Application of Low Intensity Pulsed Ultrasound for Microbial Cell Stimulation in Bioprocesses And Development of Carbon-based Silver Covered Filters for Microbial Cells Removal in Water Systems

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Biomedical Engineering

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Abstract

Renewable resources with their potential application in food industry, pharmacy, energy sector, chemistry, material science etc. can be a major player in our everyday lives. Apart from sustainability in the production, the use of bioprocesses in the energy production and waste water treatment can address the important problem of water, soil and air pollution as well as climate change, which can be essential for a higher level of life quality on the planet. Therefore, the potential use and modification of microorganisms, such as microalgae, in fermentation and as biocatalysts as well as new chemical, physical and biological methods to improve cost-efficiency of green and white biotechnology are gaining a lot of attention from modern research all over the world. One of the alternative approaches to apply in biotechnological production that can successfully improve cost-efficiency and increase yield can be ultrasound - a form of acoustic energy, characterized by frequencies of 20 kHz -1 GHz, or the range that is above the human hearing and lower than the hypersonic regime. Low-intensity pulsed ultrasound (1.5MHz, 20% duty cycle, and intensity between 20mW/cm^2 and 200mw/cm^2) has been previously used in the bioprocesses and has been proven to increase stem cell, microorganism and antibody growth. This research presents a study on the impact of Low Intensity Pulsed Ultrasound (LIPUS) as a physical method for microorganism growth and activity stimulation, which can be used in combination with all other biological and chemical methods in bioprocesses. The study showed it can be applied in algal biofuel production giving up to 20% increase in lipid yield, in bio-ethanol production using microorganisms with up to 20-30% improvement in sugars to ethanol conversion efficiency, for increasing biomass of microalgae up to 20% with potential use for food, material and pharmaceutical

applications, as well as for waste-water treatment with microorganisms. Improving the efficiency of these processes by this mechanism on an industrial scale could yield more affordable price for biofuel or other products of above mentioned bioprocesses. The study has also revealed that LIPUS showed consistent results in different bioprocesses with different microorganisms and duration of cell culture, which indicates that there is a broad spectrum of potential LIPUS applications.

Preface

This dissertation is submitted for the degree of Doctor of Philosophy at the University of Alberta. This Ph.D. thesis is based on the results of reseach performed at the department of Biomedical Engineering, University of Alberta, under the supervision of Professor Jie Chen between January 2011 and December 2016. Some of the experiments presented here were the subject of collaborations.

Chapter 2 of this thesis has been presented as an oral presentation at Graduate Research Symposium, University of Alberta, June 2013 and as a poster presentation at the 5th International Conference on Algal Biomass, Biofuels and Bioproducts in San Diego, June 2015. Later it was published as Oleksandra Savchenko, Jida Xing, Xiaoyan Yang, Quanrong Gu, Mohamed Shaheen, Min Huang, Xiaojian Yu, Robert Burrell, Prabir Patra and Jie Chen. " Algal Cell Response to Pulsed Waved Stimulation and Its Application to Increase Algal Lipid Production" Scientific Reports 7, Article number: 42003 (2017). I carried out all main experiments and measurements, performed data analysis and wrote the manuscript.

Chapter 3 of this thesis has not yet been published, but we are preparing it for submission as Savchenko, O., J. Chen, X. Yang, R.Burrell, "Determining the Effects of Low Intensity Pulsed Ultrasound (LIPUS) on the Growth of Schizochytrium in Shake Flask Culture". I am the first author on the manuscript and was responsible for all the experiments, data analysis and preparation of the manuscript.

The work presented in Chapter 4 is also in preparation for submission as Michael C. Schultz, Jian Zhang, Xian Luo, Olekesandra Savchenko, Liang Li, Michael Deyholos and Jie Chen «Impact of Low-intensity Pulsed Ultrasound on Transcript

and Metabolite Abundance in *Saccharomyces cerevisiae*». That manuscript is based on the work done in collaboration with X. Luo and Dr. L Li from the Department of Chemistry and M. Schultz from the Department of Biochemistry, University of Alberta. Gene expression analysis was performed with the help of J.Zhang from InnTech, Alberta and M. Deyholos from the Department of Biology, University of British columbia. I carried out the fermentation experiments with *S. Cerevisiae* cells and harvested cells with further preparation for the analysis and took part in manuscript preparation.

Chapter 5 of this thesis was presented at BOTANY conference in Edmonton, Canada, July 2015 and later was published as Oleksandra Savchenko, Jie Chen, Yuzhi Hao, Xiaoyan Yang, Susie Li and Jian Yang, " Three-Dimensional Coating of Porous Activated Carbons with Silver Nanoparticles and its Scale-up Design for Plant Disease Management in Greenhouses" J Plant Pathol Microbiol 2016, 7: 381. 7:10, (2016). I was responsible for al the filter material development, characterization and static testing in the lab and manuscript preparation. Big part of the testic with pathogens and dynamic scale-up testing was performed in collaboration with Jian Yang and Susie Li from Alberta Innovates Technology Futures, Vegreville, AB, Canada.

Acknowledgments

First and foremost, I would like to express my deepest gratitude to my mentor and supervisor Professor Jie Chen, who gave me this oppotrunity to walk the path towards a doctoral degree in Canada. His guidance, patience, critical evaluation of my progress, yet ever present support are greatly appreciated and without them this thesis

and my degree would not have been possible. Not only did he supervise me with my PhD research, but also he gave me opportunities to work in diverse projects to broaden my skills and knowledge and to have the experience of collaboration with industry partners as well as international experience with TUM.

I am also very thankful to Dr. Robert Burrell, the head of my department of Biomedical Engineering. I felt very privileged to have worked under his supervision and his suggestions and thought provoking discussions contributed a lot to the fruitful completion of my research, while his own research work inspired and motivated me through all the years of my PhD program and will surely remain as such in my future career.

I would like to extend my appreciation to all my supervisory committee members Dr. Scott Chang, Dr. Huazhou Li, Dr. Yang Liu, Dr. Kesen Ma, to Dr. Damase Khasa for reviewing my proposal and Alan Wilman for serving as the chair of the committee.

I would like to thank all my colleagues from BINARY lab and partners in collaboration for their help and support during our collaborative research endeavours and Dr. Paolo Lugli and Katharina Melzer of TUM for hospitality during my internship.

Special thanks and appreciation go to Maisie Goh (BME) for all her kind help and support through the years and all the kindness that comes from her heart; and to Xiaoyan Yang, who was not only my colleague, but also a great friend and only with her generous help this work has reached its fruition.

The research that is presented in this thesis was supported by NSERC research grants, BME Graduate Research Awards 2013, 2014 and U of A and TUM Exchange grant and I want to use this opportunity to express my gratitude for this funding that made the research possible.

Finally, my doctoral student life would have never been possible without my family and friends. I would like to thank my sister, who always defied the boundaries and greatly inspired me, my mom for her unconditional love, my friends Anna Kuteleva, Jessi Deringer, Kateryna Fux, Alina Krasova, Samira Farsinezhad for proofreading and correcting my thesis and proposal and for being the inspiration in my PhD life, as well as all other great friends around the world for simply being with me in all the ups and downs through the years and for their neverending support. I feel very blessed to have them in my life.

This thesis and my doctoral degree are dedicated to my dad, who never believed in me and to Serge Verba, who taught me to believe in myself.

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Chapter 1

Introduction

1.1. Ultrasound and its application

Ultrasound is a form of acoustic energy exceeding the human hearing range and below the hypersonic regime, characterized by frequencies greater than 20 kHz and below 1 GHz (Figure 1.1). (Awad, Moharram, Shaltout, Asker, & Youssef, 2012) It has been known in nature in the form of echolocation, used by several animals for navigation, helping to detect distance to objects on the way, for communication and in hunting.

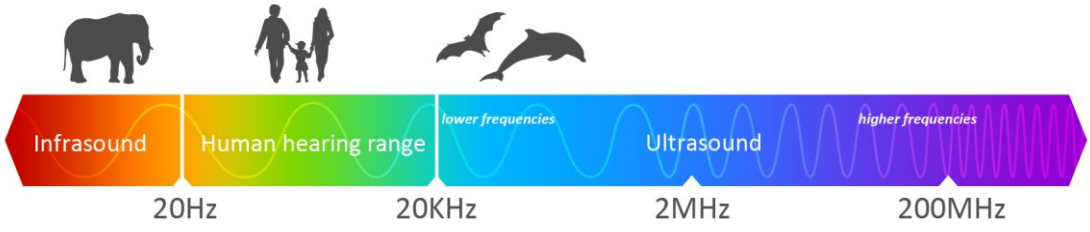

Figure 1.1. Range of ultrasonic wave frequencies

Due to the physical effects generated by this type of acoustic wave such as: mechanical agitation, shear forces, microstreams and microjets, cavitation associated with microbubbling and local heat increase, ultrasound has also found numerous applications in different industrial processes. (Ashokkumar, 2015) Also, ultrasound is a relatively cheap and low energy-consuming technology that is easy to use, which also contributes to its attractiveness for use in a variety of applications. (Awad et al., 2012) Depending on the application, high or low intensity of the ultrasound can be selected. High intensity ultrasound is defined by intensities higher than $1W/cm^2$. It is used in processes in which sonication chemically, physically or mechanically affects the medium or its contents and changes properties. Such processes include surface cleaning (sonication bath), microorganisms inactivation and disruption, medical therapy, atomization of liquids and free radical generation in chemical reactions, emulsification (with ultrasonic immersion probes) as well as machining. Low intensity ultrasound has intensities lower than $1W/cm^2$. It is used for transmitting energy through the medium without affecting the medium. Processes that use low intensity ultrasound include underwater communication and object detection, medical diagnostics, non-destructive testing, materials properties evaluation, quality control, inactivation or enhancement of enzyme activity etc. Mechanical and sheer forces that ultrasound generates increase mass transfer(Sinisterra, 1992) that can be used in bioprocesses using cells to enhance nutrients diffusion to the cell (Wu and Lin 2002), while microjets also have impact on the cell wall leading to a better release of cell products into the medium. (Ashokkumar, 2015)

The application of ultrasound in the medical field is one of the widest and most well known (Sarvazyan, 2010). It uses both high and low intensity ultrasound: low intensity is used for non-invasive diagnostic, such as ultrasound imaging, offering better clarity and more detailed information compared with other imaging techniques, e.g. imaging to find defects in tissues, or the widely used fetus imaging. At the same time, high intensity ultrasound and the local heat it generates are used in therapeutic purposes for treatment in cases of ligament, muscle and tendon injuries or for surgical purposes.

Another parameter that characterizes ultrasound is its operational frequency. Applications with frequencies higher than 100 kHz are used for guide waves for ultrasonic non-destructive testing, and medical imaging, while the higher frequencies of ultrasound are used in acoustic sensing and microscopy. To generate ultrasound wave simple electronic is used, comprised of a frequency generator, an amplifier, and a transducer- a device that converts electrical form of energy into mechanical energy.

Both physical and chemical effects of ultrasound attracted research attention to study its applications for biological processes. However, it is low intensity that is being more widely used in biological experiments due to the reduced heat generation component that is observed at higher intensities. Both low intensity continuous ultrasound (LIUS) and low intensity pulsed ultrasound (LIPUS) are finding their application in the research labs showing beneficial effect sometimes for the same process. The term “pulsed” describes the non-continuous ultrasound wave, with periods when ultrasound wave generation is on and periods when it is off. In cartilage tissue engineering both application of LIUS (H. J. Lee, Choi, Min, Son, & Park, 2006) and LIPUS (Lai et al., 2010) showed increased proliferation of stem cells and enhanced chondrogenesis. (Schumann et al., 2006)

Ultrasound has been successfully applied in drug delivery and for gene delivery into animal and plant cells (Chisti, 2003). This effect, when ultrasound is used for the non-chemical, non-viral, and non-invasive delivery of gene or protein into the cell, is called sonoporation. It utilizes the physical effect of ultrasound, which is associated with cavitation- creation and rupture of the microbubbles, that leads to microstreaming helping to move material into the cell (Lentacker, De Cock, Deckers, De Smedt, & Moonen, 2014). In the research presented in this thesis, my focus was on the application of low intensity pules ultrasound (LIPUS). This type of ultrasound was generated by commercially developed LIPUS device SonaCell^{TM}, developed by IntelligentNano Inc. (Edmonton, Alberta) for biological labs. Previous studies carried out by Dr. Jie Chen's group showed that LIPUS can increase antibody production (i.e. human anti-IL-8 antibody) by CHO cells up to 30%. (Zhao, Xing, Xing, Ang, & Chen, 2014) LIPUS has also been used in monoclonal antibody production by hybridoma cells (increased cell number and mAb production (i.e. anti-CD4) up to 70% was observed). (Xing, Yang, Xu, Ang, & Chen, 2012) Furthermore, the ultrasound can improve the viability, proliferation and differentiation of hematopoetic progenitor/stem cells. (P. Xu et al., 2012) Also the application of LIPUS could increase bio-ethanol production by fungi and yeast up to 50%(Shaheen et al., 2013). Ultrasound has many potential applications in food processing. (Ashokkumar, 2015; Feng, Barbosa-Canovas, & Weiss, 2011) It has been shown how low intensity ultrasound can be applied on lab scale in bioreactors based on juice and wine production wastewaters with yeast as microorganism and can increase biological activity and cell density of the cells with the effect still observed few hours after the sonication. (Schläfer, Sievers, Klotzbücher, & Onyeche, 2000) 200L pilot scale-up process was developed for the same microorganism utilizing food wastewaters from

juice producing company as glucose source, in which separate bioreactor was used for the ultrasound stimulation. (Schläfer, Onyeche, Bormann, Schröder, & Sievers, 2002) While optimized ultrasound conditions have shown to increase biological degradation of the organic compounds in wastewater and enhance biological activity of the microorganisms, the study has also pointed out that such beneficial effect can only be obtained in a very narrow range of ultrasound parameters, while other settings, not optimized for the process, can even decrease biological activity. Similar conclusions have been reported for other bioprocesses, stating ultrasound benefits in one range of settings, with cell damage and decrease in cell viability caused by sonication in other ranges of parameters. (Böhm et al., 2000; Francko & Thomas, 1990) Overall, ultrasound parameters such as frequency, intensity, duration, continuous or intermittent treatment, etc. cannot be generalized for many different processes yielding desired improvement and, thus, equipment and stimulation parameters adjustment have to be made every time to meet the needs of every individual application. (Awad et al., 2012; Chisti, 2003)The application of ultrasound could potentially be a physical method for use in bioprocesses along with existing biological and chemical methods. It can be applied in algal biofuel production, in bio-ethanol production using microorganisms, in biomass increase of microalgae in food, material and pharmaceutical applications, as well as in waste-water treatment. The application of LIPUS in these processes on industrial scale could significantly improve cost-efficiency of the production.

1.2. Bioprocesses and biotechnology

Biotechnology has become one of the biggest technological breakthroughs of the past century leading to significant improvements in many aspects of human life (Figure 1.2). It includes techniques and bioprocesses that use complete living organisms or their components to obtain desired products, or modify microorganisms for specific use. (Yang, 2007)

The biotechnological application of fermentation processes for food has been around for thousands of years in the process of winemaking, which utilizes microbial culture fermentation in the liquid medium. The discovery of microorganisms and further of their contribution to the wine production process and potentially their use for other bioprocesses attracted more research attention to the subject. (Giraffa & Carminati, 2008) Since the 1900s, the large scale anaerobic production of acetone, and butanol as well as aerobic production of citric acid began, marking the beginning of the fermentation industry. Shortly after such application of fermentation for the production of chemicals, production of pharmaceuticals- inculin and penicillin followed. (Demain & Vaishnav, 2009)

The modern biotechnology era started with the first plasmid gene delivery into *Escherichia coli* cell that followed after the discovery of genes and the possibility to manipulate them. Genetic engineering led to the pharmaceutical production of recombinant protein and peptides, (Demain, 2006) offering an alternative biotechnological approach to complex problem solving. It was followed by the discovery of microbial enzyme production, offering cheaper prices, higher availability and, thus, leading to a rapid growth of the enzyme industry.(Demain & Vaishnav, 2009)

Microbial fermentation processes offer certain advantages over chemical synthesis, such as cheaper and pollutant free production,(Spadiut, Capone, Krainer,

Glieder, & Herwig, 2014) as well as over bioprocesses using mammalian cell expression such as lower media cost, easier cultivation, stability of the expression system, shorter generation and growth time, etc. as well as few disadvantages. (Demain & Vaishnav, 2009)

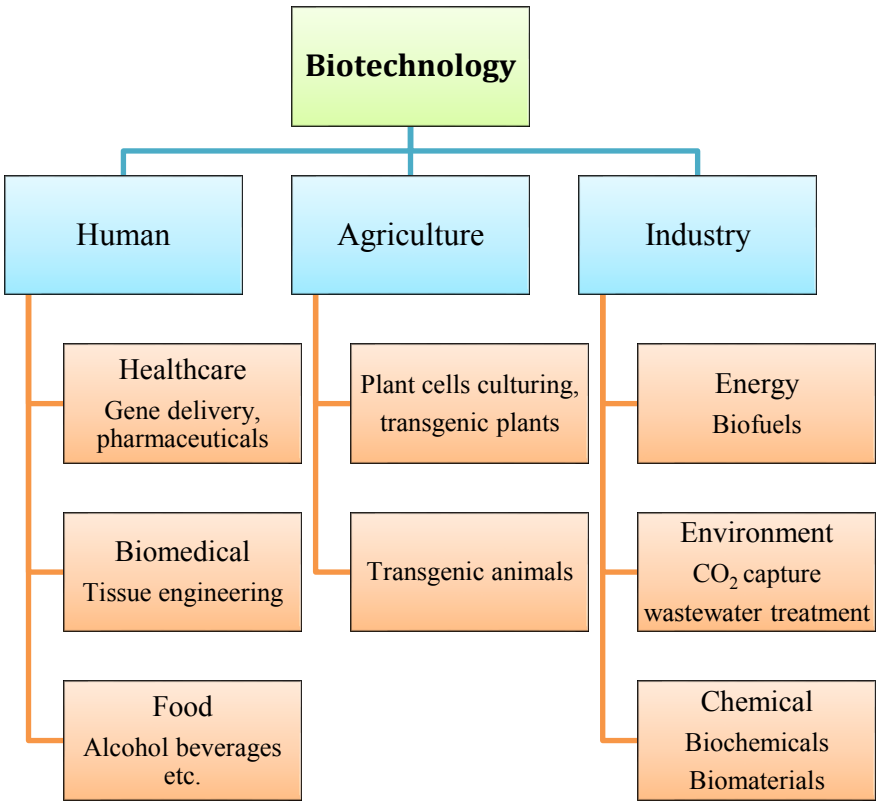

Figure 1.2. Overview of biotechnology fields

Current industrial bioprocesses use different types of microorganisms: procaryotic cells (bacteria) as well as eucaryotic (yeast, filamentous fungi) depending on the type of fermentation process, desired final product, substrate availability and process design. Plant cells, hairy root cultures and marine microorganisms recently started gaining research attention showing previously underexplored potential to be

used as microbial systems as well as for new bioactive compounds production.(Yang, 2007) In microbial protein expression technology, procaryotic bacteria are used as the system for smaller protein production, and eukaryotes - for proteins over 100 kD expression.

Escherichia coli – is probably the most widely used bacterial host system that offers a cheap and easy way for protein expression and is most prominent after Chinese hamster ovary (CHO) cells. (Terpe, 2006) It is well known for its robust, cheap and high yielding production of antibodies and recombinant proteins. Industrial application of *E.coli* started in 1980s and still remains the core host platform in many biotechnological companies due to its rapid growth and expression and ability to tolerate different conditions. It can accumulate recombinant protein up to 80% of its dry weight. Among the limitations of E.coli as production platform for proteins are the inability to express large proteins, proteins with many disulfide bonds or proteins that need post translational modifications like glycosilation. Recent studies, however, have shown that E.coli can produce small quantities of glycosilated proteins, which potentially broadens its applications.(Nothaft & Szymanski, 2010) Products such as insulin, human growth hormone, interferons were produced in the recombinant processes in *E.coli*. New emerging platforms have also allowed the secretion of human antibody fragments by *E.coli*.(Kwong & Rader, 2009)

Another useful bacterium based platform is *Bacillus*. It offers expression of enzymes: proteases and amylases that can be used for detergents and in the food industry. Among the benefits of bacilli is the cost effective production of proteins with secretion into the medium, which requires no cell rapture and makes downstream processing easy. Other proteins successfully expressed in *Bacillus* systems include heterologous interleukin-3EGF, amylases and various proteases. (Demain &

Vaishnav, 2009) The food industry uses *Lactoccocus latis* – gram-positive lactic bacteria in food fermentation for large-scale production of recombinant membrane proteins.

The alternative yeast expression platform offers many benefits such as: high yield production with rapid growth rate, cost effectiveness, high productivity, and high-density growth. Due to their defined genome, yeasts are easy to modify genetically. Compared to *E.coli*, yeast fermentation process times are longer, but yeasts can handle the production of disulfide rich proteins, glycosylated proteins, and can assist in post-translational protein folding.

Yeast product processing is similar to mammalian cells, though with shorter process time. Also, downstream processing is much easier than for mammalian cell culture. (Demain & Vaishnav, 2009) The first yeast expression system was with *Saccharomyces cerevisiae*, well known also for its use in baking. It was used for production of biopharmaceuticals such as insulin, hepatitis B vaccines, glucagon, platelet-derived growth factors, and antibody fragments. Researchers also use it as a model microorganism to study genomic changes and gene's function. (Dujon, 2010) Another yeast platform, *Pichia pastoris,* combines prokaryotic growth characteristics with the ability for posttranslational protein modifications like eukaryotes. Its advantages over *S. cerevisiae* include higher protein productivity, the ability to use methanol as carbon source and grow in strong methanol environment that is toxic for most of microorganisms, and the avoidance of hyperglycosilation. (Gerngross, 2004) It has become popular in the vaccines production, antibody fragments (Fabs), cytokines, hormones and matrix proteins. In Fabs production it offers faster development time compared to CHO based systems, offering also cheaper production. (Kunert, Gach, & Katinger, 2008)

Filamentous fungi (molds) – another eucaryotic microorganism that is also an attractive host for recombinant protein production due to its high-yielding secretion of proteins and the ability to carry out post-translational processing. Plasmids can deliver foreign genes into fungi and the genes incorporate into chromosome, giving long-term genetic stability. Bioproduction of human lactoferrin, monoclonal antibodies, recombinant enzymes, and humanized immunoglobulin full-length antibodies – are few of the processes carried out using fungi as host cells. Among widely used fungi are *Aspergillus niger, Trichoderma reesei, Aspergillus oryzae*, etc. (Chambergo & Valencia, 2016). In cases when more complex posttranslational modifications are required or for production of soluble proteins of mammalian origin, insect cells can be used as alternative for fungi host cells. (Demain & Vaishnav, 2009)

Typically, a bioprocess consists of several steps: feedstock pretreatment, fermentation or biocatalysis and downstream processing or purification of final product and separation from by-products. Aside from microorganism strain selection and genetic engineering, other factors play an important role in productivity, cost and product quality of bioprocesses, such as bioreactor design, thorough mixing for heat and mass transfer, media optimization, etc. Also fermentation processes need to be closely monitored in order to select and adjust parameters for maximum yield. Different parameters could be screened depending on the specific bioprocess and its application: glucose level, ammonia, lactate, acetone levels, biomass density, oil accumulation, ethanol level, etc. New screening methods for these parameters are being developed as the use of bioprocesses attracts more and more research attention. New types of bioreactors have been designed with integrated sensors in order to provide high-throughput screening for bioprocess optimization.(Yang, 2007) The search for methods that can optimize bioprocesses and improve cost-efficiency

continues.

In the middle of the 20th century, oil discovery led to a decrease in fermentation production of many chemicals and other products, replacing it with petroleum based production. The trend is reverting again nowadays, when the world is facing rapid depletion of fossil fuel resources simultaneously with rising energy demands and environmental pollution which prompts a search for alternatives both with regard to resources and technologies. Bioproducts and bioprocesses that use natural resources offer an environmentally benign solution to this problem, as well as to the problem of abundant agricultural and food processing waste. (Yang, 2007) Renewable resources have great potential in many industry sectors (food, pharmacy, energy, chemistry, material science). They also play important roles in our daily lives. Apart from sustainability in production, the applications of bioprocesses in energy supply and wastewater treatment are critical for controlling climate change and reducing environmental (water / soil / air) pollution (Figure 1.3). Consequently, the potential use of modified microorganisms (such as microalgae in fermentation), biocatalysts and new chemical, physical and biological methods to improve cost-efficiency of green (focused on agriculture) and white (gene-based bioprocesses) biotechnology (Bevan & Franssen, 2006) is gaining lots of research attention worldwide. One of the examples of renewable resources application in energy is the use of oleaginous plant and food crops for biofuel production.

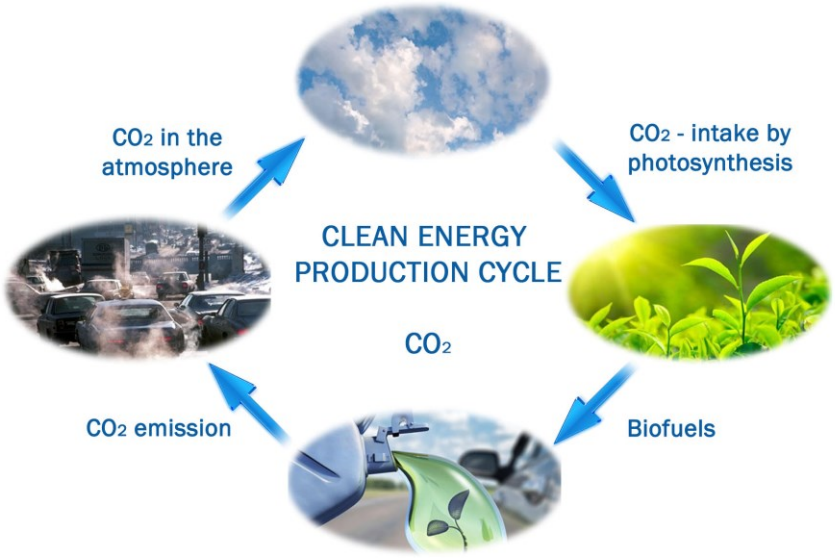

Figure 1.3. Clean energy production cycle through bioprocesses with CO_2 sequestration from atmosphere to be used further for biofuel production through photosynthesis.

In addition to biofuels, other valuable products can also be derived from biomass, such as carotenoids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)- omega-3 long-chain polyunsaturated fatty acids. Those products are important compounds of the human diet. For instance, *Schizochytrium sp.* is a microalgae that accumulates DHA or omega-3, a well-known additive for stroke prevention. A wide application of biological fermentation processes has become irreplaceable in biopharmaceutical, food, energy, wastewater treatment and chemical industries in the past decade. They are used for single cell protein production, for biochemicals, enzymatic transformation and organic compounds degradation, etc. (Schläfer et al., 2002) With such attention to the bioprocesses use, the search for cheap and efficient methods to increase the productivity in bioreactors becomes essential in order to

enhance the metabolic activity of the microorganisms and to improve the economics and manufacturing efficiency of biological processes. (Chisti, 2003)

1.3. Algae and its potential for bioprocesses and biotechnology

While the benefits of bioprocesses with fungi, yeast and bacteria have been widely reported for decades, microalgae are still a new microorganism platform, whose potential for biotechnology is yet to be fully explored. It is already obvious that microorganisms can offer many new production opportunities both through enriched biomass production and high added value biomolecules, but still many of the species out of the estimated 350000 pool (Bux, 2013) have not been fully characterized and all their versatile metabolic potential and commercial benefits are yet to be identified. (Bumbak, Cook, Zachleder, Hauser, & Kovar, 2011) The term «microalgae» typically describes a diverse group of unicellular (though colonial or filamentous also exist) procaryotic and eucaryotic microorganisms that are capable to utilize CO_2 and energy from the light in the process of photosynthesis. Algae are ubiquitous in distribution and can be found in brackish waters, in fresh water ponds and in the sea, in the soil, hot springs, etc. They also can be floating or suspended in their aquatic environment or attached to the bottom of the habitat. Three ways of algae reproduction are known: vegetative, asexual and sexual. Vegetative reproduction includes most known cell division or binary fission as well as fragmentation. Asexual reproduction takes place through various spores (e.g. zoospores in *Schitzochytrium* or alanospores in *Chlorella*) formation. Sexual reproduction is also present in most classes of algae and offers the opportunity for genetic material exchange (Bux, 2013).

The industrial use of algae is extremely diverse and economically useful in the

fields of agriculture, human consumption, pharmaceutical industry, for production of organic matter in aquatic habitats, for production of soda and potash, glue etc. Species that are known for commercial cultivation include: *Spirulina, Chlorella, Haematococcus, Dunaliella, Botryococcus, Phaeodactylum, Porphyridium, Chaetoceros, Crypthecodinium, Isochrysis, Nannochloris, Nitzschia, Schizochytrium, Tetraselmis, and Skeletonema*. (Raja, Hemaiswarya, Kumar, Sridhar, & Rengasamy, 2008) The agricultural industry utilizes soil algae and their ability to fix nitrogen, thus increasing soil fertility and crop yields. Another potential of algae – their usage as fertilizers - is successfully applied in many countries- on rice fields in China, for growing vegetables in France, Ireland, etc. In the food industry algae are widely used for fish and animal feed as well as in the human diet especially in countries like Japan. They can also be used as fodder for cattle to increase egg and milk yields, while also adding iodine and carotene to the content. In medicine it can be a source of antibiotics (Chlorellin from *Chlorella*). Also, antiviral and antibacterial properties of algae as well as high iodine content are being exploited. Algae-derived agar agar is widely used in the production of pills and creams and carrageenin has anti coagulative properties. Agar agar can also be used in food as well as in media for cultivation of many microorganisms. Algae are successfully applied for sewage treatment due to their ability to grow there, decompose organic matter from waste and convert it into valuable fertilizer. Algae grown on sewage can be further separated and used further. Algae are also known for their extensive use in biological research, e.g. in the investigation of photosynthesis and sexual reproduction.

In biotechnology, algae biomass is the main product. It is grown in photobioreactors (PBR) or in open ponds and further harvested and typically dried. Further it can be utilized in health or food industry, with up to 75% of algae biomass

annually going into the production of powders, tablets, capsules. The main species cultivated as biomass for food and health are *Spirulina* and *Chlorella*. (Raja et al., 2008)

Microalgae can also serve as important source for commercially valuable products: polyunsaturated fatty acid (PUFA) such as arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA); carotenoids such as β-carotene and astaxanthin and phycobiliproteins such as phycocyanin, that can be used as natural dye and in pharmaceuticals. (Spolaore, Joannis-Cassan, Duran, & Isambert, 2006)

Algae biotechnology as well as technology for algae cultivation are growing rapidly due to numerous advantages of microalgae such as the potential for cultivation on non-arable lands, the ability to use wastewater for nutrients, potential production of biofuel and high value products while reducing CO_2 emissions. (Rosenberg, Mathias, Korth, Betenbaugh, & Oyler, 2011) With the development of protocols of algal genetic engineering, yet new potential of microalgae in biotechnology emerges. Along with tools for genetic optimization, the search for new strains that can be grown under extreme conditions continues.

Important factors that require optimization for algal growth on a commercial scale include but are not limited to light intensity, temperature, nutrients and CO_2 availability, pH and salinity depenfing on the strain. (Bhola et al., 2011)

Recently scientific attention was mainly focused on the potential use of microalgae in biofuel production. Microalgae have the potential to become a new generation renewable and sustainable source of biofuels. Biodiesel, biomethane, biohydrogen, biobutanol, that meet main fuel requirements can be potentially derived

from microalgae with higher efficiency than oil crops.(Chisti 2007) However, such a production still needs to be optimized especially with regard to lipid extraction processes from algal biomass before it moves from lab scale to large scale production systems.

Main challenges in microalgal biotechnology that are yet to be overcome include improvements in strain selection that can be used for large scale cultivation, biochemical and genetic modification protocol development, optimization of growth and harvesting parameters as well as methods of oil and high value products extraction. (Bux, 2013) Future research should target at solving these problems in order to make algae technology economically sustainable and competitive.

1.4. Hypothesis and aims of the research:

Based on the literature reports and in studies performed by Dr.Chen's group, low intensity pulsed ultrasound (LIPUS with the frequency of 1.5MHz, 20% duty cycle, and intensity between 20mW/cm2 and 400mW/cm^2) has shown great potential as an additional approach to be applied in biotechnological production that can successfully improve cost-efficiency and increase yield.

The aim of this study was to determine the potential and limitations of the ultrasound stimulation using LIPUS in bioprocesses based on several types of microorganisms: green freshwater algae *Chlorella*, seawater *T.chuii*, protist *Schitzochytrium*.

Bioprocesses that use microorganisms and algae in particular attract a lot of research attention now and various approaches are being tested regarding their potential to increase production efficiency. LIPUS can be used along all of the other successful approaches as an additional physical tool of stimulation.

Accumulation of biomass by microalgae and lipid accumulation are two processes that are equally important and have potential for industrial application: biomass for feedstock, biomaterials, high value compounds production, water purification technologies etc, while lipid accumulation is important for biodiesel from microalgae or DHA/EPA long chain polyunsaturated fatty acids production. The biggest limitation here is that these two processes cannot be optimized at the same time. It was crucial to see whether it is possible to separate conditions for either oil increase or biomass increase. Thus, we aimed to study the effect of LIPUS stimulation on cells for higher lipid accumulation with potential use in bioenergy production. Also we wanted to see under which conditions LIPUS can be effective in stimulating higher biomass production, which has potential in many bioprocesses.

Microalgae are different from mammalian cells because of their thick cell wall. It has been reported that mammalian cells are sensitive to LIPUS, but we aimed to study which parameters needed adjustment to see LIPUS impact on the plant cells as well.

While studies on ultrasound application exist, very little is known about the mechanism of ultrasound effect on the cells. Thus, we also aimed to get a better understanding on what ultrasound does to the cells, what physiological changes can be seen and which processes are being effected. To study that, we chose the model microorganism yeast *S. cerevisiae* that has previously shown great response to ultrasound stimulation in another bioenergy related bioprocess- bioethanol production.(Shaheen et al., 2013) One of the hypotheses was that ultrasound stimulation creates microjets that stimulate changes on cell membrane surface (or on the cell wall), and thus nutrients /waste products circulate better. Another hypothesis was that ultrasound stimulates more active cell division. Finally, we wanted to see

whether the LIPUS effect can be observed not only by the increase in final products of bioprocesses, but also on the gene expression level as well as metabolomics pathways.

Chapter 2

Effect of low intensity pulsed ultrasound on lipid accumulation by algal cells (*Chlorella* and *T.chuii*)

"The fuel of the future is going to come from fruit like that sumach out by the road, or from apples, weeds, sawdust - almost anything. There is fuel in every bit of vegetable matter that can be fermented. There's enough alcohol in one year's yield of an acre of potatoes to drive the machinery necessary to cultivate the fields for a hundred years."

Henry Ford

(New York Times 1925)

2.1 Introduction

Oleaginous plants, often food crops, store large amounts of lipids in seeds and provide energy for plant growth during germination (Murphy, 2001). However, to produce biofuel on a commercial scale, a controversial "food vs. fuel" issue arises. The United States Department of Energy launched the Aquatic Species Program (ASP) in 1978 to develop renewable fuel for transportation. The program focused on producing biodiesel fuel from algae and reached the following conclusions regarding the impact of algae on resources:

a. Microalgae produce lipids with high efficiency – achieving yields up to 80% of the dry biomass using a photosynthetic mechanism for CO_2 sequestration. (Chisti 2007; Subashchandrabose et al. 2011)
b. Microalgae absorb greenhouse gases responsible for climate change (Lashof & Ahuja, 1990). They produce lipid oils at efficiencies 30 to 100 times higher than edible agricultural products, such as corn and soybeans – classified as first-generation biofuel sources (Demirbas & Fatih Demirbas, 2011).
c. Microalgae preserve arable soils (Gutzeit, Lorch, Weber, Engels, & Neis, 2005) and other natural resources (Talebi, Raut, Al-Qasimy, Al-Saadi, & Al-Balushi, 2012), and need only marginal lands for cultivation.

They are easily adapted to grow faster even under extreme environments. Pacific Northwest National Laboratory recently reported that crude algae oil was converted into aviation fuel, gasoline or diesel fuel(Hoffmann, 1998). Various studies conclude that microalgae hold great potential as the next generation sustainable source of renewable energy for the next 10 to 15 years (Abdel-Raouf, Al-Homaidan, & Ibraheem, 2012; Elliott, Biller, Ross, Schmidt, & Jones, 2015; Pittman, Dean, & Osundeko, 2011)

Simultaneously with CO_2 sequestration and algal oil generation, microalgae can also be used in wastewater treatment (Hoffmann, 1998). Wastewater contains organic carbon, nitrogen and phosphate, which support the growth of many species of microalgae in heterotrophic or mixotrophic growth conditions. Microalgae can use wastewater as its growth medium and the production of algal oils is biodegradable and non-toxic. Algal consumption of organic carbon, nitrogen and phosphate salts decreases the biological oxygen demand (BOD) in wastewater and thus makes biodiesel fuel production from algae economically feasible (Gutzeit et al., 2005; Pittman et al., 2011). Pacific Northwest National Laboratory recently reported that crude algae oil was converted into aviation fuel, gasoline or diesel fuel (Elliott et al., 2015). Various studies conclude that microalgae hold great potential as the next generation sustainable source of renewable energy for the next 10 to 15 years (Mata, Martins, & Caetano, 2010; Tang et al., 2011; Wijffels et al., 2010) with appropriate fuel grade algae growth. Recent drop in oil prices poses a short-term challenge for biofuel production, by rendering the production price of biodiesel more expensive than oil price on the market. While this trend may be temporary, biofuel production will still need federal support to make sure the industry survives at times when oil prices drop significantly. According to the implementation and finalization of the "Clean Power Plan" drafted by United States Environmental Protection Agency in August 2015, support for renewable energy becomes a global strategy in order to cut down green gas emissions.

Microalgae can grow in phototrophic, heterotrophic, or mixotrophic conditions(Subashchandrabose, Ramakrishnan, Megharaj, Venkateswarlu, & Naidu, 2013). In phototrophic growth conditions, cells capture carbon, arising from

greenhouse gases (CO_2). Microalgae efficiently convert CO_2 into algal oil, a precursor to biodiesel fuel. One hundred tons of algae can capture 180 tons of CO_2 by photosynthesis(*Accelerating the uptake of CCS: industrial use of captured carbon dioxide | Global Carbon Capture and Storage Institute*, 2011). Phototrophic growth is an elegant conversion pathway, but it is not economically feasible for commercialization (Huang, Chen, Wei, Zhang, & Chen, 2010). Industries generally favor heterotrophic growth conditions due to the faster production of cell biomass and oil content. For instance, the heterotrophic growth of *Chlorella protothecoides* can accumulate lipid concentrations as high as 55% of the cell dry mass after 144 hours of cultivation with a feedstock of corn powder hydrolysate (H. Xu, Miao, & Wu, 2006). Mixotrophic growth conditions have been reported to be even better than heterotrophic growth alone in terms of biomass productivity as well as algal oil content (Subashchandrabose et al., 2013).

Microalgae are not naturally adapted to biofuel production. Its function involves complex regulatory networks and mutual interaction of physiological processes and organelles. To identify the optimal strategy to increase algal oil production, a systems biology approach, in particular *in silico* model, was used to identify genetic and physiological modification (Rupprecht, 2009). Molecular toolkits were also used to prepare various algal strains most suitable for lipid production (Georgianna & Mayfield, 2012). In addition, methods to improve light absorption and photo-synthetic efficiency and to improve carbon fixation were reported by (Ort, Melis, & Melis, 2011),(Mussgnug et al., 2007) and (Ducat & Silver, 2012) respectively. Because microalgae involve complex lipid metabolism, different metabolic

engineering approaches were proposed to improve lipid production (Griffiths, van Hille, & Harrison, 2012; Yu et al., 2011).

Microalgae do not directly produce biofuel, but they produce lipids, such as triacyl-, diacyl- and monoacyl-glycerols, which can be transformed in methanol esters to be used as biofuel. Despite improvements in optimizing microalgal growth, the price of biofuel is approximately 1.5 to 2 times higher than that of conventional fossil fuel (Huang et al., 2010). The search for growth conditions (nutrition, temperature, oxygen level) that maximize algal biomass productivity and oil storage continues (Demirbas, 2010). Stress responses of lipid metabolism in the oleaginous microalgae have drawn increasing research attention. Increasing lipid production in the context of stress exposure, such as N-availability, temperature, high irradiance and salinity was also reported (Solovchenko et al., 2014). The growth of *Nannochloropsis* in nitrogen-replete media under a high light condition triggered a rapid acclimation of the microalgae to the high light stress in a salinity-dependent manner associated with a moderate decrease in eicosapentaenoic acid proportion of total fatty acid. The influence of microwave radiation on physiological changes (higher growth rates and biomass) was reported by (Asadi, Khavari-Nejad, Soltani, Najafi, & Molaie-Rad, 2011).

In the research presented in this chapter, I studied the effect of mechanical ultrasound wave instead of microwave, specifically pulsed wave, to improve algal lipid production efficiency. Two commonly used fresh water and seawater microalgae, *Chlorella vulgaris* and *Tetraselmis chuii*, were chosen.

2.2 Materials and Methods

Chemicals: Mowiol® 10-98, polyvinyl alcohol (PVA) with a molecular weight (M_w) of 61000, 99% linolenic acid, and Nile Red dye were purchased from Sigma-Aldrich (St. Louis, MO). BODIPY 505/515 fluorescent molecular probe was purchased from Invitrogen (Carlsbad, CA).

2.2.1 Algae preparation: *C. vulgaris*, fresh water green algae, purchased from ATCC, was grown in a modified 3N bold medium. The standard recipe of 3N bold medium was used for algal growth medium (http://www.ccap.ac.uk/media/documents/3N_BBM_V.pdf). However, the amount of nitrogen ($NaNO_3$) was decreased 3-fold (from 75g/L of $NaNO_3$ in stock solution to 25 g/L). *T. chuii*, a seawater green algae, purchased from ATCC, was grown in F/2 medium (https://ncma.bigelow.org/media/pdf/NCMA-algal-medium-f_2.pdf), prepared using concentrated F/2 medium from the kit (Bigelow Laboratory for Ocean Sciences) and artificial seawater (Tap water containing sea salt mixture from "Instant Ocean" at 35g/ L. "Instant Ocean" refers to a commonly used brand of marine salt). Filter sterilization was used to avoid precipitation. The total growth cycle was 8 days for *Chlorella* and 11 days for *T.chuii*.

2.2.2 Culture conditions: The algal strains were grown mixotrophically in a shake flask at 27°C with a shaking speed of 150 rpm. 1L of filter-sterilized medium was inoculated with exponentially growing seed culture at a concentration of 10% of the total medium. Spectrophotometry at the optical wavelength of 550nm (OD_{550}) was used, revealing that the OD measurements of the initial solution were between 0.09 and 0.1. The initial solution was divided between flasks for ultrasound treatments

under different conditions. The culture in the shake-flasks was exposed to light flux - 60 μmol photons/m^2/sec for 16-hours (light on), and 8-hours of lights off (darkness). Algae were grown for a total of 8-11 days. 15 mL of glucose (40% solution) was added to the culture (100 mL) on the 5^{th} day of the growth cycle so that the final concentration was 6% glucose in the flask. Daily measurements of OD_{550} were performed to check the cell growth. Lipid accumulation was also tested daily using the developed technique with fluorescent lipid-staining probe and flow cytometry. At the end of cultivation, the culture was harvested with centrifugation at 5000 rpm and freeze-dried for further lipid extraction.

2.2.3. Pulsed-wave generating device: Current research on increasing algal oil production primarily focuses on algae strain selection, genetically modifying algae, as well as the optimization of medium and growth conditions (Radakovits, Jinkerson, Darzins, & Posewitz, 2010)(Hannon, Gimpel, Tran, Rasala, & Mayfield, 2010). Unlike the previously mentioned biochemical approaches, we have developed a physical stimulation method, or a pulsed wave known as low-intensity pulsed ultrasound (LIPUS) with frequency of 1.5MHz, duty cycle of 20%, and intensity within 20mW/cm^2 - 400mW/cm^2 to increase lipid accumulation in algae. The system-level design, the board-level design and the set-up for ultrasound calibration are shown in Figures 2 (a), 2(b) and 2(c), respectively.

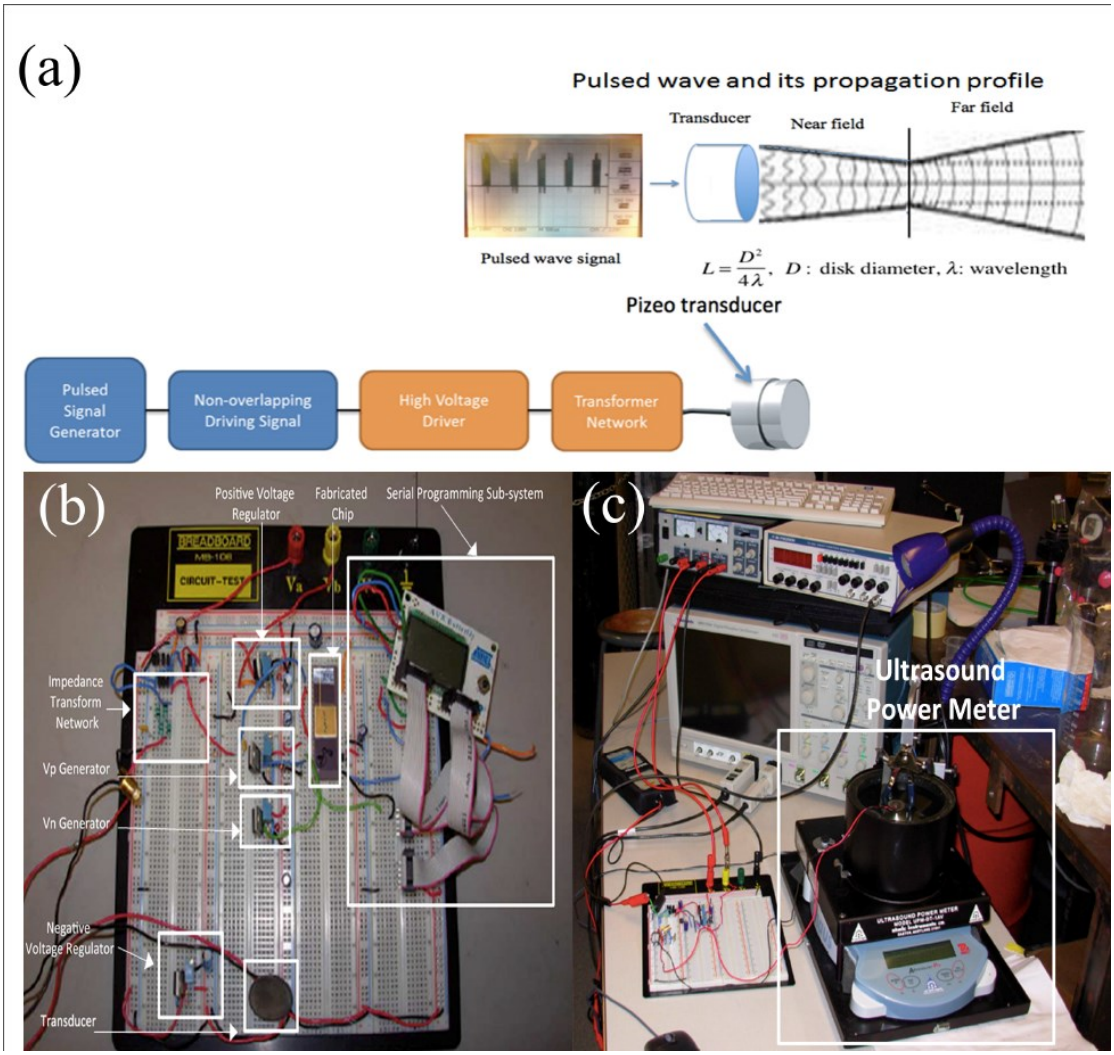

Figure 2.1: **(a)** LIPUS design consists of various modules: such as pulsed signal generation, signal amplification and impedance matching for driving piezoelectric transducers, **(b)** the detailed LIPUS design was mapped on a breadboard with individual components marked, **(c)** the ultrasound power meter was used to calibrate the output intensities of pulsed wave.

2.2.4 Experimental Set-up: The experimental set-up of the used pulsed wave stimulation is as follows. A pulsed wave device, more specifically a low-intensity pulsed ultrasound (LIPUS) device with 1.5MHz and 20% duty cycle, was designed to generate the required pulsed wave for the experiments. The profile of the pulsed wave is shown in Figure 2a. The waves were applied to the culture by placing the

culture flask in a customized water-bath applicator, which was designed specifically for individual flasks (refer to Figure 2.2(a)). In the water bath applicator, two ultrasound transducers were mounted underneath because one transducer cannot cover the bottom area of the flask. Ultrasound can propagate efficiently through aqueous solutions, but not through air. Therefore, the flask was subsequently placed in the water bath applicator (step 2 in Figure 2.2 (a)), where water serves as a propagating medium. In this case, ultrasound can efficiently penetrate without attenuation in the Y-axis (refer to Figure 2.2(b)). In addition, the flask was shaken at 150rpm during cell culturing. Statistically, cells receive equal sonication due to the constant shaking. Overall, ultrasound waves penetrate uniformly through the bottom of the flask to reach the cell culture.

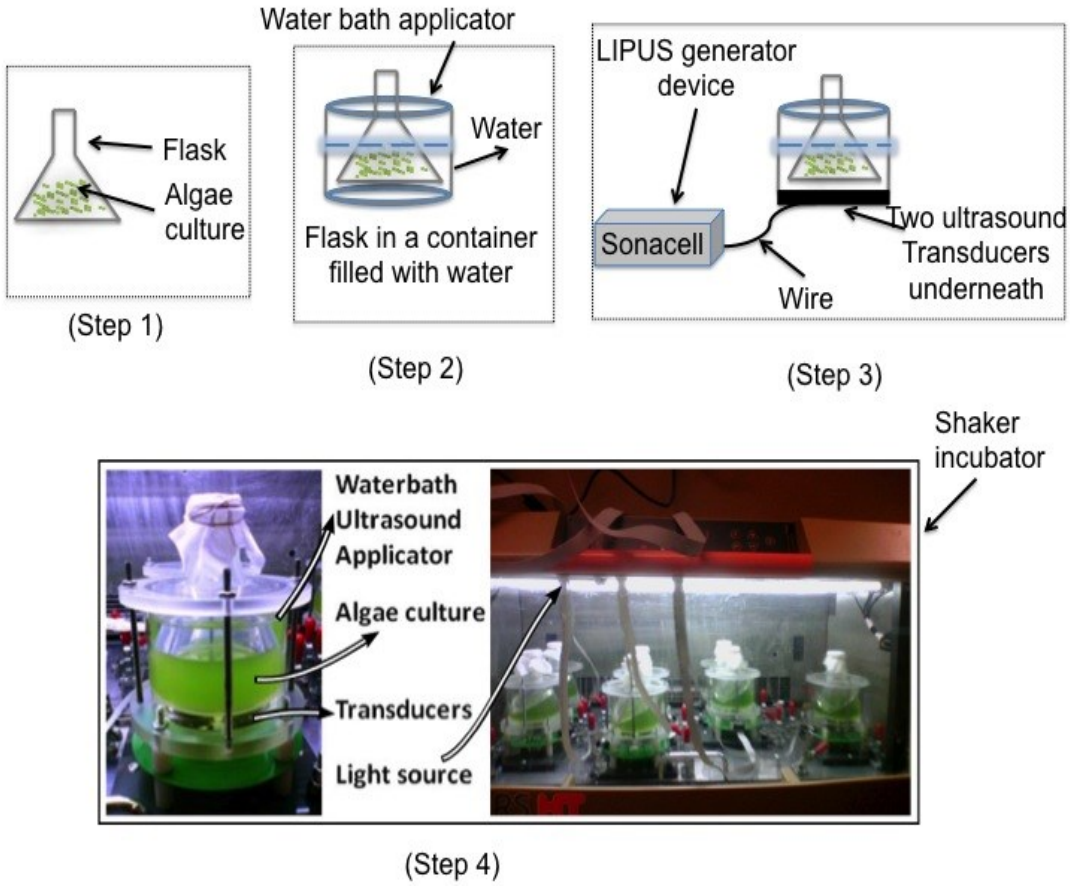

Figure 2.2.a : Experimental setup for algae cultivation in a shaker incubator with proper lighting.

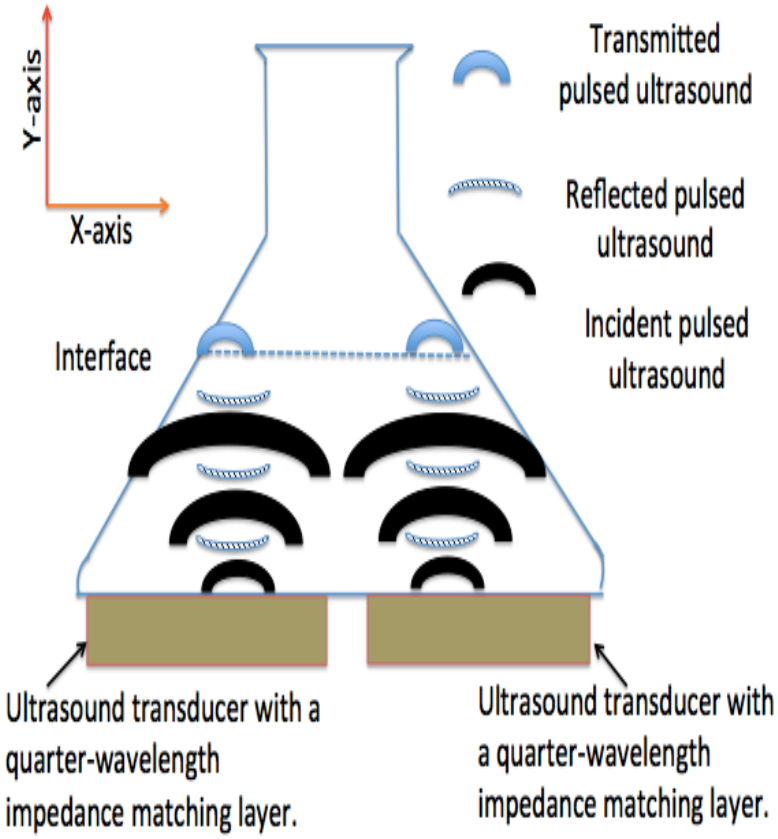

Figure 2.2.b: Schematic image of shake flask in water bath (water serves as medium for ultrasound)

These applicators together with flasks were placed into the shaker incubator (step 4 in Figure 2.2(a)). The incubator was equipped with a controlled light source (16-hour light on and 8-hour light off per day) and special holders on the floor of the shaker incubator to secure the applicators in place during shaking. The shaker was set at 150 rpm, with temperature controlled at 27^{o}C during the whole cell growth cycle. Different samples received different intensities of ultrasound stimulation (e.g. control without stimulation, 60 mW/cm^2, 80 mW/cm^2 and 100 mW/cm^2) during the cell growth cycle. The intensity used in the experiment refers to the intensity of spatial peak temporal average, or ISPTA= 40, 60 and 80mW/cm^2 (or ultrasound pressure

amplitude of 268.76, 403.15, and 537.53mPa) applied to cell stimulation. They were individually calibrated using an ultrasound power meter (Ohmic Instruments Company, Maryland, USA).

2.2.5 Algal cell lipid staining: Nile Red dye (9-diethylamino-5Hbenzo [α] phenoxazine-5-one) was prepared as a stock solution at a concentration of 0.25 mg/mL in acetone. Stock solutions of the dye were kept in the dark at -20^{o}C. Staining of the *C. vulgaris* cells was performed using a technique (Doan & Obbard, 2011), where glycerol solution served as a carrier for the dye. *Chlorella vulgaris* cell culture was diluted 10 times using deionized (DI) water. 3 mL of algae suspension was mixed with 0.8 mL of the glycerol solution (0.5g/mL) so that the final glycerol concentration was 0.1g/mL. 20 μL of Nile Red in acetone was added to the samples, which were subsequently vortexed for 1 minute and left in the dark for 10 minutes. To improve staining efficiency, samples of 1 mL were then sonicated for 30 minutes and heated to 60^{o}C in a water bath for 30 minutes. *C. vulgaris* cells have a thick cell wall rendering it very difficult to stain the cells. Because only 1 mL of sample was used, higher staining temperature could be applied and the sample was then disposed. After numerous trials, we discovered that 60^{o}C plus sonication could achieve a staining rate of 97 to 100% while keeping cells intact. Unbound dye was removed by centrifugal washing for 5 minutes at 1,500 rpm. This washing procedure was performed three times. For BODIPY staining 3 mL of cell suspension was mixed with 20 μL of 1 mM BODIPY in dimethyl sulfoxide (DMSO) solution, vortexed for 1 minute, and then sonicated for 35 minutes at 60^{o}C.

T. chuii samples were tested without dilution because their concentration was much lower than that of *C. vulgaris* samples (around 1×10^6 cells/mL). Centrifugal washing was not applied because the change in osmotic pressure would cause cell rapture. Nile red staining was performed in the same way as for *C. vulgaris*, though without sonication because *T. chuii* has thin cell wall allowing dye to easily penetrate into the cell body. The sample was heated to 40°C for 1 hour. For the BODIPY staining, 3 mL of cell suspension was mixed with 20 μL of 1 mM BODIPY in DMSO solution, vortexed for 1 minute, and then kept in the dark for 10 minutes.

2.2.6 Emulsion preparation and particle staining: Linolenic acid was used as the oil phase in an oil/water emulsion. 5mL of 6% w/v solution PVA was used as the water phase. PVA also served as a surfactant. A fixed amount (20 μL) of linolenic acid was used in the flow cytometry experiment. The mixtures were emulsified using a high-speed homogenizer (Fisher Scientific PowerGen, Model 125) for 40 minutes at 5 different speeds in the range 20,000 – 30,000 rpm for different sizes (within 2.8-3.7μm range) of the oil droplets. Particle size was estimated by the method of Dynamic Light Scattering (DLS, Malvern, Zetasizer Nano S). Stability of the obtained emulsions was tested using the spectrophotometer. Particles in the emulsion were stained using both NR and BODIPY in the same way as algae cells: 20 μL of dye solution (NR in acetone or BODIPY in DMSO) was added to 3 mL of emulsion, followed by 1 minute of vortexing, and kept for 10 min in the dark.

2.2.7 Measurements of fluorescence: Fluorescence measurements for both lipid particles in the emulsion and lipids inside the cells were performed using a flow cytometry apparatus with an argon ion laser (BD FACS Calibur, Flow cytometer,

Becton Dickinson). The FL2 (orange-red) channel was used for Nile Red fluorescence intensity measurements with excitation at 488 nm. Green fluorescence of the BODIPY was detected using the FL1 (green) channel with an excitation wavelength of 503 nm.

2.2.8 Gravimetric lipid extraction: Algae cells from the culture were collected into tubes, centrifuged and freeze-dried using a vacuum system for 24 hours. Dry biomass of each sample was mixed with a solvent composed of Chloroform:Methanol = 2:1 (S. J. Lee, Yoon, & Oh, 1998). 5 mL of solvent mixture (containing 200 mg of the dried algal sample) was sonicated for 10 minutes. Sonicated samples were centrifuged and solvents with extracted lipids were then collected into a pre-weighed dry vial. Such an extraction was repeated three times. The solvents from the vial were removed using an evaporator. Vials with dry lipids were weighed again to check the mass of the lipids.

2.2.9 Fatty acid composition analysis: To validate the fatty acid composition, GC/MS were used according to the protocol listed as follows:

(a) Preparation of FAMEs: Fatty acid methyl esters (FAMES) were prepared by 2% H_2SO_4 methanol method. 2% H_2SO_4 in methanol was prepared by mixing 2 mL of H_2SO_4 (Certified ACS plus, Fisher Scientific) with 100 mL methanol (Chroma Solv, Sigma) (Christie, 2003). A known amount of extracted total lipids was spiked with 20 µL of 1 mg/mL heptadecanoic acid (17:0, used as internal standard). The mixture was evaporated under nitrogen gas and to this 1 mL of 2% H_2SO_4 was added. The mixture was incubated at 86 °C for 1h,

cooled on ice for 5 min and subsequently neutralized by 0.5 mL 0.5% sodium chloride solution. FAMEs were extracted by the addition of 2×2 mL aliquots of hexane and then vortexed. The two layers were allowed to separate and the upper hexane layer was collected, and subjected to gas chromatographic analysis for identification and quantification of fatty acids.

(b) Gas chromatographic analysis of FAMEs: Analysis of FAMEs was performed on the Agilent 6890N gas chromatography instrument coupled with an Agilent MS-5975 inert XL mass selective detector (Agilent technologies) in Electron Impact (EI) mode. Separation of fatty acids was achieved by injecting 2 µL of the FAMEs onto an 88% - Cyanopropyl aryl-polysiloxane column, HP88 (Agilent J & W Scientific, 30×0.25 mm×0.25 µm). Splitless injection was performed with a constant carrier gas (helium) flow of 1 mL/min. Inlet temperature and transfer line temperatures were set at 200°C and 180°C respectively. Temperature programming was as follows: initial isotherm of 80°C held for 1 min, raised to 90°C at 1°C / min, 90-250°C at a rate of 6.1°C / min, and held at the final temperature for 15 min. The MS ion source temperature was 230°C and the Quadrupole temperature was 150°C. Peak identification of fatty acids in the analyzed samples was carried out by comparison of chromatogram with mass spectral library (NIST) and against the retention times and mass spectra of Supelco 37 component FAME mix (Sigma-Aldrich, St Louis, MO, USA). The final result of different fatty acid components was calculated and expressed on a molar percentage basis according to the official methods of the Association of Analytical Chemists and the American Oil Chemists' Society.

2.2.10 Statistical analysis: Experimental values were determined in three independent experiments. Each experiment has been conducted in duplicates. All values regarding measurement and percentage of lipid content were expressed as standard deviation (SD). Differences less than 0.05 ($p<0.05$) were considered statistically significant.

2.3 Results and discussion

2.3.1 Pulsed Wave for Enhancing Algae Lipid Production in *Chlorella vulgaris* and *T. chuii*

To study the effect of LIPUS, *C. vulgaris* was chosen and mixotrophic growth conditions were used because it is the most commonly adopted growth condition in industry (Mata et al., 2010). More specifically, photoautotrophic conditions were employed during the first five days of algae growth, and glucose as an organic substrate was then added on day five. Spectrophotometry with the OD_{550} as well as staining, coupled with flow cytometry, were used for measuring changes (cell growth and lipid accumulation) during the course of each experiment. LIPUS stimulation at the intensity levels of 80 mW/cm^2 and 100 mW/cm^2, for 15 minute per treatment every three hours has proven to be more efficient than the control (without ultrasound) in increasing lipid production of algal cells (refer to Figure 2.3c). The experiment was repeated three times and each experiment has been conducted in duplicates to verify the reproducibility of results.

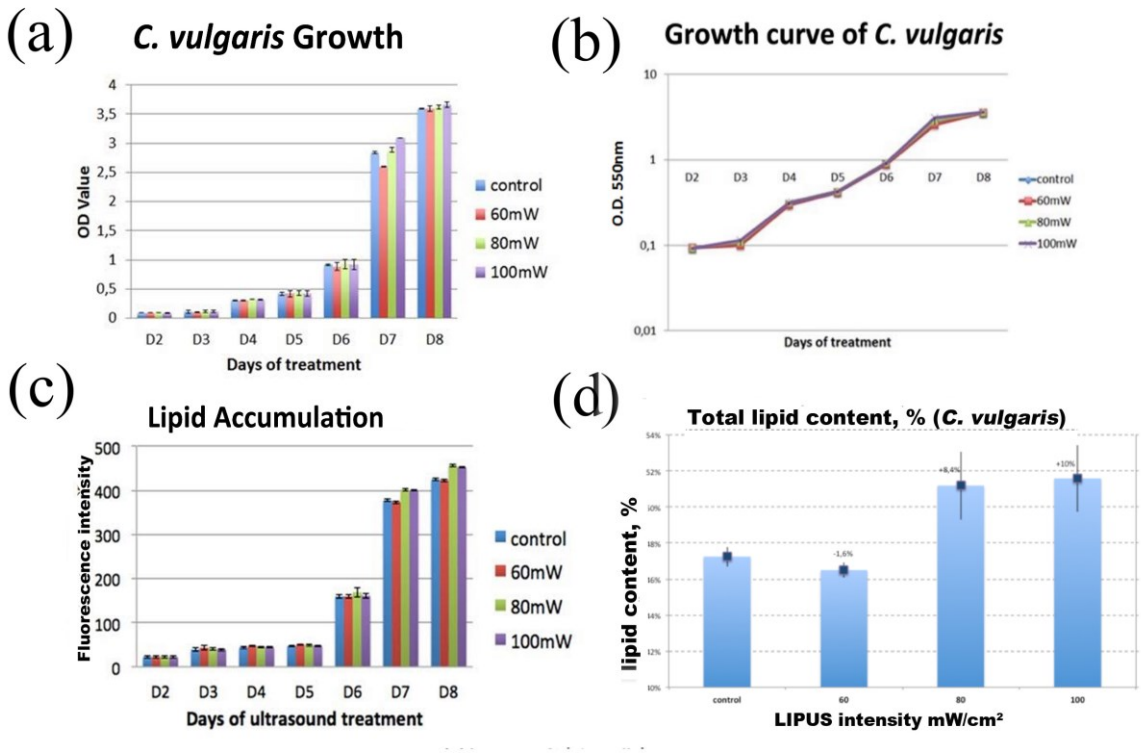

Figure 2.3: (**a**) **(b)** Growth curve for *Chlorella vulgaris* in the LIPUS stimulation experiment based on OD_{550} measurements. Since cellular growth is exponential, growth curves are presented as a semi-logarithmic plot. (**c**) Lipid accumulation curve for *Chlorella vulgaris* based on fluorescence intensity measurements using flow cytometry. Note that the mean and standard deviation were obtained in 6 replicates (n=6) (We have performed 3 independent experiments. Each experiment has been conducted in duplicates). A significant difference ($p<0.05$) was observed on Day 7 and Day 8, when comparing samples with and without ultrasound treatments. **(d)** Results for total lipid content per biomass in *Chlorella* cells with and without pulsed-wave stimulation in an 8-day cultivation cycle obtained using the extraction method.

Although no significant improvement in cell growth was observed Figure 2.3 (a,b), LIPUS stimulation yielded stable and repeatable increases in lipid production, up to 8.4% for 80 mW/cm^2 and up to 10.0% for 100 mW/cm^2 for *C. vulgaris* (refer to Figure 2.3d) because all added energies were stored as lipid content rather than contributing to cell growth. However, samples stimulated with 60 mW/cm^2 intensity

did not show significant lipid increase compared to the control. The results indicate that the intensity greater than 80 mW/cm^2 is required for the increase.

In addition to *C. vulgaris*, we have also tried to treat the seawater algae *T. chuii* using LIPUS. To measure the growth of *T. chuii*, 3 mL of culture was extracted every three days, and the OD at 550nm was determined. The optimal set of ultrasound parameters was found to be 80mW/cm^2, 5 minutes and 12 times a day. Because *T. chuii* has thinner cell walls than *C. vulgaris,* the ultrasound stimulations given were milder, or 80mW/cm^2, 5 minutes 12 times a day instead of 100mW/cm^2, 15 minutes 8 times a day. At the end of cultivation, the culture was harvested by centrifuging at 5000 rpm×g then freeze-dried for 24 hours. The dried samples were weighed to measure their biomass. Unlike *Chlorella, T. chuii* responded to LIPUS treatment not only in lipid increase, but also in higher biomass. The improvement in growth under this condition was 17% in dry biomass or, based on OD at 550nm on Day 12, – 14% increase. (Figure 2.4 (a,b)) Total lipid was extracted using the chloroform–methanol (2:1 ratio) extraction method previously reported (Folch, Lees, & Sloane-Stanley, 1957). The increase of total lipid content normalized by biomass was about 24% with this condition (refer to Figures 2.4(c) and 2.4(d)). We also used flow cytometry measurements to validate the results (refer to Figure 2.8 Conditions of 80mW/cm^2, 5 minutes and 12 times a day were found to induce the highest increase in lipid content.

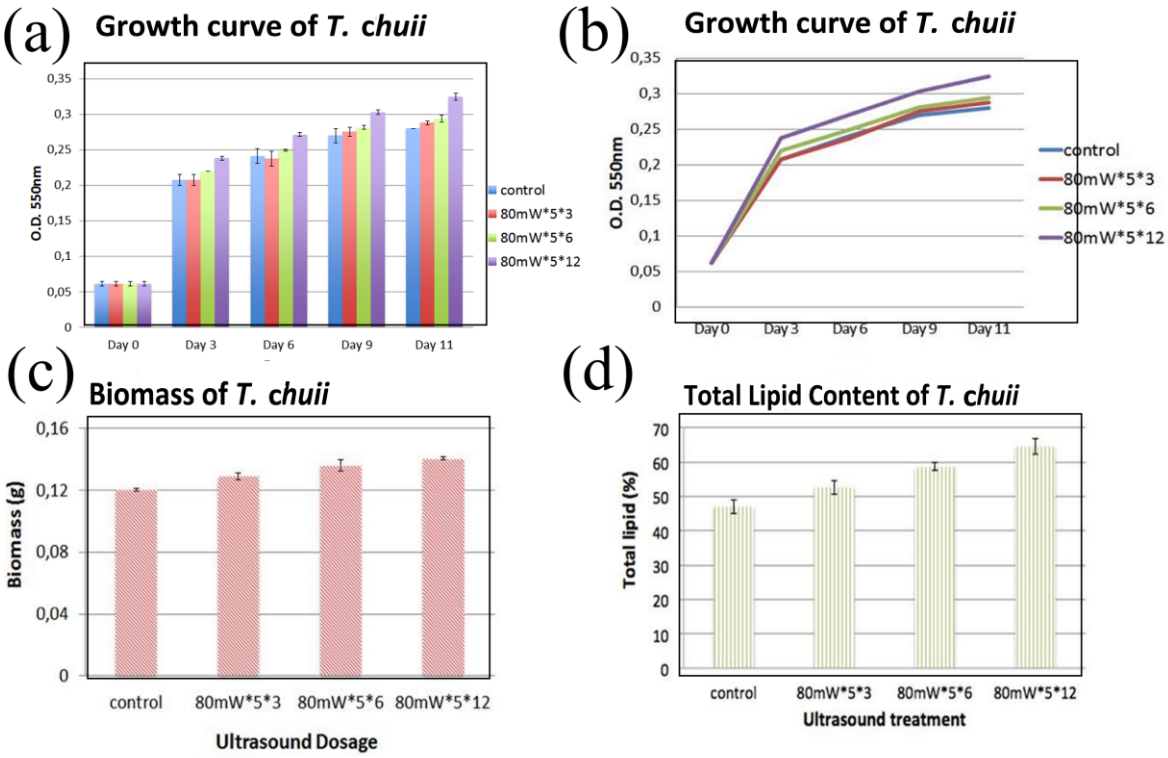

Figure 2.4: Effect of different LIPUS treatments on *T. chuii*. **(a) (b)** Growth curves of *T. chuii* in F/2 medium. *T. chuii* was grown under the conditions described in the materials and methods section. Since cellular growth is exponential, growth curves are presented as semi-logarithmic plots. **(c) (d)** Total cell biomass and lipid content per dry biomass (%) are shown for different ultrasound conditions. Note that the mean and standard deviation were obtained in 6 replicates (n=6) (We have performed 3 independent experiments. Each experiment was conducted in duplicates). Significant differences ($p<0.05$) were observed both for biomass and total lipid when comparing samples with and without ultrasound treatments.

2.3.2 Accurate and Fast Daily Screening of Algae Lipid Content

Lipid accumulation by algae cells is not a rapid process and the size of the lipids in algae cells changes gradually increasing within the 1µm range during growth cycle. This is why a method for real-time screening lipid increase needs to be accurate and sensitive (detecting even tiny fluctuations in lipid concentrations). The traditional *Gravimetric* method cannot be chosen because it takes more than 24 hours of sample

preparation and a large sample volume for accuracy. Consequently, an accurate and fast daily screening method is needed.

Daily optical density (OD) measurements can be used to monitor biomass increase, but a technique that can monitor lipid accumulation in algae cells on a daily basis has yet to be developed. Such an evaluation is very useful in finding the optimal and most cost-efficient culture condition by using a very small amount of culture sample (< 1mL). Although lipid content measurements based on the conventional *Gravimetric* extraction to determine lipid content have been widely used (Wahlen, Willis, & Seefeldt, 2011), such a method requires 24 hours of sample preparation, and therefore unsuitable for real-time monitoring. Lipid staining with fluorescent dyes has previously been reported (Greenspan, Mayer, & Fowler, 1985) as a method widely used to screen microalgae species in both fresh water and brackish water for their potential to accumulate lipids. For instance, fluorescent lipid probes, like Nile Red, coupled with the flow cytometry technique have been successfully used to stain cell lipids in order to qualitatively select the most lipid productive cells (Cooksey, K. E., Guckert, J. B., Williams, S. A., & Callis, 1987; Greenspan & Fowler, 1985) Because these cells were used for culturing after selection, cell viability was important after staining. Studies to improve staining efficiency using Nile Red dye without damaging cells have been published recently (Doan & Obbard, 2011). The research shows that the use of carriers can achieve better dye penetration into algae cells while keeping them alive. Cell viability, however, is not important for lipid accumulation measurements. Accuracy, repeatability and easy staining protocol as well as small sample culture volume are more important criteria. Although a new dye BODIPY has recently been suggested as an alternative lipid staining method for algal cells (Rumin

et al., 2015), its full potential for lipid quantification has not been fully explored. We used both Nile red and BODIPY dyes for lipid staining in order to select an appropriate dye that meets the needs of real-time lipid content measurements while yielding more reliable results.

To see which dye gives better accuracy with algae, we would have to do the FACS measurement in the stained cells every day and gravimetric extraction every day in order to see the correlation of the results between these two methods. This was not possible as the whole culture volume was small. That is why we applied the lipid droplets in oil/water emulsion as the model system to see which dye can be used more accurately. While the size of lipid droplets varies much in the cells, in the emulsion we can create a system with lipid particles of known size and see how fluorescence reflects the increase of lipid content. Particles were made using linolenic acid, one of the natural lipids accumulated in algae (e.g. *C. vulgaris*) cells (Khasanova, Gusakova, & Taubaev, 1978). To allow for easy staining and also avoid interference from confounding factors, such as how different staining protocols can affect the accuracy of results, only a thin PVA shell was used to cover lipid particles, allowing for the fast and simple staining procedure.

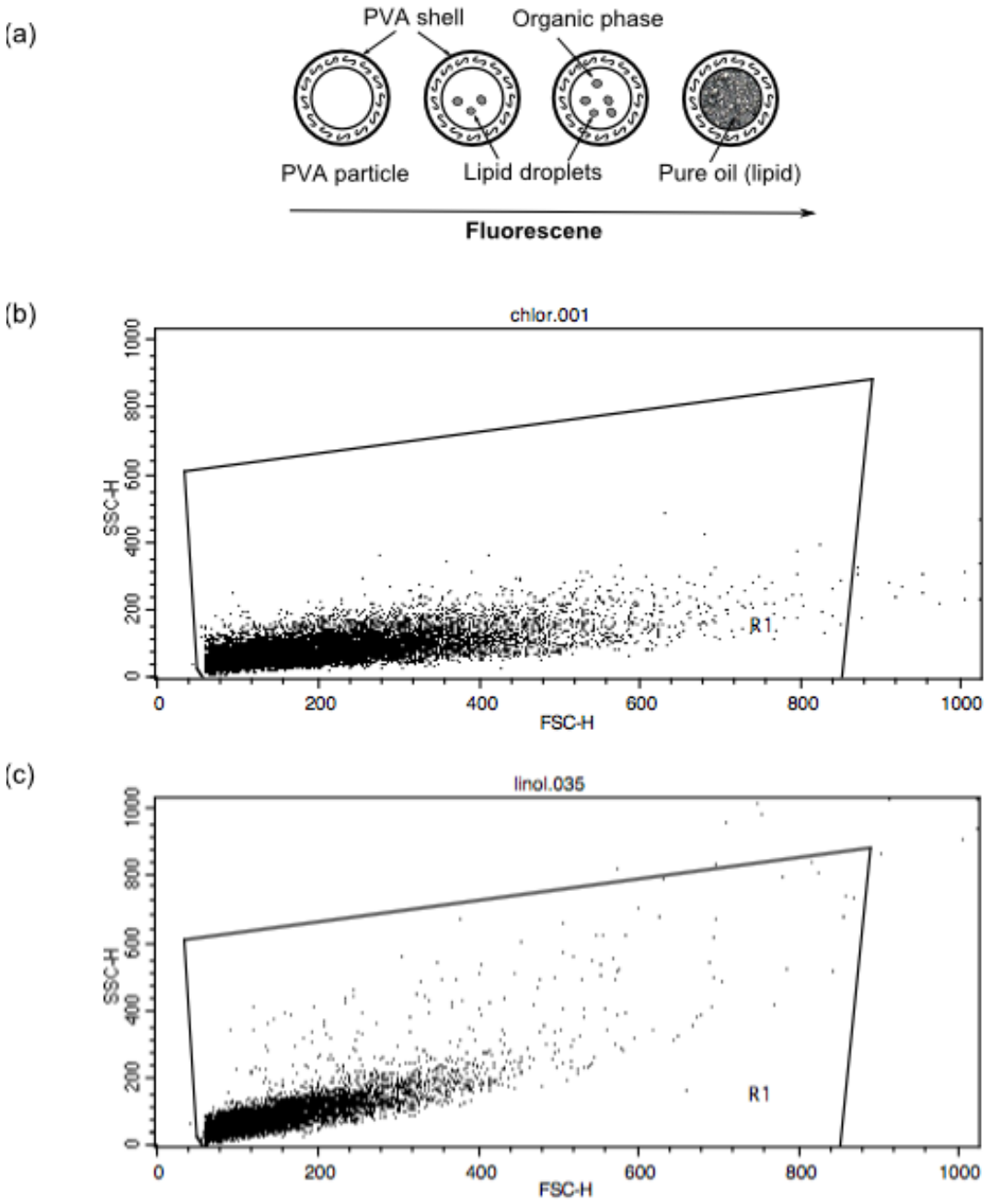

Figure 2.5 **(a)** shows a schematic diagram of the oil particle structure used as a model system for dye selection, where the oil content in the core of the particles (inside the PVA shell) increases from left to right. **(b,c)** FACS histogram plots of stained algae cells (b) and stained oil particles (c) showing that particles can be used as model system for this experiment as plots are similar; except that particle size distribution is more narrow.

We followed the algae staining protocol, but, unlike for algae, we did not need to add any carrier or additional methods like sonication or heating for the dye penetration into the lipid core. At the same time changing the speed mode of homogenizer, used for emulsification, we could change the size of the lipid droplets within one micron range like it happens in the algae cells when lipid content increases during the growth cycle and, thus, evaluate sensitivity of the method. Several emulsions with particles of different sizes within a range of 2.85 μm to 3.85 μm were prepared (the higher the speed, the smaller the size of the droplets in the emulsion). Emulsions obtained in such a way yielded a narrow size distribution of lipid particles as confirmed using DLS. Similar to how flow cytometry is used to measure fluorescence per algal cell, flow cytometry can also be used to measure fluorescence intensity per lipid particle after emulsification. The flow cytometry machine counts the number of particles and measures their fluorescence. Flow cytometry histogram plots of stained algae cell and stained oil particles are shown in Figures 2.5(b) and 2.5(c), respectively. Along the Y-axis (HSSC-H) is the side-scattered light, which is proportional to the internal complexity. Along the X-axis (FSC) is forward scattered light, which is proportional to the size or the surface of cells or particles. These two histogram plots look very similar except that particle size distribution is narrower than that of algae cells.

The experimental results showed that larger sized particles correlated to higher lipid content, indicated by higher fluorescence intensity after staining. These results were consistent with our expectation. The size of oil droplets in our emulsions was close to the size of lipid droplets in the algae cells. This can be seen on the confocal images of both stained particles and stained cells using either BODIPY or Nile Red dyes (shown in Figures 2.6(a) to 2.6(d)).

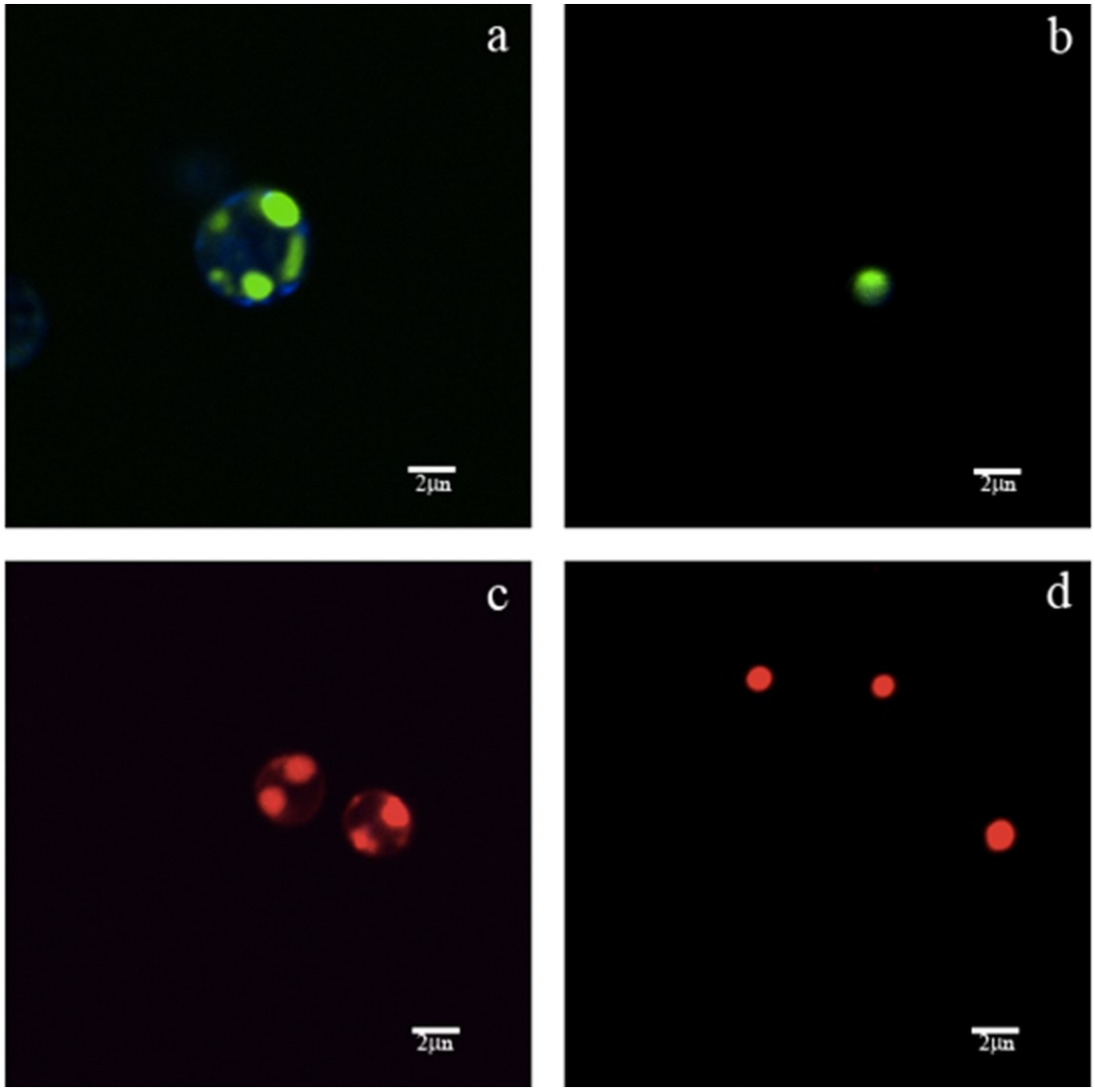

Figure 2.6: Confocal laser scanning microscopy images: (**a,c**) *C. vulgaris* cells with lipids stained by **a** – BODIPY staining, **c** –Nile Red staining. (**b,d**) Oil particles from o/w emulsion alone with lipids stained by **b** – BODIPY staining, **d** – Nile Red staining.

The size of oil particles was similar with the size of the lipids in the algae *C. vulgaris* cells at the end of their growth cycle. 10,000 particles threshold was used in flow cytometry measurement to reduce statistical deviation. Therefore, our model system with emulsions could be used for selecting the more accurate dye for lipid content measurement (BODIPY vs. Nile Red). When the emulsions were used for calibration,

repeatable and stable results were obtained by using BODIPY dye, while the Nile Red dye produced deviations too great to be used (refer to Figure 2.7).

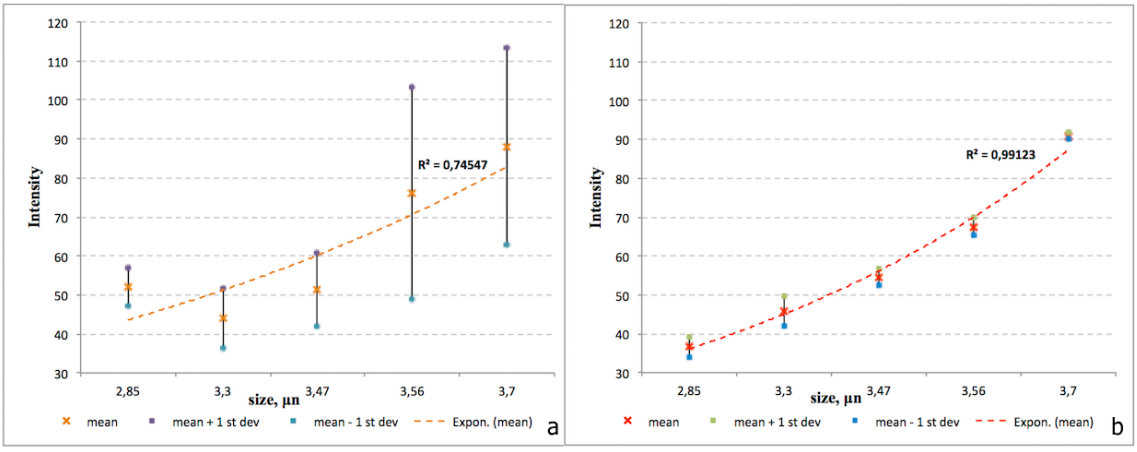

Figure 2.7. Fluorescence intensity obtained for different sizes of oil particles stained with **(a)-** Nile Red dye, **(b)-** BODIPY dyes.

To track daily lipid accumulation, flow cytometry measurements coupled with fluorescent dyes were also performed for stained cells every day and then the results were compared with those obtained using the *Gravimetric* extraction method. Two dyes were tested for lipid staining: Nile Red and BODIPY. Nile Red staining required more effort due to difficulties with dye penetration into the cells, especially for *C. vulgaris*, which has thick cell walls. For flow cytometry measurements, because we took only 1 mL sample cell viability was not a concern, when it came to proper staining. To improve staining with Nile Red we used glycerol as a carrier when working with *C. vulgaris*. In addition to the carrier, sonication with heating was used to achieve better staining. On the other hand, it was generally much easier to stain with the BODIPY dye. When used with algal cells, the green fluorescent lipid probe BODIPY allowed for much easier staining. In addition, BODIPY required less time for sonication and milder heating conditions for *C. vulgaris* sample preparation. For

the thin cell walls of *T. chuii* the BODIPY dye easily yielded perfect staining without any additional procedures by simply mixing a sample with the dye followed by 10 minutes of incubation in the dark. More importantly, the BODIPY dye gave much more stable and reliable results on the last day of the cycle that were similar to the ones obtained by the gravimetric extraction method. Based on these observations, BODIPY dye was used in real-time screening procedures to monitor the impact of ultrasound on lipid growth in algal cells. Fluorescence measurements using BODIPY showed similar increase as was observed with gravimetric extraction method on the final day of growth cycle (Figure 2.8).

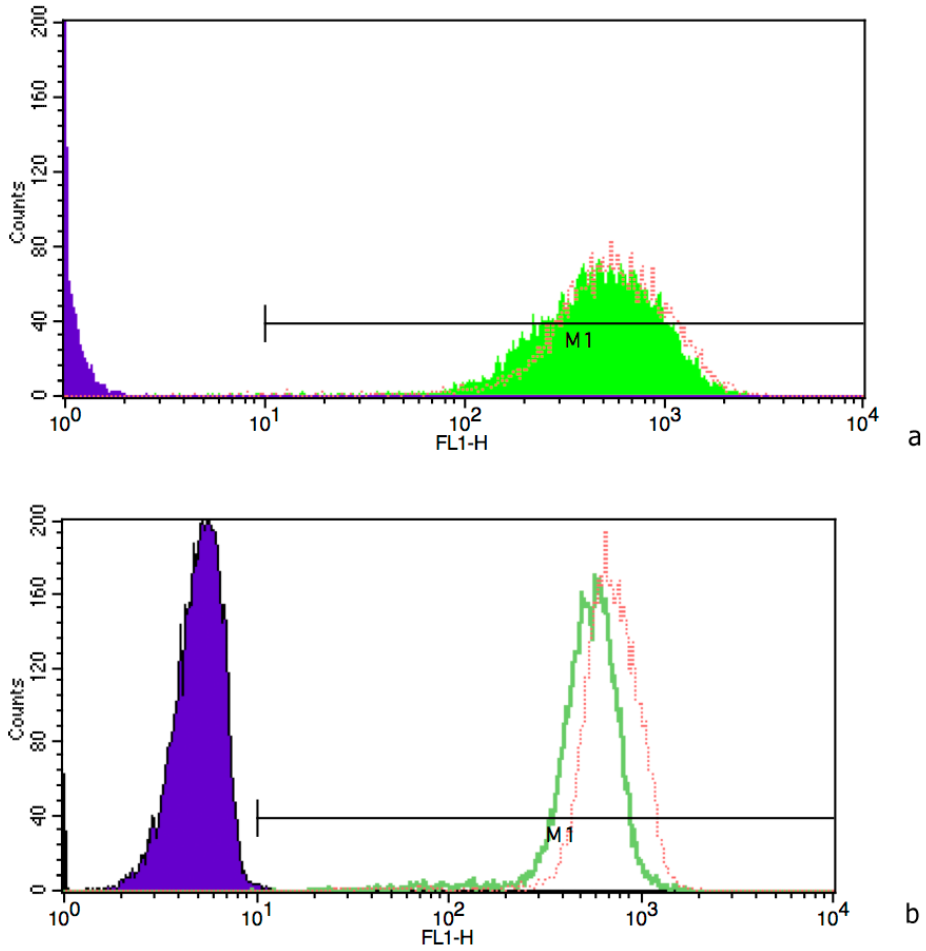

Figure 2.8. FACS histogram analysis plots for fluorescence measurements in *Chlorella* and *Tetraselmis chuii* algal cells, stained with BODIPY dye. **(a)**- results for *Chlorella* experiment, where purple peak – control(cells without staining), green

peak- stained cells of control, red- peak for sample with 100mW ultrasound stimulation. Peak shift indicates 16%increase in lipid content.
(b)- results for *Tetraselmis chuii* experiment, where purple peak- control (unstained cells), green- control-stained cells with no ultrasound stimulation, red- peak for the sample of 12 times a day 80 mW stimulation. Peak shift indicates 24% increase in lipid content

BODIPY staining coupled with flow cytometry allowed quantitative measurement of the difference between samples. However, fluorescence measurements with Nile Red had poor stability and could not be used quantitatively because some measurements showed lipid content increase up to 75% or more between samples, which could not be true according to our extraction measurements. Although the Nile Red dye was extensively used for lipid staining (Held & Raymond, 2011), in our case better repeatability and more accurate results were obtained using the BODIPY dye. These results correlated well with the results of extraction method and thus showed that our method can be used for real-time daily screening of the algae culture as we used it in our case to evaluate ultrasound parameters for better lipid growth.

2.3.3. Cost Analysis

The research presented in this thesis focuses on a new technological development. From the technological viewpoint, in future applications this design needs to be translated from bench scale (in shake flasks) to industry (in bioreactors). The price of LIPUS application and energy cost vs benefits that can be obtained is one of the arising questions. The one-time installation cost (including a LIPUS generator and an ultrasound transducer) for a 1L spinner-flask bioreactor is less than $50 US Dollars.

The routine cost to trade10 ~ 20% increase in lipid content is the cost of electricity. According to our previous discussion, the following are the treatment conditions:

- *T. chuii*: 80mW/cm^2 , 5 minutes, 12 times a day, and
- *C. vulgaris*: 100mW/cm^2, 15 minutes, 8 times a day.

The power efficiency of our LIPUS driving circuit is about 40%, and the surface area of a transducer is 3.5cm^2. Therefore, the total energy consumption of the LIPUS device to stimulate *T. chuii* is ((80mW/cm^2*3.5cm^2)/40%) *5 minutes*12 times/per day*8 days=700mJ/s*3600s/per day*8 days = 0.7J* 28800 = 20160J. Since 1kilowatt-hour (kWh) = 3600000J, then 20160J= 0.0056kWh. Similarly, the total energy needed to stimulate *C. vulgaris* using LIPUS is ((100mW/cm^2*3.5cm^2)/40%) *15 minutes*8 times/per day*8days=14Wh=0.014kWh. According to the report of electricity rates in North America (http://www.gov.mb.ca/jec/invest/busfacts/utilities/compare.html), the average cost of electricity is about $0.10 US Dollar per kWh. Or, the total cost needed for *T. chuii* is 0.0056kWh*$0.1/kWh=$0.00056, and $0.0014 for *C. vulgaris*, respectively. The benefit gain is obvious.

2.3.4 Quality of Lipids Remains Unchanged After Pulsed Wave Stimulation

Lipids extracted from *Chlorella* were tested using gas liquid chromatography to verify that ultrasound stimulation does not cause lipid composition change in the cells. GC/MS spectra of lipids extracted from LIPUS treated and control samples were compared. The results show that the fatty acid composition remains the same with or

without pulsed wave stimulations (refer to Table 2.1 and Figure 2.9 (a,b)). We have repeated the fatty acid composition GC-MS analysis for lipids from three different runs of experiment, also using results of lipids extracted in the middle (Day 4)(Figure 2.9 a) and at the end of cycle (Figure 2.9 b) to confirm that LIPUS does not alter fatty acid composition at any time of the lipid accumulation process and the main fatty acid peaks are the same (16:0; 16:2; 16:3; 18:0; 18:1; 18:2, 18:3).

Table 2.1: Lipid analysis result of samples with and without pulsed wave stimulation

	Fatty acid composition of total lipid fractions for the Control					
	(mol % of total fatty acids)					
16:0	**16:2(7,10)**	**16:3(7,10,13)**	**18:0**	**18:1(9)**	**18:2(9,12)**	**18:3(9,12,15)**
34.8±6.9	10.8±0.1	11.0±4.0	3.4±0.1	6.1±2.3	15.1±8.6	18.4±3.4
	Fatty acid composition of total lipid fractions for the Treated Sample					
	(mol % of total fatty acids)					
16:0	**16:2(7,10)**	**16:3(7,10,13)**	**18:0**	**18:1(9)**	**18:2(9,12)**	**18:3(9,12,15)**
37.3±5.7	10.7±2.2	8.4±6.2	3.2±1.2	8.5±0.7	16.1±11.4	15.4±2.3

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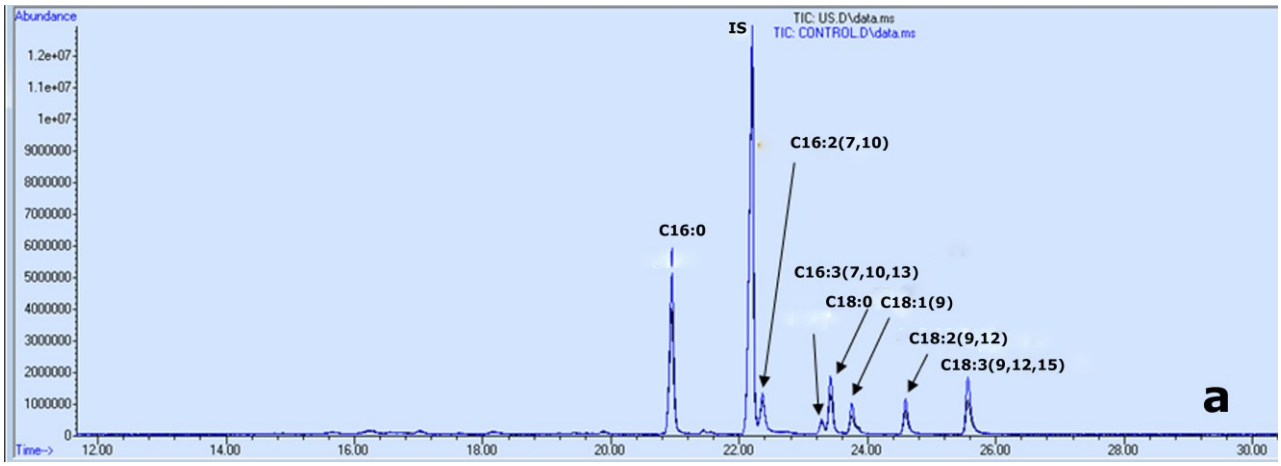


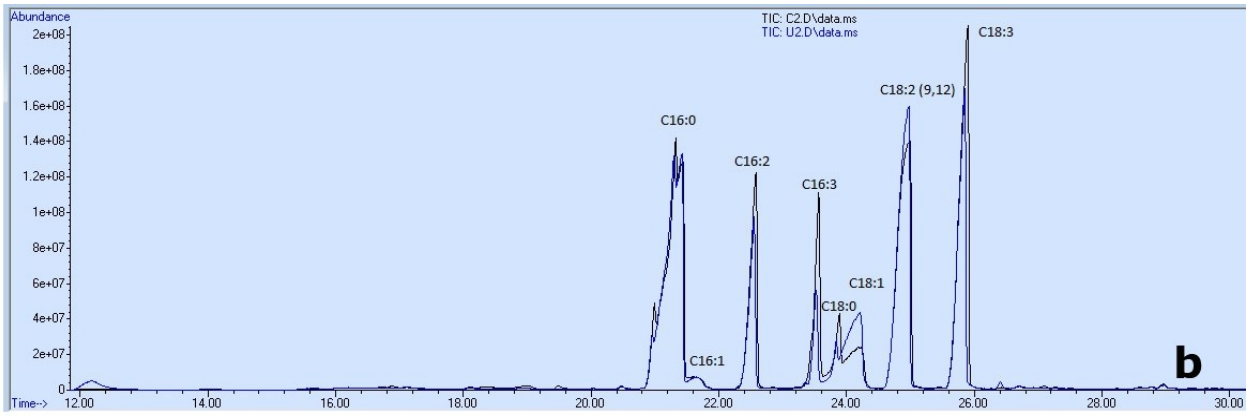


Figure 2.9: GC/MS analysis results for *Chlorella* lipids, **(a)** extracted in the middle of the cultivation cycle and **(b)** extracted at the end of the cycle to verify that there were no changes in lipid composition induced by pulsed wave stimulation. The peak identification and assignment are labeled in the figure. The blue line is the sample without pulsed wave stimulations while the blank line is the sample with pulsed wave stimulations. Both lines are overlapped with each other, which means that pulsed wave stimulations do not alter the fatty acid composition.

Test results are consistent with our expectations and confirm that LIPUS is an additional method of stimulation that can be used for improvement in biofuel production processes.

2.4. Conclusions

The use of biofuels as a partial alternative for fossil fuels is gaining great attention worldwide. Algal oil is a promising biofuel source. Unfortunately, current algal oil production processes is inefficient. To increase the conversion efficiency, Low-intensity Pulsed Ultrasound can be applied. Research results presented in this chapter showed that LIPUS can increase algal oil production by 10 to 20% in shake flask cultivation process. With the selection of the best algal strain for lipid accumulation or improvement of media, the pulsed ultrasound stimulation is a complementary physical method to induce physiological changes of algae cells, increase efficiency

and reduce cost of lipid production by microalgae. Both freshwater algae *C. vulgaris* and seawater algae *T. chuii* were studied. Accurate and simple method of lipid measurements during lipid accumulation process is required. Two different fluorescent dyes Nile Red and BODIPY, previously known doe successful lipid staining, were tested both using model system and then on algal cells in the process of growth cycle to verify which fluorophore can suit better for the purpose. BODIPY provided better accuracy of measurements compared to Nile Red dye in our experiments. Because this fluorescent staining method for monitoring daily lipid accumulation requires only small volumes of culture sample, it is ideal for research labs or manufacturers to assess the increase of algal lipids and to optimize culturing conditions.

Chapter 3

Effect of low intensity pulsed ultrasound on the growth and biomass production of *C.vulgaris* and *Schizochytrium sp.*

3.1 Introduction

With the shortage of fossil fuel resources, it comes as no surprise that major interest in algae biotechnology was drawn to algae-derived biofuels and tools to increase lipid production. Algal biomass accumulation and technologies that can be used to increase it can also be of interest for many industries. Enriched biomass can be used for food and in biomaterials industry, algae pigments can be used as natural dyes, replacing toxic synthetic alternatives, high-value chemicals (e.g. carotenoids, known for their antioxidant properties and used in cancer, obesity, hypertension prevention) can be obtained from biomass and used as nutraceuticals and pharmaceuticals. (Rawat, Ranjith Kumar, Mutanda, & Bux, 2011). l-Ascorbic acid and polysaccharides are algal derived extracellular products that are commercially valuable. (Bumbak et al., 2011) Other examples of high-value nutrient products that can also be extracted from microalgae biomass are docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)- omega-3 long-chain polyunsaturated fatty acids (LC-PUFA) ("Enhanced production of lipids containing polyenoic fatty acid by very high density cultures of eukaryotic microbes in fermentors," 2001). It has been known for decades that DHA in the human diet has many beneficial effects, such as reducing the chance of getting a stroke and preventing rheumatoid arthritis and cancers. In

infancy, DHA plays important role in eye retina and brain development (Carlson, Werkman, Rhodes, & Tolley, 1993; M. H. Li, Robinson, Tucker, Manning, & Khoo, 2009). However, previously major sources of DHA were fish and fish oil, which came with unpleasant side effects, such as pollution in the fish or bad odor and fishy taste in fish oil (M. H. Li et al., 2009; Vazhappilly & Chen, 1998),which led to a decreased DHA consumption in humans. Consequently, search for an alternative sustainable source of DHA has begun in the 1980s, and it was discovered that marine microalgae were the main microorganism producers of omega 3 LC-PUFA. As fish do not synthesize DHA themselves, but rather get it from a diet, the idea behind it was to find an alternative source of high-quality DHA- a strain of microalgae that can produce LC-PUFA without consequent fishy taste/odor (Turon, 2013). Such microalgae could be used as a fish feed, as well as autonomously as nutrition additive for human food.

However, it was not feasible back in the 1980s, because such a production faced many problems including strain screening, culture and harvesting processes parameters selection, bioreactors corrosion, etc. Since then algal research and biomass production by algae have become an interesting research topic as many solutions and improvements to bioreactor design and culture conditions for algae growth have been proposed (S. T. Wu, Yu, & Lin, 2005). Among various microalgae, *Schizochytrium sp.* attracts a lot of attention. It belongs to a group of marine microorganisms *Thraustochytrids* (microalgae or microalgae like species) (Singh, Liu, Li, & Wang, 2014) with high lipid content reaching up to 55-70% of dry biomass. The accumulated lipids have significant levels of saturated fatty acids and n-6 docosapentaenoic acid and very high level (up to 49%) of docosahexaenoic acid (DHA). Consequently, *Schizochytrium sp.* can be used for feeding clams, mussels and

fish leading to a better flesh quality in terms of fat composition (M. H. Li et al., 2009; Van Hoestenberghe et al., 2016) and the amount of polyunsaturated fatty as well as to decreased pressure on wild stocks and environment. When *Schizochytrium sp.* was used for feeding Atlantic salmon grown in fresh water, it improved muscle build-up in fish, increasing the relative amount of fillets, as well as enhanced fatty acid composition (Miller, Nichols, & Carter, 2007). Similar effect with higher weight gain and increased DHA content in fillets was observed on tilapia fish. (Sarker et al., 2016) *Schizochytrium sp*. meets the main requirements for DHA production: it can inexpensively produce a large amount of DHA in heterotrophic growth cycle with the potential to use inexpensive glucose source (Barclay, Weaver, Metz, & Hansen, 2015); it has no colorful pigments and therefore, can also be used as invisible food additive. It has very fast growth rates (6-9 doubling per day) and can grow and produce lipids under a low-salinity condition, decreasing chances for bioreactor corrosion. It is thermoresistant and successfully grows at +300C (faster biomass accumulation). DHA accumulation in *Schizochytrium* can reach up to 43% of oil content even at low salinity or 20-25% of dry biomass, reaching a production rate of 0.55 g/L.h ("Enhanced production of lipids containing polyenoic fatty acid by very high density cultures of eukaryotic microbes in fermentors," 2001). In all, *Schizochytrium sp* shows great potential to serve as an alternative source of polyunsaturated acids ("Microalgae in fish farming Advances in pikeperch research Improving technology uptake and market impact of genetic research," 2015).

Safety studies have been carried out. No toxins associated with *Schizochytrium sp.* intake were found (Fedorova-Dahms, Marone, Bauter, & Ryan, 2011) proving that it was safe to consume either separately or directly adding

extracted DHA-oil in foods ("Enhanced production of lipids containing polyenoic fatty acid by very high density cultures of eukaryotic microbes in fermentors," 2001). The DHA-enriched biomass can be used in fish feed ("Microalgae in fish farming Advances in pikeperch research Improving technology uptake and market impact of genetic research," 2015), poultry feed (producing DHA-enriched eggs) (Chin, Shen, Su, & Ding, 2006), swine, or cow feed (producing milk with DHA). With such obvious benefits, a lot of research attention was drawn into the studies on improvement of *Schizochytrium sp.* biomass growth and DHA accumulation enhancement. (Jain, Raghukumar, Sambaiah, Kumon, & Nakahara, 2007). Effects of cultivation time, pH, medium composition, temperature and salinity (Liu, Tang, Li, Daroch, & Cheng, 2014; S. T. Wu et al., 2005) have also been studied to improve DHA production by varying main nutrients intake-oxygen, phosphorus and nitrogen (Ganuza, Benítez-Santana, et al., 2008; Ganuza, Anderson, & Ratledge, 2008; Ren et al., 2014; Ren, Feng, Li, Qu, & Huang, 2013; Yaguchi, Tanaka, Yokochi, Nakahara, & Higashihara, 1997). Methods of growing *Schizochytrium sp.* on waste products, such as glycerol (Ethier, Woisard, Vaughan, & Wen, 2011; Pyle, Garcia, & Wen, 2008) distillery wastewaters (Yamasaki et al., 2006), rather than on expensive sources of carbon have also been explored (Chi, Liu, Frear, & Chen, 2009). Kollenmareth suggested that not only polyunsaturated fatty acids could be successfully derived from the algae, but also polysaccharides, carotenoids. (Jain, Raghukumar, Tharanathan, & Bhosle, 2005; "Strain of Schizochytrium limacinum useful in the production of lipids and extracellular polysaccharides and process thereof," 2008). Different approaches for algae growth process optimization have been reported, (Unagul et al., 2006; Unagul, Assantachai, Phadungruengluij, Suphantharika, & Verduyn, 2005).

Algal research now focuses on attempts to develop the best practices for high cell density algae cultivation for biomass accumulation and production of specific products for nutrition and pharmaceutical commercial applications. Ultrasound can be used for stimulation as one of the alternative approaches to successfully reduce cost, but increase yield for various biotechnological production (Ashokkumar, 2015)

Research presented in this chapter focuses on the impact of the ultrasound stimulation using the Low-Intensity Pulsed Ultrasound (LIPUS) on algae cells, more specifically on its potential to improve algal growth and biomass production. Two microalgae were studied: fresh-water *Chlorella vulgaris* in both mixotrophic and autotrophic growth conditions and marine *Schitzochytrium sp.*in heterotrophic growth cycle.

3.2 Materials and Methods

LIPUS was used to stimulate algae cells for a short period of time during their growth in the shake culture. A positive effect of LIPUS was observed on the growth of *Chlorella* and *Schyzochytrium sp.*, or more specifically the LIPUS treatment increased biomass by up to 20% (refer to further discussion).

3.2.1 Algae preparation: *C. vulgaris,* freshwater algae were grown in a modified 3N bold medium. The standard recipe of 3N bold medium was used for algal growth medium (http://www.ccap.ac.uk/media/documents/3N_BBM_V.pdf) with the amount of nitrogen ($NaNO_3$) decreased 3-fold (from 75g/L of $NaNO_3$ in stock solution to 25 g/L). 40% Glucose solution was used in the scale-up study. (Refer to 2.2.1-2.2.2. in Chapter 2) *Schizochytrium*, salt-water algae, grows in a heterotrophic condition. The medium contains glucose 20 g/L, $(NH_4)_2SO_4$ 1.2 g/L, $MgSO_4*7H_2O$ 2.5 g/L, NaCl

12.5 g/L, KH_2PO_4 0.5g/L, KCl 0.5 g/L, and $CaCl_2$ 0.1g/L. Trace element solution was added in the amount of 10 ml/L and prepared according to the following protocol: 6g/L EDTA, 0.29g/L $FeCl_3*6H_2O$, 6.84g/L H_2BO_3, 0.86g/L $MnCl_2*4H_2O$, 0.06 g/L $ZnCl_2$, 0.002 g/L $CuSO_4*$ $5H_2O$, 0.05 g/L $NiSO_4*6H_2O$, 0.005 g/L $Na_2MoO_4*2H_2O$. Both the growth medium and the trace element solution were sterilized using an autoclave. 1 mL of vitamin solution (thiamin, biotin, and cyanocobalamin, filter sterilized) was added to the final medium. Final pH was adjusted to 7, using KOH, and polyvinyl alcohol (PVA) was used as the surfactant in the amount of 0.05%.

3.2.2. Culture conditions: In the scale-up study, *Chlorella* was grown mixotrophically in a spin flask bioreactor (Figure 3.1) at 27°C with a spinning magnet to provide constant stirring and constant light. 1L of the filter-sterilized medium was inoculated with exponentially growing seed culture and initial OD_{550} after the medium was inoculated with stock culture was 0.09-0.1. The initial solution was divided between two bioreactors 640 mL each: one for ultrasound treatment and the other one for control. Algae were grown for 11 days and 95 mL of glucose (40% solution) were added to the culture (640 mL) on the 7^{th} day of the growth cycle so that the final concentration in bioreactors was 6% glucose (see Chapter 2).

In shake flasks *Chlorella* was grown autotrophically at 27°C and constant light with a shaking speed of 150 rpm. (Refer to Figure 2.2 steps 1-4 in Chapter 2). 1L of filter-sterilized medium was inoculated with exponentially growing seed culture with initial OD_{550} between 0.09 and 0.1. The initial solution was divided between flasks (100 mL) for ultrasound treatments under different conditions (control, 80 mW/cm^2 and 100 mW/cm^2). Algae were grown for a total of 11 days. At the end of

cultivation, the culture was harvested by centrifugation at 5000 rpm and freeze-dried to measure the final dry biomass.

Schitzochytrium was grown heterotrophically in darkness in a shake flask at 24°C with a shaking speed of 200 rpm. 1L of the filter-sterilized medium was inoculated with exponentially growing seed culture. Spectrophotometry at the optical wavelength of 600nm (OD_{600}) was used, revealing that the OD measurements of the initial solution were between 0.018 and 0.021. The initial solution was divided between flasks for the ultrasound treatments under different conditions. In particular, 5 flasks were for control and 5 flasks for the ultrasound treatment. 10x dilution was performed every day starting from Day 1 by replacing 90 mL of culture in a flask with fresh medium. Algae were grown for a total of 5 days.

3.2.3. Growth analysis: For *Chlorella* daily measurements of OD_{550} were performed both in scale-up bioreactors study and in shake flasks to check the cell growth. Cell count was also done for samples collected from shake flasks autotrophically grown culture to see how ultrasound stimulation affects cell division in the culture.

For *Schitzochytrium* measurements of OD_{600} were performed daily to check the cell growth. In addition to OD measurements, dry biomass, collected through filtration, was measured daily. Two samples of control and two LIPUS treated samples were filtered using pre-weighed Whatman #1 paper filters to collect biomass and were left to dry at 70 ^{o}C in the thermostat oven overnight. After this, the filters were weighed again to determine the dry biomass. Cell count was also performed daily.

3.2.4. Experimental set-up: For autotrophic shake flask experiment the same set-up was used as for mixotrophic growth cycle in Chapter 2 and described in 2.2.4. (Experimental set-up is the same as shown in Figure 2.2 in Chapter 2). A constant light was used.

For bioreactors scale-up study *Chlorella* culture was grown in spinner flasks. Ultrasound transducer was put into ethanol sterilized plastic tube with water (water serves as propagating medium for ultrasound wave) and embedded into bioreactor through one of the sidearms so that tube was immersed in the culture. Attenuation of the wave due to the thickness of the tube was taken into account and LIPUS was calibrated in a way that the final intensity in the bioreactor was as needed (section 3.2.5). Another sidearm was covered with a cloth for breathing. In control bioreactor no ultrasound was applied. (Refer to Figure 3.1). Spinner flasks were put in water-bath that was placed onto hot plates with a magnetic field to provide 27^{o}C in the culture, constant stirring, and constant light.

Figure 3.1. Experimental set-up for the scale-up study of LIPUS effect on *Chlorella's* biomass accumulation during mixotrophic growth in spinner-flask bioreactors, where left is LIPUS stimulated sample, and right is control.

For *Schitzochytrium* experiment the same experimental set up was used for ultrasound application as described in 2.2.4 in Chapter 2 and shown in Figure 2.2d. The shaker was set at 200 rpm, with temperature controlled at 24^{o}C during the whole cell growth cycle. Constant shaking was applied to assure equal LIPUS stimulation.

3.2.5. Ultrasound treatment: The same ultrasound generating device as described in Chapter 2, section 2.2.3 was used, generating the pulsed wave at the frequency of 1.5MHz, duty cycle of 20% (200μs LIPUS on and 800μs LIPUS off), and intensity within 80mW/cm^2 - 400mW/cm^2. (Refer to Figure 2.1 in Chapter 2).

In shake flask *Chlorella* experiments the intensities that previously showed the best effect on *Chlorella* lipid accumulation (Refer to Chapter 2) were used – 80 mW/cm^2 and 100 mW/cm^2, but this time for biomass stimulation. 8 times a day 15 minutes stimulation was applied daily during 11 days growth cycle.

For the scale-up *Chlorella* experiments we selected one intensity that gave better results in 100 mL shake flasks- 100 mW/cm^2 . However, only one transducer could be used in our bioreactors, while the volume was increased 6.4 times, so we adjusted both intensity and doubled treatment time to cover the 640 mL volume. The final stimulation parameters were: 110 mW/cm^2 and 30 min 8 times per day.

For *Schitzochytrium* experiment we did not have any LIPUS experience before so different intensities were tested (within the range of 80-400 mW /cm^2) to determine the optimal LIPUS treatment intensity. Previous experiments showed that intensities around 60-100 mW/ cm^2 could improve cell growth of mammalian and some other algal cells (Xing et al., 2012; P. Xu et al., 2012), but higher intensities were required for this strain. Ultrasound was applied from the beginning of the experiment on Day

0 – Day 1 of the growth cycle and then at the end of the cycle starting on Day 4 through Day 5. Continuous sonication during whole culture cycle has proven to be inefficient (refer to our discussion later). LIPUS was applied 10 times per day for 20 minutes.

3.2.6. Fluorescent microscopy: For microscopy *Schitzochytrium* cells were spun down using a centrifuge and fixed in 1% paraformaldehyde. Slides were pre-treated with poly-L-lysine to help the cells attach to the slides. After that a drop of previously fixed cells was put on a slide and treated with ProLong® Gold Antifade Mountant media with DAPI in it to stain the nuclei and prevent from fading. After that cover slip was put on the slide and samples were studied using the fluorescent microscope.

3.3 Results and Discussion

Chlorella study

3.3.1 Application of LIPUS for biomass increase by *Chlorella vulgaris*. Scale-up study in spinner flask bioreactors

In the *Chlorella* experiments with 100 mL shake flasks (refer to Chapter 2) the main focus was on using ultrasound for the better lipid accumulation by the cells. All the parameters of the experiment were adjusted for that purpose. Mixotrophic growth was chosen, with the autotrophic growth of the cells for the first 5 days, followed by addition of glucose to increase lipid content and cell harvesting few days after – just after the cells had turned glucose into oils, but before they started eating their own lipids due to nutrient deprivation. That experiment was efficient for lipid increase, but

either no significant increase of biomass was observed, which makes sense, as it is only possible to design a process that is optimal either for lipid or biomass increase.

At the same time during those experiments observations showed there were more cells in the sonicated samples compared to the control ones. Thus the hypothesis was that it should be possible to reflect that in biomass as well. Higher cell numbers with lower final dry biomass in sonicated samples could be explained by a simple fact that cells were just smaller, and the assumption was that giving them few more days to grow could lead to a sacrifice in oil increase, but to biomass increase instead. It was also interesting to see whether we could observe the same effect on a bigger scale. It is very important to translate the LIPUS stimulation technology to bioreactors in the end so we combined two studies. The experiment was run similarly to the one described in Chapter 2 for lipid increase in *Chlorella*. LIPUS was applied in bioreactors of 640 mL with the same mixotrophic growth cycle, but with longer cycle to let the cells grow. The most efficient ultrasound parameters from that experiment were selected- 100 mW/cm^2 and 8 times per day 15 min treatment, but because of higher volume and different bioreactor design ultrasound intensity and duration had to be adjusted to 110 mW/cm^2 and 30 min*8 times per day to show effect. Growth cycle was also modified and glucose was added on Day 7 instead of Day 5, as cells were generally growing slower in bioreactors. Harvesting was done on Day 11. With such set-up and parameters up to 20% increase was achieved in biomass production (Refer to Figure 3.2), while no significant lipid increase was observed. This was consistent with our expectations and the purpose to show that LIPUS can be used successfully for both lipid and biomass increase but process parameters have to be optimized depending on the initial purpose.

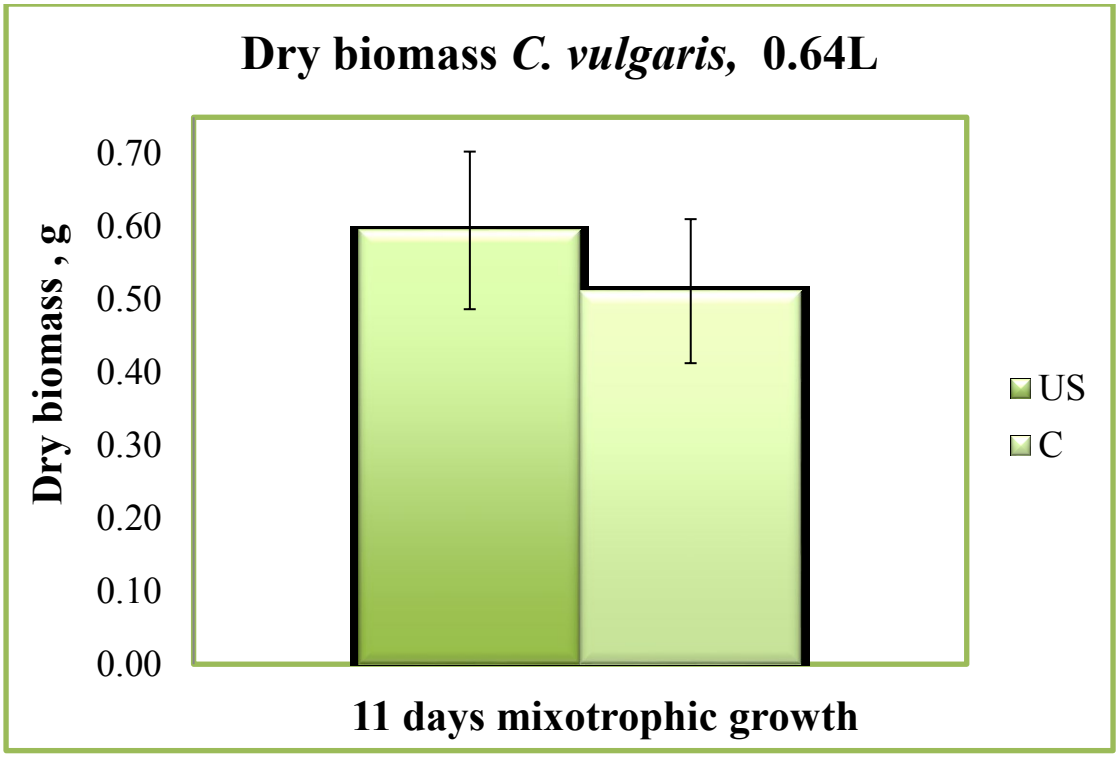


Figure 3.2. Results for total dry biomass produced by *Chlorella* cells with (US) and without (C) pulsed-wave stimulation in 11 days cultivation cycle in scale-up bioreactors study.

High error bars are due to the experiment run not in triplicates, but in 3 separate experiments and, while all three showed over 10% increase, final biomass of one of them was higher than the other two. Low biomass is explained by the design of the bioreactor that is far from optimal for aerobic cultivation- only one sidearm of the bioreactor was providing oxygen and it was insufficient for such amount of culture as well as insufficient light. The main purpose of the experiment was to see whether LIPUS effect remained with the culture volume increase and it did, further optimization of the process could be done along with successful ultrasound application.

3.3.2 Application of LIPUS for biomass increase by *Chlorella vulgaris* in shake flasks

Another experiment was run in 100 mL shake flasks with Chlorella cells, but with completely autotrophic growth and also extended cycle with LIPUS stimulation to see its effect on biomass. The set-up and ultrasound stimulation parameters were as described in Chapter 2. The growth cycle was extended to 11 days. This experiment also showed LIPUS efficiency for inducing higher biomass production as up to 28% increase in biomass was observed for 100 mW/cm^2 stimulated sample compared to the control. (Figure 3.3)

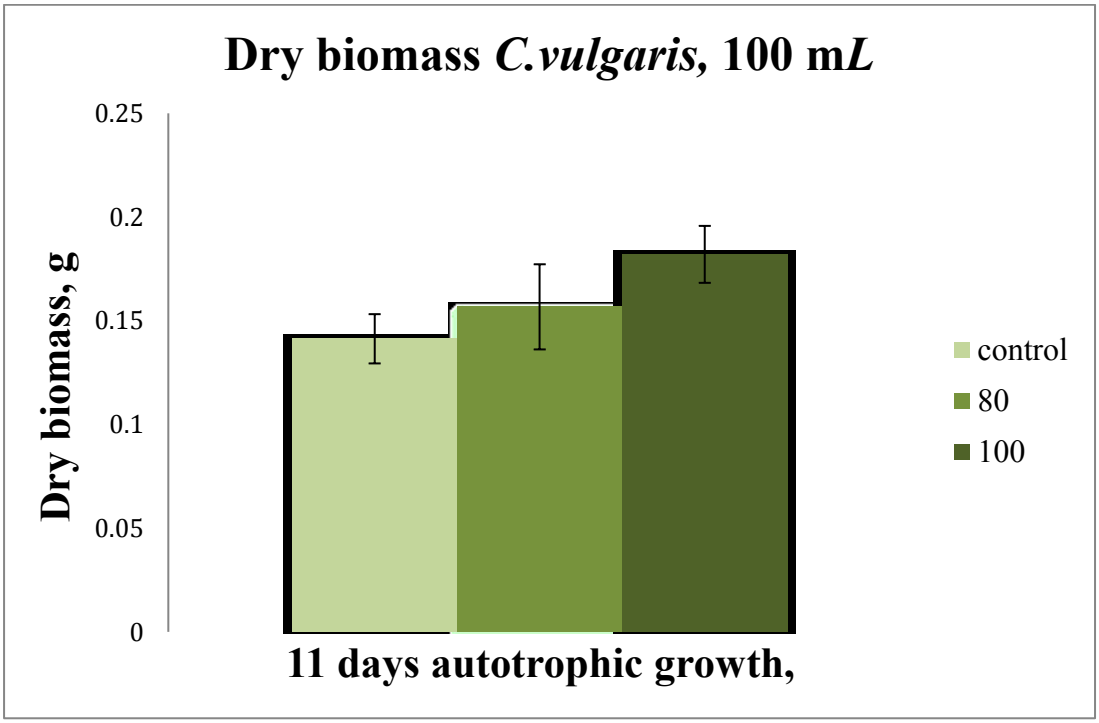


Figure 3.3. Results for total dry biomass produced by *Chlorella* cells with (80 mW/cm^2 and 100 mW/cm^2) and without (control) pulsed-wave stimulation in 11 days cultivation cycle in shake flasks under autotrophic growth conditions

Overall, our results show that it is possible to find optimal parameters of LIPUS stimulation depending on the initial purpose- to increase lipid or biomass

accumulation. Also, it was shown that successful transfer of the technology application from small flask to bioreactor was possible without the loss of the desired effect. Two different types of growth- more used in industry mixotrophic or cheaper autotrophic growth were tested with the same 11 days cycle and LIPUS stimulation and in both experiments significant repeatable biomass increase was observed.

Schitzochytrium study

3.3.3 Application of LIPUS for improved growth of *Schitzochytrium sp.* in shake flasks

In this study we wanted to determine whether LIPUS has a positive effect on the growth of *Schizochytrium sp*, which can lead to a potential cost-efficiency improvement in the process of cultivation of Schitzochytrium for DHA production. Although *Schitzochytrium* can grow in a wide range of temperature and pH (from 5 to 11) conditions, pH=7 was chosen for our experiments, because it was reported to promote better DHA production. Another factor to be considered is the ability of *Schizochytrium* cells to produce ectoplasmic nets leading to cell binding. It is important for the ultrasound effect to have separate cells, not clusters so that the wave stimulation can have the same effect on each single cell. Our preliminary experiments showed that cell aggregation prevents LIPUS from making any impact on cells. To remove the clustering, we have applied different methods, including increasing shaking speed, adding beads to the culture (mechanical separation), and introducing a small amount of surfactant (0.1% PVA) that can cover the cell and prevent the cells from aggregation. Mechanical separation with beads was too harsh on this microorganism and destructive for the cells, and could not be considered for the purpose. At the same time significant improvements in the cell growth were observed

with the addition of the surfactant. By simply adding 0.1% PVA, much better growth was achieved, compared to all other samples (control, control+ beads, stimulated cells with LIPUS of intensities either 250 mW/cm^2 or 300 mW/cm^2). We further decreased the amount of surfactant even to 0.05%, which could still separate cells in order to get the desired stimulation effect from the ultrasound wave. The cell separation not only helps LIPUS stimulation, but also has an obvious positive effect on the cell growth due to better nutrient availability for every cell even without additional ultrasound stimulation (Refer to Figure 3.4) and in our further research we combined effect of surfactant with LIPUS stimulation.

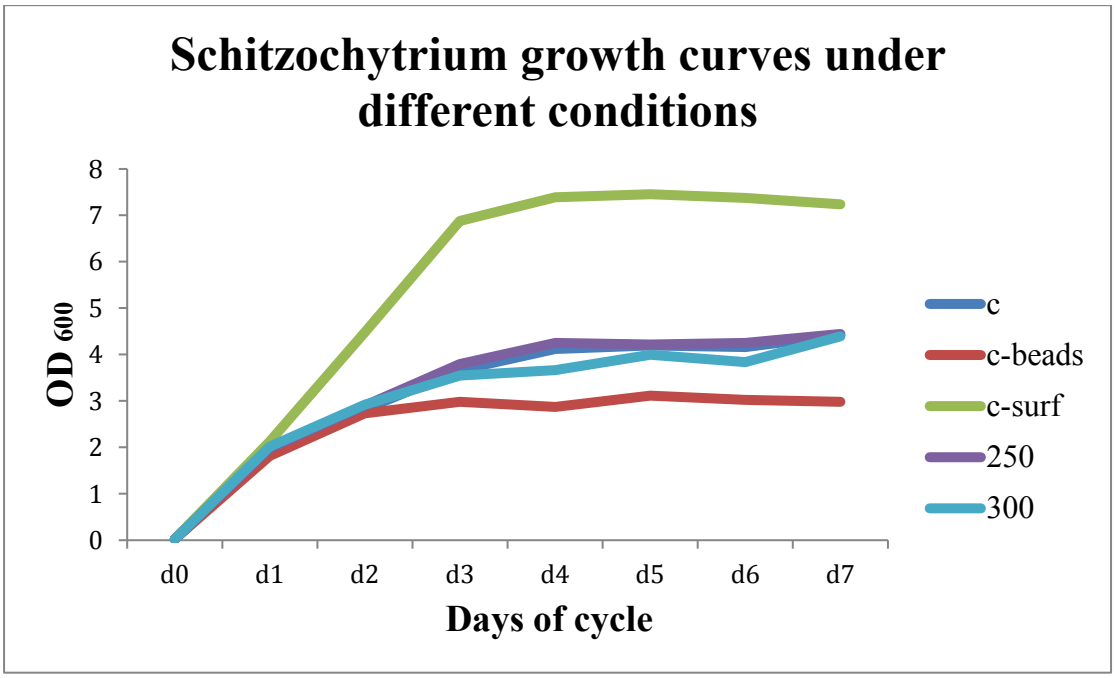
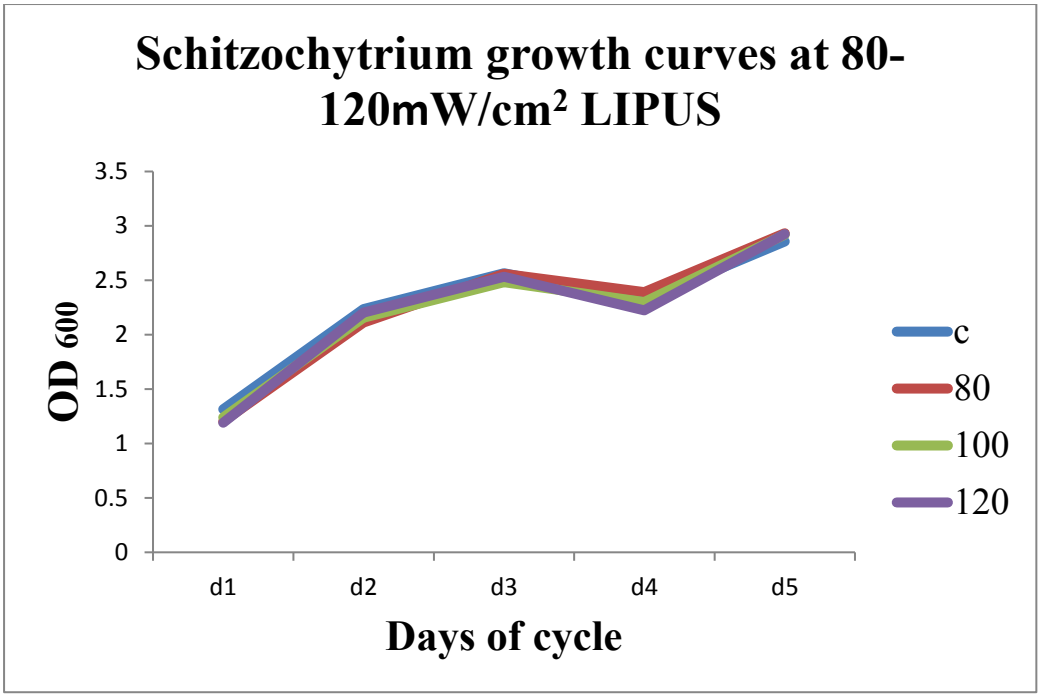


Figure 3.4. Effect of different parameters on the growth of the *Schizochytrium* culture in a shake flask in order to prevent cell clustering, where **c**- control (*Shitzochytrium* culture), **c-beads** – cell culture with addition of beads for mechanical separation, **c-surf** – cell culture with the addition of 0.1% PVA as surfactant, and **250** and **300** refers to mW/cm^2 intensity of LIPUS stimulation in samples without surfactant, but with 12 times a day treatment for 15 min. While mechanical separation with beads

was destructive for the cells, addition of surfactant significantly improved the growth due to cell separation.

3.3.4 Selecting Optimal LIPUS Stimulation Condition for *Schitzochytrium* growth

One important parameter to determine for ultrasound experiments is the intensity of LIPUS. Previous studies have shown that LIPUS can stimulate the growth of mammalian cells at about 60-80 mW/cm^2, and around 80-110 mW/cm^2 were efficient for algae and yeast cells. Because mammalian cells do not have a cell wall, they can respond to lower ultrasound intensity stimulation. *Schizochytrium* is an algae-like microorganism, and we started with ultrasound at the intensity of 80 mW/cm^2, because this intensity was effective for another marine algae *T. chuii* (Chapter 2) and for *Chlorella*. We then tried to search for an optimal stimulation intensity for *Schizochytrium sp.* However, cells did not respond to the treatment with any noticeable effects, when ultrasound intensity of 80mW/cm^2, 100 mW/cm^2 and 120 mW/cm^2 was used. Although some improvements in growth were observed at 160 mW and 250 mW/cm^2, we did not observe any significant increase in final biomass until the intensity of ultrasound reached 300-400 mW/cm^2. After many trials, we chose 10 times per day and 20 minutes stimulation with 400 mW/cm^2.



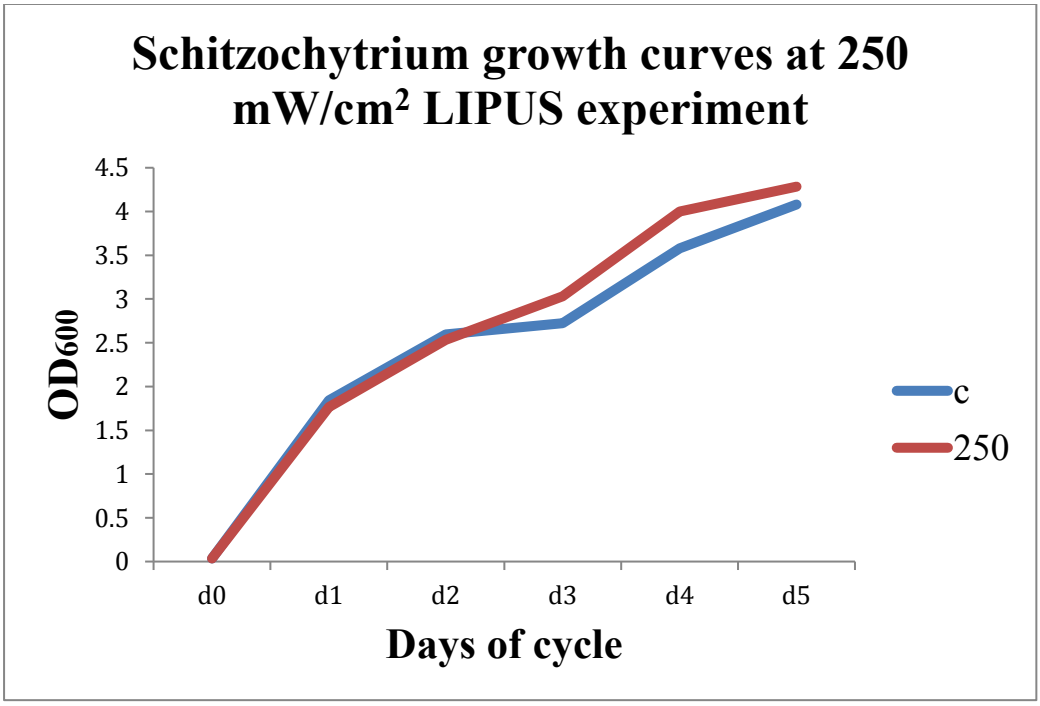


Figure 3.5. Growth curves of *Shitzochytrium* in shake flasks with LIPUS treatment at 80 mW/cm^2, 100 mW/cm^2, 120 mW/cm^2 and 250 mW/cm^2 and without stimulation (control). No significant difference was observed until intensity reached 250 mW/cm^2.

Another distinctive feature of *Schizochytrium sp* is their reproduction mechanism not by binary or multiple fissions, but by zoospore formation. Typically zoospore formation cycle in *Schizochytrium* takes approximately 2.5 days. This was an important factor to be considered for ultrasound application. We have noticed that if ultrasound stimulation was applied during the growth phase, when there were many (mostly) zoospores in the culture, a significant increase in both OD and final cell biomass was achieved at the end of the experiment. However, if experiment was started with the inoculum at the growth phase, when mostly single cells were present in the culture, ultrasound stimulation did not give significant increase at the end. That is why it is important to monitor the stock culture to see when is a good time to start experiments (or LIPUS stimulations) before experiment starts. The same reason gives explanation to inefficient continuous (ultrasound stimulation every day for 5 consecutive days) stimulation for *Schizochytrium*, while it worked for other cells and microorganisms. It takes 2.5 days for the cells to form new zoospores and that is why LIPUS in our experiment was applied with 3 days interval (D_0-D_1 and D_4-D_5). We started the experiment with many zoospores in the stock on D0. When zoospores formation cycle has passed, we applied a second round of ultrasound stimulation on D4-D5. We learned from our previous discussion for different cell lines that LIPUS stimulation could increase cell division. Therefore, LIPUS stimulation should be re-applied when cells are entering a new cycle of division through zoospores.

Three main parameters were observed during the growth cycle of *Schyzochytrium sp.*: the amount of cells per 1 mL of culture, optical density at 600 nm (OD_{600}), and dry biomass using filtration through pre-weighed Whatman paper filters. While for *Chlorella* and *T.chuii* in our previous experiments and for algae in general typically daily OD measurements are sufficient to check the increase of biomass,

Schyzochytrium culture is very difficult to sample and simple OD_{600} readings were not completely reliable. *Schizochytrium sp.* cells tend to accumulate a lot of fat (up to 65-80% of their mass), and, thus, cell distribution within the flask medium can be uneven with the fattest cells coming to the surface. In addition, surfactant (though a small amount was added), could enhance this effect and add more sampling errors leading to inaccurate OD readings. To avoid high deviations, it was necessary to track all three parameters daily (optical density, cell amount, and dry biomass) in order to evaluate the LIPUS effect on culture growth. To decrease operational errors, we also increased the number of flasks: n=6 and higher was used for OD curves, and cell count was performed at the same time. To obtain the most accurate information about biomass increase in response to LIPUS stimulations, we collected all culture and filtered it so that the dry biomass could be weighed every day. In such a way, all cells, regardless of their fat content and distribution in the medium, were collected.

Obvious effects of sonication were observed after the first round of stimulations between D0 and D1. In particular, cell amount increased by 25-50%, OD readings increased by 20-25%, and dry biomass by 20-25% on D1. In the following days, however, this effect of stimulation was not very obvious. Several reasons can be attributed for this outcome.

One was high cell culture density, which prevented LIPUS wave to effectively penetrate and stimulate individual cells. Due to a very high growth rate of *Schizochytrium sp,* x20 times biomass increase was observed after the first day of LIPUS stimulation. It is likely that high cell density decreased LIPUS effect on cells in the following days. To avoid such an impact, we have tested two different approaches: one, where we stimulated the cells without dilution; the other one, where we applied the same LIPUS stimulation but with x10 times dilution of the samples in

shake flasks on each day of cell cycle starting from D1. While non-diluted samples stopped showing any response to LIPUS stimulation after the first day because of high cell density, diluted samples, on the other hand, responded well to LIPUS stimulation in terms of higher biomass accumulation. This discovery gave us the anticipated result that LIPUS indeed has a positive effect on cell growth. Researchers reported previously that very high cell density can prevent DHA accumulation, so diluted culture along with LIPUS can give both higher biomass accumulation without loss in DHA production. To further confirm our findings, we monitored the cell growth using microscopy to compare the diluted vs. non-diluted samples after LIPUS stimulations. Some interesting phenomena were observed. We started monitoring stock culture 2 days before inoculation so that on D0 most cells in the inoculum were zoospores (transferred from the stock). To avoid lag phase in our experiment we started adding fresh medium to stock culture two days prior to inoculation, so that the cells on D0 could be stimulated and grow exponentially right from the start of the experiment. In the successful experiment(leading to final significant dry biomass increase and run with 10 times dilution) in stock two days before inoculation we observed mostly zoospores with 10+ cells per spore, with the addition of new medium on the next day we also observed zoospores but bigger with 15+ cells per spore and we inoculated our medium with big spores on D0. In control samples on D1 still zoospores, though smaller prevailed in the culture, on D2 and D3 still were mostly spores and only on D4 and D5 mostly single cells were observed. Different picture was observed for LIPUS treated samples, where cell cycle ran faster due to ultrasound and already after 1st round of stimulation on D1 a lot of single cells were present along with small zoospores in the culture, and D2-D4 single cells prevailed unlike in control and on D4 when second round of sonication was applied it stimulated new

round of zoospores formation and, consequently, more zoospores than single cells were present in ultrasound stimulated culture on D5. This explains why we saw both higher OD and dry biomass for ultrasound stimulated samples, but cell amount was higher for control. Higher cell number, but with smaller single cells equals less in biomass compared to the smaller amount of much bigger zoospores.

Our microscopy observations also explain why we had a bigger increase in cell number on D1 after 1st LIPUS stimulation, while OD and dry biomass, though also increased, compared to the control samples, but not to such an extent. On D1 smaller zoospores are prevailing in control samples, and single cells in sonicated, and with biomass increase observed, much more single cells are needed to reflect that, compared to zoospores.

An experiment that was started from the different growth phase of inoculum (single cells) did not give significant biomass increase after 5 days of cultivation. In non-diluted samples starting from D1 all cells were single cells (Figure 3.6) and did not form zoospores during our 5 days of observation. Thus we chose to run experiments using dilution and monitor stock so that the inoculum contained at least 50% of zoospores on D0. In that case, LIPUS stimulation could lead to significantly higher biomass at the end.

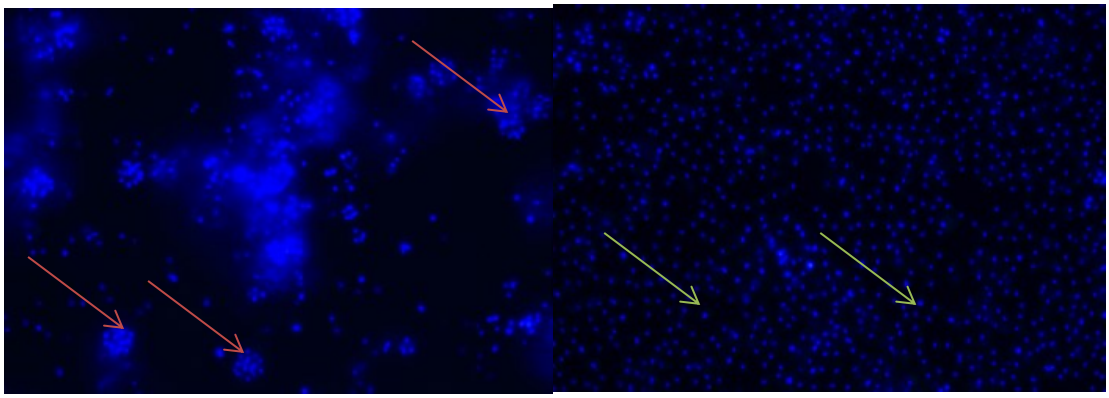


Figure 3.6. Microscopy images of cells collected on D_2 from LIPUS treated samples without dilution (right) and with 10 times dilution (left). Single cells can be seen in

the culture without dilution (shown with green arrows on the right image), while prevailing zoospores along with some single cells are seen in the image of the diluted culture (shown with red arrows on the left image).

Previous studies by our group with different algal cells (green algae *Chlorella*, seawater *T.chuii*) as well as mammalian cells showed that ultrasound can enhance cell division. The same result was observed for *Schizochytrium* cells in this study. In particular, after the 1st day of stimulation, LIPUS treated samples showed 25-50% increase in cell amount compared to the control one. This can also be attributed to the fact that *Schizochytrium* cells divide by zoospores and, thus, one cell can produce 6 to 20 new cells in one division. Microscopy study also revealed that the sizes of LIPUS treated zoospore samples were larger than that of the control samples, producing more cells in one division due to the sonication.

Although cell amount in the sonicated samples at the end of the experiment was not higher, (Figure 3.7) the most important thing is that higher dry biomass was achieved, compared to the control. It is easily explained by the above-mentioned differences in the types of cells comprising cell culture on the day of harvest – mostly single cells in control flasks and mostly zoospores in LIPUS treated samples.

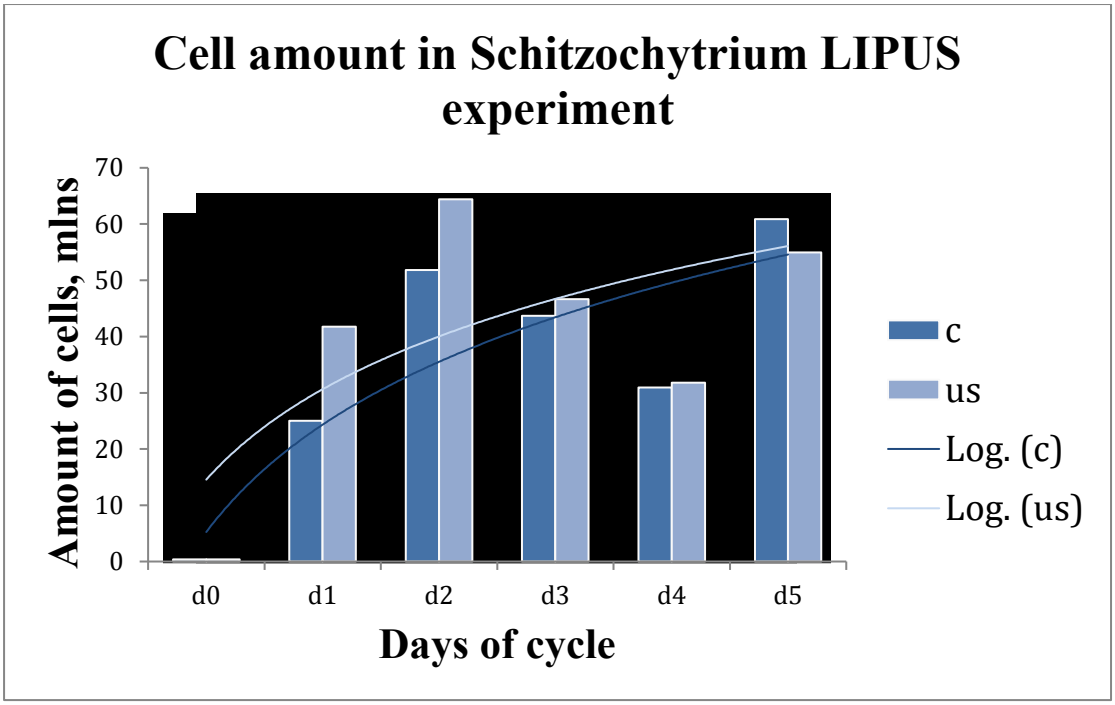


Figure 3.7. Effect of LIPUS treatments at 400 mW/cm^2 (US) on cell amount per mL of *Schizochytrium sp.* culture compared to non-treated sample (control - C).

The OD_{600} measurements (refer to Figure 3.8) also showed the increase in biomass due to the LIPUS stimulation. The LIPUS treated samples also showed higher OD value on D_1 after 24 hours of first LIPUS treatment and on D_5 after D_4-D_5 ultrasound stimulation. During D2 through D4 there was no ultrasound stimulation applied. Continuous stimulation during the whole cycle did not yield biomass increase in the end.

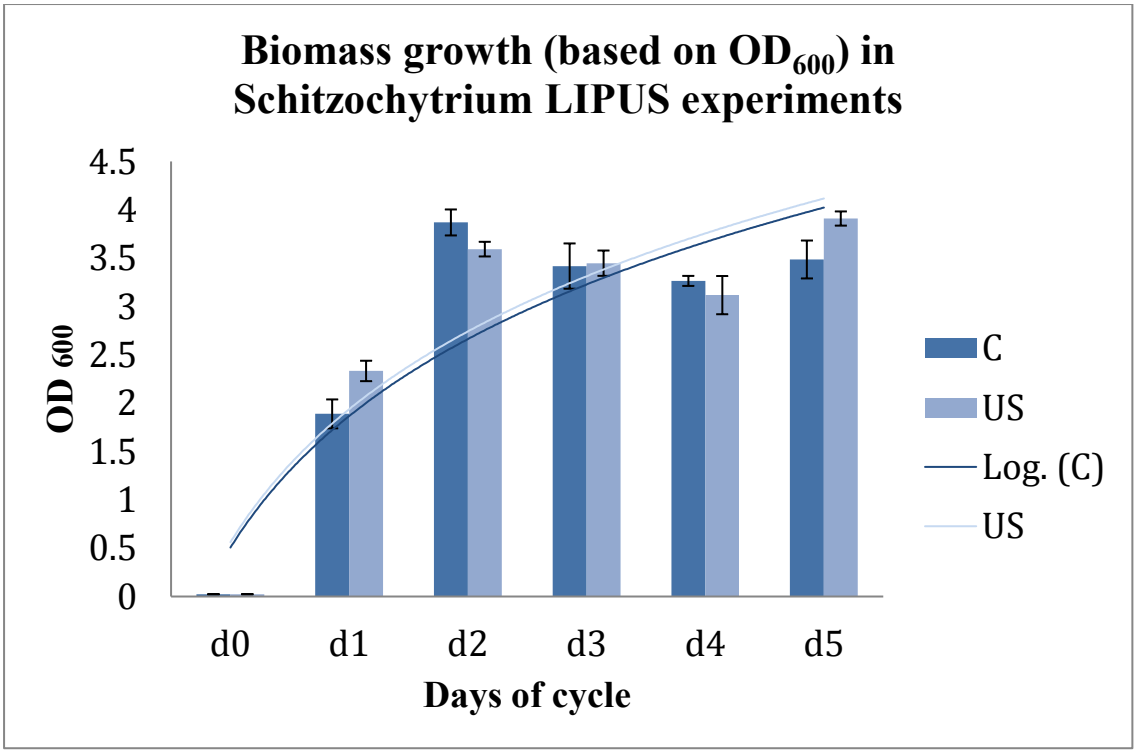


Figure 3.8. The growth of *Schizochytrium sp*. with (US) and without (C-control) LIPUS stimulations based on OD_{600} measurements.

The culture was harvested on Day 5 and up to 20% increase in dry biomass was observed (refer to Figure 3.9). This finding shows the potential of using LIPUS stimulation for biomass increase and growth stimulation of *Schizochytrium sp*.

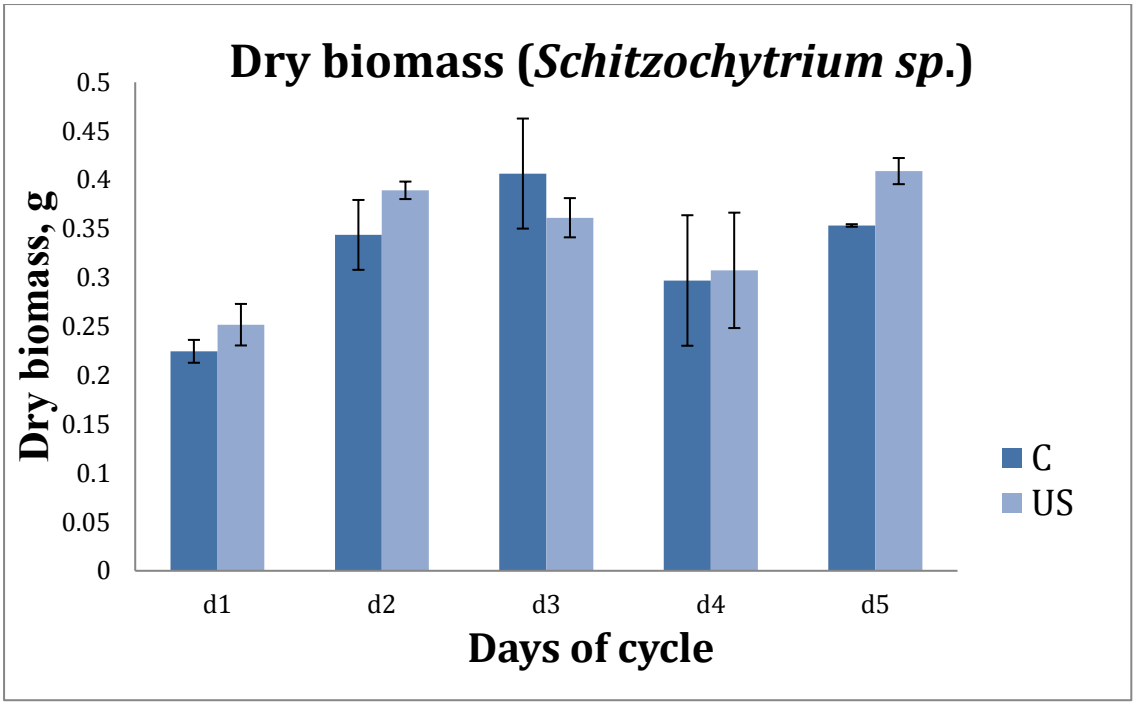


Figure 3.9. Effect of LIPUS treatments of *Schizochytrium sp.* biomass during 5-day growth cycle with the LIPUS stimulation and without (the control). Up to 20% increase was observed based on the data for dry biomass due to LIPUS treatment of the samples.

3.4. Conclusions

Low intensity pulsed ultrasound can be used to increase biomass production of microalgae with the potential to be applied to improve cost-efficiency of microalgae cultivation for DHA, carotenoids, pigments and other biomass-derived chemicals production, as well as for better enriched biomass growth, which can further be used as additive in human nutrition as well as for fish and cattle feed. We have screened several ultrasound conditions in terms of intensity (80-400 mW/cm^2) and duration of treatment 5-30 minutes as well as continuous and non-continuous stimulation during the whole growth cycle on the example of two different algae- *Chlorella* and

Schitzochytrium sp. that have different mechanisms of division and types of growth. In both cultivation processes we have observed that stimulation led to increase in final biomass production up to 20%. Both increase in cell amounts and OD values after 1st round of sonication for the treated samples also confirmed that LIPUS increases cell division and biomass accumulation in the culture. It could be further developed to be embedded in the large-scale systems, such as open ponds, raceways, and photo-bioreactors, for microalgae biomass production.

Chapter 4

Potential explanations on mechanism of ultrasound effect on the cells

4.1. No harmful effect of ultrasound stimulation on cells was detected

It has been shown in the previous two chapters that LIPUS treatment can have a beneficial effect on the cell culture and stimulate both better growth or higher lipid accumulation by algae cells, depending on the purpose of the process. However, little is known about how exactly LIPUS affects the cells. It can be said that LIPUS can improve the flow of nutrients to the cells as well as metabolites from the cells due to microstreaming. To see whether any changes can be observed on the cells we performed Scanning Electron Microscopy (SEM) of *Chlorella* cells without treatment and cells collected and fixed right after a round of ultrasound stimulation. (Refer to Figure 4.1 a-b) Some morphological differences are clearly observed between the samples. Cells after treatment had much more shrinking on the surface compared to control one, while cells without treatment, though also had a shrinking cell wall, still looked much smoother on the surface. Such wrinkles or membrane folds could effectively increase membrane specific surface area for respiration and cell uptake (the cell surface recovers after stimulation is removed). Previous studies by our group showed that 40mW/cm^2 to 60mW/cm^2 stimulation intensity was required for mammalian cells, and higher intensities were needed for plant cells because of the thick cell wall. For thick cell walls more ultrasound treatment is required to achieve the same effect.

It is important to note that LIPUS did not have a harmful effect on the treated microorganisms. Previous studies by our group with LIPUS application on mammalian cells followed by viability studies did not show a decrease in viability of cells. In our studies higher cell amount was observed in the treated samples compared to control as well as the better growth of the microorganisms, which clearly proves that no damage to the cells comes from ultrasound if the right treatment intensity and duration were selected. Lipids, extracted from algae, also showed that higher accumulation of algal oil due to LIPUS did not cause a change in lipid composition. (Refer to Chapter 2). When applied in the study of LIPUS application for wastewater treatment with microorganisms, enhancement of community stability was observed in the culture due to ultrasound effect.

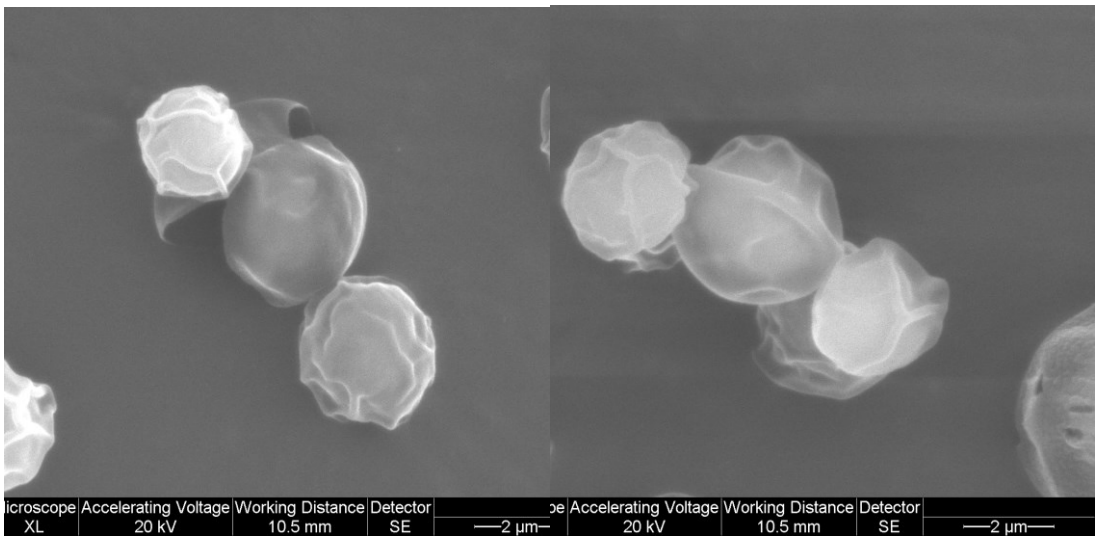


Figure 4.1. **(a)** SEM images of *Chlorella vulgaris* cells without LIPUS treatment (control)

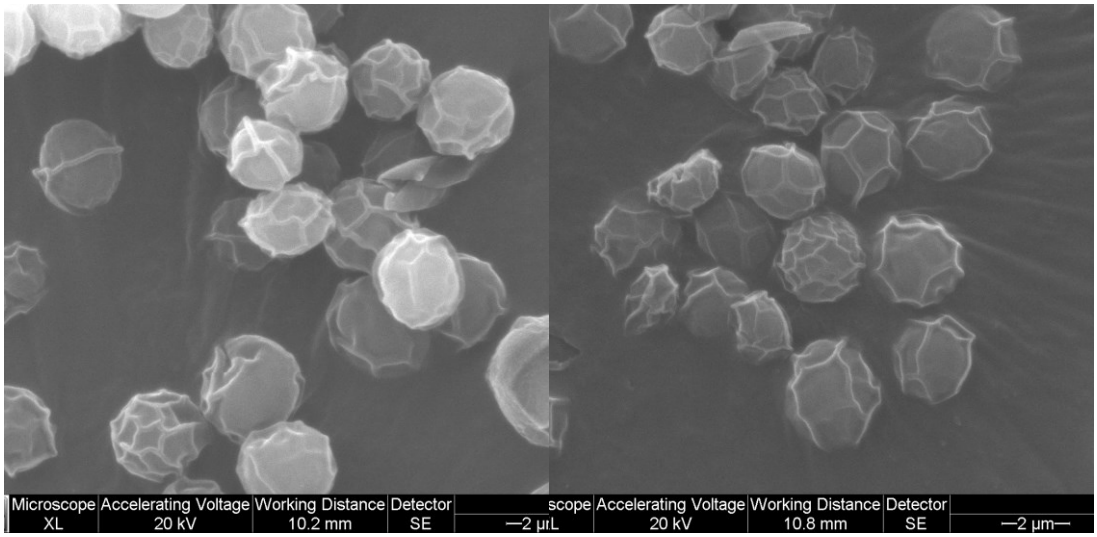


Figure 4.1. **(b)** SEM images of *Chlorella vulgaris* cells collected after LIPUS treatment. Left: ultrasound intensity 80 mW/cm^2, and right: ultrasound intensity 100 mW/cm^2.

4.2. Physical effect of LIPUS

It is known that ultrasound can induce sheer stress on the cells. Considering the culture medium as a non-compressible Newtonian fluid, the shear stress (*F/A*) is proportional to the velocity gradient (*dv/dy*) (shown in Figure 4.2).

Or,

$$\eta = \frac{F/A}{dv/dy},$$

where η is the viscosity of the culture medium. The cell of area *A* is moved with the constant velocity *v* on a layer of the culture medium with the thickness of *y*. The force (*F*) is the drag exerted by the liquid under LIPUS stimulation (refer to Figure 4.2). The shear stress varies depending on the viscosity, the thickness of cell wall and the contact area between cells and culture medium.

The shear stress induced by ultrasound can also cause microstreaming that induces air microbubbles around living cells due to an abundance of dissolved atmospheric gasses in the cell culture media. These microbubbles, encapsulated between the bilayer of the cell membrane, can increase cell permeability when they burst. Based on previous studies by our group (Xing et al., 2012), Lactate Dehydrogenase (LDH) plays an important role in cellular respiration, and it was used to test cell permeability changes. An increase in LDH uptake was observed in pulsed-wave treated cells vs. the control. Suitable amounts of shear stress induced by LIPUS, which can vary for different cell type, can increase protein expression (Shaheen et al., 2013; Zhao et al., 2014). The same effect affects algal cells, or ultrasound can increase cell permeability and help cell metabolism, and thus biomass / lipid content accumulation can be achieved. However, the degree of this effect depends on the thickness of the cell wall. *Chlorella vulgaris* has thicker cell walls than *T. chuii*, hence reduced shear stress explains why the LIPUS stimulation is less efficient in producing lipid compared to *T. chuii*. However, increasing shear stress too much can lead to cell death. Therefore, screening for the appropriate ultrasound simulation condition is crucial.

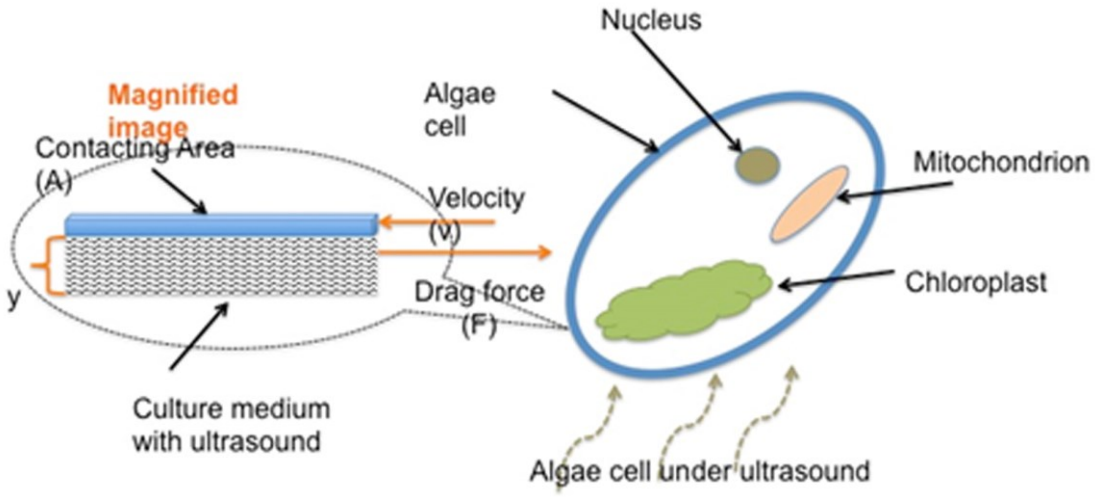


Figure 4.2. Schematic image of proposed mechanism for algae cells ultrasound stimulation. Appropriate amounts of shear stress induced by LIPUS can promote algal lipid or biomass accumulation.

4.3 LIPUS stimulation leads to more active cell division

The effect of ultrasound on the cell amount per 1 mL of sample was also observed in experiments with *Chlorella* and *Schitzochytrium*.

For *Chlorella* significant increase in cell division was detected both during the experiments for lipid accumulation (Refer to table 4.1) as well as during autotrophic 11 days cycle (Refer to Figure 4.3).

In mixotrophic *Chlorella* growth the main focus was on lipid increase and cells were collected few days after glucose was added. No biomass increase was observed, though cell amount was higher in sonicated samples, which could be explained by the smaller size of the cells. It is consistent with the hypothesis that ultrasound stimulates more active cell division and cells after division need time to grow to their normal size.

Table 4.1. Cell count results for *Chlorella* cells on Day 3 and Day 8 of 8-days growth cycle for samples with ultrasound stimulation (60 mW/cm^2, 80 mW/cm^2 and 100 mW/cm^2) and control (no stimulation) showing cell number increase due to LIPUS application.

Sample	Day 3	Day 8
Control	$1{,}85*10^6$	$1{,}82*10^8$
60mW/cm^2	$3{,}04*10^6$	$1{,}70*10^8$
80mW/ cm^2	$4{,}02*10^6$	$2{,}23*10^8$
100mW/ cm^2	$4{,}39*10^6$	$2{,}20*10^8$

In the autotrophic growth cycle the same trend was observed: enhanced cell division in sonicated samples and, because cells had enough time to grow in size, increased cell amount was also reflected in higher biomass.

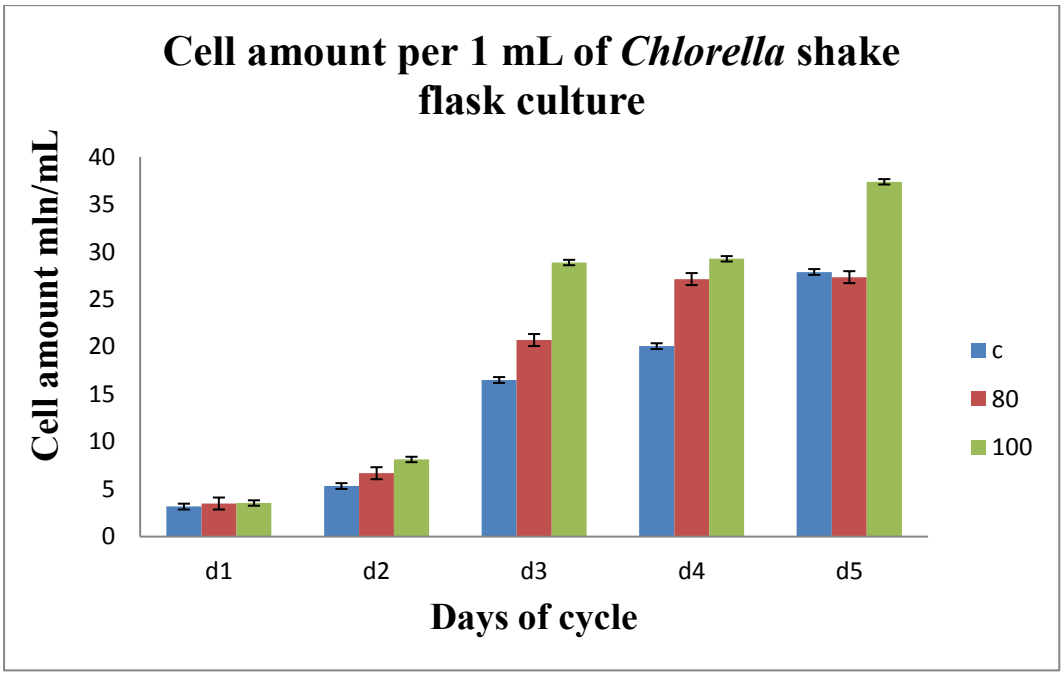


Figure 4.3. Amount of cells per 1 mL of culture in autotrophic shake flask growth of *Chlorella* experiment with ultrasound stimulation (80 mW/cm2 and 100 mW/cm2 samples) and without stimulation (control) showing that LIPUS enhances cell division

For *Schizochytrium* after 1st stimulation on Day 1 sonicated samples with 400 mW/cm2 showed over 25% increase in cell number, compared to control.(Refer to Table 4.2).

Table 4.2. Cell count results for *Schitzochytrium* cells in LIPUS experiment during first 2 days of the cycle, where LIPUS was applied during D0-D1 for treated samples and for control (no stimulation), showing cell number increase due to LIPUS application.

Sample	Day 1	Day 2
Control	$2.5*10^7$	$5.18*10^8$
LIPUS	$4.175*10^7$	$6.44*10^8$

The growth rate μ (Refer to Table 4.3) was calculated for first two days of culture for LIPUS treated samples and for control using the formula:

$$\boldsymbol{\mu = \frac{lnOD2 - lnOD1}{(t2 - t1)}}$$

It can be seen that LIPUS stimulation increased growth rate in stimulated samples on D1, but after the stimulation was stopped the effect did not remain.

Table 4.3. Growth rate, calculated for Day 1 and Day 2 of Schitzochytrium growth experiment with LIPUS stimulation and with no stimulation (control)

Growth rate (μ)/h	Control	US
Day 1	0.176	0.197
Day 2	0.03	0.018

Based on these observations, it can be said that ultrasound treatment stimulates more active cell division in cell culture.

4.4. Impact of low-intensity pulsed ultrasound on gene expression and metabolism of *Saccharomyces cerevisiae*

Several studies performed by our group (LIPUS for algae stimulation, reported in previous chapters; LIPUS for antibody production and for stem cells proliferation (Refer to (Xing et al., 2012; P. Xu et al., 2012; Zhao et al., 2014)) have proven that ultrasound can effectively stimulate cells for better growth and affect some enzymes, stimulating higher activity. To study ultrasound effect deeper, we performed gene expression study of the samples with and without LIPUS treatment, and study of metabolites of the abovementioned samples. For these studies we chose cells of *Saccharomyces cerevisiae* yeast- another microorganism that showed significant response to LIPUS stimulation previously in the experiments run by our group (Shaheen et al., 2013). The yeast cells were selected because *S. cerevisiae* is known as model microorganism in many studies due to its fully known genome and well-studied molecular pathways. In our previous studies LIPUS stimulation with

intensities of 80 mW/cm^2 and 100 mW/cm^2 led to significant increase in both biomass production by yeast cells and more efficient glucose to ethanol conversion by cell enzymes. Based on these results (Shaheen et al., 2013), we treated cells with the same ultrasound intensities for further gene expression and metabolite studies.

4.4.1. Materials and Methods

4.4.1.1. Yeast Strain Culture and Ultrasound Stimualtion

Saccharomyces cerevisiae (ATCC#: 96581) was purchased from the American Type Culture Collection (American Type Culture Collection (ATCC), Manassas, VA 20108, USA). *Saccharomyces cerevisiae* was used for ethanol production in the study of ultrasound impact on fermentation and was routinely grown on YPD (yeast extract-peptone-dextrose) agar plates (pH 4.5) at 30^{o}C for 48 hours. The YPD medium contained yeast extract (3.0 g/L), proteose peptone (5.0 g/L), glucose (10.0 g/L) and agar (20.0 g/L).

Inoculum preparation: The inoculum for the fermentation was prepared by inoculating 100 mL of YPD medium with one or two colonies from the YPD agar plate and by growing the yeast cells for 24 hours at 30^{o}C at 180 rpm×g in a rotary shaker. The seed culture was harvested by centrifuging for 5 minutes at 3000 rpm×g, washing with 0.9% NaCl solution, then resuspending in 10 mL of fermentation broth, which was used to inoculate the fermentation medium as 1% of the total volume.

Fermentation medium: Glucose solution (150 g/L) supplemented with detoxifying solution containing $(NH_4)_2HPO_4$ (0.5g/L), $MgSO_4.7H_2O$ (0.03 g/L), NaH_2PO_4 (1.38 g/L) and yeast extract (1 g/L). The pH was adjusted to 5.5 with 2 M NaOH before sterilization. Autoclaving time was 15 minutes.

Fermentation conditions: The fermentation was carried out in 250 mL Erlenmeyer culture flasks containing 100 mL of fermentation medium at 30ºC for 5 days in a rotary shaker at 180 rpm×g. LIPUS (1.5MHz, 20%duty cycle) was applied to the culture by placing the culture flask in a water-bath chamber (designed for individual flask (Refer to Figure 2.2.a in Chapter 2)) equipped with ultrasound transducer. The ultrasound-generating device generated the ultrasound. The water bath was used to hold the flask in the shaker during the culture cycle. The stimulation condition of 5 minutes per treatment and 12 times treatments every day was selected based on the previous results with *Saccharomyces cerevisiae*. Ultrasound conditions were selected as follows: 80mW/cm^2 × 5min × 12 times/day, 100mW/cm^2 × 5min × 12 times/day while the control stands for no LIPUS treatment.

4.4.1.2. RNA expression analysis of *Saccharomyces cerevisiae*

RNA Sequencing: RNA was extracted from triplicate yeast cultures under three conditions: no ultrasound treatment (control), the ultrasound treatments of intensities at either 100 mW/cm^2 or 80 mW/cm^2, using a Qiagen RNA plant mini kit (RNeasy Plant Mini Kit (50), 79904, QIAGEN Inc. Toronto, Ontario). The RNA was made into a TrueSeq Paired End 100bp library and sequenced on an Illumina HighSeq 2000 system. The sequence was mapped to the Ensemble *Saccharomyces cerevisiae* genome annotated with genes and transcripts using CLC Genomics Workbench 7.0.3. An RNA-Seq analysis was performed on the data and an experiment comparing all 9 samples (triplicate yeast cultures under three conditions) was conducted.

Statistics Study: An ANOVA was calculated on the data, and those samples with an ANOVA p-value less then 0.05 were made into a subset for which the remaining

analysis was completed. A principle components analysis was performed on the samples, as well as a clusters analysis and a series of T-tests by comparing all three groups of samples. The Yeast Gene IDs from the ensemble published genome (http://uswest.ensembl.org/Saccharomyces_cerevisiae/Info/Index) were used to determine a set of gene ontology (GO) annotations for the dataset and fold differences in the original normalized comparison were used along with the GO annotations to create a set of charts using ReVigo (http://revigo.irb.hr/) in order to visualize the areas of greater increased or decreased activity and the general trends that the GO data was showing.

4.4.1.3. Metabolomic profiling of *Saccharomyces cerevisiae*

Chemicals and reagents: LC-MS grade solvent (acetonitrile, methanol and water) was purchased from Thermo Fisher Scientific (Edmonton, AB, Canada). Glass beads (0.5 mm diameter) were purchased from Biospec Products. ^{13}C-dansyl Chloride was synthesized using the protocol published previously (Guo & Li, 2009). All the other reagents and chemicals were purchased from Sigma-Aldrich Canada (Markham, ON, Canada).

Cell lysis and metabolites extraction: 100 µL of 50% MeOH and 0.5 mL of glass beads were added yeast pellets in a 1.5 mL microcentrifuge tube. Five-round one-minute periods of glass bead beating were performed for cell lysis. After lysis, 800 µL of 50% MeOH were added for metabolites extraction. Cell debris were removed by centrifugation (Eppendorf Centrifuge 5417R) at 16,000 x *g* at 4°C for 10 min, and the supernatant was transferred to another microcentrifuge tube and dried down in a Speed Vac (Savant SC110A). The dried extract was re-dissolved in LC-MS grade water, and stored at -80 °C freezer.

Dansylation labeling: 25 µL of the metabolite extract was mixed with 12.5 µL of acetonitrile (ACN) and 12.5 µL of sodium carbonate -sodium bicarbonate buffer. The solution was then mixed with 25 µL of 18 mg/mL ^{12}C-dansyl chloride in ACN for light labeling, or 18 mg/mL ^{13}C-dansyl chloride solution in ACN for heavy labeling. The reaction was carried out at 40°C for 1 hr. After 1 hour, the reaction mixture was cooled down in an ice-water bath and 5 µL of 250 mM NaOH were added to quench the excess dansyl chloride. The solution was then incubated at 40°C for another 10 min. Finally, 25 µL of 425mM formic acid in 1:1 ACN: H_2O (v/v) were added to consume excess NaOH and to acidify the solution.

Sample Normalization: A sample normalization step was performed before LC-MS analysis (Y. Wu & Li, 2012). The total concentration of labeled metabolites was quantified by LC-UV in order to use the same amount of samples for metabolome comparison. 2 µL of the labeled solution was injected onto a Phenomenex Kinetex C18 column (2.1 mm × 5 cm, 1.7 µm particle size, 100 Å pore size) linked to a Waters ACQUITY UPLC system (Waters, Milford, MA) for step-gradient LC-UV. Mobile phase A was 5% (v/v) ACN in water with 0.1% (v/v) formic acid added, and mobile phase B was acetonitrile with 0.1% (v/v) formic acid added. The step-gradient used for LC-UV was: t=0 min, 100% A; t=1 min, 100% A; t=1.1 min, 5% A; t=2.5 min, 5% A; t=3min, 100% A; t=6 min, 100% A. The flow rate was 450 µL/min. The UV detector was operated at 338 nm.

LC-MS: The ^{12}C-/^{13}C-mixtures were injected onto Dionex Ultimate 3000 UHPLC system (ThermoScientific, SunnyValve, CA) linked to a Bruker Maxis Impact

Quadrupole Time-of-flight (Q-TOF) mass spectrometer (Bruker, Billerica, MA). Separations were performed on an Agilent reversed phase Eclipse Plus C18 column (2.1 mm × 10 cm, 1.8 μm particle size, 95 Å pore size). Mobile phase A was 5% (v/v) ACN in water with 0.1% (v/v) formic acid added, and mobile phase B was acetonitrile with 0.1% (v/v) formic acid added. The chromatographic conditions were: t = 0 min, 20% B; t = 3.5 min, 35% B; t = 18 min, 65% B; t = 21 min, 95% B; t = 26 min, 95%; t=34 min, 95% B. The flow rate was 180 μL/min. The mass spectrometer conditions were as follows: capillary voltage, 4500 V; end plate offset, 500V; dry temperature, 230°C; spectra rate, 1.0 Hz; nebulizer, 1.0 bar; dry gas, 8 L/min. All MS spectra were obtained in the positive ion mode.

Data Processing and Analysis: The raw data was exported as a .csv file, which included m/z, peak intensity, peak width and retention time. The peak pairs extraction, peak filter and peak ratio calculations were conducted by a software tool IsoMS. (Zhou, Tseng, Huan, & Li, 2014) The multiple files were aligned by each feature's retention time and accurate mass. The missing values in the feature were filled by Zerofill script. (Huan & Li, 2015) Principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and pathway enrichment analysis were all performed by website based statistical tool MetaboAnalyst (www.metaboanalyst.ca) (Tseng & Li, 2014). Volcano plots were generated by Origin 2015. Metabolites positive identification was done, based on retention time and accurate mass match to a DnsID library (www.mycompoundid.org) (Huan et al., 2015). Metabolites putative identification was performed based on accurate mass match to the metabolites the human metabolome database (HMDB) (www.hmdb.ca) (Wishart et al., 2013) and the evidence-based metabolome library in MyCompoundID (MCID)

(www.mycompoundid.org) (L. Li et al., 2013) with one reaction. MCID library is composed of 8,021 known human endogenous metabolites and 375,809 predicted metabolites from one metabolic reaction.

4.4.2. Results and Discussion

Studying Signal Pathways of S. cerevisiae

A distinct difference between the sample stimulated with 100mW/cm^2 LIPUS and other two samples (Control and 80mW/cm^2) was noticed after principal components analysis of the gene expression was done. Sample treated with 80mW/cm^2 showed less distinct difference from the control (Figure 4.5). Higher intensity of ultrasound showed more effect on this level.

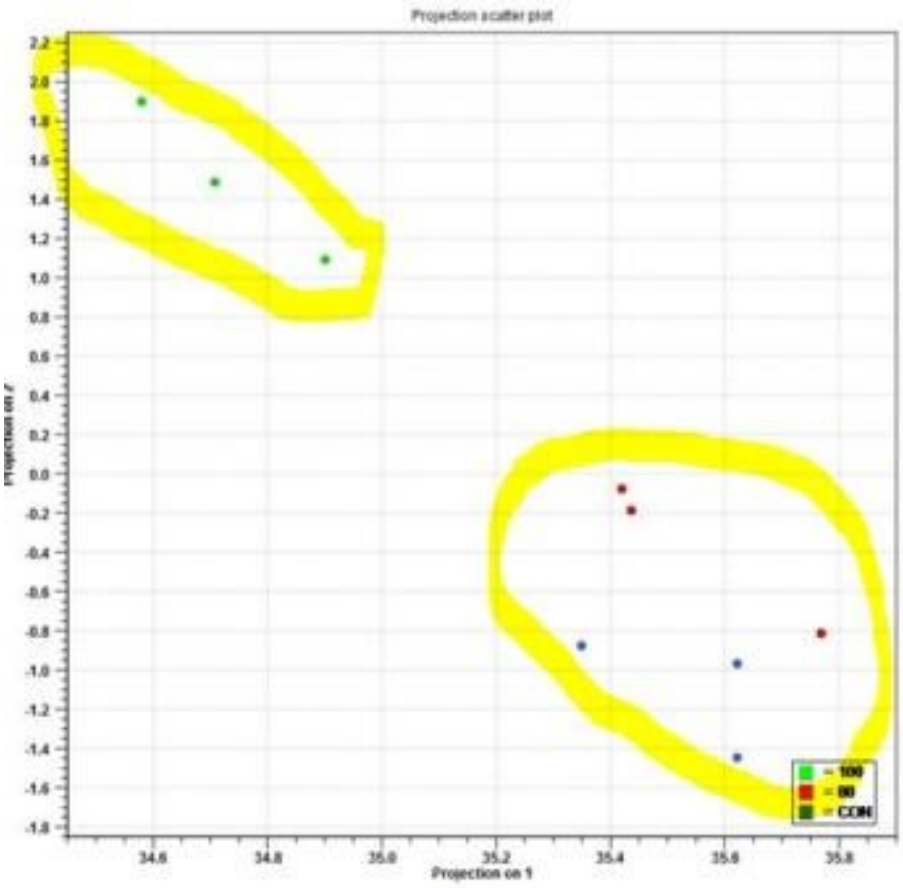
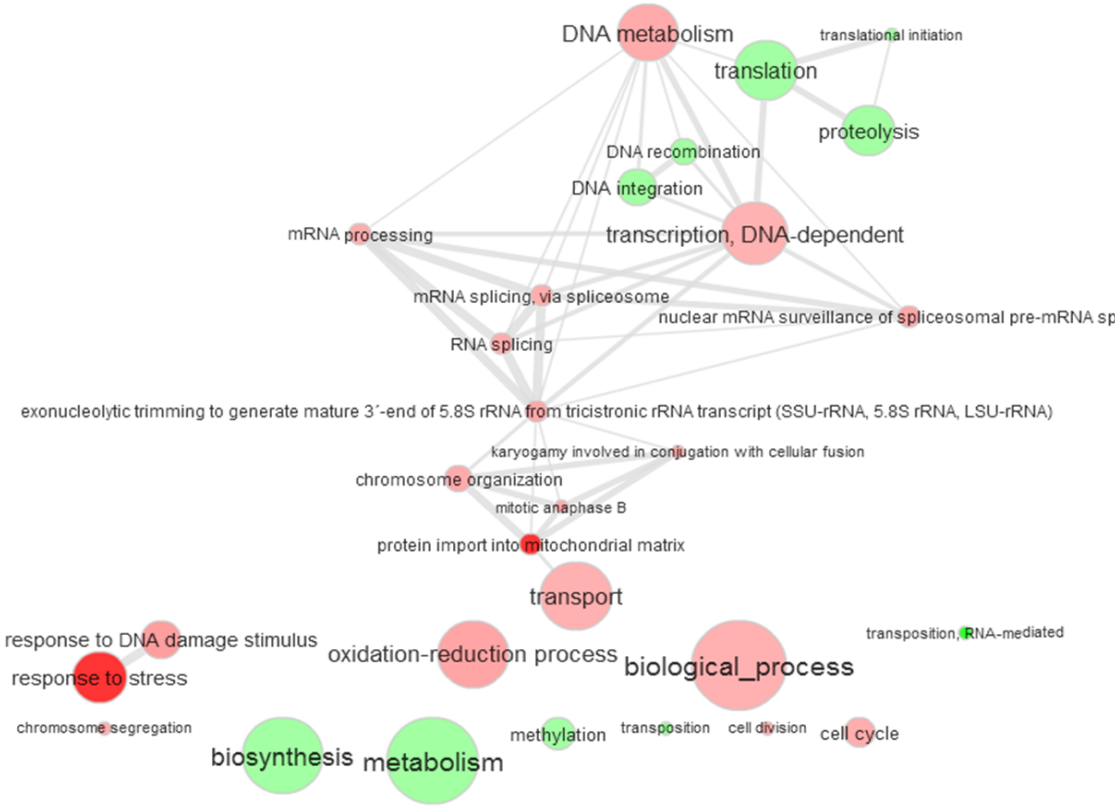


Figure 4.5: PCA analysis of ANOVA statistically significant transformed expression values, which shows obvious separation of 100mW/cm^2 (green dots) treated group from control and 80 mW/cm^2 (blue and red dots).

According to the ANOVA results, genes that showed 2 fold activity change were subjects to T-tests for each combination of pairs and the results of tests were presented in a set of REVIGO graphs for better data visualization. (Figure 4.6)



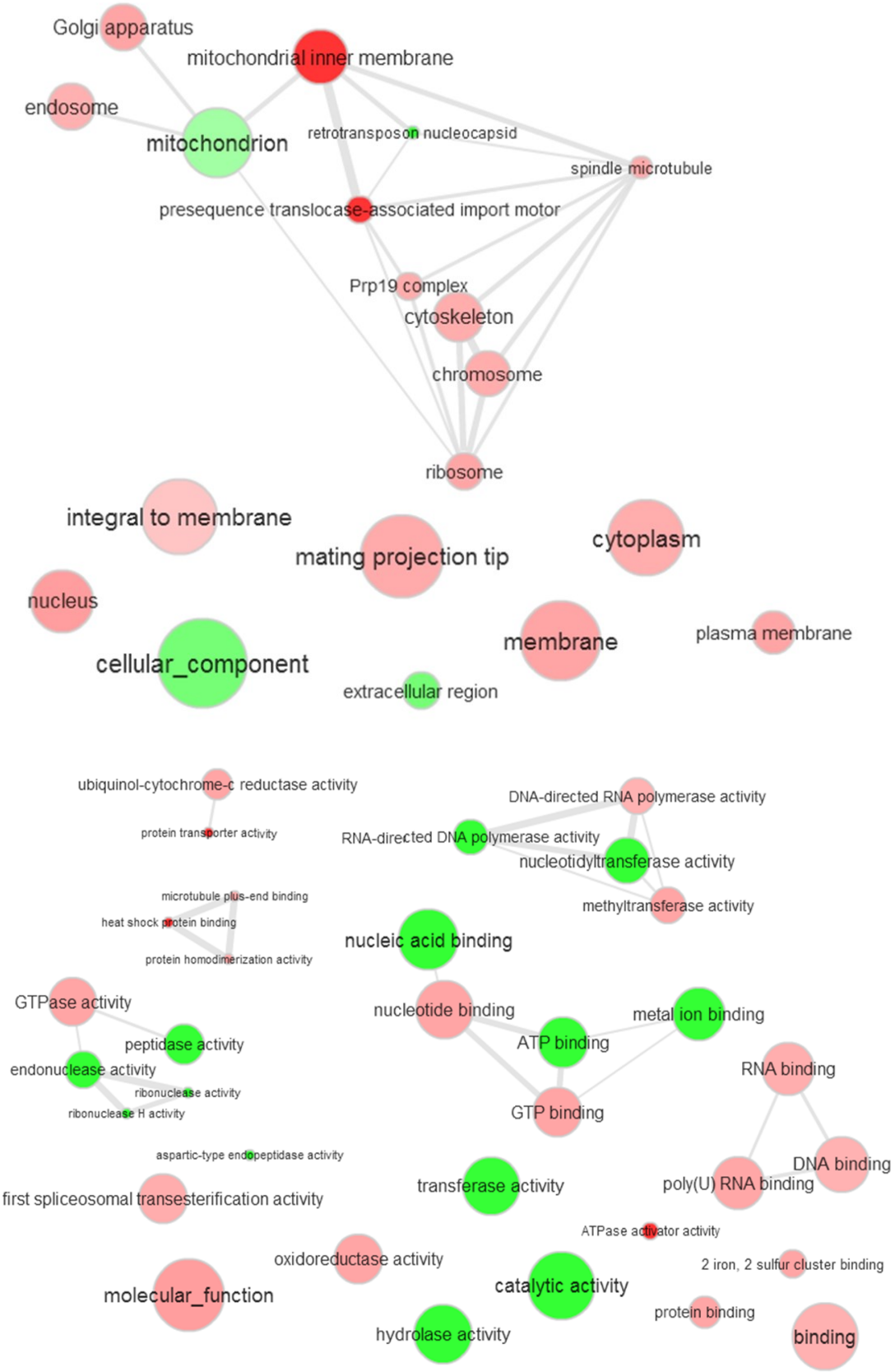

Golgi apparatus
mitochondrial inner membrane
endosome
mitochondrion
retrotransposon nucleocapsid
spindle microtubule
presequence translocase-associated import motor
Prp19 complex
cytoskeleton
chromosome
ribosome
integral to membrane
mating projection tip
cytoplasm
nucleus
membrane
plasma membrane
cellular_component
extracellular region
ubiquinol-cytochrome-c reductase activity
protein transporter activity
DNA-directed RNA polymerase activity
RNA-directed DNA polymerase activity
nucleotidyltransferase activity
methyltransferase activity
microtubule plus-end binding
heat shock protein binding
protein homodimerization activity
nucleic acid binding
GTPase activity
peptidase activity
endonuclease activity
ribonuclease activity
ribonuclease H activity
nucleotide binding
metal ion binding
ATP binding
RNA binding
GTP binding
aspartic-type endopeptidase activity
first spliceosomal transesterification activity
transferase activity
DNA binding
poly(U) RNA binding
ATPase activator activity
oxidoreductase activity
2 iron, 2 sulfur cluster binding
molecular_function
catalytic activity
protein binding
binding
hydrolase activity

Figure 4.6. REVIGO graphs of GO annotated biological processes, cellular components, and molecular functions. Increased fold shown in green, decreased in red. The intensity of the color reflects the fold change.

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Analysis of REVIGO graphs (Figure 4.6) showed that in biological processes and molecular functions there was a decrease in the fold change of 100 mW/cm^2 sample compared to the control (decrease shown in red). While for cellular components there was increase in the fold change observed (shown in green) compared to the control. The size of the dot reflects how frequently the GO term is found in the list of annotations - the larger the size of the dot- the more often it is found. Color intensity reflects the fold change (increased expression is presented in more intense green, while decreased is shown in darker red).

Such visualization shows that the strongest positive fold changes of molecular functions (increases in expression, shown in green) are observed in catalytic activities and nucleic acid binding. Also, a strong decrease in accessions that can be associated with stress response and the mitochondrial inner membrane can be seen.

REVIGO maps also implied that protein import into the mitochondrial matrix to be the most active area of change. The pre-sequence translocase-associated import motor, nuclear mRNA surveillance of spliceosomal pre-mRNA splicing, stress response, and nucleotide binding also showed larger amounts of activity. These areas are consistent between both measures in REVIGO: the frequency of GO terms and the larger absolute value of the fold change.

Overall, LIPUS specifically increased protein synthesis, and not just simply cell growth. In addition, some genes involved in cell division were affected as well as . arabinose and xylose, which could be cell wall as part of cell division, or maybe just increase in consumption of energy (glycogen).

Metabolomic Profiling of S. cerevisiae

The response of a biological system to environmental influence can be reflected in the level of metabolites. Metabolomic profiling can be used to characterize levels of all the small molecules. Chemical isotopic labeling liquid chromatography mass spectrometry (CIL-LC-MS) has been successfully used for metabolomic profiling and has been used in this study to identify changes in metabolites level due to LIPUS in yeast cells. The traditional workflow for metabolomic profiling of yeast includes cell lysis, metabolite extraction, metabolite identification/quantification by analytical platforms, and data processing.

The procedure was done according to the following protocol: *S. cerevisiae* cells were resuspended in 50% MeOH and lysed by glass-beads-assisted lysis. The extracts were then dried and re-dissolved in water, and each individual sample was subject to 12C-dansylation labeling. The total concentration of dansylation labeled metabolites was determined by LC-UV. Based on the total concentration, equal amount of each individual samples were taken and mixed to generate a pooled sample, which was further labeled with ^{13}C-dansyl chloride. After that, each ^{12}C-dansylation labeled individual sample was mixed with an equal amount of the ^{13}C-dansylation labeled pooled sample, and the ^{12}C- /^{13}C-mixture was injected into LC-MS for analysis. Statistical tools were used to analyze the metabolomic data and to determine the dysregulated metabolites of *S. cerevisiae*. The metabolites identification analysis is based on retention time and accurate mass match to different libraries.

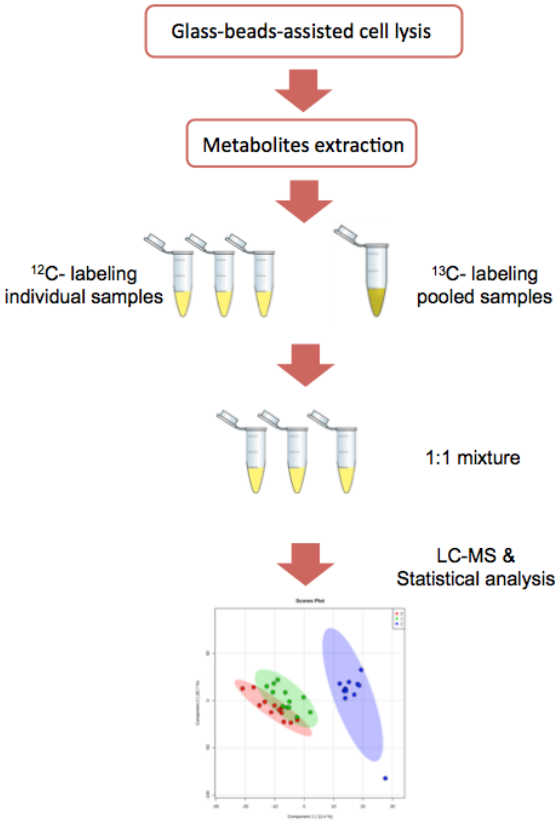

Figure 4.7. Schematic image of the workflow for CIL LC-MS metabolomic profiling of LIPUS treated and control samples of yeast cells.

As the first step, the multivariate principal component analysis (PCA) was done to visualize all the information regarding yeast metabolome data and, according to the analysis, obvious separation of 100 mW/cm^2 treated sample from control was observed. (Figure 4.8a) In the plot, the 80mW/cm^2 treated samples and the control overlap, while the 100 mW/cm^2 treated samples are separated well on the principal component 2 (PC 2) from other two. This implies that metabolome of 100 mW/cm^2 treated samples experienced larger alternation compared to the control, and more significant than the 80 mW/cm^2 treated samples. It was also confirmed by the predominance of partial least squares-discriminant analysis (PLS-DA) (Figure 4.8 b).

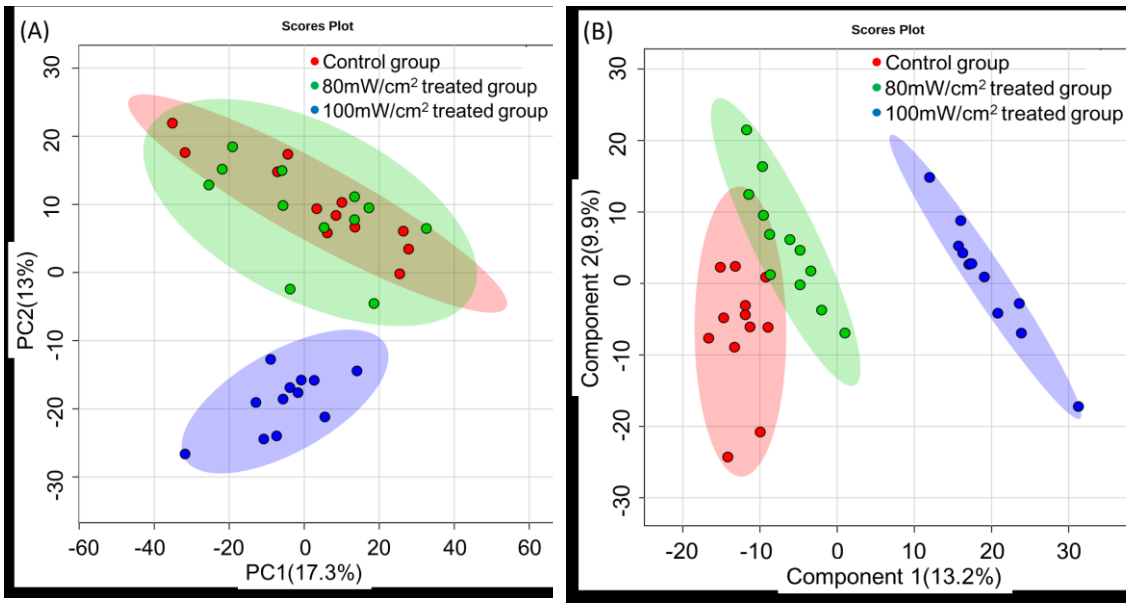

Figure 4.8: (A) PCA score plot of 80 mW/cm^2 treated samples, 100 mW/cm^2 treated sample and the control. (B) PLS-DA score plot of 80 mW/cm^2 treated samples, 100 mW/cm^2 treated samples and the control. The data points were from experimental replicates on biological triplicate samples. Both analysis score plots show obvious separation of 100 mW/cm^2 treated group.

The volcano plots that reflect dysregulated metabolites of 80 mW/cm^2 versus control were shown in Figure 4.9b and 100 mW/cm^2 versus the control were shown in Figure 4.9a; the threshold of p-value was 0.05. According to volcano plots 434 upregulated metabolites and 229 downregulated metabolites were observed for 100 mW/cm^2, while only 87 upregulated metabolites and 36 downregulated for 80 mW/cm^2. The number of dysregulated metabolites of 80 mW/cm^2 treated samples, thus, was much less. The result further confirms that the higher intensity of ultrasound led to more significant impact on metabolism of *S. cerevisiae* cells.

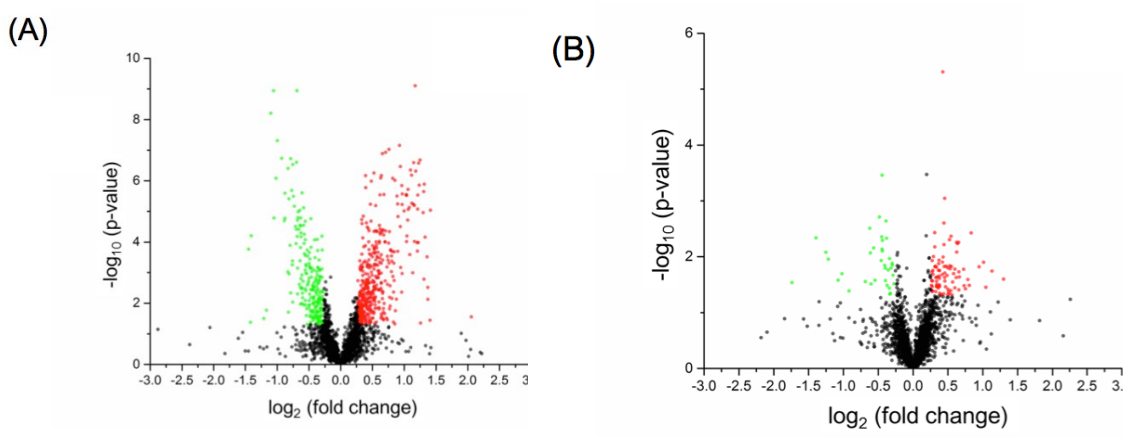

Figure 4.9. (A) Volcano plot of 100 mW/cm^2 treated samples versus the control, (B) Volcano plot of 80 mW/cm^2 treated samples versus the control. The up-regulated or down-regulated metabolites by at least 1.2-fold, with p-values of smaller than 0.05, are marked in red and green, respectively.

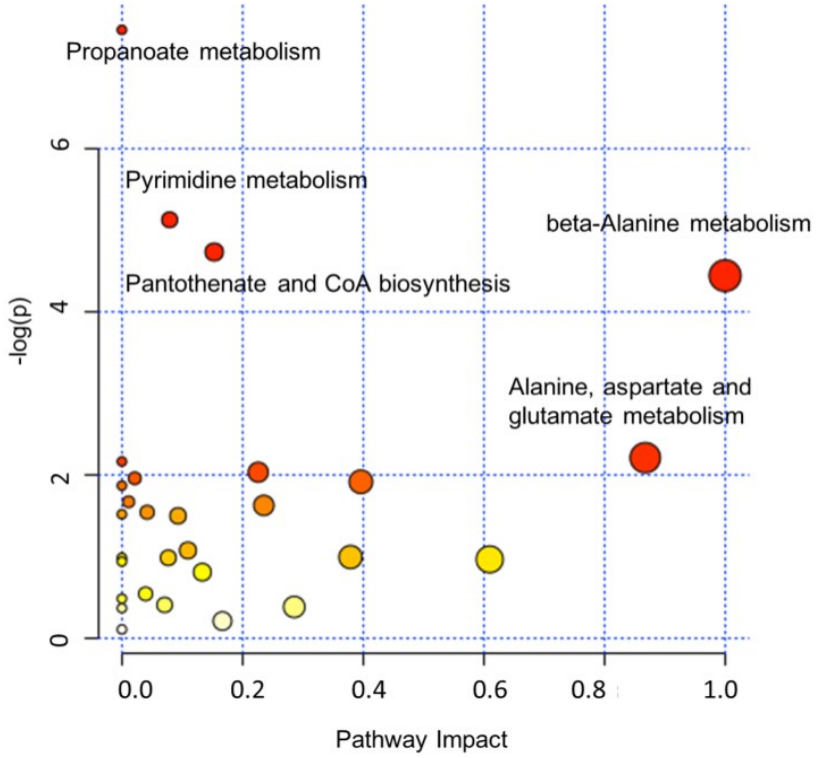

Figure 4.10. Analysis of metabolic pathway enrichment results overview.

Metabolomic profiling includes all metabolites level alternation in *S. cerevisiae* after LIPUS treatment. To understand what metabolic pathways were

associated with those metabolites changes, the identified metabolites were input into the Pathway Analysis tools in Metaboanalyst, which is integrated with enrichment analysis and pathway topology analysis. The pathway enrichment analysis gives quantitative analysis, directly based on metabolites concentration, and topological analysis is used for the structure of pathways. Figure 4.10 presents combined enrichment and topological analysis in overview of pathway analysis. The y-axis represents the p-value calculated from pathway enrichment analysis, while x-axis represents the pathway impact values from topological analysis. Darker red color reflects larger p-value, and larger node radius represents larger impact values.

The most noticeable changes in pathways metabolomics data are associated with biosynthesis of pantothenate and coenzymeA (CoA), amino acid metabolism/biosynthesis and aminoacyl-tRNA biosynthesis. In biosynthesis of pantothenate and CoA metabolism, dysregulation of β-alanine and pantothenic acid-metabolic intermediates in CoA biosynthesis was detected. CoA plays an important role in oxidation of fatty acids and carbohydrates. The gene chip data showed dysregulations of branched-chain amino acid aminotransferase and ketol-acid reductoisomerase. The accumulation of β-alanine and pantothenic acid may be associated with the dysregulation of the two upstream enzymatic processes. (Figure 4.11 shows pathways that are associated with dysregulated metabolites).

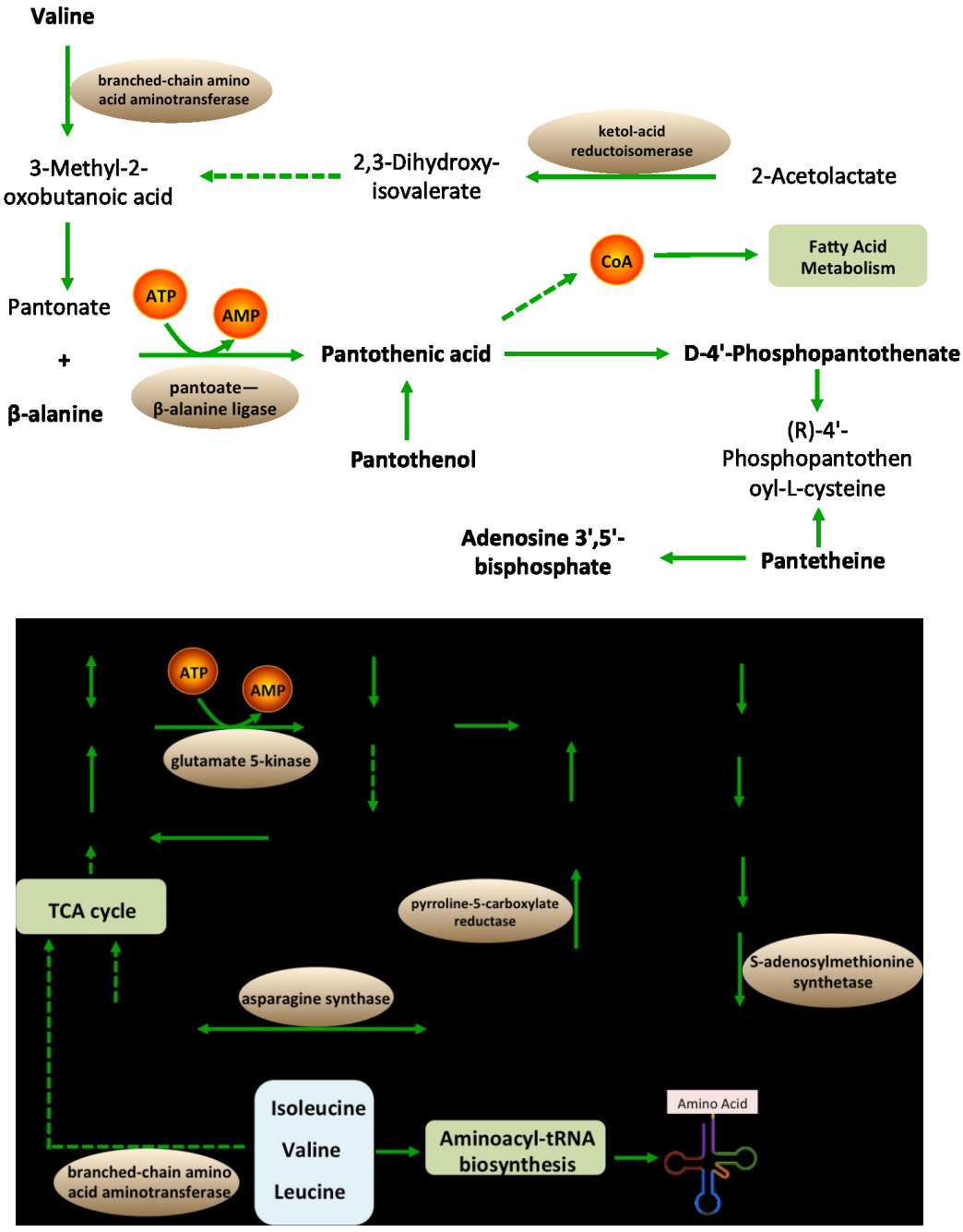

Figure 4.11. Two of the pathways affected by LIPUS as suggested by metabolomic study and pathway analysis.

Dysregulation of amino acid metabolism and aminoacyl-tRNA biosynthesis was also detected. Dysregulation of asparagine synthase may lead to asparagine accumulation. The dysregulation of aminoacyl-tRNA biosynthesis is associated

with protein synthesis (refer to Figure 4.11). The enrichment pathway analysis indicates that LIPUS has impact on energy metabolism, amino acid metabolism and protein synthesis.

4.5. Conclusions

Mechanism of LIPUS effect on cells is complicated and still to be clarified and described in more details. Our experiments showed that LIPUS has no harmful effect on the cells and with the correct treatment parameters does not lead to decreased cell viability. The physical effect of LIPUS is associated with microbubbling, microstreaming and cavitation and shear stress and is dependent on the viscosity of the medium, thickness of cell wall and contact area. Our experimental results on algae cells of *Chlorella* and *Schitzochytrium* show that LIPUS stimulates more active cell division and increases the growth rate of the cells in the culture. A separate study on *Cerevisiae* yeast cells was carried out to see whether changes caused by LIPUS could be identified on gene expression level or metabolites level. That study confirmed that LIPUS effect cannot be attributed to changes only in one or two genes, enzymes or metabolites. LIPUS effect was detected on energy metabolism, amino acid and protein synthesis and catabolism, and the cell division of *S. cerevisiae* cells. The effect may be due to the stress response of *S. cerevisiae* cells to LIPUS stimulation.

Chapter 5

Water purification using active carbon based filters covered with silver nanoparticles and its potential application in agriculture and bioprocesses

5.1. Introduction

One of the challenges that a lot of bioprocesses face is the large amount of water needed for biotechnological production and costly methods of its purification. Recycling of water or its use for other processes could be a solution, but such systems that re-circulate water are prone to contamination and pathogens are often being introduced into the system with water. One of the examples is hydroponic vegetable production, where re-circulation of water leads to pathogens spreading quickly once they get into water systems and causing the yield losses of agricultural products, significantly affecting their production. Highly destructive plant diseases, such as late blight, have become substantial risks to greenhouse tomatoes because of their presence in farm fields and home gardens. Pathogens can be introduced from dugout water used for greenhouse irrigation. The standard practice in modern vegetable production uses recirculated nutrient solution that further increases risk by spreading disease from infected plants throughout the entire greenhouse. Various techniques

have been applied to treat recirculated nutrient solution in greenhouses, including heat treatment, ozone treatment, UV disinfection, H_2O_2 treatment, biofilters etc. Biofilters are used in greenhouses to treat recirculated nutrient solutions, but the main purpose of the system is to convert ammonia to nitrogen gas and remove carbon dioxide and various organic contaminants, and it has less effect on removing pathogens (Berghage, 1996; Tyson, Simonne, Treadwell, White, & Simonne, 2008). Better water treatment solutions are needed for plant disease management in the hydroponic-grown vegetables. Silver (Ag+) has been studied for use in disinfection of various harmful microorganisms because of its multiple modes of inhibition (Dibrov, Dzioba, Gosink, & Häse, 2002; Maiti, Krishnan, Barman, Ghosh, & Laha, 2014; Mazurak, Burrell, Tredget, Clandinin, & Field, 2007; Richter et al., 2015; Safavi, 2011). Recently, several promising reports on using silver nanoparticles (AgNPs) against plant pathogenic fungi have been published (Jo, Kim, & Jung, 2009; Karumuri, Oswal, Hostetler, & Mukhopadhyay, 2013; Kim et al., 2009, 2012; Lamsal et al., 2011; Nasrollahi, Pourshamsian, & Mansourkiaee, 2011; Prabhu & Poulose, 2012; Safavi, 2011; Wani & Shah, 2012; Zahir, Bagavan, & Kamaraj, 2012). The mechanisms and potential application of synthesized AgNPs as alternatives to pesticides have been reviewed (Al-samarrai, 2012; Sahayaraj & Rajesh, 2011), but there have been few attempts to bring AgNP technology to practical use.

Nanotechnology is an emerging branch of science and has been applied in clinical methodologies including imaging, diagnostic, therapeutics, drug delivery and tissue engineering (B, 2011; Vijaya Shanti B; Mrudula T; Naga Deepth CH; Sree Venkateshwarlu Y, 2011). It has great potential applications in controlled environment agriculture (CEA), an advanced and intensive form of hydroponically based agriculture (Joseph & Morrison, 2006). AgNPs have attracted much attention in

agricultural application such as reduction of the root diseases incidents and improvement of the growth and health of various plants (Tien An, Dong, Hanh, Nhi, & Vu, 2010). It can also be used to clean ground water. Argonide in USA used 2 nm diameter aluminum oxide nanofibres (NanoCeram) as a water purifier that can remove viruses, bacteria and protozoan cysts from water (http://nanotechweb.org/articles/news/3/4/7). BASF is one of the largest chemical producers in the world and has devoted a significant proportion of its nanotechnology research fund to water purification techniques. Many countries in Europe, Asia and America have worked on the development of nanoscale materials for water purification (Joseph & Morrison, 2006). Nanotechnology has been used in municipal and rural water treatment to remove bacteria. Nanosilver-coated polypropylene water filter has been designed and evaluated to treat *Escherichia coli* contaminated drinking water (Heidarpour, Ghani, & Ahmadun, 2010). AgNP-coated materials were investigated for the removal of bacteria from ground water (Mpenyana-Monyatsi, Mthombeni, Onyango, & Momba, 2012). Potentially, these nano-materials can be applied to hydroponic production to control plant diseases. Furthermore, they can be applied to treat dugout water for plant irrigation in greenhouses in rural areas.

With regard to the environmental consequences of AgNP, so far no universal conclusion has been drawn about the toxicity of AgNP. The adverse effect in humans of chronic exposure to silver includes but not limited to argyria or argyrosis of the skin and/or the eyes. Many studies show that only the release of ionic silver has been found to be toxic, but the release alone cannot be accounted for the toxic side effects. In the aqueous condition, the ionic silver tends to form silver chloride and silver sulphide, which are highly stable.

This chapter presents a procedure to develop a new water filter material using the silver nanoparticles-coated substrate. Activated carbon (AC) has been successfully used for water purification over the past decades as a cheap and efficient filter material. We have combined the antibacterial properties of silver with absorbent properties and large surface area of activated carbon for water purification. The novel approach introduces silver nanoparticles both to the inner and outer surface of active carbon and covers the pores, which significantly increases active contact surface of material with water and improves efficiency of filtration. The potential application of such materials in filtration was tested against fungal and bacterial pathogens, most often occurring in greenhouse vegetables.

5.2. Materials and Methods

5.2.1. Sample Preparation

Two types of AC materials were tested - bigger size AquaSorb® 1500 (Jacobi) from Bituminous coal, water washed, size 0.6-2.36 mm; and smaller size AC - CR1230C-AW (Carbon Resources manufacturer), from coconut shell, acid washed, size 0.6-1.7 mm.

Two types of coating modes have been studied in order to achieve better killing effects – single coating and double coating. Sample preparation was done according to the following protocols:

Single coating: A range of $AgNO_3$ concentrations were tested to determine the optimum concentration. $AgNO_3$ solution (3 mL) of different concentrations (3, 8, 11, 17, 20, 23, 33 g/L) was mixed with 2 g of active carbon, gently shaken and left overnight to penetrate into pores and be

absorbed by the carbon particles. After that, remains of the solution were removed, and 10% HCl was used in the analytical reaction for Ag+ detection to prove that all Ag was absorbed and none was left in the solution. Three millilitre of $NaBH_4$ solution (5g/L) was added for the reduction reaction into above AC and shaken evenly. Samples were then washed using deionized (DI) water.

Double coating: The single coated sample was used. Two grams (wet weight) of the previously coated sample were mixed with 1.6 mL of $AgNO_3$ solution (10.8 g/L concentration). Samples were shaken gently and left for an hour at room temperature. Samples were then washed using DI water. A total of 1.6 mL $NaBH_4$ (6 g/L) solution was quickly added to the sample and shaken until bubbles disappeared. Samples were then washed three times with DI water and treated for removal of remaining liquid.

Three types of sample-drying treatments were evaluated: (a) removal of the liquid using a pipette; (b) freeze-drying with vacuum; and (c) drying in the oven (60°C) for 2-4 hours. AC mixed with DI water without $AgNO_3$ was used as a control.

5.2.2. Sample Characterization

The coated materials were characterized using standard methods such as Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), and X-ray diffraction analyses.

SEM: AgNP treated activated carbon particles were mounted on the stabs and air-dried at room temperature in the dark, then coated with evaporative carbon in Leica EM SCD005. Samples were observed with a Field Emission scanning electron microscope (JEOL 6301F).

TEM: Samples of AC pre-treated with AgNP were air-dried and then embedded in the plastic and thin cuts (80-100 nm) were made using glass knife. TEM images were taken using a Philips-FEI Morgagni 268 instrument, operated at 80 kV.

X-Ray analysis was performed on the Rigaku Ultima IV Powder X-Ray diffractometer. Samples were air-dried at room temperature and prepared in the same way as for the SEM imaging.

ICP-MS: Detection of silver (Ag^+) ions in the filtrates was performed using ICP-Mass. Samples (0.5 g) were mixed with 2 mL of DI water and shaken overnight. After that water was filtered through 0.22 μm millipore filter to remove remains of carbon and then was tested for Ag^+.

5.2.3. Evaluation of the Developed Filter Materials

Common fungal pathogens, *Botrytis* sp., *Fusarium* spp., *Pythium* spp., *Rhizoctonia* sp., and *Sclerotinia* sp. as well bacteria (*Paenibacillus* sp. and *Pseudomonas* sp.) were collected from greenhouse cucumbers, lettuce, tomatoes and peppers. Infected plant tissue was surface disinfected in 0.5% bleach, rinsed in sterile distilled water and placed on potato dextrose agar (PDA). Fungal isolates were purified by single spore/single colony culture technique and stored at 4°C.

5.2.3.1. Screening Test: Preliminary efficacy of AgNPs treated AC samples was tested against yeast cell culture on the lab scale (*Saccharomyces cerevisiae*) to determine the best drying condition and optimum AgNP concentration at different treatment duration as well as for selection of AC material. One colony of two-day-old *S. cerevisiae* culture was transferred from yeast extract-peptone-dextrose (YPD) agar

plate to 100 mL of YPD broth and incubated on a shaker at 180 rpm at 30°C for 24 hours. One mL of diluted (OD_{600} = 0.1) overnight grown yeast culture was added to 20 mL vial containing 0.5g of AgNPs-AC (wet weight). The mixture was shaken for 3 to 24 hours, and plated on YPD agar plates and incubated for 24 hours.

5.2.3.2. AgNP-AC against pathogenic bacteria and fungi (static test): Activated carbon treated with sliver nanoparticles (20 g/L or 23 g/L) were evaluated for their effect against a bacterium *Pseudomonas sp.,* and a fungus *Fusarium oxysporum*. A 0.5 g of each sample was added to 5 mL spore suspension (approximately 3×10^6 spores/mL) in a test tube. AC treated with water was included as a control. Tubes were incubated on a shaker at 200 rpm at room temperature. A drop (10 µL) of the culture mix was plated to a PDA plate after growing for 1 h and 24 h, respectively, with four plates per treatment. Plates were then incubated for 3 days at 25°C in an incubator and the colonies per plate were counted and recorded to calculate the killing efficiency of filter materials.

The percent of colony forming units (CFU) reduction efficiency was calculated based on the CFU counts of bacteria on control plate:

*Efficiency (%) = (CFU of Control – CFU of Treatment) / CFU of Control * 100*

5.2.3.3. AgNP-AC against pathogen suspension (dynamic test): Smaller size carbon (CR1230C-AW) had larger surface area and showed better results on the small scale, and thus we used it for the scale-up tests. Sample preparation was performed using the same protocol as at a smaller scale. Dynamic-flow scale-up testing was

performed using bacterium *Paenibacillus polymyxa* with untreated AC as the control. A cylinder column (100 mL) was filled with active carbon (CR1230C-AW) pre-treated with single or double coating of AgNP. Bacterial (*Paenibacillus*) suspension with concentration about 10^5 CFU/mL was filtered through the column at a flow rate 70-75 mL/h. The test was run non-continuously for 6 days in a total of 6 hours per day. Filtrates were collected hourly for first 3 days, then every 3 hours on day 4 and every 6 hours on day 5 and day 6. Collected samples were plated on the PDA plates and total CFU per plate were counted after incubation for two days. This test was repeated three times.

The percent of antibacterial efficiency was calculated based on the original bacterial concentration:

*Efficiency (%) = (1 - bacterial concentration in filtrate / original bacterial concentration)*100*

5.3. Results and Discussion

Multiple tests with different AgNP concentrations showed that efficacy of samples against yeast *S. cerevisiae* increased with the increase of silver concentration from 3 g/L to 23 g/L (data not shown here), and a decrease in efficiency with further increase of the concentration was observed. It was not efficient at all when the concentration reached 33 g/L. The optimal efficacy was reached at concentration of 20-23 g/L (Figure 5.1), and declined further. Based on these results, samples with the concentration of AgNPs between 20 g/L and 23 g/L were chosen for further testing against other pathogens (*Pseudomonas* and *Fusarium*).

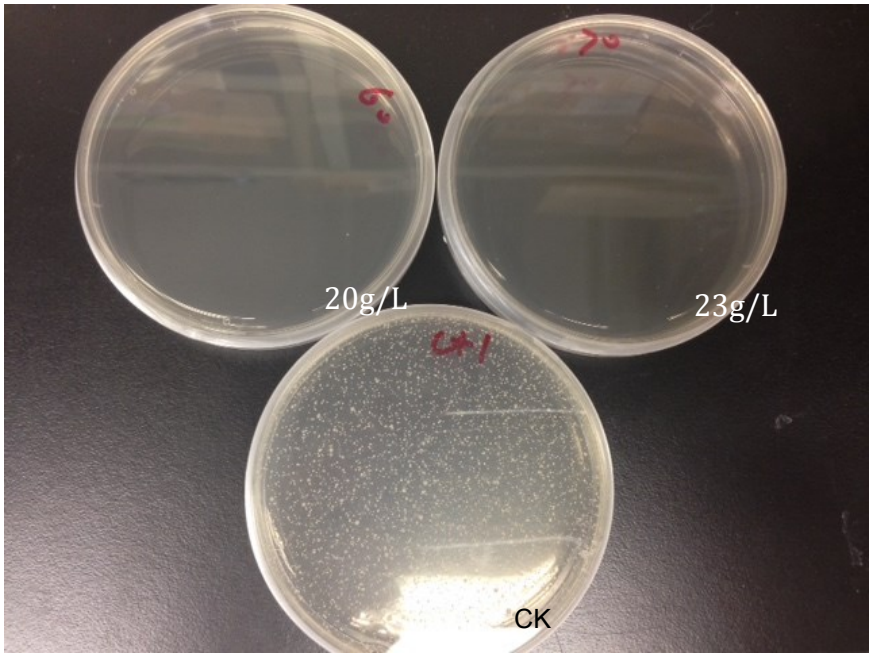

Figure 5.1. The efficiency of the AgNP-AC prepared at two optimal concentrations (20g/L and 23 g/L) against *S. cerevisiae* yeast cells. AC without AgNP coating was used as the control (CK). We can clearly see AgNP-AC killed all *S. cerevisiae* cells.

Our preliminary results showed that, although AC coated with AgNPs is effective in killing microorganisms, different sample preparation methods could have different impacts. We tested three drying methods in the sample preparation and tested the samples against *S. cerevisiae* cell culture. Results showed that all three drying methods left some killing potential for AgNP-treated AC samples to kill the yeast cells. However, the efficacy of treated AC samples significantly decreased with the use of vacuum or an oven for drying while samples that had only liquid removed by pipetting were active and showed good killing results. Consequently, we used pipetting as a drying method for further experiments performed.

Time (treatment duration) for antimicrobial efficiency by filter material is another important factor to consider. While 20 to 24 hours of treatment gave excellent results, shorter time did not (data not shown here). Therefore, we tested another type of AC

material with smaller particle size and larger surface area to solve this problem. Another approach that we tested was second coating of the material. Results showed that smaller size carbon had better cell killing efficacy. While for larger sized AC, 20 to 24 hours of treatment were required; 3 hours were enough for smaller sized AC. The larger surface area of the carbon with smaller particle size can easily explain this phenomenon. Second coating also improved results with the larger sized AC, but smaller AC-AgNP still showed better results (Figure 5.2). Overall, up to 99% killing of *S. cerevisiae* cells were observed with both single and double-coated sample treatments.

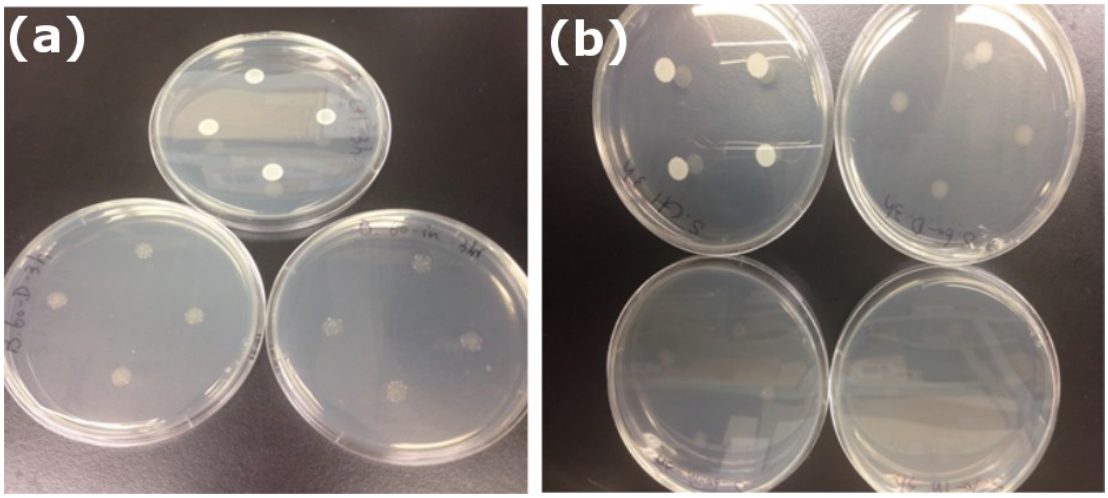

Figure 5.2. Screening test of AgNP-treated AC samples against yeast *S. cerevisiae* cells. **(a)** Bigger sized AgNP-AC sample with single and double-coating. Upper plate-control, bottom (left to right) treated with double-coated sample and with single coated after 3-hour treatment, respectively. Only single colonies were observed. **(b)** Smaller sized AC with no treatment (upper left – control), upper right- double-coated AC, bottom row - single coated ACs. With smaller sized AC, both single-coated and double-coated samples showed excellent cell killing (almost no cells left after 3 hours treatment, compared to the control).

5.3.1 Characterization of AgNP coated AC

Scanning Electron Micrographs (SEM) showed the distribution of silver particles that were quite evenly coated on the surface and inside pores of activated carbon (Figures 5.3(a), 5.4(b)) while there were no silver particles found on the untreated AC (Figures 5.3(b) and 5.4(a)). The silver particles had different sizes (Figure 5.3(c)) and can be found inside porous area (Figure 5.3(d)), or become aggregated (Figure 5.3(e)) and form larger particles (Figure 5.3(f)). The micrograph samples with double and single coating have similar appearance (Figures 5.3-5.4), or there was no obvious difference between samples of smaller and larger sized AC (In both cases, we used the same concentration of silver to coat them). X-ray analysis confirmed presence of silver nanoparticles on the surface of treated active carbon (Figure 5.4(d)), and no silver particles on untreated AC (Figure 5.4(c)).

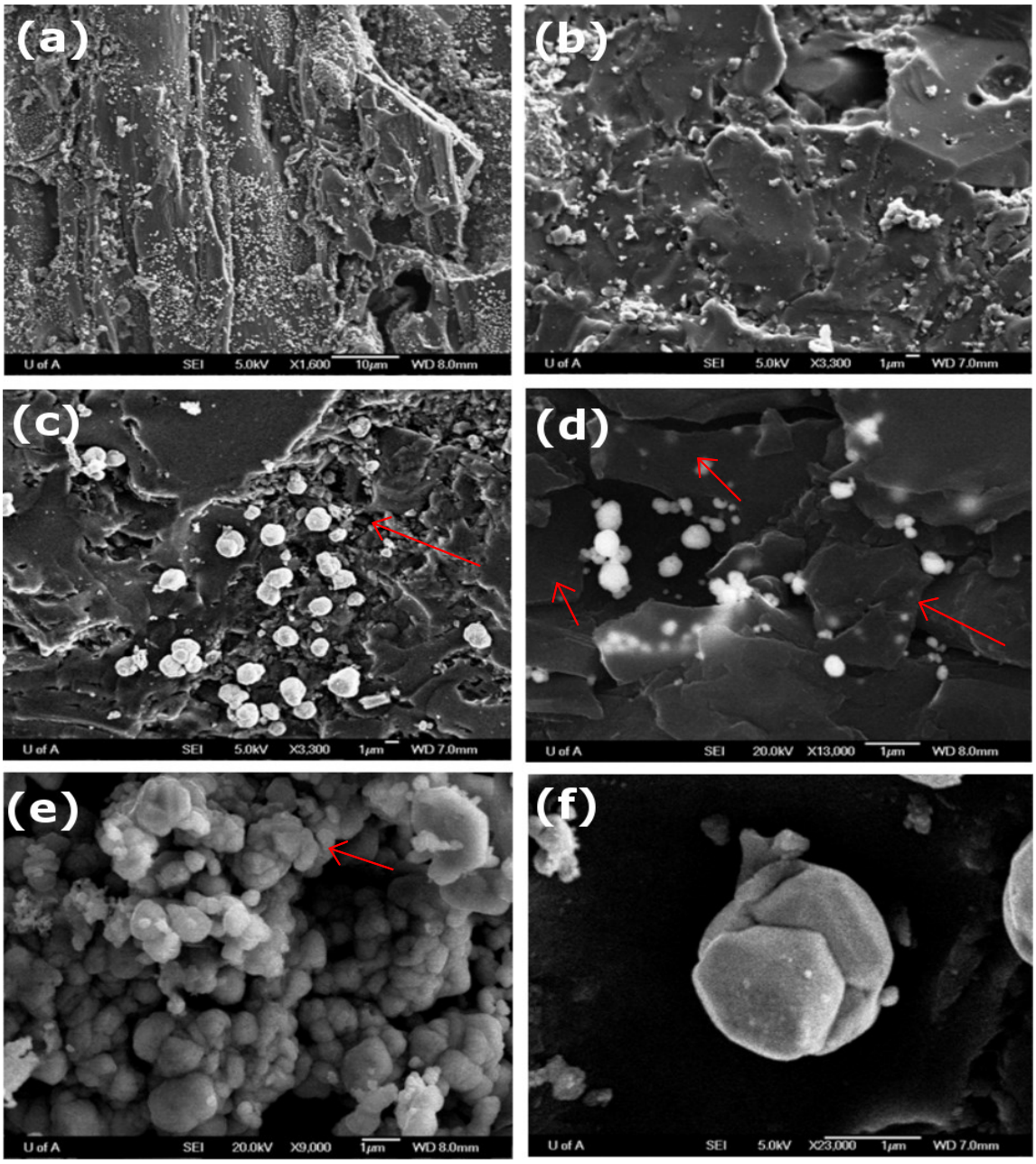

Figure 5.3. Scanning Electron Micrographs (SEM) of single-coated samples: **(a)** Silver nanoparticle (20 g/L)-treated activated carbon (AC); **(b)** untreated AC; and **(c) – (f)** AgNPs (23 g/L) treated AC. AgNPs are shown with red arrows.

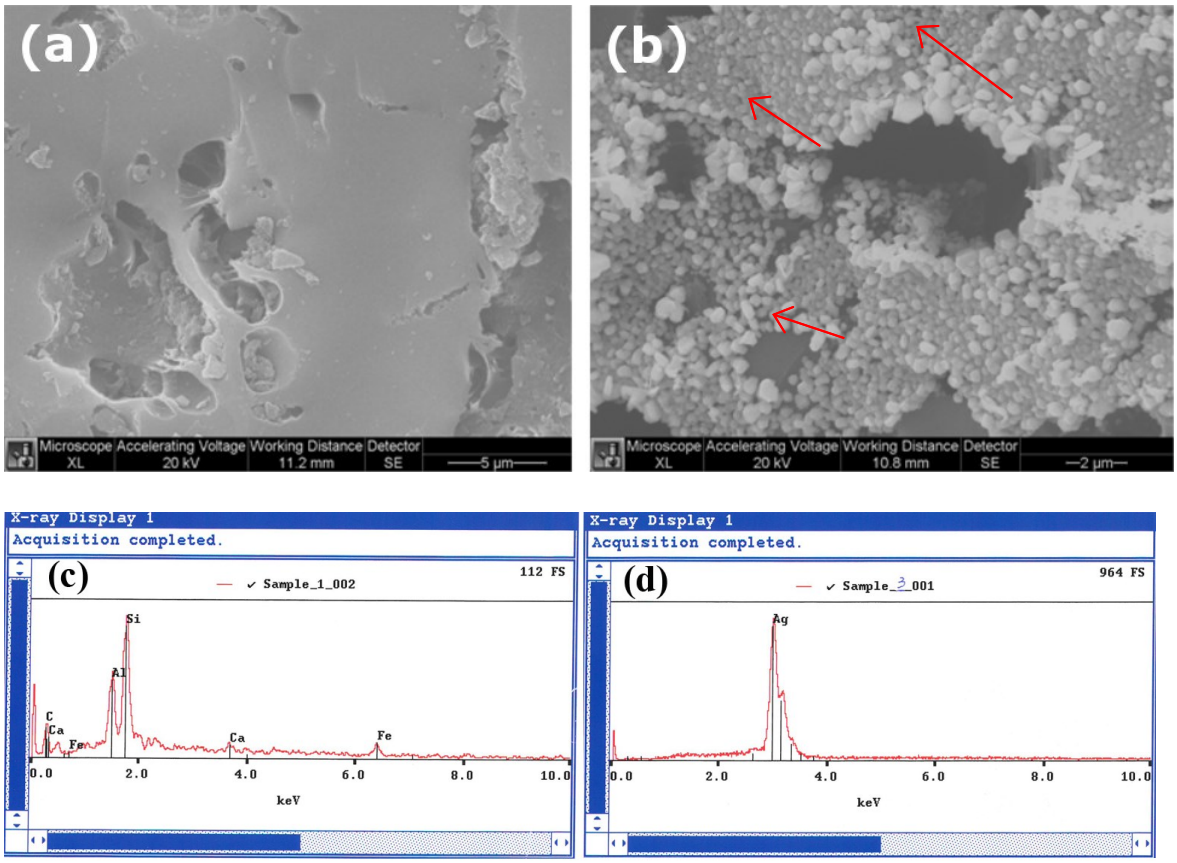

Figure 5.4. Scanning Electron Micrographs (SEM) of double-coated sample of CR1230C-AW carbon (20 g/L) **(a)** control, **(b)** AgNP on the surface of AC and inside the pore. AgNPs are shown with red arrows. X-ray analysis showing the major elements in the samples: **(c)** Untreated AC had high levels of silicon, aluminum and some carbon, calcium and iron; **(d)** AgNP treated AC had high level of silver.

Transmission Electron Microscopy (TEM) images showed that, while carbon structure was destroyed in the process of cutting in preparation for TEM, silver nanoparticles were still present both on the surface and in the pores of carbon (Figure 5.5). There was no visible difference between samples of single coated and double-coated AC-AgNPs. Though some silver particles fall off during the cutting, from Figure 5.5(d) we can see how the silver particles remain attached to the surface of AC on the edge of AC and plastic. These particles were within the range of 20-100 nm

with round shaped, and could be distinguished on the micrographs by much higher density, compared to that of carbon.

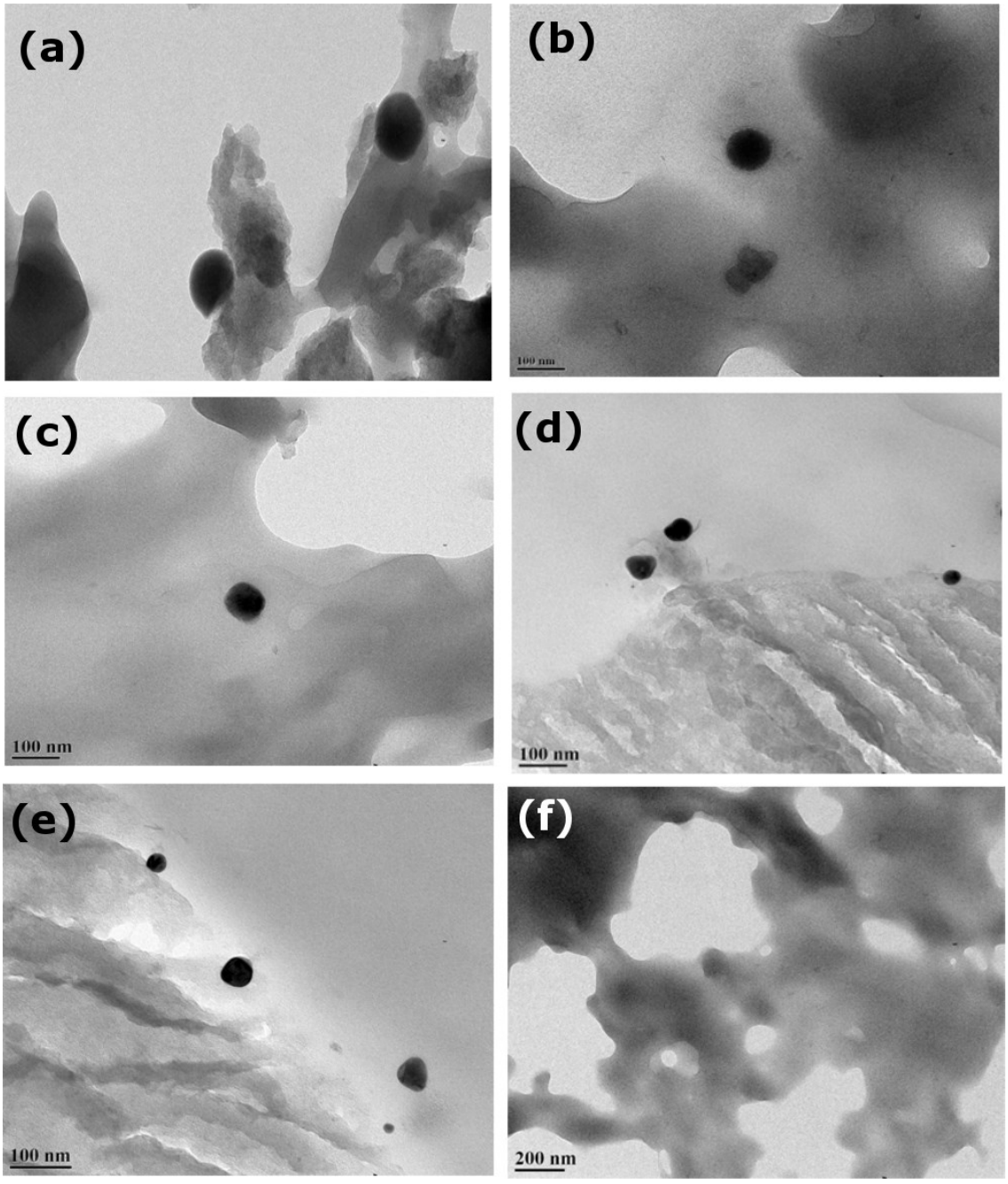

Figure 5.5. Transmission Electron Microscopy images: Silver nanoparticle (20 g/L)-treated activated carbon (AC) sample **(a)-(b)** bigger sized Aquasorb carbon with single coating, and **(c)-(e)** smaller sized CR1230C-AW with double coating, (**f**) control (No nanoparticles coating). Pictures show silver nanoparticles on the surface **(a), (d), (e)** and embedded in the structure of AC **(b) and (c)**.

High anti-bacterial efficiency of the developed material suggested that it might have broader application including drinking water filtration. However, it is still unclear what the impact of AgNPs on human is. Therefore, we designed another experiment to see whether AgNPs were present in the eluent after filtration. We used an ICP-MS detection method. For larger size AC, silver ions were not detected in the control or the sample (data not shown here). However, in smaller size AC samples, silver ions were detected in the filtrated water. Furthermore, double-coated samples (SD) had more silver ions (0.0273 ppm) in the filtrate than single coated samples (SS) (0.00354 ppm) (Table 1). However, in both cases silver ions in the filtrates were far below the normal level for water treated using silver (50 µg/ L – 0.05ppm) (Shatkin, 2008; WHO, 1996; "WHO | The world health report 2007 - A safer future: global public health security in the 21st century," n.d.).

Table 5.1. ICP-MS testing results of silver in filtrate

Analyte	**Ag (ppm)**
Detection Limits (DL)	0.00001
Control	<DL
SD	0.0273
SS	0.00354

5.3.2 Testing of filter materials against plant pathogens

Our main objective was to develop a material, which is effective against major pathogens in agricultural hydroponic production. After the preliminary screening

studies (for killing *S. cerevisiae yeast cells*), tests against a bacterium *Pseudomonas* sp. and a fungus *Fusarium* sp. from greenhouses were performed. Both (20g/L and 23 g/L) AgNP-coated ACs completely inhibited the growth of *Pseudomonas* after one-hour treatment while the ACs significantly reduced the CFU counts after one hour treatment and almost completely killed the *Fusarium* spores after 24 hours of treatment (Table 2, Figures 5.6 and 5.7). The bacterium is more sensitive to Ag^+ than the fungus. The possible reason may be due to the fact that bacteria are procaryotes while *Fusarium* – eukaryotes and procaryotes.

Table 5.2. Bioassay of AgNPs-coated activated carbon against *Pseudomonas* and *Fusarium* treated for one or 24 hours, and also counted three days after plating and incubating at 25°C.

Microbe	Treatment	Mean CFU per Plate (n = 4)	
		1 hour	24 hour
Pseudomonas	20 g/L	0.0	0.0
	23 g/L	0.0	0.0
	0 g (CK)	102.3	95.0
Fusarium	20 g/L	12.5	0.5
	23 g/L	66.8	0.0
	0 g (CK)	500.0	472.8

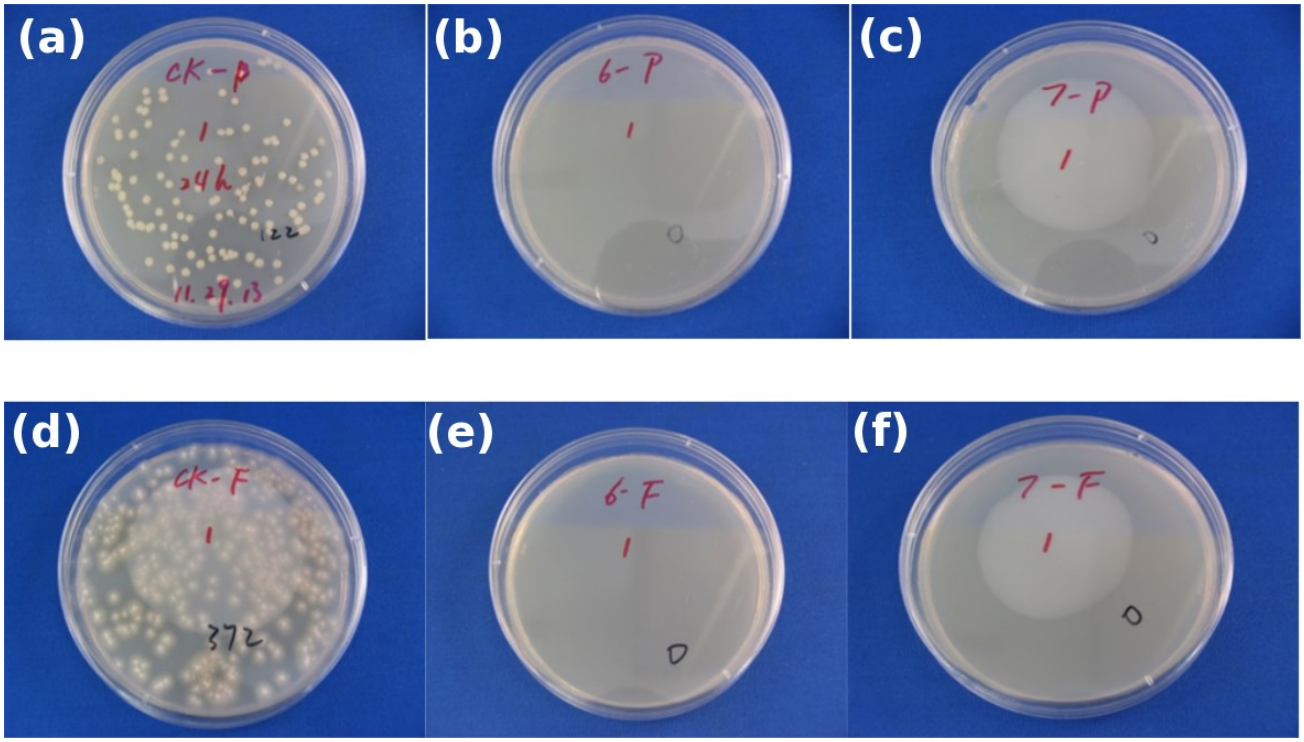

Figure 5.6. *Pseudomonas* sp. treated with AgNP-coated AC for 24 hours on a shaker at 200rpm at 25°C, 3 days after plating. **(a)** control; **(b), (c)** filtered with 20 g/L and 23 g/L treated AgNP-AC, respectively.
Fusarium oxysporum treated using AgNP-coated AC for 24 hours on a shaker at 200 rpm at 25°C, 3 days after plating. **(d)** control; **(e), (f)** filtered water with 20 g/L and 23 g/L treated AgNP-AC, respectively.

Our testing results show that the developed AgNP-AC material was very efficient in killing living cells of pathogenic bacterial and fungal species in the small scale setting in the lab. The next step was to test the developed material in dynamic flow on a bigger scale with the expectation to implement the design in greenhouses.

5.3.3 Dynamic scale-up testing results

As smaller size carbon (CR1230C-AW) had larger surface area and showed better results on the small scale, we used it for the scale-up tests. Sample preparation was

performed using the same protocol as for the smaller-scale tests. Dynamic testing was performed using *Paenibacillus* as pathogen bacteria and untreated AC as the control. Scale-up tests were performed with the following system for dynamic tests (Figure 5.7). The results showed up to 90% killing efficiency in the dynamic scale-up tests (Figures 5.7-5.8).

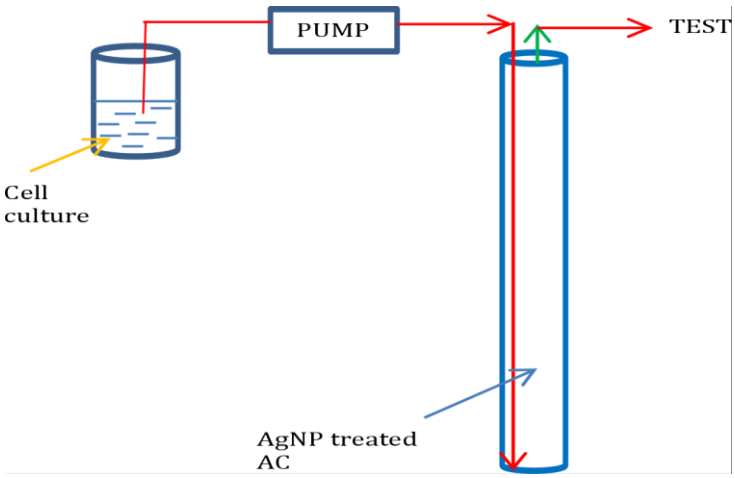

Figure 5.7. Schematic drawing of a filtration system used for scale-up dynamic test of the AgNP treated AC material. *Paenibacillus* spore suspension was pumped at a flow rate of 70-75 mL/h to the bottom of the 100 mL column filled with the developed material and the filtrated water samples were collected on the top of the column for plating on agar and further CFU count.

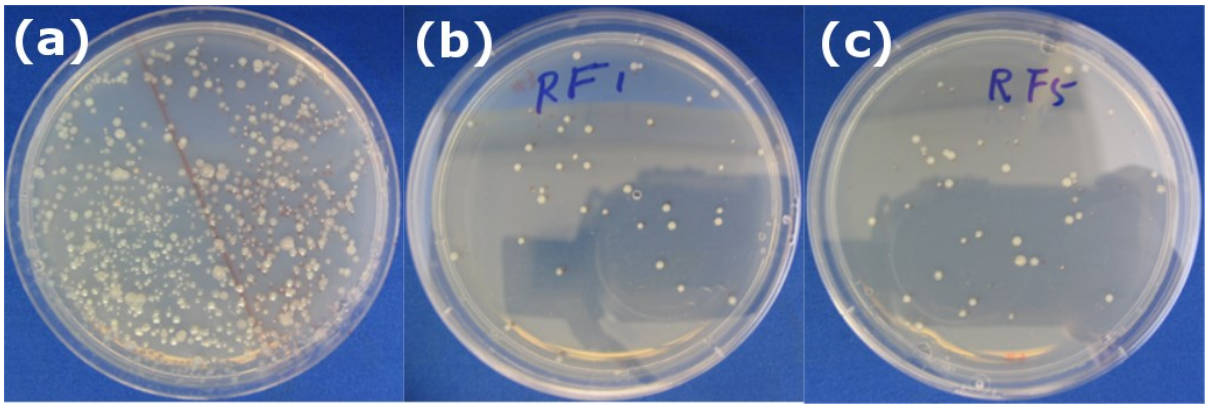

Figure 5.8. Agar plates two days after plating with the filtrated water collected from the dynamic scale-up test. **(a)** control, original bacterial suspension; **(b), (c)** filtrates collected after one hour and 5 hours of filtration through AgNP-AC material, respectively.

Results for the scale-up dynamic flow test using *Paenobacillus* spore suspension showed that in both cases, either for single-coated or for double-coated filteration material. There were over 95% pathogen cells killed after 29 hours and more than 80% after the system ran for around 40 hours. The pathogen killing efficiencies were decreasing over the period of testing (Figure 5.9). Flow rate did not change significantly during the whole filtration process (data not shown here).

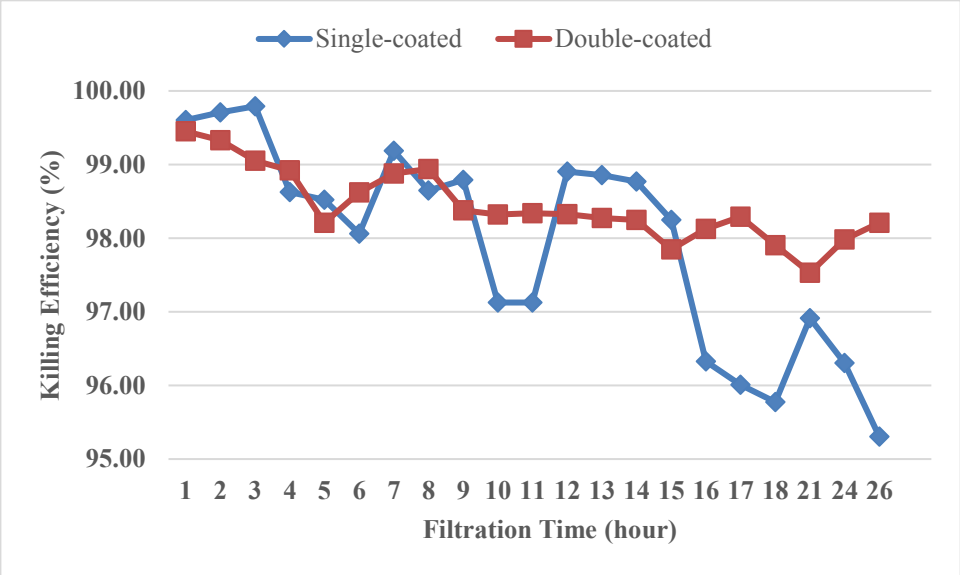

Figure 5.9. Efficiency test using the scale-up (100 ml) dynamic flow system to treat *Paenibacillus* spore suspension with single- vs. double-coated material.

Overall, dynamic test showed that AgNPs-coated AC material effectively killed pathogen cells during the filtration. AC with single- or double-coated AC had similar bacterial killing efficiency. However, double-coated AgNP-AC has longer life span than single-coated AgNP-AC materials.

In the final filtration test we used commercial filtration cartridge and filled it with our developed AgNP-AC material. Based on the result of dynamic test for 100 ml (Fig.5.9), we chose double-coated material for commercial sized scale up test. A

total of 1.7 L AC (CR1230C-AW) was double-coated with AgNPs, and then was filled in a commercial RFF-Series Refillable Cartridge (200701, 2.5 × 20 inch, AXEON Water Technologies) that was then inserted into a Slim Line Series Filter Housing (207296, AXEON Water Technologies). Bacterial (*Paenibacillus*) suspension with a concentration about 10^4 CFU / mL was filtered through the filter device. A pump (Mandel Watson 505U) was set at 2 rpm and a flow rate at about 800 mL per hour (Figure 5.10). Filtrate samples were collected hourly and plated on PDA plates, incubated at 25°C in the dark. CFU was counted 2 days after plating. A cartridge filled with uncoated AC (CR1230C-AW) was used as a control and tested using the same procedure.

Figure 5.10. A large scale-up filtration set up, using a commercial filtration cartridge, but filled with AgNPs-coated AC and then put into a housing unit.

In the commercial-cartridges scale up filter test, more than 99% of initial bacterial population were killed in the first hour and continued for 11 hours with the AgNP-coated AC substrate. The efficiency remained at 97% after 30 hours of filtration (Figure 5.11(a)). In the control (filter with AC substrate without AgNP

coating), it was observed that bacteria also reduced more than 90% in the beginning, but the efficiency rapidly dropped to about 30% after 10 hours filtration (Figure 5.11(b)) because the bacteria gradually blocked the porous carbon. Consequently, the AC only filter has a very short life span. Results demonstrated that addition of AgNPs to the filter substrate could significantly increase the filter life span and antimicrobial efficiency. Again, no Ag+ was detected in the filtrated water.

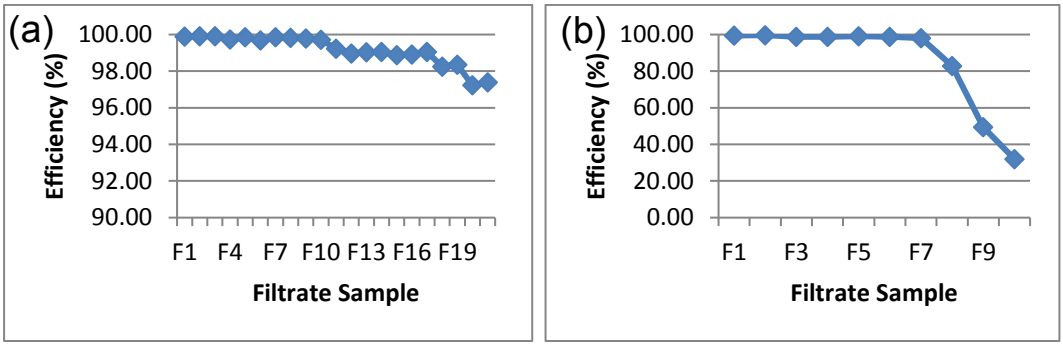

Figure 5.11. Summary of the scale-up test using commercial cartridge filled with 1.7 L AgNP double-coated AC material against a bacterium Paenibacillus. (a) Filtrate samples were collected hourly for the first 17 hours, then collected every 3 hours between 18 to 21 hours; (b) Control: Filter cartridge filled with uncoated AC, filtrate samples were collected hourly for the first 10 hours. Filtrates were plated on PDA and incubated at 25°C for two days.

Growth chamber trial

Cucumber crown root showed discoloration four weeks after the plants were watered with *Pythium* contaminated NS without filtration while slight discoloration was observed on plants filtered with AC without AgNP treatment. There was no root infection observed on plants watered with AgNP-AC treated NS and control plants (Figure 5.12). This growth chamber trial demonstrated that the filter treatment reduced the Pythium root disease on cucumbers although the difference on root infection was mild between treatments.

Figure 5.12. Six-week-old plants watered with *Pythium*-contaminated nutrient solution (NS) filtered through AgNP-AC filter (Treated) and watered with *Pythium*-contaminated NS filtered through AC without AgNP coated filter (CK) in the growth chamber trial (a); cucumber crown root showed discoloration watered with *Pythium* contaminated NS filtered with AC without AgNPs treatment (b) and healthy cucumber roots watered with *Pythium* contaminated NS filtered with AC with AgNPs treatment (c).

5.4. Conclusions

Silver nanoparticles have previously shown a great potential for killing many pathogenic microorganisms. In this study we have combined its disinfecting properties with cheap, efficient and commonly used filter material - active carbon. We developed a new filter material to address the problem of pathogen contaminating water in greenhouses. Our method of depositing silver nanoparticles on active carbon particles allowed having the 3D coating of the active carbon with nanoparticles covering both inside and outside surfaces of the pores, offering better anti-microbial efficiency during water filtration. The preparation of the material is easy and our tests showed that pathogen killing efficiency could reach as high as 90-99%. We have also tested the material both in lab-scale setting and scale-up dynamic setting for 100 mL

and in the commercial large size cartridge. In our tests, we used four different types of microorganisms, three of them are typical pathogens in greenhouses (*Pseudomonas* sp. (G-negative bacterium), *Fusarium* oxysporum (fungus), *Paenibacillus* (a G-positive bacterium)). All tests showed the developed AgNP-AC has a great potential to kill pathogens in greenhouse water. Our solution is an alternative strategy to overcome pesticide resistance developed by pathogens, and is also an alternative strategy to treat contaminated water in greenhouses to reduce pesticide related health risks and environmental contaminations.

Chapter 6

Conclusions and Future work

Due to significant environmental pollution and the shortage of fossil-based resources, that have been providing most of the energy, as well as the main source of raw material for many industries for many years, modern research is more and more turning its attention to bioprocesses and biotechnology. Such processes are environmentally friendly, sustainable and can rely on renewable resources that were the main base of raw material for thousands of years before the discovery of oil and oil based products. Microalgae –are the prospective microorganisms, whose great potential for biotechnology has recently been discovered. Among the reasons microalgae are attractive are: their ability to sequester CO_2 from the atmosphere, reducing pollution; their large scale production can be done on non-arable lands; they can use wastewater for growth, simultaneously removing heavy metals and organic matter; they are not among human-edible crops; they can be grown under quite extreme conditions with very high speed of biomass production; lipids, accumulated by microalgae can be used as biofuels, solving yet another very important problem.

Among the processes that can successfully use microalgae are wastewater purification, biomaterials production, high-value chemicals, pharmaceuticals and nutriceuticals production, biodiesel and biogas production as well as accumulation of biomass to be used as feedstock for cattle, fish, and poultry, resulting in enriched fillets, eggs and milk. A lot of research has been done recently in order to make the microalgae and microorganism-based bioprocesses feasible. The methods that have been applied include biological methods such as system biology, genetic engineering,

bio-refining for optimizing cell strains; chemical methods of adjusting media and culture conditions; physical methods like applying microwave, screening for optimal temperature and light conditions, the design of bioreactors, etc.

While high-frequency ultrasound has been previously successfully used in biotechnology for cell rapture, in extraction processes for better release of desired final products, this thesis was focused on the potential application of low intensity pulsed ultrasound in bioprocesses. LIPUS – a pulsed wave at the frequency of 1.5MHz and the duty cycle of 20%- is a new form of mechanical stimulation that can be successfully applied in bioprocesses in addition to all other methods for improvement. Two different applications of LIPUS were studied: ultrasound-mediated higher lipid accumulation that has great potential for biodiesel production and higher biomass accumulation after ultrasound treatment, that has the potential for food, biomaterials, nutraceuticals, pharmaceuticals and feedstock production. Several different microalgae were studied (*Chlorella, T.chuii, Schytzochytrium*) and all three showed a response to the ultrasound treatment. Treatment conditions such as duration, intensity, continuity of cycle cannot be optimized for both processes- biomass and lipid accumulation at the same time and have to be adjusted depending on the main application as well as have to be selected for every microorganism separately because cell wall thickness, size of cell, type of cell division and growth cycle vary and have an impact on LIPUS treatment efficiency. Our results showed that up to LIPUS can increase lipid production by microalgae up to 20% through sheer stress, without altering the lipid composition. At the same time, it can also be successfully applied for biomass production enhancement and can also increase it up to 20%. Both small scale (100 mL flasks) and scale up to small bioreactors were studied and in both cases LIPUS treatment resulted in significant improvement. Mixotrophic, autotrophic and

heterotrophic algae growth cycle were tested with regard to algae response to LIPUS because all three types of microorganism growth are applied in industrial biotechnology and all three types of growth could be enhanced by the ultrasound stimulation. The total electricity cost needed for the stimulations in a one-liter bioreactor is only one-tenth of a US penny, showing that LIPUS can successfully improve cost-efficiency of various bioprocesses.

While research shows the potential of cell stimulation with LIPUS, little is known about the mechanism of its effect on the cells. Attempts to clarify the mechanism were performed and showed that if LIPUS parameters are chosen appropriately for the cell strain, it causes no damage to the cells and does not decrease cell viability. At the same time, LIPUS has caused increased cell amount in algae culture, stimulating more active cell division and enhancing cell growth. SEM images of the cells showed the increase in membrane folds on cell surface caused by ultrasound, which may enhance transport rate of oxygen and nutrients to the cells as well as the transport rate of waste products away from the cells.

Both gene expression study and the metabolomic study showed that LIPUS alters several genes and pathways as well as metabolism. Several processes are most likely to be affected by LIPUS according to the studies: biosynthesis of CoA, amino acid metabolism, aminoacyl-tRNA biosynthesis, which are associated with protein synthesis.

A separate study has been carried out addressing another significant challenge of bioprocesses- water purification and recycling. Filters, based on cheap and efficient active carbon and covered with silver nanoparticles both on the outer and inner surface have been developed to be applied as an antibacterial step in water filtration.

The developed filters have been tested in greenhouses for hydroponic vegetable production and showed great efficiency against major pathogens.

Several directions of future work for LIPUS application in bioprocesses should be mentioned. First of all, it is the conversion of bench scale bioreactors to industrial scale. Many different changes occur in the bioreactor when its size increases and, while ultrasound obviously can still be applied, adjustment of process parameters and development of suitable bioreactor design so that LIPUS stimulation could be incorporated are still needed.

Another direction is LIPUS application for bacteria stimulation in wastewater treatment. Preliminary studies have shown that LIPUS stimulation in such process causes better community stability of the microorganism culture, enhances ammonia removal, stimulates higher bacteria number and biomass density. Large scale study of this process, as well as longer observation, are needed to study the effect of ultrasound on the sludge in the process and develop parameters for LIPUS to be successfully applied in wastewater treatment technology.

The full cycle bio-ethanol production from the straw can also benefit from LIPUS stimulation. Tons of agricultural waste is being produced every year worldwide that can serve as the lignucellulosic source for bio-ethanol. Enzymatic technology for such production is being developed using several different microorganisms in the process in order to make the production environmentally friendly as old chemical methods cause water and air contamination. LIPUS stimulation can be used for each of 3 stages: stimulation of microorganisms for lignin removal, treatment of fungus cells for better cellulose digestion and treatment of yeast cells for more efficient fermentation of sugars to bioethanol. In such a way LIPUS can

significantly increase efficiency and reduce the cost of the process by up to 40% making technology more feasible and competitive on the market.

In this thesis, LIPUS has been successfully applied for increased biomass production of Schitzochytrium sp. cells. These microorganisms are now being studied with the potential to use as a source of omega-3- valuable nutrient required for human health. Application of LIPUS for enhanced oil (DHA) accumulation by this species of algae can also significantly improve cost efficiency of the technology.

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