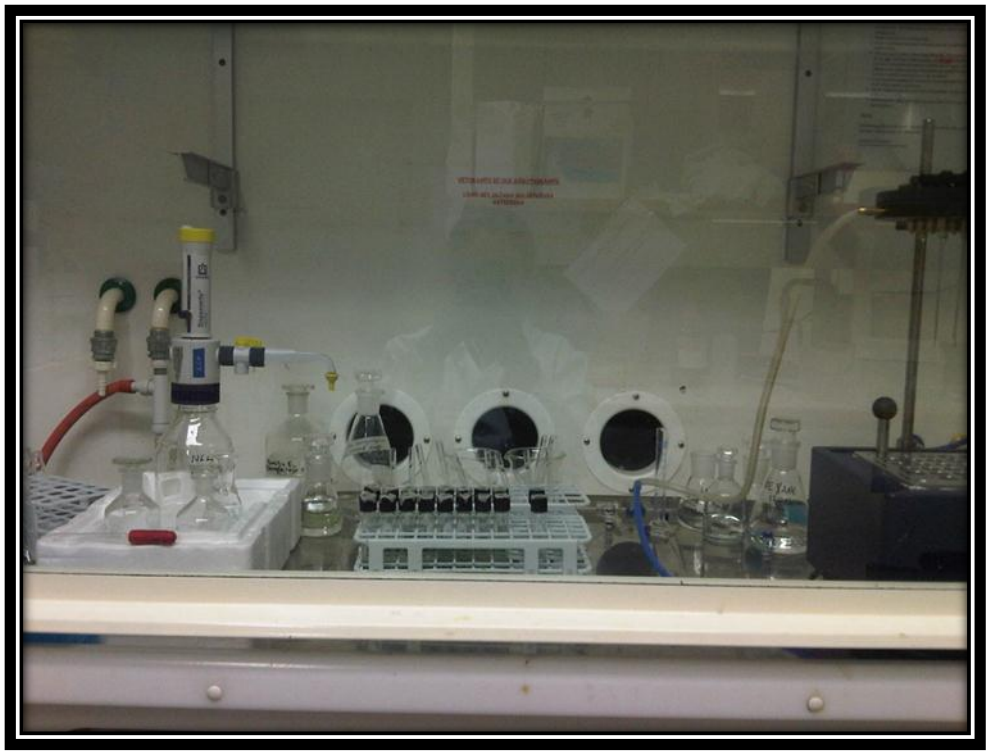

Fig.13 Solvent extraction of lipids.

3.9 Lipid analysis

The samples were filtered (whatman SPARTAN 13/ 0,2 RC ; 0,2 um) and total lipids were determined gravimetrically. Samples were divided in to two parts. Half of the sample was used for analysis and another one was stored in freezer to be used in case of any error. Hexane was evaporated under nitrogen stream and 1 mL of toluene, 20µL of BHT and 2 mL of methylation reagent (1 % sulphuric acid: methanol solution) was added in the sample tubes. Tubes were then closed under nitrogen stream and vortex (30 s) and incubated 16 hours in warm water bath (50 ^{0}C). The tubes were cooled to room temperature and 3mL of NaCL (5 % in MQ water) and 5 mL of hexane was added in the tubes. These tubes were then again closed under nitrogen stream, vortex and centrifuged (1500 rpm, 2 min). The organic upper layer was collected to other test tube and this process was repeated by addition of 5mL hexane followed by vortexing and centrifuging steps. The combinated organic layers was washed with 3.5 mL 2 % potassium bicarbonate and dried with anhydrous sodium sulphate. The samples were then evaporated under the nitrogen flow. 1 mL of hexane was added to the tubes and sample was then removed to GC vials and diluted (X100) to other GC vial before fatty acid analysis. (38)

Samples were analyzed with GC-MS equipped with an auto sampler (AOC-20s, Shimadzu). The column (Agilent Technologies, DB 23) with length 30 m, internal diameter 0.250 mm and width of the stationary phase 0.15 µm was used. Helium was used as a carrier gas (rate 1 mL/ min, split less). Sample injection temperature was 250 ^{0}C, and the temperature program was as follows: Initial column temperature was 60 ºC where it was held for 1 min, temperature increase to 110 ºC at the rate of 20 ºC / min, 110 ºC – 185 ºC at the rate of 3 ºC / min 185 ºC-

195ºC at the rate of 0.50 ºC / min and 195 ºC – 250 ºC at the rate of 25 ºC / min where it was held for 2 min. Fatty acids were identified with comparison of retention times and mass spectra to chromatogram peaks of known fatty acids in standard solution (Supelco ^{TM} 37 Component FAME Mix, Sigma). The internal standard (Methyl nonadecanoate, analytical standard, Sigma) C19:0 was prepared by diluting with hexane. The standard mix was then prepared in concentrations of 250ng/µL, 50ng/µL, 25ng/µL, 2.5ng/µL and internal standard was added to calibration standard (final concentration in FAME mix, 6 ng µL). The fatty acids in the samples were quantified by peak areas on the chromatogram..

4 RESULTS

1. Characteristics of waste water

Concentrations of main nutrients (nitrate, ammonium, phosphate, total nitrogen) and COD from wastewater obtained from the Kujala waste treatment plant are presented in table 3. The wastewater used in these experiments was pressed from mixture of new municipal organic waste and partly composted garden waste. The nutrient concentrations in 100% wastewater are quite high and thus require dilutions before being used for algae cultivation so as the growth is not suppressed. Hence, 1% dilutions have been used to grow algae.

Table 3 Nutrient concentration in original and diluted (1 %) wastewater from Kujala

Nutrients	**Concentration in wastewater (100%) (**mgL^{-1}**)**	**Concentration in 1% wastewater (**mgL^{-1}**)**
NITRATE	124	1.24
AMMONIUM	4280	42.8
PHOSPHATE	278	2.78
TOTAL NITROGEN	14800	148
COD	6400	64

4.2 Growth in the laboratory cultures

In the laboratory experiments tendency of growth in individual and mixed cultures was almost equal during the growth but there were differences in biomass yield (Fig. 14). In the individual populations *C.pyrenoidosa* had highest and *Selenastrum* lowest biomass concentration in the end of cultivation (1.2±0.3 gL^{-1} and 0.7±0.1 DW). Biomass concentration in *E.gracilis* culture reached 1.0 ±0.04 gL^{-1} DW. The growth rates have been calculated from the biomass growth curve with *C.pyrenoidosa* at 0.11d^{-1}, *Selenastrum* showing a growth rate of 0.10 d^{-1} and

E.gracilis with growth rate of 0.14 d^{-1} is seen. The mixed populations however show growth pattern in consistency with individual populations. In the mixed population of all three species *(C.pyrenoidosa, E.gracilis* and *Selenastrum)* and in the mixed population of *E.gracilis* and *Selenastrum* strains biomass in the end of the cultivation was equal with single culture of *E.gracilis.* The combination of *C.pyrenoidosa* with *E.gracilis* and *C.pyrenoidosa* with *Selenastrum* showed biomass concentrations 0.7±0.1 and 0.8±0.1 gL^{-1} DW). All the cultures showed exponential growth in the first 5 or 6 days of the experiment. The growth rates were similar for the populations of *C.pyrenoidosa* and *E.gracilis* and *Selenastrum* and *E.gracilis* at 0.13 d^{-1} . The population of *C.pyrenoidosa* and *Selenastrum* showed a growth rate of 0.16 d^{-1} and all the three strains together showed growth rate of 0.2 d^{-1}(Table 4). Growth of strains appeared to be stable after logarithmic growth phase except of slight rise in the end. The mixed population of *E.gracilis* and *Selenastrum* however, showed a stable growth during this period. The single and mixed cultures show a stress on day 12 with *E.gracilis* and *Selenastrum* being an exception. Towards the end of cultivation, all the cultures seem to show highest biomass concentrations.

Table 4 Growth rates in Laboratory scale

SPECIES	**GROWTH RATES (d^{-1})**
C.pyrenoidosa	0.11
E.gracilis	0.10
Selenastrum	0.14
C.pyrenoidosa and E.gracilis	0.13
C.pyrenoidosa and Selenastrum	0.16
E.gracilis and Selenastrum	0.13
C.pyrenoidosa, E.gracilis and Selenastrum	0.2

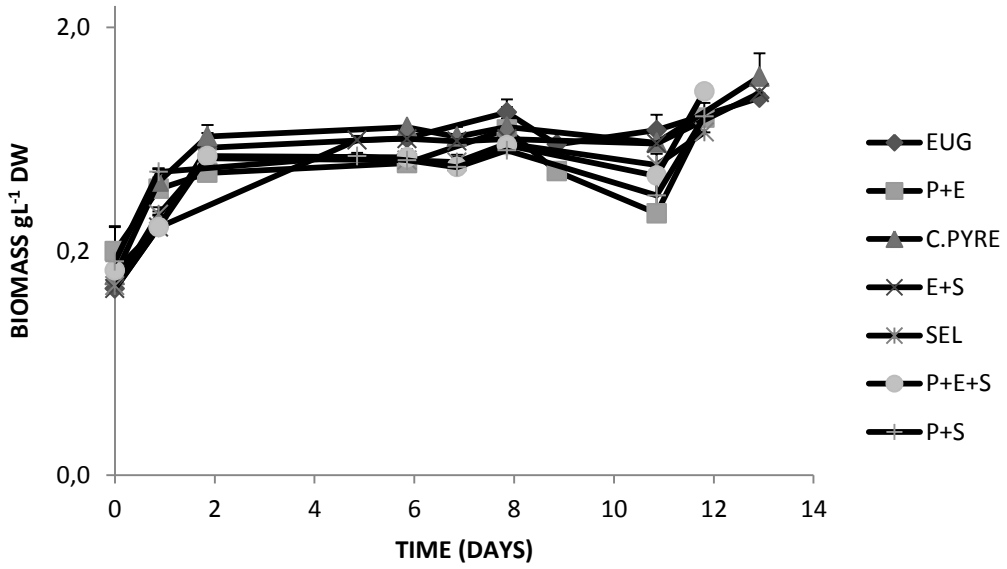

Fig.14 Growth of different combinations of algae species in wastewater in Laboratory scale (gL^{-1}).

Cell concentrations in the end of the cultivation in laboratory cultures are shown in Fig. 15. *C.pyrenoidosa* had the maximum amount of cells as an individual population (8.68E+06 cells mL^{-1}). The mixed culture of *C.pyrenoidosa* and *E.gracilis* shows that the cells of *C.pyrenoidosa* are better adaptive to wastewaters in mixed culture. This can also be seen in case of mixed culture of *C.pyrenoidosa and Selenastrum sp.* where *C.pyrenoidosa* out competes *Selenastrum*. *E.gracilis* and *Selenastrum* in a combination show survival of both the species but cell concentration of *Selenastrum* strain was lower than when it was cultured alone (2.29E+06 and 2.75E+05 cells mL^{-1}). In presence of all the three species, *Selenastrum* again shows a minimal survival rate and disappeared in the end of the cultivation (day 7 onwards). However, also in this case the co-existence of *E.gracilis* and *C.pyrenoidosa* is seen. *E.gracilis* showed a healthy cell population under microscope during the growth in laboratory scale in every culture combinations.

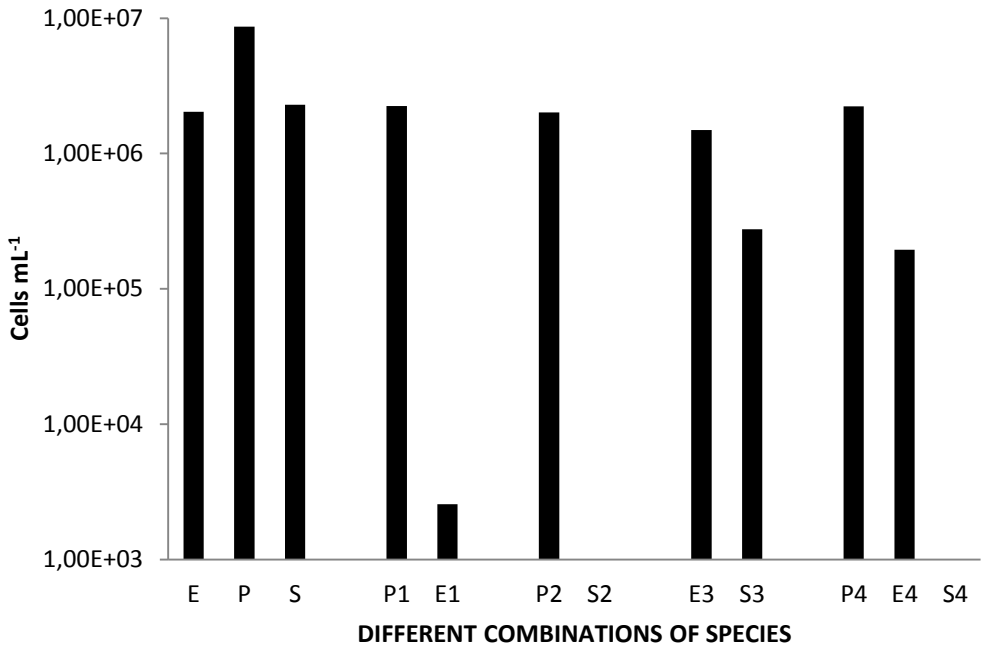

Fig. 15 Cell concentrations (Cells mL^{-1}) in end of laboratory scale cultivation. (Here, E *is E.gracilis*, P is *C.pyrenoidosa*, and S is *Selenastrum* sp.)

4.3 Growth in photobioreactor

Considering the survival of species in laboratory experiments, three combinations that included *E.gracilis* as a single population and in mixed culture with *C.pyrenoidosa* and mixed culture with *Selenastrum* were selected to cultivation experiments in photobioreactor. These populations in the reactors were cultivated for a period of 2 weeks and the biomass concentrations in the end of the cultivation have been shown in Fig. 16. The combination of *E.gracilis* and *Selenastrum* showed a rapid growth showing high biomass in the end of cultivation at 0.72±0.1 gL^{-1} DW. This biomass was comparable to the single population of *E.gracilis* that shows highest biomass in the end of cultivation at 0.8±0.1 gL^{-1}. Both mixed cultures showed highest biomass at day 5 (0.76±0.03 gL^{-1} DW and 0.81±0.02 gL^{-1} DW). The culture of *E.gracilis* and *Selenastrum* showed a slight drop from day 5 onwards. *E.gracilis*, on the other hand showed a rise towards the end of cultivation. The growth rates of the cultures indicated that

E.gracilis and C.pyrenoidosa had attained the growth rate of 0.3 d-1. *E.gracilis* and *Selenastrum* showed growth rate of 0.46 d^{-1}, *E.gracilis* in single culture grew at the rate of 0.37 d^{-1}.During the growth, *E.gracilis* population seemed to be very healthy under the microscope. *E.gracilis* cells being very big showed dominance over other species under the microscope. Cell concentrations were calculated by microscopy from the last days of cultivation. (No other cells were seen) The cell concentrations of *E.gracilis* in individual population show comparable results with presence of *Selenastrum.* The population of *E.gracilis* is however is very less when it grew in combination with *C.pyrenoidosa* (Fig 17).

Table 5 Growth rates of algae species in reactors.

SPECIES	GROWTH RATE(d^{-1})
E.gracilis	0.37
C.pyrenoidosa and E.gracilis	0.30
E.gracilis and Selenastrum	0.46

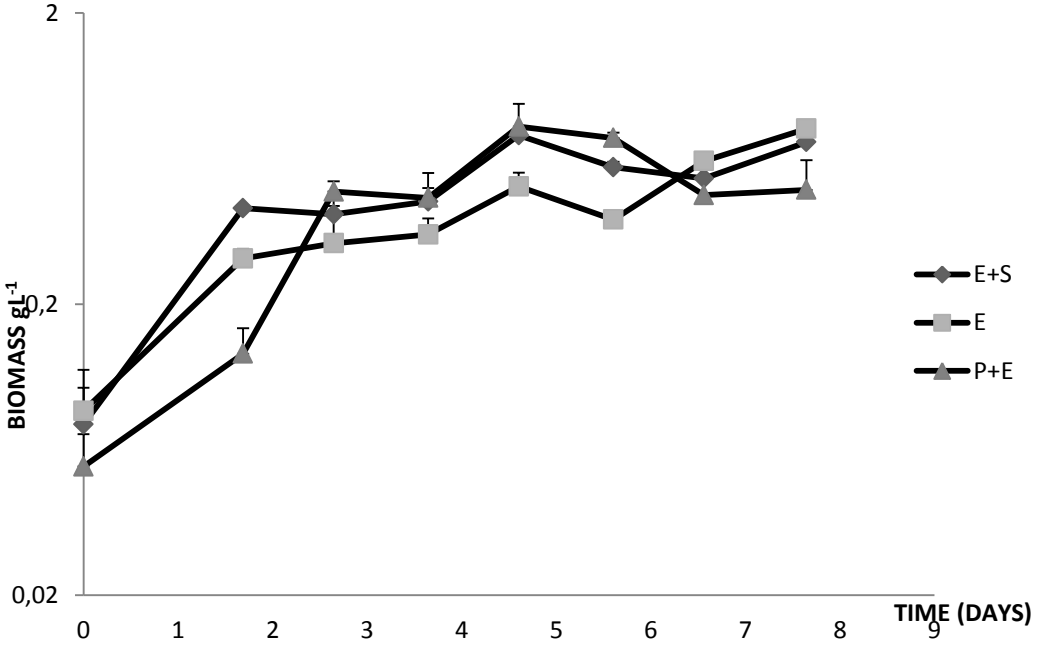

Fig.16 Growth of different combinations of algae species in wastewater in photobioreactor.

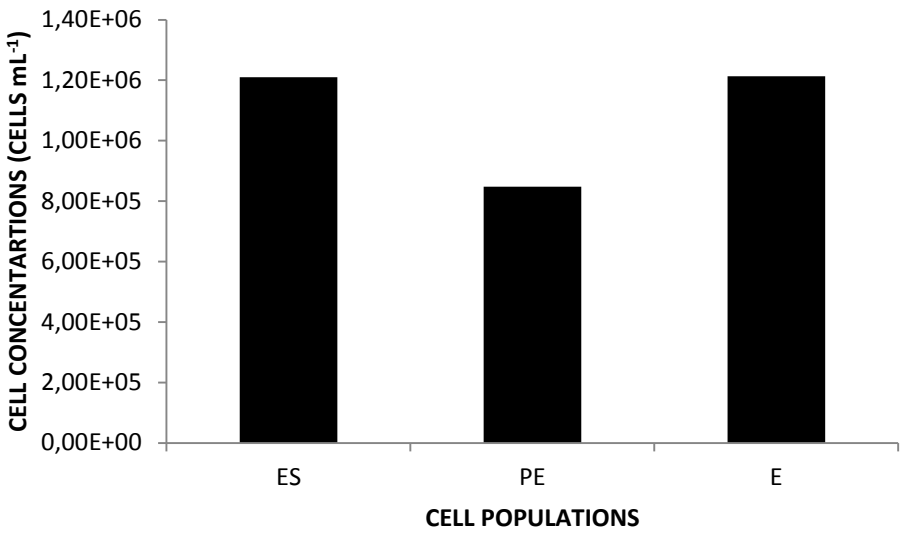

Fig.17 Cell concentrations of *E.gracilis* (Cells mL^{-1}) in the end of reactor cultivation. (Here, E *is E.gracilis*, P is *C.pyrenoidosa*, and S is *Selenastrum* sp.)

4.4 Reduction of nutrients in photobioreactor experiments

Nutrient reduction during the growth was obtained from the single and mixed population cultivations in the photobioreactor (Fig.18, 19 and 20). The populations show a similar pattern of nutrient consumption during the growth of the different combination of species. There is drop in concentrations of nitrate, ammonium, phosphate and total nitrogen during the growth in reactors. Phosphate and nitrate show overlapping results in all the cultures. Nitrate shows slight increase over days 2 and 3 and drops towards the end of cultivation. Phosphate levels show drop at day 3 with rise at day 4 and drop again towards the end of cultivation. Total nitrogen and ammonium show a drastic drop over the cultivation phase in all the cultures. COD in the all the populations shows a similar pattern of rise and drop. It however attains a stable value of 182 mgL^{-1} by the end of cultivation.

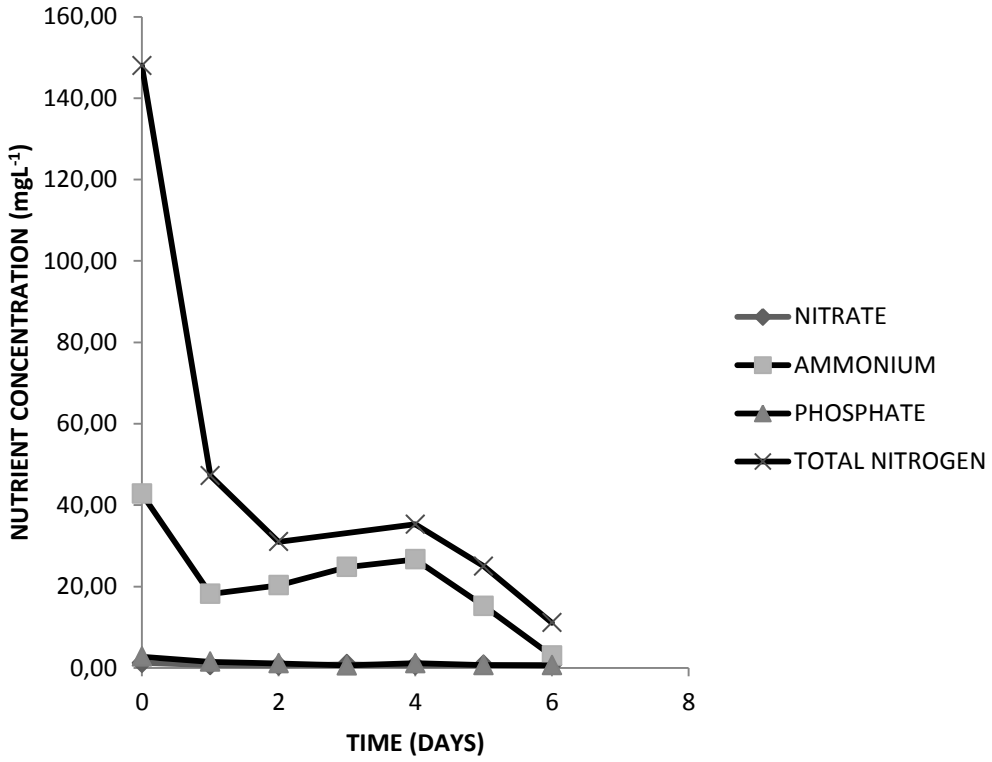

Fig 18 Nutrient analysis of waste water with *E.gracilis and C.pyrenoidosa.*

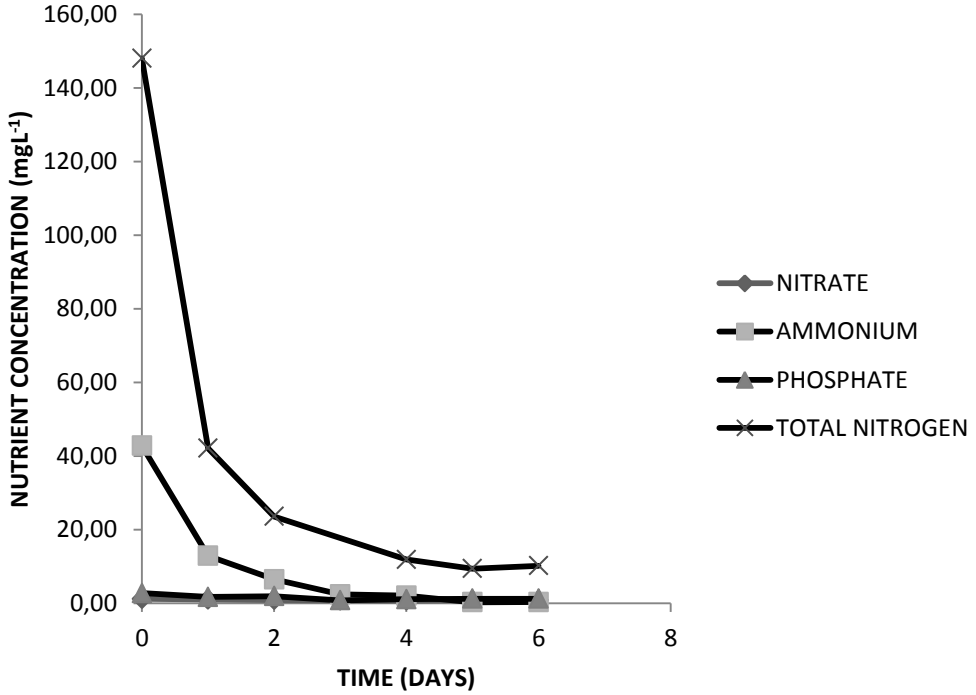

Fig 19 Nutrient analysis of waste water with *E.gracilis and Selenastrum.*

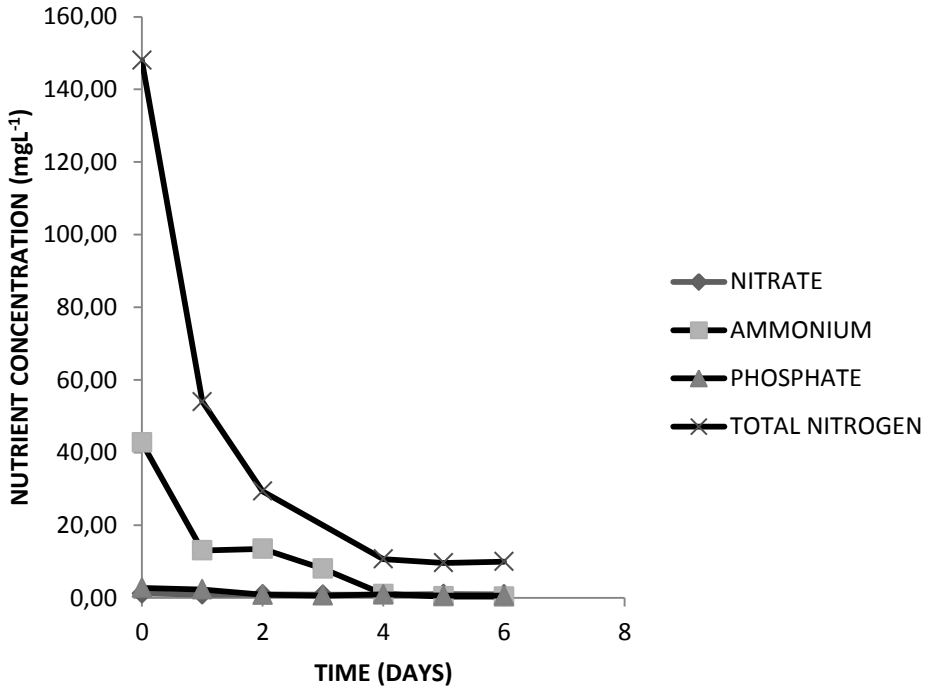

Fig 20 Nutrient analysis of waste water with *E.gracilis*

Total nutrient removal from the wastewaters in different cultivations is presented in fig 21. The ammonium removal was attained highest by the combination of *Selenastrum* and *E.gracilis* (99.18%) and *E.gracilis* alone followed closely by *E.gracilis and C.pyrenoidosa* (92.99%). The nitrate removal was seen maximum by *E.gracilis* and *C.pyrenoidosa* (41.61%) followed by *E.gracilis* combination with *Selenastrum* (30.56%) and individual population (22.42%). The phosphate removal was, however, seen maximum by combination of *E.gracilis* (78.42%) followed by *E.gracilis* and *C.pyrenoidosa* (76.26%) and least by *E.gracilis* and *Selenastrum* (54.32%).

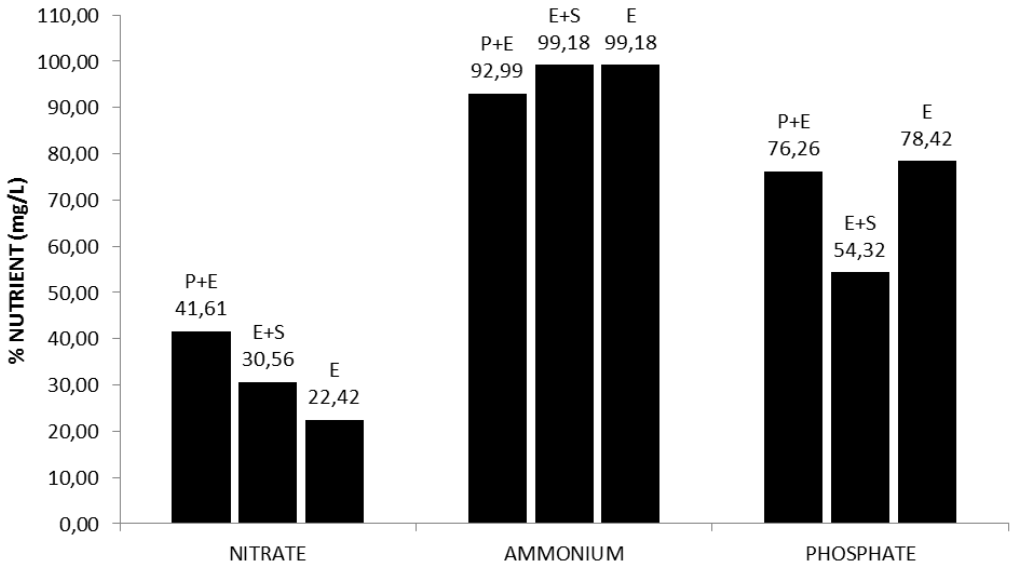

Fig 21 Percentage nutrient removal from wastewater by different algae populations. (Here, E *is E.gracilis*, P is *C.pyrenoidosa*, and S is *Selenastrum* sp.)

4.5 Total lipid content and fatty acid profile of populations in the photobioreactor experiments

The total lipid content of algae was analyzed by gravimetric determination and their comparison with the sum of individual fatty acids from last four days of cultivation has been show in in Fig 22. The mixed culture of *E.gracilis* and *Selenastrum* showed high lipid fatty acid contents as compared to all other cultures during these 4 days. The mixed culture of *E.gracilis* and *C.pyrenoidosa* shows low amount of lipids over the days. This mixed culture shows higher total lipid content than the single population of *E.gracilis* in day 7 in both the FA and gravimetric analysis.

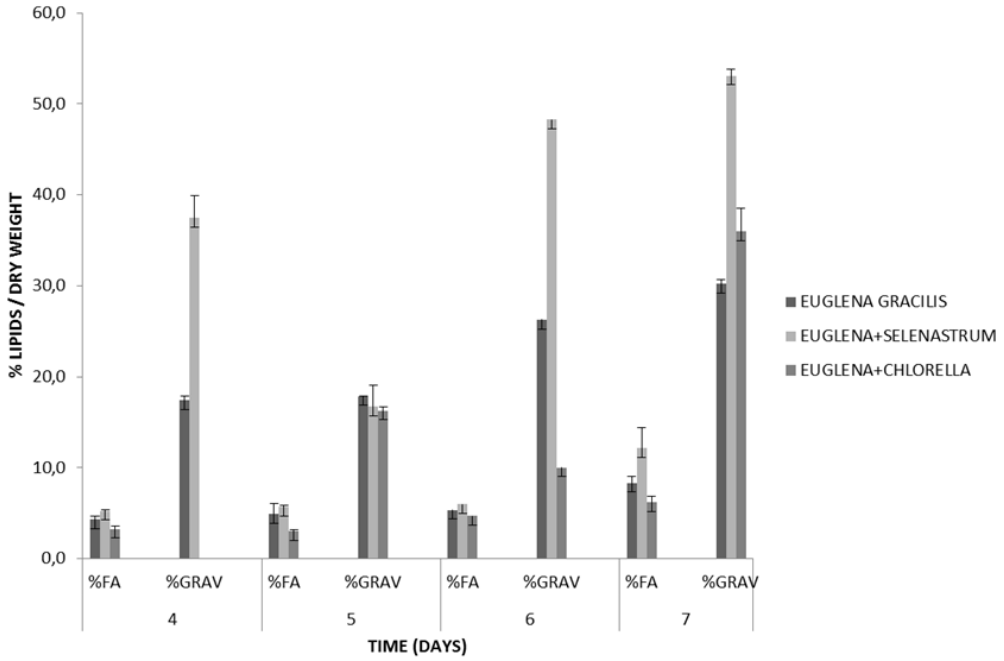

Fig. 22 Comparison of sum of individual fatty acids and total lipid content determined gravimetrically.

The monoculture and mixed culture of *E.gracilis* were analyzed for lipid content and characterization to find suitability for bio-diesel production. The fatty acid profile was analyzed from the last days of cultivation in reactors. The results are presented in Table 6, 7 and 8. In the FAME profile of the single population of *E.gracilis* the content of saturated fatty acids shows higher accumulations in day 2 (40.44±4.73% DW) and day 3 (41.35±0.62% DW) but drops again on day 4 (33.18±2.35% DW) in the same level than in day 1 (37.98±2.45% DW). The monounsaturated fatty acids decrease over day 2 and 3 (from 11.27±0.46% DW to 7.82±0.14% DW) and increase again in the end (12.80±1.23% DW). The content of polyunsaturated fatty acids is constant over all the days and show minor changes (approx. 51% DW).The total FA content is maximum at day 4 (8.32±0.73) which is nearly double from day 1 (4.32±0.30% DW). This profile shows major accumulations of the total FA, saturated FAs and PUFAs in days 2

and day 3. This FA content is least when compared with the other cultures (*E.gracilis and Selenastrum* showing the highest content)

Table 6 FAME profile of *E.gracilis*. Proportions of individual FAs, saturated, monounsaturated and polyunsaturated FAs and sum of identified FAs (% DW ±SD of the two measurements). ND, not detected.

	E.GRACILIS							
DAYS	1		2		3		4	
C 12:0	1.01	±0.19	2.61	±0.33	2.44	±0.03	0.63	±0.18
C 13:0	1.21	±0.12	1.91	±0.23	3.01	±0.10	2.26	±0.20
C 14:0	6.94	±0.18	7.45	±0.47	7.17	±0.24	6.82	±0.09
C 15:0	1.61	±0.12	2.15	±0.35	2.75	±0.07	2.09	±0.14
C 16:0	14.70	±0.74	14.21	±0.75	15.25	±0.09	17.40	±1.32
C 16:1	3.17	±0.07	3.23	±0.26	2.99	±0.05	2.52	±0.20
C 17:0	2.22	±0.21	2.37	±0.53	2.12	±0.03	1.06	±0.11
C 17:1	3.83	±0.14	N.D		N.D		N.D	
C 18:0	4.61	±0.36	4.65	±0.91	4.10	±0.04	2.13	±0.21
C 18:1N9c	4.26	±0.25	4.86	±0.67	4.83	±0.08	10.29	±1.03
C 18:2N6C	5.48	±0.14	6.28	±0.22	8.43	±0.06	7.65	±5.43
C 18:3N3	22.32	±2.20	22.10	±4.25	20.50	±0.66	23.74	±2.51
C 20:0	3.68	±0.34	3.34	±0.76	2.97	±0.02	N.D	
C 20:2	3.03	±0.21	3.06	±0.41	3.05	±0.02	2.55	±0.25
C 20:3N6	2.94	±0.24	2.77	±0.44	2.48	±0.02	1.51	±0.14
C 21:0	2.00	±0.19	1.74	±0.40	1.54	±0.01	0.78	±0.09
C 20:4N6	4.81	±0.06	4.51	±0.08	4.32	±0.03	4.15	±0.35
C 20:3N3	2.56	±0.24	2.48	±0.43	2.46	±0.00	2.12	±0.18
C 20:5N3	4.85	±0.05	5.21	±0.10	4.98	±0.00	5.34	±0.59
C 22:6n3	4.76	±0.22	5.06	±0.14	5.09	±0.05	4.10	±0.51
Total FAs% DW	4.32±0.30		4.87±1.19		5.33±0.27		**8.32±0.73**	
SATURATED FA	37.98±2.45		**40.44±4.73**		**41.35±0.62**		33.18±2.35	
MONOUNSATURATED FA	11.27±0.46		8.09±0.93		7.82±0.14		**12.80±1.23**	
POLYUNSATURATED FA	50.75±3.36		**51.47±6.06**		**51.32±0.84**		51.14±9.97	

The mixed culture of *E.gracilis* and *C.pyrenoidosa* show a different profile from that of single population of *E.gracilis.* Here, the saturated fatty acid content is higher during the first three days (44.13±2.65, 44.97±0.34, 40.88±1.46% DW respectively)but then shows a sharp drop in day 4 (28.18±3.51% DW). The monounsaturated is maximally accumulated on day 1 (20.85±1.02), later, fatty

acids show a drop over days 2 (10.82±0.37% DW) and 3 (8.36±0.22% DW) but increase towards day 4 (17.25±2.59% DW). The content of polyunsaturated fatty acids show increase over all the days being maximum on day 4 (51.93±4.80% DW).The total FA content shows a similar pattern to that of E.gracilis profile. It is the maximum on day 4 (6.17±0.71% DW) which is roughly the double from its content on day 1 (3.25±0.29% DW).

Table 7 FAME profile of *E.gracilis and C.pyrenoidosa*. Proportions of individual FAs, saturated, monounsaturated and polyunsaturated FAs and sum of identified FAs (% DW ±SD of the two measurements). ND, not detected.

	E.GRACILIS GRACILIS and C.PYRENOIDOSA PYRENOIDOSA							
	1		2		3		4	
C 12:0	1.14	±0.12	3.53	±0.06	2.91	±0.06	0.54	±0.20
C 13:0	1.62	±0.14	2.42	±0.00	2.01	±0.07	0.95	±0.01
C 14:0	4.19	±0.21	6.45	±0.16	6.89	±0.15	5.39	±1.00
C 15:0	2.38	±0.15	2.80	±0.02	2.28	±0.09	1.51	±0.13
C 16:0	14.51	±0.67	12.13	±0.03	13.39	±0.41	12.96	±1.13
C 16:1	4.07	±0.04	4.17	±0.13	3.12	±0.08	2.32	±0.04
C 17:0	3.65	±0.23	3.47	±0.02	2.62	±0.13	1.58	±0.30
C 17:1	10.64	±0.72	N.D		N.D		N.D	
C 18:0	7.40	±0.49	6.72	±0.01	5.12	±0.25	3.19	±0.51
C 18:1N9c	6.14	±0.27	6.65	±0.24	5.24	±0.14	14.93	±2.56
C 18:2N6C	4.58	±0.10	5.28	±0.24	4.24	±0.19	7.02	±1.43
C 18:3N3	10.73	±1.54	16.80	±1.10	23.30	±1.32	25.41	±1.78
C 20:0	6.00	±0.41	4.89	±0.03	3.71	±0.19	N.D	
C 20:2	4.22	±0.27	3.91	±0.06	3.71	±0.11	2.52	±0.27
C 20:3N6	N.D		N.D		3.01	±0.13	2.20	±0.39
C 21:0	3.24	±0.22	2.56	±0.01	1.94	±0.10	2.06	±0.23
C 20:4N6	4.56	±0.00	4.22	±0.31	3.99	±0.07	3.57	±0.10
C 20:3N3	N.D		N.D		2.72	±0.15	2.19	±0.32
C 20:5N3	4.95	±0.07	5.20	±0.49	5.18	±0.05	4.66	±0.12
C 22:6n3	5.98	±0.19	6.77	±0.96	5.09	±0.05	4.37	±0.38
Total FAs% DW	3.25±0.29		2.99±0.19		4.67±0.25		**6.17±0.71**	
SATURATED	**44.13±2.65**		**44.97±0.34**		40.88±1.46		28.18±3.51	
MONOUNSATURATED	**20.85±1.02**		10.82±0.37		8.36±0.22		17.25±2.59	
POLYUNSATURATED	35.02±2.17		42.17±3.16		51.25±2.07		**51.93±4.80**	

The combination of E.gracilis and Selenastrum show higher accumulation of saturated fatty acids on day 1 (38.15±1.86% DW) while dropping over days 2 (37.64±3.11% DW) and 3 (37.62±1.53% DW) and being the least on day 4 (30.29±1.20% DW).The monounsaturated fatty acids show maximum accumulation on day 1 (13.33±0.24% DW) followed by a pattern of drop over day 2 (11.69±0.64% DW), day 3 (7.00±0.34% DW) and day 4 (11.22±1.31% DW). The content of polyunsaturated fatty acids show increase from day 1 (48.52±2.34% DW) to day 2 (50.67±3.86% DW) and day 3 (55.38±2.44% DW) being maximum on day 4 (56.68±1.68% DW).The total FA content shows a similar profile as compared to the above mentioned cases being maximum on day 4 (12.14±2.27% DW) which is nearly twice to its content on day 1 (5.22±0.11% DW). This FA content is the highest as compared to all other cultures used for lipid analysis.

Table 8 FAME profile of *E.gracilis and Selenastrum*. Proportions of individual FAs, saturated, monounsaturated and polyunsaturated FAs and sum of identified FAs (% DW ±SD of the two measurements). ND, not detected.

	E.GRACILIS and SELENASTRUM							
	1		2		3		4	
C 12:0	1.11	±0.18	2.18	±0.19	2.02	±0.20	0.43	±0.02
C 13:0	1.27	±0.11	1.47	±0.16	2.42	±0.09	1.62	±0.02
C 14:0	7.70	±0.07	7.34	±0.35	6.91	±0.07	6.24	±0.14
C 15:0	1.64	±0.05	1.69	±0.23	2.35	±0.07	1.93	±0.04
C 16:0	15.12	±0.51	15.38	±0.57	15.03	±0.20	16.68	±0.53
C 16:1	3.68	±0.01	3.11	±0.12	2.78	±0.09	2.44	±0.02
C 17:0	2.01	±0.16	1.88	±0.32	1.76	±0.18	0.91	±0.15
C 17:1	5.42	±0.01	4.40	±0.20	N.D		N.D	
C 18:0	4.24	±0.33	3.75	±0.58	3.43	±0.34	1.80	±0.26
C 18:1N9c	4.23	±0.21	4.18	±0.32	4.22	±0.26	8.78	±1.29
C 18:2N6C	4.73	±0.03	5.21	±0.10	8.14	±0.04	11.18	±0.10
C 18:3N3	21.55	±1.42	24.01	±2.68	25.27	±1.74	26.12	±0.82
C 20:0	3.29	±0.29	2.61	±0.46	2.44	±0.26	N.D	
C 20:2	2.96	±0.16	2.74	±0.25	3.08	±0.11	2.59	±0.08
C 20:3N6	2.78	±0.17	2.46	±0.29	2.15	±0.15	1.32	±0.15
C 21:0	1.76	±0.17	1.36	±0.24	1.26	±0.13	0.68	±0.05
C 20:4N6	5.28	±0.10	5.06	±0.08	4.90	±0.12	4.53	±0.12
C 20:3N3	2.35	±0.19	2.09	±0.24	2.32	±0.11	2.03	±0.13
C 20:5N3	4.74	±0.13	5.01	±0.03	5.35	±0.10	5.41	±0.10
C 22:6n3	4.12	±0.14	4.08	±0.21	4.17	±0.08	3.51	±0.16
Total FAs% DW	5.22±0.11		5.66±0.21		6.00± 0.04		**12.14±2.27**	
SATURATED	**38.15±1.86**		37.64± 3.11		37.62± 1.53		30.29±1.20	
MONOUNSATURATED	**13.33±0.24**		11.69±0.64		7.00±0.34		11.22±1.31	
POLYUNSATURATED	48.52±2.34		50.67±3.86		55.38±2.44		**56.68±1.68**	

5 DISCUSSIONS

Algae species (*C.pyrenoidosa*, *Selenastrum* and *E.gracilis*) in single populations thrive in the wastewater and showed efficient growth in the laboratory scale experiments. In the mixed populations, however, not all the combinations have shown survival. This can be seen in case of mixed culture of *C.pyrenoidosa* and *Selenastrum.* The *Selenastrum* cells started to disappear from the cultures after they were grown together for a week. Also in pure cultures cell amount of *Selenastrum* was lower than cell amounts of *C.pyrenoidosa* strain. The mixed cultures of *C.pyrenoidosa* and *E.gracilis* showed survival of both the strains. However, in this case also the cells of *C.pyrenoidosa* were presented in larger quantities as compared to *E.gracilis* cells. This can be attributed to the fact that small cells have a rapid metabolism and ability to uptake nutrients faster than bigger cells. *C.pyrenoidosa*, for this reason maybe showed more efficient growth than other species (*E.gracilis* and *Selenastrum*) when present in mixed culture with them. *E.gracilis* however showed a more dynamic growth at reactor scale. The reactors provided a regulated pH environment which could be beneficial for *E.gracilis*. In laboratory scale experiments the pH was not controlled and was higher (8±2). This could be a reason for weaker growth of *E.gracilis* in laboratory scale. The mixed culture of *E.gracilis* and *Selenastrum* showed a better adaptivity to each other in laboratory scale as compared to other cultures. In the presence of all the three species, *Selenastrum* cells were not seen by the end of laboratory scale experiments. However, the cells of *E.gracilis* and *C.pyrenoidosa* survive together. This can again be attributed to the fact that *E.gracilis* has growth rates comparable to that of *C.pyrenoidosa* (0.10 d^{-1}) in laboratory scale.

In the reactor scale experiments, the results show better success in *E.gracilis* growth. The individual population of *E.gracilis* and in mixed cultures of this strain with *Selenastrum* and *C.pyrenoidosa* show a profuse growth of *E.gracilis* cells. The growth was maybe favored owing to the physical environment provided by the reactors. When observed under the microscope, the *E.gracilis* cells were intact and looked healthy. During the cultivation period, mixed populations of *E.gracilis* cells with the other species showed growth of mainly *E.gracilis* cells only from day 3 onwards. It is also possible that *E.gracilis* cells excrete some compounds that might be unsuitable for growth of any other species but that is not very plausible because other species survived well in laboratory scale.

In the reactor scale single population of *E.gracilis* had higher biomass during the cultivation as compared to the combination *of E.gracilis* with *Selenastrum* and *E.gracilis* with *C.pyrenoidosa*. The single culture of *E.gracilis* cells reaches the maximum biomass by the end of cultivation (0.8 ± 0.1 $gL^{-1)}$. This is however contradictory to the presence of only *E.gracilis* cells in the mixed populations as well seen by microscope. The reason could be secretion of some compounds by the other species which were beneficial for the growth of *E.gracilis* cells. However, the presence of other species enhances the biomass productivities in large scale set up. There is also a possibility that cells of other species are present in very low concentrations that could not be determined by microscopy.

There are significant differences seen between gravimetrically determined total lipid content and sum of individual FAs. There is a possibility that some lipids were evaporated during the methylation and have not been accounted by the GC-MS but the methylation process was tested before experiments with standard olive oil and conversion of gravimetric determination versus sum of individual fatty

acids of about 70 % was found at the time of 16 hours. In the methylation process fatty acids are diversed from glycerol backbone and for the calculation of exact recovery information, lipid class composition would be needed. Conversion of triacylglycerols, if compared to gravimetric results is near to one but conversion of mono- or diglycerides or other lipid classes much more lower (38). Thus it can be concluded that there were no significant loss of FAs. Also, the gravimetric method cannot be regarded as a very precise form of calculating lipid content as it can determine other compounds as well.

Algal feedstocks have generated considerable discussion in the literature with numerous review or perspective articles often containing supporting or critical statements regarding the feasibility of algae-derived biodiesel being published (28). However, it may be noted in this connection that algal oils enriched in omega-3 polyunsaturated fatty acids (PUFAs) have been of interest for other applications due to the recognized health benefits of these fatty acids. Saturated FA chains are problematic for cold flow properties and polyunsaturated FA chains are problematic for oxidative stability. In order to improve biofuel properties, therefore, a fatty acid composition is desirable and compromises on biofuel properties, *i.e.*, offers acceptable cold flow and oxidative stability simultaneously. (28)The fatty acid profiles of the individual population of *E.gracilis* shows difference when in mixed populations for presence of saturated, monounsaturated and polyunsaturated fatty acids. The single population of *E.gracilis* shows high content of saturated fatty acids in day 2 and day 3 as compared to its combination with *Selenastrum*. The content of monounsaturated fatty acids is constant amongst all the cultures with *E.gracilis and C.pyrenoidosa* showing the highest content.

The polyunsaturated fatty acids show the maximum content in the entire FAME profile (~50%) during the cultivation phase. This fatty acid content would likely exhibit poor cold flow and oxidative stabilities. There were 20 different fatty acids identified from *E.gracilis* alone and its combination with *C.pyrenoidosa* and *Selenastrum*. Among these the palmitic acid (C 16:0) and linolenic acid (C 18:3) are abundant. The presence of PUFAs, mainly the linoleic acid (C18:3n3) in this study, makes the algal oil a preferred component for infant formulas and nutritional supplements and food additives (42). The high content of C18:3n3 is an essential FA for mammals including humans and C20:5n3 (EPA) and C22:6n3 (DHA) are also required to get from nutrition because the synthesis of these fatty acids is not efficient.

This study also predicts that there is potential for reducing the nutrient load from wastewater streams majorly ammonium content followed by phosphate content. It is a well known fact that typically phosphorus or nitrogen is the limiting factor of growth. When one or the other is used (in this case nitrogen) it starts to limit biomass growth. Algae need for nitrogen: phosphorus is 16:1. So, phosphorus is needed in lower amounts. Ammonium is more useful for algae than nitrate, so its consumption is more effective and all the species cannot use nitrate at all. Not all forms of nitrogen can be readily used by algae especially nitrogen bounded with particulate organic matter. In general, algae and aquatic plants directly utilize inorganic forms of nitrogen such as nitrates, nitrites, and ammonia (45). In all these cultures, consumption of nitrate follows a similar pattern of consumption. It shows a constant uptake by the algae cells during the growth, but not in large quantities.

From the growth curves, it can be seen that efficient growth stops when ammonium is used. In the end of cultivation period biomass is growing only pure culture of E.gracilis. But still there are few differences in the nutrient uptake in the different cultures, even though only cells from *E.gracilis* are seen and not from any other strain. There may be a possibility of presence of cells of other strains in very small quantities that lead to minor variations in the nutrient consumption.

6 CONCLUSION

The experiments show suitability of the single and mixed populations of strains to the wastewater in the laboratory scale and reactor scale. Results showed that biomass yield in the mixed cultures was mainly lower than pure cultures but there was enhancement in lipid production when E.gracilis and Selenastrum were cultured together.

However results indicate that cultivation in mixed populations can reach higher lipid production but fatty acid profile of E.gracilis is not optimal for biodiesel applications.

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