Treatment of Nitrogenous Wastewater by Using Microalgae and Its Utilization for the Production of Value-Added Products

THESIS

Submitted in partial fulfilment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

Mr. Ram Indrajit Chavan

Under the Supervision of

Dr. Srikanth Mutnuri

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI
2020

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

CERTIFICATE

This is to certify that the thesis entitled **"Treatment of Nitrogenous Wastewater by Using Microalgae and Its Utilization for The Production of Value-Added Products"** submitted by **Mr. Ram Indrajit Chavan**, ID no. **2014PHXF0406G** for the award of PhD of the institute embodies original work done by him under my supervision.

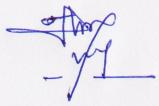
Signature of Supervisor

Dr. Srikanth Mutnuri,
Professor and Head,
Dept. of Biological Sciences
Date: 01-06-2020
Place: BITS Pilani, Goa

Head
Dept of Biological Science
BITS, PILANI - K. K. Birla Goa Campus

DECLARATION

This is to certify that the material embodied in the present work, entitled "TREATMENT OF NITROGENOUS WASTEWATER BY USING MICROALGAE AND ITS UTILIZATION FOR THE PRODUCTION OF VALUE-ADDED PRODUCTS" is based on my original research work. It has not been submitted, in part or full, for any other diploma or degree of any university/Institution, Deemed to be University and college/Institution of National Importance. References from other works have been duly cited at relevant places.

Signature of the candidate: 

Name in capital block letters: RAM INDRAJIT CHAVAN

Date: 01/06/2020

Acknowledgement

It is my pleasure to convey my gratitude to all those who supported and helped me in many ways during the completion of this thesis.

I am greatly indebted to my guide, Dr. Srikanth Mutnuri, Professor, Department of Biological Sciences, BITS Pilani, KK Birla Goa Campus, for his guidance, valuable suggestions, constructive criticism, patience, help and constant encouragement. His scientific experience, enthusiasm and consistency have influenced me throughout the successful completion of this research investigation.

I would like to thank Vice-Chancellor Prof. Souvik Bhattacharyya; Director, Prof. G. Raghurama; Ex-Director, Prof. K. E. Raman, Associate Dean AGSRD, Prof. Bharat Deshpande, Ex Associate Dean AGSRD, Prof. P. K. Das; Associate Dean SRCD, Prof. Meenal Kowshik, Dean SRCD, Prof. S. Bhand; for all their support.

I am thankful to my Doctoral Advisory Committee (DAC) members, Prof. Judith Bragança and Prof. S. Baral for their valuable insights, help and guidance throughout my thesis.

My sincere thanks to the faculty members of the Department of Biological Sciences, Dr. Anasuya Ganguly, Prof. Vijayashree Nayak, Dr. Indrani Talukdar, Dr.Malbika Biswas, Prof. Utpal Roy, Prof. Dibakar Chakrabarty, Dr. Sumit Biswas, Dr. Angshuman Sarkar, Dr. Veeky Baths, Dr. Sukanta Mondol, Dr. Kundan Kumar, Dr. Raviprasad Aduri, Dr. Arnab Banerjee, Dr. Sandhya Mehrotra and Dr. Rajesh Mehrotra for their cooperation throughout this study.

I am grateful to Prof. N. N. Ghosh, Professor, Department of Chemistry, BITS Pilani, KK Birla Goa Campus for his valuable help in XRD analysis.

I owe my deepest gratitude to my colleagues Dr. Vidhya Prabhudessai, Dr. Meghnath Prabhu., Dr. Rajesh Pasumarthi, Dr. Vaishnavi Unde, Dr. Gowdaman, Dr. Lalita Baragi, Dr. Monsoor, Ms.Anumeha Vats, Mr. Anant Yadav, Mr. Guruprasad Talekar, Ms.Priya Sharma Mr. Ravikiran Shet, Ms. Rajashree Yaragal and Mr. Swapnil Punyapawar, for their timely help, support, encouragement and motivation throughout this research.

I am deeply indebted to my dear friends and research scholars for their timely help, Dr. Aashu, Dr. Om Prakash, Dr. Yogesh, Dr. Ajay, Dr. Bhakti, Dr. Shruti, Dr. Kabilan, Dr. Ramya, Dr. Geethanjali, Dr. Gautam Krishnan, Mrs. Deepthi, Dr. Ram Ko., Dr. Pallavee, Dr. Jigni, Dr. Akriti, Ms. Nupur, Mrs. Mansi, Dr. Ansie, Dr. Kshipra Naik, Dr. Ketaki Dr. Ankush, Dr. Priyanka, Dr. Archana, Ms. Prajakta, Ms. Pranjita, Ms. Pooja, Ms.

Reshma, Ms. Chitra, Ms. Nupur, Mr. Joseph, Mr. Nilesh and all the research scholars of the Biological Sciences Department for all the good times and fun, apart from the unconditional support and motivation.

I am grateful to BITS Pilani KK Birla Goa Campus for providing financial support during this study.

My special thanks to Mr. Mahaling Lamani, who has helped me in sampling during the entire period of the study. I thank Mrs. Kamna Uphadhay and Mr. Mahadeo Shetkar and for their timely help during the study.

I would like to thank Ms. Megha Shinge and Ms. Jyoti Singh for their help in Raman Spectroscopy and FAMEs analysis.

Finally, I would not have been able to complete this thesis without the understanding and support of my parents; Aai, Tatya and family members, Bhaoji, Manisha tai, Shyam, Anita vahini and Rasika. Thank you all and I will be forever grateful to you all. Above all, I praise God, the almighty for providing me this opportunity and granting me the capability to proceed successfully.

Ram Indrajit Chavan

DEDICATED TO MY FAMILY

No words can express my gratitude for them, for they have always supported and encouraged me.

CONTENTS

TABLE OF CONTENTS

PAGE NO

Chapter II: Tertiary treatment of domestic wastewater by *Spirulina platensis* integrated with microalgal biorefinery

Chapter V: Demonstration of pilot scale integrative treatment of nitrogenous industrial effluent for struvite and algal biomass production

Abstract

Since India is moving towards more developing economy, there is a rapid increase in urbanization and industrialization. As reported by Central Pollution Control Board (CPCB-India;2017), 62000 MLD of Domestic Wastewater (DW) is being generated and the installed treatment capacity is 23000 MLD leading to 39000 MLD untreated water ending up in rivers and oceans. CPCB also depicts that out of 800 STPs throughout country, only 520 are operational further reducing the treatment capacity to 20000 MLD. In India, wastewater treatment is preferably performed by Upflow Anaerobic Sludge Blanket Reactor (UASB), Waste Stabilization Pond (WSP) and Activated Sludge Process (ASP). These treatment systems suffer numerous disadvantages including high capital costs (200-450 USD/m^3), annual maintenance and operation cost (20-60 USD/m^3), land requirement (2-14 m^2/m^3), sensitivity to wastewater composition and lack of technical staff.

Wastewater treatment systems are classified on the basis of energy requirement; active (High energy demand) and passive (low energy demand). These days, Sequencing Batch Reactor (SBR) system is widely used but its removal efficiency varies; carbon (65-90%), nitrogen (35-92%) and phosphorous (20-95%). Power requirement for SBR is also high (0.3 kWh/m^3) and it does not generate any value-added product. It is important to use hybrid technologies that can be established at pre-existing Sewage Treatment Plants (STPs) without major modification to available infrastructure in order to increase long term pollutant removal efficiency as well as to generate some value-added products. Microalgal Treatment System (MTS) can be integrated with SBR for simultaneous wastewater treatment and resource recovery in terms of microalgal biomass. The biomass generated can be used to extract lipids, to produce ethanol via fermentation and to generate electricity via anaerobic digestion. This study has investigated the treatment of DW by integrating Microalgal Treatment System (MTS) to active treatment system (SBR) and one passive treatment system (Vertical Flow Constructed Wetland-VFCW). Fertilizer industrial wastewater was treated by integrating MTS to struvite production.

Second and third chapter deals with the tertiary treatment of DW by using MTS integrated to SBR. The main aim of these studies was to explore parallel nutrient removal from secondary treated wastewater by microalga *Spirulina platensis* and *Chlorella vulgaris* and their biogas production potential. DW was initially treated with SBR and SBR effluent was further phycoremediated by using two individual microalga species; *Spirulina platensis* and *Chlorella vulgaris*. The observed removal efficiency of COD, PO_4-P, NH_4-N and TKN of *S. platensis* and *C. vulgaris* were 18%, 14%, 17%, 16% and 31%, 40%, 36% and 38 %, respectively. *S. platensis* and *C. vulgaris* biomass was observed to contain 26.65 % and 16.45 % lipids,

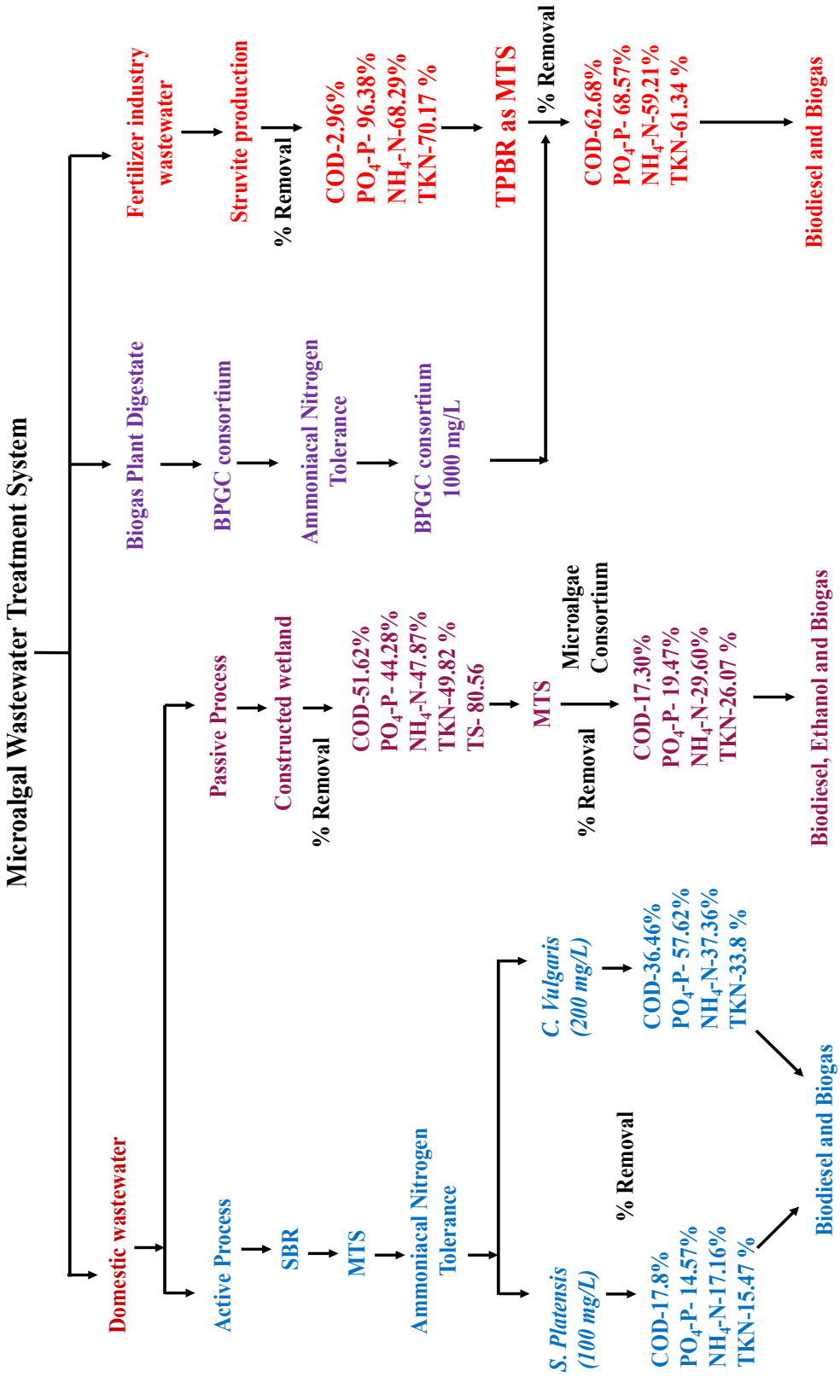
respectively. The maximum biogas production (mL/g VS) was observed at 2 g VS/L. *S. platensis* and *C. vulgaris* was observed to produce 320 mL/g VS and 450 mL/g VS biogas, respectively. These studies have also investigated the ammoniacal nitrogen tolerance of *Spirulina platensis* (100 mg/L) and *Chlorella vulgaris* (200 mg/L). Effect of different pre-treatment methods (thermal, chemical, sonication and thermo-chemical) has also been studied. The biomass and biomass extract (before and after pre-treatments) were also analyzed for solubilization of complex compounds. Thermally pre-treatment of *S. platensis* and *C. vulgaris* biomass increases biogas production by 8.5% and 6.6 %, respectively. These studies have successfully demonstrated that microalgal cultivation in wastewater can be easily adopted in currently available wastewater treatment plants without any major modifications of existing available infrastructure.

Fourth chapter of the thesis deals with integration of MTS to first stage vertical flow constructed wetland and microalgal treatment system for treatment of raw DW. SBR technology has certain limitations; requirement of high level of sophistication and maintenance, plugging of aeration devices, requirement of post equalization phase post SBR treatment and chances of discharging floating or settled sludge during fill and decant phase. DW often contains solid matter limiting its direct use as a medium for microalgal growth. These limitations can be overcome by adopting hybrid treatment system; Vertical Flow Constructed Wetland (VFCW) and MTS. The main aim of this study is to treat DW in a hybrid Vertical Flow Constructed Wetland (VFCW-4.2 m^2) and Microalgal Treatment System (MTS-1m^2). The objective is not only to treat DW but also to produce value added products from microalgal biomass. The DW was initially treated by VFCW and the VFCW effluent was further phycoremediated by MTS. *Canna indica* was used for wetland vegetation and resident microalgal consortium from VFCW effluent was used in MTS. The VFCW and MTS were operated at 1 m^3/day (HRT-0.25 m^3/m^2-day, OLR-400 g/m^2-day) and 0.3 m^3/day (HRT-0.03 m^3/m^2-day, OLR-400 g/m^2-day), respectively. The integrated system was observed to remove 68.9% COD, 77.4% NH_4-N, 75.8% TKN and 63.6% PO_4-P. The harvested Naive Biomass (NB) was observed to contain 16.7 % of lipids (W/W). The Residual Biomass after Lipid Extraction (RBLE) was used as a substrate for ethanol production. The observed yield of ethanol using RBLE as a substrate was 33.4 %. NB, RBLE, and Residual Biomass after Lipid and Sugar Extraction (RBLSE) indicated net biomethane yield (mL/g VS) of 211.8, 134.6 and 107.7, respectively. This study demonstrated an initial attempt of demonstrating hybrid wastewater treatment system for the production of value-added products in terms of biofuel.

Fifth chapter of the thesis deals with the demonstration of pilot scale integrative treatment of nitrogenous industrial effluent for struvite and algal biomass production. Wastewater from

fertilizer industries is rich in ammoniacal nitrogen and orthophosphates increasing risks of eutrophication and disorders like blue baby syndrome at an alarming level. This study aims at recovering nutrients from fertilizer industry effluent in the form of microalgal biomass to produce various bioproducts. This study demonstrates the integration of pilot scale struvite production from fertilizer industrial wastewater in air-agitated reactor and phycoremediation of residual wastewater. The parameters required for the production of high yield and better quality of struvite were optimized. The microalgal BPGC consortium was isolated from digestate from anaerobic digester and adapted to tolerate 1000 mg/L of NH_4-N using synthetic wastewater rich in NH_4-N. Pilot-scale struvite production was carried out in the air-agitated reactor (1 m^3 capacity) in batch mode and phycoremediation of residual effluent was carried out in tubular photobioreactor (200 L capacity) in fed batch mode. Pilot-scale struvite crystallization produced 60 kg of struvite from 1 m^3 of effluent. During struvite precipitation, 2.96 % of COD , 68.29 % of NH_4-N and 96.38 % of PO_4-P were recovered. The residual effluent was further phycoremediated by the microalgal consortium. During phycoremediation, 62.68 % of COD, 59.21 % of NH_4-N and 68.57 % of PO_4-P were recovered in terms of microalgal biomass. It led to production of biomass that can produce lipids, methane, and ω-3 fatty acids. Due to integration, 64.58 % COD, 87.31 % NH_4-N, 89.0 % TKN (Total Kjeldahl Nitrogen) and 98.79 % of PO_4-P was removed. In brief, the integration of struvite production and microalgae cultivation can be used as an effective treatment system for fertilizer industry wastewater.

Graphical abstract



LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND SYMBOLS

STP- Sewage Treatment Plant

°C- Degree Celsius

MBR- Membrane Bioreactor

SBR-Sequencing Batch Reactor

COD-Chemical Oxygen Demand

BOD-Biological Oxygen Demand

PO_4-P- Orthophosphates

NH_4-N- Ammoniacal Nitrogen

TKN-Total Kjeldahl Nitrogen

MLD-Million Litres per Day

ORP-Open Raceway Pond

TPBR-Tubular PhotoBio-Reactor

MTS-Microalgal Treatment System

VFCW-Vertical Flow Constructed Wetland

CSTP-Conventional Sewage Treatment Plant

CPCB-Central Pollution Control Board

TS-Total Solids

VS-Volatile Solids

NO_3-N- Nitrate Nitrogen

VFA-Volatile Fatty Acids

TDS-Total Dissolved Solids

TSS-Total Suspended Solids

NC-Negative Control

RDW-Residual Domestic Wastewater

mg/L-Milligrams per litre

DW-Domestic Wastewater

NRCD-National River Conservation Directorate

MoEFCC- Ministry of Environment, Forest and Climate Change

ASP-Activated Sludge Process

UASB- Upflow Anaerobic Sludge Blanket Reactor

CW-Constructed Wetland

FW- Fertilizer industry Wastewater

MAP-Magnesium Ammonium Phosphate

FAME-Fatty Acid Methyl Esters

BMP-Biomethanation Potential

TW-Treated Water

SPR- *Spirulina platensis* Residual biomass after lipid extraction

ASTM- American Society for Testing and Materials

SBRTW- Sequential Batch Reactor Treated Water

HRT- Hydraulic Retention Time

EPA- Environmental Protection Agency

NB-Naive Biomass

RBLE- Residual Biomass after Lipid Extraction

RBLSE- Residual Biomass after Lipid and Sugar Extraction

BPGC-BITS Pilani Goa Campus

SW-Synthetic Wastewater

BBM-Bolds Basal Medium

RFW- Residual Fertilizer Wastewater

Introduction and Review of Literature

1.1 Introduction

1.1.1 Potential of microalgae in wastewater treatment

Algae have recently received a lot of attention as a new biomass source for the production of renewable energy. Some of the main characteristics which set algae apart from other biomass sources are that algae (can) have a high biomass yield per unit of light and area, can have a high oil or starch content, do not require agricultural land, fresh water is not essential, and nutrients can be supplied by wastewater and CO_2 by combustion gas. The first distinction that needs to be made is between macroalgae (or seaweed) versus microalgae. Microalgae have many different species with widely varying compositions and live as single cells or colonies without any specialization. This makes their cultivation easier and more controllable.

It is estimated, that one ton of algal biomass would produce net renewable fuel sufficient to abate a similar amount of fossil CO_2, based on a reasonable mix of natural gas, oil and coal. Therefore, the potential of microalgae for Greenhouse gas (GHG) abatement is the product of productivity times the total aggregate scale of processes that is hectares of ponds. Thus, a global deployment of 10 million hectares of algal ponds would abate 1 gigaton of fossil CO_2 emissions. Adjustments would be required for other GHG credits (e.g. for energy savings in wastewater treatment), or if the fossil energy source being abated were an advanced natural gas power plant vs. a current coal power plants, for examples. However, overall, an estimate of 100 tons fossil CO_2 abatement per hectare of algal ponds appears to be a reasonable initial global estimate.

Although 10 million hectares of algal ponds would be an ambitious long-term goal, this scale is similar to that of ponds used globally in shrimp or fish aquaculture, and a small fraction of the several hundred million hectares of rice paddy fields used for rice cultivation. Most importantly, microalgae production systems could use land and water resources not suitable for agriculture or aquaculture (e.g. saline, brackish, waste waters), and, in any event, their water use efficiency (tons of water per ton output) would be much higher than any terrestrial crop.

The major limitations of this technology are not land, water or CO_2 resources, but the technical feasibility and economic competitiveness of microalgae processes compared to other alternatives, including crop production and forestry, for examples. Integrating algal GHG abatement with other large-volume co-processes and coproducts, assures that microalgae will make a significant contribution to different sustainable development goals.

In India 34% of household sewage flows virtually untreated into waterways. The fate of the key nutrients (chiefly nitrogen and phosphorus) that are present in sewage are wasted, either

by discharging them untreated into the environment. Most of the population is dependent on *septic tanks* for decentralized sewage treatment. Septic tanks do not sanitise wastewater. Moreover, the effluent from septic tanks still contains most of the sewage nutrients. Centralized sewage treatment relies instead on conventional activated sludge (CAS), which achieves sufficiently low carbon, nitrogen and phosphorus effluent levels, but is not cost-effective, hardly achieves recovery, requires electricity equivalent to a fossil fuel consumption of 85 kWh per Inhabitant Equivalent (IE) per year and has an operational CO_2 footprint of 80 kg CO_2 IE/year. Projected water and nutrient shortages and the need to lower greenhouse gas emissions force us to rethink wastewater treatment for a sustainable future by production of value- added products.

Microalgae have proven to be significant in recovery of pollutants from wastewater while generating high value biomass for extraction of various value-added products (Biodiesel, Ethanol, pharmaceutical products and Biomethane) (Prokop, Bajpai, and Zappi 2015). Wastewater treatment using microalgae can be achieved by either Open Raceway Ponds (ORPs) or closed photobioreactors (Tubular Photo-Bioreactor-TPBR).

Wastewater generated from households, municipal corporations, agro-industries and other sources generally consists of organic matter, N (ammoniacal nitrogen being dominant) and P compounds and can be easily colonized by variety of microalgae species (N.F.Y.Tam and Y. S. Wong 1996). Tertiary treatment of wastewater was carried out using microalgae post-secondary treatment (Martin, de la Noüe, and Picard 1985). Recently, it was reported that many microalgal species can be grown mixotrophically to assimilate organics from wastewater (Nascimento et al. 2011; Singh, Reynolds, and Das 2011).

There are two major limitations of microalgal wastewater treatment systems. Firstly, high solid content in wastewater increasing turbidity leading to loss of light penetration affecting their growth and decreasing their phycoremediation efficiency. Secondly, microalgae are sensitive to high ammonia concentration in wastewater (Collos and Harrison 2014a; Mustafa, Phang, and Chu 2012; Przytocka-Jsiak 1976). The first limitation can be overcome by integrating microalgal Treatment System (MTS) to SBR or Vertical Flow Constructed Wetlands (VFCWs). Whereas, the second limitation can be solved by using specific ammoniacal nitrogen (NH_4-N) tolerant algal strains depending on initial NH_4-N concentration present in wastewater. *Chlorella* sp. and *Spirulina* sp. have tolerance limit of 200 and 100 mg/L of NH_4-N and can be used for treatment of varieties of wastewater.

Recently, use of microalgal consortium is gaining importance for treatment of wastewater as maintaining unialgal culture in wastewater is difficult (Chinnasamy et al. 2010; Prokop et al. 2015; Singh et al. 2011; Subashchandrabose et al. 2011). Use of algal consortium is

preferred over unialgal strains in wastewater treatment as it is robust in nature and not very much sensitive to changes in wastewater composition (Samorì et al. 2013; Subashchandrabose et al. 2011).

Fertilizer manufacturing industries generates huge amount of effluents rich in NH_4-N (5000 mg/L) and PO_4-P (4000 mg/L) and less carbon content. In this context, Conventional Sewage Treatment Plants (CSTPs) cannot be directly adopted for effluent treatment. Microalgal strains also cannot grow in this effluent due to NH_4-N toxicity. So, attempts should be made to recover NH_4-N and PO_4-P as struvite and the residual effluent can be used for the growth of NH_4-N tolerant microalgal consortium. In this context, the focus the thesis was not only to increase the long-term pollutant removal efficiency by integrating MTS to currently available treatment systems but also to generate value-added products (lipids, biogas, ethanol, struvite etc.)

1.2 Literature review

1.2.1 Origin, nature and scope of problem

Sewage treatment plants (STPs) are mainly focused on Activated Sludge Process (ASP) for the removal of Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) and to less extent on N and P removal from wastewater. As per the reports published by AQUA-STAT and Sato et al (2013), globally, 90,000 MLD of municipal wastewater generated daily, 70 % of wastewater accounted for domestic wastewater and 30 % for industrial manufacturing sector (Sato et al. 2013; Wichelns, Drechsel, and Qadir 2015). Out of this, 80 % of wastewater was generated by global urban population and 20 % by global rural population. Out of this huge quantity of wastewater, only 60 % is being treated globally (Wichelns et al. 2015). Numerous advanced wastewater treatment systems were designed and widely used over time including Membrane Bioreactor (MBR), Sequencing Batch Reactor (SBR) etc. The STPs are focused on safe disposal of water rather than treating a wastewater as a valuable resource for the production of value-added products. Both of these systems suffer several advantages and disadvantages.

The mechanisms of currently available technologies for wastewater treatment include adsorption, ion exchange, activated sludge, electrochemical, and filtration (Clara et al. 2005; Kightlinger et al. 2014). This suffers several disadvantages that include requirement of an external supply of carbon, operation and maintenance cost, need of technical staff, sensitivity to wastewater composition, limited wastewater handling capacity and generation of large quantity of harmful sewage sludge (Luo et al. 2014). Treatment of wastewater is depreciated in countryside areas of India due to the improper design of treatment plant, lack of financial

resources, lack of technical staff, and poor maintenance (Yadav, Chazarenc, and Mutnuri 2018). Most of the treatment facilities do not treat wastewater to reach wastewater discharge standards and not maintained to its proper functioning ability (Konnerup, Koottatep, and Brix 2009).

Even if ammoniacal nitrogen is preferred form nitrogen by microalgae, at elevated concentrations, alkaline pH and in presence of urea, it is toxic to cells and may also inhibit cell growth and/or cell death (Azov and Goldman 1982). Ammonia toxicity is well known phenomenon with respect to higher plants and algae. Ammonia toxicity mechanisms suggests the disturbances in ionic balance, intracellular pH, hormonal balance, changes in membrane permeability via saturation of membrane lipids, photodamage of electron transport system and photosystems etc. (Britto and Kronzucker 2002).

The study of ammoniacal nitrogen tolerance of microalgae is of particular interests because, a) almost all types of wastewater contains ammoniacal nitrogen, b) little information is available about the problems resulting from microalgal cultivation at high ammoniacal nitrogen, c) little is known about the concentration at which ammonia toxicity becomes effective and d) the use of ammonium fertilizers in cultivation medium instead of nitrate fertilizers to reduce microalgal biomass production costs. Different microalgal species exhibit different ammoniacal nitrogen tolerance limits depending on other conditions. Most of the studies focuses on the ammoniacal nitrogen tolerance studies of microalgae in presence of high P and organic matter (Collos and Harrison 2014b; González, Cañizares, and Baena 1997; Kim et al. 2010; Li, Y. F. Chen, et al. 2011; N.F.Y.Tam and Y. S. Wong 1996; Przytocka-Jsiak 1976). Present study investigates the ammoniacal nitrogen tolerance limits of two microalgae (*Spirulina platensis* and *Chlorella vulgaris*) and microalgal consortium under limitations of P and organic matter. In this context, attempts were made to treat domestic wastewater initially by SBR/constructed wetland and MTS by using *S. platensis* and *C. vulgaris*. Fertilizer industrial effluent was treated by struvite crystallization followed by MTS using ammoniacal nitrogen tolerant BPGC microalgal consortium.

1.2.2 Characteristics of domestic and industrial wastewater

The characteristics of the wastewater generated varies greatly on source of wastewater i.e. household, municipal or industrial wastewater (textile, leather, paper, agro-industries, food processing and fertilizer production industry). Municipal wastewater often contains high organic matter (BOD and COD) as compared to inorganics (carbonates, nitrogen compounds and phosphorous compounds) (Rana et al. 2017; Sonune, Mungal, and Kamble 2015; Tanimu et al. 2014). However, fertilizer industries wastewater consists of high amounts of inorganics

(nitrogen compounds and phosphorous compounds) and negligible organic matter (Ferhan Cecen 1996). Wastewater composition decides the selection of wastewater treatment technology. The factors affecting the characteristics of wastewater are quality of water supplied, habits of peoples, people equivalent water use and type of sewerage system. The important characteristics of wastewater are; pH, temperature, COD, Total Solids (TS), Volatile Solids (VS), color, odor, nitrogen content, phosphorous content, organic matter and metals (Tchobanoglous, Burton, and David Stensel 1991). The characteristics of municipal wastewater is depicted in Table 1.1

Table 1.1 Characteristics of municipal wastewater

Parameters (mg/L)	Concentration		
	Low strength	Medium strength	High strength
COD	250	430	800
BOD	110	190	350
TKN	20	40	70
PO_4-P	4	7	12

Adopted from Tchobanoglous et al. 1991.

As per the annual report published by Ministry of Fertilizers, Government of India, there are 61 (31-Urea, 21-Diammonium Phosphate and 9- Ammonium Sulphate) fertilizer production units in country. The effluent from fertilizer industry may contain 50-140000 mg/L of COD, 6-4000 mg/L of NH_4-N and 200-5000 mg/L of PO_4-P (Bhandari, Sorokhaibam, and Ranade 2016; Thakre and Khan 2017).

1.2.3 Wastewater generation and treatment-Indian and global scenario

As reported by Central Pollution Control Board (CPCB-India, 2013), 38.25 million cubic meters per day domestic wastewater (DW) is being generated in Class I and Class II cities in India and current treatment capacity for municipal corporations is only 11,787 MLD corresponding to only 31 % of DW generation. CPCB studies also depict that out of 269 DW treatment plants in India, only 231 are operational further reducing the treatment capacity to 21 % of the amount of sewage being generated (J.S. Kamyotra and R.M. Bhardwaj 2011). As per the reports published by AQUA-STAT and Sato et al (2013), globally, 90,000 MLD of municipal wastewater generated daily, 70 % of wastewater accounted for domestic wastewater and 30 % for industrial manufacturing sector (Sato et al. 2013; Wichelns et al. 2015). Out of this, 80 % of wastewater was generated by global urban population and 20 % by global rural population. Out of this huge quantity of wastewater, only 60 % is being treated globally (Wichelns et al. 2015).

The state wise sewage generation installed treatment capacity and number of polluted river stretches is depicted in Fig. 1.1.

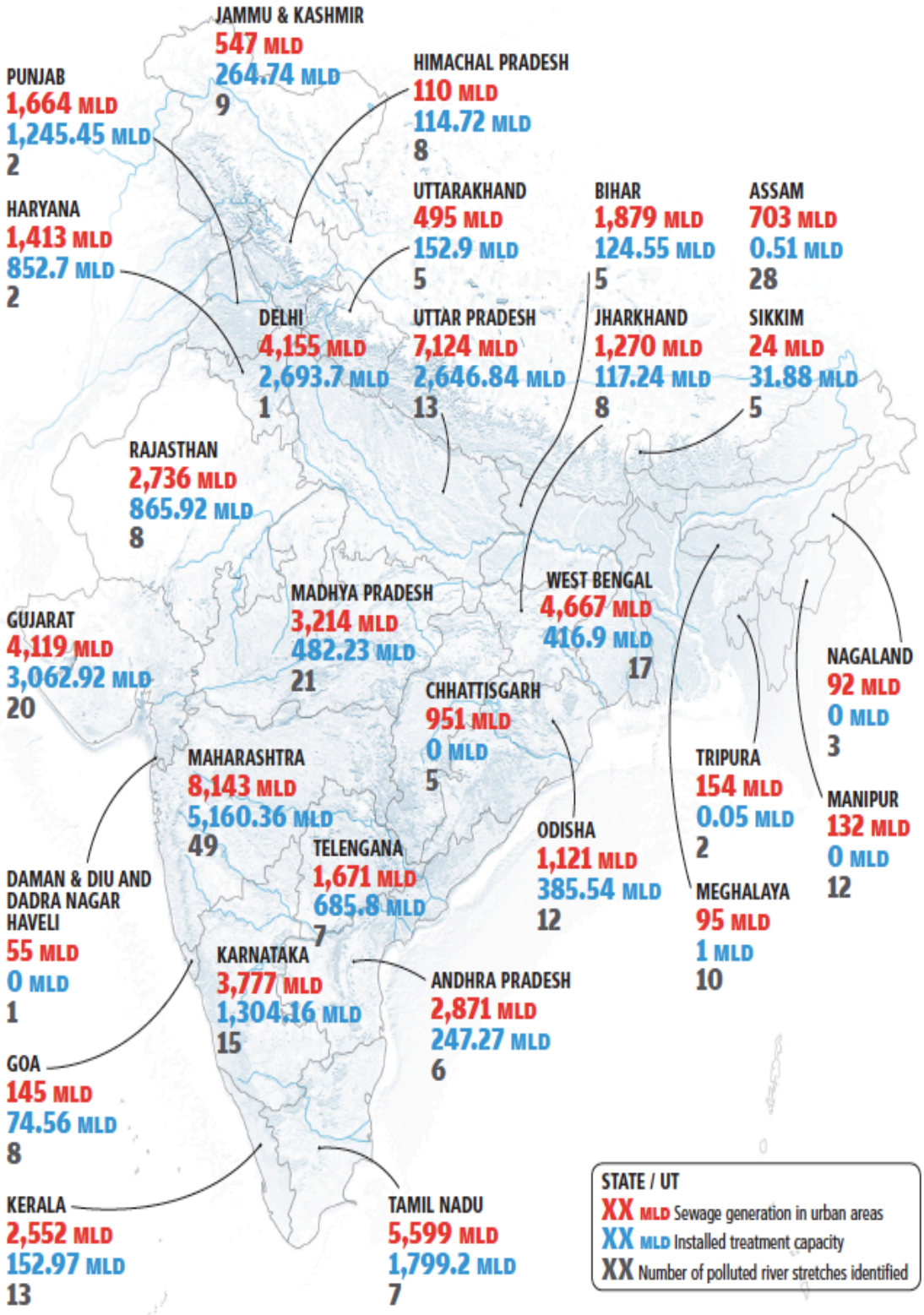


Fig.1.1 The state wise sewage generation, installed treatment capacity and number of polluted river stretches (Adopted from S. Sengupta and S. Narain, 2018 (Sushmita Sengupta and Sunita Narain 2018).

1.2.4 Challenges in wastewater treatment

In 2014, Vishal et al has reported the status of wastewater treatment in India (Table 1.2) (Bhawan et al. 2015).

Table 1.2 Status of wastewater treatment in India

Status	Number of STPs	Treatment Capacity (MLD)
Operational	520	19000
Non-operational	80	1240
Under construction	145	2530
Proposed	72	630
Total	817	23400

Source; Vishal et al 2015.

Wastewater can be classified in two categories; domestic wastewater (DW) and industrial wastewater. The domestic wastewater can be treated by active (SBR) or passive treatment systems (constructed wetland). The active and passive treatment systems can remove BOD, COD efficiently but the removal of N and P is not satisfactory. Therefore, these treatment systems need to be integrated with Microalgal Treatment System (MTS) to remove residual N and P from wastewater. Industrial wastewater cannot be directly treated by active or passive treatment systems because of their composition. For ex. fertilizer industry wastewater can be treated by struvite crystallization to remove ammonia and phosphorous in first stage and the residual wastewater can be treated by MTS in later stage.

There are numerous challenges in wastewater treatment and vary depending on effluent discharge standards, regional sources of water and socio-economic conditions (Hosomi 2016). Therefore, it is difficult to identify a common challenge in all types of wastewater treatment systems. Nevertheless, it is important to design high-performance and cost-effective treatment system. Few important challenges in wastewater treatment systems are described below.

a. Availability of technical staff.

b. Social awareness for water use, waste generation, handling and disposal.

c. Designing, long term operation and maintenance of high-performance and cost-effective treatment system.

d. Challenges in safe sludge disposal.

e. Recovery of value-added products from the wastes.

f. Wastewater treatment system design that can be adapted in future to integrate with other advanced treatment systems.

1.2.5 Conventional wastewater treatment systems

There are various levels of treatments in conventional wastewater treatment (Topare, Attar, and Manfe 2011). Preliminary treatment includes the removal of wastewater constituents causing maintenance and operational problems in wastewater unit operations. It deals with the removal of debris, rags, grits by screening, floatation and skimming. Primary treatment removes the suspended solids and part of organic matter from wastewater using clarifiers. Secondary treatment uses the alternating aerobic and anaerobic phases to remove biodegradable organics and suspended solids via biological decomposition. Secondary treatment can be achieved by using aeration tanks (ASP, Oxidation Ponds (OP) and Aerated Lagoons (AL). Tertiary treatment processes involve the further removal of N and P compounds, dissolved organic matter, and toxic compounds. Tertiary treatment can be achieved by coagulation, ion exchange, flocculation, filtration and reverse osmosis (Topare et al. 2011). Secondary and tertiary treatment of wastewater can be carried out by using microalgae (Christenson and Sims 2011; Gonçalves, Pires, and Simões 2017; Lim, Chu, and Phang 2010; Singh et al. 2011). Quaternary treatment deals with the removal of pathogens by chlorination. The steps and unit operations involved in conventional wastewater treatment process is depicted in Fig. 1.2.

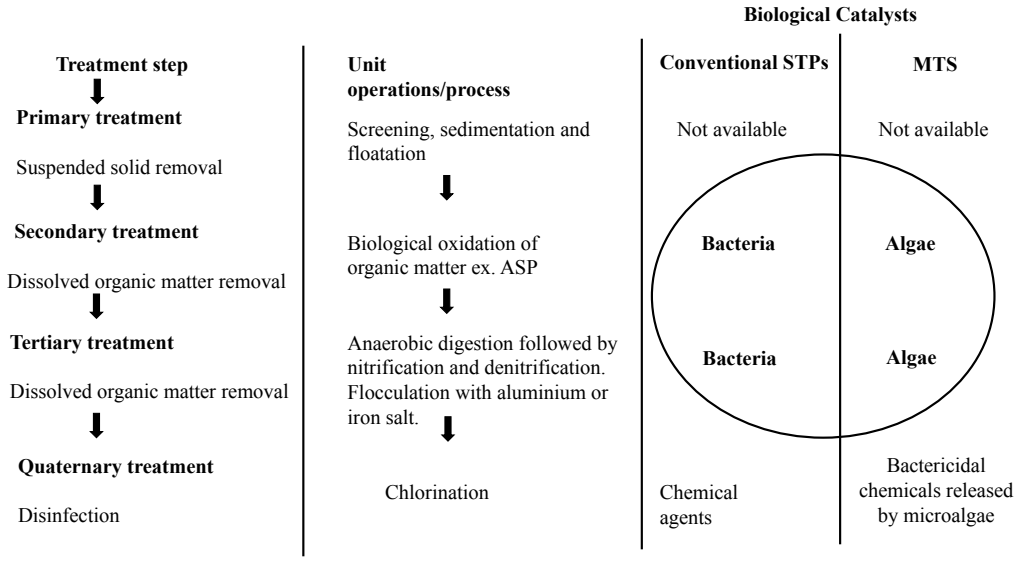


Fig. 1.2 Steps and unit operations involved in conventional wastewater treatment process and the catalyst used (Source: Adopted and modified from Gonçalves et al, 2017 (Gonçalves et al. 2017)).

1.2.6 Wastewater treatment technologies – current status in India

The efficiency of wastewater treatment can be determined by characteristics to wastewater streams and treated water after reviewing the wastewater discharge standards (Topare et al.

2011). Wastewater treatment process involves many unit processes/operations; physical, chemical and biological.

Physical unit operations include screening, mixing, sedimentation, filtration, flocculation and gas transfer operations. Chemical unit operations include adsorption, precipitation and transformations. Biological unit operations mainly used for removal of organic matter wherein it is converted to gases and generate biomass that can be easily separated by settling or filtration.

In India, under National River Conservation Directorate (NRCD) funded scheme, the generated wastewater was treated by three treatment technologies: Natural systems (oxidation ponds, waste stabilization ponds and aerated ponds), Conventional Treatment (Activated Sludge Process (ASP), extended aeration, trickling filters, cyclic ASP, Waste Stabilization Pond (WSP), Upflow Anaerobic Sludge Blanket Reactor (UASB) and Advanced technologies (sequential batch reactor (SBR), fluidized aerobic bed reactor and biofilters) (Sh. J.S. Kamyotra, Dr. D.D. Basu, R.M. Bhardwaj,A K Sinha, A K Sinha, Vishal Gandhi, Garima Dublish 2013). NRCD stated that UASB is the preferred treatment system over other treatment technologies. Table 1.3 depicts the different wastewater treatment systems widely used in India for wastewater treatment and the costs associated with it.

Table 1.3 Different wastewater treatment systems widely used in India for wastewater treatment and the costs associated with it

STP Type	Capital cost (USD/m^3)	Maintenance and Operation cost (USD/m^3)	Land requirement (m^2/m^3)
UASB	441	20	14
WSP	20	50	12-14
ASP	186	47	1.5

Source; Singh and Srivastava, 2011.

Most of the STPs in India are based on ASP where the wastewater treatment is carried out by microbial sludge. The process mechanism relies on efficient and long-term aeration. Microorganisms assimilate/bio-transform the organic matter and dissolved pollutants from wastewater during aerobic phase. The microorganisms forms clumps and settles down at bottom along with sludge and the clean water can be removed and collected from top (Eckenfelder, Grau, and International Association on Water Pollution Research and Control. Conference (1992 : Washington 1998; Hreiz, Latifi, and Roche 2015). ASP is efficient in removal of organic matter, nitrogen and phosphorous compounds. However, ASP suffers with some disadvantages such as sludge disposal problem, requirement of skilled labours and

high operational cost (Albert B. Pincince et al. 1997; Hansen, Thøgersen, and Rogalla 2007). ASP requires constant supply of energy (0.5 kWh/m^3) for aeration and water pumping (Bodík, I., Kubaská 2013). Power shortage hamper the treatment capacity and efficiency of ASP system (Soares et al. 2017). The technology selection for wastewater treatment depends on availability of space, quality and quantity of wastewater. The classification of available biological wastewater treatment systems is depicted in Fig. 1.3.

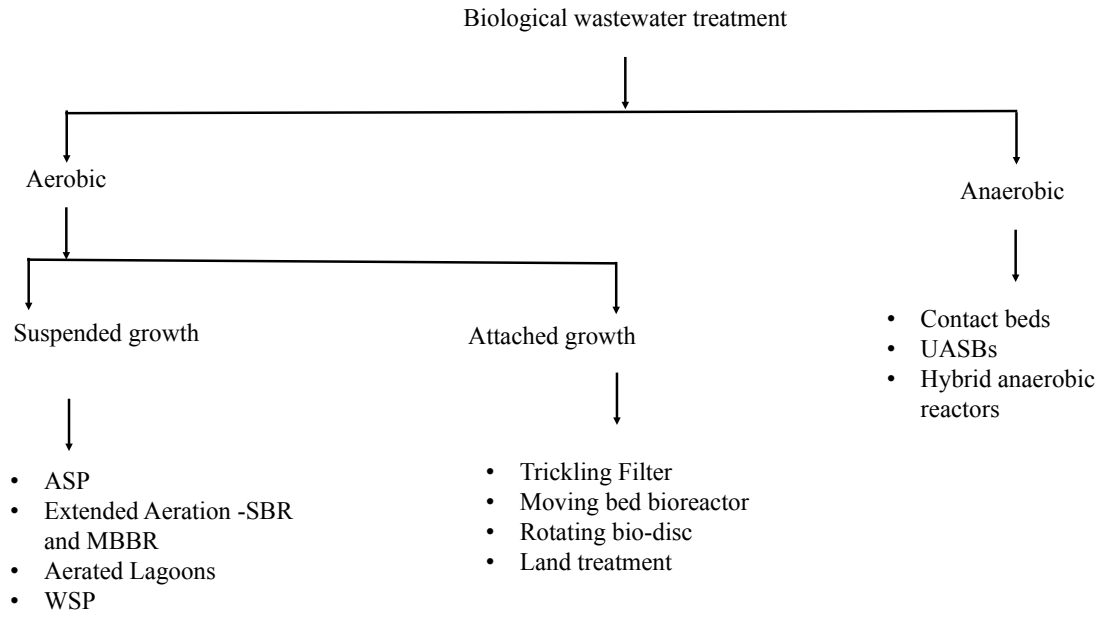


Fig. 1.3 Classification of biological wastewater treatment systems

Membrane Bioreactors (MBRs) are very efficient in wastewater treatment and require less land requirement. However, use of membrane technology is restricted due to high capital, maintenance and operational costs, requirement of highly trained technical staff, complexity of the process, use of harmful chemicals for cleaning and membrane fouling (Judd 2008; Lo, McAdam, and Judd 2015; Santos, Ma, and Judd 2011). Another frequently used technology for wastewater treatment is Fixed Aerated Bed (FAB) that requires matrices for the growth of microorganism. These matrices provide large surface area per unit volume of the reactor. Different matrix manufacturers suggest different quantity of matrix to be used per unit of reactor volume and the size, shape, dimensions and available surface area also varies. The FAB also require continuous aeration and proper design optimization with respect to input and output flow of water (Lo et al. 2015; Suresh Kumar D. and Dr. Sekaran V. 2013) . FAB is also sensitive to variation in pH, temperature and dissolved oxygen concentration (Ye et al. 2016). All these requirements make standardization of process difficult.

Solid organic substrates from wastewater can be considered for anaerobic digestion for the production of methane (Chandler et al. 1980) . Variety of substrates can be used as feed in anaerobic digestion like sorted municipal solid wastes, wood, grasses, vegetables and biomass etc. (Nallathambi Gunaseelan 1997). There are varieties of compounds present in digester feed causing digester failure; ammonia, heavy metals, organics and sulfides (Chen, Cheng, and Creamer 2008). Due to the compositional variations in inoculum and substrate it is difficult to predict Biomethanation potential of substrate accurately. Anaerobic digestion often suffer low methane yield and process instability limiting its use as widely applied technology (Chen et al. 2008). Anaerobic digesters leads to generation of digestate that can be used as organic fertilizers after subjected to some pretreatment methods, however, its NH_3 emission potential is high, and there is great risk of environmental hazards (Nkoa 2014). Table. 1.4 depicts the salient features of currently available treatment systems in India.

Recently, SBRs are getting widespread acceptance as treatment system due to its operational flexibility and efficient process control ability (Dutta and Sarkar 2015). SBR has several advantages over other treatment system; flexibility of operation and control, minimal footprint and cost effectivity. However, it suffer several disadvantages such as requirement of high level sophistication and maintenance, frequent fouling of aeration systems, possible discharge of sludge during decant phase and requirement of equalization of treated water before use (Dutta and Sarkar 2015; Sh. J.S. Kamyotra, Dr. D.D. Basu, R.M. Bhardwaj,A K Sinha, A K Sinha, Vishal Gandhi, Garima Dublish 2013; Singh and Srivastava 2011).

Table 1.4 Salient features of currently available treatment systems in India.

	*ASP	*MBR	*SBR	*MBBR/*FAB	*ASFF
Working Principle	Continuous aeration	ASP coupled with ultrafiltration-continuous	Modified ASP-Batch process	ASP in presence of support matrix-continuous	ASP with fixed submerged media
Advantages	Good COD and BOD removal	Good effluent quality	High COD, BOD, N and P removal	Good COD and BOD removal, less footprint	Less footprint as compared to ASP
Disadvantages	High footprint	Highly expensive	High sophistication, clogging of aeration devices	Hard to troubleshoot operational problems, sensitivity to wastewater characteristics	Media clogging, no proper growth of bacteria
Power requirement	0.46 kWh/m^3 (Bodík, I., Kubaská 2013)	0.7 kWh/m^3 (Özçimen and Inan 2015)	0.33 kWh/m^3 (Gu et al. 2017)	0.38 kWh/m^3 (Li, Luo, and He 2016)	0.17 kWh/m^3 (Stillwell et al. 2010)

* ASP-Activated Sludge Process, MBR-Membrane Bioreactor, SBR-Sequencing Batch reactor, MBBR-Moving Bed Biofilm Reactor, FAB-Fluidized Aerobic Bed Reactor and ASFF- Aeration Submerged Fixed Film.

The limitations of currently available wastewater treatment technologies are;

- Sensitivity to sudden changes in quality and quantity of wastewater (Hreiz et al. 2015).
- Require high BOD to COD ratio (>0.6) (Fux et al. 2002; Hreiz et al. 2015).
- Problems associated with sludge separation, handling and safe disposal (Nilsson and Dahlström 2005; Sezgin 1982).
- Requirement of high capital, maintenance and operation cost and high land requirement (Singh and Srivastava 2011).
- Operated by civil engineers rather than a biologist (Chatterjee, Ghangrekar, and Rao 2016).
- Lack of potential to generate value-added product (Gonçalves et al. 2017).

The limitations of currently available wastewater treatment systems can be overcome by adopting MTS. Microalgae have proven to be useful in treatment of varieties of wastewater including domestic (Kosaric, Nguyen, and Bergougnou 1974; Ruiz-Marin, Mendoza-Espinosa, and Stephenson 2010; Wang et al. 2010), municipal (Li, Y.-F. Chen, et al. 2011; Wang et al. 2010), textile effluents (Lim et al. 2010), anaerobic digestate (Park et al. 2010), dairy wastewater (Woertz et al. 2011), piggery wastewater (Park and Craggs 2007; Zhu et al. 2013), fishpond water (Konnerup et al. 2011), poultry litter (Singh et al. 2011) and anaerobic digestate (Park et al. 2010) suggesting that they are less sensitive to wastewater composition and has ability to adapt to the changes in wastewater characteristics (Min et al. 2011; Zhu et al. 2013). Numerous studies has also reported that microalgae can be grown autotrophically (Yun et al. 1997) in wastewater with less COD/BOD ratio and high N/C ratio (Fux et al. 2002; Park et al. 2010; Samorì et al. 2013). Microalgal wastewater treatment has proven to be economical and ecofriendly over currently available wastewater treatment systems (Abdel-raouf, A.A.Al-Homaidanb, and I.B.M.Ibraheembc 2014; Cai, Park, and Li 2013; Mishra and Mishra 2017; Molazadeh et al. 2019; Rawat et al. 2011). Civil engineers and STP operators can be easily trained for microalgal wastewater treatment systems. Numerous studies have reported the potential of microalgal biomass for the extraction of biodiesel (Converti et al. 2009; D'Oca et al. 2011; Fakhry and Maghraby 2013; Halim, Danquah, and Webley 2012; Johnson and Wen 2009; Li, Y.-F. Chen, et al. 2011; Schenk et al. 2008), biogas (Calicioglu and Demirer 2015; Craggs et al. 2014; Passos, Garca, and Ferrer 2013; Varol and Ugurlu 2016; Yadav et al. 2016), ethanol (Harun, Danquah, and Forde 2009; Ho et al. 2013; Hossain, Basu, and Mamun 2015; Valderrama et al. 2002) and phytochemicals (Bhagavathy, Sumathi, and Jancy Sherene Bell 2011; Panja et al. 2014; Prakash, @ Antonisamy, and Jeeva 2011;

Raposo and de Morais 2015) in combined biorefinery concept (Chavan and Mutnuri 2018; Cherubini 2010; Fon-Sing et al. 2016; Jong et al. 2011; Mussgnug et al. 2010).

1.2.7 Constructed wetlands as treatment system

The alarming increase in the volume of raw domestic wastewater being generated is imparting pressure on wastewater treatment plants. Constructed Wetlands (CWs) are treatment systems that mimic the natural wetlands that use vegetation, soil and associated microorganisms to increase the water quality. CWs are gaining widespread attention as an alternative technology for sewage treatment. CWs can efficiently treat varieties of wastewater including DW, storm water, leachate, polluted river, rural runoff and industrial effluents (Abou-Elela and Hellal 2012; Badhe et al. 2014; Saeed and Sun 2013). CWs are found to be efficient in removal of organic and in-organic pollutants, solids, nitrogenous and phosphate compounds , trace elements and various other pharmacological contaminants (Cui et al. 2010; Saeed and Sun 2013). The main factors influencing the long term pollutant removal efficiency of CWs are plant species, microbial biofilm and construction media (Li et al. 2008). The other critical operational factors influencing the treatment performance of CWs are hydraulic loading rates, design and construction of CWs, water depth, water retention time and feeding mode (Wu et al. 2014).

There are several advantages of CWs over conventional treatment systems including low maintenance and operation costs and potential application in developing countries. In VFCWs, treatment mechanisms to remove phosphorous, nitrogen, organic matter and solids includes: a) Physical treatment - filtration, settling, volatilization and sedimentation b) Chemical-oxidation, ion exchange, adsorption and precipitation c) Biological - bacterial degradation, bacterial consumption, nitrification, denitrification, plant uptake, predation, biodegradation and phytoremediation (Kadlec and Wallace 2009). In the selection of CWs vegetation preference should be given to the plant species naturally occurring to the area where CWs are being constructed.

Even if CWs have advantages over conventional treatment systems in terms of organic, inorganic carbon and solids removal, it suffers from several limitations. The removal nitrogenous and phosphate compounds are not efficient in CWs (Abdelhakeem, Aboulroos, and Kamel 2016). The main removal mechanism of PO_4-P is adsorption, and it may leach out during the time course. Therefore, it is essential to integrate treatment VFCWs to Microalgal Treatment System (MTS) to increase overall long-term pollutant removal efficiency.

In VFCWs, as the oxygen diffusion rate are not uniform throughout the system, the rate of nitrification is often high ; the rates of denitrification and NH_3 volatilization are low. To

increase the long-term pollutant removal efficiency of VFCW, it is essential that nitrification should be followed by denitrification. However, this is not the case. Nitrification is carried out by obligate aerobes (in presence of oxygen) and denitrification is carried out by facultative anaerobes (in absence of oxygen). Nitrifying microorganisms include *Nitrosomonas* sp., *Nitrobacter* sp., *Nitrosococcus* sp., *Actinomycetes* sp. and fungi. Denitrifying microorganisms consists *Paracoccus* sp., *Micrococcus* sp., *Pseudomonas* sp. *Thiobacillus* sp. and *Achromobacter* sp. The rate of nitrification depends on ammonification rate (requires high O_2 and low carbon concentration), NH_4-N flux into aerobic zone (roots and air-water interface) and dissolved O_2 concentration. However, denitrification rate depends on nitrification rate (requires low O_2 and high carbon concentration) and flux of NO_3-N into anaerobic zone. As nitrification occurs in confined areas (root rhizospheres and air-water interface) rates are often limited in VFCW.

1.2.8 Struvite crystallization

Fertilizer industry Wastewater (FW) is composed mainly of ammoniacal nitrogen (NH_4-N), nitrate nitrogen (NO_3-N), orthophosphate (PO_4-P) and low carbon content (less C: N ratio) leading to the emission of potential greenhouse gas, i.e. nitrous oxide (N_2O) (Kampschreur et al. 2009). Utilization of water contaminated with nitrate leads to health disorders in human infants known as blue baby syndrome (Knobeloch et al. 2000) and excess of phosphorous leaching to water bodies causes eutrophication leading to algal blooms resulting in loss of aquatic life via anoxic conditions (Conley et al. 2009; Correll 1998; Ryther and Dunstan 1971; Vollenweider 1968). Therefore, it is necessary to remove these pollutants from wastewater/effluent before discharging to water bodies or reusing. Wastewater treatment includes removal of solid matter and dissolved pollutants to produce effluent that can be recycled or reused. There are different technologies available namely aerobic, anaerobic, membrane separation and adsorption for removal of NH_4-N, COD depending on wastewater composition (Cheremisinoff 2002).

Efficient removal of nitrogen and phosphorous from wastewater is a challenge for treatment process. Possible solution to this is to recover nitrogen and phosphorous from wastewater in the form of struvite. Struvite is an equimolar crystalline mixture of magnesium, ammonium, and phosphate (MAP). There are several advantages of struvite production systems over conventional nitrogen and phosphorous removal technologies, i.e. its precipitation can be controlled easily leading to a solid mass of slow release fertilizers, no sludge formation and most importantly pollutants can be recovered and considered as nutrients. Several technologies exist for pilot scale controlled struvite recovery from wastewater including

stirred tank, air agitated and fluidized bed reactors (KS Le Corre, Valsami-Jones, and Hobbs 2009). Since C: N ratio will be less in FW, biological wastewater treatment cannot be adopted easily for effective removal of N and P compounds by nitrification and denitrification by microorganisms as they require carbon as a source of energy (Meyer et al. 2005). Besides this, conventional wastewater treatment generates large amount of sludge leading to problems associated with nutrient recovery in the form of fertilizer, sludge handling and disposal (Ahluwalia and Goyal 2007). As struvite precipitation will remove only NH_4-N, TKN and PO_4-P, microalgal production in residual wastewater after struvite precipitation could be a better alternative for wastewater treatment as well as using the biomass for the production of value added products.

1.2.9 Integration of wastewater treatment to microalgal biorefinery

1.2.9.1 Biofuels

Recently, the world is facing the challenges of global warming, energy crisis and sudden climate change. To overcome all these challenges, industries, academia and governments are relying on sustainable and pollution free energy production and consumption. The main route of all these problems is increasing number of on road vehicles emitting CO_2, CO, NO_x, particulate matter and other unused hydrocarbons in the atmosphere contributing to air pollution. Almost all of these vehicles either use fossil fuels (petrol or diesel) as an energy source. CO_2 emissions have been increased to 41 % from1990 to 2008 (Le Quéré et al. 2009). The UK energy Research Center have concluded that the fossil fuel reserves will be depleted by 2030 (Sorrell et al. 2009). New oil and gas reserves are constantly being found in arctic circle replenishing the global oil reserves. The increased CO_2 emissions from burning fossil fuels is directly related to climate change such as heat wave, heavy precipitation, rise in sea level and various natural catastrophes.

Biofuels can be defined as the fuels that can be produced from biomass using various bio-transformation processes. The biomass can be anything having stored solar energy in the form of carbon ex. wood, sugarcane etc. These fuels can be looked as the better substitute for fossil fuels in terms of sustainability. Fossil fuels including coal, petrol and natural gas are not considered as sustainable biofuels as they are the alternate forms of carbon fixed in tens of thousands of years ago. Biofuels can be classified in three broad categories based on the source of biomass; first generation biofuels, second generation biofuels and third generation biofuels. First generation biofuels include biodiesel and ethanol via transesterification of lipids or sugar fermentation produced from food crops (palm, canola, corn and potato) grown on arable land. Second generation biofuels include the ethanol produced from lignocellulosic

materials (agricultural residues, woody plants and waste plant materials (wood, jatropha and switch grass). The evolution of biofuels is depicted in Fig.1.4.The first and second-generation biofuels competes for arable land thus leading to possible decrease in food production for ever increasing human population and obvious increase in food price. Third generation biofuels (microalgal biofuels) can confront these challenges as they do not compete for arable land for production.

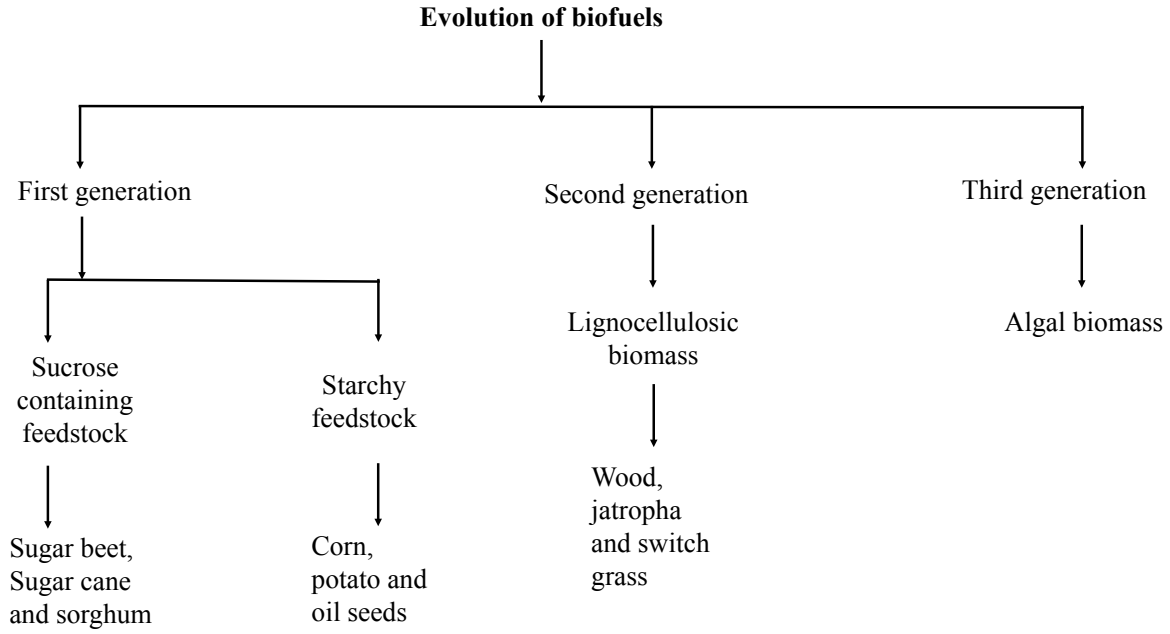


Fig. 1.4 Evolution of Biofuels (Halim et al. 2012).

Microalgae are unicellular or multicellular autotrophic micro-organisms having ability to store solar energy in the form of lipids, carbohydrates and proteins in high concentrations as a constituent of their biomass. They do not show true leaf, stem and root like structures. *Cyanobacteria* are prokaryotic while all the members of *Chlorophyta* are eukaryotic. They can be grown in shallow lagoons, ponds, closed reactors, sea-based systems or in photobioreactor. The factors to be considered in microalgal cultivation systems are; a) Selection of species/strain, b) Nutrient availability, c) Light d) Source of organic carbon (for mixotrophic growth) e) Microalgae cultivation system (open raceways, photobioreactor, polybags etc.) and f) Molecular biology and genetic engineering. Microalgal biofuels are gaining immense attention and interests as third generation biofuels as they do not compete for farmland for their cultivation, high biomass generation per unit area as compared to other energy crops, high lipid content, can be grown in salty and wastewater reducing cost of cultivation and can utilize flue or exhaust gases as a carbon source.

1.2.9.2 Microalgal production systems:

Microalgae can utilize CO_2 from atmosphere as carbon source, energy from sunlight and nutrients from aquatic environment for their growth under natural conditions. Therefore, artificial production systems should be designed to mimic the natural growth conditions. The major limiting factor for natural production system is uniform availability of light throughout the year. The limitations to light availability can be confronted with the use of fluorescent light for continuous algal production. Microalgae can utilize carbon from three major sources, namely; a) CO_2 from air, b) CO_2 from industry flue gases and c) CO_2 generated during carbonates dissolved in water. Other inorganic nutrients required for microalgal production consists of nitrogen and phosphorous. Nitrogen can be supplied in the form of urea and phosphorous can be supplied in terms of phosphates.

Microalgal production systems can be classified in three broad categories on the basis of energy source, namely, photoautotrophic cultivativation, heterotrophic cultivation and mixotrophic cultivation (Brennan and Owende 2010). These broad categories are subdivided into subcategories on the basis of structure and design of microalgal production systems as depicted in Fig. 1.5. The basic structural design of different types of microalgal production systems are described in Fig.1.6.

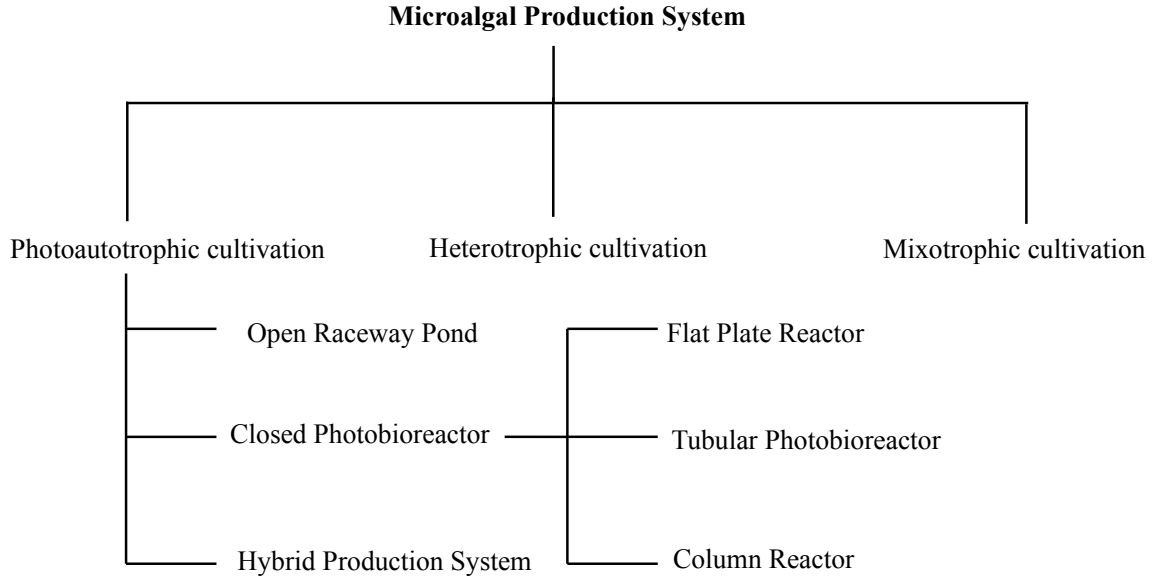
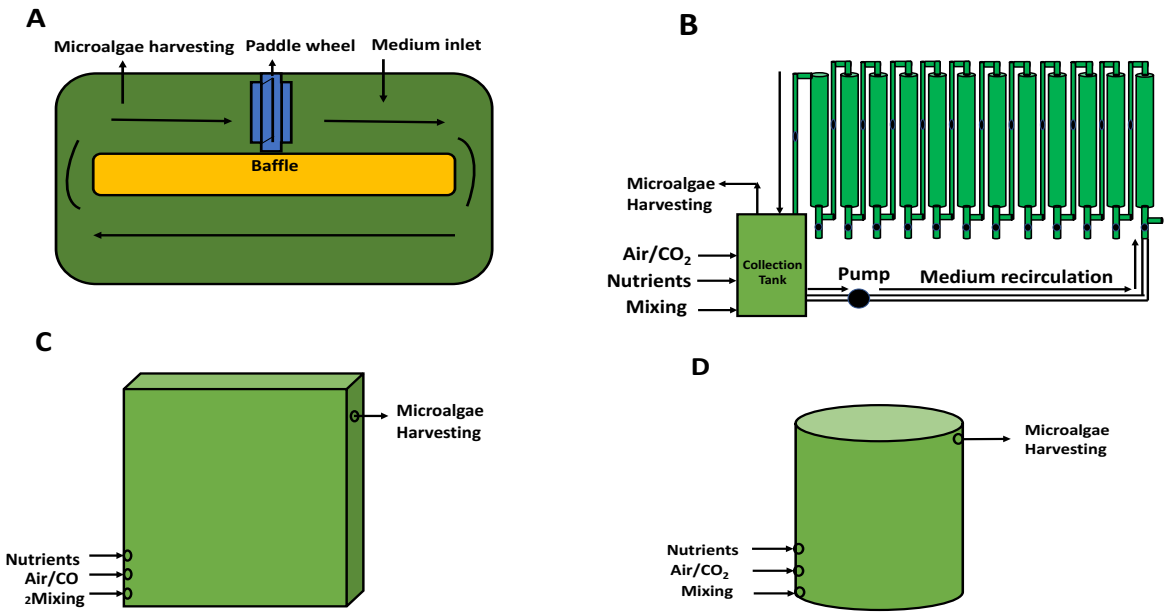


Fig. 1.5 Classification of microalgal production systems (Adopted from Brennan and Owende; 2010 (Brennan and Owende 2010)).



1.6 Basic structural design of different types of microalgal production systems (A-Open raceway pond, B-Tubular photobioreactor, C-Flat panel photobioreactor and D-Column photobioreactor).

1.2.9.2.1 Photoautotrophic cultivation systems

These microalgal production systems basically uses natural sunlight as a source of energy. This production system is most technically and economically feasible.

1.2.9.2.1.1 Open raceway ponds

Open raceway pond microalgal production systems are most widely used for large scale algal cultivation (Fig. 1.6A). They are typically consisting of oval shaped, closed loop recirculation water tanks made of concrete. These ponds can be operated at the depth of 0.2 to 0.5 meters. Mixing and aeration can be performed by continuously operating paddlewheels. The nutrients can be fed in front of paddlewheel and the harvesting point at the other end. These production systems do not compete with agricultural crops for farmland as they can be constructed anywhere in non-agricultural land. The maintenance and operation costs for open raceway ponds are less. However, these production systems suffer inherent threat of contamination by bacteria and other microalgal species. This can be confronted by using monoalgal cultures that needs extreme conditions for their growth (*Spirulina platensis* needs highly alkaline environment for their growth checking the growth of others).

1.2.9.2.1.2 Closed photobioreactor system

To overcome the problem of contamination in microalgal cultivation in open system closed photobioreactor systems have been developed. Due to the use of closed photobioreactor, it is possible to control the experimental environment that can be used for cultivation of monoalgal cultures for production of value-added products. On the basis of structure and design, closed photobioreactors are further subdivided into three categories.

1.2.9.2.1.2.1 Tubular Photobioreactor

This kind of algal production system include either vertical or horizontally inter-connected transparent glass or acrylic tubes. These tubes are connected to external tank. The biomass can be harvested from external tank. Moreover, the mixing and nutrient replenishment is also carried out in external tank. Well mixed medium can be fed back into the tubular photobioreactor using centrifugal water pumps as shown in Fig. 1.6B. Tubular photobioreactors provides large surface areas to capture light, can be used for outdoor cultivation and can provide high biomass productivities. However, it suffers from some operating issues, namely, frequently observed wall growth, fouling, large land requirement during scaling it up and gradients of dissolved oxygen, carbon dioxide, pH and nutrients along the length of tubes.

1.2.9.2.1.2.2 Flat plate Photobioreactor

This form of close photobioreactor system consists of rectangular transparent tank with smaller widths. This reactor systems provides several adavantages including large illumination surface area, easy sterilization ability, suitability for outdoor cultivation, low oxygen build-up and easy cleaning. However, these production systems also suffer several limitations like difficulties in scaling up, problems associated with temperature control, fouling and some degree of wall growth. It is equipped with different ports for medium inlet, aeration, mixing and harvesting microalgal biomass as shown in Fig. 1.6C.

1.2.9.2.1.2.3 Column Photobioreactor

Column photobioreactors consists of transparent cylindrical tanks equipped with several ports for medium inlet, mixing, air sparging and biomass harvesting as shown in Fig. 1.6D. These reactor systems provide efficient mixing with high volumetric transfer rates and the environmental conditions can be easily controlled. The main advantage of this kind of reactor include the ability to aerate the reactor from inside and internal illumination. These reactors are widely used for bio mitigation of CO_2 emissions using microalgae.

1.2.9.2.1.2.4 Hybrid Systems

Hybrid systems integrates closed photobioreactor systems to open raceway ponds providing ability to carry out two different growth stages sequentially. Closed systems are used to maintain aseptic environment to avoid contamination from other algal species and continuous cell division. Further, the microalgal culture is fed into open raceway pond to provide desired nutrient stress to accumulate desired product in microalgal biomass. For example, nitrogen limitation enhances lipid content of microalgal cells (Rodolfi et al. 2009).

1.2.9.2.2 Heterotrophic cultivation

Heterotrophic microalgal cultivation uses organic carbon sources (glucose, sucrose, bicarbonates etc.) instead of sunlight as a source of energy in open raceway ponds or in closed photobioreactors. These production systems do not rely on sunlight leading to easy scaling up. Moreover, as the use of organic carbon increases lipid content, this production system usually used for production of biodiesel from microalgal biomass (Miao and Wu 2006).

1.2.9.2.3 Mixotrophic cultivation

Many microalgal species are able to grow mixotrophicaly indicating that they have potential to use either organic carbon or light as a source of energy depending on the availability. As they can metabolize organic carbon, meaning that the microalgal growth is not strictly dependent on light availability. The microalgal organisms with mixotrophic potential are *Spirulina platensis* and *Chlamydomonas reinhardtii* (Chen, Zhang, and Guo 1996).

1.2.9.3 Microalgal biofuels

Cultivation of microalgae using different nutrient streams is relatively easy, however, recovery of biomass is limiting factor. Biomass harvesting process accounts to 30 % of production costs. There are several methods to separate biomass from liquid, namely, flocculation, sedimentation, gravity settling, filtration and centrifugation (Brennan and Owende 2010). Among all these biomass recovery methods, filtration is technical and economically viable method and can be scaled up easily. The choice biomass recovery methods depend on the size of microalgal culture under study, for example, for smaller algal cells (< 30 uM size) membrane filtration and microfiltration are technically viable method and for harvesting of algal cells like *S. platensis* (> 70 uM size) filtration can be employed (Petruševski et al. 1995).During filtration, use of filter aids (diatomaceous earth and cellulose) can improve the filtration efficiency by avoiding clogging of filter pores.

Once the biomass is harvested, methods should be employed to remove the moisture to get dry biomass pellets. Various drying techniques are in existence including sun drying, spray drying, drum drying, hot air drying and freeze drying. Sun drying method is relatively cheap and viable, but it suffers the disadvantages like requirement of large surface area, long drying time and possibility of loss of metabolite from biomass. Other methods can confront these disadvantages but are relatively expensive and need technical staff to handle the drying process.

The dried cell pellets need to be broken apart by mechanical, enzymatic, moist heat under pressure or chemical methods to extract intracellular metabolites like lipids and pigments. For example, to extract lipids from microalgal cells, solvent (Chloroform: Methanol 2:1) extraction, is generally used in Soxhlet extraction.

The harvested biomass should be stored in cool and dry place until it can be used as a substrate for extraction of biofuels. There are several approaches that can be applied to extract different kinds of biofuels from microalgal biomass as shown in Fig. 1.7.

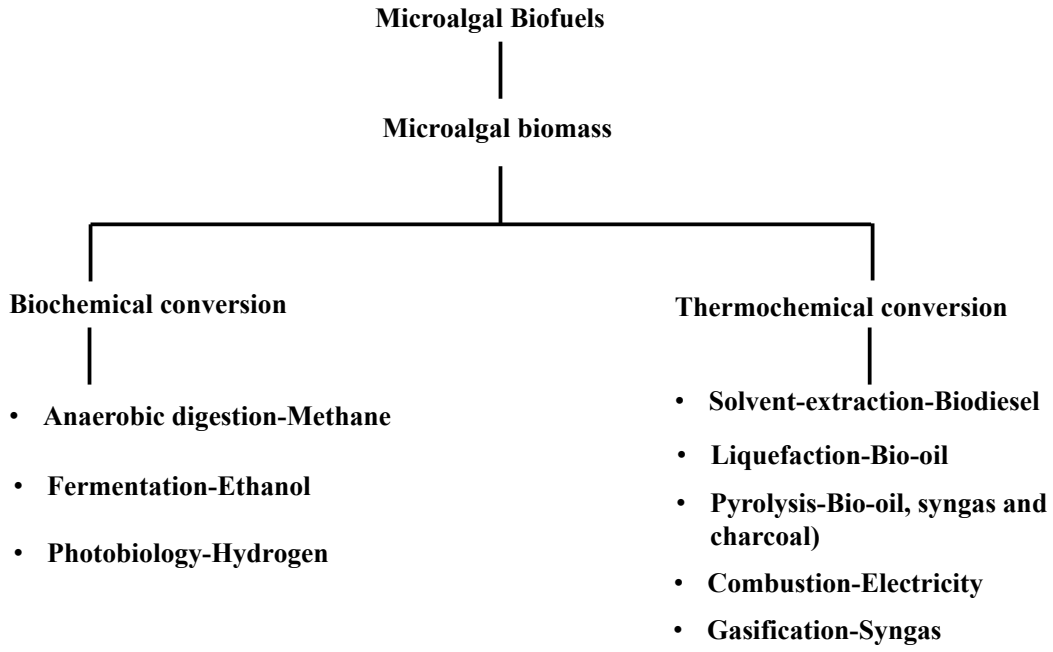


Fig. 1.7 General overview of biofuel production from microalgal biomass (Adopted from Sukahara and Sawayama;2005 (Tsukahara K 2005).

1.2.9.3.1 Thermochemical conversion

Thermochemical processes encompass the thermal degradation of organic matter present in biomass to obtain variety of fuel products. It includes gasification, liquefaction, pyrolysis and direct combustion as shown in Fig.4. The choice should be made depending on the target fuel.

1.2.9.3.1.1 Gasification

The microalgal biomass undergoes partial oxidation at (800-1000 ºC) in presence of O_2 and water to generate low calorific (4-6 MJ/M^3) syngas (a mixture of CO, H_2, CO_2 and CH_4) that can be burned in gas engines (McKendry 2002). Many researchers have explored gasification potential of *S. platensis* and *Chlorella vulgaris* to convert carbon component to methane and nitrogen component to ammoniacal fertilizer (Minowa and Sawayama 1999).

1.2.9.3.1.2 Thermochemical liquefaction

Thermochemical liquefaction can directly convert wet algal biomass directly to liquid fuel, bio-oil. The process is carried out at low temperature (300-400 ºC) and high pressure (5-10 MPa) in presence of catalyst and hydrogen. The process uses the water activity at subcritical temperature to break large molecules with less density to small molecules with relatively higher energy density (Patil, Tran, and Giselrød 2008). Many researchers have investigated the production of bio-oil from microalgal biomass using thermochemical liquefaction. For example, Dote et al has observed 64 % (W/W) bio-oil yield using *Botryococcus braunii* biomass with positive energy balance (Dote et al. 1994).

1.2.9.3.1.3 Pyrolysis

Pyrolysis covers the utilization of microalgal biomass for production of charcoal, syngas and bio-oil at temperature of 300-500 ºC in absence of air. Flash pyrolysis involves the use of hot vapor stream for one second indicating the viable possibility of liquid biofuel production from algal biomass with biomass to liquid fuel conversion efficiency of 95 % (Stevens, Clark, and Deswarte 2008). The bio-oils produced via pyrolysis often contains solids, acidic pH, unstable compounds, viscous in nature and dissolved water. Therefore, the process needs to be upgraded for catalytic hydrogenation and remove alkalis (Demirbaş 2001). Miao and Wu has demonstrated the potential production of bio-oil using fast pyrolysis from *Chlorella prothothecoides* biomass with 57 % of oil yield (Miao and Wu 2004).

1.2.9.3.1.4 Direct combustion

Microalgal biomass can be burnt in presence of O_2 to release the stored chemical energy in the form of heat. The heat can be used in boilers and the steam generated can be used to run turbines producing electrical energy that can be stored. During the conversion of chemical energy to stored electrical energy, some fraction of energy might be lost. The biomass should have less than 50 % (W/W) moisture to be used as a substrate for combustion (McKendry 2002).

1.2.9.3.1.5 Solvent extraction

Microalgal biomass is rich in lipids. These lipids can be extracted by using different solvents, namely, hexane, ethanol, chloroform and methanol either individually or in combination in Soxhlet system depending on the fatty acid composition of algal lipids (Amos Richmond 2004). The extracted lipids are subjected to transesterification in presence of acidic or basic catalyst where the algal lipids are finally converted to fatty acid methyl esters (FAMEs) in presence of methanol. During transesterification triglycerides (TAGs) are converted to diglycerides, monoglycerides and finally to esters. The FAMEs can be used as a biodiesel. Francisco et al has reported that *C. vulgaris* with lipid content of 27 % dry weight (43 % saturated and 42 % monounsaturated fatty acids) can be effectively used as feedstock of for biodiesel production (Francisco et al. 2010).

1.2.9.3.2 Biochemical conversion

The microalgal biomass can be biochemically converted to fuels via anaerobic digestion, alcoholic fermentation and light driven bio-hydrogen production.

1.2.9.3.2.1 Anaerobic digestion

Anaerobic digestion is the process of converting organic matter present in biomass to biogas containing CH_4, CO_2 and other gases in trace amounts (for example H_2S) in absence of O_2. Anaerobic digestion can be employed for biomass with high moisture content (80-90 %) and as well as biomass with less moisture content as low as 5 % (McKendry 2002). Anaerobic digestion process occurs in three sequential phases, namely, hydrolysis, fermentation and methanogenesis. During hydrolysis, the large, complex molecules are broken down into smaller molecules like sugars. These sugars are then fermented to alcohols, volatile fatty acids and acids by fermentative anaerobes. These acids and alcohols are further metabolized by methanogens to CH_4 (60-70 %), and CO_2 (30-40 %), while leaving the nutrient rich liquid digestate that can be used as soil fertilizer or can be recycled by to nutrient stream for microalgal cultivation (Sialve et al. 2009).

There are certain challenges in anaerobic digestion of microalgal biomass as mentioned below. The ideal C/N ratio for anaerobic digestion for efficient production of methane is 25 (Dioha et al. 2013). Microalgal biomass often have less C/N (3 to 17) ratio (Geider and Roche n.d.) leading to fast acidification of reactors; however, if algal biomass is co-digested anaerobically with other substrate with high C/N ratio can solve this problem. Microalgal biomass also contains high amounts of proteins which release ammonia during fermentation phase of anaerobic digestion inhibiting variety of anaerobic microorganisms. Dogan and

Demirer has observed the biogas yield of 238 mL/ g VS (volatile solids) from anaerobic digestion of Chlorellae vulgaris biomass (Doğan-Subaşı and Demirer 2016).

1.2.9.3.2.2 Alcoholic fermentation

Fermentation process involves the biochemical conversion of carbohydrates (sugars, starch and cellulose) to alcohol by using fermentative yeast, *Saccharomyces cerevisiae*. Microalgal biomass can be utilized as a substrate for ethanol production via alcoholic fermentation. Fermentation processes end up in dilute broth containing 10-15 % of ethanol. These dilute broths are then subjected to distillation and the ethanol vapors are condensed at low temperature to obtain concentrated ethanol solution (95 % V/V). The residual solids at the end of fermentation can be fed for gasification, anaerobic digestion or as organic fertilizer (Demirbaş 2001; McKendry 2002). Previuously, *C. vulgaris* (37 % starch content on dry weight basis) has been used for production of ethanol via yeast fermentation with 65 % of conversion efficiency (Hirano et al. 1997).

1.2.9.3.2.3 Photocatalytic hydrogen production

Hydrogen (H_2) gas is a clean, efficient and high calorific value energy source. Under anaerobic conditions, microalgae can produce hydrogen during CO2 fixation stage of photosynthesis. The hydrogen thus produced can serve the dual role, either as electron donor or as an electron acceptor. During photosynthesis inside microalgal cells, the water molecule is broken down into hydrogen ions and oxygen, these hydrogen ions are then converted to hydrogen gas by Hydrogenase enzymes in anaerobic conditions (Cantrell et al. 2008).

1.2.9.4 Sustainability of microalgal biofuels

Microalgal biofuels can be produced in sustainable manner by two sequential approaches as mentioned below.

1. Use of wastewater for microalgal cultivation.
2. Sequential extraction of multiple products from microalgal biomass-a biorefinery concept.

1.2.9.4.1 Use of wastewater for microalgal cultivation

Wastewater treatment potential of various microalgae have been very well studied by many researchers by using different reactor systems. In general, the wastewater consists of organic and inorganic carbon, ammoniacal nitrogen, total kjeldahl nitrogen, orthophosphates and dissolved carbon dioxide making the natural environment for microalgal growth. Microalgal growth systems can use these components from wastewater as nutrients for their growth

while minimizing the use of fresh water for cultivation and the reducing the cost of microalgal biomass production. The factors influencing potential of wastewater treatment by using microalgae include, abiotic (light), physical and chemical factors (temperature, nutrient availability, O2, CO_2, pH, salinity and toxic chemicals), biotic factors (pathogens-bacterial, fungi and viruses, predation by zooplankton and competition between species) and operation factors (mixing, dilution rate, depth and harvesting frequency) (Larsdotter 2006). *Chlorellae pyrenoidosa* have been successfully utilized for treatment of settled domestic sewage for removal of 93.7 % nitrogen and 80 % of phosphorous with retention time of 13 days (Tam and Wong 1989). The different wastewater treatment systems and potential of MTS to integrate with available infrastructure is depicted in Fig. 1.8

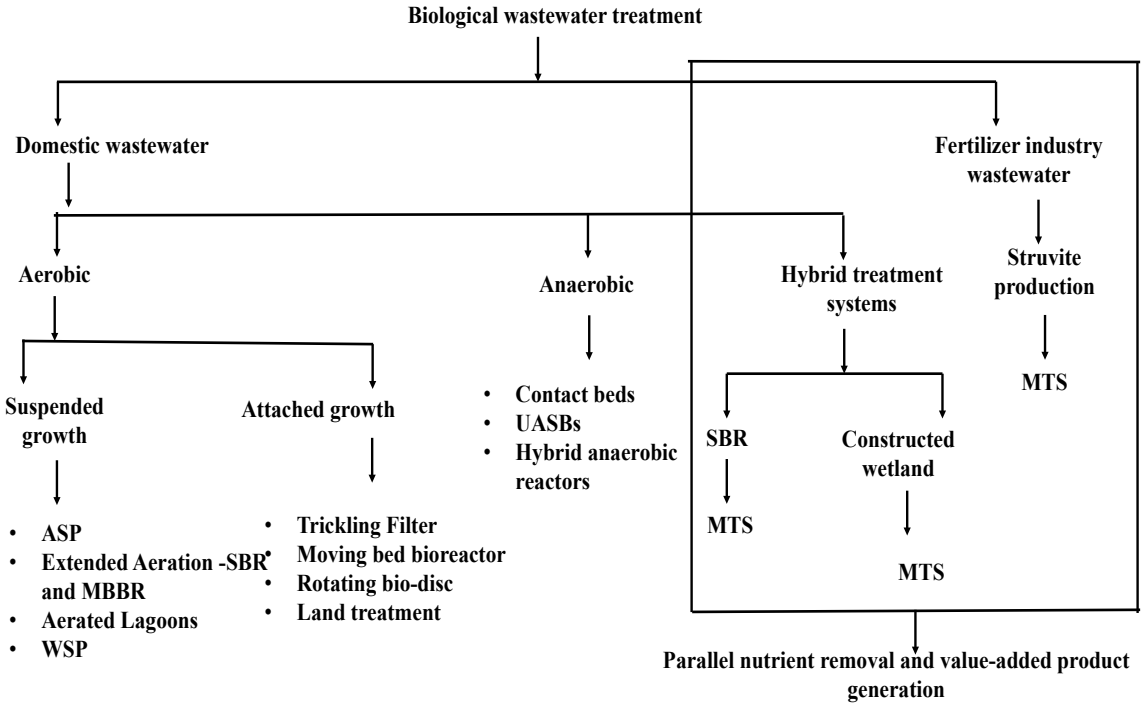


Fig. 1.8 The different wastewater treatment systems and potential of MTS to integrate with available infrastructure (modified from Goncalves et al, 2017 (Gonçalves et al. 2017)).

1.2.9.4.2 Microalgal biorefinery

Microalgal biomass generally consists of lipids, carbohydrates and proteins that can be extracted and utilized for production of variety of value-added products sequentially. True sustainable biofuels from microalgal biomass produced using wastewater can be obtained by using every component of biomass. For example, after lipid extraction 65 % of residual biomass is generated (Zhu 2014). The ideal sequence to use microalgal biomass in a

biorefinery concept is to extract lipids (for biodiesel production), extract carbohydrates, starch and cellulose (for ethanol generation via fermentation), extract leftover complex carbohydrates, lipids and proteins (biogas generation via anaerobic digestion) and the residual biomass slurry can be dried and used as organic fertilizer or soil amendment (Zhu et al. 2014). Chavan and Mutnuri has successfully demonstrated the integration of wastewater treatment of primary treated municipal wastewater with *S. platensis* cultivation for production of biodiesel and biogas in a biorefinery concept (Chavan and Mutnuri 2018). The concept of microalgal biorefinery is proposed in figure 1.9.

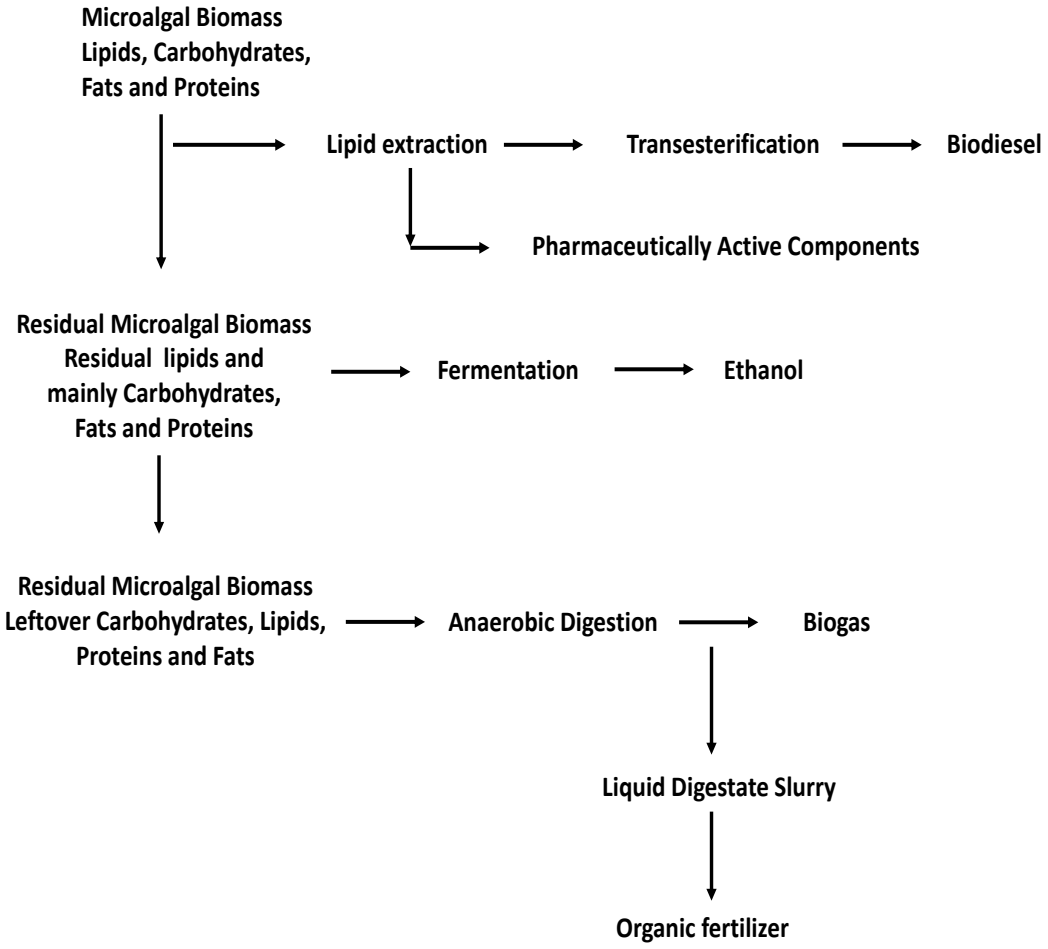


Figure 1.9 Proposed idea of microalgal biorefinery to sequential extraction of multiple products from microalgal biomass.

1.3 Gaps in research

Numerous studies focusing on wastewater treatment by using microalgae are available. However, there are certain loopholes that are described below.

- The composition of wastewater (COD, NH_4-N, TKN, PO_4-P) vary depending on many factors like source of wastewater, drainage system and human habits. Many studies have focused on bioremediation of municipal or industrial wastewater with initial NH_4-N concentrations from 40-100 mg/L. Fertilizer industry wastewater is generally rich in NH_4-N, PO_4-P and has less organic matter. As dissolved ammonia is toxic to algal cells, it is essential to study the ammoniacal nitrogen tolerance studies of individual algal species or algal consortium at NH_4-N concentrations higher than 500 mg/L .
- The literature regarding the anaerobic digestion of biomass for the production of biogas is rich. However, the studies which are dealing with the determination of optimal loading rate of algal biomass for biogas production and effect of different biomass pre-treatment methods on biomass are scarce.
- Various studies have carried out the wastewater treatment by using microalgae and the production of single value-added product at a lab scale and not in the actual field. The studies focused on extraction of multiple products in sequential manner from algal biomass in biorefinery concept are scarce.
- The studies regarding integration of SBR, constructed wetland or struvite crystallization to microalgal treatment system for wastewater treatment are not available.

1.4 Aims and objectives of research work

The main focus of this study was to study the feasibility and potential of MTS in wastewater treatment to be integrated with currently available technologies (SBR, constructed wetland and struvite production) for parallel nutrient removal and production of value-added products. As domestic and fertilizer industrial wastewater vary in NH_4-N composition and variation in microalgal biomass composition, there should be an emphasis on microalga/consortium selection for wastewater treatment.

The proposed objectives of the study are given below.

- **Isolation and enrichment of different microalgae in different wastewaters.**

  The wastewater composition varies based on the nature of source of wastewater. Domestic wastewater and fertilizer industry wastewater mainly vary with respect to carbon, nitrogen and phosphorous content. Microalgae species, either individual or in

the form of consortium was enriched in particular type of wastewater. Our initial focus was on enrichment of microalgae (*S. platensis* and *C. vulgaris*), which is commonly available, frequently used in wastewater treatment and occasionally present in domestic wastewater. The microalgae consortium (BPGC) was isolated from anaerobic digestate having high NH_4-N tolerance. The ammoniacal nitrogen tolerance studies of *S. platensis*, *C. vulgaris* and BPGC consortium was also studied. *S. platensis* and *C. vulgaris* was used for the treatment of domestic wastewater and ammoniacal nitrogen tolerant BPGC consortium was used for the treatment of fertilizer industry wastewater.

- **Biomethane potential of different microalgae isolated in combination with ethanol production or other beneficial products.**

  The potential of *S. platensis*, *C. vulgaris* and BPGC consortium for the production of different value-added products (biogas, ethanol and biodiesel) was also studied. The effect of different biomass pre-treatments (thermal, chemical, sonication and thermochemical) on biogas production from microalgal biomass was also studied.

- **Photobioreactor development for wastewater treatment using isolated microalgae.**

  Efficiency of MTS depends on availability of light as source of energy. Tubular Photobioreactors (TPBRs) have high surface area to volume ratio. Pilot scale (200 L capacity) TPBR was constructed in campus premises and was used for the treatment of fertilizer industry wastewater by using BPGC consortium isolated in previous studies.

- **Pilot scale industrial waste water treatment by selected microalgae.**

  Fertilizer industry wastewater was rich in nitrogen and phosphorous and has less carbon content. Therefore, conventional treatment technologies cannot be used for treatment of such type of wastewater. Fertilizer industry wastewater treatment was carried out by integrating MTS to struvite production and various value-added products were produced from microalgal biomass.

Chapter II

Tertiary treatment of domestic wastewater by *Spirulina platensis* integrated with microalgal biorefinery

2.1 Introduction

Organic and inorganic compounds are released into the atmosphere by means of domestic, agricultural and industrial wastewater pollution. In conventional wastewater treatment plants, about 15–20% total nitrogen load is recirculated to the main stream with the return partially treated water from the previous stage (Fux et al. 2002). This leads to a successive increase in nitrogen load to the wastewater, further decreasing the total nitrogen removal efficiency of wastewater treatment. Drinking nitrate-contaminated water can cause blue baby syndrome in infants (Fan and Steinberg 1996; Knobeloch et al. 2000). The slow flux of phosphorus to aquatic systems causes eutrophication that leads to algal bloom and harmful anoxic effects, destroying aquatic life (Carpenter 2005) and grazing animals as well as sometimes human life (Wase, discovery, and 2008 n.d.). Therefore, it would be beneficial to remove high loads of nitrogen and phosphorus from wastewater. Several approaches have been previously employed for phosphorus removal including microbial adsorption, constructed wetlands, and precipitation; however, each of these has its own benefits and deleterious effects (de-Bashan and Bashan 2004).

Spirulina platensis is a spirally shaped, gram-negative, nontoxic, multicellular blue-green cyanobacterium. It usually occurs in alkaline water rich in carbonate and bicarbonate. Many bacteria are known for their virulence and pathogenicity; however, *S. platensis* is known for its potential nutrient value across the world. *S. platensis* is also known as a rich source of vitamins A, K1, K2 and B12. *S. platensis* is a better candidate for tertiary treatment of wastewater because of its less stringent growth requirements and because it is easy to harvest due to its filamentous shape. Further, treatment of effluent with this microalga results in various benefits, i.e. cost reduction in microalgae cultivation coupled to production of high-value algal biomass (Mahmoud et al. 2015). *S. platensis* can be grown on almost all types of wastewaters, such as secondarily treated wastewater, sago starch factory wastewater, piggery wastewater and anaerobically treated swine wastewater (Cheunbarn and Peerapornpisal 2010; Christenson and Sims 2011; Kosaric, Nguyen, and Bergougnou 1974; Olguín et al. 2003; Phang et al. 2000). *S. platensis* biomass is rich in amino acids, proteins, carbohydrates, vitamins and carotenoids (Belay, Kato, and Ota 1996). It can be used as potential animal feed. Due to the toxic nature of the wastewater, the occasional presence of heavy metals and the difficulty in maintaining unialgal culture, microalgae grown on wastewater have attracted less attention as a food supplement, but they can be used as a substrate for biofuel and energy production in terms of ethanol and biogas production (Converti et al. 2009; RR, GM*, and SV 2015).

Liquid biofuels are divided into three major categories based on the substrate and production technology used (Hossain, Basu, and Mamun 2015). First-generation biofuels (ethanol and biodiesel) were produced using food crops (sugarcane, vegetables, oil seeds, maize, etc.), conflicting with food production and food prices. First-generation biofuels were replaced by second-generation biofuels where waste cooking oil, non-edible plant seed oil (e.g. Jatropha, soybean and Camalina sativa), waste vegetable oil, animal fats, etc. can be used as the substrate (Nagarajan et al. 2013). Second-generation biofuels overcame the problems associated with their first-generation counterparts (for example, cereal and sugar crops like Arundo donax, Miscanthus sp., etc.); then an increase in fuel demand created the need for a consistent supply of substrate, leading to the third-generation biofuel production technologies such as microalgae-based biodiesel.

During the current study, high-rate algal ponds were used for large-scale municipal wastewater treatment using *S. platensis* (Cheng et al. 2012; Park et al. 2010). However, long-term cultivation of microalgae in open ponds was limited by contamination by protozoa and other algae (Radmann, Reinehr, and Costa 2007). The advantages of photo-bioreactors in this process are quite obvious: they offer cultivation under a wide variety of conditions and are recalcitrant to growth of other algae or contamination by undesirable microorganisms and grazers (Moheimani and Borowitzka 2007; Posten 2009).

Several studies have shown that *Spirulina sp.* can be used either for wastewater treatment or for wastewater remediation coupled with biomass production as a source of value-added products. In the current study, we focused mainly on the integrated approach of using algae in wastewater treatment/reclamation and microalgal biorefinery. *S. platensis* biomass can be successfully used as a substrate for biogas production (Varol and Ugurlu 2016). Its low lipid content (<30% cell dry weight) and high carbohydrate content make it an attractive candidate for anaerobic digestion. Generally, pre-treatment of microalgal biomass improves the production and yield of biogas. To make the biomass amenable for biogas production, several pre-treatment methods such as acid treatment, heat, alkali, ultrasonic and microwave pre-treatment are routinely used (Passos et al. 2014, 2016; Passos, Garca, and Ferrer 2013). However, only a limited number of studies have attempted to obtain multiple products from *S. platensis* biomass in a sequential manner by using treated wastewater as a growth medium.

In present study, treated wastewater from the BITS Pilani, KK Birla Goa Campus's Sequencing Batch Reactor (SBR) was used for *S. platensis* cultivation, and subsequently the harvested biomass was used for biodiesel production followed by biogas production. Different pretreatment methods were studied to determine whether they increase biogas yield.

Biomethane potential assays were carried out at 0.1-L scale followed by 5.0-L scale. The effect of different loading rates was also studied.

2.2 Materials and Methods

2.2.1 Source of treated wastewater

BITS Pilani, KK Birla Goa campus, Goa, India, has an SBR for treatment of in-house-generated wastewater. It can treat 250 m^3 of wastewater per day. The overall treatment process includes primary treatment (for removal of solids) followed by secondary treatment in the SBR. This SBR-treated wastewater is then stored in a collection tank and used for gardening. We conducted pilot scale tertiary treatment of wastewater using microalgae. The average composition of SBR outlet, i.e. treated wastewater (TW), is given in Table 2.1).

2.2.2 Microorganism and inoculum preparation

The *S. platensis* culture used in this study was a kind gift from Dr. S. Ramachandran, Biological Sciences, BITS Pilani, Dubai campus. First, laboratory-scale studies were performed to obtain the limit of tolerance for ammoniacal nitrogen (100 mg/L in current study). The system was then scaled to a 30-L glass aquarium equipped with aeration in modified Zarrouk's medium. The cultures were maintained at 30 ± 2°C and illuminated with 40-W fluorescent lamps (Philips) to obtain a photon-flux density of 41.22 ± 2.15 umol/m^2-s, with a 12 h light/dark photoperiod. The light intensity was measured with a light meter (Lutron-LX101A). The microalgal biomass was then harvested by centrifugation. The microalgal pellet obtained was washed thrice with distilled water to remove salts and other debris and stored as a pellet at 4 °C for 2 h, before being used as the inoculum in the pilot-scale treatment of wastewater.

Biomass production during the experiments was analyzed by measuring absorbance at 680 nm and on a dry weight basis. The microalgal culture was sampled on a daily basis for absorbance measurements at 680 nm. The dry weight measurement was performed with minor modification. Pandey et al. used screen-printing paper as a filter membrane while this study uses silk cloth for microalgae filtration (Pandey, Tiwari, and Mishra 2010). The culture was centrifuged every 3 days to obtain a microalgal pellet. The pellet was washed thrice with distilled water and centrifuged at 8000 rpm for 5 min, and the supernatant was decanted. The pellet was then subjected to drying overnight at 60 °C to obtain dry weight.

2.2.3 Batch ammoniacal nitrogen tolerance studies of *S. platensis*

Synthetic Wastewater (SW) was prepared as per the following composition; Sodium carbonate- 2610 mg/L, potassium dihydrogen phosphate- 69.84 mg/L, ammonium chloride- 38.23 g/L and sodium nitrate- 447.46 mg/L. This will produce SW-1000 (NH_4-N concentration is 1000 mg/L) with a final concentration of CO_3^{-2}-1478.26 mg/L, PO_4-P-15.92 mg/L, NH_4-N-1000 mg/L and NO_3-N-172.6 mg/L, respectively. This SW is then further diluted with distilled water to obtain different concentrations (1:10 to 10/10) of NH_4-N. All these ammoniacal nitrogen tolerance studies were carried out at the scale of 200 mL in conical flasks of 500 mL capacity. The pH was adjusted to 8.0. Each flask was then inoculated with 25 mg of freshly harvested *S. platensis* biomass. The flasks were irradiated with 42 µmol photons/m^2-s by using 40 W fluorescent tubes with 16:8 hours of alternating light/dark photoperiod at temperature of 30°C. The negative controls were placed to understand the ammonia stripping at alkaline pH. The samples were removed periodically after every 3 days, centrifuged at 8000 rpm for 15 minutes. The biomass was dried at 40°C overnight in a hot air oven to obtain dry weight. The growth curve was obtained by plotting biomass concentration with respect to time. The growth rate was calculated using following formula,

$$Growth\ rate\ (day^{-1}) = (lnN_2 - lnN_1) \div (t_2 - t_1)$$

Where,N_2 and N_1= biomass concentration at t_2 and t_1, respectively.

2.2.4 Growth of microalgae in treated wastewater

An open raceway pond (ORP) of 300 L capacity was used to cultivate microalgae using treated wastewater from the in-house SBR. Fresh *S. platensis* biomass was harvested from *S. platensis* cultivated in lab-scale clear glass aquariums using Zarrouk's medium. Initially the ORP was filled with 200 L treated water, and freshly harvested *S. platensis* biomass (40 g wet weight basis) was inoculated and mixed thoroughly to obtain a homogeneous suspension. The ORP was equipped with an Alternating Current (AC) synchronous motor (Srijan-SYN1101-Pune) with the propeller rotating at 15 rpm to serve as a mixing apparatus. The maximum depth of wastewater in the pond was kept constant at 15 cm, the temperature was 33 °C and the initial pH of wastewater was 6.3. The ORP was filled with SBR wastewater to 200 L, the *S. platensis* biomass was added to it and the experiment was conducted in batch mode for 6 days. From the seventh day onward, the experiment was continued in fed-batch mode by removing 33 L of microalgal suspension and adding 33 L of fresh treated wastewater on daily basis. The overall experimental setup is depicted in Fig. 2.1.

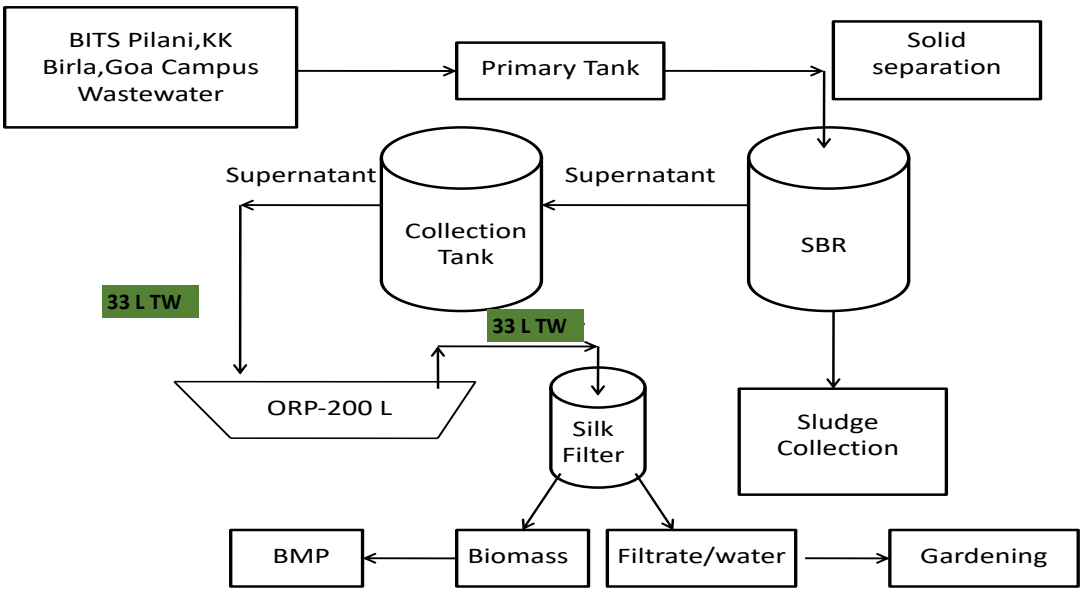


Fig. 2.1 Schematic representation of experimental setup for microalgae cultivation using treated wastewater (TW) as a medium for biofuel and biogas production; ORP (Open Raceway Pond) ; BMP (Biomethanation Potential) .

2.2.5 Analysis of pollutant removal

The wastewater pollutant parameters considered here are chemical oxygen demand (COD), phosphorus (PO_4-P), ammoniacal nitrogen (NH_4-N) and total Kjeldahl Nitrogen (TKN). These parameters were analyzed every 3 days as per standard operating procedures described by APHA (American Public Health Association) 2005 (Eaton et al. 2005). The percentage removal of pollutants was calculated using equation below;

$$\%\, removal = (Initial\ Conc. - Final\ Conc.) \div Initial\ Conc.$$

A three-point sampling method was employed as follows:

(1) Compositional analysis of microalgal suspension in ORP

(2) Compositional analysis of fresh TW to be added to ORP

(3) Compositional analysis of microalgal suspension in ORP after removal of 50 L suspension and addition of 50 L fresh TW.

2.2.6 Biomass characterization

The *S. platensis* biomass cultivated using TW as a medium was harvested using silk cloth, scraped out, air dried, and stored in a cool and dry place until further processing. The advantages of silk cloth are its small pore size and easy scraping of microalgal biomass, and that it is very cost effective. It was then subjected to biomass characterization including total

solids (TS), volatile solids (VS), ash, and proteins (Eaton et al. 2005; Victoria González López et al. 2010). Total carbohydrate values were determined spectrophotometrically using the phenol-sulfuric acid method with D-glucose as the standard (Krishnaveni, Sadavisam, and Balasubramanian 1984). Cellulose, hemicellulose and lignin was determined by Fibraplus (Pelican equipments, Chennai). Elemental analysis of biomass was performed by Elementar (varioMICRO).

2.2.7 Biodiesel production

S. platensis culture was harvested after 6 days of cultivation, on a daily basis, and lyophilized using vacuum freeze-drying equipment (ALPHA1-2LD, Martin Christ, Osterode am Harz, Germany) for 24 h. After drying, the cell pellets were weighed and stored at 20 °C. Lipid extraction from dried biomass was performed using the modified Bligh and Dyer method with chloroform and methanol (2:1 v/v) (Johnson and Wen 2009). The extracted mixture was sonicated at 55 Hz (with a Transonic model 460/H, Elma, Singen, Germany) at room temperature. The lipid extracts were dried in a rotary evaporator and weighed. The total lipid content was expressed as % dry weight.

Approximately 10 mg of the total lipids were transesterified into methyl esters using 2.5 mL of 2 (%) H_2SO_4-methanol (v/v). The detailed fatty acid methyl ester (FAME) analysis was performed using the Agilent 7890-5975 GC–MS system (Agilent Technologies Inc. Santa Clara, CA, USA) using DB5 column (inner diameter 0.18–0.32 m, length 12–60 m, film 0.1–1 uM, temperature 60–325/350°C, inert and nonpolar column consisting of (5%-phenyl)-methylpolysiloxane; Agilent Technologies Inc., USA). The oven temperature was set at 150 °C for 1 min, then heated to 230 °C with a heating rate of 2.9 °C per minute with a holding time of 1 min and total time of 30 min. One microliter of the transesterified sample was injected into a gas chromatograph. The individual FAME peaks were identified by comparison with the retention time of peaks of reference FAMEs (FAME Mix, C8:0–C24:0, Sigma Aldrich).

2.2.8 Biomethanation potential (BMP) assay

Biomethanation potential (BMP) assays were performed in serum bottles of 0.13-L capacity with an actual working volume of 0.1 L, and in 5-L reactors with 4.0 L working volume and 1.0 L headspace. All experiments were conducted in duplicate. The experiments were conducted

at different loading rates (from 1 g VS/L to 5 g VS/L), with 1 mL micronutrient stock ($FeCl_2.4H_2O$-2, $CoCl_2.6H_2O$-0.5 g/L, $MnCl_2.4H_2O$-0.1 g/L, $NiCl_2.6H_2O$- 0.1 g/L, $ZnCl_2$-0.05

g/L, H_3BO_3-0.05 g/L, Na_2SeO_3- 0.05 g/L, $CuCl_2.2H_2O$-0.04 g/L and $Na_2MoO_4.2H_2O$-0.01 g/L), 1 mL macronutrient stock (NH_4Cl-26.6 g/L, KH_2PO_4-10 g/L, $MgCl_2.6H_2O$-6 g/L and $CaCl_2.2H_2O$-3 g/ L), 5 mL bicarbonate (50 g/L) and 80 mL of anaerobic digester slurry as inoculum (for composition see Table 2.1), and 13 mL of distilled water for each serum bottle (Yadav et al. 2016).The bottles were sealed and sparged with N_2 gas. Endogenous biogas production was also studied, using a control without substrate. The volume of biogas was measured daily by the water displacement method (Richa Kothari1*, Virendra Kumar1, and and Vineet Veer Tyagi2 2011), and biogas composition was analyzed by gas chromatography. The biogas production was corrected for blank biogas production. The samples were analyzed with a gas chromatograph (GC-7610, Chemito) equipped with a thermal conductivity detector. H_2 was used as the carrier gas. The oven, injector and detector temperatures were 80, 150 and 250°C, respectively (Prabhu and Mutnuri 2016). The same procedure was followed for BMP assay at the 5 L scale. Two substrates were used for BMP assays, whole S. platensis biomass as is or using residual biomass pellet after lipid extraction. To increase the BMP, several pre-treatments were used on the biomass pellet.

2.2.9 Biomass pre-treatments

To increase the production of biogas from the residual microalgal biomass, different pre-treatment methods were employed as follows;

1. Thermal: the substrate (microalgal biomass) was autoclaved at 15 psi or 121°C for 20 min.
2. Sonication: wet biomass was subjected to sonication at 20 watts for 5 min continuously in an ice bath.
3. Chemical: the substrate was treated with 0.5 M NaOH.
4. Thermochemical: the substrate was first autoclaved at 15 psi for 20 min and then treated with 0.5 M NaOH.

After pre-treatments, the biomass was separated, microalgae extract was further analyzed. The pre-treated biomass was again subjected to BMP assays at different VS ratios in serum bottles and 5-L reactors as well.

2.2.10 Statistical analysis of data

All the experiments were carried out with two biological replicates and two technical replicates. All the values represent mean value and mean standard error at n = 2. The data was checked for normality and homogeneity of variance. The percentage data was arcsine transformed. One-way ANOVA was carried with treatments as a fixed factor on growth rate and the removal of NH_4-N, TKN, COD and PO_4-P. All statistical analysis (comparison

between treatments and means) was performed using IBM SPSS data analysis software package (IBM-USA).

2.3 Results and Discussion

2.3.1 Composition of treated water and BMP inoculum

The TW-SBR outlet composition is depicted in Table. 2.1. It was observed that SBR is not working satisfactorily and the effluent does not meet wastewater discharge standards specified by CPCB and European Protection Agency (EPA). Further treatment process needs to be used to treat SBR effluent to meet wastewater discharge standards.

Table 2.1 Average composition of SBR outlet and BMP inoculum

Parameters (mg/L)	TW-SBR outlet	BMP Inoculum
COD	129.32 ± 14.37	13430.87 ± 187.35
PO_4-P	6.36 ± 0.63	429.33 ± 12.32
NH_4-N	37.54 ± 5.58	1137.21 ± 23.44
NO_3-N	4.73 ± 0.32	Not Determined
TKN	45.65 ± 3.75	17560.34 ± 68.38
pH	6.32 ± 0.57	8.63 ± 0.34
TS %	Not Determined	11.36 ± 1.21
VS %	Not Determined	8.21 ± 0.45

TW-Treated Water, SBR- Sequential Batch Reactor, COD-Chemical Oxygen Demand, PO_4-P-Orthophosphates, NH_4-N-Ammoniacal Nitrogen, NO_3-N-Nitrate Nitrogen, TKN-total Kjeldahl Nitrogen, TS-Total Solids and VS-Volatile Solids. All the values represent mean and standard error at n=2.

2.3.2 Ammoniacal nitrogen tolerance of *S. platensis*

There is significant difference ($p<0.05$) in growth of *Spirulina* sp. in SW 50 and SW 100. It was observed that *S. platensis* can tolerate at initial NH_4-N concentration of upto 100 mg/L. The observed growth rates were 0.17 and 0.18 per day at initial NH_4-N concentration of 50 mg/L and 100 mg/L respectively.

In case of pollutant removal, there is significant difference ($p<0.05$) between pollutant removal at SW 50 and SW 100 and their respective controls. It was observed that, *S. platensis*

has removed 40 % COD, 90 % PO_4-P and 62 % NH_4-N and 59 % TKN and 85 % COD, 89 % PO_4-P and 59 % NH_4-N and 55 % TKN from SW50 and SW100, respectively. The observed removal in controls was 10 % COD, 11 % PO_4-P and 23 % NH_4-N and 10% TKN and 10 % COD, 9 % PO_4-P and 23 % NH_4-N and 17 % TKN from SW50 and SW100, respectively. The literature regarding the studies of *S. platensis* at high concentration of NH_4-N is very limited and fragmentary. However, Canizares and Dominguez has reported the treatment of 50 % diluted swine wastewater (600 mg/L COD, 3.97 mg/L PO_4-P and 85 mg/L NH_4-N) by using *Spirulina maxima* with removal of 23 % PO_4-P and 31 % NH_4-N (Cañizares and Domínguez 1993). The removal efficiencies are higher in present study as compared to removal observed in Canizares and Dominguez study. These differences in removal efficiencies might be attributed to the use of *S. platensis* in present study as compared to *S. maxima* in Canizares and Dominguez study. Przytocka has suggested that the ammoniacal nitrogen tolerance depends on individual algal species and culture conditions (Przytocka-Jsiak 1976). The results of ammoniacal nitrogen tolerance studies of *S. platensis*. is depicted in Fig. 2.2.

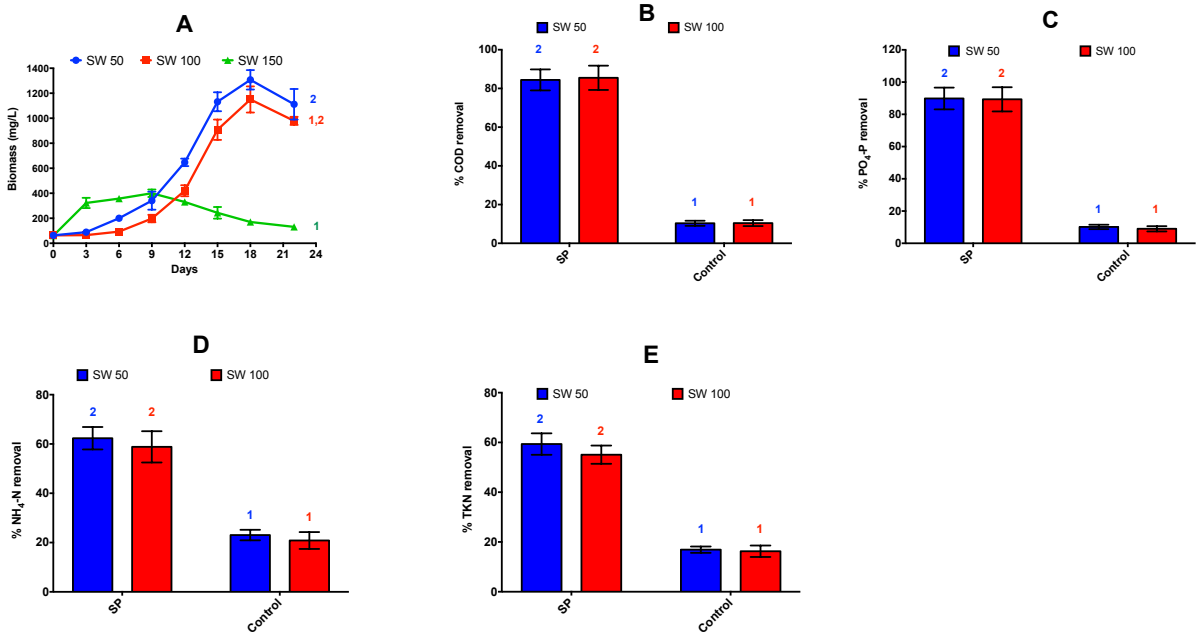


Fig.2.2 Ammoniacal nitrogen tolerance of studies of *Spirulina platensis* (SP); A- Growth of *Spirulina* sp. at different concentration of NH_4-N; B-E- percentage removal of pollutants (COD, PO_4-P, NH_4-N, and TKN) by *Spirulina platensis* and intrinsic removal by controls at different concentration of NH_4-N. The numbers above the bars denote significant groups ($p<0.05$) based on Tukey's post hoc tests. All the values represent mean value and mean standard error at n =2.

At alkaline pH, the ammonium ion (NH_4^+) will be dissociated to NH_3 (Awoke Guadie et al. 2014; Collos and Harrison 2014a). NH_4-N above the concentration of 2 mM is toxic to microbial cells; it causes the loss of photosynthetic pigments, inhibits photosynthetic electron transport system and make the system anaerobic (Abeliovich and Azov 1976).). In present study, it was observed that *Spirulina* sp. can tolerate ammoniacal nitrogen upto concentration 100 mg/L (12 mM), respectively. Previously, Kim et al has reported that *Chlorella* sp. can tolerate ammoniacal nitrogen up-to 0.03 M. the reason behind the less ammoniacal nitrogen tolerance exhibited by the *Spirulina* sp. in present study as compared to observations of Kim et al might be attributed to the absence of organic carbon in present study as compared to the presence of organic carbon in their study. The ammoniacal nitrogen tolerance limits of microalgae can be induced by growing them in a medium with gradual increase in ammoniacal nitrogen concentration. Lightfoot et has reported that ammoniacal nitrogen tolerance in microalgae (cyanobacterium *Synechococcus* PCC6301) can be induced by gene expression from bacteria (*Escherichia coli* glutamate dehydrogenase gene) (Lightfoot, Baron, and Wootton 1988). Collos and Harrison has described the ammoniacal nitrogen tolerance limits of different classes of microalgae; Chlorophyceae (39 mM), Cyanophyceae (13 mm), Dinophyceae (3.6 mM), Diatomophyceae (2.5 mm) and Raphidophyceae (1.2 mm) (Collos and Harrison 2014b).

The possible mechanisms of ammoniacal nitrogen tolerance is can be explained by classical and recent hypothesis (Esteban et al. 2016). Classical hypothesis states that; during the presence of high ammoniacal nitrogen in surroundings, the expression of variety of oxidases increases, NH_4^+ assimilation increases, tolerance to external pH acidification increases and respiration rates also increases. However, as per recent hypothesis, there is increase in the expression of proteins involved in facilitated diffusion of NH_3, nitrate and auxin signaling pathways, aquaporins, GDP mannose-pyrophosphorylase. It is possible that one or more of these ammoniacal nitrogen tolerance mechanisms are active in *S.platensis* in present study; either individual or in combination. Further studies need to be carried out at genetic and molecular level to understand the actual mechanism of ammoniacal nitrogen tolerance in microalgae.

2.3.3 Growth of microalgae in treated water

Since the TW was not a sterile medium, other microalgal contaminants were observed with *S. platensis* as the dominant one, as seen in Fig. 2.3. *S. platensis* was cultivated efficiently on treated wastewater in an ORP using pollutants present in TW as nutrients (Fig. 2.4). The average sunlight intensity was 418.12 ±15 µmoles/m^2/s during experimentation. The average temperature of the microalgal culture was observed to be 28°C. During the initial phases of growth, up to 6 days, microalgae was grown in batch mode whereby the pH increases from 6.32 to 8.0, and once the growth regime shifted from lag phase to log phase, it is subjected to fed-batch mode. The use of a high pH, 8.0, was preferred to discourage the growth of other microalgae. During fed-batch cultivation, 30 L of microalgal culture was harvested and replaced by an equal amount of TW. The microalgal biomass was harvested and processed as described previously to study the impact on different pollutants.

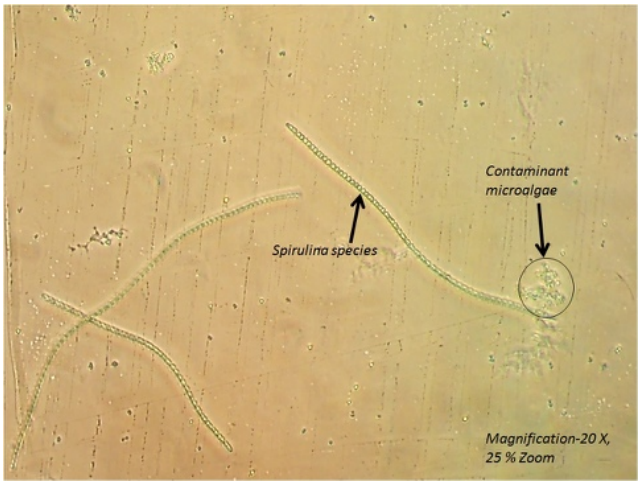


Fig. 2.3 Growth of *S.platensis* as dominant microalgae in Treated Water (TW).

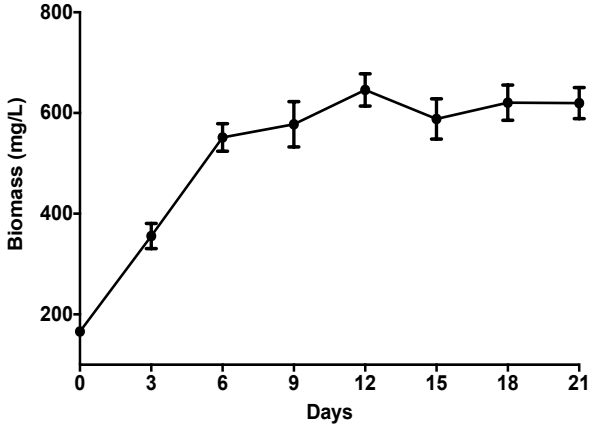


Fig. 2.4. Growth of *S.platensis* in Treated Water (TW)

2.3.4 Biomass characterization

The average biomass concentration was observed to be **0.61 ± 0.13 mg/L**, which is higher than the average *S.platensis* yield (0.12 g/L) obtained using turtle breeding wastewater as the medium (Sunja Cho a et al. 2011). This increase in biomass could be attributed to the less ammoniacal nitrogen and total nitrogen in our TW as compared to turtle breeding wastewater. The harvested biomass was then air dried and characterized for different parameters as shown in Tables 2.2 and 2.3. However, fiber composition results do not exactly match the results observed by Vacek et al.(Vacek 2010), as the biomass under study was a mixed culture, and due to changes in wastewater composition. Contamination by protozoa and other algae resulted in an increase in ash content, neutral detergent fiber, acid detergent fiber and soluble fiber but a decrease in acid detergent lignin.

Table 2.2 Biomass characterization of *S.platensis* cultivated in TW and biomass after pre-treatment methods

Parameters	Control	Thermal	Sonication	Chemical	Thermochemical
Moisture %	5.2 ± 0.34	10.52 ± 0.07	11.31 ± 0.07	11.47 ± 0.13	11.68 ± 0.32
TS %	94.8 ± 2.31	89.48 ± 0.07	88.71 ± 0.07	88.55 ± 0.13	88.32 ± 0.32
VS %	83.92 ± 1.02	67.28 ± 0.95	63.99 ± 0.41	64.93 ± 0.69	64.32 ± 0.96
VS/TS	0.89 ± 0.95	0.75 ± 0.11	0.72 ± 0.19	0.73 ± 0.13	0.73 ± 0.15
Ash %	16.08 ± 0.21	8.68 ± 0.21	9.24 ± 0.12	9.83 ± 0.15	9.23 ± 0.31
Protein %	41.24 ± 2.06	29.37 ±1.47	28.32 ±1.4	23.56 ± 1.18	35.21 ± 1.76
Carbohydrate %	34.78 ± 1.74	21.32 ± 1.06	20.45 ± 1.02	29.35 ± 1.47	19.23 ± 0.96

*TS-Total Solids and VS-Volatile solids.

Table 2.3 Fibre and elemental composition of *S.platensis* Biomass

S. No.	Fibre Type	% Content
1	NDF	10.23 ±0.845
2	ADF	6.69 ± 0.12
3	ADL	2.47 ± 0.02
4	Soluble fraction	89.77 ± 4.67
5	Crude fibre	3.55 ± 0.38
6	Cellulose	1.56 ± 0.03
7	Hemicellulose	23.19 ± 2.35
8	Lignin	2.27 ± 0.25
9	N:C:H:S	10.1:42.47:6.88:0.81

NDF-Neutral detergent fibre, ADF-Acid detergent fibre, ADL-Acid detergent lignin.

2.3.5 Analysis of Pollutant removal

The removal pattern and removal efficiency of various pollutants is shown in Fig.2.5 and Table 2.4 respectively. In terms of pollutant removal rates, there is a variation between our observations and what has been reported in the literature, and this can be attributed to a shorter residence time (4 days in the current study).

The total COD during 26 days of experimentation was reduced from 240.43 g to 197.452 g with total removal of **17.88%** and a removal rate of 2.86 g/day. This COD removal efficiency is lower than the COD removal efficiency of 23% found when *S. platensis* was used to treat of swine wastewater treatment effluent in batch mode with 12 days retention time, as observed by Cheunbarn et al. (Cheunbarn and Peerapornpisal 2010). The COD removal rate (mg/L-day) is higher in the present study (14.33) compared to 0.27 as observed by Cheunbarn et al. (Cheunbarn and Peerapornpisal 2010).

The total PO_4-P was reduced from 11.91 g to 10.17 g with total removal of **14.57%,** which is lower in comparison to Cheunbarn et al.'s removal efficiency of 67%; however, the observed removal rate of 0.58 mg/L-day is higher than the removal rate of 0.39 mg/L-day as observed by Cheunbarn et al. (Cheunbarn and Peerapornpisal 2010).

The total NH_4-N quantity was reduced from 69.88 g to 57.57 g with a total removal of **17.16%.** This removal efficiency was lower than the removal efficiency observed by Cheunbarn et al. (Cheunbarn and Peerapornpisal 2010), of 92 %; however, our observed removal rate of 4.1 mg/L-day is higher than their removal rate of 1.0 mg/L-day.

TKN was reduced from 85.29 g to 72.09 g with a total removal of **15.47%** at a removal rate of 4.4 mg/L-day, which is higher in comparison to the removal rate of 3.8 mg/L-day observed by Cheunbarn et al.(Cheunbarn and Peerapornpisal 2010). This decrease in pollutant removal efficiency might be for three possible reasons: Cheunbarn et al. used a retention time of 12 days, with 10% dilution of wastewater including external addition of $NaHCO_3$ (8 g/L) and $NaNO_3$ (1.5 g/L), and they used a batch-mode treatment system as opposed to the semi-continuous operation of the present study. The increase in rate of removal of pollutants might be attributed to composition of wastewater serving as good medium for growth in terms of micronutrients and macronutrients as well.

The pollutant concentrations in ORPs are remained high, mainly due to two reasons; i) the values reported here are related to pollutant concentration when fresh TW-SBR effluent was added and ii) the retention time in the present study was low (only 6 days).

The COD/N ratio influences growth competition between auto trophic and heterotrophic microorganisms (Hanaki, Wantawin, and Ohgaki 1990). It is also reported that the COD/N ratio mainly influence aerobic treatment systems as compared to anaerobic treatment systems (Randall, Clifford W., James Lang Barnard, and H. David Stensel 1992). The less pollutant removal is observed in the present study due to high COD/N ratio-2.8. It is reported that, as COD/N ratio increases from 1 to 4; nitrification decreases due to decrease in nitrifying bacterial population (Carrera, Vicent, and Lafuente 2004).

It was observed that, the treated water was composed of, on average (mg/L), COD-100, NH_4-N-31, TKN-38 and PO_4-P-6. The removal of all these pollutants is not significant ($p>0.05$). This does not meet the wastewater discharge standards as specified by CPCB-India and EPA. This less treatment efficiency is attributed to the less HRT of 6 days in MTS. It was previously observed that the long-term cultivation of microalgae in wastewater leads to growth of larvae and mosquitoes and frequent visits of birds to MTS, raising the issue of hygiene. Therefore, compromises were made between long HRT and treatment efficiency. However, use of secondary treated wastewater (TDS-1225, COD-480, NH_4-N-118, PO_4-P 49 mg/L) for irrigation of agricultural land suggested increase in soil organic matter, soil PO_4-P and decrease in soil pH (Mohammad and Mazahreh 2003). Thus, reducing the need of organic and inorganic chemical fertilizer application to land. It is necessary to consider this output water for quaternary treatment before being used for agricultural irrigation.

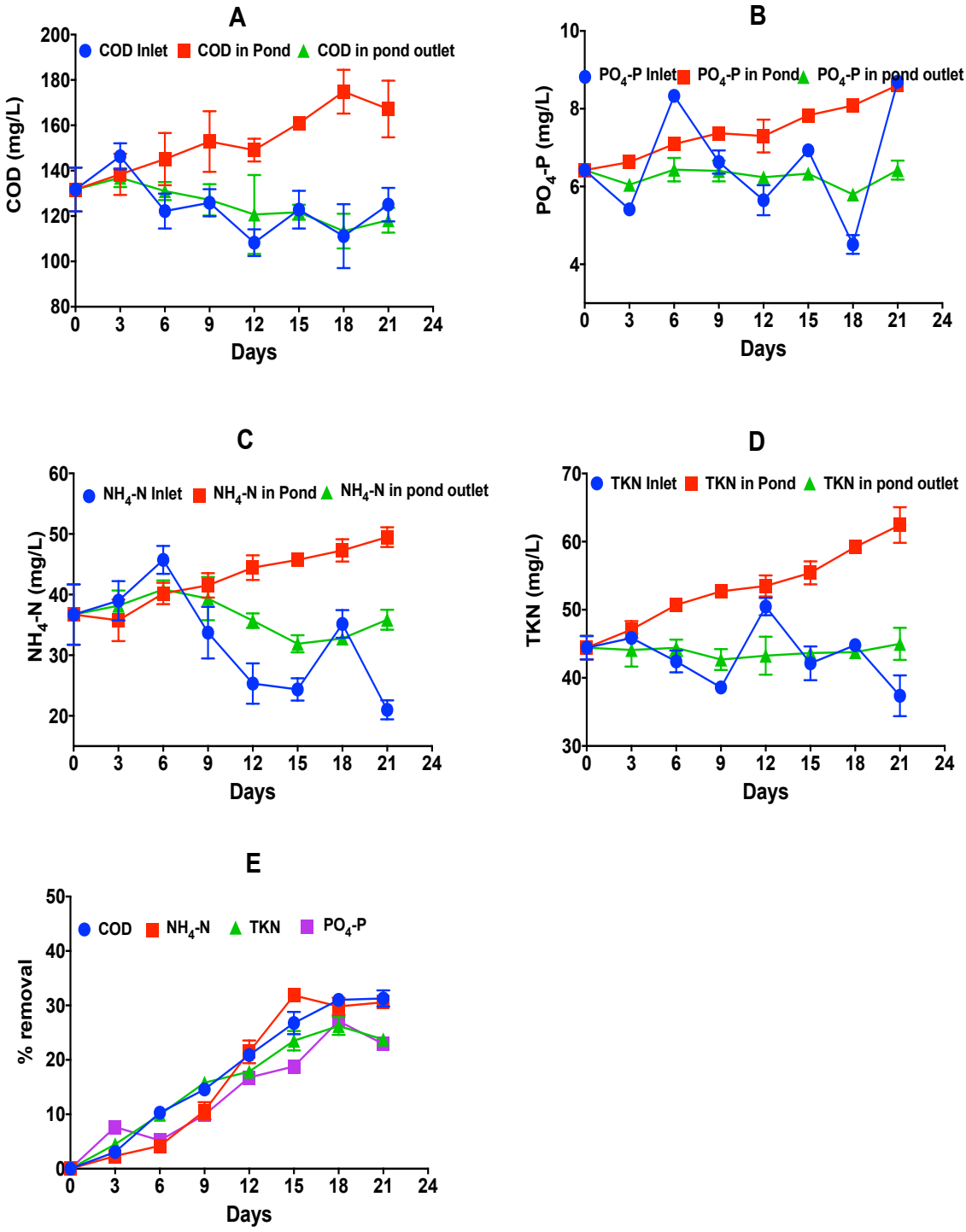


Fig. 2.5 Pollutant removal from Treated Water (TW) using *S. platensis*; A- COD profile, B- PO_4-P profile, C- NH_4-N profile, D-TKN profile and E-% pollutant removal;(COD-Chemical Oxygen Demand, PO_4-P-Orthophosphates, NH_4-N-Ammoniacal Nitrogen, TKN-total Kjeldahl Nitrogen). All the values represent mean and standard error at n=4.

Table 2.4. Removal efficiency of various pollutants from treated wastewater

Parameters	COD	PO_4-P	NH_4-N	NO_3-N	TKN
Total Inlet (g)	240.43	11.91	69.89	8.56	85.30
Total Outlet (g)	197.45	10.17	57.56	6.55	72.10
Total Removal (g)	42.98	1.73	12.31	2.01	13.20
Total % Removal	17.88[#]	14.57[#]	17.16[#]	23.49[#]	15.47[#]
Removal rate (mg/L-day)	14.33	0.58	4.10	0.67	4.40

[#] No significant removal.

2.3.6 Analysis of FAMEs

The profile of fatty acids and their physico-chemical properties are depicted in Table 2.5. The crude lipid content was found to be 26.65% (dry weight of biomass) which comprises 95.19% pure lipids. Two fatty acids were observed, hexadecanoic acid (comprising 81.83% crude lipid and 20.76% algae by dry weight) and 6-octadecenoic acid (comprising 13.36% crude lipid and 3.39% algae by dry weight). Viscosity is an important property of fuel as high viscosity interferes with fuel injection in engines (Hoekman et al. 2012). As the degree of saturation and the length of the fatty acid increases, viscosity increases. The range of viscosity values observed here is 4.38–4.51 mm^2/s with an average value of 4.44 mm^2/s, which is in accordance with the viscosity values required by the American Society for Testing and Materials (ASTM), i.e. 1.9–6.0 mm^2/s (Gouw and Vlugter 1964). Cetane number is used to determine the ignition quality of fuel. Fuels with lower cetane number show a delay in ignition and require more time for fuel combustion. On the other hand, fuels with a high cetane number fuels provide smooth ignition and operate more smoothly. Fuels with a low cetane number produce more particulate exhaust, reducing fuel efficiency as well as energy recovery. Fuels having C16:1, C18:1 and C14:0 in the ratio 5:4:1 have low oxidation potential and they reduce the ignition delay period (Knothe 2005). In the current investigation the ratio of C16 and C18:1 was observed to be 6.125, which indicates an increase in oxidation potential. In view of this, the C18:1 concentration of *S. platensis* needs to be increased by changing the medium composition. The current study indicates 81.83% saturated FAMEs, indicating the high stability of the fuel.

Table 2.5 Fatty acid methyl esters composition from *S.platensis* biomass and their physico-chemical properties

fatty acid	C	% lipid	% dry weight	Molecular weight (g/mol)	Melting Point °C	Boiling Point °C	Kinematic Viscosity (mm^2/S)	Cetane Number	Oil stability Index (h)	Density (g/cm^3)
Hexadecanoic acid	C=16	81.83	20.76	256.42	62.9	351	4.38	86	>40	0.853
Octadecenoic acid	C=18:6	13.36	3.39	282.47	29.8	360	4.51	59	2.5	0.895
	Total %	95.19	24.15	*Crude Lipid -26.65 % (Dry Weight)						

The physico-chemical properties of FAMEs are adopted from (Fakhry and El Maghraby 2013)

2.3.7 Biomethanation potential assays

Trends in cumulative biogas production by *S. platensis* biomass as a substrate for 16 days of experimentation are shown in Fig. 2.6 and Table 2.6. The serum bottles were loaded with different VS ratios, i.e. from 1 g VS/L to 5 g VS/L, to determine the optimal loading rate. All the experiments were conducted in batch mode. The experiments were continued until biogas production decreased and remained constant. The maximum yield of biogas was observed at a loading rate of 2 g VS/L, with a biogas yield of 320 mL/g VS with 67.02% methane. This yield of biogas and biomethane content was lower compared to 526.78 mL/g VS and 70.54% methane observed by El-Mashad et al. using *S. platensis* algae in combination with enzymatically saccharified switchgrass to increase the C:N ratio (El-Mashad 2015). This decrease might be due to the mixed microalgae culture with complex cell walls and a lower C:N ratio (i.e. 7:1) in the present study, since no enzymatically saccharified switchgrass or other biomass was added to improve the C:N ratio. For higher biogas production from any substrate the C:N ratio should be 25. Pre-treatment is recommended as a necessary step for biogas production (Cho et al. 2013a). The details of pre-treatments and their results are shown in Fig. 2.6 and Table 2.6. Different pre-treatment methods were carried out at a 2 g VS/L biomass loading. Total biogas produced is corrected against the negative control, and his corrected volume of biogas is used to calculate the biogas yield per unit of VS added. Among the four pretreatments employed, thermal pre-treatment and sonication pre-treatment gave higher biogas yield (i.e. 395 and 375 mL/g VS) as compared to control (340 mL/g VS), with 55 and 35 mL/g VS increase, respectively, per gram of volatile solids. Thermal pre-treatment of biomass showed an 8.53% increase in biogas production and a 16.18% increase in biogas yield based on VS ratio, indicating better substrate utilization, and a 26.12% increase in methane content, indicating better product formation. All the pre-treatments showed an increase in methane content compared to control, but there is no significant variation among pre-treatments. Cho et al. showed that thermal pre-treatment of biomass at 120°C gave 405 mL CH_4/g VS in presence of continuous shaking, which is higher than our biogas yield of 290 mL of CH4/g VS; this decrease might be due to the manual shaking of 3 times for mixing during our experiment (Cho et al. 2013b), although our results are comparable to those of Alzate et al. (Alzate et al. 2012). The increase in biogas volume and methane content using thermal pretreated biomass might be due to an increase in substrate solubilization (Honglay Chen and Oswald 1998). There is no significant difference between biogas production by chemically pretreated and thermochemically pre-treated biomass as compared to control; this might be due to a severe change in pH and destruction of substrate.

At 5-L scale with a loading rate of 2 g VS/L, we observed a biogas yield of 407.18 mL/g VS in thermal pre-treatment as compared to 318.4 mL/g VS in the control, leading to a 27.88% increase. The methane content was also increased from 62.38% to 72%, resembling the results at 0.1-L scale.

The trends in cumulative biogas production by *S. platensis* biomass at different loading rates for 16 days of experimentation are shown in Fig.2.6 and Table 2.6.

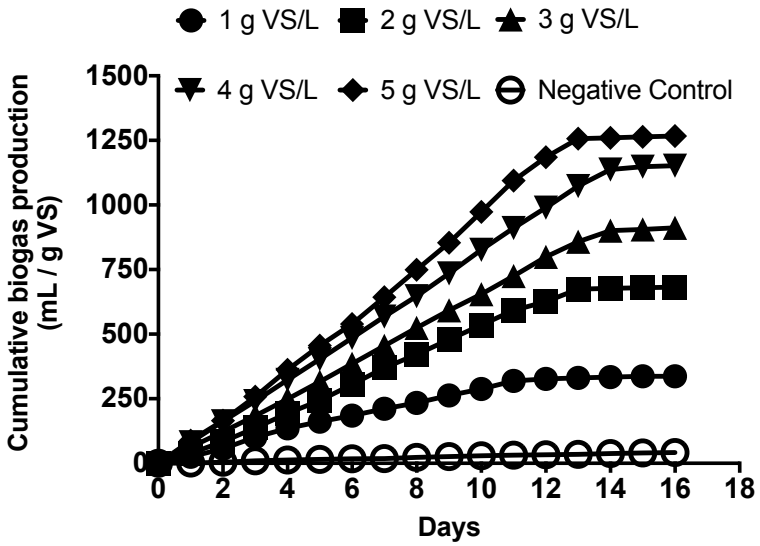


Fig. 2.6 Biogas production from *S. platensis* biomass at different loading rates

Table. 2.6 Results of Biogas production from *S.platensis* grown on treated wastewater

Method	Scale	Duration (s)	Loading rate (gVS/L)	Biogas Yield (mL/gVS)	% Methane
No treatments	0.1 L	16	1	295 ± 7.5	65.51 ± 2.67
			2	320 ± 5	67.02 ± 1.32
			3	290 ± 2.7	64.98 ± 2.22
			4	277.5 ± 4.75	68.25 ± 3.16
			5	245 ± 5	69.08 ± 1.58
Control	0.1 L	22	2	340 ± 3.38	58.19 ± 4.35
Sonication				375 ± 4.28	71.42 ± 3.62
Thermal				395 ± 3.64	73.39 ± 4.28
Chemical				345 ± 4.63	76.94 ± 1.34
Thermochemical				337.5 ± 6.48	73.69 ± 2.67
Control	5 L	35	2	318.4 ± 8.45	62.38 ± 1.89
Thermal				407.18 ± 7.26	72.05 ± 2.49
SPR	0.1 L	22	2	165.0 ± 5.39	62.38 ± 2.12
SPR Thermal	0.1L	22	2	230.0 ± 3.28	59.87 ± 5.87

*SPR-*S.platensis* residual biomass after lipid extraction, SPR Thermal-thermally treated *S.platensis* residual biomass after lipid extraction

The Effect of different pretreatments on biogas production are depicted in Fig. 2.7. and Table 2.6.

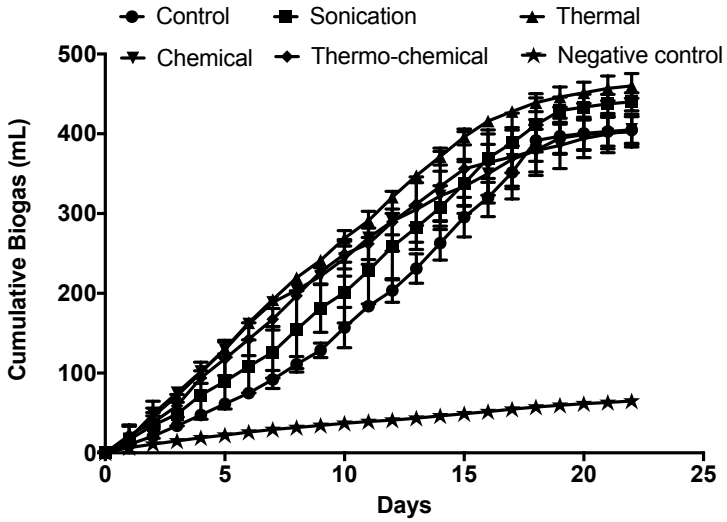


Fig. 2.7 Effect of different pretreatments on biogas production from *S. platensis* biomass at loading rate of 2 g VS/L. The values are normalized to loading rate of 1 g VS/L.

The results of biogas production at 5 L scale using thermally pretreated *S.platensis* biomass is depicted in Fig. 2.8.

The effect of different pre-treatments and characteristics of microalgae extract are shown in Fig. 2.8 and Table 2.7. To estimate the release of proteins, carbohydrates, COD, TKN and ammonium nitrogen from biomass, a known amount of microalgal biomass (by dry weight) was dissolved in distilled water and subjected to the pre-treatments, and the biomass was separated by centrifugation at 8000 rpm for 20 min to obtain microalgae extract. For the control, biomass was dissolved in water, mixed in a shaker at 120 rpm and 30°C and then centrifuged to obtain microalgae extract. The extract is further characterized for the release of biomolecules and increase in their solubility. All treatments increased the release of compounds from biomass to a great extent as compared with the control, but thermal pre-treatment showed the highest release. Thermal pre-treatment increased the proteins, carbohydrate, soluble COD, TKN and NH_4-N by 350.12%, 290.54%, 219.02%, 137.88% and 519.58%, respectively. As this implies, we used microalgae extract containing pre-treated biomass as a substrate for a biomethane potential assay based on calculations of VS content. Microalgae extract can be used as a valuable supplement in the preparation of complex laboratory media to improve the growth and metabolism of common laboratory microorganisms (Kightlinger et al. 2014).

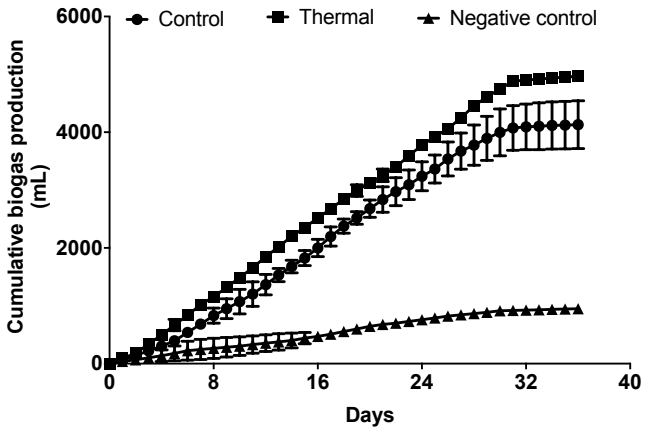


Fig. 2.8 Biogas production at 5 L scale using thermally pretreated *S.platensis* biomass

The effect of different pre-treatments and characteristics of microalgae extract are shown in Table 2.7.

Table 2.7.Characterization of microalgae extract after pretreatments

Parameters/Methods	Control	Thermal	Sonication	Chemical	Thermochemical
Protein (mg/g)	42.91 ± 2.31	193.15 ± 4.63	195.92 ± 3.18	199.14 ± 5.43	170.1 ± 6.18
Carbohydrate (mg/g)	19.56 ± 1.06	76.39 ± 2.14	75.77 ± 3.21	73.96 ± 4.35	77.41 ± 3.92
Soluble COD (mg/g)	3789.55 ± 135.21	12089.75 ± 140.32	11197.1 ±149.87	11627.55 ± 180.26	12545.39 ± 210.16
TKN- (%)	0.227 ± 0.10	0.54 ± 0.16	0.55 ± 0.11	0.58 ± 0.16	0.55 ± 0.11
NH_4-N (mg/L-g)	5.31 ± 0.89	32.9 ± 0.73	39.9 ± 0.25	49 ± 0.28	49.7 ± 0.19

The residual *S. platensis* biomass after total lipid extraction was also used as a substrate for biogas production (Fig. 2.9). It can produce 165.0 ± 5.39 mL of biogas per g VS/L, with an average methane content of 62.38 ± 2.12%. The residual biomass after thermal pretreatment produced 230.0 ± 3.28 mL biogas per g VS/L with average methane content of 59.87 ± 5.87%, indicating a 25.93% increase in total methane yield. The decrease in biogas production was caused by the decrease in C : N ratio due to lipid extraction in previous steps.

Fig. 2.9 depicts Biogas production from SPR (*S.platensis* after lipid extraction) and SPR Thermal (Thermally treated *S.platensis* after lipid extraction).

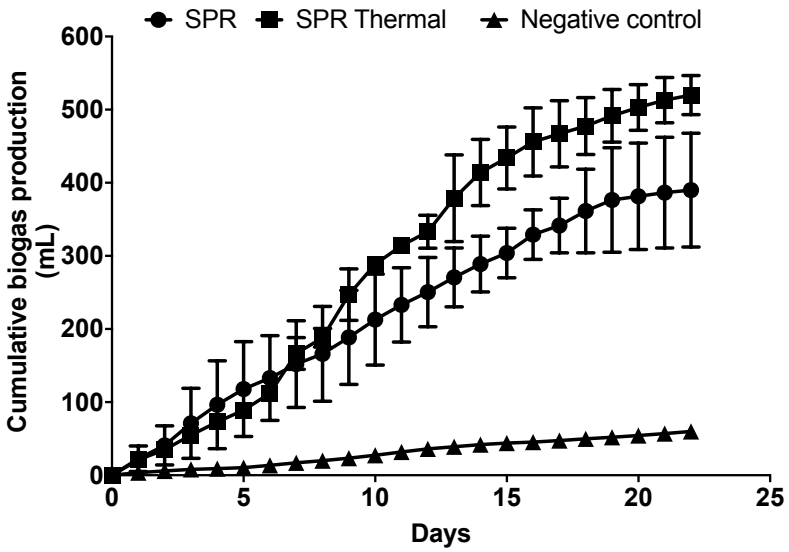


Fig. 2.9 Biogas production from SPR (*S.platensis* after lipid extraction) and SPR Thermal (Thermally treated *S.platensis* after lipid extraction)

2.4 Conclusion

This study brings possible insights into the sustainable production of microalgae biomass using secondarily treated wastewater to produce value-added products such as free radical scavengers, lipids and biomethane in a sequential manner. This will lead to maximum utilization of the microalgal biomass. Moreover, the wastewater can be considered a valuable resource medium for microalgae cultivation rather than waste as such, leading to a reduction in the cost of wastewater treatment and reclamation in terms of raising possible revenue through value added bio-products and bioenergy. *S. platensis* can tolerate 100 mg/L of NH_4-N.

Chapter III

Phycoremediation and biogas production potential of *Chlorella vulgaris* grown in secondary treated wastewater

3.1 Introduction

The increase in volume of wastewater beyond the installed treatment capacity of sewage treatment plants is quite common and is leading to the release of untreated water into rivers, wells, and groundwater. On the other hand, the fresh water resources are shrinking, exerting a need to look for alternative wastewater treatment systems to treat and reuse of the water. Drinking of water contaminated with nitrate can cause Blue Baby Syndrome in human infants and release of phosphates to water bodies leads to eutrophication (Booker, Cooney, and Priestley 1996; Knobeloch et al. 2000).

Currently available technologies for wastewater treatment include adsorption, ion exchange, activated sludge, electrochemical, and membrane filtration (Clara et al. 2005; Kightlinger et al. 2014). This suffers several disadvantages that include requirement of an external supply of carbon, operation and maintenance cost, need of technical staff, sensitivity to wastewater composition, limited wastewater handling capacity and generation of large quantity of harmful sewage sludge (Luo et al. 2014). Treatment of wastewater is depreciated in countryside areas of India due to the improper design of treatment plant, lack of financial resources, lack of technical staff, and poor maintenance (Yadav, Chazarenc, and Mutnuri 2018). Most of the treatment facilities do not treat wastewater to reach wastewater discharge standards and not maintained to its proper functioning ability (Konnerup, Koottatep, and Brix 2009).

As reported by Central Pollution Control Board (CPCB) India, 38.25 million cubic meters per day domestic wastewater (DW) is being generated in Class I and Class II cities in India and current treatment capacity for municipal corporations is only 11,787 MLD corresponding to only 31 % of DW generation. CPCB studies also depict that out of 269 DW treatment plants in India, only 231 are operational further reducing the treatment capacity to 21 % of the amount of sewage being generated (J.S. Kamyotra and R.M. Bhardwaj 2011). AQUA-STAT has reported that, globally, 90000 MLD of municipal wastewater was being generated and only 60 % is being treated (Sato et al. 2013; Wichelns, Drechsel, and Qadir 2015).

Chlorella vulgaris is single celled, photosynthetic and nonmotile green microalgae (2-10 µm in size) belonging to Chlorophyta division. *C. vulgaris* reproduce asexually by nonmotile autospores (Manisha 2007). It was found to contain 25-58 % proteins, 5-40 % lipids, and 40-61 % carbohydrates as cellular composition (Ana Cláudia Freitas Margarites 2014; Becker 1994, 2007; Illman, Scragg, and Shales 2000). The cell wall of *C. vulgaris* is composed of complex polysaccharides including cellulose, hemicellulose, lignin and minerals (Abo-Shady, Mohamed, and Lasheen 1993; Northcote, Goulding, and Horne 1958; Safi et al. 2014).

C. vulgaris has been used for treatment of different wastewater including textile, domestic sewage, municipal, agricultural, anaerobic digester effluent and recalcitrant wastewater with 45-97 % N, 28-96 % P and 60-90 % COD removal potential (Lau, Tam, and Wong 1996; Lim, Chu, and Phang 2010; Park et al. 2010; Valderrama et al. 2002; Yun et al. 1997). The microalgae cultivation systems may include closed (cuvette, container, stirred vessel, natural water, raceway pond and inclined surface water) or open systems (plastic sleeves, fermenter like tank, tubular and laminar glass tanks) (Pulz and Gross 2004). However, open raceway ponds have several advantages over other cultivation methods; low costs for construction, maintenance and operation, easy to scale up and the potential to integrate with wastewater treatment plants (Z. J. Pei et al. 2013). Numerous researchers have demonstrated the use of *C. vulgaris* biomass for biomethane production (Babaee and Shayegan 2011; Calicioglu and Demirer 2015; Doğan-Subaşı and Demirer 2016; Elango et al. 2007; Mendez et al. 2015). Biomass pre-treatment methods (thermal, chemical, sonication and thermochemical) can increase biogas production(Alzate et al. 2012; Campo G., Rıggıo V.A., Cerutti A., Ruffino B., Panepinto D. 2018; Cho et al. 2013a; Lee et al. 2014).

This present study focusses on phycoremediation potential of *C. vulgaris* for treatment of secondary treated wastewater and the utilization of biomass for sustainable biogas production. The effects of different biomass pre-treatments on biogas production also been explored. In conclusion, this study demonstrates the alternative wastewater treatment process that can be incorporated in current wastewater treatment systems without any major modification to available infrastructure.

3.2 Materials and Methods

3.2.1 Collection and analysis of Sequential Batch Reactor Treated Water (SBRTW)

BITS Pilani, KK Birla Goa campus has a Sequential Batch Reactor (SBR) as a sewage treatment system inside the campus. It can treat 250 m^3 of wastewater daily. However, over the period of time, due to increase in population, volume of wastewater increased to 300 m^3 daily. Due to the increase in wastewater beyond the installed treatment capacity of SBR, the treatment efficiency of SBR was reduced, COD was not removed efficiently. Therefore, attempts were made to further phycoremediate Sequential Batch Reactor Treated Water (SBRTW) using Open Raceway Pond (ORP). SBRTW was analyzed for COD, PO_4-P, NH_4-N and TKN as per standard methods for examination of water and wastewater (Eaton et al. 2005).

3.2.2 Selection of microalga and inoculum preparation

Chlorella vulgaris was selected for phycoremediation of SBRTW. It was received as gift from Dr. Pradeep Dhamole from BITS Pilani, Hyderabad campus. *C. vulgaris* was inoculated in Bold's Basal Medium (BBM) (pH=7.0) and incubated at 30°C for 10 days at light intensity of 50 μmoles photons/m^2-s with 12-hour light/dark cycle. Mixing of culture was performed by manual shaking, thrice a day. This microalgal culture was further scaled up in 20 L aquarium, harvested by silk cloth filtration and used as inoculum for phycoremediation of SBRTW.

3.2.3 Batch ammoniacal nitrogen tolerance studies of *C. vulgaris*

Synthetic Wastewater (SW) was prepared as per the following composition; Sodium carbonate- 2610 mg/L, potassium dihydrogen phosphate- 69.84 mg/L, ammonium chloride- 38.23 g/L and sodium nitrate- 447.46 mg/L. This will produce SW-1000 (NH_4-N concentration is 1000 mg/L) with a final concentration of CO_3^{-2}-1478.26 mg/L, PO_4-P-15.92 mg/L, NH_4-N-1000 mg/L and NO_3-N-172.6 mg/L, respectively. This SW is then further diluted with distilled water to obtain different concentrations (1:10 to 10/10) of NH_4-N. All these ammoniacal nitrogen tolerance studies were carried out at the scale of 200 mL in conical flasks of 500 mL capacity. The pH was adjusted to 8.0. Each flask was then inoculated with 25 mg of freshly harvested *C. vulgaris* biomass. The flasks were irradiated with 42 μmol photons/m^2-s by using 40 W fluorescent tubes with 16:8 hours of alternating light/dark photoperiod at temperature of 30°C. The negative controls were placed to understand the ammonia stripping at alkaline pH. The samples were removed periodically after every 3 days, centrifuged at 8000 rpm for 15 minutes. The biomass was dried at 40°C overnight in a hot air oven to obtain dry weight. The growth curve was obtained by plotting biomass concentration with respect to time. The growth rate was calculated using following formula,

$$Growth\ rate\ (day^{-1}) = (lnN_2 - lnN_1) \div (t_2 - t_1)$$

Where,N_2 and N_1= biomass concentration at t_2 and t_1, respectively.

3.2.4 Phycoremediation of SBRTW

Phycoremediation of SBRTW was carried out in open raceway pond (ORP). The ORP was operated at working volume of 200 L at depth of 0.15 m. Paddle wheel rotating at 20 RPM was used as mixing apparatus. The average light intensity, pH and temperature was 850 μmoles/m^2-s, 6.8 and 30°C, respectively. The ORP was filled with 200 L SBRTW and

inoculated with *C. vulgaris* (50 g wet weight). The ORP was operated in batch mode for 6 days and seventh day onwards, it was operated in fed-batch mode. During Fed-batch mode, 33.3 L of culture from ORP was removed and it was replaced with fresh 33.3 L of SBRTW. The Hydraulic Retention Time (HRT) of SBRTW was maintained at 6 days. The water Samples were analyzed for COD, PO_4-P, NH_4-N and TKN as per standard methods for examination of water and wastewater (Eaton et al. 2005).

Three-point sampling was used to study the phycoremediation of SBRTW;

1. Composition of microalgal culture in ORP
2. Composition of fresh SBRTW to be added to ORP
3. Composition of microalgal culture after ORP

After every three days, known volume of microalgal suspension from ORP was subjected to centrifugation at 1000 RPM for 10 minutes. The biomass was dried in oven at 40°C overnight and dry weight was measured. Growth curve was obtained by plotting biomass concentration (mg/L) with respect to time. The supernatant was stored at -4°C and analyzed for COD, PO_4-P, NH_4-N and TKN. The pollutant removal efficiency was calculated using formulae mentioned below.

% removal = [(A-B)/A] X 100

Where,

A=initial concentration

B=Final concentration

The overall methodology of experiment is described in Fig. 3.1.

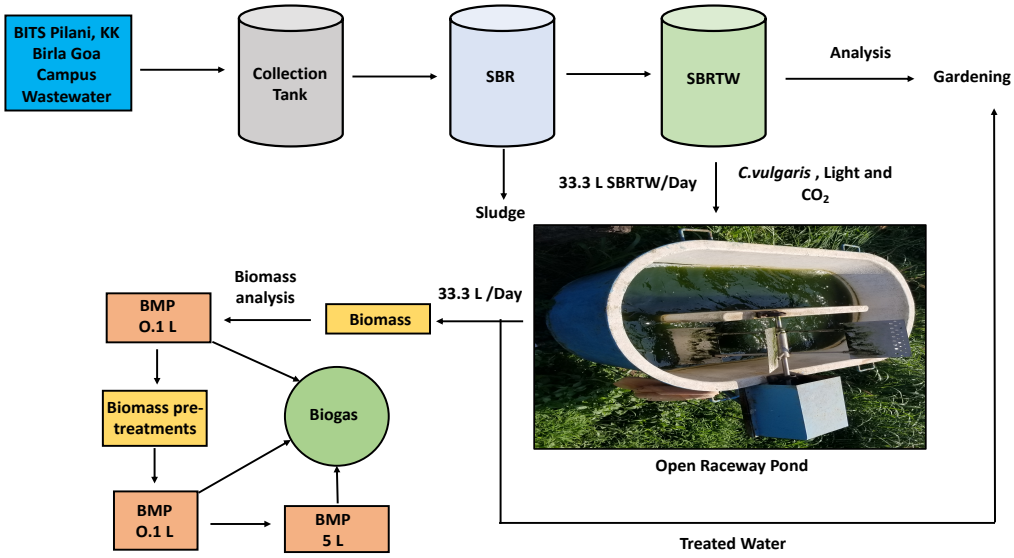


Fig. 3.1 Overall methodology of experiment (SBR-Sequential Batch Reactor, SBRTW-Sequential Batch Reactor Treated Water and BMP-Biomethanation Potential)

3.2.5 Biomass analysis

The biomass was analyzed for moisture, Total Solids (TS), Volatile Solids (VS) and ASH. Total carbohydrates content and protein content was analyzed spectrophotometrically by using phenol-sulphuric acid method and Lowry's method, respectively (Dubois et al. 2009; González López et al. 2010). The total lipids were extracted and quantified as per Folch et al (Folch, Lees, and Stanley 1957). Fibraplus FES-04 (Pelican equipments, Chennai) was used to determine the cellulose, hemicellulose and lignin content of microalgal biomass. FTIR analysis of biomass was performed as per method described by Vidyadharani et al (Vidyadharani and Dhandapani 2016).

3.2.6 Biomass pre-treatments

The biomass was subjected to different pre-treatment methods to understand their effect on Biomethanation Potential (BMP). Known mass of biomass was dissolved in known volume of distilled water and the pretreatment were performed. The pre-treatments were described below,

1. Thermal pre-treatment- microalgal biomass was autoclaved at 15 psi for 20 minutes.
2. Chemical- microalgal biomass was treated with 0.5 M NaOH at 30°C overnight.
3. Sonication- The biomass was sonicated at 20 watts for 5 minutes in ice bath.
4. Thermochemical- The biomass was treated with 0.5 M NaOH followed by autoclaving at 15 psi for 20 minutes.

The biomass was separated from the liquid by centrifugation at 10000 RPM for 10 minutes. The supernatant and biomass (after dying at 50°C overnight) was stored in cool and dry place. The biomass and supernatant were analyzed separately. During anaerobic digestion studies, the biomass along with the liquid was used as substrate.

3.2.7 BMP of microalgal biomass

Anaerobic digestion of microalgal biomass was performed in serum bottles with total volume of 0.13 L and working volume of 0.1 L. BITS Pilani, KK Birla Goa Campus has anaerobic digester running on food wastes collected from institute cafeteria. The liquid digestate from this digester was used as inoculum for the BMP assay. BMP assays were carried out in duplicates. In each bottle, 1 mL micronutrient stock, 1 mL macronutrient stock, 5 mL 5 % (W/V) $NaHCO_3$, 13 mL of distilled water and 80 mL of BMP inoculum was added (Chavan and Mutnuri 2018). This combination was referred as negative control. Initially, BMP assays were carried out at different loading rates (1-5 g VS/L) in serum bottles to optimize the loading rate. The bottles were properly sealed properly and N_2 gas was sparged to make

system anaerobic. The biogas production was measured on daily basis using water displacement method and methane composition was analyzed by using gas chromatography (Thermofischer Trace-1110) using packed sphaerocarb column. The injector and detector temperature were set at 150°C and 200°C, respectively. The oven ramp program was set as 50°C for 1 minute and 5°C rise per minute for 10 minutes.

The yield of biogas was determined by using following formulae,

Net Biogas production (mL) (A) = (B-C)

Net biogas yield (mL/ g VS) (D) = A / g VS added

Net Methane yield = D X % methane

Where,

B= Total Biogas produced, C= Total biogas produced by negative control.

The loading rate giving maximum yield of methane was selected for further BMP studies. Later, known amount of biomass was subjected to different pre-treatment methods and used as substrate for BMP assay. The pretreatment method and the loading rate giving maximum yield of methane was used to study BMP assay at 5 L reactor volume.

3.2.8 Statistical analysis of data

All the experiments were carried out with two biological replicates and two technical replicates. All the values represent mean value and mean standard error at n = 2. The data was checked for normality and homogeneity of variance. The percentage data was arcsine transformed. One-way ANOVA was carried with treatments as a fixed factor on growth rate and the removal of NH_4-N, TKN, COD and PO_4-P. All statistical analysis (comparison between treatments and means) was performed using IBM SPSS data analysis software package (IBM-USA).

3.3. Results and Discussion

3.3.1 Elemental composition of SBRTW

It was observed that the SBRTW is rich in pollutants indicating that the SBR is not working efficiently. The SBRTW does not meet the standard wastewater discharge limits specified by Environmental Protection Agency (EPA)-USA and Central Pollution Control Board (CPCB)-India. These pollutants can be utilized as nutrients by *C. vulgaris*. Microalgae have been considered as pollutant scavengers for variety of wastewaters including domestic, agricultural and industrial wastewater. Microalgae can assimilate organic and inorganic forms of C, N and P along with some hydrocarbons and antibiotics (Fon-Sing et al. 2016).

The composition of SBRTW, ORP outlet and inoculum used for BMP is depicted in Table 3.1. It was observed that the SBRTW is rich in pollutants that can be further used as nutrients by *C. vulgaris*.

Table 3.1 Elemental composition of SBRTW and inoculum used for BMP

Parameter (mg/L)	SBRTW	ORP Outlet	BMP Inoculum
COD	121.34 ±13.29	72.94 ± 6.72	14520.48 ± 135.21
PO_4-P	8.69 ± 0.54	5.40 ± 1.14	489.77 ± 10.94
NH_4-N	42.61 ± 6.83	22.59 ± 3.57	1213.56 ± 55.39
TKN	81.23 ± 5.41	22.41 ± 4.72	17560.34 ± 68.37
pH	6.72 ± 0.48	7.5 ± 0.4	8.30 ± 0.79
TS %	Not Determined	Not Determined	13.55 ± 2.36
VS %	Not Determined	Not Determined	7.91 ± 0.62

SBRTW- Sequential Batch Reactor Treated Water, BMP-Biomethanation Potential, ORP-Open Raceway Pond, COD-Chemical Oxygen Demand, PO_4-P- Orthophosphate, NH_4-N-Ammoniacal Nitrogen, NO_3-N-Nitrate Nitrogen, TKN-Total Kjeldahl Nitrogen, TS- Total Solids and VS-Volatile Solids. All the values represent mean and standard error at n=4.

3.3.2 Ammoniacal nitrogen tolerance of *Chlorella* sp.

There is significant difference ($p<0.05$) in growth of *C. vulgaris* in SW 100 and SW 300, but there is no significant difference ($p>0.05$) in growth of *Chlorella* sp. in SW 100 and SW 200. It was observed that *C. vulgaris* can grow well in NH_4-N concentrations from 100 mg/L to 200 mg/L with growth rate of 0.1 per day and 0.12 per day, respectively. One study has suggested that the *C. vulgaris* can tolerate NH_4-N concentrations upto 195 mg/L (Przytocka-Jsiak 1976). Previous studies had reported that, *C. vulgaris* can tolerate NH_4-N concentrations upto 1000 mg/L but the growth is limited at NH_4-N concentration less than 20 mg/L or higher than 200 mg/L (Collos and Harrison 2014). Tam and Wong et al has reported that *C. vulgaris* can attain growth rate of growth rate of 0.22 per day at NH_4-N concentrations from 80 mg/L to 150 mg/L at initial PO_4-P concentration of 53 mg/L and pH adjusted to 7.0 on regular basis (N.F.Y.Tam and Y. S. Wong 1996) . In present study, the decrease in growth rate at given NH_4-N concentrations is less due to increase in pH over time, use of NH_4Cl as source NH_4^+ and PO_4-P and COD limitations. Previously, it was reported that *Chlorella* sp. isolated from waste stabilization ponds can be grown at 10 mM NH_4-N at temperature of

25°C and pH-9.0, where 40 % ammonia is present in toxic NH_3 form (Konig et al. 1987). In present study, the pH at the end of experiment was 9.2, and initial NH_4-N concentrations were 6 mM and 12 mM at NH_4-N concentrations of 100 mg/L and 200 mg/L, respectively. Azov and Goldman has reported previously that at alkaline pH >8, and NH_4-N > 29 mg/L may lead to inhibition of photosynthesis in microalgae (Azov and Goldman 1982). This indicates that, during experimentation period, *Chlorella* sp. under study was adapted to tolerate NH_4-N at 100 mg/L.

In case of pollutant removal, there is significant difference ($p<0.05$) between pollutant removal at SW 100 and SW 200 and their respective controls. It was observed that, *C. vulgaris* has removed 84 % COD, 68 % PO_4-P and 49 % NH_4-N and 53% TKN and 81 % COD, 78 % PO_4-P and 51 % NH_4-N and 54 % TKN from SW100 and SW200, respectively. The observed removal in controls was 11 % COD, 10 % PO_4-P and 20 % NH_4-N and 18% TKN and 13 % COD, 11 % PO_4-P and 18 % NH_4-N and 16 % TKN from SW100 and SW200, respectively. These results are well in agreement with the studies carried out by Jiang et al (Lin et al. 2007). Previously, it was reported that *Chlorella pyrenoidosa* can be used to treat 10 % diluted landfill leachate with initial concentration of COD-130 mg/L, NH_4-N-135 mg/L and PO_4-P-0.6 mg/L with observed removal of 57 % COD, 60 % NH_4-N and 65 % PO_4-P (Lin et al. 2007). Jiang et al has also observed the removal of 10 % COD, 13 % PO_4-P and 15 % NH_4-N in controls. The results of ammoniacal nitrogen tolerance studies of *C. vulgaris* is depicted in Fig. 3.2.

At alkaline pH, the ammonium ion (NH_4^+) will be dissociated to NH_3 (Awoke Guadie et al. 2014; Collos and Harrison 2014b). NH_4-N above the concentration of 2 mM is toxic to microbial cells; it causes the loss of photosynthetic pigments, inhibits photosynthetic electron transport system and make the system anaerobic (Abeliovich and Azov 1976).). In present study, it was observed that *Chlorella* sp. can tolerate ammoniacal nitrogen upto concentrations 200 mg/L (12 mM), respectively. Previously, Kim et al has reported that *Chlorella* sp. can tolerate ammoniacal nitrogen up-to 0.03 M. the reason behind the less ammoniacal nitrogen tolerance exhibited by that *Chlorella* sp. and *Spirulina* sp. in present study as compared to observations of Kim et al might be attributed to the absence of organic carbon in present study as compared to the presence of organic carbon in their study. The ammoniacal nitrogen tolerance limits of microalgae can be induced by growing them in a medium with gradual increase in ammoniacal nitrogen concentration.

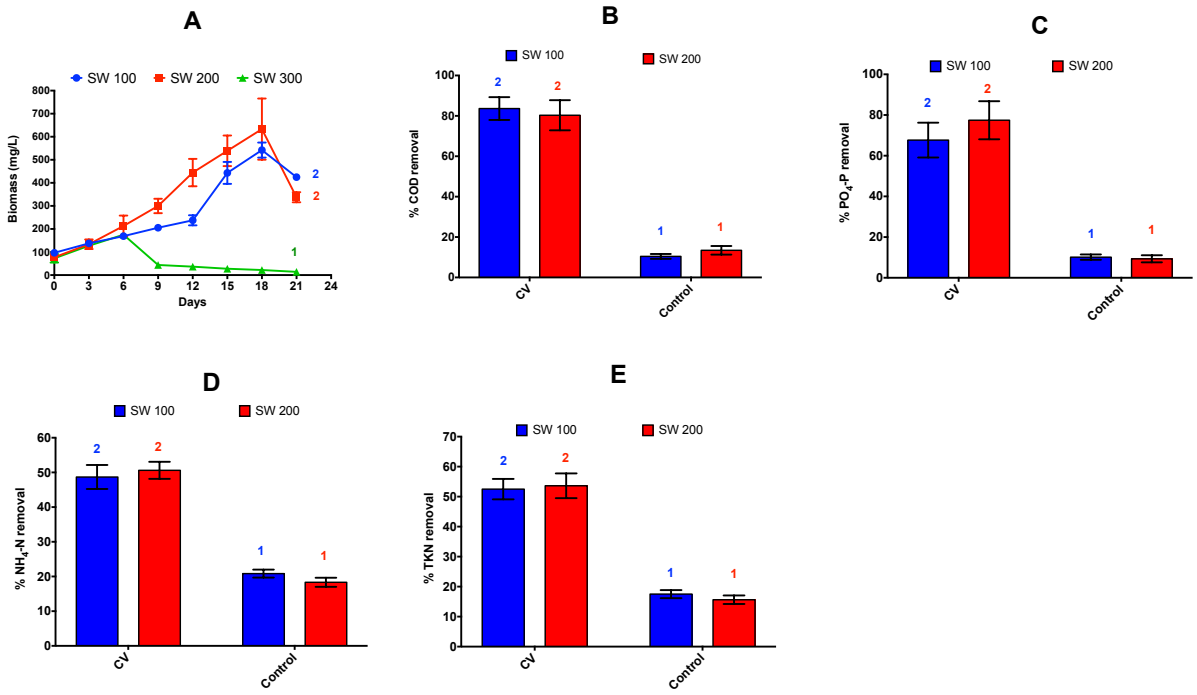


Fig. 3.2 Ammoniacal nitrogen tolerance of studies of *Chlorella vulgaris* (CV); A- Growth of *Chlorella* sp. at different concentration of NH_4-N; B-E-percentage removal of pollutants (COD, PO_4-P, NH_4-N, and TKN) by *Chlorella vulgaris* and intrinsic removal by controls at different concentration of NH_4-N. The numbers above the bars denote significant groups ($p<0.05$) based on Tukey's post hoc tests. All the values represent mean value and mean standard error at n = 2.

Lightfoot et has reported that ammoniacal nitrogen tolerance in microalgae (cyanobacterium *Synechococcus* PCC6301) can be induced by gene expression from bacteria (*Escherichia coli* glutamate dehydrogenase gene) (Lightfoot et al. 1988). Collos and Harrison has described the ammoniacal nitrogen tolerance limits of different classes of microalgae; Chlorophyceae (39 mM), Cyanophyceae (13 mm), Dinophyceae (3.6 mM), Diatomophyceae (2.5 mm) and Raphidophyceae (1.2 mm) (Collos and Harrison 2014a).

The possible mechanisms of ammoniacal nitrogen tolerance is can be explained by classical and recent hypothesis (Esteban et al. 2016). Classical hypothesis states that; during the presence of high ammoniacal nitrogen in surroundings, the expression of variety of oxidases increases, NH_4^+ assimilation increases, tolerance to external pH acidification increases and respiration rates also increases. However, as per recent hypothesis, there is increase in the expression of proteins involved in facilitated diffusion of NH_3, nitrate and auxin signaling pathways, aquaporins, GDP mannose-pyrophosphorylase. It is possible that one or more of these ammoniacal nitrogen tolerance mechanisms are active in *C. vulgaris* in present study;

either individual or in combination. Further studies need to be carried out at genetic and molecular level to understand the actual mechanism of ammoniacal nitrogen tolerance in microalgae.

3.3.3 Phycoremediation of SBRTW

Fig. 3.3 indicates that *C.vulgaris* is dominant microalgae in SBRTW. Growth of *C. vulgaris* in SBRTW and Pollutant removal is depicted in Fig. 3.4 and Table 3.2 represent the pollutant removal from SBRTW using *C. vulgaris.*

The observed growth rate and doubling time was **0.36/day** and **0.44** days, respectively. Numerous studies have reported growth rate and doubling time of *C. vulgaris* ranging from 0.165-1.0 per day and 0.5- 1.5 per day, respectively on different growth medium and different types of wastewater (MASalem 2015; Saad H. Ammar 2016; Wang et al. 2010, 2015). The biomass yield was observed to be 260 mg/L.

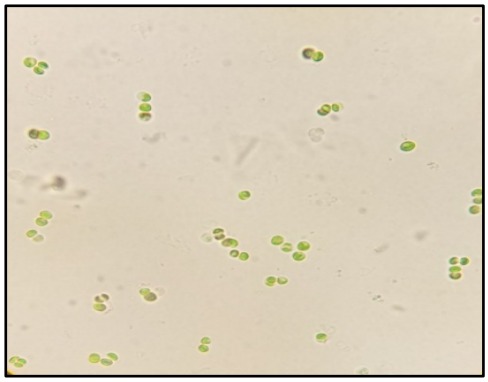


Fig. 3.3 *C.vulgaris* as dominant microalgae in SBRTW.

Table 3.2 represent the pollutant removal from SBRTW using *C. vulgaris.* During phycoremediation using *C. vulgaris*, **31.21 %** (31.33 g) COD was reduced with removal rate of 7.49 mg/L-day. Numerous studies has reported COD removal of 40-90 % and COD removal rates of 17-150 mg/L-day in different wastewater with HRT of 10-16 days (Min et al. 2011; Wang et al. 2010, 2015). The COD removal is less in present study as compared to previous studies. This may be attributed to less HRT of 6 days in present study. The COD removal rate may be increased by increasing the initial microalgal inoculum during phycoremediation studies. However, present study suggests that *C. vulgaris* was able to use organic and inorganic carbon including carbon dioxide as carbon source. Previously it was reported that *Chlorella* sp. strain VJ79 can grow autotrophically, heterotrophically and mixotrophically depending on source of carbon in growth medium (Lalucat, Imperial, and Parés 1984). Martinez et al has also reported that *Chlorella* sp. can grow mixotrophically and

can utilize light, organic carbon sources (glucose and organic acids) and inorganic carbon dioxide; organic carbon is utilized preferably over inorganic carbon (Martínez et al. 1997).

C. vulgaris was observed to remove **39.47 %** (3.35 g) of PO_4-P with removal rate of 0.8 mg/L-day. Various researchers have reported PO_4-P removal of 40- 90 % with removal rates of 0.78 to 12.25 mg/L-day using *C. vulgaris* in different kinds of wastewaters with pH range of 6.5 to 9. The PO_4-P removal efficiency in present study is similar to PO_4-P removal efficiencies reported earlier; however, it can be increased by increasing HRT and amount of initial microalgal inoculum. It was stated that PO_4-P from wastewater can be utilized by two main mechanisms; microalgal utilization and PO_4-P precipitation at alkaline conditions (Li et al. 2011). In present study, as the pH did not increase beyond 8.0, all the PO_4-P removal may be attributed to microalgal utilization.

It was observed that, *C. vulgaris* have removed **35.89 %** (12 g) NH_4-N with removal rate of 2.86 mg/L-day in present study. Various other studies have observed NH_4-N removal of 40-94 % with removal rate of 2.78 to 5.72 mg/L-day (Li et al. 2011; Wang et al. 2010). The NH_4-N removal rate is matching with available literature, but the removal efficiency is less as compared to reported values. This suggests that the removal efficiency can be increased by increasing HRT. Microalgae can utilize inorganic Nitrogen (NH_4-N, NO_3-N and NO_2-N) and simple organic Nitrogen (urea and amino acids) from wastewater depending on availability; NO_3-N is preferred Nitrogen source (Matusiak 1976). The removal mechanisms of NH_4-N from wastewater by microalgae include microalgal absorption and NH_3 stripping at pH>9.0, presence of abundant urea and elevated temperature (Matusiak 1976). In present study, the temperature did not exceed to 30°C and the pH was observed to be < 8.0; suggesting that NH_3 is not significant and NH_4-N removal was attributes to microalgal absorption only.

Observed TKN removal and removal rate in present study was **38 %** (22.52 g) and 5.36 mg/L-day, respectively. Previously it was reported that *C. vulgaris* grown on varieties of wastewater can achieve TKN removal of 40-80 % with removal rate of 4.45-7.43 mg/L-day (Li et al. 2011; Wang et al. 2010). The TKN removal efficiency and removal rate were similar to previously reported values but can be increased by increasing initial inoculum concentration and HRT. TKN removal is observed to be greater than NH_4-N removal in present study suggesting that *C. vulgaris* can utilize NH_4-N, NO_3-N and NO_2-N as Nitrogen source (Martínez et al. 1997). Further research needs to be carried out in this regard.

Table 3.2 Pollutant removal from SBRTW using *C. vulgaris*.

Parameter	COD	PO_4-P	NH_4-N	TKN
Total Inlet (g)	100.73 ±13.45	8.48 ± 1.56	33.48 ± 3.59	59.28 ± 5.94
Total Outlet (g)	69.29 ± 7.37	5.13 ± 0.82	21.46 ± 2.87	36.75 ± 7.65
Total removed (g)	31.33 ± 5.62	3.35 ± 0.64	12.01 ± 1.75	22.52 ± 2.17
% Removal	31.21 ± 3.41#	39.47 ± 5.27#	35.89 ± 6.52*	38.0 ± 4.82*
Removal rate (mg/L-day)	7.49 ± 1.08	0.8 ± 0.12	2.86 ± 0.31	5.36 ± 0.67

No significant removal, * Significant removal

(COD-Chemical Oxygen Demand, PO_4-P- Orthophosphate, NH_4-N-Ammoniacal Nitrogen, NO_3-N-Nitrate Nitrogen, TKN-Total Kjeldahl Nitrogen). All the values represent mean and standard error at n=2.

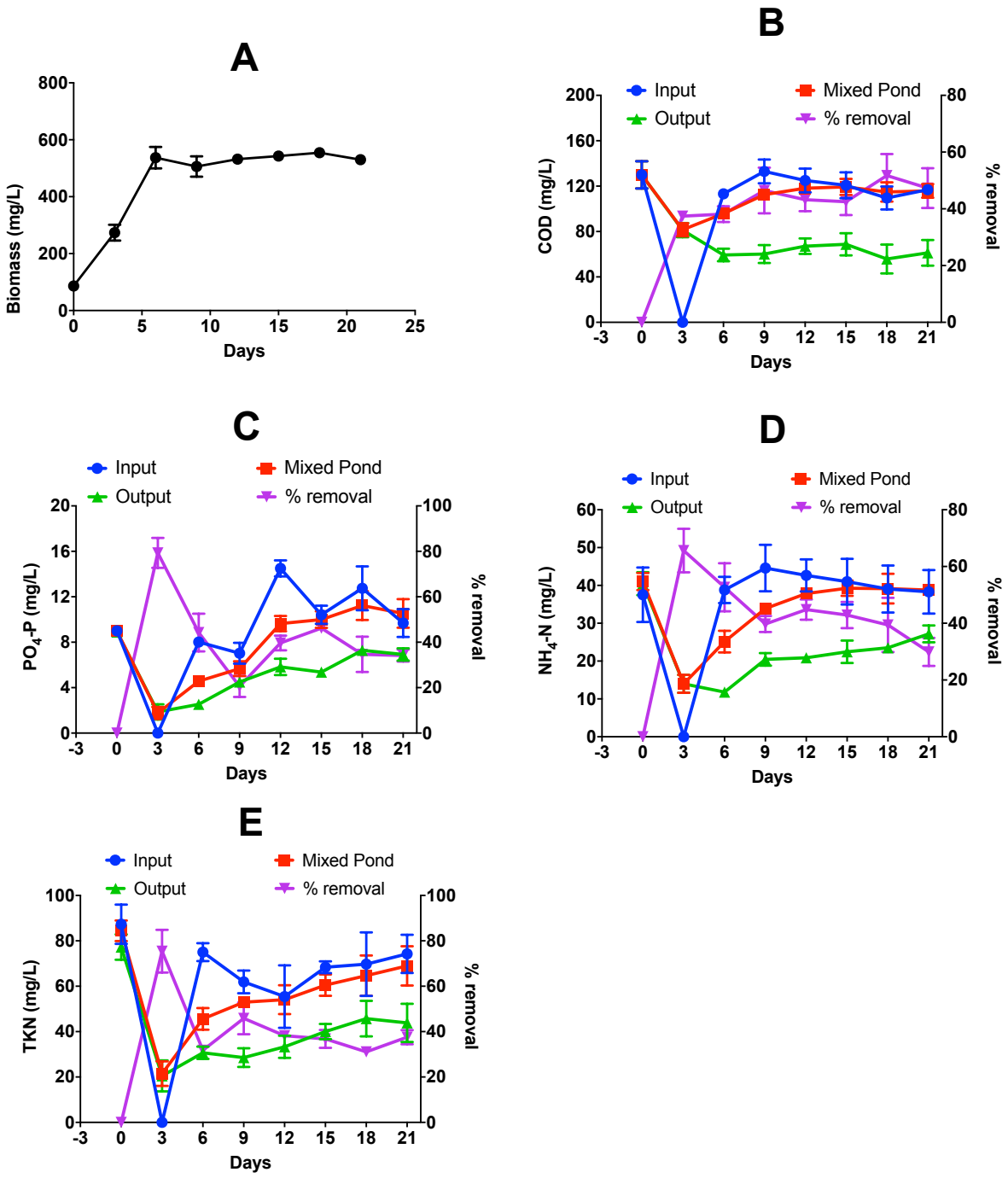


Fig. 3.4 Growth of *C. vulgaris* in SBRTW and Pollutant removal; A-growth curve, B-COD profile, C- PO_4-P profile, D- NH_4-N profile and E- TKN profile.

(COD-Chemical Oxygen Demand, PO_4-P- Orthophosphate, NH_4-N-Ammoniacal Nitrogen, NO_3-N-Nitrate Nitrogen, TKN-Total Kjeldahl Nitrogen). All the values represent mean and standard error at n=2.

It was observed that the *C. vulgaris* can significantly remove pollutants from SBRTW, however it does not meet the wastewater discharge limits as specified by Environmental Protection Agency-USA (EPA) and Central Pollution Control Board (CPCB, India). This

decrease in efficiency was attributed to short HRT of 6 days. We have observed that, increase in HRT leads to larval growth and frequent visits of birds to treatment site, raising the issue of human health and hygiene. However, the treated water can be used for agricultural irrigation after disinfection. Previously it was observed that, the use of treated water for agriculture increases the soil organic content, nitrogen and phosphorous content, thus reducing the use of chemical fertilizers (Mohammad and Mazahreh 2003).

3.3.4 Biomass analysis

Table 3.3 represent fibre composition of *C. vulgaris* biomass.

Table 3.3 Fibre composition of *C.vulgaris*

Fibre Type	% Content
Soluble fibre	76.21 ± 7.82
Crude fibre	3.79 ± 2.69
Cellulose	4.57 ± 0.94
Hemicellulose	15.58 ± 2.63
Lignin	3.64 ± 1.04

(NDF-Neutral detergent fibre, ADF-Acid detergent fibre, ADL-Acid detergent lignin)

FTIR spectrum of *C.vulgaris* is depicted in Fig. 3.5. and tentative assignment of bands is depicted in Table 3.4.

C. vulgaris biomass consists of 76 % soluble fibre and 23. 79 % of crude fibre. The crude fibre consists of cellulose, 4.57 %; hemicellulose, 15.58 % and Lignin, 3.64 %; cellulose/hemicellulose =0.29 . The literature regarding fibre composition of *C. vulgaris* is not very well documented. However, it was reported that *Chlorella pyrenoidosa* consists of 0.3 % cellulose and 0.5 % hemicellulose (Gai et al. 2015). It was also reported that *Chlorella* sp. contains 8.6 % hemicellulose and 15.4 % cellulose; cellulose/hemicellulose =1.79 (Northcote et al. 1958). The lower cellulose/hemicellulose ratio (0.29) was obtained in present study as observed (1.79) by Northcote et al. Lower the cellulose/hemicellulose ratio, higher the biodegradability of biomass and can be used as a substrate for anaerobic digestion (Ghosh, Henry, and Christopher 1985). The presence of lignin in *Chlorella* sp. was not always clear. Zhu et al reported that the lignin is not present in *Chlorella* sp. (Zhu et al. 2014). However, Zhou et al has reported the presence of lignin in biomass in less amount (Zhou et al. 2012). Cellulose, hemicellulose, and lignin play an important role as fibrous barrier to protect the cells from external environment (Abo-Shady et al. 1993; Safi et al. 2014). The high lignin content provides the robustness to algal cell walls.

FTIR spectrum of *C.vulgaris* (Fig.3.5) confirmed the presence of alkanes, alkenes, aromatics, lipids, proteins, carbohydrates, alcohols and carboxylic acids (Table 3.4). These results are well in agreement with Dilek and Vidyadharani et al (Dilek (Yalcin) Duygu, 2012; Vidyadharani and Dhandapani 2016).

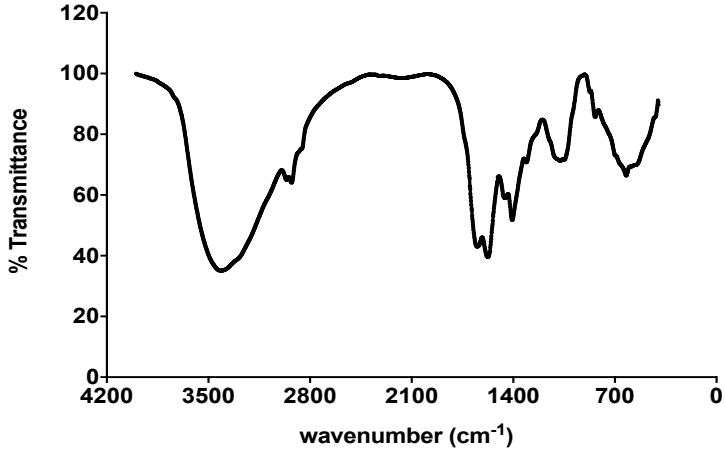


Fig. 3.5 FTIR spectrum of *C.vulgaris*

Table 3.4 Tentative assignment of bands observed in FTIR spectrum of *C. vulgaris*.

Main peak (cm^{-1})	Typical Band Assignment	Wavenumber range (cm^{-1})
3414	Water v(O-H) stretching, protein v(N-H) stretching	3029-3639
2929	Lipid-Carbohydrate, mainly $v_{as}(CH_2)$ and $V_s(CH_2)$ stretching	2809-3012
1656	Protein Amide I bond, mainly v(C=O) stretching	1583-1709
1577	Protein Amide II bond, mainly ó(N-H) bending and v(C-N) stretching	1481-1585
1465	Proteins $ó_{as}(CH_2)$ and (CH_3) bending of methyl, and Lipid $ó_{as}(CH_2)$ bending of methyl	1425-1477
1403	Protein $ó_s(CH_2)$ and $ó_s(CH_3)$ bending of methyl, Carboxylic acid v_s(C-O) of COO groups of carboxylates and Lipids ós($N(CH_3)_3$) bending of methyl	1357-1423
1078	Carbohydrate v(C-O-C) of polysaccharides, nucleic acids and other P containing compounds, Vs(>P=O) stretching of phosphodiester bonds	1072-1099
867	C-H “oop”, aromatics	675-900
622	C-Br stretch, alkyl halides	515-690

3.3.5 Biomass pre-treatments

Table 3.5 represent composition of *C.vulgaris* before and after pre-treatments and Table 3.6 represent composition of *C. vulgaris* extract before and after pre-treatments.

It was found to contain **34 %** proteins, **16 %** lipids and **38 %** carbohydrates. Previously, it was observed that *C. vulgaris* can accumulate 5-40 % lipids inside the cells, this found to be true in our studies (Becker 1994). However, it was found that the *C. vulgaris* may contain 37-58 % proteins and 51-61 % carbohydrate (Becker 2007; Illman et al. 2000; Kumar, Dasgupta, and Das 2014). This decrease in proteins and carbohydrate content in our study may be attributed to the presence of high nitrogen and orthophosphates in SBRTW as compared to absence of orthophosphates in the cultivation medium their studies. Previously, it was observed that N and P limitation can increase the proteins, lipids and carbohydrate content of biomass (Ana Cláudia Freitas Margarites 2014; Illman et al. 2000). The observed VS content is well in agreement with Calicioglu et al (Calicioglu and Demirer 2015).

.It was observed that during pre-treatments VS, proteins, lipids and carbohydrate content of biomass is decreased and proteins, carbohydrates, COD, TKN and NH4-N were increased in microalgal extract as compared to control. This was observed due to solubilization of biomass during different pre-treatments (Alzate et al. 2012; Cho et al. 2013b). The observed order of biomass solubilization with respect to different pre-treatment methods as compared to control was thermal > thermochemical > sonication > chemical. It was reported that the increase in biomass solubilization increases biogas production (Alzate et al. 2012; Calicioglu and Demirer 2015).

Table 3.5 Characterization of *C.vulgaris* biomass after pre-treatments

Method	Control	Thermal	Sonication	Chemical	Thermochemical
Moisture %	6.8 ± 0.86	9.26 ± 0.13	10.86 ± 0.28	12.17 ± 0.796	13.23 ± 1.39
TS %	93.2 ± 3.53	90.74 ± 0.86	89.14 ± 4.16	87.83 ± 2.73	86.77 ± 0.32
VS %	85.71 ± 2.55	67.42 ± 2.32	73.29 ± 2.51	72.39 ± 0.69	69.54 ± 3.46
VS/TS	0.92 ± 0.82	0.74 ± 0.19	0.82 ± 0.23	0.82 ± 0.14	80.14 ± 2.58
Ash %	14.9 ± 0.15	32.58 ± 0.16	26.71 ± 0.27	27.61 ± 0.97	30.46 ± 0.295
Protein %	30.26 ± 1.29	19.38 ± 2.13	20.87 ± 3.49	18.26 ± 2.23	20.91 ± 3.12
Lipids %	16.45 ± 2.43	ND	ND	ND	ND
Carbohydrate %	38.31 ± 3.28	21.49 ± 3.29	19.86 ± 3.54	18.56 ± 3.28	17.89 ± 1.88

TS-Total Solids, VS- Volatile Solids and ND-Not determined. All the values represent mean and standard error at n=4

Table 3.6 Characterization of *C.vulgaris* extract after pre-treatments

Method	Control	Thermal	Sonication	Chemical	Thermochemical
Lipids %	28.32 ± 2.45	2.15 ± 0.12	3.18 ± 0.41	2.27 ± 0.36	2.78 ± 0.46
Protein mg/g	38.56 ± 3.42	128.64 ± 5.88	125.86 ± 6.58	89.64 ± 6.32	135.89 ± 20.37
Carbohydrate mg/g	29.38 ± 1.89	82.39 ± 6.53	79.63 ± 5.43	74.59 ± 5.33	78.41 ± 5.63
Soluble COD/g	4128.89 ± 143.93	9845.27 ± 220.89	7895.19 ± 189.35	7658.43±250.68	9586.17 ± 175.66
TKN %	0.38 ± 0.28	0.69 ± 0.28	0.61 ± 0.23	0.53 ± 0.21	0.61 ± 0.09
NH_4-N mg/L-g	6.43 ± 0.87	29.35 ± 1.28	26.58 ± 0.81	24.53 ± 1.20	28.32 ± 0.22

COD- Chemical Oxygen Demand, TKN- Total Kjeldahl Nitrogen and NH_4-N- Ammoniacal Nitrogen. All the values represent mean and standard error at n=4.

3.3.6 Biogas production

Biogas production from *C. vulgaris* biomass at different loading rate, scale of operation and the effect of different biomass pre-treatments is depicted in Fig. 3.6 and Table 3.7.

Maximum biogas production was observed at loading rate of **2 g VS/L** followed by 3 g VS/L with biogas yield of **452 mL/g VS** and 386 mL/g VS, respectively with increase of **31. 5 %** and **12.61 %,** respectively. The range of biogas yield and methane yield at different loading rates were found to be 340-452 mL/ g VS and 185- 224 mL/ g VS, respectively. Previous studies have reported that the optimum VS loading for anaerobic digestion was 1.4-2.9 g VS/L with biogas yield of 250- 360 ml /g VS while using vegetable waste as substrate (Babaee and Shayegan 2011; Elango et al. 2007). Numerous studies have also reported the biogas yields ranging from 238- 630 mL/ g VS while using different substrates (microalgae, food wastes, dairy manure and vegetable wastes) for anaerobic digestion (Agyeman and Tao 2014; Calicioglu and Demirer 2015, n.d.; Doğan-Subaşı and Demirer 2016). At high VS loading > 3 gVS/L, there was increase in cumulative biogas production but yield of biogas was not increased. This might be due to the effect of accumulation of various toxic metabolites including aromatics, ammonia, volatile fatty acids (Hecht and Griehl 2009). Further research needs to be carried out to confirm this assumption.

Effect of different pre-treatments on biogas production from microalgal biomass was studied at optimum loading rate of **2 g VS/L**. Biomass pre-treatments increase the solubilization of biomass components leading to their bioavailability to be used as nutrients by anaerobic microorganisms (Alzate et al. 2012; Calicioglu and Demirer 2015). The biogas yield (mL/gVS) obtained by untreated, thermal, sonication, chemical and thermo-chemical treated biomass was 350, 482, 360, 374 and 430, respectively. The maximum biogas increase was observed in biomass pretreated with thermal; 60 % followed by thermochemical; 28 %, sonication; 24% and chemical method; 18 %. It was reported previously that thermal pretreated biomass can produce 29- 70 % higher biogas as compared to untreated biomass (Alzate et al. 2012; Campo G., Rıggıo V.A., Ceruttı A., Ruffino B., Panepınto D. 2018; Cho et al. 2013a; Passos and Ferrer 2014), this found to be true in present study as well. Sonication pre-treated biomass was reported to produce 6-130 % more biogas (384 mL/ g VS) as compared to untreated biomass (Alzate, Munoz, et al., 2012; Lee, Chantrasakdakul, et al., 2014). However, maximum biogas yield obtained by sonication treated biomass in present study is less as compared to previous observation in Lee et al study ; this might be due to the use of high sonication dose (2500 J/mL) as compared to 300 J/mL in present study (Lee et al. 2014). Thermochemical pretreatment can increase biogas production up to 44 % (Campo G., Rıggıo V.A., Ceruttı A., Ruffıno B., Panepınto D. 2018). In present study,

thermochemically pre-treated biomass produced 28 % more biogas as compared to untreated biomass. Anaerobic digestion studies at 5 L scale using untreated and thermally pre-treated biomass replicated the results of 0.1 L scale studies. This indicate that the anaerobic digestion can be further scaled up to understand more about the feasibility and cost effectivity of both processes; thermal pre-treatment and anaerobic digestion.

Table 3.7 Biogas production from *C.vulgaris* at different loading rates, different scales of experiment and effects of biomass pre-treatment methods

Sr. NO.	Method	Scale	Duration (Days)	Loading rate (g VS/L)	Biogas Yield (mL/g VS)	Methane yield (mL/g VS)
1	No treatments	0.1 L	29	1	343.5 ± 25.32	185.49 ± 20.34
				2	452 ± 35.36	289.28 ± 14.59
				3	386.83 ± 27.8	224.36 ± 30.24
				4	346.38 ± 51.73	207.82 ± 27.36
				5	341.5 ± 17.48	194.65 ± 13.48
2	Control	0.1 L	30	2	350 ± 23.47	174 ±19.75
	Thermal				482.25 ± 29.32	279.71 ± 12.85
	Sonication				359.75 ± 28.5	215.85 ± 17.68
	Chemical				374.75 ± 10.84	206.11 ± 9.92
	Thermochemical				430.87 ± 20.52	224.06 ± 18.75
3	Control	5 L	33	2	370.65 ± 25.29	180.45± 14.73
	Thermal				470.56 ± 10.25	285.55 ± 11.61

All the values represent mean and standard error at n=2.

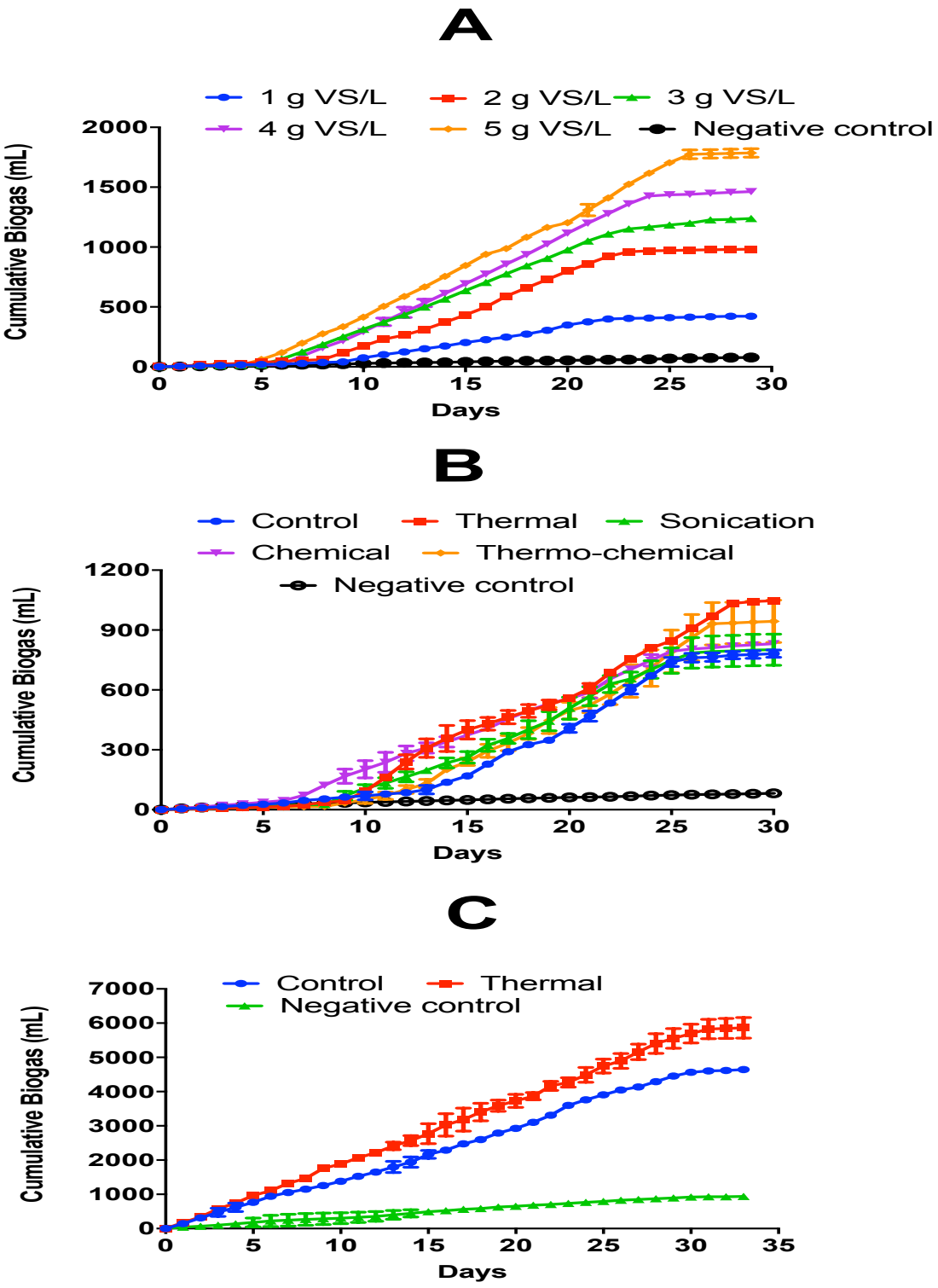


Fig. 3.6 Biogas production from *C.vulgaris* A-Biogas production at different loading rate, B-Effects of biomass pre-treatment methods on biogas production and C-Comparison of biogas production from fresh biomass and thermally pre-treated biomass at 5 L scale. (VS-Volatile solids; all the values represent mean and standard error at n= 2).

3.4 Conclusion

Most of the conventional wastewater treatment facilities focuses mainly on COD and BOD removal, however *C. vulgaris* was found to be effective in removal of COD, Nitrogen and Phosphorous. It can remove 30-40 % of these pollutants from wastewater. TKN removal is higher compared to NH_4-N removal suggesting that NH_4-N is not the preferred N source. Moreover, microalgal treatment systems can be installed at sewage treatment plants without any major modification to available infrastructure. Thermal pretreatment of biomass can increase the biogas production up to 60 %. Integration of microalgal cultivation with wastewater treatment may serve two major purposes; handling of wastewater and sustainable generation of clean energy fuel, biogas. It was also observed that NH_4-N tolerance of C. vulgaris can be increased by adaptation in a growth medium with gradual increase in NH_4-N concentration.

Chapter IV

Integration of microalgal biorefinery to vertical flow constructed wetland and microalgal treatment system for treatment of raw domestic wastewater

4.1 Introduction

In India there is huge gap between the installed sewage treatment capacity and the required sewage treatment capacity. One of the major limitation is the operational costs. Decentralized hybrid treatment systems could be a solution to this which will focus apart from treating wastewater in producing value added products. Constructed Wetlands (CWs) are treatment systems that mimic the natural wetlands that use vegetation, soil and associated microorganisms to increase the water quality. CWs are gaining widespread attention as an alternative technology for sewage treatment as it can efficiently treat varieties of wastewater including DW, storm water, leachate, polluted river, rural runoff and industrial effluents (Abou-Elela and Hellal 2012; Saeed and Sun 2013). CWs are found to be efficient in removal of organic and in-organic pollutants, solids, nitrogenous and phosphate compounds , trace elements and various other pharmacological contaminants (Cui et al. 2010; Saeed and Sun 2013). The main factors influencing the long-term pollutant removal efficiency of CWs are plant species, microbial biofilm and construction media. Other critical operational factors influencing the treatment performance of CWs are hydraulic loading rates, design and construction of CWs, water depth, water retention time and feeding mode. In the selection of CWs vegetation preference should be given to the plant species naturally occurring to the area where CWs are being constructed.

Even if CWs have advantages over conventional treatment systems in terms of organic, inorganic carbon and solids removal, it suffers from several limitations. The removal nitrogenous and phosphate compounds is not efficient in CWs (Abdelhakeem, Aboulroos, and Kamel 2016). Nitrogen in reduced form can be converted to oxidized form; the rates of denitrification and NH_3 volatilization are less. Adding to this, the main removal mechanism of PO_4-P is adsorption, and it may leach out during the time course.

To increase overall long-term pollutant removal efficiency and also have an economic advantage, VFCWs can be combined with other technologies , such as Microalgal Treatment System (MTS). Microalgal consortium or purified microalgal strains can be used to efficiently for wastewater treatment using Open Raceway Pond (ORP), and the biomass can be used to produce various value-added products (Pittman, Dean, and Osundeko 2011; Samorì et al. 2013). The valuable products may include asthaxanthin, phycocyanin, methane, electricity, hydrogen, pigments, lipids, fatty acids, carotenoids, polysaccharides, etc. (Brennan and Owende 2010; Pulz Wolfgang Gross 2004). Microalgal biomass mainly consists of lipids and carbohydrates. These biomass components can be sequentially extracted and used for production of different value-added products. For example, the extracted lipids can be transesterified to produce biodiesel. In general, Naive Biomass (NB) after lipid extraction can

generate 65 % of residual biomass which is rich in carbohydrates (starch and cellulose), leftover lipids and some proteins (Zhu 2014). The Residual Biomass after Lipid Extraction (RBLE) can be saccharified and can be used as a substrate for fermentative ethanol production. The Residual Biomass after Extraction of Lipids and Sugars (RBLSE) can produce of biogas via anaerobic digestion (Chavan and Mutnuri 2018). The microalgal biomass should be used to extract multiple products sequentially for sustainable microalgal biomass production.

The main objectives of this study are: (i) treatment of DW by hybrid treatment system including VFCW and MTS (ii) Harvesting of high value microalgal biomass and (iii) extraction of value-added products (lipids, ethanol and biomethane) from microalgal biomass. As India is a tropical country, the rate of phytoremediation and phycoremediation will be high due to abundant solar energy throughout the year. Therefore, the attempts were made to investigate the resource recovery from DW and its transformation to valuable algal biomass to develop a process that is cost effective and has less maintenance and operational costs.

4.2 Materials and Methods

4.2.1 Collection and Analysis of DW

DW was collected from on-campus sewage treatment plant located at Birla Institute of Technology and Science (BITS) Pilani, KK Birla Goa Campus (15.3911° N, 73.8782° E). DW was analyzed for Chemical Oxygen Demand (COD), Phosphate Phosphorous (PO_4-P), Total Kjeldahl Nitrogen (TKN), Ammonium Nitrogen (NH_4-N), and Total Solids (TS) as per standard methods for the examination of water and wastewater (Eaton et al. 2005).

Schematic representation of integrative treatment of DW by VFCW and microalgal treatment system is depicted in Fig. 4.1.

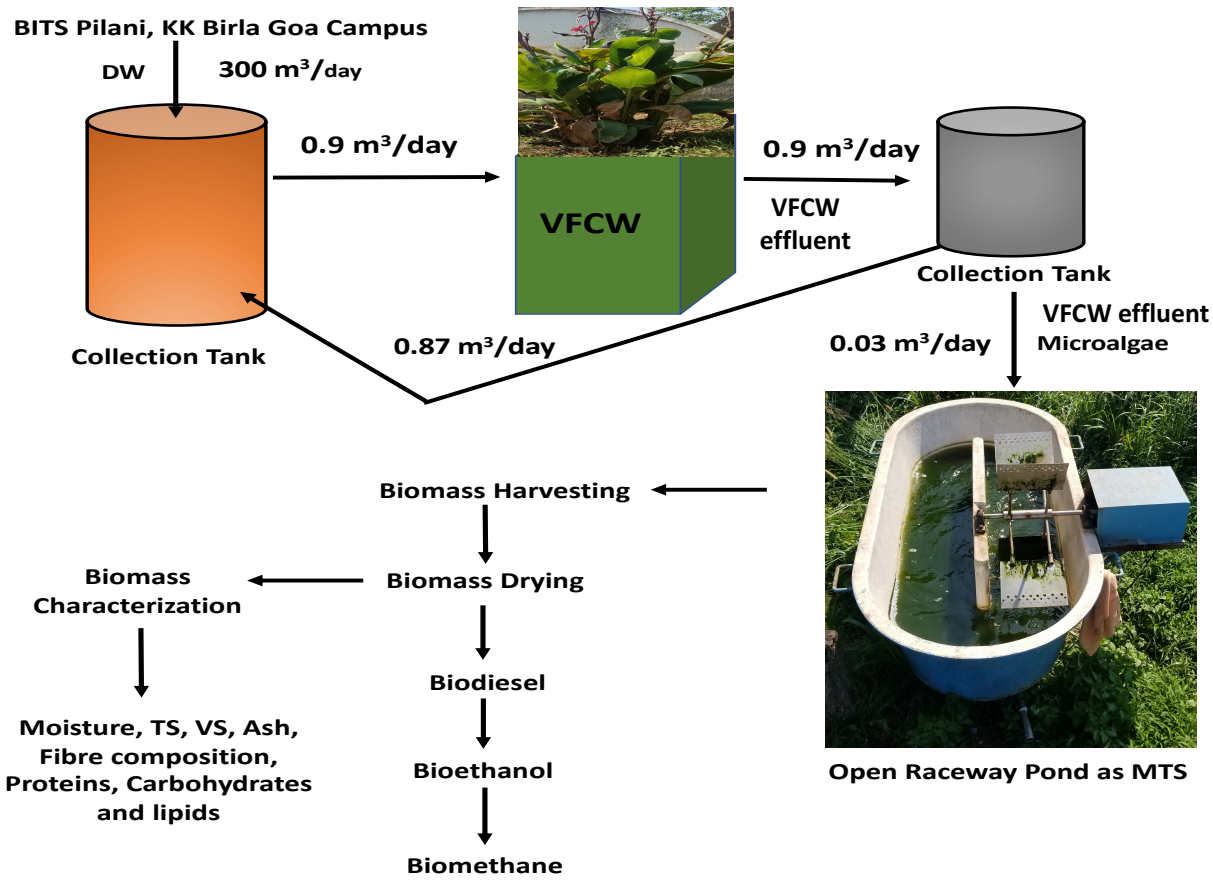


Fig.4.1 Schematic representation of integrative treatment of Domestic Wastewater (DW) and VFCW effluent by integration of Vertical Flow Constructed Wetland (VFCW) and Microalgal Treatment System (MTS).

4.2.2 Selection of wetland vegetation

In the present study, *Canna indica* was selected for wetland vegetation because it is native to Goa, it is vigorous in growth, provide aesthetic appeal to constructed wetland and hence can be easily accepted by public (Konnerup, Koottatep, and Brix 2009; Yadav, Chazarenc, and Mutnuri 2018). Fresh *Canna indica* plantlets (15 cm in height) were collected from on-campus nursery and kept submerged in still water until planted in the wetland. VFCW was partitioned into 3 equal filter beds; 5 *Canna indica* plantlets were planted equidistantly in each filter bed (Total plantlets=15). After 25 days, once the plantlets formed the roots and started to grow, the wetland was subjected to experimentation.

4.2.3 Operation and initiation of VFCW

This study was carried out by using previously existing VFCW in campus premises, and it was operated with minor modification (Yadav et al. 2018). Yadav et al (2018) has used two stage VFCWs in series for treatment of DW, however, in present study the second stage VFCW was replaced by MTS.

In brief, the gravels of different sizes were used as media (from bottom level; 20 cm (10-20 mm), 20 cm (10-15 mm) and 50 cm (2-8 mm)). The wetland was being used for 2.5 years continuously. As the life span of *Canna indica* is around 9-10 months (Mercedes Ciciarelli 2012), even if the VFCW was well established, the old plants were replaced with fresh plantlets. The surface area of VFCW was 4.2 m^2. The study was started in March 2017. The VFCW was fed with 300 L/day of DW in the morning (9 AM) using 1 HP water pump (flow rate-150 L/min), and the outlet was closed. In the evening (6 PM), the outlet was opened, the effluent from VFCW was collected in a collection tank. This operation was continued for 25 days for proper development of plants and biofilms. As Goa is situated in tropical climate zone, the evapotranspiration rates were high, and the optimum loading rate was found to be 60-70 g COD/m^2-day. To meet this requirement, once the plants were properly developed, VFCW was fed with 1 m^3/day DW (using 1 HP water pump with flow rate of 150 L/min and retention time of 5 minutes) with Hydraulic Loading Rate (HLR) of 0.25 m/day. The VFCW effluent was collected in a collection tank. The experiment was continued for 30 days and samples were collected after every three days and analyzed regularly for COD, NH_4-N, TKN, PO_4-P, and TS. The experimental trial was carried out for 30 days to understand the initial performance, characterization, feasibility and potential of VFCW and MTS system in integrative manner.

Inoculation of VFCW: The VFCW was not inoculated by any specific microorganisms. The microbes from DW was accumulated in VFCW bed during feeding.

4.2.4 Integration of VFCW to Microalgal Treatment System (MTS)

Open Raceway Pond (ORP) was used as MTS. Paddle wheels (connected of alternating current single-phase synchronous motor with reducing gear) revolving at speed of 20 RPM was used as mixing equipment(APS Lifetech, Pune). The ORP was operated at maximum depth of 20 cm. Initially, 0.2 m^3 of VFCW effluent was fed in the ORP, and it was operated at batch mode for 6 days (from day 0 to day 6) to enrich the resident microalgae consortium. Resident microalgae consortium has been previously used for the treatment of domestic wastewater (Rawat et al. 2011). Onward from day 6 to day 30, the ORP was operated in fed-batch mode by harvesting 30 L of microalgae culture from ORP followed by addition of 30 L of fresh VFCW effluent from collection tank every day with a retention time of 6 days. The average

light intensity and temperature during experimentation was found to be 925 μmol/m^2-s and 30 °C, respectively. The microalgal biomass was harvested using silk cloth as a filter. Silk cloth has a small pore size and the silk fibers do not attach to biomass during scrapping of the biomass cake. The biomass was subjected to drying in oven at 60 °C overnight, ground into fine powder using kitchen blender and stored at 4ºC until further use. The fractions of liquid samples after biomass harvesting were subjected to centrifugation for 15 minutes at 10000 RPM. The clear supernatant was separated and analyzed for COD, NH_4-N, TKN, and PO_4-P. TS analysis was performed before biomass separation from liquid culture. Three-point sampling was employed to analyze the composition of; a) microalgal culture present initially in ORP, b) fresh VFCW effluent added to ORP and c) microalgal culture in ORP after removal of 30 L microalgal culture and addition of 30 L of fresh VFCW effluent (Chavan and Mutnuri 2018).

4.2.5 Analysis of microalgae consortium biomass

The microalgal biomass was analyzed for TS, moisture, Volatile Solids (VS) and ash content (Eaton et al. 2005). The microalgal protein content was analyzed by Lowry's method (López, García, and Fernández 2010). Total carbohydrates of biomass was analyzed as per method described by Krisnaveni et al (Krishnaveni, Sadavisam, and Balasubramanian 1984). The fiber composition was estimated by the Van Soest method using semi-automated Fibraplus FES04E (Pelican Equipments, Chennai). Elementar-varioMICRO was used to analyze elemental composition of microalgal biomass (Germany).

4.2.6 Lipid extraction and analysis of FAMEs (Fatty Acid Methyl Esters)

Total lipids were extracted from 50 g of Naive Biomass (NB) and 300 mL of a mixture of chloroform and methanol (2:1) using Soxhlet extraction (Fakhry and Maghraby 2013). The extracted lipid-solvent mixture was phase separated by adding 100 mL of 1 % NaCl. The lower chloroform layer containing lipids was separated and evaporated under vacuum at 40ºC. The lipid residue was weighed and stored at -4ºC till further use. Transesterification of extracted lipids and analysis of FAMEs was performed as described previously by Chavan et al (Chavan and Mutnuri 2018). The Residual Biomass after Lipid Extraction (RBLE) was air dried and stored at -4ºC until further use.

4.2.7 Ethanol production

4.2.7.1 Selection and identification of yeast strain

The yeast used in this study is usually used for ethanol production from rice at 40 °C in local areas of north-east region of India. This culture was received as gift from Mr. Motilal Yadav (Resident of North-East India). We identified the yeast strain and it was found to be hotspring yeast RND13 strain, which is widely used for ethanol fermentation with high efficiency. It was revived by suspending 1 g yeast pellets in 0.5 L of sterile Luria broth (2 g/L) (Himedia Labs-India), incubated at 30ºC in shaking incubator for 2 days. This revival step was repeated 5 times to obtain the pure culture of yeast. The yeast was maintained on Luria agar plates. The yeast biomass was separated from yeast broth after 16 hours of growth by centrifugation at 8000 RPM. The yeast genomic DNA was extracted according to method used by Cheng et al (Cheng, Cheng, and Jiang 2006). The ~ 2kb 18s rDNA nucleotide region was amplified using PCR polymerase and subjected for sequencing. The sequence data was used to create phylogenetic tree using weighbor software to identify the yeast species and closest neighbors. The 18S rRNA genes were amplified by using universal fungal primers NS1 (5'-GTAGTCATAKGCTNGTCTS-3') and C-18L (5'GARACCTDGTTAVGACTY3') (UENO, URANO, and KIMURA 2002).

4.2.7.2 Sachharification of RBLE

The Residual Biomass after Lipid Extraction (RBLE) (40 g/L) was treated with 1 % (V/V) H_2SO_4 for 30 minutes at 140ºC. The mixture was allowed to cool, subjected to centrifugation at 1000 RPM for 5 minutes. The supernatant was separated and stored at -4ºC after pH adjusted to 7.0 using 2N NaOH. The reducing sugar analysis was performed by the DNS method (Fu et al. 2010). The residual biomass after sequential extraction of lipids followed by sugars (RBLSE) was air dried and stored at -4ºC till further use.

4.2.7.3 Fermentation

The liquid extract obtained after Sachharification of RBLE was inoculated with 3 % (V/V) of yeast culture from the exponential growth phase. The fermentation was carried out for 72 hours in a shaker incubator at 120 RPM and the temperature was maintained at 40ºC. The solids were removed from fermented broth by centrifugation for 10 minutes at 8000 RPM. The clear supernatant was analyzed for ethanol content using HPLC (M. Castellari, E. Sartini, U. Spinabelli, C. Riponi 2001).

4.2.8 Biomethanation Potential (BMP) assay

Glass vials (100 mL working volume and 30 mL headspace) were used to perform BMP assays at 1 g VS/L loading. Three kinds of biomass were considered for BMP assay; a) NB, b) RBLE and c) RBLSE. Each vial contains 60 mL of anaerobic digester slurry (Table 4.1), 1 mL micronutrient stock, 1 mL macronutrient stock, 5 mL of 5 % $NaHCO_3$ and 32 mL distilled water (Prabhu and Mutnuri 2016). The respective vials were inoculated with respective biomass with 1 g VS/L loading. The vials were sealed with a rubber stopper and aluminum caps. N_2 gas was sparged through the vials to maintain the anaerobic atmosphere. The negative control (NC) was devoid of biomass as a substrate. Water displacement method was used to quantify the biogas produced. Methane composition of biogas was analyzed by using gas chromatography using thermal conductivity detector as per method described previously (Chavan and Mutnuri 2018). The kinetics of biogas production was determined by using the following equations;

A. Net Biogas Production (mL) = W-X
B. Net Biogas Yield (mL/ g VS) = A/ Y
C. Net Methane Yield (mL/g VS) = B * Z
Where,
W = Total biogas produced from substrate (mL)
X = Total biogas produced by negative control (mL)
Y = g VS added to reactor
Z = % methane composition of biogas

4.2.9 Statistical analysis of data

All the experiments were carried out with two biological replicates and two technical replicates. All the values represent mean value and mean standard error at n = 2. The data was checked for normality and homogeneity of variance. The percentage data was arcsine transformed. One-way ANOVA was carried with treatments as a fixed factor on growth rate and the removal of NH_4-N, TKN, COD and PO_4-P. All statistical analysis (comparison between treatments and means) was performed using IBM SPSS data analysis software package (IBM-USA).

4.3 Results and Discussion

4.3.1. Analysis of DW

The compositional analysis of DW is mentioned in Table 4.1. It consisted of **1738 ± 80 mg/L COD, 39.5 ± 2.0 mg/L NH_4-N, 166.5 ± 2.9 mg/L TKN, 45.7 ± 1.3 mg/L PO_4-P and 1004 ± 23 mg/L of TS.** These results are in well agreement with Yadav et al (Yadav et al. 2018). The COD and PO4-P value of DW reported here is unusually high. In India, low to medium strength DW is generated. The value reported here is even higher than the high-strength wastewater generated in some of the western countries. There might be couple of reasons for these deviations as mentioned below.

1. The studies were carried out during Summer 2017. Goa is a tropical state and the rate of evapotranspiration is high.
2. The campus has SBR as its centralized wastewater treatment system. The wastewater from the campus (hostels, mess, staff quarters, administration buildings etc.) is collected in single collection tank by using underground channels made of concrete canals, PVC pipes and metal pipes. At many places these wastewater transport systems pass nearby septic tanks. The chances of leakage of septic water from these tank to the transport channels is highly likely.
3. During summer, there was water scarcity. At the same time, few social and cultural events were happening inside campus premises increasing population density thus increasing the concentration of these pollutants in wastewater.

4.3.2 Treatment of DW with VFCW

The trends in pollutant removal from DW using VFCW is depicted in Fig. 4.2 and the average composition of DW and VFCW effluent is depicted in Table 4.1. The mass balance of pollutants and removal efficiency using VFCW is depicted in Table 4.2.

Table 4.1. Average Composition of Domestic Wastewater (DW), VFCW effluent, VFCW effluent post Microalgal Treatment System (MTS) and Biomethanation Potential (BMP) inoculum.

Parameters (mg/L)	DW	VFCW effluent	MTS Discharge	BMP Inoculum
COD	1738 ± 80	841 ± 26	327 ± 25	12437 ± 548
NH_4-N	39.5 ± 2.0	20.6 ± 0.9	7.2 ± 0.62	1325.6 ±167.4
TKN	166.5 ± 2.9	80.4 ± 5.5	23.1 ± 1.8	18389.4 ±1209.6
PO_4-P	45.7 ± 5.3	25.5 ± 1.0	12.1 ± 0.5	538.4 ± 34.1
TS	1004 ± 23	195 ± 7	1969 ± 324	1246 ± 216
pH	6.5	6.8	7.2	7.5

*COD-Chemical Oxygen Demand, NH_4-N-Ammonium Nitrogen, TKN-Total Kjeldahl Nitrogen, PO_4-P- Phosphate Phosphorous, TS-Total Solids, DW-Raw Domestic Sewage and VFCW-Vertical Flow Constructed Wetland. (All values represent mean values and mean standard error at n=4; each sampling point was analyzed by two biological replicates and each biological replicate was considered for two technical replicates).

It was observed that the COD input from DW to VFCW during 30 days of operation was 46.9 ± 3.4 Kg. Out of this, VFCW has removed **24 ± 1.2 Kg (51.6 ± 3.4 %).** This COD removal efficiency is in well agreement with Yadav et al with 53 % of COD removal while using *Canna indica* as wetland vegetation in drum experiment (Yadav et al. 2018). Previously, 90 % of COD removal from domestic sewage was observed by integration of Upflow-anaerobic Sludge Blanket Reactor (UASB), VFCW and Horizontal Flow constructed Wetland (HFCW) (Álvarez et al. 2017). Yadav et al has observed 90 % of COD removal from raw domestic sewage by integrating two stage VFCWs in series (Yadav et al. 2018).

It was observed that the NH_4-N input from DW during 30 days of operation was 1.0 ± 0.1 Kg. Out of this, VFCW has removed **0.5 ± 0.2 Kg (47.8 ± 4.3 %).** The removal of NH_4-N may be attributed to the mineralization of nitrogen compounds and ammonia nitrification by bacteria (*Nitrobacter* sp. and *Nitrosomonas* sp.) followed by plant uptake (Mayo and Bigambo 2005). This NH_4-N removal efficiency (47.8 ± 4.3 %) is less as compared to NH_4-N removal efficiency (54 %) as observed by Yadav et al in single stage VFCW (Yadav et al. 2018). The decrease in NH_4-N removal efficiency in present study might be attributed to use of *Canna indica* as wetland vegetation in present study as opposed to use of *Typha angustata* used for wetland vegetation by Yadav et al (Yadav et al. 2018). The observed NH_4-N removal rate (g/m^2-day) by VFCW was 4.3 ± 0.7. Nitrification potential was not studied in present study, but

denitrification may be carried out by *Nitrosomas europaea* and *Nitrosomonas eutropha* in aerobic condition in CWs (Helmer et al. 1999).

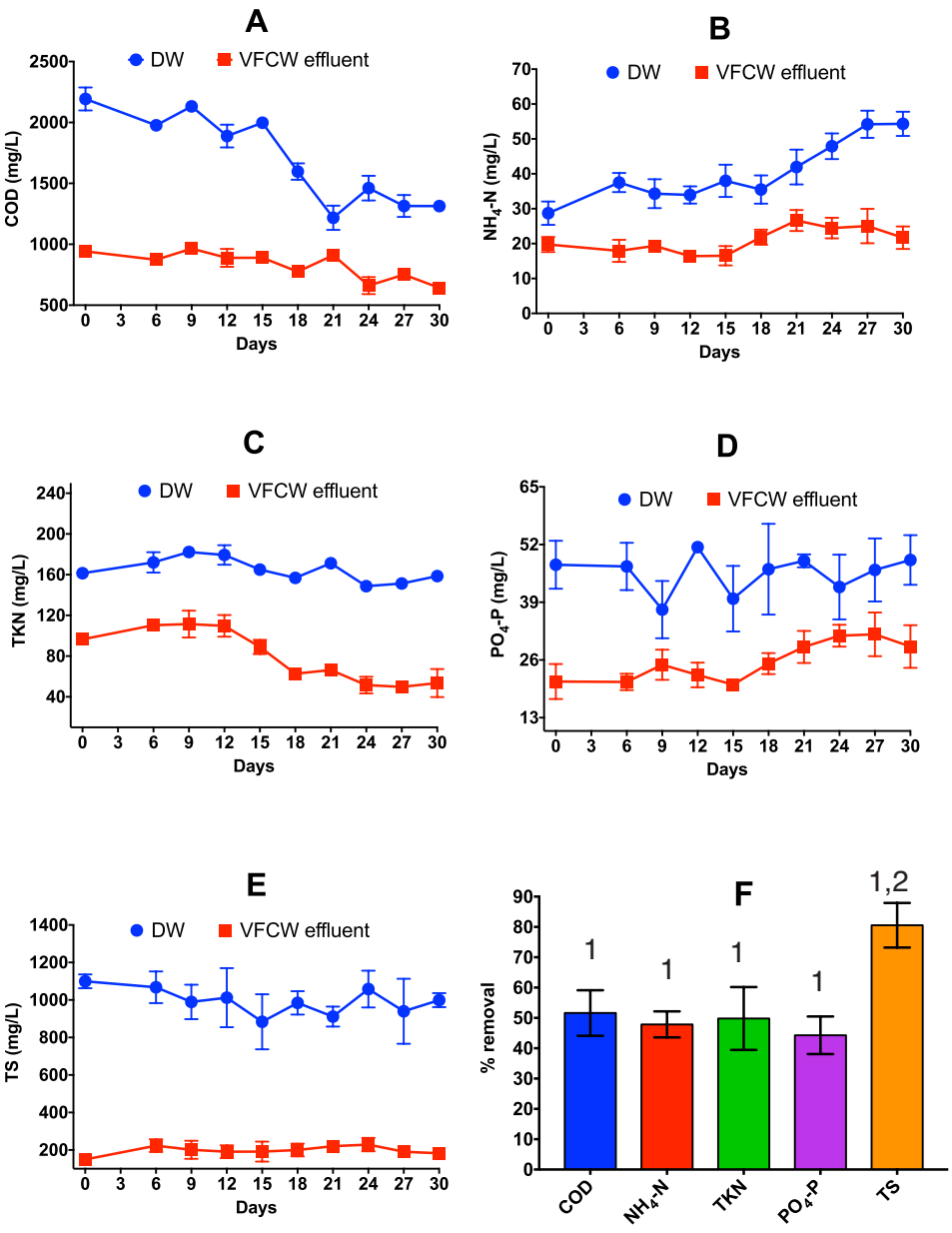


Fig.4.2. Trends in pollutant concentration and overall % removal from Domestic Wastewater (DW) using Vertical Flow Constructed Wetland (VFCW). *COD-Chemical Oxygen Demand, NH_4-N-Ammonium Nitrogen, TKN-Total Kjeldahl Nitrogen, PO_4-P- Phosphate Phosphorous, TS-Total Solids, DW- Domestic Wastewater and VFCW-Vertical Flow Constructed Wetland. The numbers above the bars denote significant groups ($p<0.05$) based on Tukey's post hoc tests. All the values represent mean value and mean standard error at n = 2. (All values represent mean values and mean standard error at n=2; two biological replicates, each with two technical replicate).

Table 4.2 Pollutant removal by Vertical Flow Constructed Wetland (VFCW).

Parameter	COD	NH_4-N	TKN	PO_4-P	TS
Input (Kg)	46.9 ± 3.4	1.0 ± 0.1	4.5 ± 0.2	1.2 ± 0.2	27.1 ± 2.1
Removal by VFCW (Kg)	**24 ± 1.2**	**0.5 ± 0.2**	**2.3 ± 0.2**	**0.5 ± 0.1**	**21.5 ± 1.7**
% Removal by VFCW	**51.6 ± 3.4**	**47.8 ± 4.3**	**51.6 ± 2.3**	**44.2 ± 3.5**	**79.3 ± 3.4**
VFCW removal rate (g/m^2-day)	190.46 ± 21.5	4.3 ± 0.7	18.25 ± 1.3	3.96 ± 0.8	238.17 ± 13.2
VFCW removal rate (mg/L-day)	**897.4 ± 146.7**	**18.9 ± 2.1**	**86.0 ± 7.4**	**20.2 ± 2.1**	**797.2 ± 135.7**

*COD-Chemical Oxygen Demand, NH_4-N-Ammonium Nitrogen, TKN-Total Kjeldahl Nitrogen, PO_4-P- Phosphate Phosphorous, TS-Total Solids. (All values represent mean values and mean standard error at n=4: each sampling point was analyzed by two biological replicates and each biological replicate was considered for two technical replicates).

It was observed that the TKN input from DW during 30 days of operation was 4.5 ± 0.2 Kg. Out of this, VFCW has removed **2.3 ± 0.2 Kg (51.6 ± 2.3 %)**. This TKN removal efficiency (51.6 ± 2.3 %) is higher than TKN removal efficiency as observed by Yadav et al (34 %) but is less than TKN removal efficiency as observed by Kantawanichkul et al (88 %) (Kantawanichkul et al. 1999; Yadav et al. 2018). This difference might be due to use of *Canna indica* as wetland vegetation in the present study as opposed to *Typha angustata* used by Yadav et al and *Vetiveria zizanioides Nash* by Kantawanichkul et al in their studies. Kantawanichkul et al has used 4 hours retention time in their studies while, in present study, no retention time was used. In present study, the observed TKN removal rate (g/m^2-day) by VFCW was 18.25± 1.3. *Canna indica* plants are also vigorous in growth in tropical climate that increases removal rate of pollutants from DW (Konnerup et al. 2009).

It was observed that the PO_4-P input from DW during 30 days of operation was 1.2 ± 0.2 Kg. Out of this, VFCW has removed **0.5 ± 0.1 Kg (44.2 ± 3.5 %)**. This PO_4-P removal efficiency (44.2 ± 3.5 %) is higher as compared to PO_4-P removal efficiency as observed by Yadav et al (10%) while using *Typha angustata* as plant vegetation in drum experiment to treat domestic sewage (Yadav et al. 2018). This difference might be due to use of *Canna indica* as wetland vegetation in the present study as opposed to *Typha angustata* used by Yadav et al. The observed PO_4-P removal rate (g/m^2-day) by VFCW was 3.96 ± 0.8. The removal of Nitrogen and phosphorous in constructed wetland was observed to be around 50 % and removal of COD

and TS was observed to be around 90 % (Verhoeven and Meuleman 1999; Vymazal 2007). Previously it was stated that 5 % of applied Phosphorous in CWs can be assimilated by plants , 15 to 25 % can be transformed and used by microorganisms (Kuschk et al. 2003), suggesting that physical filtration, deposition and adsorption are contributing to remaining 20 % Phosphorous removal in present study.

It was observed that the TS input from DW during 30 days of operation was 27.1 ± 2.1 Kg. Out of this, VFCW has removed **21.5 ± 1.7 Kg (79.3 ± 3.4 %)**. This TS removal efficiency (79.3 ± 3.4 %) is higher as compared to TS removal efficiency as observed by Yadav et al (61 %). This difference in TS removal efficiency might be attributed to initial TS concentration present in DW. In the present study, observed initial TS concentration was 1000 mg/L as compared to initial TS concentration of 650 mg/L in studies carried out by Yadav et al (Yadav et al. 2018). However, TS removal efficiency (79.3 ± 3.4 %) in the present study is similar with TS removal efficiency (86 %) as observed in studies carried out by Xu et al (Xu et al. 2014). The observed TS removal rate (g/m^2-day) by VFCW was 238.17 ± 13.

The role of plant vegetation, soil components and microbial communities of constructed wetlands in pollutant removal is not always clear (Sklarz et al. 2009). Previous studies suggest that wetland vegetation might increase pollutant removal efficiency by pollutant assimilation, increase in transport of oxygen, releasing enzymes and growth regulators for the enrichment of microbial communities in wetlands. Plants and microbes play a major role in transformation of organics and mineralization of nutrients. The literature suggesting the differences in pollutant removal by physical components and biological components in wetlands is limited. Previously, it was reported by Kuschk et al (2003), 5 % of Phosphorous and 5-10 % of Nitrogen from domestic wastewater can be assimilated by plants (Kuschk et al. 2003). This study has also reported that 22-33 % of Nitrogen and 21 to 30 % of Phosphorous from wastewater can be removed by plant and microbial communities in wetlands. This study also suggests 79-93 % of Phosphorous removal and 65-92 % of Nitrogen removal can be achieved by filtration by physical components, plant vegetation and microbial communities in CWs and these removal efficiencies are in correlation with growth of plants (Kuschk et al. 2003). The selection of plant species in CWs is also important as there are differences in removal efficiencies of different pollutants using different plants (Brisson and Chazarenc 2009). On the other hand, few studies has reported that the equal pollutant removal efficiencies can be achieved with or without plant vegetation in wetlands (Gross, Kaplan, and Baker 2007; Lahav et al. 2001). Sklarz et al has studied unplanted Recirculating Vertical Flow Constructed Wetland (RVFCW) for removing pollutants from wastewater (Sklarz et al. 2009). Sklarz et al has observed that during single

batch run of domestic wastewater in RVFCW, removal of TS, COD, NH_4-N- and TKN was 30, 28, 30.6 and 9.3 %, respectively.

4.3.3 Microalgal treatment System

Fig. 4.3 depicts that *C.vulgaris* was the dominant algal species present in VFCW effluent. The pollutant removal from VFCW effluent using MTS is depicted in Table 4.3 and Fig. 4.4 respectively. The average composition of VFCW effluent post MTS is depicted in Table 4.1. The growth pattern of microalgae in ORP using VFCW effluent is shown in Fig.4.5.

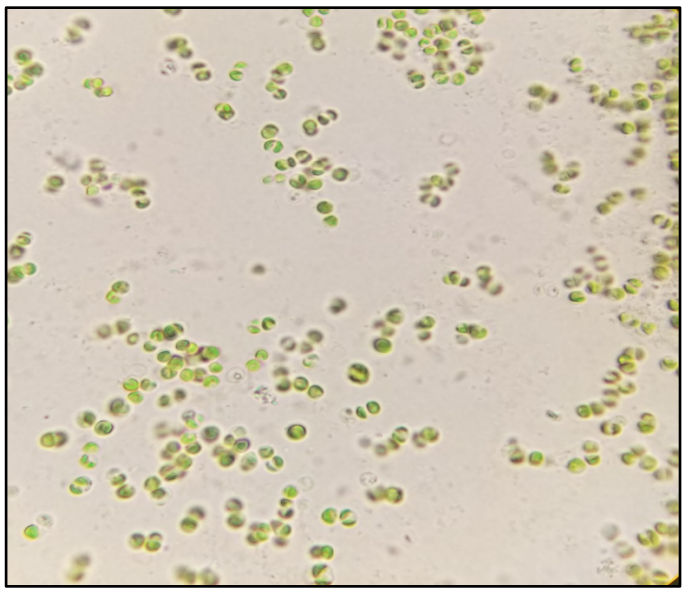


Fig. 4.3 *C.vulgaris* as the dominant algal species present in VFCW effluent.

Table 4.3. Pollutant removal by Microalgal Treatment System (MTS)

Parameters	% Removal	Removal rate (mg/L-day)	Removal rate (mg/m^2-day)
COD	35.7 ± 3.2	42.7 ± 4.7	124.8 ± 13.5
NH_4-N	56.7 ± 4.7	1.6 ± 0.3	4.9 ± 0.3
TKN	56.2 ± 5.6	6.4 ± 0.8	18.8 ± 2.1
PO_4-P	34.9 ± 4.2	1.1 ± 0.1	3.3 ± 0.7

*COD-Chemical Oxygen Demand, NH_4-N-Ammonium Nitrogen, TKN-Total Kjeldahl Nitrogen, PO_4-P- Phosphate Phosphorous. (All values represent mean values and mean standard error at n=4, each sampling point was analyzed by two biological replicates and each biological replicate was considered for two technical replicates).

During 30 days of operation, the observed COD removal was **35.7 ± 3.2 %.** The observed removal rates; mg/L-day and mg/m^2-day were 42.7 ± 4.7 and 124.8 ± 13.5 respectively. The % COD removal was not in agreement as observed by Li et al (Li et al. 2011). Li et al have observed 90.8 % COD removal from raw centrate in laboratory photobioreactor with 14 days of retention time as opposed to 6.6 days in the current study in an open field without any control systems. However, the COD removal rate of 42.7 ± 4.7 mg/L-day in the present study is higher as compared to 5.9 mg/L-day as observed by Li et al (Li et al. 2011). This increase might be attributed to the use of naturally occurring resident microalgae present in DW.

During 30 days of operation, the observed NH_4-N removal was **56.7 ± 4.7 %.** The observed removal rates; mg/L-day and mg/m^2-day were 1.6 ± 0.3 and 4.9 ± 0.3 respectively. Li et al have observed 93.9 % NH_4-N removal from raw centrate in laboratory photobioreactor with 14 days of retention time as compared to 6.65 days in the current study in an open field without any control systems. However, the NH_4-N removal rate of 1.6 ± 0.3 mg/L-day in the present study is higher as compared to 0.2 mg/L-day as observed by Li et al (Li et al. 2011). This increase might be attributed to the use of naturally occurring resident microalgae present in DW.

During 30 days of operation, the observed TKN removal was **56.2 ± 5.6 %.** The observed removal rates; mg/L-day and mg/m^2-day were 6.4 ± 0.8 and 18.8 ± 2.1 respectively. Li et al have observed 89 % TKN removal from raw centrate in laboratory photobioreactor with 14 days of retention time as opposed to 6.65 days in the current study in an open field without any control systems. However, the TKN removal rate of 6.4 ± 0.8 mg/L-day in the present study is higher as compared to 0.30 mg/L-day as observed by Li et al (Li et al. 2011). This increase might be attributed to the use of naturally occurring resident microalgae present in DW. However, the removal rate is in agreement as previously described in treatment of urban wastewater by *Chlorellae vulgaris* (Cabanelas et al. 2013).

During 30 days of operation, the observed PO_4-P removal was **34.9 ± 4.2 %.** The observed removal rates; mg/L-day and mg/m^2-day were 1.1 ± 0.1 and 3.3 ± 0.7 respectively. Li et al have observed 80.9 % PO_4-P removal from raw centrate in laboratory photobioreactor with 14 days of retention time as compared to 6.65 days in the current study in an open field without any control systems. However, the removal rate agrees with Li et al.

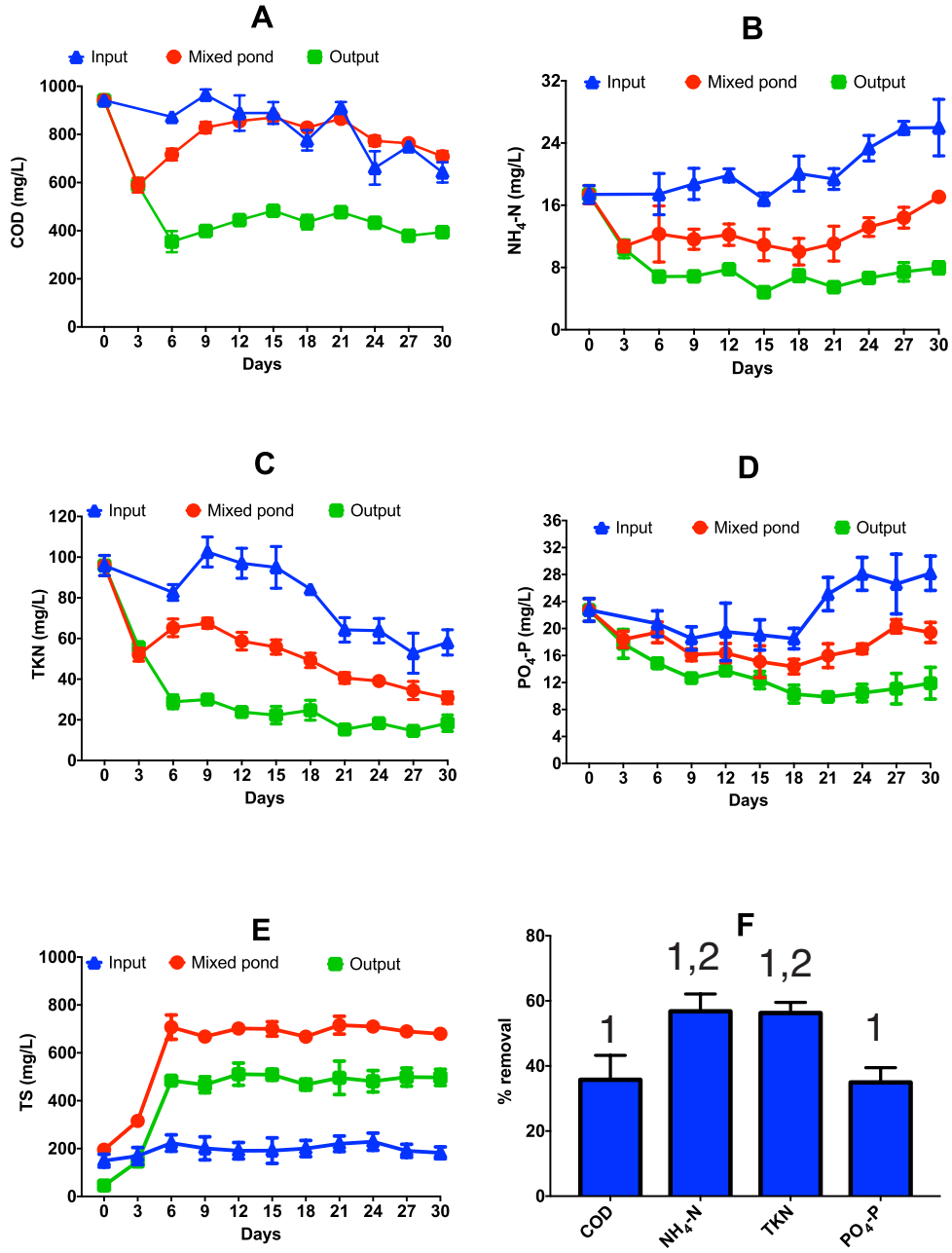


Fig. 4.4 Trends in pollutant concentration and overall % removal Pollutant removal by Microalgal Treatment System (MTS). *COD-Chemical Oxygen Demand, NH_4-N-Ammonium Nitrogen, TKN-Total Kjeldahl Nitrogen, PO_4-P- Phosphate Phosphorous, TS-Total Solids. The numbers above the bars denote significant groups ($p<0.05$) based on Tukey's post hoc tests. All the values represent mean value and mean standard error at n = 2.

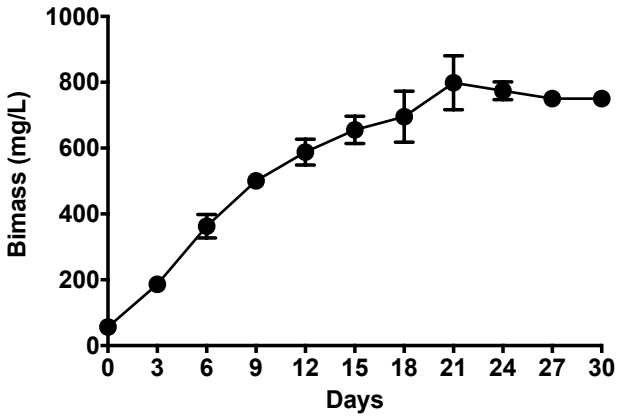


Fig 4.5. Growth of microalgae using VFCW effluent in ORP in fed-batch mode. (All values represent mean values and mean standard error at n=4).

4.3.4 Comparison of VFCW-MTS with other existing treatment systems

The comparison of the treatment efficiency of VFCW-MTS and other existing systems like Extended Aeration (EA), Sequential Batch Reactor (SBR), Membrane Bio-Reactor (MBR) and Moving Bed Biofilm Reactor (MBBR) is depicted in Table.4.4. The integrated system was observed to remove 68.9% COD, 77.4% NH_4-N, 75.8% TKN and 63.6% PO_4-P. The pollutant removal efficiencies (%) of EA, SBR, MBBR and MBR were reported to be; EA (67 % COD (Colmenarejo et al. 2006), 75 % NH_4-N (Colmenarejo et al. 2006), 72 % TKN (Kutty et al. 2014) and 83% PO_4-P (Mehrdadi and Azimi 2006)), SBR (48 % COD (Lackner and Horn 2013), 90 % NH_4-N (Lackner and Horn 2013), 95 % TKN (Lu et al. 2009) and 86 % PO_4-P (Lu et al. 2009)), MBBR (38 % COD (Lackner and Horn 2013) , 88 % NH_4-N (Lackner and Horn 2013), 74 % TKN (Tomaszek and Grabas 2007) and 89 % PO_4-P (Kermani et al. 2009)) and MBR (91 % COD (Turken et al. 2019), 92 % NH_4-N (Wang et al. 2012), 40 % TKN (Turken et al. 2019)and 5 % PO_4-P (Turken et al. 2019)). It was observed that the treatment efficiency of VFCW-MTS system in present study is similar to the reported treatment efficiency of EA, SBR, MBBR and MBR. However, VFCW-MTS system has low maintenance and operation and it can generate high value microalgal biomass as end product which can be used to extract multiple products (FAMEs, ethanol and biomethane) having commercial value: it does not only reduce the cost on wastewater treatment but also generate some revenues, making the treatment system cost effective.

Table. 4.4 Comparison of the treatment efficiency of VFCW-MTS and other existing systems like Extended Aeration (EA), Sequential Batch Reactor (SBR), Membrane Bio-Reactor (MBR) and Moving Bed Biofilm Reactor (MBBR)

Technology type	% Removal			
	COD	NH_4-N	TKN	PO_4-P
EA	67 (Colmenarejo et al. 2006)	75 (Colmenarejo et al. 2006)	72 (Kutty et al. 2014)	83 (Mehrdadi and Azimi 2006)
SBR	48 (Lackner and Horn 2013)	90 (Lackner and Horn 2013)	95 (Lu et al. 2009)	86 (Lu et al. 2009)
MBBR	38 (Lackner and Horn 2013)	88 (Lackner and Horn 2013)	74 (Tomaszek and Grabas 2007)	89 (Kermani et al. 2009)
MBR	91 (Turken et al. 2019)	92 (Wang et al. 2012)	40 (Turken et al. 2019)	5 (Turken et al. 2019)
VFCW and MTS	68.9	77.4	75.8	63.6

VFCW-Vertical Flow Constructed Wetland, MTS-Microalgal Treatment System.

It was observed that, the treated water at the end of VFCW and MTS was composed of, on average (mg/L), COD-327, NH_4-N-7.2, TKN-23.1 and PO_4-P-12.1. This does not meet the standard wastewater discharge standards as specified by CPCB-India and EPA. This less treatment efficiency is attributed to the less HRT of 6 days VFCW effluent in MTS. It was previously observed that the long-term cultivation of microalgae in wastewater leads to growth of larvae and mosquitoes and frequent visits of birds to MTS, raising the issue of hygiene. Therefore, compromises were made between long HRT and treatment efficiency. However, use of secondary treated wastewater (TDS-1225, COD-480, NH_4-N-118, PO_4-P 49 mg/L) for irrigation of agricultural land suggested increase in soil organic matter, soil PO_4-P and decrease in soil pH (Mohammad and Mazahreh 2003). Thus, reducing the need of organic and inorganic chemical fertilizer application to land. It is necessary to consider this output water for quaternary treatment before being used for agricultural irrigation.

Constructed Wetlands (CW) were found to be effective in wastewater treatment. However, they suffer many problems their practical use as a treatment system. CWs are sensitive to variations in climatic conditions and temperature, occasional problems of saturation and blockage, requirement of large areas, problems with proper design and construction of CW, choice of plant vegetation, variation in microbial communities in CWs with respect to CW depth and wastewater being treated (HUANG Jin-lou, Qin, and XU Lian-huang 2013). These

factors play and important role in deciding the long-term pollutant efficiency, scalability and shelf life of CW.

4.3.5 VFCW-MTS Footprint

The major limitation in the application of the CWs as treatment system is the requirement of large area to achieve effective treatment (Ilyas and Masih 2017). The footprint may be increased further in case of hybrid CWs (Foladori et al. 2012). EPA recommends that the CWs with footprint of 1 m^2/PE is sufficient to achieve desired effluent quality (Ilyas and Masih 2017). However, this is not always true; footprint required for storm water treatment, secondary and tertiary treatment wetland is 0.5, 1-10 and 5 m^2/PE, respectively (Vymazal 2011). The CWs with high footprint might have high removal of organic matter and solids, but the removal of dissolved pollutant might be low (Babatunde 2008). The present study has demonstrated that hybrid CW (VFCW-MTS) with reduced footprint of 1.35 m^2/PE can achieve good removal of solids and organic matter and sufficient removal of dissolved pollutants.

4.3.6 Biomass composition

The results of biomass composition are well in agreement as described previously (Chavan and Mutnuri 2018) except the lipid composition. Chavan et al has observed 26.6 % lipid with *Spirulina platensis* as compared to 16.7 % with microalgae consortium in present study. This decrease might be attributed to the TS attached to microalgal biomass during harvesting. However, this lipid content is in agreement with lipid content of 8.5-18.4 % in Class Chlorophyceae (Brown and Jeffrey 1992). The protein content of 22.3 % was observed in microalgal biomass in present study which is in agreement with protein content of 15-25.6 %, as observed in previous study (Brown and Jeffrey 1992). Total carbohydrate content of biomass was observed to be 37.8 %, previously it was stated that, microalgal biomass contains 5-51 % of carbohydrates on dry weight basis (Brown and Jeffrey 1992; Ho et al. 2013). In present study, microalgal biomass was observed to contain 75 % VS. The composition of biomass is depicted in Table.4.5.

Table 4.5 Compositional analysis of Biomass:

Parameter	Naive Biomass
Moisture %	10.2 ± 4.5
TS %	84.7 ± 5.2
VS %	75.4 ± 4.8
VS/TS	0.8 ± 0.1
Ash %	24.5 ± 1.3
Proteins (mg/gm)	223.7 ± 45.6
Carbohydrates (mg/gm)	378.5 ± 37.2
Lipids (%)	16.7 ± 3.5
TKN (%)	0.4 ± 0.1
Fiber Type	
Soluble fraction (%)	78.7 ± 5.6
Crude fibre (%)	21.5 ± 2.6
Cellulose (%)	5.6 ± 0.3
Hemicellulose (%)	7.8 ± 1.4
Lignin (%)	1.2 ± 0.2
N:C:H: S	9.5:48.6:7.9:1.4

*TS-Total Solids, VS-Volatile Solids, TKN-Total Kjeldahl Nitrogen. (All values represent mean values and mean standard error at n=4).

4.3.7 FAMEs analysis

FAMEs composition of biomass is depicted in Table. 4.6.

Table. 4.6 FAMEs composition of microalgal biomass:

Name	Type	Chemical formula	Molar Mass (g/mol)	% FAMEs	% Crude lipids
Tetradecanoate	Saturated	$C_{14}H_{28}O_2$	228.37	3.83	0.64
Hexadecanoic acid	Saturated	$C_{16}H_{32}O_2$	256.43	16.22	2.72
Heptadecanoic acid	Saturated	$C_{17}H_{34}O_2$	270.45	0.99	0.17
9-Octadecene	Unsaturated	$C_{18}H_{36}$	252.49	1.7	0.29
1-Octadecene	Unsaturated	$C_{18}H_{37}$	252.49	4.55	0.76
9-Octadecenoic acid	Unsaturated	$C_{18}H_{32}O_2$	282.47	2.53	0.42

*FAME-Fatty Acid Methyl Esters; calculated on the basis of biomass dry weight.

It was observed that the biomass contains **16.7 ± 3.5 %** of crude lipids with transesterification efficiency of 29.8 %, resulting in 4.8 % of pure FAMEs. The yield of lipids is not in agreement as per previously observed using *Chlorella pyrenoidosa* with ultra-sonication (D'Oca et al.

2011) because of TS attached to microalgal biomass during harvesting in present study and Soxhlet extraction method. However, the % composition of fatty acids with respect to crude lipids is well in agreement with previous study (D'Oca et al. 2011). The FAME fraction was observed to contain Tetradecanoic acid, Hexadecanoic acid, Heptadecanoic acid, 9-Octadecene, 1-Octadecene and 9-Octadecenoic acid. The resulting saturated to unsaturated fatty acid ratio was observed to be 2.4.

Previous study has described the physico-chemical properties of FAMEs derived from microalgal biomass (Fakhry and Maghraby 2013). The properties of FAMEs including Viscocity, cetane number and oxidation potential are important to be used as fuel. Viscocity influences the fuel injection of fuel in the combustion engines. AS the degree of unsaturation and length of fatty acid increases, viscocity increases. The viscocity values of FAMEs in current study is in range of 3.3-4.5 mm^2/s with an average of 4.0 mm^2/s which is in agreement with the ideal viscocity values (1.9 -6 mm^2/s) of fuels as suggested by American Society for Testing of Materials (ASTM) (Gouw and Vlugter 1964). Cetane number describes the autoignition properties of fuels. Fuels with high cetane number burns quickly, smoothly and releases more energy. On the other hand, low cetane fuels delay ignition, release particulate matter and leads to incomplete combustion. The cetane value of commercial diesel is 45-55. In present study, the range of cetane numbers of FAMEs is observed to be 59-86 with an average of 65.8, suggesting the suitability of FAMEs to be used as fuel. The ideal ratio of FAMEs to be used as a fuel is C16/C18:1/C14 is 5/4/1, such fuel will be having low oxidation potential and thus reduces ignition delay period in combustion engines (Knothe 2005). The observed C16/C18:1/C14 ratio in present study was 4.2/2.2/1, suggesting that the concentration of C16 and C18:1 need to be increased by alternation in medium conditions.

4.3.8 Ethanol Production

18 S rDNA sequence determination and phylogenetic analysis of fermentative yeast used in the present study revealed that the yeast strain is **RND13**. As per the phylogenetic analysis, it is observed that RND13 strain is closely related to *Candida glabrata* and *Kluyveromyces delphensis* which are closely related to *Saccharomyces cerevisiae*. The details regarding yeast identification are mentioned in **supplementary material I (Appendix I).**

RND13 has been previously used for ethanol production using glucose as a substrate (UENO et al. 2002). It was observed that **3.88 g/L** (84.22 mMol) of ethanol produced (Ethanol yield-0.26 g/g) with an initial concentration of 15.12 g/L of reducing sugars. In previous study, ethanol yield of 1% (W/V) (21.7 mMol) at initial sugar concentration of 15.2 g/L was obtained by using *Spirulina* sp. (Hossain, Basu, and Mamun 2015). This corresponding increase in

ethanol yield may be attributed to the use of yeast by Hossain et al and RND 13 in present study. It suggests that RND13 can efficiently ferment sugars at 40 °C. Harun and Danquah has reported ethanol yield of 1 g/L using *Chlorococcum humicola* acid treated biomass at loading rate of 10 g/L (Rasit et al. 2015)(Harun and Danquah 2011). However, these results are not in agreement with the results obtained by Ueno et al. Ueno et al have observed an ethanol yield of 0.46 g/g with a total production of 70 g/L ethanol (1519.4 mMol). The variations might be attributed to the initial sugar concentration of 150 g/L and the quantity of initial yeast inoculum (5 % W/V) as opposed to initial sugar concentration of 15.1 g/L and the quantity of initial yeast inoculum (3 % V/V) in present study. The observed specific yield of ethanol over reducing sugar consumed was 33.4 % (Molar yield=1.31). This experimental yield of ethanol is 64.9 % of theoretical ethanol yield over glucose as substrate.

4.3.9 Biomethane production

The results of biogas production from naive biomass, RBLE and RBLSE are depicted in Table. 4.7 and Fig.4.6.

Table 4.7 Biomethane production from biomass

	NB	RBLE	RBLSE	NC
Loading rate (g VS/L)	1.00	1.00	1.00	0.00
Total Biogas (mL)	455.0 ± 45.1	344.5±38.9	304.0 ± 30.2	77.5± 10.2
Corrected Biogas (mL)	377.5 ± 25.4	267.0±28.6	226.5 ± 18.7	
Net Biogas yield (mL/g VS)	377.5 ± 20.2	267.0±16.7	226.5 ± 12.6	
% Methane	56.1 ± 3.2	50.4±8.2	47.5 ± 9.3	
Net Methane yield (mL/g VS)	211.8±19.24	134.6±13.6	107.7 ± 7.3	

*VS-Volatile Solids, NB-Naive Biomass, RBLE-Residual Biomass after lipid Extraction, RBLSE-Residual Biomass after Extraction of Lipids and Sugars, NC-Negative control without any substrate. (All values represent mean values and mean standard error at n=4).

The BMP experiments were carried out at a loading rate of 1 g VS/L. In case of NB as a substrate, observed biogas and methane yield (mL/g VS) were **377.5** and **211.8** respectively. Previously, Cherubini has reported the methane yield (230-410 mL/g Vs) from freshwater and marine microalgal biomass (Cherubini 2010). Net biogas yield observed in the present study is lower than net biogas yield observed by Mussgnug et al using *Arthrospira platensis* (481 mL/g VS) as a substrate. This decrease in biogas yield might be attributed to a mixed culture of

biomass and contamination by adhered TS in our study as compared to use of pure culture of microalga as a substrate and controlled atmosphere reactor during biomethane production studies carried out by Mussgnug et al (Mussgnug et al. 2010). However, the biogas yield in the present study is higher as compared to biogas production studies carried out by Mussgnug et al using *Chlorella kessleri* (335 mL/g VS) and *Scenedesmus obliquus* (287 mL/g VS). These studies indicated that the net biogas yield varies depending on the use of different microalga as a substrate. In the case of RBLE as a substrate, observed biogas and methane yield (mL/g VS) were 267 and 134.6. In the case of RBLSE as a substrate, observed biogas and methane yield (mL/g VS) were 226.5 and 107.78.

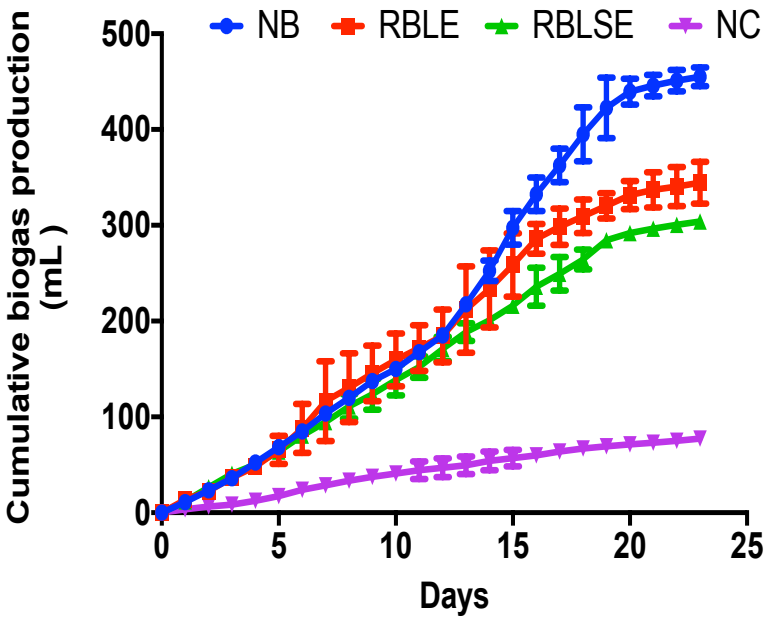


Fig. 4.6 Biomethane production from microalgal biomass:

*NB-Naive Biomass, RBLE-Residual Biomass after lipid Extraction, RBLSE-Residual Biomass after Extraction of Lipids and Sugars, NC-Negative control without any substrate (The loading rate employed here is 1 g VS/L). (All values represent mean values and mean standard error at n=4).

4.4 Conclusion

Much of the algal production research is limited to laboratory facilities. Though many research institutes and private companies have moved from laboratory to pilot scale, the major problems concerning the availability of nutrients and water, climatic conditions, microalgal integrity and contamination by larvae restricted further scaling up. Overcoming these challenges in microalgae cultivation and harvesting will benefit both the fields; biofuels and wastewater treatment. However, the attempts for hybrid treatment using wastewater as a resource to produce algal biofuels are very limited. Furthermore, the existing facilities at the wastewater treatment plant can be used for the controlled and sustainable production of microalgae. This study demonstrates that the wastewater treatment facility can be integrated with commercial microalgal production system without any major modification to pre-existing infrastructure that will reduce capital costs and produce value-added products. Previous studies mentioned that the constructed wetland requires footprint of 2-4 m^2/person. Our study has demonstrated that at hydraulic loading rate of 150 L/person, the footprint required by integration of treatment vertical flow wetland and microalgal treatment system can be reduced to 1.35 m^2/person. During the course of study, 1 Kg of microalgal biomass was harvested that can produce 0.16 Kg lipids, 0.26 Kg ethanol and 0.2 kWh of energy. This study presents an initial attempt to demonstrate two stage hybrid wastewater treatment system consuming less footprint and having potential of production of value-added products in terms of biofuel.

Chapter V

Demonstration of pilot scale integrative treatment of nitrogenous industrial effluent for struvite and algal biomass production

5.1 Introduction

As per the report published by Ministry of fertilizers, Govt. of India in 2013, the projected demand of N and P based fertilizers is for Urea-33754×10^6 Kg, DAP (Diammonium Phosphate)-12764×10^6 Kg, NP/NPKs-11841×10^6 Kg and SSP (Single Superphosphate)-6476×10^6 Kg (Lal et al. 2017). In India, the number of production units and their installed production capacities (× 10^{11} Kg) for Urea, DAP and SSP production are 30, 12 and 85 respectively with production capacities (× 10^{11} Kg) of 215, 83 and 77, respectively (Lal et al. 2017). As per studies carried out by Lu and Tian, out of total global consumption of N and P fertilizers per unit agricultural land, China ranks highest (N-31 %, P- 27 %) followed by India (N-15%, P-13%), USA (N-11%, P-10%), Brazil (N-3%, P-11%) and others (N-37 %, P-37 %) (Lu and Tian 2017).

Chemical fertilizer industries produce N and P based fertilizers along with some complex fertilizers including Urea, Ammonium Nitrate, Ammonium Sulphate, Diammonium Phosphate, Single Superphosphate etc. The main production process includes fractional distillation of air to produce free N_2 gas that reacts with free H_2 gas in presence of iron as catalyst at temperature of 450°C and 200 atmospheric pressure to generate NH_3 gas to produce liquid ammonia after condensation. The liquid ammonia is then reacted with oxygen at temperature of 800°C in presence of platinum catalyst to produce nitric acid. The produced ammonia and nitric acid can be used to produce ammonium nitrate and urea. The ammonia can also be used with phosphoric acid and sulphuric acid to produce diammonium phosphate and ammonium sulphate, respectively. Chemical fertilizer industry needs large quantity of water. During the production process of these fertilizers, large quantity of wastewater can be generated that can be estimated to 250 m^3/hour with ammonium levels of 2700 mg/L (Lotfi and Rostamy-malkhalifeh 2014). Phosphoric acid production plants can generate wastewater containing PO_4-P in the range of 4000 mg/L to 7500 mg/L (Bossler et al. 2009).

Fertilizer industry Wastewater (FW) is composed mainly of ammoniacal nitrogen (NH_4-N), nitrate nitrogen (NO_3-N), orthophosphate (PO_4-P) and low carbon content (less C: N ratio) leading to the emission of potential greenhouse gas, i.e. nitrous oxide (N_2O) (Kampschreur et al. 2009). Utilization of water contaminated with nitrate leads to health disorders in human infants known as blue baby syndrome (Knobeloch et al. 2000) and excess of phosphorous leaching to water bodies causes eutrophication leading to algal blooms resulting in loss of aquatic life via anoxic conditions (Conley et al. 2009; Correll 1998; Ryther and Dunstan 1971; Vollenweider 1968). Therefore, it is necessary to remove these pollutants from

wastewater/effluent before discharging to water bodies or reusing. Wastewater treatment includes removal of solid matter and dissolved pollutants to produce effluent that can be recycled or reused. There are different technologies available namely aerobic, anaerobic, membrane separation and adsorption for removal of NH_4-N, PO_4-P and chemical oxygen demand (COD) depending on wastewater composition (Cheremisinoff 2002).

Efficient removal of nitrogen and phosphorous from wastewater is a challenge for treatment process. Possible solution to this is to recover nitrogen and phosphorous from wastewater in the form of struvite. Struvite is an equimolar crystalline mixture of magnesium, ammonium, and phosphate (MAP). There are several advantages of struvite production systems over conventional nitrogen and phosphorous removal technologies, i.e. its precipitation can be controlled easily leading to a solid mass of slow release fertilizers, no sludge formation and most importantly pollutants can be recovered and considered as nutrients.

Struvite precipitates in a 1:1:1 molar ratio following the general equation (with n = 0, 1, or 2): $Mg^{2+} + NH^{+}_4 + H_nPO^{3-n}_4 + 6H_2O \rightarrow MgNH_4PO_4 \cdot 6H_2O + nH^+$ (KS Le Corre, E. Valsami-Jones, et al. 2009).

The occurrence and development of struvite crystals follows two chemical stages: nucleation (crystal birth) and crystal growth (development of crystals until equilibrium) (Jones 2002). Predicting or controlling these mechanisms is complex, as it is controlled by a combination of factors including the crystal state of initial compounds, thermodynamic of liquid-solid equilibrium, phenomena of matter transfer between solid and liquid phases , and kinetics of reaction (Ohlinger, Young, and Schroeder 1999), as well as several physico-chemical parameters such as pH of the solution from which struvite may precipitate, supersaturation (Doyle and Parsons 2002), mixing energy (Mullin 2001), temperature, and presence of foreign ions (Le Corre et al. 2009).

Several technologies exist for pilot scale controlled struvite recovery from wastewater including stirred tank, air agitated and fluidized bed reactors (KS Le Corre, E Valsami-Jones, and Hobbs 2009). Since C: N ratio will be less in FW, biological wastewater treatment cannot be adopted easily for effective removal of N and P compounds by nitrification and denitrification by microorganisms as they require carbon as a source of energy (Meyer et al. 2005). Besides this, conventional wastewater treatment generates large amount of sludge leading to problems associated with nutrient recovery in the form of fertilizer, sludge handling and disposal (Ahluwalia and Goyal 2007). As struvite precipitation will remove only NH_4-N, TKN and PO_4-P, microalgal production in residual wastewater after struvite

precipitation could be a better alternative for wastewater treatment as well as using the biomass for the production of value added products.

Use of microalgae for wastewater treatment exploits fast algal growth and ability to assimilate pollutants from wastewater in terms of biomass as they grow. Most of the eukaryotic microalgae can utilize organic nutrients and carbon (Larsdotter, Jansen, and Dalhammar 2007). Microalgae can effectively remove P and N from wastewater to produce high-value biomass that can be used to produce or extract varieties of value added products including ethanol, methanol, nutraceuticals, biogas, biodiesel and much more (Jong et al. 2011).

This research represents the integrative approach of struvite precipitation from FW and use of Residual Fertilizer Wastewater (RFW) (remained after struvite separation) as a growth medium for ammoniacal nitrogen tolerant microalgal cultivation in a tubular photobioreactor to produce high value biomass. It was observed that ≈ 60 Kg of struvite can be recovered from 1 m^3 of FW. Due to the integration of struvite precipitation followed by microalgal production, 64.58 % COD, 87.31 % NH_4-N, 89.0 % TKN and 98.79 % of PO_4-P is recovered. In brief, the integration of struvite production and microalgae cultivation can be used as an effective treatment system for fertilizer industry wastewater.

5.2 Materials and Methods

5.2.1 Collection and analysis of wastewater sample

FW from fertilizer producing industry from Goa was collected in a polypropylene tank. The fertilizer industry under study has zero waste policy. In brief, they do not follow conventional wastewater treatment onsite, but the wastewater generated is collected in collection tank for settling and the liquid is reused for manufacturing of chemical fertilizers.

The wastewater was analyzed for PO_4-P, NH_4-N, TKN, pH and COD (Eaton et al. 2005). Total carbon (TC) was analyzed by TOC Analyzer (Shimadzu TOC-L). The overall methodology of experiment is depicted in fig 5.1.

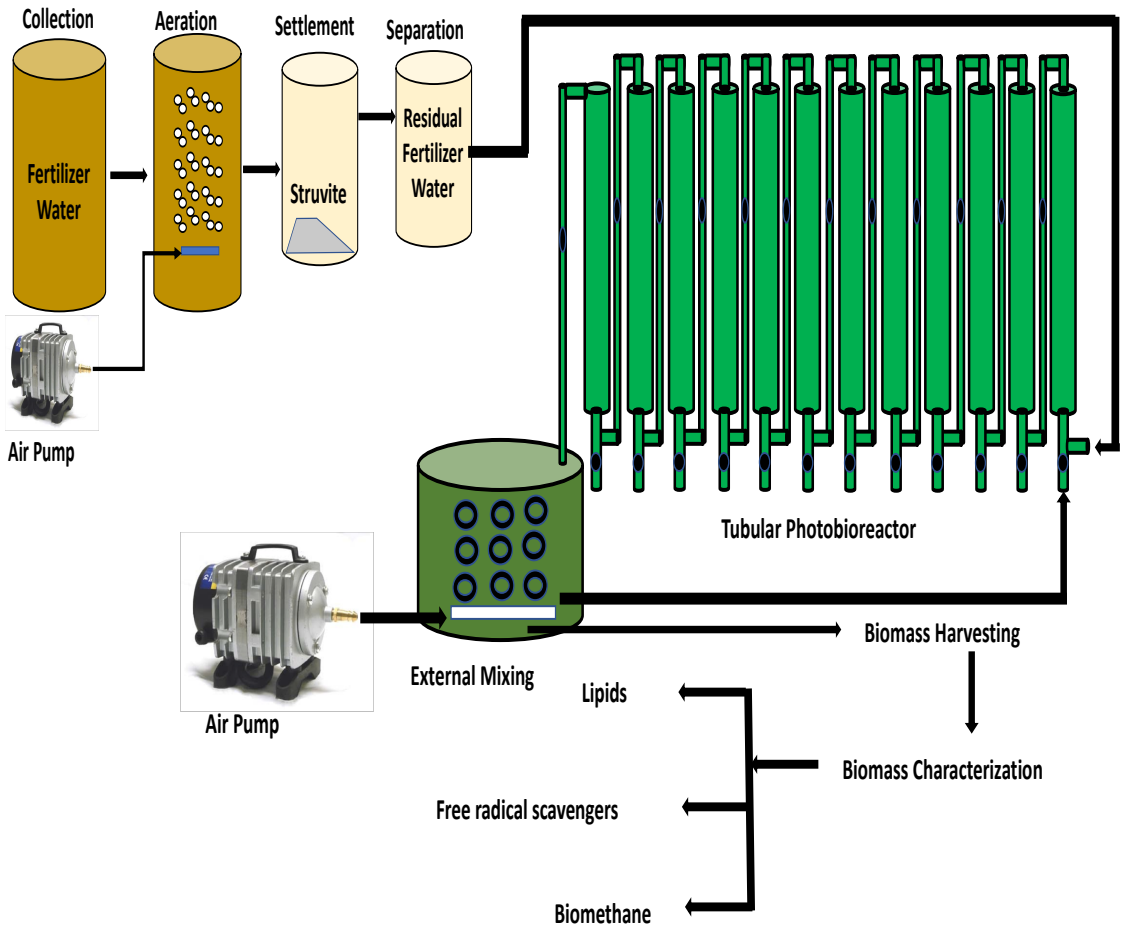


Fig. 5.1 Schematic representation of experimental study.

5.2.2 Ammoniacal nitrogen tolerance studies of BPGC consortium

5.2.2.1 Isolation and Identification of BPGC consortium

The anaerobic digestion slurry was collected from biogas plant situated in BITS Pilani, KK Birla Goa Campus. The anaerobic digester runs on a food waste generated in the campus. Three mL of this slurry was inoculated in 500 mL of Bolds Basal medium. The culture was irradiated with 42 µmol photons/m^2-s by using 40 W fluorescent tubes with 16:8 hours of alternating light/dark photoperiod. After every 8 days, the culture was sub-cultured to fresh medium for 5 passages to obtain algal consortium which was named as BPGC.

The steps followed to identify the composition of BPGC consortium is mentioned below. A minimum number of microorganisms were identified by using t-RFLP. The 1.8 Kb 18s r-DNA fragment was amplified using high fidelity PCR polymerase with 6-carboxyflurescein amidite (FAM). The PCR products were restriction digested with a 4-base cutter HpaII

enzyme. The fluorescent labelled fragments were size separated on ABI 3500xL automated sequencer (Applied Biosystems) using an internal size standard (LIZ-500). t-RFLP electropherograms were analyzed with GeneMapper software version 4.1 (Applied Biosystems). The number of peaks obtained in profiles was an approximate representation of some microalgae present in the sample. The primer sequences used were given below,

Forward primer- 5'- GTAAGCTCGGCGGCCGCGTAGTCATATGCTTGTCTC -3'

Reverse Primer- 5'-GTAAGCTCGGCGGCCGCGAAACCTTGTTACGACTT -3'

The PCR product was cloned at Not I site in the pBlueScript vector. Positive clones were screened by colony PCR. The clones were sequenced bi-directionally using forward and reverse primer. The sequence data were aligned and analyzed to identify the microalga. The followed during identification of composition of BPGC consortium were t-RFLP, PCR, cloning, sequencing and phylogenetic analysis via sequence alignment **(Supplementary material II-Appendix II)**.

5.2.2.2 Batch ammoniacal nitrogen tolerance studies of microalgae

Synthetic Wastewater (SW) was prepared as per the following composition; Sodium carbonate- 2610 mg/L, potassium dihydrogen phosphate- 69.84 mg/L, ammonium chloride- 38.23 g/L and sodium nitrate- 447.46 mg/L. This will produce SW-1000 (NH_4-N concentration is 1000 mg/L) with a final concentration of CO_3^{-2}-1478.26 mg/L, PO_4-P-15.92 mg/L, NH_4-N-1000 mg/L and NO_3-N-172.6 mg/L, respectively. This SW is then further diluted with distilled water to obtain different concentrations (1:10 to 10/10) of NH_4-N. All these ammoniacal nitrogen tolerance studies were carried out at the scale of 200 mL in conical flasks of 500 mL capacity. The pH was adjusted to 8.0. Each flask was then inoculated with 25 mg of freshly harvested algal biomass of BPGC consortium. The flasks were irradiated with 42 µmol photons/m^2-s by using 40 W fluorescent tubes with 16:8 hours of alternating light/dark photoperiod at temperature of 30°C. The negative controls were placed to understand the ammonia stripping at alkaline pH. The samples were removed periodically after every 3 days, centrifuged at 8000 rpm for 15 minutes. The biomass was dried at 40°C overnight in a hot air oven to obtain dry weight. The growth curve was obtained by plotting biomass concentration with respect to time. The growth rate was calculated using following formula,

$$Growth\ rate\ (day^{-1}) = (lnN_2 - lnN_1) \div (t_2 - t_1)$$

Where, N_2 and N_1= biomass concentration at t_2 and t_1, respectively.

5.2.3 Lab scale struvite crystallization

Initial studies for struvite crystallization were performed at 200 mL scale. Two magnesium sources were tested; $MgCl_2.6H_2O$ and $Mg(OH)_2$ (Wu and Bishop 2004). In case of $MgCl_2.6H_2O$ as a source of Mg^{2+}, initially the pH of wastewater was adjusted to 9.0 by using 6N NaOH solution, and different concentrations of $MgCl_2.6H_2O$ (1 % to 5 % W/V) were used (Nelson, Mikkelsen, and Hesterberg 2003). In case of $Mg(OH)_2$ as a supplemental source of Mg^{2+}, there was no necessity of pH adjustment as hydroxyl ions increase the pH. After addition of Mg^{2+} source, the mixtures were stirred using a magnetic stirrer for 60 minutes and the crystals were separated by miracloth filtration (22-25 µm pore size, Merck). The crystalline cake was air dried at room temperature and confirmed for the quality of struvite by X-ray diffraction technique (Prabhu and Mutnuri 2014). The filtrate (Residual Fertilizer Wastewater (RFW)) from each experiment was analyzed for PO_4-P, NH_4-N, TKN, pH, and COD. RFW was then considered for growth studies of BPGC consortium and removal of nutrients were further analyzed. The concentration of $MgCl_2.6H_2O$ (% W/V) that gave a better yield, quality of struvite, better nutrient removal and highest microalgal growth was used in pilot-scale struvite crystallization. For pilot studies, 5 % $MgCl_2.6H_2O$ (% W/V) was used as a source of Mg^{2+}.

5.2.4 Pilot scale struvite crystallization

Pilot-scale struvite precipitation was carried out in 1.3 m^3 polypropylene tank by using 1 m^3 FW. The pH and $MgCl_2$ concentration were selected from the lab scale struvite crystallization experiment. The mixture was aerated by using an air compressor (12 m^3 $hour^{-1}$) for 1 hour followed by 3 hours settling time (Suzuki et al. 2007). The crystallized struvite was then separated, filtered by using nylon cloth, dried in sunlight, subjected for XRD pattern analysis and dry weight determination. The RFW was collected in a tank, analyzed for composition and used as a medium for microalgae growth in Tubular PhotoBioReactor (TPBR). Crystallization efficiency, precipitation efficiency and phosphate conversion was calculated with minor modification as performed by Battistoni et al. (Battistoni et al. 2001). The equations are given below:

$$crystallization\ efficiency\ (\%) = [\![(A - B) \div A]\!] \times 100$$

$$Precipitation\ efficiency\ (\%) = [\![(B - C) \div A]\!] \times 100$$

$$Phosphate - MAP\ conversion\ (\%) = [\![(A - C) \div A]\!] \times 100$$

Where, A-Total PO_4-P in the reactor, B-Total PO_4-P out from the reactor and C-Soluble PO_4-P out from the reactor.

5.2.5 Construction and operation of TPBR

Pilot-scale TPBR was fabricated at BITS Pilani, KK Birla Goa Campus. The reactor consists of 12 tubular acrylic columns connected to each other by UPVC pipe (2.54 cm outer diameter). The acrylic material had 80 % light transmittance efficiency. The dimensions of each column are 10 cm outer diameter, 178 cm height and 3 mm thickness that can carry 13.98 L of RFW as a medium. The total working volume of the reactor is 167.76 L. The medium was aerated externally by using air pump 85 L/hour in a tank (100 L capacity) with 50 L of RFW. The aerated RFW is then recirculated in the reactor (150 L/hour). The reactor was operated at the dilution rate of 37.46 L/day with a hydraulic retention time (HRT) of 6 days. Sampling was done after every three days. The reactor was operated in fed batch mode. The biomass was filtered by using polyester-silk cloth (E.S.Bejor et al. 2013), air dried and analyzed for composition and the treated water was analyzed for pollutants.

5.2.6 Biomass characterization

Air dried biomass was stored in cool and dry place. Total Solids (TS) and Volatile Solids (VS) content were measured as per standard methods for the examination of water and wastewater (Eaton et al. 2005). Total carbohydrates were analyzed by a Phenol-Sulphuric acid method (Dubois et al. 2009). The protein content of dry microalgal biomass was measured by Lowry's method (López, García, and Fernández 2010).

5.2.7 Extraction of Lipids and analysis of fatty acid methyl esters (FAMEs)

Total microalgal lipids were extracted by using a modified Folch's method (Folch, Lees, and Stanley 1957). Ten grams of dried microalgal biomass was extracted in Soxhlet apparatus for 10 hours by using a mixture of Chloroform: Methanol (2:1) followed by washing with 20 mL of 5 % NaCl solution thrice and the solvent was evaporated in rotary vacuum evaporator after phase separation. The lipid content was measured by using the gravimetric method.

Analysis of FAMEs was performed with minor modifications suggested by Prateepchaikul et al (Prateepchaikul, Allen, and Leevijit 2007). Ten mg of the total lipids were transesterified into methyl esters using 2.5 mL of 2 % H_2SO_4-methanol (v/v). The detailed fatty acid methyl esters (FAMEs) analysis was performed using the Agilent 7890-5975 GC–MS system (Agilent Technologies Inc. Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and a DB5 column. The oven temperature ramp program was set at 150°C for 1 min, heated at 2.9°C/min up to 230°C, where it was held for 1 min, with a total run time of 30 min. One microliter of the sample was injected into GC. The individual FAME peaks in the algal

lipid sample were identified based on their retention time comparing it to the retention time of the peaks of known FAMEs in the reference solution (FAME Mix, C8:0–C24:0, Sigma Aldrich).

5.2.8 Anaerobic digestion of microalgal biomass

Biomethanation potential (BMP) assays were conducted for three types of biomass; a) Original Naive Biomass (NB), b) Residual Biomass After Lipid Extraction (RBLE). NB was characterized for TS, VS, ash, carbohydrates and proteins. BMP assays were conducted in duplicates in serum bottles (130 mL capacity) with a working volume of 100 mL. The inoculum for BMP experiment was collected from previously existing biogas plant in campus running on food waste as substrates. The inoculum was carried to the laboratory in a closed container, incubated at 30°C and monitored for gas production. Once gas production ceased, it was used as inoculum to carry out BMP assay with microalgal biomass as substrate. Each bottle was fed with 1 mL micronutrient stock, 1 mL macronutrient stock, 5 mL of 5 % sodium bicarbonate stock, 13 mL distilled water, 80 mL of inoculum and required amount of biomass as per desired loading rate (g VS/L). Two bottles were kept as a negative control to study endogenous biogas production and two bottles for positive control with 1 mL of absolute ethanol to confirm the activity of inoculum. The bottles were sealed and sparged with N_2 gas to make the system anaerobic. The experiments were carried out at loading rate of 1 g VS/L. The biogas volume was measured by using water displacement method (Elaiyaraju and Partha 2014) and quality of gas was analyzed by using gas chromatography (GC) (Prabhu and Mutnuri 2016). In brief, the GC was equipped with a thermal conductivity detector (TCD) and packed stainless steel sphaerocarb column (length-2 m and diameter-1/8). Hydrogen gas was used as a carrier. The temperatures at the injector and detector were maintained at 150 °C and 183 °C respectively. The biogas samples were heated in oven from 60 °C to 120 °C at the rate of 5 °C per minute.

5.2.9 Statistical analysis of data

All the experiments were carried out with two biological replicates and two technical replicates. All the values represent mean value and mean standard error at n = 2. The data was checked for normality and homogeneity of variance. The percentage data was arcsine transformed. One-way ANOVA was carried with treatments as a fixed factor on growth rate and the removal of pH, NH_4-N, TKN, TC, PO_4-P and struvite yield. All statistical analysis

(comparison between treatments and means) was performed using IBM SPSS data analysis software package (IBM-USA).

5.3 Results and Discussion

5.3.1 Ammoniacal Nitrogen tolerance of BPGC Consortium

5.3.1.1 Isolation and identification of BPGC consortium

BPGC was isolated from anaerobic digestate slurry (Table 5.1) from previously existing biogas plant from the campus.

Table 5.1 The composition of Biogas plant digestate

Parameter	Biogas digestate/BMP Inoculum
COD (mg/L)	12540.87 ± 139.98
PO_4-P (mg/L)	479.26 ± 50.58
NH_4-N (mg/L)	1134.56 ± 104.05
TKN (mg/L)	12560.34 ± 109.48
pH	8.23 ± 0.09
TS %	10.29 ± 2.02
VS %	9.28 ± 2.02

(*COD-Chemical Oxygen demand, PO_4-P-Orthophosphates, NH_4-N-Ammoniacal Nitrogen, TKN-total Kjeldahl Nitrogen, TS-Total Solids and VS-Volatile Solids). All the values represent mean value and mean standard error at n = 2.

BPGC consortium was found to contain five major microalgae; ***Chlorella pyrenoidosa*****,** ***Micractinium pusillum*****,** ***Actinastrum hantzschii*****,** ***Micractinium*** **sp. and** ***Chlorella coloniales*****.** The sequences of 18s rDNA were submitted to NCBI GenBank, and their accession numbers are MH121171, MH121172, MH121173, MH121174 and MH121175 respectively.

5.3.1.2 Ammoniacal nitrogen tolerance of BPGC consortium

The results of ammoniacal nitrogen tolerance studies BPGC consortium are depicted in fig. 5.2.

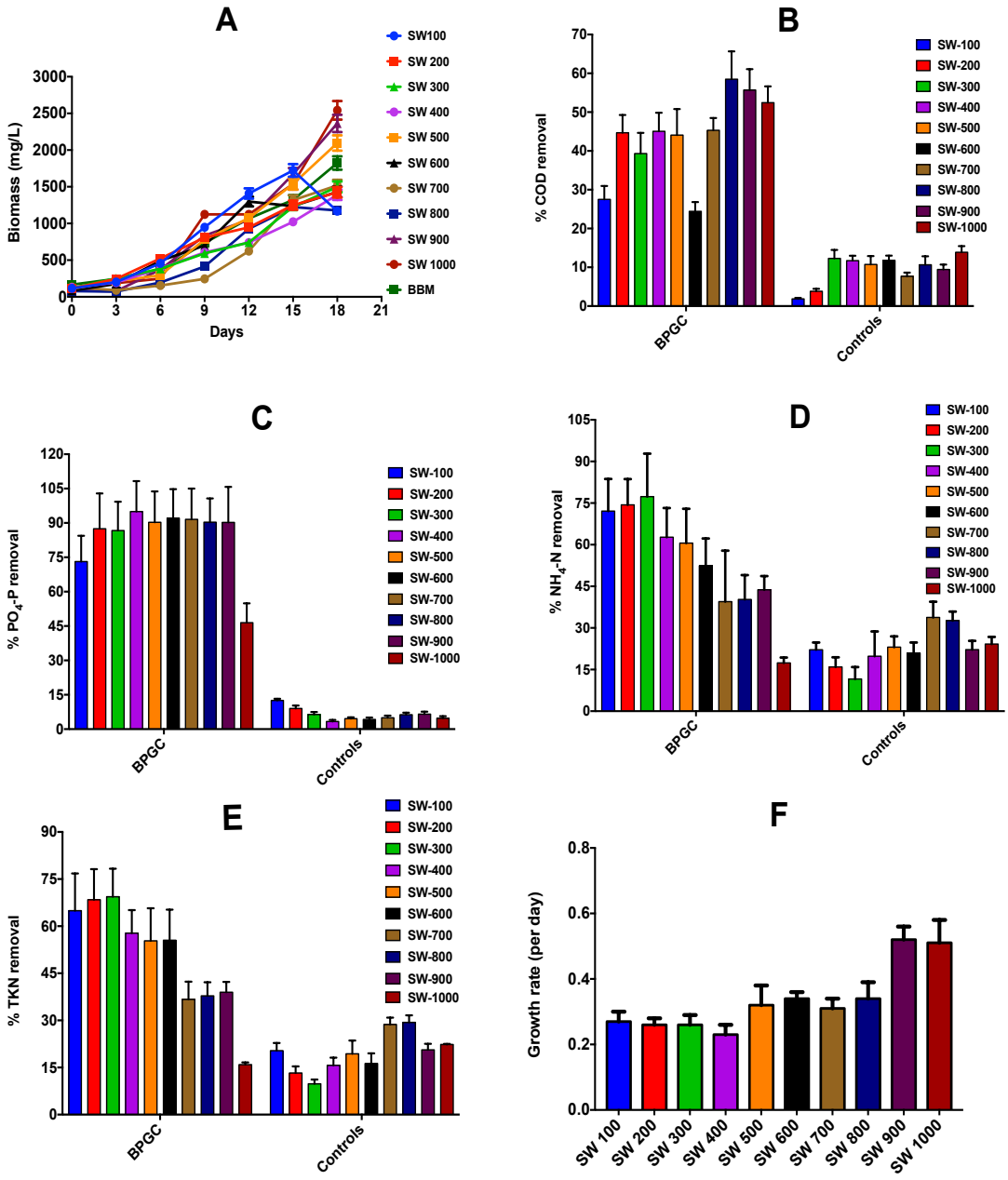


Fig. 5.2 Ammoniacal nitrogen tolerance of studies of BPGC consortium; A-Growth curve of BPGC consortium in SW at different concentrations of NH_4-N, B-E- percentage removal of COD, PO_4-P, NH_4-N and TKN from SW at different concentrations of NH_4-N by BPGC consortium and controls, respectively, F- growth rate of BPGC consortium in SW at different concentrations of NH_4-N.

The observed values of growth parameters in SW-900 were specific growth rate (**0.52/day**) and biomass productivity (**127 mg/L-day**). BPGC showed better growth profile in SW-900 (0.59 M NH_4-N) as compared to SW-1000 (0.6 M NH_4^+) and BBM. This might be due to high ammonia toxicity in SW-1000 and the lack of NH_4-N in BBM. There is no much difference in BPGC consortium growth rate from SW-100 to SW-800 as compared to the growth rate in BBM indicating that it can use NO_3^--N and/or NH_4-N as a source of nitrogen. There is significant difference in BPGC growth rate in SW-900 ($p<0.05$) and SW-1000 ($p<0.05$) as compared to growth rate in BBM. Microalgae usually cannot grow in anaerobic plant digestate because of high ammoniacal nitrogen, high solid content leading to loss of light penetration and presence of an anaerobic atmosphere. High ammoniacal nitrogen beyond 2 mM leads to loss of pigment making the system anaerobic and inhibiting the growth of microalgae (Abeliovich and Azov 1976). BPGC indicated unusual ammoniacal tolerance limit of up to 0.06 M NH_4^+-N which is higher as compared to tolerance of *Chlorella* sp., i.e. 0.03 M NH_4^+-N as observed by Kim et al (Kim et al. 2012). This tolerance may be attributed to its natural adaptation to ammoniacal nitrogen rich anaerobic digestate from where it was isolated.

The ammonia toxicity is attributed to dissolved NH_3 at pH>9 and free NH_4^+ at pH<8 (Collos and Harrison 2014). Previously, Lightfoot et al has induced ammonium tolerance in cyanobacterium *Synechococcus* PCC6301 by expression of the *Escherichia coli* glutamate dehydrogenase gene (Lightfoot, Baron, and Wootton 1988). Esteban et al has described classical and recent hypothesis of ammonium tolerance in plants (Esteban et al. 2016). The classical hypothesis states that the ammonium tolerance in plants might be attributed to the increase in alternative oxidase, increase in NH_4^+ assimilation, decrease in sensitivity to external pH acidification and increase in respiration rates. However, according to recent hypothesis, plants can tolerate high NH_4^+ by increasing facilitated diffusion of NH_3, increased regulation of non-orthodox aquaporins, increase in the expression of GDP mannose-pyrophosphorylase, increase in nitrate and auxin signaling. It is very likely that at least one or more in combination of above-mentioned ammonium tolerance hypothesis is responsible for ammonium tolerance of BPGC consortium. Compositional identification of BPGC consortium shows that it consists of microalga from class Chlorophyceae. Previously it was stated by Collos and Harrison that the alga from class Chlorophyceae has high ammoniacal nitrogen tolerance (up to 39000 uM) than alga from class Cyanophyceae (13000 uM), Dinophyceae (3600 uM), Diatomophyceae (2500 uM) and Raphidophyceae (1200 uM) (Collos and Harrison 2014).

It was observed that BPGC was effective in treating **SW-900** compared to other synthetic wastewater combination with reference to all parameters such as COD, NH_4-N, TKN and PO_4-P. In SW-900, the observed removal rates (mg/L-day) for COD, NH_4-N, TKN, and PO_4-P were found to be 1.73, 34.85, 22.34 and 0.87 respectively. To study the intrinsic removal of COD, NH_4-N, TKN and PO_4-P by BPGC alone and stripping effect of NH_4-N at high pH, negative controls without BPGC were also kept for each of medium combinations from SW100 to SW1000. It was observed that in most of the cases all these parameters were reduced over a period of time, but the reduction was not significant, except for NH_4-N and TKN. BPGC indicated maximum COD removal in SW800 and SW900. i.e. 69.14 % and 65.11 % respectively and the lowest in SW100 i.e. 27.28 %. These results indicate that COD removal from SW is growth dependent. The observations of COD removal are depicted in Fig.5.2B. There is no significant difference in NH_4-N stripping in controls carrying SW700, SW800, and SW900 but the average removal via stripping was found to be 29.53 %. In SW900, BPGC indicated 43.73 % removal of NH_4-N, which is less than 72.11 %, 74.38 %, 77.33 %, 62.70 % and 60.59 % NH_4-N removal in SW100, SW200, SW300, SW500 respectively. This observation indicated that BPGC can remove NH_4-N efficiently up to 500 mg/L of NH_4-N and at higher concentration the wastewater needs to be diluted with other clean water to achieve higher removal efficiency. So, compromise should me made either to use clean water to dilute the wastewater or to stay with less NH_4-N removal.

The decrease in TKN was found to be due to decrease in NH_4-N. This might be due to use of NH_4-N as the preferred source of nitrogen and the selection pressure imposed by high NH_4-N concentration present in its natural habitat. The maximum removal of TKN was found in SW300, i.e. 69.39 %, and the lowest was found in SW1000. Average TKN removal from SW300 to SW900 was observed to be 47.02 %. There is no significant loss in PO_4-P by controls indicating that PO_4-P removal was exclusively dependent on BPGC growth. There is no significant difference in % PO_4-P removal by BPGC from SW200 to SW900 with an average PO_4-P removal of 90.44 %. As expected, the lowest PO_4-P removal was observed in SW1000 corresponds to 46.45 % due to slow growth of BPGC.

5.3.2 Lab scale struvite crystallization from FW

XRD patterns of standard struvite and struvite obtained by using different concentrations of $MgCl_2$ and $Mg(OH)_2$ are depicted in Fig.5.3.

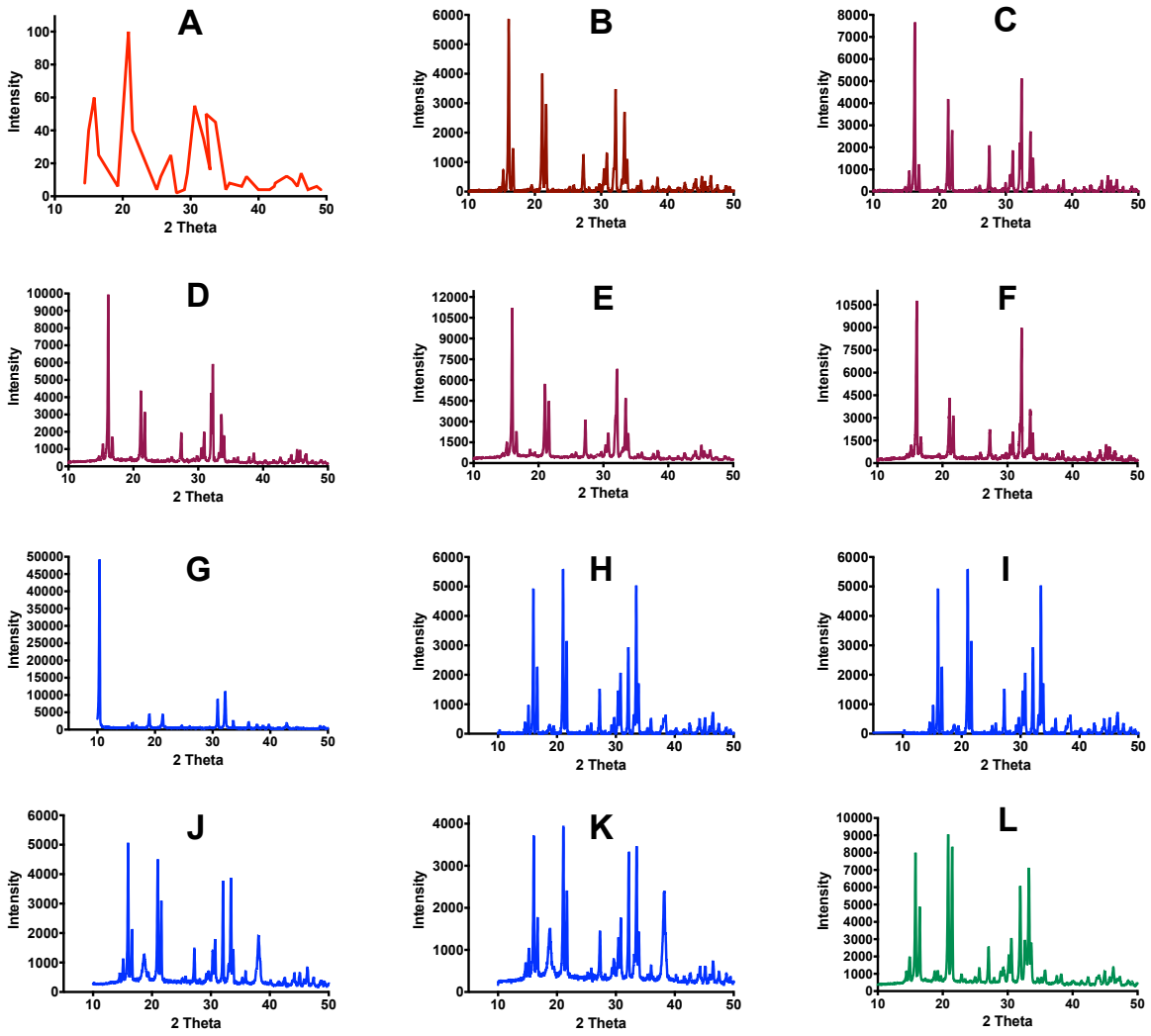


Fig. 5.3 Comparison of XRD patterns of standard struvite, struvite produced from FW at different concentrations of $MgCl_2$ and $Mg(OH)_2$ and struvite produced at pilot scale; A. Standard struvite. B, C, D, E and F-XRD pattern of struvite synthesized using 1%, 2%, 3%, 4% and 5% $MgCl_2$ respectively. G, H, I, J and K- XRD pattern of struvite synthesized using 1%, 2%, 3%, 4% and 5% $Mg(OH)_2$. L-XRD pattern of struvite produced from Fertilizer industry Wastewater (FW) using 5 % $MgCl_2$ at pilot scale.

FW was found to be rich in NH_4-N, PO_4-P, TKN and COD (Table 5.4). Due to toxic effects of high concentrations of NH_4-N, it cannot be directly used for the cultivation of BPGC microalgae. Therefore, it was necessary to reduce NH_4-N to the level of 1000 mg/L. The removal of NH_4-N can be carried out traditionally by using adsorption to clays and zeolites, wherein it can possibly remove the NH_4-N hindering nutrient recovery (Rožić et al. 2000). Another way that can efficiently remove NH_4-N, TKN, and PO_4-P in the same reaction step is

to precipitate in the form of struvite. Struvite can be easily separated and can be used as a slow release fertilizer and the residual water can be used for microalgal cultivation.

The XRD pattern of struvite synthesized by using $MgCl_2$ and $Mg(OH)_2$ confirmed that the product crystallized was struvite. The effective pH for better quality of struvite crystals was observed to be 9.0 to 9.6 (Prabhu and Mutnuri 2014). The struvite synthesized using $MgCl_2$ and $Mg(OH)_2$ was found to be crystalline and amorphous in nature, respectively. The removal of NH_4-N, TKN, PO_4-P and struvite yield increased with increase in the concentration of both $MgCl_2$ and $Mg(OH)_2$. In case of 5 % $Mg(OH)_2$, maximum struvite yield (91.05 g/L) was obtained from FW which is high as compared to maximum struvite yield (61.63 g/L) obtained by using 5 % $MgCl_2$. The struvite formed by using $Mg(OH)_2$ was slimy in texture, creating problems in filtration, drying and it can precipitate nonspecific ions as well, so in further studies, use of 5 % $MgCl_2$ was preferred over 5 % $Mg(OH)_2$.

Maximum nutrient removal in the form of struvite was observed in case of 5 % $Mg(OH)_2$ followed by 5 % $MgCl_2$. In case of 5 % $MgCl_2$, observed % removal of TKN, NH_4-N, PO_4-P and struvite yield were 58.33, 64.46, 96.44 and 61.63 g/L respectively. In case of 5 % $Mg(OH)_2$, observed the % removal of TKN, NH_4-N, PO_4-P and struvite yield were 74.81, 77.54, 99.98 and 91.05 g/L respectively.

The results of pH variation, TKN, NH_4-N, PO_4-P removal during struvite crystallization from FW are shown in Fig. 5.4A-5.4D respectively. The results of struvite yield and % nutrient removal in terms of TKN, NH_4-N, PO_4-P using different concentrations of $MgCl_2$ and $Mg(OH)_2$ is depicted in Fig.5.4E-5.4F.

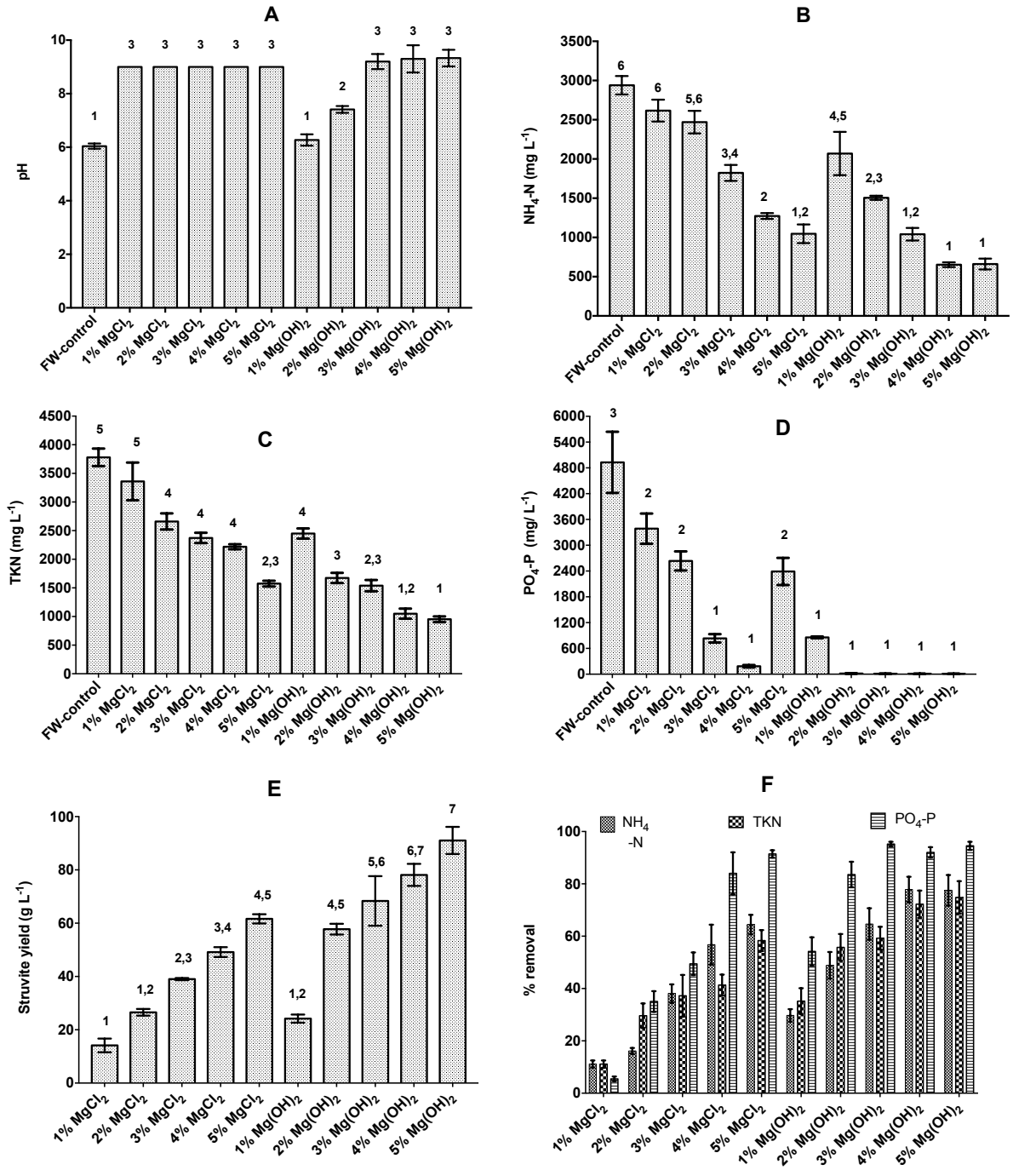


Fig. 5.4 Nutrient removal from Fertilizer industry Wastewater (FW) during struvite crystallization by using different concentrations of $MgCl_2$ and $Mg(OH)_2$.

A - pH of Residual Ferilizer industry Wastewater (RFW) compared to FW, B - Residual NH_4-N in RFW after struvite crystallization, C – Residual TKN in RFW after struvite crystallization, D – Residual PO_4-P in RFW after struvite crystallization, E - Struvite yield (g /L), F – Removal (%) of TKN, NH_4-N and PO_4-P from FW during struvite crystallization using different concentrations of $MgCl_2$ and $Mg(OH)_2$. PO_4-P-Orthophosphates, NH_4-N-

Ammoniacal Nitrogen and TKN-Total Kjeldahl Nitrogen. The numbers above the bars denote significant groups ($p<0.05$) based on Tukey's post hoc tests. All the values represent mean value and mean standard error at n = 2.

5.3.3 Use of RFW for BPGC consortium cultivation

Comparison of nutrient removal from FW and RFW during struvite crystallization BPGC consortium, respectively at different concentrations of $MgCl_2$ and $Mg(OH)_2$ is depicted in Fig.5.5.

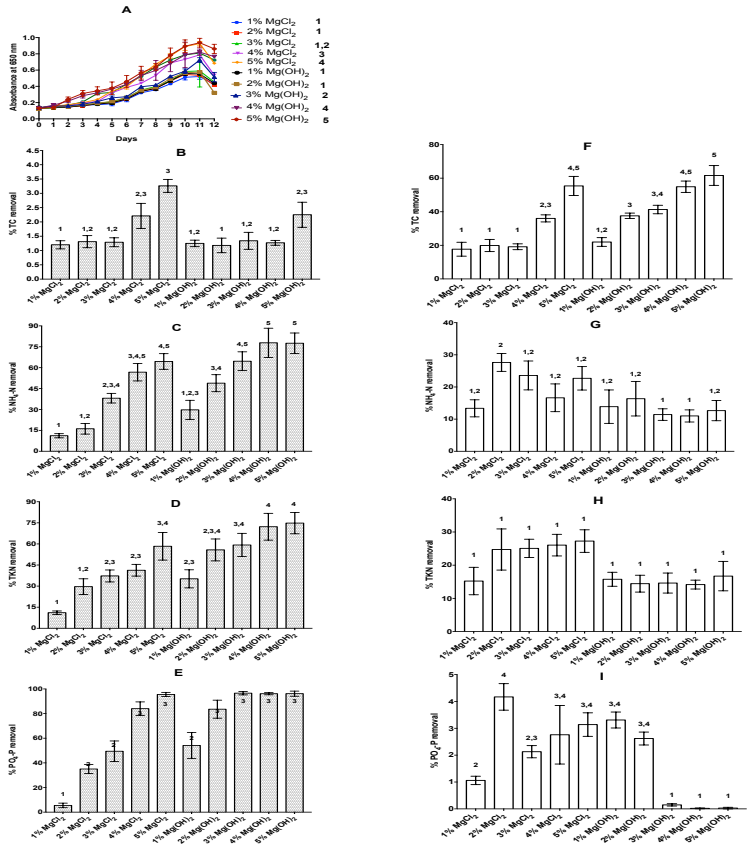


Fig. 5.5 Comparison of nutrient removal from Fertilizer industry Wastewater (FW) and Residual Fertilizer industry Wastewater (RFW) during struvite crystallization and by BPGC consortium, respectively at different concentrations of $MgCl_2$ and $Mg(OH)_2$

A - Growth curve of BPGC consortium in RFW after struvite crystallization; B,C,D and E – Removal (%) of TC, NH_4-N, TKN and PO_4-P during struvite crystallization; F,G,H and I- Removal (%) of TC, NH_4-N, TKN and PO_4-P by BPGC consortium. PO_4-P-Orthophosphates, NH_4-N-Ammoniacal Nitrogen and TKN-Total Kjeldahl Nitrogen. The numbers above the bars denote significant groups ($p<0.05$) based on Tukey's post hoc tests. All the values represent mean value and mean standard error at n = 2.

The pH of FW was dropped to 7.2 ± 0.6 after struvite crystallization, and no pH adjustment was performed further. The residual filtrate after struvite crystallization in each experiment was used as a medium for BPGC microalgae cultivation, and the respective growth patterns were depicted in Fig.5.5A. It was observed that BPGC can grow well in all RFW, but the growth is better in RFW after struvite crystallization using 5 % $MgCl_2$ and 5 % Mg $(OH)_2$. The growth parameters of BPGC and initial TKN: the PO_4-P ratio of RFW are mentioned in Table 6.2. There is significant difference in growth rate and doubling time of BPGC consortium in RFW after struvite crystallization using 5 % $MgCl_2$, 4 % $Mg(OH)_2$ and 5 % $Mg(OH)_2$ as compared to growth in BBM medium. The maximum growth rate (per day) of BPGC was observed in RFW using 5 % $Mg(OH)_2$ followed by RFW using 5 % $MgCl_2$, i.e. 0.44 and 0.37, respectively at TKN: PO_4-P ratios of 90.84 and 8.5 respectively. The results are not in exact agreement with Xin et al with *Scenedesmus* sp. at different N: P ratios (Xin et al. 2010). They have observed a growth rate of 0.81 and 0.26 at N/P ratio of 8 and 100, respectively. These deviations might be attributed to the higher initial concentrations of TKN and PO_4-P in our experiments. At high N/P ratio the growth rate was high as compared to low N: P ratio, indicating that PO_4-P is not acting as a limiting nutrient and BPGC can tolerate high amount TKN and or NH_4-N in a given range of our experiments.

Better % COD removal was observed in RFW with struvite crystallization using $Mg(OH)_2$ as compared to % COD removal observed in RFW with struvite crystallization using $MgCl_2$. The highest COD removal (81.09 %) was observed in RFW with struvite crystallization using 5 % $Mg(OH)_2$. In case of $MgCl_2$, highest COD removal of 76.63 % was observed in RFW with struvite crystallization using 5 % $MgCl_2$ followed by 70.53 % COD removal in RFW with struvite crystallization using 4 % $MgCl_2$. These removal rates are in agreement with COD removal rates observed by Zu et al in the treatment of piggery wastewater by using *Chlorella zofingiensis* at respective initial COD concentration. It was also observed that high N/P ratio increases COD removal.

TKN removal of 66.37 % was observed in RFW with struvite crystallization using 5 % $Mg(OH)_2$. In the case of $MgCl_2$, highest 65.45 % TKN removal was observed in RFW with struvite crystallization using 5 % $MgCl_2$. The highest % NH_4-N removal of 63.76 % was observed in RFW with struvite crystallization using 5 % $MgCl_2$. In case of $Mg(OH)_2$, highest NH_4-N removal of 56.22 % was observed in RFW with struvite crystallization using 5 % $Mg(OH)_2$.

The highest PO_4-P removal of 88.41 % was observed in RFW with struvite crystallization using 5 % $MgCl_2$. In case of $Mg(OH)_2$, highest PO_4-P removal of 87.18 % was observed in

RFW with struvite crystallization using 5 % $Mg(OH)_2$. Krenz et al observed that, at high N:P ratios, P will act as limiting nutrient and removal of P will be more as compared to N (Krenz Iii et al. 2009). This was found to be true in our experiments as well.

The comparison of TC, NH_4-N, TKN and PO_4-P removal by BPGC consortium and after struvite crystallization is depicted in Fig.5.4B-5.4I respectively. As expected, microalgae play a major role in TC removal. However, removal of TC after struvite crystallization is not significant. In contrast, removal of NH_4-N, TKN, and PO_4-P were significant after struvite crystallization as compared to removal of these nutrients by BPGC consortium.

Growth parameters of BPGC consortium in RFW at lab scale is shown in Table 5.2.

Table 5.2 Growth kinetic parameters of BPGC consortium in RFW after struvite crystallization at lab scale

*RFW	Growth Rate (per day)	Biomass Productivity (mg/L-day)	*TKN/PO_4-P ratio
1 % $MgCl_2$	0.2±0.01	33.28±1.1	0.68±0.01
2 % $MgCl_2$	0.19±0.02	33.62±1.03	0.79±0.2
3 % $MgCl_2$	0.22±0.03	39.38±3.79	0.90±0.19
4 % $MgCl_2$	0.35±0.03	121.69±11.01	2.66±0.18
5 % $MgCl_2$	0.37±0.04	145.66±5.88	8.50±1.3
1 % $Mg(OH)_2$	0.2±0.02	34.89±0.93	1.02±0.39
2 % $Mg(OH)_2$	0.22±0.01	38.45±4.51	1.95±0.04
3 % $Mg(OH)_2$	0.24±0.00	49.80±6.5	94.36±2.0
4 % $Mg(OH)_2$	0.36±0.02	127.79±7.1	93.50±4.07
5 % $Mg(OH)_2$	0.44±0.04	160.39±3.5	90.84±1.95

* RFW-Residual Fertilizer industry Wastewater, PO_4-P-Orthophosphates, TKN-total Kjeldahl Nitrogen. (All the values represent mean value and mean standard error at n = 4).

The % removal of COD, TKN, NH_4-N, PO_4-P from RFW using BPGC is shown in Table 5.3.

Table 5.3 lab scale nutrient removal from RFW using BPGC consortium

RFW	% COD removal	% TKN removal	% NH_4-N removal	% PO_4-P removal
1 % $MgCl_2$	13.30 ± 1.20	17.13 ± 1.0	15.03 ± 2.31	1.13 ± 1.0
2 % $MgCl_2$	20.85 ± 3.14	35.14 ± 2.99	32.91 ± 2.49	6.43 ± 2.2
3 % $MgCl_2$	25.86 ± 4.12	39.93 ± 4.05	38.06 ± 2.96	4.22 ± 1.1
4 % $MgCl_2$	70.53 ± 6.11	44.38 ± 3.26	38.48 ± 3.87	17.25 ± 2.02
5 % $MgCl_2$	76.63 ± 3.12	65.45 ± 5.21	63.76 ± 1.89	88.41 ± 4.81
1 % $Mg(OH)_2$	16.30 ± 4.7	24.32 ± 1.82	19.72 ± 3.04	7.22 ± 1.08
2 % $Mg(OH)_2$	25.32 ± 1.17	32.62 ± 2.79	31.95 ± 3.95	15.96 ± 2.02
3 % $Mg(OH)_2$	42.01± 2.01	35.88 ± 3.09	32.28 ± 5.85	48.90 ± 3.98
4 % $Mg(OH)_2$	55.13 ± 3.0	50.98 ± 4.01	49.54 ± 3.08	48.96 ± 4.91
5 % $Mg(OH)_2$	81.09 ± 4.03	66.37 ± 2.14	56.22 ± 2.41	87.18 ± 3.09

RFW-Residual Fertilizer industry Wastewater, COD-Chemical Oxygen demand, PO_4-P-Orthophosphates, NH_4-N-Ammoniacal Nitrogen, TKN-total Kjeldahl Nitrogen. (All the values represent mean value and mean standard error at n = 2).

5.3.4 Pilot scale struvite synthesis

FW had acidic pH. As observed in lab scale struvite crystallization studies, pilot-scale struvite crystallization was carried out by adjusting pH to 9.0 with NaOH which was followed by the addition of 5 % $MgCl_2$. $MgCl_2$ was selected over $Mg(OH)_2$ to reduce the cost of struvite being produced. XRD pattern of struvite crystallized from FW at pilot scale was found to be identical to the XRD pattern of struvite observed in lab scale studies using 5 % $MgCl_2$. The XRD pattern of struvite crystallized from FW at pilot scale was depicted in Fig.5.3L and was confirmed to be struvite by comparing with XRD pattern of synthetic struvite ICDD card no. 15-0762. The elemental composition of FW used for struvite crystallization and RFW is given in Table 5.4.

Table 5.4 Characteristics of FW (Fertilizer industry Water), RFW (Residual Fertilizer industry Water) and % removal of nutrient during pilot-scale struvite crystallization by using 5 % $MgCl_2$.

Parameter (mg/L)	FW	RFW	% removal during struvite
COD	1157.39 ±125.68	1123.29 ± 223.45	2.95 ± 0.56
NH_4-N	2940.10 ± 135.42	932.26 ± 89.67	68.29 ±23.45
TKN	3782.39 ± 224.7	1123.79 ± 179.41	70.17 ±3.47
PO_4-P	5116.39 ± 234.56	185.43 ± 12.34	96.38 ± 3.43
pH (unit)	6.04 ± 0.05	7.20 ± 0.2	

COD-Chemical Oxygen demand, PO_4-P-Orthophosphates, NH_4-N-Ammoniacal Nitrogen and TKN-total Kjeldahl Nitrogen. (All the values represent mean value and mean standard error at n = 2)

The yield of struvite obtained was **60.43 Kg/m^3**. The observed crystallization efficiency, precipitation efficiency and Phosphate to MAP conversion efficiency were found to be 96.38 %, 0.12 %, and 96.49 % respectively. These results are not in agreement with Battistoni et al (Battistoni et al. 2001). Battistoni et al. has observed crystallization efficiency (65 %), precipitation efficiency (11 %) and Phosphate to MAP conversion efficiency (100 %). The mass (mg/L) ratio PO_4-P: Mg used in our study was 1.24 as compared to 1.21 in an experiment carried out by Battistoni in experiment no. 3, set H. Another reason for this could be an initial load of nutrients in anaerobic supernatant used by Battistoni et al and FW in our study. In the experiment carried out by Battistoni et al, the mass (mg/L) ratio of Mg: NH_4-N: PO_4-P was 1:16.23:1.21 as compared to 1:0.3:1.24 in our study.

The results of nutrient removal from FW during struvite crystallization were depicted in Table 5.4. **It was observed that, after struvite crystallization, Percentage removal of COD, NH_4-N, TKN, and PO_4-P were 2.95 %, 68.29 %,70.17 % and 96.38 % respectively.** Removal of PO_4-P is in agreement with the results obtained by Jaffer et al. during struvite production in sewage treatment plant by using centrifugal liquor as substrate (Jaffer et al. 2002). The % removal of NH_4-N during struvite crystallization is in agreement

with % NH_4-N removal of 65 % as observed by Altinbas et al from the biologically treated opium alkaloid wastewater (Altinbas, Ozturk, and Aydin 2002).

5.3.5 Nutrient removal from RFW by BPGC consortium using TPBR

The results of BPGC growth, COD, NH_4-N, TKN, PO_4-P removal from RFW by BPGC consortium using TPBR are depicted in Fig.5.6A-5.6E respectively.

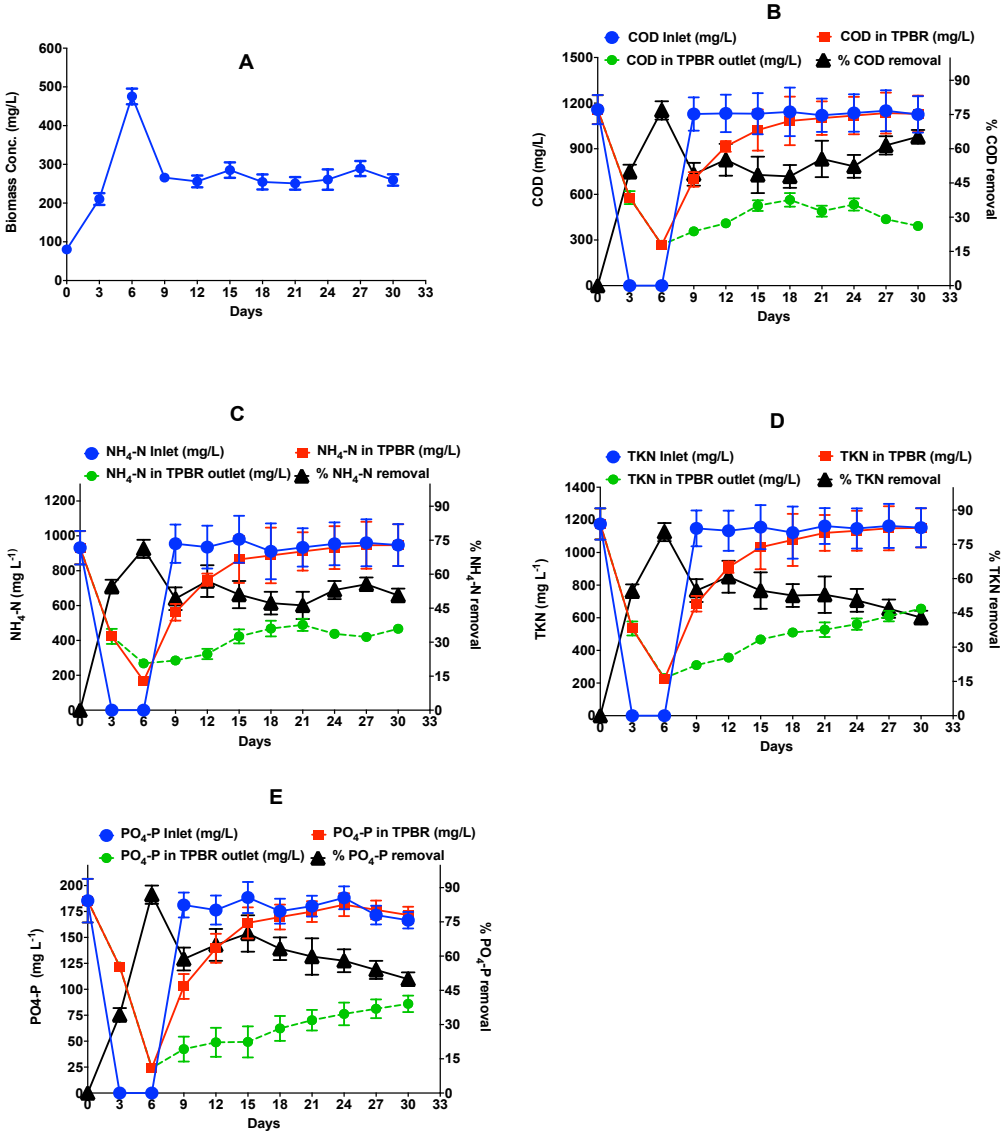


Fig. 5.6 Results of growth and nutrient removal from RFW (Fertilizer industry Wastewater after struvite crystallization by using 5 % $MgCl_2$) by using BPGC in TBPR (Tubular Photobioreactor); A - Growth curve of BPGC consortium in RFW, B - COD removal, C - NH_4-N removal, D - TKN removal, E - PO_4-P removal. (COD-Chemical Oxygen demand, PO_4-P-Orthophosphates, NH_4-N-Ammoniacal Nitrogen and TKN-Total Kjeldahl Nitrogen). (All the values represent mean value and mean standard error at n = 2).

The biomass yield was observed to be **290 mg/L**. It was observed that after 6 days of batch cultivation, **BPGC has removed 76.81 % of COD, 71.21 % of NH_4-N, 80.39 % of TKN and 86.9 % of PO_4-P.** After 6 days, TPBR was operated in a fed batch mode and it was observed that the percentage removal of all these nutrients was fairly constant. The total mass balance of these nutrients during 30 days of operation was described in Table 5.5. BPGC has removed 713.34 g (62.68 %) COD with a removal rate of 17.83 mg/L-day. During 30 days of cultivation, 559.15 g (59.21 %) of NH_4-N was removed with a removal rate of 13.98 mg/L-day. Total TKN removal was observed to be 61.34 % with the removal of 707.5 g TKN with a removal rate of 11.79 mg/L-day. Total PO_4-P removal was observed to be 68.57 % with the removal of 123.36 g PO_4-P with a removal rate of 20.56 mg/L-day. The percentage removal (TKN = 83-99 % and PO_4-P = 99%) is not in agreement with the percentage removal observed by Xin et al while using *Chlorella* Sp. in the treatment of municipal wastewater (Xin et al. 2010). These deviations might be due to high retention time (14 days) as used by Li et al against 6 days in our study and the composition of wastewater as well; RFW has NH_4-N:PO_4-P: COD ratio (mass basis, mg/L) of 5.03:1:6.05 as compared to 1:2.57:27.92 in their study. However, these removal efficiencies are well in agreement as observed by Ruiz-Marin et al in the treatment of urban wastewater by using *Chlorella vulgaris* (Ruiz-Marin, Mendoza-Espinosa, and Stephenson 2010).

The mass balance of Nutrient removal by BPGC consortium from RFW using TPBR is depicted in Table.5.5

Table 5.5 Nutrient removal by BPGC consortium from RFW using tubular photobioreactor

Parameter	COD	NH_4-N	TKN	PO_4-P
Total Inlet (g)	1138.12 ± 80.57	944.32 ± 65.90	1153.52 ± 105.47	179.90 ± 20.45
Total Outlet (g)	424.79 ± 23.57	385.17 ± 27.83	445.97 ± 31.45	56.55 ± 5.92
Total recovered (g)	713.34 ± 40.49	559.15 ± 42.16	707.55 ± 42.36	123.36 ± 12.45
Recovery Rate (g/day)	3.57 ± 0.25	2.80 ± 0.51	23.58 ± 3.62	4.11 ± 1.21
% Recovery	**62.68 ± 6.71**	**59.21 ± 4.69**	**61.34 ± 8.37**	**68.57 ± 7.34**
Recovery rate (mg/L-day)	17.83 ± 2.59	13.98 ± 1.53	11.79 ± 1.94	20.56 ± 4.51

COD-Chemical Oxygen demand, PO_4-P-Orthophosphates, NH_4-N-Ammoniacal Nitrogen, and TKN-total Kjeldahl Nitrogen. (All the values represent mean value and mean standard error at n = 2).

5.3.6 Biomass analysis

BPGC consortium biomass was observed to contain 35.63 % of proteins, 39.65 % of carbohydrate, and 19.47 % of lipids. These results are in close proximity with composition of proteins in *C.vulgaris* biomass as observed by Ahmad et al but not for carbohydrate and lipids (Ahmad, Khan, and Yasar 2013). Ahmad et al observed that *C. vulgaris* biomass contains 38.56 % proteins, 15.33 % carbohydrates, 42.53 % lipids. These deviations might be due to presence of high NH_4-N in wastewater in our study as compared to nitrate in their study. Nitrogen limitation enhances lipid accumulation in microalgae (Zhu et al. 2013).
Table 5.6 describes the composition of BPGC consortium biomass

Table 5.6 BPGC consortium biomass characterization

Properties	% dry weight
Moisture	6.8 ± 0.11
TS	92.82 ± 3.52
VS	85.39 ± 4.68
Ash	14.61 ± 0.67
Proteins	**35.63 ± 2.31**
Carbohydrates	**39.65 ± 5.31**
Lipids	**19.47 ±2.39**

TS-Total Solids and VS-Volatile Solids. (All the values represent mean value and mean standard error at n = 4).

5.3.7 Analysis of FAMEs

FAMEs composition of BPGC consortium biomass cultivated in RFW is depicted in Table 5.7.

Table 5.7 FAMEs composition of BPGC consortium biomass cultivated in Residual Fertilizer Water (RFW) in Tubular Photobioreactor (TPBR).

Type	Fatty acid	% Crude lipid	% FAME	% Biomass	S:UN ratio
C16:2	7,10-Hexadecadienoic acid***	4.40±0.31	4.04±0.11	0.79±0.07	0.57±0.01
C16:3	7,10,13-Hexadecatrienoic acid***	12.25±1.23	11.25±0.87	2.19±0.26	
C16:1	9-Hexadecenoic acid**	5.45±0.12	5.01±0.91	0.97±0.05	
C16:0	Hexadecanoic acid*	33.52±2.31	30.79±3.28	6.00±0.65	
C16:2	8,11-Octadecadienoic acid***	16.20±1.26	14.88±1.83	2.90±0.21	
C18:3	9,12,15-Octadecatrienoic acid***, #	20.04±3.41	18.41±2.68	3.58±0.82	

*-Saturated fatty acid, **-monounsaturated fatty acid, ***-polyunsaturated fatty acid, #-essential fatty acid. S-Saturated fatty acids, UN-Unsaturated fatty acids (All the values represent mean value and mean standard error at n = 4).

BPGC consortium was found to contain C16:0 in 30.79 % of total FAMEs and 6 % of biomass dry weight. The polyunsaturated FAME portion consists of C16:2, C16:3, C16:1 and C18:3. The saturated to unsaturated fatty acid ratio was observed to be 0.57. The ratio of saturated to unsaturated fatty acids helps in determination of intrinsic oxidative stability of biofuels. Higher the unsaturation; less is the stability (Hoekman et al. 2012). The FAME portion is expected to have less oxidation stability. However, C18:3 (alpha-linolenic acid-an essential fatty acid) is present in abundance (3.58 % dry weight). The culture growth phase and the nutrient condition affects the ratio of saturated and unsaturated fatty acid in *Nanochloropsis oculata*; saturated and unsaturated fatty acid tends to increase in stationary phase (Huerlimann, Heimann, and Nys 2010). In our study, the ratio of saturated to unsaturated fatty acids was less (0.57) due to harvesting of the cultures in an exponential growth phase. The saturated fatty acid portion can also be increased by using organic carbon sources. However, the observed FAME composition is in agreement to observations of Schenk et al (Schenk et al. 2008). They have observed that at low C: N ratio, the proportion of tri-enoic fatty acids increases at the expense of mono-enoic acid in heterotrophic cultivation *Chlorella sorokiniana*. A good quality of biodiesel should have 5:4:1 mass ratio

of C16:1, C18:1 and C14:1 respectively as suggested by Schenk et al (Schenk et al. 2008). In our study, C18:1 and C14:1 were not present, this might be due to absence of organic carbon in the RFW. Their synthesis can be stimulated by adding 100 mM Sodium bicarbonate or 5 mM glucose during culture conditions, as observed by Cho et al in cultivation of *Chlorella* sp. 227 in presence of different carbon sources (Cho et al. 2011).

5.3.8 Anaerobic digestion of BPGC consortium biomass

The results of Biomethanation potential of BPGC consortium biomass is depicted in Fig. 5.7

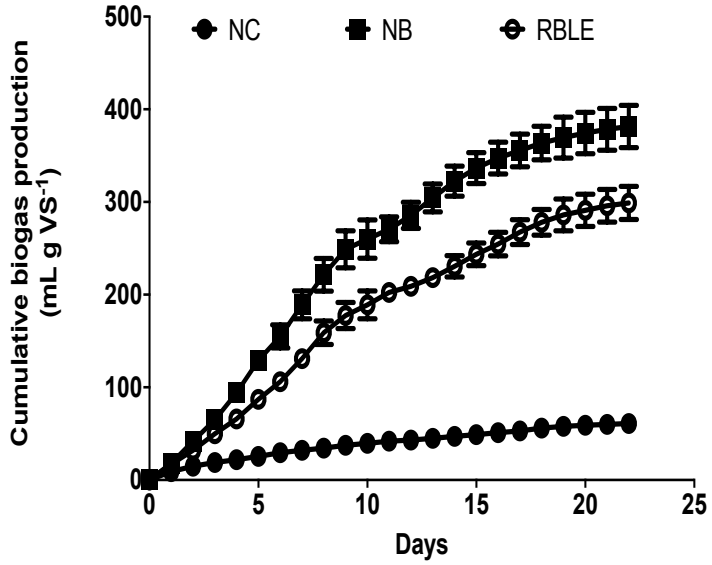


Fig. 5.7 Biomethanation potential (BMP) assay of BPGC consortium biomass; Cumulative biogas production from BPGC consortium biomass; NC- Negative control, NB-Naive Biomass, RBLE-Residual Biomass after Lipid Extraction. All the values represent mean value and mean standard error at n = 2.

BMP assays were performed at a loading rate of 1 g VS/L using two types of biomass, i.e. NB, and RBLE. Negative control produced 61 mL of biogas with 48.99 % methane and methane yield of 28.99 mL g/VS. **NB** produced **320.5 mL** of biogas with an average methane content of 62.32 % corresponding to 199.74 mL of methane. **RBLE** produced **238.0** mL of biogas with an average methane content of 59.32 % corresponding to 141.18 mL of methane. It was observed that methane yield decreases while using RBLE biomass by 29.32 % as compared to NB biomass. This decrease might be attributed to the loss of some simple sugars and fatty acids during extraction of total lipids leading to residual biomass rich in complex sugars like cellulose. The yield of methane (199.74 mL g/VS) in NB biomass is less as compared to the methane yield (320 mL g/VS) obtained from *Spirulina* sp. as observed by

Chen et al (Chen 1987).The corresponding decrease might be due to the presence of 35.65 % proteins in NB biomass. During anaerobic digestion, proteins release a large quantity of ammonia inhibiting the methanogens and failing the anaerobic digestion process. The possible strategy to minimize the inhibitory effect of ammonia is to co-digest the microalgal biomass with nitrogen poor substrate with high C: N ratio (Heaven, Milledge, and Zhang 2011).

5.4 Conclusion

Direct wastewater treatment of ammoniacal nitrogen rich wastewater from fertilizer industries by conventional biological systems will not be cost effective. So, an integrated approach focusing towards nutrient recovery and biomass generation will be beneficial. Our study show the potential of struvite precipitation and microalgal production in treating the fertilizer industry wastewater. The consortium isolated from biogas plant digestate (BPGC consortium) can tolerate up to 1000 mg/L of NH_4-N. These individual microalga or microalgae consortium can be selected to treat variety of wastewater depending on the initial composition of wastewater. Ammoniacal nitrogen exerts high toxic effects on microalgal system limiting their treatment capabilities. Therefore, struvite crystallization is necessary to reduce the toxicity of ammoniacal nitrogen on microalgae. It serves as an added advantage in production of slow release fertilizer. The microalgal biomass is a value added product as it is rich in proteins, lipids, and carbohydrates. Combining struvite production and microalgal cultivation with wastewater treatment will help fertilizer industries to earn extra revenue thus making wastewater treatment process cost effective.

Summary of Results and Conclusion

Summary of results and conclusion

Wastewater can be classified in two groups depending on the source of generation; Domestic Wastewater (DW) and industrial wastewater/effluents. As per the reports published by AQUA-STAT and Sato et al (2013), globally, 90,000 MLD of municipal wastewater generated daily, 70 % of wastewater accounted for DW and 30 % for industrial manufacturing sector. Out of this, 80 % of wastewater was generated by global urban population and 20 % by global rural population. Out of this huge quantity of wastewater, only 60 % is being treated globally. Numerous widely used advanced wastewater treatment systems were designed over time including Membrane Bioreactor (MBR), Sequencing Batch Reactor (SBR) etc. Both conventional and advanced STPs are aiming at removal of pollutants and not on the recovery of nutrients from the wastewater. Both of these systems suffer several advantages and disadvantages. Therefore, it is essential to design hybrid treatment system that can be established at current STPs without any major modifications and to recover the nutrients from the wastewater.

Wastewater treatment systems can be classified on the basis of energy requirement; active (High energy demand) and passive (low energy demand). These days, Sequencing Batch Reactor (SBR) system is widely used but its removal efficiency varies; carbon (65-90%), nitrogen (35-92%) and phosphorous (20-95%). Power requirement for SBR is also high (0.3 kWh/m^3) and it does not generate any value-added product. It is important to use hybrid technologies that can be established at pre-existing Sewage Treatment Plants (STPs) without major modification to available infrastructure in order to increase long term pollutant removal efficiency as well as to generate some value-added products. Microalgal Treatment System (MTS) can be integrated with SBR for simultaneous wastewater treatment and resource recovery in terms of microalgal biomass. This study has investigated the treatment of DW by integrating MTS to active treatment system (SBR) and passive treatment system (constructed wetland). The biomass generated was used to extract lipids, which can further be used to produce ethanol via fermentation or to generate electricity via anaerobic digestion.

DW was treated in two ways by adopting active treatment process (SBR) and passive treatment process (VFCWs). Organic matter from the wastewater can be efficiently removed by SBR but the removal of NH_4-P and PO_4-P is not satisfactory in many cases. The conventional wastewater treatment systems focus mainly on removal of pollutants from wastewater and not on the generation of value-added products. These days, wastewater is considered as a resource

to extract valuable products and therefore, STPs are known as Water Resource Recovery Facilities (WRRFs). In view of this, we have demonstrated that MTS can be integrated to SBR or VFCW at previously existing STPs to remove organic and inorganic pollutants from wastewater.

Use of *S. platensis* and *C. vulgaris* to treat secondary treated wastewater (post SBR treatment) has demonstrated their potential in simultaneous wastewater treatment and resource recovery in the form of high value biomass. DW was initially treated with SBR and SBR effluent was further phycoremediated by using two individual microalga species; *Spirulina platensis* and *Chlorella vulgaris*. The observed removal efficiency of COD, PO_4-P, NH_4-N and TKN of *S. platensis* and *C. vulgaris* were 18%, 14%, 17%, 16% and 31%, 40%, 36%, 38 %, respectively. *S. platensis* and *C. vulgaris* biomass was observed to contain 26.65 % and 16.45 % lipids, respectively. The maximum biogas production (mL/g VS) was observed at 2 g VS/L. *S. platensis* and *C. vulgaris* was observed to produce 320 mL/g VS and 450 mL/g VS biogas, respectively. Effect of different pre-treatment methods (thermal, chemical, sonication and thermo-chemical) has also been studied. The biomass and biomass extract (before and after pre-treatments) were also analyzed for solubilization of complex compounds. Thermally pre-treatment of *S. platensis* and *C. vulgaris* biomass increases biogas production by 8.5% and 6.6 %, respectively. It was also observed that the ammoniacal nitrogen tolerance of *S. platensis* and *C. vulgaris* is 100 mg/L and 200 mg/L, respectively. These studies have successfully demonstrated that microalgal cultivation in wastewater can be easily adopted in currently available wastewater treatment plants without any major modifications of existing available infrastructure.

Active treatment processes have certain limitations; requirement of high level of sophistication and maintenance, plugging of aeration devices, requirement of post equalization phase post treatment and chances of discharging floating or settled sludge during fill and decant phase. To overcome these limitations, passive wastewater treatment system was designed and demonstrated for the treatment of raw DW. DW often contains solid matter limiting its direct use as a medium for microalgal growth. This limitation was overcome by adopting hybrid treatment system; Vertical Flow Constructed Wetland (VFCW) and MTS. The main aim of this study was to treat DW in a hybrid Vertical Flow Constructed Wetland (VFCW-4.2 m^2) and Microalgal Treatment System (MTS-1m^2). VFCW uses natural mechanisms like filtration, absorption, adsorption and assimilation by plants and microorganisms to remove organic and inorganic pollutants. The objective was not only to treat DW but also to produce value added

products from microalgal biomass. The DW was initially treated by VFCW and the VFCW effluent was further phycoremediated by MTS. *Canna indica* was used for wetland vegetation and resident microalgal consortium from VFCW effluent was used in MTS. The VFCW and MTS were operated at 1 m^3/day (HRT-0.25 m^3/m^{2-}day, OLR-400 g/m^2-day) and 0.3 m^3/day (HRT-0.03 m^3/m^{2-}day, OLR-400 g/m^2-day), respectively. The integrated system was observed to remove 68.9% COD, 77.4% NH_4-N, 75.8% TKN and 63.6% PO_4-P. The harvested Naive Biomass (NB) was observed to contain 16.7 % of lipids (W/W). The integrated system of VFCW and MTS has low footprint-1.35 m^2/person. The Residual Biomass after Lipid Extraction (RBLE) was used as a substrate for ethanol production. The observed yield of ethanol using RBLE as a substrate was 33.4 %. NB, RBLE, and Residual Biomass after Lipid and Sugar Extraction (RBLSE) indicated net biomethane yield (mL/g VS) of 211.8, 134.6 and 107.7, respectively. This study demonstrated an initial attempt of demonstrating hybrid wastewater treatment system for the production of value-added products in terms of biofuel.

Fertilizer industry wastewater was rich in ammoniacal nitrogen, phosphorus but deficient in organic matter. Conventional wastewater treatment systems cannot be used to treat such wastewater. Microalgal treatment systems can be effectively used for simultaneous treatment and production of value-added products from microalgal biomass. However, ammoniacal nitrogen exerts toxicity to microalgal cells beyond 28 mg/L of undissociated ammonia. Therefore, individual microalgae or consortium need to be adapted to high ammoniacal nitrogen concentration. Present study also investigated the ammoniacal nitrogen tolerance and wastewater treatment efficiency of microalgal consortium (BPGC) using ammoniacal nitrogen rich synthetic medium. The observed ammoniacal nitrogen tolerance limits BPGC consortium is1000 mg/L. The individual microalgal species present in BPGC consortium were also identified by 18 S rDNA sequencing. BPGC consortium was found to contain five major microalgae; *Chlorella pyrenoidosa*, *Micractinium pusillum*, *Actinastrum hantzschii*, *Micractinium* sp. and *Chlorella coloniales*.

This study also demonstrates the integration of pilot scale struvite production from fertilizer industrial wastewater in air-agitated reactor to phycoremediation of residual wastewater. The optimized pH and $MgCl_2$ concentration for struvite production were 9.0 and 5 % W/V, respectively. The microalgal consortium (BPGC consortium) was isolated from anaerobic plant digestate and adapted to tolerate 1000 mg/L of NH_4-N using synthetic wastewater rich in NH_4-N. Pilot-scale struvite production was carried out in the air-agitated reactor (1 m^3 capacity) in batch mode and phycoremediation of residual effluent was carried out in tubular

photobioreactor (200 L capacity) in fed batch mode. Pilot-scale struvite crystallization produced 60 kg of struvite from 1 m^3 of effluent. During struvite precipitation, 2.96 % of COD , 68.29 % of NH_4-N and 96.38 % of PO_4-P were recovered. The residual effluent was further phycoremediated by the microalgal consortium. During phycoremediation, 62.68 % of COD, 59.21 % of NH_4-N and 68.57 % of PO_4-P were recovered in terms of microalgal biomass. Due to integration, 64.58 % COD, 87.31 % NH_4-N, 89.0 % TKN (Total Kjeldahl Nitrogen) and 98.79 % of PO_4-P was removed. It led to production of biomass that was used for production of biodiesel, methane, and ω-3 fatty acids. In brief, the integration of struvite production and microalgae cultivation can be used as an effective treatment system for fertilizer industry wastewater.

The potential of microalgal biorefineries integrated to wastewater treatment is depicted in the table below.

Table -The potential of microalgal biorefineries integrated to wastewater treatment.

Wastewater Type	Microalgae	Biomass yield (g/m^3)	Lipid yield (g/m^3)	Biogas yield ($L/m^{3)}$	Ethanol yield (g/m^3)	Struvite yield (Kg/m^3)
SBR effluent	*S. platensis*	600	160	161.28	NA	NA
SBR effluent	*C. vulgaris*	260	42.77	77.35	NA	NA
VFCW effluent	*Consortium*	320	53.44	110.656	83.2	NA
Fertilizer Industry effluent	*BPGC Consortium*	290	56.46	78.88	NA	60

- **Future Prospects:** Future research can be on demonstrating at a full scale the integration of Microalgal treatment systems with other active or passive treatments system for producing multiple value-added products so as to make the wastewater treatment system sustainably viable. Focus can also be on studying ammoniacal nitrogen tolerance behavior of BPGC consortium at genetic level and protein expression profiles.

References

Abdel-raouf, N., A.A.Al-Homaidanb, and I.B.M.Ibraheembc. 2014. "Microalgae and Wastewater Treatment." *Saudi Journal of Biological Sciences* 19(3):257–75.

Abdelhakeem, Sara G., Samir A. Aboulroos, and Mohamed M. Kamel. 2016. "Performance of a Vertical Subsurface Flow Constructed Wetland under Different Operational Conditions." *Journal of Advanced Research* 7(5):803–14.

Abeliovich, A. and Y. Azov. 1976. "Toxicity of Ammonia to Algae in Sewage Oxidation Ponds." *Applied and Environmental Microbiology* 31(6):801–6.

Abo-Shady, A. M., Y. A. Mohamed, and T. Lasheen. 1993. "Chemical Composition of the Cell Wall in Some Green Algae Species." *Biologia Plantarum* 35(4):629–32.

Abou-Elela, Sohair I. and Mohamed S. Hellal. 2012. "Municipal Wastewater Treatment Using Vertical Flow Constructed Wetlands Planted with Canna, Phragmites and Cyprus." *Ecological Engineering* 47:209–13.

Agyeman, Fred O. and Wendong Tao. 2014. "Anaerobic Co-Digestion of Food Waste and Dairy Manure: Effects of Food Waste Particle Size and Organic Loading Rate." *Journal of Environmental Management* 133:268–74.

Ahluwalia, Sarabjeet Singh and Dinesh Goyal. 2007. "Microbial and Plant Derived Biomass for Removal of Heavy Metals from Wastewater." *Bioresource Technology* 98(12):2243–57.

Ahmad, Farooq, Amin U. Khan, and Abdullah Yasar. 2013. "The Potential of Chlorella Vulgaris for Wastewater Treatment and Biodiesel Production." *Pakistan Journal of Botany* 45(SPL.ISS):461–65.

Albert B. Pincince, Brandon G. Braley, Karla H. Sangrey, and Roderick D., and Reardon. 1997. "Minimizing Costs of Activated-Sludge Systems." *Water Environment Research* 69(3):326–30.

Altinbas, M., I. Ozturk, and A. F. Aydin. 2002. "Ammonia Recovery from High Strength Agro Industry Effluents." *Water Science and Technology* 45(12):189–96.

Álvarez, J. A., C. Ávila, P. Otter, R. Kilian, D. Istenič, M. Rolletschek, P. Molle, N. Khalil, I. Ameršek, V. K. Mishra, C. Jorgensen, A. Garfi, P. Carvalho, H. Brix, and C. A. Arias. 2017. "Constructed Wetlands and Solar-Driven Disinfection Technologies for Sustainable Wastewater Treatment and Reclamation in Rural India: SWINGS Project." *Water Science and Technology* 76(6):1474–89.

Alzate, M. E., R. Munoz, F. Rogalla, F. Fdz-Polanco, and S. I. P??rez-Elvira. 2012. "Biochemical Methane Potential of Microalgae: Influence of Substrate to Inoculum Ratio, Biomass Concentration and Pretreatment." *Bioresource Technology* 123:488–94.

Amos Richmond, ed. 2004. *Handbook of Microalgal Culture*. Blackwell Science.

Ana Cláudia Freitas Margarites, Jorge Alberto Vieira Costa. 2014. "Increment of Carbohydrate Concentration of Chlorella Minutissima Microalgae for Bioethanol Production." *Int. Journal of Engineering Research and Applications* 4(11):80–86.

Awoke Guadie, Siqing Xia, Wei Jiang, Lijie Zhou, Zhiqiang Zhang, Slawomir W. Hermanowicz, Xiaoyin Xu, and Shuang Shen. 2014. "Enhanced Struvite Recovery from Wastewater Using a Novel Cone-Inserted Fluidized Bed Reactor." *Journal of Environmental Sciences* 26:765–74.

Azov, Y. and Joel C. Goldman. 1982. "Free Ammonia Inhibition of Algal Photosynthesis in Intensive Cultures." *Applied and Environmental Microbiology* 43(4):735–39.

Babaee, Azadeh and Jalal Shayegan. 2011. "Effect of Organic Loading Rates (OLR) on Production of Methane from Anaerobic Digestion of Vegetables Waste." Pp. 411–17 in *Bioenergy Technology (BE)*. Linköping- Sweden.

Babatunde, Authors. 2008. "Title Constructed Wetlands for Environmental Pollution Control : A Review of Developments, Research and Practice in Ireland." *Environment International* 34(1):116–26.

Badhe, Neha, Shaswati Saha, Rima Biswas, and Tapas Nandy. 2014. "Role of Algal Biofilm in Improving the Performance of Free Surface, up-Flow Constructed Wetland." *Bioresource Technology* 169:596–604.

Battistoni, P., D. E. Angelis, P. Pavan, M. Prisciandaro, and F. Cecchi. 2001. "Phosphorus Removal From a Real Anaerobic." *Water Research* 35(9):2167–78.

Becker, E. W. 2007. "Micro-Algae as a Source of Protein." *Biotechnology Advances* 25(2):207–10.

Becker, E. W. (E. Wolfgang). 1994. *Microalgae : Biotechnology and Microbiology*. Cambridge University Press.

Belay, Amha, Toshimitsu Kato, and Yoshimichi Ota. 1996. "Spirulina (Arthrospira): Potential Application as an Animal Feed Supplement." *Journal Of Applied Phycology* 8:303–11.

Bhagavathy, S., P. Sumathi, and I. Jancy Sherene Bell. 2011. "Green Algae Chlorococcum Humicola-a New Source of Bioactive Compounds with Antimicrobial Activity." *Asian Pacific Journal of Tropical Biomedicine* 1(1):S1–7.

Bhandari, Vinay M., Laxmi Gayatri Sorokhaibam, and Vivek V. Ranade. 2016. "Industrial Wastewater Treatment for Fertilizer Industry—A Case Study." *Desalination and Water Treatment* 57(57):1–11.

Bhawan, Parivesh, Arjun Nagar, Shashi Shekhar, and Vishal Gandhi. 2015. *Inventorization Of Sewage Treatment Plants*. Delhi.

Bodík, I., Kubaská, M. 2013. "Energy and Sustainability of Operation of a Wastewater Treatment Plant." *Environment Protection Engineering* 39(2):15--24.

Booker, NA, EL Cooney, and AJ Priestley. 1996. "Ammonia Removal from Sewage Using Natural Australian Zeolite." *Water Science and Technology* 34(9):17–24.

Bossler, John F., Ronald Travis, Clarke Veach, and Douglas M Spolarich. 2009. "Treatment Of Phosphate Fertilizer Plant Waste Water In Florida For Discharge And Re-Use Purposes." Pp. 1–12 in *Clearwater convention papers*. Florida: American Institute of Chemical Engineers (AIChE)- Florida-USA.

Brennan, Liam and Philip Owende. 2010a. "Biofuels from Microalgae-A Review of Technologies for Production, Processing, and Extractions of Biofuels and Co-Products." *Renewable and Sustainable Energy Reviews* 14(2):557–77.

Brennan, Liam and Philip Owende. 2010b. "Biofuels from Microalgae-A Review of Technologies for Production, Processing, and Extractions of Biofuels and Co-Products." *Renewable and Sustainable Energy Reviews* 14(2):557–77.

Brisson, J. and F. Chazarenc. 2009. "Maximizing Pollutant Removal in Constructed Wetlands: Should We Pay More Attention to Macrophyte Species Selection?" *Science of The Total Environment* 407(13):3923–30.

Britto, Dev T. and Herbert J. Kronzucker. 2002. "$NH4^+$ Toxicity in Higher Plants: A Critical Review." *Journal of Plant Physiology* 159(6):567–84.

Brown, Malcolm R. and S. W. Jeffrey. 1992. "Biochemical Composition of Microalgae from the Green Algal Classes Chlorophyceae and Prasinophyceae. 1. Amino Acids, Sugars and Pigments." *Journal of Experimental Marine Biology and Ecology* 161(1):91–113.

Cabanelas, Iago Teles Dominguez, Jésus Ruiz, Zouhayr Arbib, Fábio Alexandre Chinalia, Carmen Garrido-Pérez, Frank Rogalla, Iracema Andrade Nascimento, and José A. Perales. 2013. "Comparing the Use of Different Domestic Wastewaters for Coupling Microalgal Production and Nutrient Removal." *Bioresource Technology* 131:429–36.

Cai, Ting, Stephen Y. Park, and Yebo Li. 2013. "Nutrient Recovery from Wastewater Streams by Microalgae: Status and Prospects." *Renewable and Sustainable Energy Reviews* 19:360–69.

Calicioglu, Ozgul and Goksel N. Demirer. 2015. "Integrated Nutrient Removal and Biogas Production by Chlorella Vulgaris Cultures." *Journal of Renewable and Sustainable Energy* 7(3):033123,1-14.

Calicioglu, Ozgul and Goksel N. Demirer. n.d. "Parallel Nutrient Removal and Biogas Production by Chlorella Vulgaris Cultures." *International Scientific Journal* 1–8. Retrieved March 15, 2019

Campo G., Rıggıo V.A., Ceruttı A., Ruffıno B., Panepınto D., Zanettı Mc. 2018. "Thermal, Alkali And Thermo-Alkali Pretreatments Applied On Monospecific Microalgal Biomass To Improve Anaerobic Biogas Production." *Desalination and Water Treatment* 127:165–70.

Cañizares, R. O. and A. R. Domínguez. 1993. "Growth of Spirulina Maxima on Swine Waste." *Bioresource Technology* 45(1):73–75.

Cantrell, Keri B., Thomas Ducey, Kyoung S. Ro, and Patrick G. Hunt. 2008. "Livestock Waste-to-Bioenergy Generation Opportunities." *Bioresource Technology* 99(17):7941–53.

Carpenter, S. R. 2005. "Eutrophication of Aquatic Ecosystems: Bistability and Soil Phosphorus." *Proceedings of the National Academy of Sciences* 102(29):10002–5.

Carrera, Julián, Teresa Vicent, and Javier Lafuente. 2004. "Effect of Influent COD/N Ratio on Biological Nitrogen Removal (BNR) from High-Strength Ammonium Industrial Wastewater." *Process Biochemistry* 39(12):2035–41.

Chandler, J. A., W. J. Jewell, J. M. Gossett, P. J. Van Soest, and J. B. Robertson. 1980. "Predicting Methane Fermentation Biodegradability." *Biotechnol. Bioeng. Symp.; (United States)* 10.

Chatterjee, Pritha, M. M. Ghangrekar, and Surampalli Rao. 2016. "Low Efficiency of Sewage Treatment Plants Due to Unskilled Operations in India." *Environmental Chemistry Letters* 14(3):407–16.

Chavan, Ram and Srikanth Mutnuri. 2018a. "Tertiary Treatment of Domestic Wastewater by Spirulina Platensis Integrated with Microalgal Biorefinery." *Biofuels* 1–12.

Chavan, Ram and Srikanth Mutnuri. 2018b. "Tertiary Treatment of Domestic Wastewater by *Spirulina Platensis* Integrated with Microalgal Biorefinery." *Biofuels* 1–12.

Chen, Feng, Yiming Zhang, and Siyuan Guo. 1996. "Growth and Phycocyanin Formation of Spirulina Platensis in Photoheterotrophic Culture." *Biotechnology Letters* 18(5):603–8.

Chen, Paris Honglay. 1987. "Factors Influencing Methane Fermentation of Micro-Algae." University of California, Berkeley (USA).

Chen, Ye, Jay J. Cheng, and Kurt S. Creamer. 2008. "Inhibition of Anaerobic Digestion Process: A Review." *Bioresource Technology* 99(10):4044–64.

Cheng, Chieh Lun, Pei Yi Che, Bor Yann Chen, Wen Jhy Lee, Chiu Yue Lin, and Jo Shu Chang. 2012. "Biobutanol Production from Agricultural Waste by an Acclimated Mixed Bacterial Microflora." *Applied Energy* 100:3–9.

Cheng, Hairong, Hai-Rong Cheng, and Ning Jiang. 2006. "Extremely Rapid Extraction of DNA from Bacteria and Yeasts." *Biotechnology Letters* 28(1):55–59.

Cheremisinoff, Nicholas P. 2002. *Handbook of Water and Wastewater Treatment*

Technologies. Butterworth-Heinemann.

Cherubini, Francesco. 2010. "The Biorefinery Concept: Using Biomass Instead of Oil for Producing Energy and Chemicals." *Energy Conversion and Management* 51(7):1412–21.

Cheunbarn, Siraporn and Yuwadee Peerapornpisal. 2010. "Cultivation of Spirulina Platensis Using Anaerobically Swine Wastewater Treatment Effluent." *International Journal of Agriculture and Biology* 12(4):586–90.

Chinnasamy, Senthil, Ashish Bhatnagar, Ronald Claxton, and K. C. Das. 2010. "Biomass and Bioenergy Production Potential of Microalgae Consortium in Open and Closed Bioreactors Using Untreated Carpet Industry Effluent as Growth Medium." *Bioresource Technology* 101(17):6751–60.

Cho, S., D. Lee, T. T. Luong, S. Park, Y. K. Oh, and T. Lee. 2011. "Effects of Carbon and Nitrogen Sources on Fatty Acid Contents and Composition in the Green Microalga, Chlorella Sp. 227." *Journal of Microbiology and Biotechnology* 21(10).

Cho, Sunja, Seonghwan Park, Jiyun Seon, Jaechul Yu, and Taeho Lee. 2013a. "Evaluation of Thermal, Ultrasonic and Alkali Pretreatments on Mixed-Microalgal Biomass to Enhance Anaerobic Methane Production." *Bioresource Technology* 143:330–36.

Cho, Sunja, Seonghwan Park, Jiyun Seon, Jaechul Yu, and Taeho Lee. 2013b. "Evaluation of Thermal, Ultrasonic and Alkali Pretreatments on Mixed-Microalgal Biomass to Enhance Anaerobic Methane Production." *Bioresource Technology* 143:330–36.

Christenson, Logan and Ronald Sims. 2011. "Production and Harvesting of Microalgae for Wastewater Treatment, Biofuels, and Bioproducts." *Biotechnology Advances* 29(6):686–702.

Clara, M., B. Strenn, O. Gans, E. Martinez, N. Kreuzinger, and H. Kroiss. 2005. "Removal of Selected Pharmaceuticals, Fragrances and Endocrine Disrupting Compounds in a Membrane Bioreactor and Conventional Wastewater Treatment Plants." *Water Research* 39(19):4797–4807.

Collos, Yves and Paul J. Harrison. 2014a. "Acclimation and Toxicity of High Ammonium Concentrations to Unicellular Algae." *Marine Pollution Bulletin* 80(1–2):8–23.

Collos, Yves and Paul J. Harrison. 2014b. "Acclimation and Toxicity of High Ammonium Concentrations to Unicellular Algae." *Marine Pollution Bulletin* 80(1–2):8–23.

Colmenarejo, M. F., A. Rubio, E. Sánchez, J. Vicente, M. G. García, and R. Borja. 2006. "Evaluation of Municipal Wastewater Treatment Plants with Different Technologies at Las Rozas, Madrid (Spain)." *Journal of Environmental Management* 81(4):399–404.

Conley, D. J., HW Paerl, R. W. Howarth, DF Boesch, SP Seitzinger, KE Havens, C. Lancelot, and GE Likens. 2009. "Controlling Eutrophication: Nitrogen and Phosphorus." *Science*

323(5917):1014–15.

Converti, Attilio, Alessandro A. Casazza, Erika Y. Ortiz, Patrizia Perego, and Marco Del Borghi. 2009. "Effect of Temperature and Nitrogen Concentration on the Growth and Lipid Content of Nannochloropsis Oculata and Chlorella Vulgaris for Biodiesel Production." *Chemical Engineering and Processing: Process Intensification* 48(6):1146–51.

Le Corre, K. S., E. Valsami-Jones, P. Hobbs, and S. A. Parsons. 2009. "Phosphorus Recovery from Wastewater by Struvite Crystallization: A Review." *Critical Reviews in Environmental Science and Technology* 39(6):433–77.

Correll, David L. 1998. "The Role of Phosphorus in the Eutrophication of Receiving Waters: A Review." *Journal of Environment Quality* 27:261–66.

Craggs, R., J. Park, S. Heubeck, and D. Sutherland. 2014. "High Rate Algal Pond Systems for Low-Energy Wastewater Treatment, Nutrient Recovery and Energy Production." *New Zealand Journal of Botany* 52(1):60–73.

Cui, Lihua, Ying Ouyang, Qian Lou, Fengle Yang, Ying Chen, Wenling Zhu, and Shiming Luo. 2010. "Removal of Nutrients from Wastewater with Canna Indica L. under Different Vertical-Flow Constructed Wetland Conditions." *Ecological Engineering* 36(8):1083–88.

D'Oca, Marcelo G. Montes, Carolina V. Viêgas, Juliana S. Lemões, Eriksen K. Miyasaki, Joaquín A. Morón-Villarreyes, Ednei G. Primel, and Paulo C. Abreu. 2011. "Production of FAMEs from Several Microalgal Lipidic Extracts and Direct Transesterification of the Chlorella Pyrenoidosa." *Biomass and Bioenergy* 35(4):1533–38.

De-Bashan, Luz E. and Yoav Bashan. 2004. "Recent Advances in Removing Phosphorus from Wastewater and Its Future Use as Fertilizer (1997?2003)." *Water Research* 38(19):4222–46.

Demirbaş, Ayhan. 2001. "Biomass Resource Facilities and Biomass Conversion Processing for Fuels and Chemicals." *Energy Conversion and Management* 42(11):1357–78.

Dilek (Yalcin) Duygu,. 2012. "Fourier Transform Infrared (FTIR) Spectroscopy for Identification of Chlorella Vulgaris Beijerinck 1890 and Scenedesmus Obliquus (Turpin) Kützing 1833." *African Journal of Biotechnology* 11(16):3817–24.

Dioha, I. J., CH Ikeme, T. Nafi, N. I. Soba, and Yusuf Mbs. 2013. Effect Of Carbon To Nitrogen Ratio On Biogas Production. *International Research Journal of Natural Sciences*, *1*(3), 1-10.

Doğan-Subaşı, Eylem and Göksel N. Demirer. 2016. "Anaerobic Digestion of Microalgal Chlorella Vulgaris Biomass as a Source of Biogas and Biofertilizer." *Environmental Progress & Sustainable Energy* 35(4):936–41.

Dote, Yutaka, Shigeki Sawayama, Seiichi Inoue, Tomoaki Minowa, and Shin-ya Yokoyama. 1994. “Recovery of Liquid Fuel from Hydrocarbon-Rich Microalgae by Thermochemical Liquefaction.” *Fuel* 73(12):1855–57.

Doyle, JD and SA Parsons. 2002. “Struvite Formation, Control and Recovery.” *Water Research* 36(16):3925–40.

Dubois, Michel, K. A. Gilles, J. K. Hamilton, P. A. Rebers, Fred Smith, Rose J. Randall, A. Lewis, Jana Spura, Lorenz Christian Reimer, Patricia Wieloch, Kerstin Schreiber, Sebastian Buchinger, and Dietmar Schomburg. 2009. “Colorimetric Method for Determination of Sugars and Related Substances.” *Analytical Biochemistry* 394(2):192–201.

Dutta, Aparna and Sudipta Sarkar. 2015. “Sequencing Batch Reactor for Wastewater Treatment: Recent Advances.” *Current Pollution Reports* 1(3):177–90.

E.S.Bejor, C. Mota, N.M.Ogarekpe, K.U.Emerson, and J.Ukpata. 2013. “Low-Cost Harvesting of Microalgae \nbiomass from Water.” *International Journal of Development and Sustainability* 2(1):1–11.

Eaton, AD, M. A. H. Franson, L. S. Clesceri, E. W. Rice, and A. E. Greenberg. 2005. *Standard Methods for Examination of Water and Wastewater.* 21st ed. edited by A. Eaton, M. A. H. Franson, L. S. Clesceri, E. W. Rice, and A. E. Greenberg. Washington DC-USA: American Public Health Association (APHA)-American Water Works Association (AWWA).

Eckenfelder, W. Wesley (William Wesley), Petr. Grau, and D. C. .. International Association on Water Pollution Research and Control. Conference (1992 : Washington. 1998. *Activated Sludge Process Design and Control : Theory and Practice*. Technomic Pub.

El-Mashad, Hamed M. 2015. “Biomethane and Ethanol Production Potential of Spirulina Platensis Algae and Enzymatically Saccharified Switchgrass.” *Biochemical Engineering Journal* 93:119–27.

Elaiyaraju, P. and N. Partha. 2014. “Biogas Production from Co-Digestion of Orange Peel Waste and Jatropha de-Oiled Cake in an Anaerobic Batch Reactor.” *African Journal of Biotechnology* 11(14):3339–45.

Elango, D., M. Pulikesi, P. Baskaralingam, V. Ramamurthi, and S. Sivanesan. 2007. “Production of Biogas from Municipal Solid Waste with Domestic Sewage.” *Journal of Hazardous Materials* 141(1):301–4.

Esteban, Raquel, Idoia Ariz, Cristina Cruz, and Jose Fernando Moran. 2016. “Mechanisms of Ammonium Toxicity and the Quest for Tolerance.” *Plant Science* 248:92–101.

Fakhry, Eman M. and Dahlia M. El Maghraby. 2013. “Fatty Acids Composition and Biodiesel

Characterization of Dunaliella Salina." *Journal of Water Resource and Protection* 5:894–99.

Fakhry, Eman M. and Dahlia M. El Maghraby. 2013. "Fatty Acids Composition and Biodiesel Characterization of Dunaliella Salina." *Journal of Water Resource and Protection* 5:894–99.

Fan, A. M. and V. E. Steinberg. 1996. "Health Implications of Nitrate and Nitrite in Drinking Water: An Update on Methemoglobinemia Occurrence and Reproductive and Developmental Toxicity." *Regulatory Toxicology and Pharmacology : RTP* 23(23):35–43.

Ferhan Cecen. 1996. "Investigation of Partial and Full Nitrification Characteristics of Fertilizer Wastewaters in a Submerged Biofilm Reactor." *Water Science and Technology* 34(11):77–85.

Foladori, P., A. R. C. Ortigara, J. Ruaben, and G. Andreottola. 2012. "Influence of High Organic Loads during the Summer Period on the Performance of Hybrid Constructed Wetlands (VSSF + HSSF) Treating Domestic Wastewater in the Alps Region." *Water Science and Technology* 65(5):890–97.

Folch, Jordi, M. Lees, and G. H. Sloane Stanley. 1957. "A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues." *J Biol Chem* 226(1):497–509.

Fon-Sing, Sophie, Florian Delrue, Pablo Álvarez-Díaz, Jean-François Sassi, and Gatien Fleury. 2016. "The Environmental Biorefinery: Using Microalgae to Remediate Wastewater, a Win-Win Paradigm." *Energies* 9(132):1–9.

Francisco, C., D. ´. Ebora, B. Neves, Eduardo Jacob-Lopes, and Telma T. Franco. 2010. "Microalgae as Feedstock for Biodiesel Production: Carbon Dioxide Sequestration, Lipid Production and Biofuel Quality." *Journal of Chemical Technology & Biotechnology* 85(3):395–403.

Fu, Chun-Chong, Tien-Chieh Hung, Jing-Yi Chen, Chia-Hung Su, and Wen-Teng Wu. 2010. "Hydrolysis of Microalgae Cell Walls for Production of Reducing Sugar and Lipid Extraction." *Bioresource Technology* 101(22):8750–54.

Fux, Christian, Marc Boehler, Philipp Huber, Irene Brunner, and Hansruedi Siegrist. 2002. "Biological Treatment of Ammonium-Rich Wastewater by Partial Nitritation and Subsequent Anaerobic Ammonium Oxidation (Anammox) in a Pilot Plant." *Journal of Biotechnology* 99(3):295–306.

Gai, Chao, Yuanhui Zhang, Wan-Ting Chen, Peng Zhang, and Yuping Dong. 2015. "An Investigation of Reaction Pathways of Hydrothermal Liquefaction Using Chlorella Pyrenoidosa and Spirulina Platensis." *Energy Conversion and Management* 96:330–39.

Geider, Richard and Julie La Roche. n.d. “European Journal of Phycology Redfield Revisited: Variability of C:N:P in Marine Microalgae and Its Biochemical Basis.” *European Journal of Phycology* 37(1):1–17.

Ghosh, S., M. P. Henry, and R. W. Christopher. 1985. “Hemicellulose Conversion by Anaerobic Digestion.” *Biomass* 6(4):257–69.

Gonçalves, Ana L., José C. M. Pires, and Manuel Simões. 2017. “A Review on the Use of Microalgal Consortia for Wastewater Treatment.” *Algal Research* 24:403–15.

González, LE, RO Cañizares, and S. Baena. 1997. “Efficiency of Ammonia and Phosphorus Removal from a Colombian Agroindustrial Wastewater by the Microalgae Chlorella Vulgaris and Scenedesmus Dimorphus.” *Bioresource Technology* 60(3):259–62.

González López, Cynthia Victoria, María Del, Carmen Cerón García, Francisco Gabriel, Acién Fernández, Cristina Segovia Bustos, Yusuf Chisti, José María, and Fernández Sevilla. 2010. “Protein Measurements of Microalgal and Cyanobacterial Biomass.” *Bioresource Technology* 101(19):7587–91.

Gouw, T. and J. Vlugter. 1964. “Physical Properties of Fatty Acid Methyl Esters. I. Density and Molar Volume.” *Journal of the American Oil Chemists’ Society* 41(2):142–45.

Gross, Amit, Drora Kaplan, and Katherine Baker. 2007. “Removal of Chemical and Microbiological Contaminants from Domestic Greywater Using a Recycled Vertical Flow Bioreactor (RVFB).” *Ecological Engineering* 31(2):107–14.

Gu, Yifan, Yue Li, Xuyao Li, Pengzhou Luo, Hongtao Wang, Zoe P. Robinson, Xin Wang, Jiang Wu, and Fengting Li. 2017. “The Feasibility and Challenges of Energy Self-Sufficient Wastewater Treatment Plants.” *Applied Energy* 204:1463–75.

Halim, Ronald, Michael K. Danquah, and Paul A. Webley. 2012. “Extraction of Oil from Microalgae for Biodiesel Production: A Review.” *Biotechnology Advances* 30(3):709–32.

Hanaki, Keisuke, Chalermraj Wantawin, and Shinichiro Ohgaki. 1990. “Effects of the Activity of Heterotrophs on Nitrification in a Suspended-Growth Reactor.” *Water Research* 24(3):289–96.

Hansen, R., T. Thøgersen, and F. Rogalla. 2007. “Comparing Cost and Process Performance of Activated Sludge (AS) and Biological Aerated Filters (BAF) over Ten Years of Full Sale Operation.” *Water Science and Technology* 55(8–9):99–106.

Harun, Razif and Michael K. Danquah. 2011. “Influence of Acid Pre-Treatment on Microalgal Biomass for Bioethanol Production.” *Process Biochemistry* 46(1):304–9.

Harun, Razif, Michael K. Danquah, and Gareth M. Forde. 2009. “Microalgal Biomass as a Fermentation Feedstock for Bioethanol Production.” *Journal of Chemical Technology & Biotechnology* 85:199–203.

Heaven, Sonia, John Milledge, and Yue Zhang. 2011. "Comments on 'Anaerobic Digestion of Microalgae as a Necessary Step to Make Microalgal Biodiesel Sustainable.'" *Biotechnology Advances* 29(1):164–67.

Hecht, C. and C. Griehl. 2009. "Investigation of the Accumulation of Aromatic Compounds during Biogas Production from Kitchen Waste." *Bioresource Technology* 100(2):654–58.

Helmer, C., S. Kunst, S. Juretschko, M. C. Schmid, K. H. Schleifer, and M. Wagner. 1999. "Nitrogen Loss in a Nitrifying Biofilm System." *Water Science and Technology* 39(7):13–21.

Hirano, Atsushi, Ryohei Ueda, Shin Hirayama, and Yasuyuki Ogushi. 1997. "CO2 Fixation and Ethanol Production with Microalgal Photosynthesis and Intracellular Anaerobic Fermentation." *Energy* 22(2–3):137–42.

Ho, Shih-Hsin, Shu-Wen Huang, Chun-Yen Chen, Tomohisa Hasunuma, Akihiko Kondo, and Jo-Shu Chang. 2013. "Bioethanol Production Using Carbohydrate-Rich Microalgae Biomass as Feedstock." *Bioresource Technology* 135:191–98.

Hoekman, S. Kent, Amber Broch, Curtis Robbins, Eric Ceniceros, and Mani Natarajan. 2012. "Review of Biodiesel Composition, Properties, and Specifications." *Renewable and Sustainable Energy Reviews* 16(1):143–69.

Honglay Chen, Paris and William J. Oswald. 1998. "Thermochemical Treatment for Algal Fermentation." *Pergamon Environment International* 24(8):889–97.

Hosomi, Masaaki. 2016. "New Challenges on Wastewater Treatment." *Clean Technologies and Environmental Policy* 18(3):627–28.

Hossain, Md. Nahian Bin, Joyanta Kumar Basu, and Mohammad Mamun. 2015. "The Production of Ethanol from Micro-Algae Spirulina." *Procedia Engineering* 105:733–38.

Hreiz, Rainier, M. A. Latifi, and Nicolas Roche. 2015. "Optimal Design and Operation of Activated Sludge Processes: State-of-the-Art." *Chemical Engineering Journal* 281:900–920.

Huang Jin-Lou, Chen Qin, And Xu Lian-Huang. 2013. "Problems and Countermeasures in the Application of Constructed Wetlands." *Huan jing ke xue= Huanjing kexue* 34.1 (2013): 401-408

Huerlimann, Roger, Kirsten Heimann, and Rocky De Nys. 2010. "Growth, Lipid Content, Productivity, and Fatty Acid Composition of Tropical Microalgae for Scale-up Production." *Biotechnology and Bioengineering* 107(2):245–57.

Illman, A., A.Scragg, and S. .. Shales. 2000. "Increase in Chlorella Strains Calorific Values When Grown in Low Nitrogen Medium." *Enzyme and Microbial Technology* 27(8):631–35.

Ilyas, Huma and Ilyas Masih. 2017. “Intensification of Constructed Wetlands for Land Area Reduction: A Review.” *Environ Sci Pollut Res* 24(13):12081–91.

J.S. Kamyotra and R.M. Bhardwaj. 2011. *Municipal Wastewater Management in India*.

Jaffer, Y., T. A. Clark, P. Pearce, and S. A. Parsons. 2002. “Potential Phosphorus Recovery by Struvite Formation.” *Water Research* 36(7):1834–42.

Johnson, Michael B. and Zhiyou Wen. 2009. “Production of Biodiesel Fuel from the Microalga Schizochytrium Limacinum by Direct Transesterification of Algal Biomass.” *Energy and Fuels* 23(10):5179–83.

Jones, AG. 2002. *Crystallization Process Systems*.

Jong, Ed De, Adrian Higson, Patrick Walsh, and Maria Wellisch. 2011. *Biobased Chemicals - Value Added Products from Biorefineries*. *IEA Bioenergy, Task42 Biorefinery*, *34*.

Judd, Simon. 2008. “The Status of Membrane Bioreactor Technology.” *Trends in Biotechnology* 26(2):109–16.

Kadlec, Robert H. and Scott D. Wallace. 2009. *Treatment Wetlands*. 2nd ed. Taylor and Francis Group,LLC.

Kampschreur, Marlies J., Hardy Temmink, Robbert Kleerebezem, Mike S. M. Jetten, and Mark C. M. van Loosdrecht. 2009. “Nitrous Oxide Emission during Wastewater Treatment.” *Water Research* 43(17):4093–4103.

Kantawanichkul, S., S. Pilaila, W. Tanapiyawanich, W. Tikampornpittaya, and S. Kamkrua. 1999. “Wastewater Treatment by Tropical Plants in Vertical-Flow Constructed Wetlands.” *Water Science and Technology* 40(3):173–78.

Kermani, Majid, Bijan Bina, Hossein Movahedian, Mohammad Mehdi Amin, and Mahnaz Nikaeen. 2009. “Biological Phosphorus and Nitrogen Removal from Wastewater Using Moving Bed Biofilm Process.” *Iranian Journal of Biotechnology* 7(1):19–27.

Kightlinger, Weston, Kai Chen, Azadeh Pourmir, Daniel W. Crunkleton, Geoffrey L. Price, and Tyler W. Johannes. 2014. “Production and Characterization of Algae Extract from Chlamydomonas Reinhardtii.” *Electronic Journal of Biotechnology* 17(1):14–18.

Kim, J., BP Lingaraju, R. Rheaume, and JY Lee. 2010. “Removal of Ammonia from Wastewater Effluent by Chlorella Vulgaris.” *Tsinghua Science & Technology* 15(4):391–96.

Kim, Wonduck, Jang Min Park, Geun Ho Gim, Sang-Hwa Jeong, Chang Min Kang, Duk-Jin Kim, and Si Wouk Kim. 2012. “Optimization of Culture Conditions and Comparison of Biomass Productivity of Three Green Algae.” *Bioprocess and Biosystems Engineering* 35(1–2):19–27.

Knobeloch, L, B. Salna, A. Hogan, and J. Postle. 2000. “Blue Babies and Nitrate-Contaminated

Well Water." *Environmental Health Perspectives* 108(7):675–78.

Knobeloch, Lynda, Barbara Salna, Adam Hogan, Jeffrey Postle, and Henry Anderson. 2000. "Blue Babies and Nitrate-Contaminated Well Water." *Environmental Health Perspectives* 108(7):675–78.

Knothe, Gerhard. 2005. "Dependence of Biodiesel Fuel Properties on the Structure of Fatty Acid Alkyl Esters." *Fuel Proces Technol* 86:1059– 1070.

Konig, A., H. W. Pearson, and S. A. Silva. 1987. "Ammonia Toxicity to Algal Growth in Waste Stabilization Ponds." *Water Science and Technology* 19(12):115–22.

Konnerup, Dennis, Thammarat Koottatep, and Hans Brix. 2009. "Treatment of Domestic Wastewater in Tropical, Subsurface Flow Constructed Wetlands Planted with Canna and Heliconia." *Ecological Engineering* 35(2):248–57.

Konnerup, Dennis, Ngo Thuy, Diem Trang, and Hans Brix. 2011. "Treatment of Fishpond Water by Recirculating Horizontal and Vertical Flow Constructed Wetlands in the Tropics." *Aquaculture* 313(1–4):57–64.

Kosaric, N., H. T. Nguyen, and M. A. Bergougnou. 1974a. "Growth of Spirulina Maxima Algae in Effluents from Secondary Waste???Water Treatment Plants." *Biotechnology and Bioengineering* 16(7):881–96.

Kosaric, N., H. T. Nguyen, and M. A. Bergougnou. 1974b. "Growth of Spirulina Maxima Algae in Effluents from Secondary Wastewater Treatment Plants." *Biotechnology and Bioengineering* 16(7):881–96.

Krenz Iii, Robert J., Stephen S. Warrner, Derrick L. Douros, Danyelle N. Dehner, Nicole L. Heath, Jason D. Allen, and Charles L. Pederson. 2009. "N:P Ratios and Temperature Affect Phosphate Uptake Rate in Phytoplankton Assemblages of a Cooling Water Reservoir." *Transactions of the Illinois State Academy of Science* 102(1):45–54.

Krishnaveni, S., S. Sadavisam, and T. Balasubramanian. 1984. "Phenol Sulphuric Acid Method for Total Carbohydrate." P. 229 in *Biochemical Methods*. Vol. 15, edited by A. M. S. Sadasivam. New Delhi: New Age International.

KS Le Corre, E Valsami-Jones, and P. Hobbs. 2009. "Phosphorus Recovery from Wastewater by Struvite Crystallization: A Review." *Critical Reviews in Environmental Science and Technology* 39(6):433–77.

KS Le Corre, E. Valsami-Jones, P. Hobbs, and S. A. Parsons. 2009. "Phosphorus Recovery from Wastewater by Struvite Crystallization: A Review." *Critical Reviews in Environmental Science and Technology* 39(6):433–77.

Kumar, Kanhaiya, Chitralekha Nag Dasgupta, and Debabrata Das. 2014. "Cell Growth Kinetics of Chlorella Sorokiniana and Nutritional Values of Its Biomass." *Bioresource*

Technology 167:358–66.

Kuschk, P., R. A. Müller, H. Moormann, M. Kästner, O. Bederski, A. Wießner, U. Kappelmeyer, and U. Stottmeister. 2003. "Effects of Plants and Microorganisms in Constructed Wetlands for Wastewater Treatment." *Biotechnology Advances* 22(1–2):93–117.

Kutty, S. R. M., M. H. Isa, A. Nasiru, I. U. Salihi, and H. Ezerie. 2014. "Potential of the Compact Extended Aeration Reactor (CEAR) as an Integrated System to Biologically Degrade Municipal Sewage According to Malaysian Regulatory Limits: Design, Process and Performance." *Transactions on Ecology and The Environment* 186:1743–3541.

Lackner, Susanne and Harald Horn. 2013. "Comparing the Performance and Operation Stability of an SBR and MBBR for Single-Stage Nitritation-Anammox Treating Wastewater with High Organic Load." *Environmental Technology* 34(10):1319–28.

Lahav, O., E. Artzi, S. Tarre, and M. Green. 2001. "Ammonium Removal Using a Novel Unsaturated Flow Biological Filter with Passive Aeration." *Water Research* 35(2):397–404.

Lal, Sanglur, Rekha Sharma, BS Hedaoo, and Digamber Singh. 2017. *Indian Fertilizers Scenario 2017*. Economics and Statistics wing, Department of fertilizers, Ministry of chemicals and fertlizers- New Delhi (India).

Lalucat, J., J. Imperial, and R. Parés. 1984. "Utilization of Light for the Assimilation of Organic Matter in Chlorella Sp. VJ79." *Biotechnology and Bioengineering* 26(7):677–81.

Larsdotter, K., J. la Cour Jansen, and G. Dalhammar. 2007. "Biologically Mediated Phosphorus Precipitation in Wastewater Treatment With Microalgae." *Environmental Technology* 28(9):953–60.

Larsdotter, Karin. 2006. "Wastewater Treatment with Microalgae – a Literature Review." *Vatten* 62:31–38.

Lau, P. S., N. F. Y. Tam, and Y. S. Wong. 1996. "Wastewater Nutrients Removal by Chlorella Vulgaris: Optimization Through Acclimation." *Environmental Technology* 17(2):183–89.

Lee, Kwanyong, Phrompol Chantrasakdakul, Daegi Kim, Mingeun Kong, and Ki Young Park. 2014. "Ultrasound Pretreatment of Filamentous Algal Biomass for Enhanced Biogas Production." *Waste Management* 34(6):1035–40.

Li, Jian, Shuai Luo, and Zhen He. 2016. "Cathodic Fluidized Granular Activated Carbon Assisted-Membrane Bioelectrochemical Reactor for Wastewater Treatment." *Separation and Purification Technology* 169:241–46.

Li, Jianbo, Yue Wen, Qi Zhou, Zhao Xingjie, Xie Li, Silu Yang, and Tao Lin. 2008. "Influence of Vegetation and Substrate on the Removal and Transformation of Dissolved Organic

Matter in Horizontal Subsurface-Flow Constructed Wetlands." *Bioresource Technology* 99(11):4990–96.

Li, Yecong, Yi-Feng Chen, Paul Chen, Min Min, Wenguang Zhou, Blanca Martinez, Jun Zhu, and Roger Ruan. 2011a. "Characterization of a Microalga Chlorella Sp. Well Adapted to Highly Concentrated Municipal Wastewater for Nutrient Removal and Biodiesel Production." *Bioresource Technology* 102(8):5138–44.

Li, Yecong, Yi-Feng Chen, Paul Chen, Min Min, Wenguang Zhou, Blanca Martinez, Jun Zhu, and Roger Ruan. 2011b. "Characterization of a Microalga Chlorella Sp. Well Adapted to Highly Concentrated Municipal Wastewater for Nutrient Removal and Biodiesel Production." *Bioresource Technology* 102(8):5138–44.

Li, Yecong, Yi Feng Chen, Paul Chen, Min Min, Wenguang Zhou, Blanca Martinez, Jun Zhu, and Rog Ruan. 2011. "Characterization of a Microalga Chlorella Sp. Well Adapted to Highly Concentrated Municipal Wastewater for Nutrient Removal and Biodiesel Production." *Bioresource Technology* 102(8):5138–44.

Lightfoot, D. A., A. J. Baron, and J. C. Wootton. 1988. "Expression of the Escherichia Coli Glutamate Dehydrogenase Gene in the Cyanobacterium Synechococcus PCC6301 Causes Ammonium Tolerance." *Plant Molecular Biology* 11(3):335–44.

Lim, Sing-Lai, Wan-Loy Chu, and Siew-Moi Phang. 2010. "Use of Chlorella Vulgaris for Bioremediation of Textile Wastewater." *Bioresource Technology* 101(19):7314–22.

Lim, Sing Lai, Wan Loy Chu, and Siew Moi Phang. 2010. "Use of Chlorella Vulgaris for Bioremediation of Textile Wastewater." *Bioresource Technology* 101(19):7314–22.

Lin, L., G. Y. S. Chan, B. L. Jiang, and C. Y. Lan. 2007. "Use of Ammoniacal Nitrogen Tolerant Microalgae in Landfill Leachate Treatment." *Waste Management* 27(10):1376–82.

Lo, C. H., E. McAdam, and S. Judd. 2015. "The Cost of a Small Membrane Bioreactor." *Water Science and Technology* 72(10):1739–46.

López, CVG, MCC García, and FGA Fernández. 2010. "Protein Measurements of Microalgal and Cyanobacterial Biomass.Pdf." *Bioresource Technology* 101(19):7587–91.

Lotfi, Farhad Hosseinzadeh and Mohsen Rostamy-malkhalifeh. 2014. "Characteristics of Effluent from a Chemical Fertilizer Industry in Southern Nigeria." *Journal of Applied Science, Engineering and Technology* 7(1):211–17.

Lu, Chaoqun and Hanqin Tian. 2017. "Global Nitrogen and Phosphorus Fertilizer Use for Agriculture Production in the Past Half Century: Shifted Hot Spots and Nutrient Imbalance." *Earth Syst. Sci. Data* 9:181–92.

Lu, Liang, Shuai Zhang, Hui Li, Zhaode Wang, Jinjin Li, Zhijian Zhang, and Jun Zhu. 2009.

"A Reformed SBR Technology Integrated with Two-Step Feeding and Low-Intensity Aeration for Swine Wastewater Treatment." *Environmental Technology* 30(3):251–60.

Luo, Yunlong, Wenshan Guo, Huu Hao Ngo, Long Duc Nghiem, Faisal Ibney Hai, Jian Zhang, Shuang Liang, and Xiaochang C. Wang. 2014. "A Review on the Occurrence of Micropollutants in the Aquatic Environment and Their Fate and Removal during Wastewater Treatment." *Science of The Total Environment* 473–474:619–41.

M. Castellari, E. Sartini, U. Spinabelli, C. Riponi, and S. Galassi. 2001. "Determination of Carboxylic Acids, Carbohydrates, Glycerol, Ethanol, and 5-HMF in Beer by High-Performance Liquid Chromatography and UV–Refractive Index Double Detection." *Journal of Chromatographic Science* 39:235–38.

Mahmoud, Eman A., Laila A. Farahat, Zeinab K. Abdel Aziz, Nesreen A. Fatthallah, Rawheya A. Salah, and El Din. 2015. "Evaluation of the Potential for Some Isolated Microalgae to Produce Biodiesel." *Egyptian Journal of Petroleum* 24:97–101.

Manisha, S. C. Agrawal. 2007. "Growth, Survival and Reproduction InChlorella Vulgaris AndC. Variegata with Respect to Culture Age and under Different Chemical Factors." *Folia Microbiologica* 52(4):399–406.

Martin, C., J. de la Noüe, and G. Picard. 1985. "Intensive Cultivation of Freshwater Microalgae on Aerated Pig Manure." *Biomass* 7(4):245–59.

Martínez, Ma. E., F. Camacho, J. M. Jiménez, and J. B. Espínola. 1997. "Influence of Light Intensity on the Kinetic and Yield Parameters of Chlorella Pyrenoidosa Mixotrophic Growth." *Process Biochemistry* 32(2):93–98.

MASalem, Olfat. 2015. *Kinetic Study for Growth of Phormedium Sp. and Chlorella Vulgaris*. *International Journal of ChemTech Research*, *8*(9), 284-289.

Matusiak, K. 1976. "Studies on the Purification of Wastewater from the Nitrogen Fertilizer Industry by Intensive Algal Cultures. I. Growth of Chlorella Vulgaris in Wastes." *Acta Microbiologica Polonica* 25(3):233–42.

Mayo, A. W. and T. Bigambo. 2005. "Nitrogen Transformation in Horizontal Subsurface Flow Constructed Wetlands I: Model Development." *Physics and Chemistry of the Earth, Parts A/B/C* 30(11–16):658–67.

McKendry, Peter. 2002. "Energy Production from Biomass (Part 3): Gasification Technologies." *Bioresource Technology* 83(1):55–63.

Mehrdadi, N. and AA Azimi. 2006. "Determination of Design Criteria of an H-IFAS Reactor in Comparison with an Extended Aeration Activated Sludge Process." *Iranian Journal of Environmental Health Science & Engineering* 3(1):53–64.

Mendez, Lara, Ahmed Mahdy, Mercedes Ballesteros, and Cristina González-Fernández. 2015.

"Chlorella Vulgaris vs Cyanobacterial Biomasses: Comparison in Terms of Biomass Productivity and Biogas Yield." *Energy Conversion and Management* 92:137–42.

Mercedes Ciciarelli, Mara de las. 2012. "Life Cycle in Natural Populations of Canna Indica L. from Argentina." Pp. 101–16 in *Phenology and Climate Change*, edited by D. X. Zhang. InTech.

Meyer, Rikke Louise, Raymond Jianxiong Zeng, Valerio Giugliano, and Linda Louise Blackall. 2005. "Challenges for Simultaneous Nitrification, Denitrification, and Phosphorus Removal in Microbial Aggregates: Mass Transfer Limitation and Nitrous Oxide Production." *FEMS Microbiology Ecology* 52(3):329–38.

Miao, Xiaoling and Qingyu Wu. 2004. "High Yield Bio-Oil Production from Fast Pyrolysis by Metabolic Controlling of Chlorella Protothecoides." *Journal of Biotechnology* 110(1):85–93.

Miao, Xiaoling and Qingyu Wu. 2006. "Biodiesel Production from Heterotrophic Microalgal Oil." *Bioresource Technology* 97(6):841–46.

Min, Min, Liang Wang, Yecong Li, Michael J. Mohr, Bing Hu, Wenguang Zhou, Paul Chen, and Roger Ruan. 2011. "Cultivating Chlorella Sp. in a Pilot-Scale Photobioreactor Using Centrate Wastewater for Microalgae Biomass Production and Wastewater Nutrient Removal." *Applied Biochemistry and Biotechnology* 165(1):123–37.

Minowa, T. and S. Sawayama. 1999. "A Novel Microalgal System for Energy Production with Nitrogen Cycling." *Fuel* 78(10):1213–15.

Mishra, Neha and Neetu Mishra. 2017. "Microalgae for Integrated Biomass Production and Phycoremediation Utilization of Microalgae for Integrated Biomass Production and Phycoremediation of Wastewater." *J. Algal Biomass Utln* 8(4):95–105.

Mohammad, Munir J. and N. Mazahreh. 2003. "Changes in Soil Fertility Parameters in Response to Irrigation of Forage Crops with Secondary Treated Wastewater." *Communications in Soil Science and Plant Analysis* 34:1281–94.

Moheimani, Navid Reza and Michael A. Borowitzka. 2007. "Limits to Productivity of the Alga Pleurochrysis Carterae (Haptophyta) Grown in Outdoor Raceway Ponds." *Biotechnology and Bioengineering* 96(1):27–36.

Molazadeh, Marziyeh, Hossein Ahmadzadeh, Hamid R. Pourianfar, Stephen Lyon, and Pabulo Henrique Rampelotto. 2019. "The Use of Microalgae for Coupling Wastewater Treatment With CO2 Biofixation." *Frontiers in Bioengineering and Biotechnology* 7:42.

Mullin, JW. 2001. *Crystallization* Elsevier.

Mussgnug, J. H., V. Klassen, A. Schlüter, and O. Kruse. 2010. "Microalgae as Substrates for

Fermentative Biogas Production in a Combined Biorefinery Concept." *Journal of Biotechnology* 150(1):51–56.

Mustafa, Emienour Muzalina, Siew Moi Phang, and Wan Loy Chu. 2012. "Use of an Algal Consortium of Five Algae in the Treatment of Landfill Leachate Using the High-Rate Algal Pond System." *Journal of Applied Phycology* 24(4):953–63.

N.F.Y.Tam and Y. S. Wong. 1996. "Effect of Ammonia Concentrations on Growth of Chlorella Vulgaris and Nitrogen Removal from Media." *Bioresource Technology* 57:45–50.

Nagarajan, Sanjay, Siaw Kiang Chou, Shenyan Cao, Chen Wu, and Zhi Zhou. 2013. "An Updated Comprehensive Techno-Economic Analysis of Algae Biodiesel." *Bioresource Technology* 145:150–56.

Nallathambi Gunaseelan, V. 1997. "Anaerobic Digestion of Biomass for Methane Production: A Review." *Biomass and Bioenergy* 13(1–2):83–114.

Nascimento, V. M., G. F. Bueno, P. L. M. Aquino, and Del Bianchi. 2011. "Study of the Removal of Chemical Oxygen Demand of the Cheese Whey and Wastewater from Dairy Industry Using Spirulina Platensis." *Holos Environment* 11(1):161.

Nelson, NO, RL Mikkelsen, and DL Hesterberg. 2003. "Struvite Precipitation in Anaerobic Swine Lagoon Liquid: Effect of PH and Mg: P Ratio and Determination of Rate Constant." *Bioresource Technology* 89(3):229–36.

Nilsson, Chris and Hanna Dahlström. 2005. "Treatment and Disposal Methods for Wastewater Sludge in the Area of Beijing, China / Behandlings- Och Hanteringsmetoder För Avloppsslam i Pekingområdet, Kina." Lund University, Sweden.

Nkoa, Roger. 2014. "Agricultural Benefits and Environmental Risks of Soil Fertilization with Anaerobic Digestates: A Review." *Agronomy for Sustainable Development* 34(2):473–92.

Northcote, D. H., K. J. Goulding, and R. W. Horne. 1958. "The Chemical Composition and Structure of the Cell Wall of Chlorella Pyrenoidosa." *Biochemical Journal* 70(3):391–97.

Ohlinger, Kurt N., Thomas M. Young, and Edward D. Schroeder. 1999. "Kinetics Effects on Preferential Struvite Accumulation in Wastewater." *Journal of Environmental Engineering* 125(8):730–37.

Olguín, Eugenia J., Sonia Galicia, Gabriel Mercado, and Teresa Pérez. 2003. "Annual Productivity of Spirulina (Arthrospira) and Nutrient Removal in a Pig Wastewater Recycling Process under Tropical Conditions." *Journal of Applied Phycology* 15(2–3):249–57.

Özçimen, Didem and Benan Inan. 2015. "An Overview of Bioethanol Production From Algae." Pp. 141–62 in *Biofuels - Status and Perspective*, edited by Krzysztof Biernat.

Rijeka,croatia: InTech.

Pandey, JP, A. Tiwari, and RM Mishra. 2010. “Evaluation of Biomass Production of Spirulina Maxima on Different Reported Media.” *J. Algal Biomass Utln* 1(3):70–81.

Panja, S., D. Chaudhuri, N. B. Ghate, and N. Mandal. 2014. “Phytochemical Profile of a Microalgae Euglena Tuba and Its Hepatoprotective Effect against Iron-Induced Liver Damage in Swiss Albino Mice.” *Journal of Applied Microbiology* 117(6):1773–86.

Park, J. B. K. and R. J. Craggs. 2007. “Nutrient Removal in Wastewater Treatment High Rate Algal Ponds with Carbon Dioxide Addition.” *Water Science & Technology\r* 55:257–64.

Park, J., HF Jin, BR Lim, KY Park, and K. Lee. 2010. “Ammonia Removal from Anaerobic Digestion Effluent of Livestock Waste Using Green Alga Scenedesmus Sp.” *Bioresource Technology* 101(22):8649–57.

Park, J., HF Jin, BR Lim, KY Park, K. Lee-Bioresource technology, and undefined 2010. 2010. “Ammonia Removal from Anaerobic Digestion Effluent of Livestock Waste Using Green Alga Scenedesmus Sp.” *Bioresource Technology* 101:8649–57.

Passos, F., E. Uggetti, H. Carrère, and I. Ferrer. 2014. “Pretreatment of Microalgae to Improve Biogas Production: A Review.” *Bioresource Technology* 172:403–12.

Passos, Fabiana and Ivet Ferrer. 2014. “Microalgae Conversion to Biogas: Thermal Pretreatment Contribution on Net Energy Production.” *Environmental Science and Technology* 48(12):7171–78.

Passos, Fabiana, Joan Garca, and Ivet Ferrer. 2013. “Impact of Low Temperature Pretreatment on the Anaerobic Digestion of Microalgal Biomass.” *Bioresource Technology* 138:79–86.

Passos, Fabiana, Andrea Hom-Diaz, Paqui Blanquez, Teresa Vicent, and Ivet Ferrer. 2016. “Improving Biogas Production from Microalgae by Enzymatic Pretreatment.” *Bioresource Technology* 199:347–51.

Patil, Vishwanath, Khanh-Quang Tran, and Hans Ragnar Giselrød. 2008. “Towards Sustainable Production of Biofuels from Microalgae.” *International Journal of Molecular Sciences* 9(7):1188–95.

Petruševski, B., G. Bolier, A. N. Van Breemen, and G. J. Alaerts. 1995. “Tangential Flow Filtration: A Method to Concentrate Freshwater Algae.” *Water Research* 29(5):1419–24.

Phang, S. M., M. S. Miah, B. G. Yeoh, and M. A. Hashim. 2000. “Spirulina Cultivation in Digested Sago Starch Factory Wastewater.” *Journal of Applied Phycology* 12(3/5):395–400.

Pittman, Jon K., Andrew P. Dean, and Olumayowa Osundeko. 2011. “The Potential of Sustainable Algal Biofuel Production Using Wastewater Resources.” *Bioresource Technology* 102(1):17–25.

Posten, Clemens. 2009. “Design Principles of Photo-Bioreactors for Cultivation of Microalgae.” *Engineering in Life Sciences* 9(3):165–77.

Prabhu, M. and S. Mutnuri. 2014. “Cow Urine as a Potential Source for Struvite Production.” *International Journal of Recycling of Organic Waste* 3:49.

Prabhu, Meghanath S. and Srikanth Mutnuri. 2016. “Anaerobic Co-Digestion of Sewage Sludge and Food Waste.” *Waste Management & Research* 34(4):307–15.

Prakash, Justella Wilfred, Johnson Marimuthu Antonisamy, and Solomon Jeeva. 2011. “Antimicrobial Activity of Certain Fresh Water Microalgae from Thamirabarani River, Tamil Nadu, South India.” *Asian Pacific Journal of Tropical Biomedicine* 1(2):S170–73.

Prateepchaikul, Gumpon, Michael L. Allen, and Theerayut Leevijit. 2007. “Methyl Ester Production From High Free Fatty Acid Mixed Crude Palm Oil.” *Songklanakarin Journal of Science Technology* 29(2007):1551–61.

Prokop, Aleš, Rakesh K. Bajpai, and Mark E. Zappi. 2015. *Algal Biorefineries. Volume 2, Products and Refinery Design*. edited by A. P. · R. K. Bajpai and M. E. Zappi. LA , USA: Springer International Publishing AG Switzerland.

Przytocka-Jsiak, M. 1976. “Growth and Survival of Chlorella Vulgaris in High Concentrations of Nitrogen.” *Acta Microbiologica Polonica* 25(3):287–89.

Pulz, O. and W. Gross. 2004. “Valuable Products from Biotechnology of Microalgae.” *Applied Microbiology and Biotechnologyiotechnology* 65(6):635–648.

Pulz Wolfgang Gross, Otto. 2004. “Mini-Review Valuable Products from Biotechnology of Microalgae.” *Appl Microbiol Biotechnol* 65(6):635–48.

Le Quéré, Corinne, Michael R. Raupach, Josep G. Canadell, and Gregg Marland. 2009. “Trends in the Sources and Sinks of Carbon Dioxide.” *Nature Geoscience* 2:831–36.

Radmann, E. M., C. O. Reinehr, and J. A. V Costa. 2007. “Optimization of the Repeated Batch Cultivation of Microalga Spirulina Platensis in Open Raceway Ponds.” *Aquaculture* 265(1–4):118–26.

Rana, Rajender Singh, Prashant Singh, Vikash Kandari, Rakesh Singh, Rajendra Dobhal, and Sanjay Gupta. 2017. “A Review on Characterization and Bioremediation of Pharmaceutical Industries’ Wastewater: An Indian Perspective.” *Applied Water Science* 7(1):1–12.

Randall, Clifford W., James Lang Barnard, and H. David Stensel, Eds., ed. 1992. *Design and Retrofit of Wastewater Treatment Plants for Biological Nutritient Removal*. Vol 5. Technomic Publishing Company, Inc.

Raposo, Maria Filomena de Jesus and Alcina Maria Miranda Bernardo de Morais. 2015. “Microalgae for the Prevention of Cardiovascular Disease and Stroke.” *Life Sciences*

125:32–41.

Rasit, Nazaitulshila, Azni Idris, Razif Harun, and Wan Azlina Wan Ab Karim Ghani. 2015. "Effects of Lipid Inhibition on Biogas Production of Anaerobic Digestion from Oily Effluents and Sludges: An Overview." *Renewable and Sustainable Energy Reviews* 45:351–58.

Rawat, I., R. Ranjith Kumar, T. Mutanda, and F. Bux. 2011. "Dual Role of Microalgae: Phycoremediation of Domestic Wastewater and Biomass Production for Sustainable Biofuels Production." *Applied Energy* 88(10):3411–24.

Richa Kothari1*, Virendra Kumar1, and and Vineet Veer Tyagi2. 2011. "Assessment Of Waste Treatment And Energy Recovery From Dairy Industrial Waste By Anaerobic Digestion." *The IIOAB Journal* 2(1):1–6.

Rodolfi, Liliana, Graziella Chini Zittelli, Niccolò Bassi, Giulia Padovani, Natascia Biondi, Gimena Bonini, and Mario R. Tredici. 2009. "Microalgae for Oil: Strain Selection, Induction of Lipid Synthesis and Outdoor Mass Cultivation in a Low-Cost Photobioreactor." *Wiley InterScience (Www.Interscience.Wiley.Com). Biotechnol. Bioeng* 102:100–112.

Rožić, M., Š. Cerjan-Stefanović, S. Kurajica, V. Vančina, and E. Hodžić. 2000. "Ammoniacal Nitrogen Removal from Water by Treatment with Clays and Zeolites." *Water Research* 34(14):3675–81.

RR, Siva Kiran, Madhu GM, and Satyanarayana SV. 2015. "Spirulina in Combating Protein Energy Malnutrition (PEM) and Protein Energy Wasting (PEW) - A Review." *Journal of Nutrition Research* 3(1):62–79.

Ruiz-Marin, Alejandro, Leopoldo G. Mendoza-Espinosa, and Tom Stephenson. 2010. "Growth and Nutrient Removal in Free and Immobilized Green Algae in Batch and Semi-Continuous Cultures Treating Real Wastewater." *Bioresource Technology* 101(1):58–64.

Ryther, J. H. and W. M. Dunstan. 1971. "Nitrogen Phosphorus and Eutrophication in the Coastal Marine Environment." *Sci.* 171(3975):1008–13.

Saad H. Ammar. 2016. "Cultivation of Microalgae Chlorella Vulgaris in Airlift Photobioreactor for Biomass Production Using Commercial NPK Nutrients." *Al-Khwarizmi Engineering Journal* 12(1):90–99.

Saeed, Tanveer and Guangzhi Sun. 2013. "A Lab-Scale Study of Constructed Wetlands with Sugarcane Bagasse and Sand Media for the Treatment of Textile Wastewater." *Bioresource Technology* 128:438–47.

Safi, Carl, Bachar Zebib, Othmane Merah, Pierre-Yves Pontalier, and Carlos Vaca-Garcia. 2014. "Morphology, Composition, Production, Processing and Applications of Chlorella

Vulgaris: A Review." *Renewable and Sustainable Energy Reviews* 35:265–78.

Samorì, Giulia, Chiara Samorì, Franca Guerrini, and Rossella Pistocchi. 2013. "Growth and Nitrogen Removal Capacity of Desmodesmus Communis and of a Natural Microalgae Consortium in a Batch Culture System in View of Urban Wastewater Treatment: Part I." *Water Research* 47(2):791–801.

Santos, A., W. Ma, and S. J. Judd. 2011. "Membrane Bioreactors: Two Decades of Research and Implementation." *Desalination* 273(1):148–54.

Sato, Toshio, Manzoor Qadir, Sadahiro Yamamoto, Tsuneyoshi Endo, and Ahmad Zahoor. 2013. "Global, Regional, and Country Level Need for Data on Wastewater Generation, Treatment, and Use." *Agricultural Water Management* 130:1–13.

Schenk, Peer M., Skye R. Thomas-Hall, Evan Stephens, Ute C. Marx, Jan H. Mussgnug, Clemens Posten, Olaf Kruse, and Ben Hankamer. 2008. "Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production." *BioEnergy Research* 1(1):20–43.

Sezgin, Mesut. 1982. "Variation of Sludge Volume Index with Activated Sludge Characteristics." *Water Research* 16(1):83–88.

Sh. J.S. Kamyotra, Dr. D.D. Basu, R.M. Bhardwaj,A K Sinha, A K Sinha, Vishal Gandhi, Garima Dublish, Anita Pal. 2013. *Performance Evaluation of Sewage Treatment Plants under NRCD*. Delhi.

Sialve, Bruno, Nicolas Bernet, Olivier Bernard, and Olivier Bernard Anaerobic. 2009. *Anaerobic Digestion of Microalgae as a Necessary Step to Make Microalgal Biodiesel Sustainable. Anaerobic Digestion of Microalgae as a Necessary Step to Make Microalgal Biodiesel Sustainable 4 5*. Vol. 27. Elsevier.

Singh, Manjinder, David L. Reynolds, and Keshav C. Das. 2011. "Microalgal System for Treatment of Effluent from Poultry Litter Anaerobic Digestion." *Bioresource Technology* 102(23):10841–48.

Singh, Mohini and R. K. Srivastava. 2011. "Sequencing Batch Reactor Technology for Biological Wastewater Treatment: A Review." *Asia-Pacific Journal of Chemical Engineering* 6(1):3–13.

Sklarz, M. Y., A. Gross, A. Yakirevich, and M. I. M. Soares. 2009. "A Recirculating Vertical Flow Constructed Wetland for the Treatment of Domestic Wastewater." *Desalination* 246(1–3):617–24.

Soares, RB, MS Memelli, … RP Roque-International Journal of, and undefined 2017. 2017. "Comparative Analysis of the Energy Consumption of Different Wastewater Treatment Plants." *International Journal of Architecture, Arts and Applications* 3(6):79.

Sonune, NA, NA Mungal, and SP Kamble. 2015. "Study of Physico-Chemical Characteristics

of Domestic Wastewater in Vishnupuri, Nanded, India." *Int.J.Curr.Microbiol.App.Sci* 4(1):533–36.

Sorrell, Steve, Jamie Speirs, Roger Bentley, Adam Brandt, and Richard Miller. 2009. *An Assessment of the Evidence for a Near-Term Peak in Global Oil Production A Report Produced by the Technology and Policy Assessment Function of the UK Energy Research Centre*.

Stevens, Christian V, James H. Clark, and Fabien EI Deswarte. 2008. *Introduction to Chemicals from Biomass*. York,UK.

Stillwell, Ashlynn, David Hoppock, Michael Webber, Ashlynn S. Stillwell, David C. Hoppock, and Michael E. Webber. 2010. "Energy Recovery from Wastewater Treatment Plants in the United States: A Case Study of the Energy-Water Nexus." *Sustainability* 2(4):945–62.

Subashchandrabose, Suresh R., Balasubramanian Ramakrishnan, Mallavarapu Megharaj, Kadiyala Venkateswarlu, and Ravi Naidu. 2011. "Consortia of Cyanobacteria/Microalgae and Bacteria: Biotechnological Potential." *Biotechnology Advances* 29(6):896–907.

Sunja Cho A, Thanh Thao Luong a, Dukhaeng Lee a, You-Kwan Oh b, and ⇑ Taeho Lee a. 2011. "Reuse of Effluent Water from a Municipal Wastewater Treatment Plant in Microalgae Cultivation for Biofuel Production." *Bioresource Technology* 102:8639–45.

Suresh Kumar D. and Dr. Sekaran V. 2013. "FBBR and MBBR Bioreactors for Sewage Treatment." *International Journal of Environmental Engineering and Management,* 4(4):73–88.

Sushmita Sengupta and Sunita Narain. 2018. *STATE OF INDIA'S ENVIRONMENT 2018*. Delhi: DownToEarth Annual.

Suzuki, Kazuyoshi, Yasuo Tanaka, Kazutaka Kuroda, Dai Hanajima, Yasuyuki Fukumoto, Tomoko Yasuda, and Miyoko Waki. 2007. "Removal and Recovery of Phosphorous from Swine Wastewater by Demonstration Crystallization Reactor and Struvite Accumulation Device." *Bioresource Technology* 98(8):1573–78.

Tam, N. F. Y. and Y. S. Wong. 1989. "Wastewater Nutrient Removal by Chlorella Pyrenoidosa and Scenedesmus Sp." *Environmental Pollution* 58(1):19–34.

Tanimu, Musa I., Tinia I. Mohd Ghazi, Razif M. Harun, and Azni Idris. 2014. "Effect of Carbon to Nitrogen Ratio of Food Waste on Biogas Methane Production in a Batch Mesophilic Anaerobic Digester." *International Journal of Innovation, Management and Technology* 5(2):116–19.

Tchobanoglous, George, Franklin L. Burton, and H. David Stensel. 1991. "Wastewater Engineering: An Overview." Pp. 1–24 in *Wastewater Engineering Treatment and Reuse*.

Metcalf & Eddy, Inc.

Thakre, Chitesh Y. and Arif Khan. 2017. “Impact of Fertilizer Industry Effluent on Land Environment: A Review.” *International Research Journal of Engineering and Technology* 4(5):1005–8.

Tomaszek, J. A. and M. Grabas. 2007. “Carbon and Nitrogen Removal in a Biofilm Reactors System with Raw Wastewater Stream Distribution.” *Environmental Technology* 28(5):555–63.

Topare, Niraj S., S. J. Attar, and Mosleh M. Manfe. 2011. *Sewage/Wastewater Treatment Technologies : A Review*.

Tsukahara K, Sawayama S. 2005. “Liquid Fuel Production Using Microalgae.” *Journal of the Japan Petroleum Institute* 48(5):251–59.

Turken, Turker, Borte Kose-Mutlu, Selin Okatan, Gamze Durmaz, Mehmet C. Guclu, Serkan Guclu, Suleyman Ovez, and Ismail Koyuncu. 2019. “Long-Term MBR Performance of Polymeric Membrane Modified with Bismuth-BAL Chelate (BisBAL).” *Environmental Technology (United Kingdom)* 40(15):2011–17.

Ueno, Ryohei, Naoto Urano, and Shigeru Kimura. 2002. “Effect of Temperature and Cell Density on Ethanol Fermentation by a Thermotolerant Aquatic Yeast Strain Isolated from a Hot Spring Environment.” *Fisheries Science* 68(3):571–78.

Vacek, Jan. 2010. “Nitrogen Content , Dietary Fiber , and Digestibility in Algal Food Products.” *Czech Journal of Food* 28(1):27–35.

Valderrama, Luz T., Claudia M. Del Campo, Claudia M. Rodriguez, Luz E. de- Bashan, and Yoav Bashan. 2002. “Treatment of Recalcitrant Wastewater from Ethanol and Citric Acid Production Using the Microalga Chlorella Vulgaris and the Macrophyte Lemna Minuscula.” *Water Research* 36(17):4185–92.

Varol, Ayhan and Aysenur Ugurlu. 2016. “Biogas Production from Microalgae (Spirulina Platensis) in a Two Stage Anaerobic System.” *Waste and Biomass Valorization* 7(1):193–200.

Verhoeven, Jos T. .. and Arthur F. .. Meuleman. 1999. “Wetlands for Wastewater Treatment: Opportunities and Limitations.” *Ecological Engineering* 12(1–2):5–12.

Victoria González López, Cynthia, María Del Carmen, Cerón García, Francisco Gabriel, Acién Fernández, Cristina Segovia Bustos, Yusuf Chisti, José María, and Fernández Sevilla. 2010. “Protein Measurements of Microalgal and Cyanobacterial Biomass.” *Bioresource Technology* 101:7587–91.

Vidyadharani, G. and R. Dhandapani. 2016. “Fourier Transform Infrared (FTIR) Spectroscopy for the Analysis of Lipid from Chlorella Vulgaris.” *Applied Biology*

61:16753–56.

Vollenweider, R. A. 1968. *Scientific Fundamentals of the Eutrophication of Lakes and Flowing Waters, with Particular Reference to Nitrogen and Phosphorus as Factors in Eutrophication.* Vol. 3.

Vymazal, Jan. 2007. "Removal of Nutrients in Various Types of Constructed Wetlands." *Science of The Total Environment* 380(1–3):48–65.

Vymazal, Jan. 2011. "Constructed Wetlands for Wastewater Treatment: Five Decades of Experience." *Environmental Science and Technology* 45(1):61–69.

Wang, Liang, Min Min, Yecong Li, Paul Chen, Yifeng Chen, Yuhuan Liu, Yingkuan Wang, and Roger Ruan. 2010. "Cultivation of Green Algae Chlorella Sp. in Different Wastewaters from Municipal Wastewater Treatment Plant." *Applied Biochemistry and Biotechnology* 162(4):1174–86.

Wang, Yue, Wanqian Guo, Hong-Wei Yen, Shih-Hsin Ho, Yung-Chung Lo, Chieh-Lun Cheng, Nanqi Ren, and Jo-Shu Chang. 2015. "Cultivation of Chlorella Vulgaris JSC-6 with Swine Wastewater for Simultaneous Nutrient/COD Removal and Carbohydrate Production." *Bioresource Technology* 198:619–25.

Wang, Zixing, Xiaochen Xu, Zheng Gong, and Fenglin Yang. 2012. "Removal of COD, Phenols and Ammonium from Lurgi Coal Gasification Wastewater Using A2O-MBR System." *Journal of Hazardous Materials* 235–236:78–84.

Wase, NV, PC Wright-Expert opinion on drug discovery, and undefined 2008. n.d. "Systems Biology of Cyanobacterial Secondary Metabolite Production and Its Role in Drug Discovery." *Taylor & Francis*.

Wichelns, Dennis, Pay Drechsel, and Manzoor Qadir. 2015. "Wastewater: Economic Asset in an Urbanizing World." Pp. 3–14 in *Wastewater*, edited by P. Drechsel, M. Qadir, and D. Wichelns. Dordrecht: Springer Netherlands.

Woertz, I., A. Feffer, … T. Lundquist-Journal of Environmental, and Undefined 2009. 2011. "Algae Grown on Dairy and Municipal Wastewater for Simultaneous Nutrient Removal and Lipid Production for Biofuel Feedstock." . . *J Environ Eng* 135(11):1158–1123.

Wu, Q. and PL Bishop. 2004. "Enhancing Struvite Crystallization from Anaerobic Supernatant." *Journal of Environmental Engineering and Science* 3(1):21–29.

Wu, Shubiao, Peter Kuschk, Hans Brix, Jan Vymazal, and Renjie Dong. 2014. "Development of Constructed Wetlands in Performance Intensifications for Wastewater Treatment: A Nitrogen and Organic Matter Targeted Review." *Water Research* 57:40–55.

Xin, Li, Hu Hong-ying, Gan Ke, and Sun Ying-xue. 2010. "Effects of Different Nitrogen and Phosphorus Concentrations on the Growth, Nutrient Uptake, and Lipid Accumulation of

a Freshwater Microalga Scenedesmus Sp." *Bioresource Technology* 101(14):5494–5500.

Xu, Jiabo, Yonghai Shi, Genyu Zhang, Jianzhong Liu, and Yazhu Zhu. 2014. "Effect of Hydraulic Loading Rate on the Efficiency of Effluent Treatment in a Recirculating Puffer Aquaculture System Coupled with Constructed Wetlands." *Journal of Ocean University of China* 13(1):146–52.

Yadav, Anant, Florent Chazarenc, and Srikanth Mutnuri. 2018. "Development of the 'French System' Vertical Flow Constructed Wetland to Treat Raw Domestic Wastewater in India." *Ecological Engineering* 113:88–93.

Yadav, Anant, Piyush Choudhary, Neelam Atri, Sebastian Teir, and Srikanth Mutnuri. 2016a. "Pilot Project at Hazira, India, for Capture of Carbon Dioxide and Its Biofixation Using Microalgae." *Environmental Science and Pollution Research* 23(33):22284–22291.

Yadav, Anant, Piyush Choudhary, Neelam Atri, Sebastian Teir, and Srikanth Mutnuri. 2016b. "Pilot Project at Hazira, India, for Capture of Carbon Dioxide and Its Biofixation Using Microalgae." *Environmental Science and Pollution Research* 23(22):22284–91.

Ye, Yaoli, Nicole LaBarge, Hiroyuki Kashima, Kyoung-Yeol Kim, Pei-Ying Hong, Pascal E. Saikaly, and Bruce E. Logan. 2016. "An Aerated and Fluidized Bed Membrane Bioreactor for Effective Wastewater Treatment with Low Membrane Fouling." *Environmental Science: Water Research & Technology* 2(6):994–1003.

Yun, Yeoung-Sang, Sun Bok Lee, Jong Moon Park, Choong-Il Lee, and Ji-Won Yang. 1997. "Carbon Dioxide Fixation by Algal Cultivation Using Wastewater Nutrients." *Journal of Chemical Technology & Biotechnology* 69(4):451–55.

Z. J. Pei, Q. Wu, E. Mao, Y. Shen, and W. Yuan. 2013. "Microalgae Mass Production Methods." *Transactions of the ASABE* 52(4):1275–87.

Zhou, Na, Yimin Zhang, Xiaowu Gong, Qinhong Wang, and Yanhe Ma. 2012. "Ionic Liquids-Based Hydrolysis of Chlorella Biomass for Fermentable Sugars." *Bioresource Technology* 118:512–17.

Zhu, L. D., E. Hiltunen, E. Antila, J. J. Zhong, Z. H. Yuan, and Z. M. Wang. 2014. "Microalgal Biofuels: Flexible Bioenergies for Sustainable Development." *Renewable and Sustainable Energy Reviews* 30:1035–46.

Zhu, Liandong. 2014. "The Combined Production of Ethanol and Biogas from Microalgal Residuals to Sustain Microalgal Biodiesel: A Theoretical Evaluation." *Biofuels, Bioproducts and Biorefining* 8(1):7–15.

Zhu, Liandong, Zhongming Wang, Qing Shu, Josu Takala, Erkki Hiltunen, Pingzhong Feng, and Zhenhong Yuan. 2013. "Nutrient Removal and Biodiesel Production by Integration of Freshwater Algae Cultivation with Piggery Wastewater Treatment." *Water Research*

47(13):4294–4302.

Zhu, Shunni, Yajie Wang, Wei Huang, Jin Xu, Zhongming Wang, Jingliang Xu, and Zhenhong Yuan. 2014. “Enhanced Accumulation of Carbohydrate and Starch in Chlorella Zofingiensis Induced by Nitrogen Starvation.” *Applied Biochemistry and Biotechnology* 174(7):2435–45.

Appendix

Appendix I

Supplementary Material I

Identification of Yeast strain

Step 1. DNA extraction from fungal sample
DNA was extracted from fungal samples by using Fungal Genomic DNA Isolation kit (RKT13,Chromous Biotech-Banglore). The extracted DNA was run on 1 % Agarose gel.

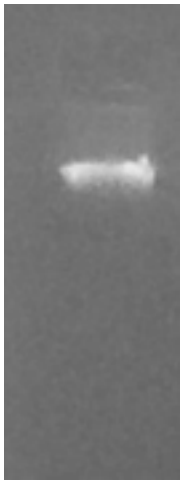


Fig.1 Extracted genomic DNA band on 1 % Agarose gel.

Step 2 . PCR amplification of 18s rDNA region from genomic DNA

2.1 PCR Amplification conditions:

DNA: 1 µl (100ng)
Forward Primer -400ng
Reverse Primer -400ng
dNTPs (10mM each) -4µl
10X C hrom Taq DNA Polymerase Assay Buffer -10 µl
Chrom Taq DNA Polymerase Enzyme (3U/ µl) -1 µl
Water -70 µl
Total reaction volume: 100 µl

2.2 PCR profile:

94 °C	94 °C	55 °C	72 °C	72 °C
5 min	30 seconds	30 seconds	2 minutes	7 minutes
	35 cycles			

2.3 Electrophoresis of PCR Product:

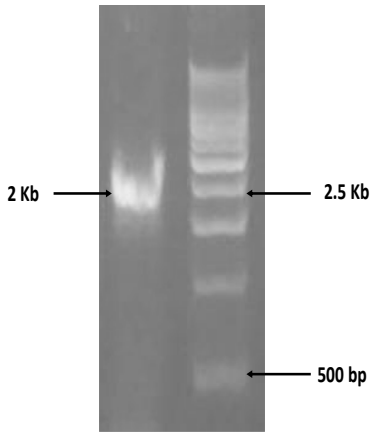


Fig.2. Electrophoresis of PCR product (PCR amplification of 18s rDNA region from fungal sample. The size of PCR amplified product is ~ 2kb).

Step 3. Sequencing of PCR product:

3.1 Sequencing Reaction:

The Sequencing mix Composition and PCR Conditions are as follows:
10µl Sequencing Reaction
• Big Dye Terminator
Ready Reaction Mix : 4µl
• Template (100ng/ul) :1µl
• Primer (10pmol/λ) :2µl
• Milli Q Water :3µl

3.2 PCR Conditions: (25 cycles):

Initial Denaturation : 96°C for 1min
Denaturation : 96°C for 10 sec
Hybridization : 50 °C for 5 sec
Elongation : 60 °C for 4 m

3.3 Aligned Sequence Data (1666 bp):

TAAAGGGCAAATTTATACAGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTT
ATTTGATAGTTCCTTTACTACATGGTATAACTGTGGTAATTCTAGAGCTAATACATGC
TTAACATCTCGACCTCTTGGAAGAGATGTATTTATTAGATAAAAAATCAATGTCTTC
GGACTTTTTGATGATTCATAATAACTTTTCGAATCGCATGGCCTTGTGCTGGCGATGG
TTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGTGGCCTACCATGGTT
TCAACGGGTAACGGGGAATAAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGC
TACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAG
GTAGTGACAATAAATAACGATACAGGGCCCATTCGGGTCTTGTAATTGGAATGAGT
ACAATGTAAATACCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCC
GCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTA
GTTGAACTTTGGGCCTGGGTGGCCGGTCCGATTTTTTCGTGTACTGGAATGCACCCG

GGCCTTTCCTTCTGGCTAACCCCAAGTCCTTGTGGCTTGGCGGCGAACCAGGACTTTT
ACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCGTATTGCTCGAATATATTAGCATGG
AATAATGGAATAGGACGTTTGGTTCTATTTTTGTTGGTTTCTAGGAACCATCCGTAA
ATGATTAATAGGGACGGTCGGGGGGCATCAGTATTCAATTGTCAGAGGTGAAATTCT
TGGATTTATTGAAGACTAACTACTGCGAAAGCATTTGCCAAGGACGTTTTCATTAAT
CAAGAACGAAAGTTAGGGGATCGAAGATGATCAGATACCGTCGTAGTCTTAACCAT
AAACTATGCCGACTAGGGATCGGGTGGTGTTTTTTTAGTGACCCACTCGGCACCTTA
CGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTA
AAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCA
ACACGGGGAAACTCACCAGGTCCAGACACAATAAGGATTGACAGATTGAGAGCTCT
TTCTTGATTTTGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCT
GCTTAATTGCGATAACGAACGAGACCTTAACCTACTAAATAGTGGTGCTAGCATTTG
CTGGTTGTCCACTTCTTAGAGGGACTATCGGTTTCAAGCCGATGGAAGTTTGAGGCA
ATAACAGGTCTGTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGG
AGCCAGCGAGTCTAACCTTGGCCGAGAGGTCTTGGTAATCTTGTGAAACTCCGTCGT
GCTGGGGATAGAGCATTGTAATTATTGCTCTTCAACGAGGAATTCCTAGTAAGCGCA
AGTCATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTAGT
ACCGATTGAATGGCTTAGTGAGGCCTCAGGATCTGCTTAGAAGAGGGGGCGACTCC
ACTTCAGAGACGGGGA

Result:

Sl. No.	Organism Name	Accession No.	% Match
1	Candida glabrata strain SZ2	KT229542.1	99%
2	Candida glabrata strain CBS138	CR380958.2	99%
3	Candida glabrata	AY046237.1	99%
4	Candida glabrata strain CBS 138	AY198398.1	99%
5	Candida edaphicus	AB247500.1	99%
6	Candida glabrata	X51831.1	99%
7	Candida glabrata strain N2	AY218893.1	99%
8	Hot spring yeast RND13	AB071282.1.	99%
9	Kluyveromyces delphensis strain CBS2170.	AY198400.1	99%
10	Candida glabrata	AY083230.1	99%

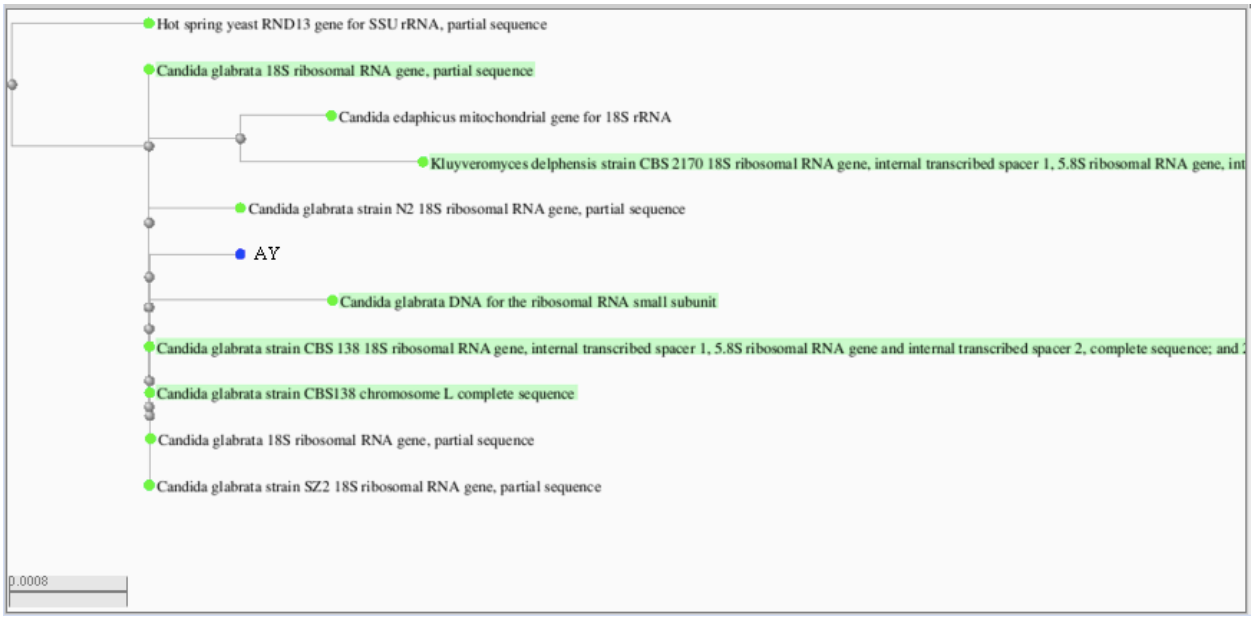


Fig.3. Phylogenetic Tree

Instrument and Chemistry Details:

Sequencing Machine : ABI 3500XL Genetic Analyzer
Chemistry : Big Dye Terminator version 3.1”
Cycle sequencing kit.
Polymer & Capillary Array : POP_7 polymer 50 cm Capillary Array.
Analysis protocol : BDTv3-KB-Denovo_v 5.2
Data Analysis : Seq Scape_ v 5.2 Software
Reaction Plate : Applied Biosystem Micro Amp
Optical 96-Well Reaction plate

Overall Methodology:

• The ~ 2kb 18srDNA region was amplified using high –fidelity PCR polymerase.
• The PCR product was sequenced bidirectionally.
• The sequence data was analyzed to identify the culture and its closest neighbors.

Final Result:

1. The Sample was found to be closest to Candida glabrata strain SZ2 18S ribosomal RNA gene, partial sequence Sequence ID: gb|KT229542.1|

2. The next closest homologue was found to be Candida glabrata strain CBS138 chromosome L complete sequence Sequence ID: emb|CR380958.2|

Ethanol production from microalgal biomass

Table1 shows the concentration of ethanol(g/L) and their respective peak area (volts).

Table 1. Ethanol standard

Conc. (g/L)	Area (Volts)
1	413493600.79
2	826975701.58
3	1220195632.37
4	1653966233.16

Fig. 4 shows the concentration of ethanol(g/L) and their respective peak area (volts)- standard curve of ethanol:

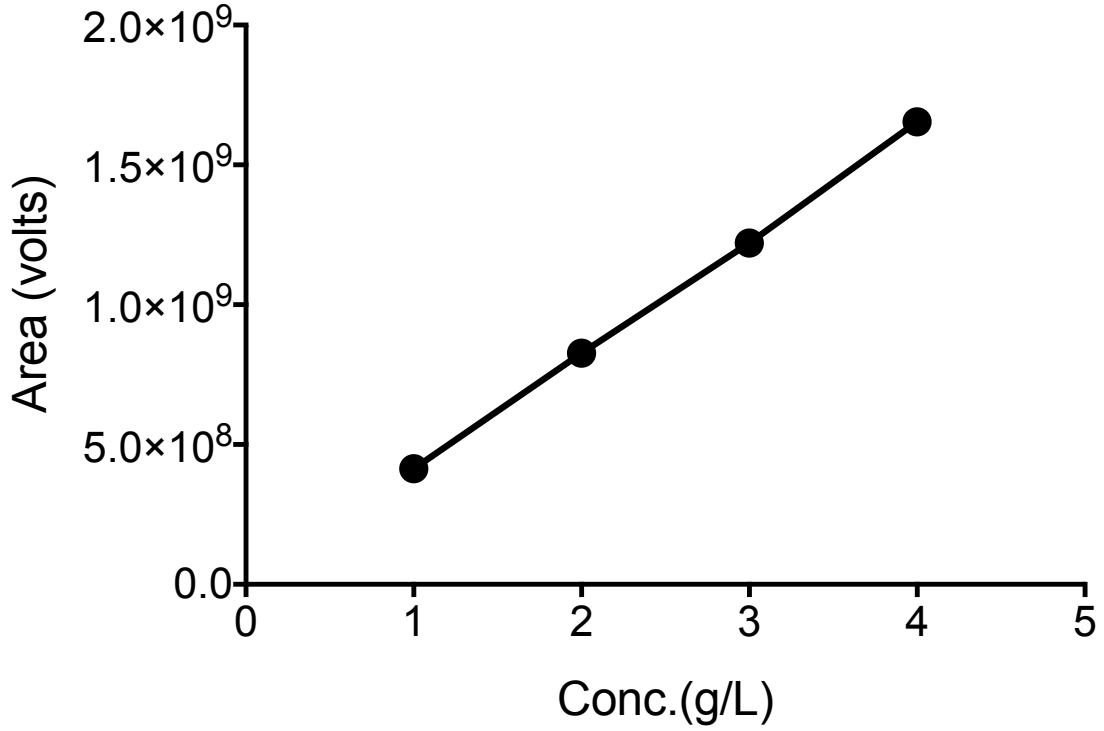


Fig.4 Standard curve of ethanol

Fig. 5 depicts the and Table. 2 depicts the analysis of ethanol content of sample.

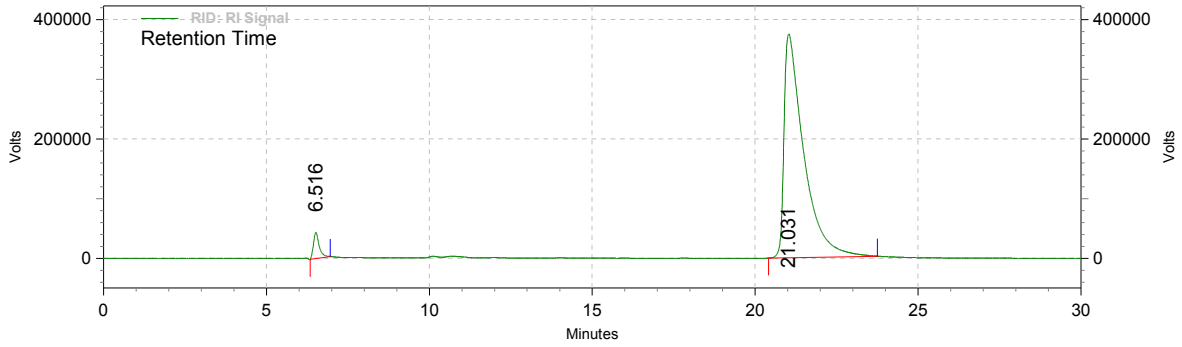


Fig. 5 Ethanol analysis by HPLC

Table 2 Result of ethanol analysis by HPLC

Retention Time	Area	Area %	Height	Height %
6.516	53523172	3.29	4384340	10.46
21.031	1571290883	96.71	37511100	89.54
Totals	1624814055	100.00	41895440	100.00

Appendix II

Supplementary Material II

To estimate minimum number of organism present in the BPGC consortium by t-RFLP analysis.

Steps Followed:

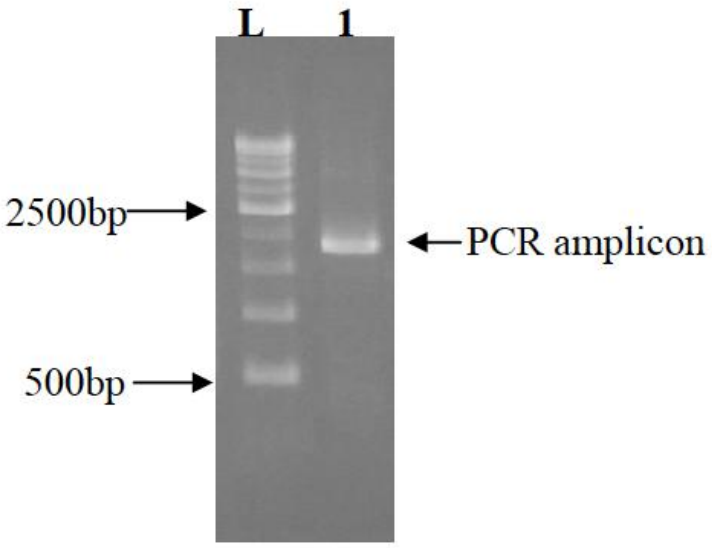
- Genomic DNA was extracted from BPGC consortium.
- The ~1800bp 18s rDNA region was amplified using high–fidelity PCR polymerase with 6-carboxyfluorescein (FAM)-labeled primers.
- The PCR products were subjected to restriction digestion with a 4-base cutter (HpaII).
- The fluorescent labeled fragments were size separated on an ABI 3500xL automated sequencer (Applied Biosystems) using an internal size standard (LIZ- 500).
- t- RFLP electropherograms were analyzed with GeneMapper software version 4.1 (Applied Biosystems).
- The numbers of peaks obtained in profiles are an approximate representation of minimum number of bacteria present in the sample.

PCR Reaction mix:

- Template (100ng/µl gDNA) -1.0 uL
- Forward Primer (100ng/µl) - 1.0 uL
- Reverse primer (100ng/µl) - 1.0 uL
- dNTPs (10mM) - 1.0 uL
- 10X Taq Assay Buffer -2.5 uL
- Taq Enzyme (3U/µl) - 0.25 uL
- Water -0.25 uL
- Total reaction volume -18.25 uL

PCR Cycle Condition:

94 ºC	94 ºC	50 ºC	72 ºC	72 ºC
5 min	30 sec	30 sec	90 sec	7 min
	35 cycles			



Pic: PCR Amplicon loaded on 1% Agarose Gel

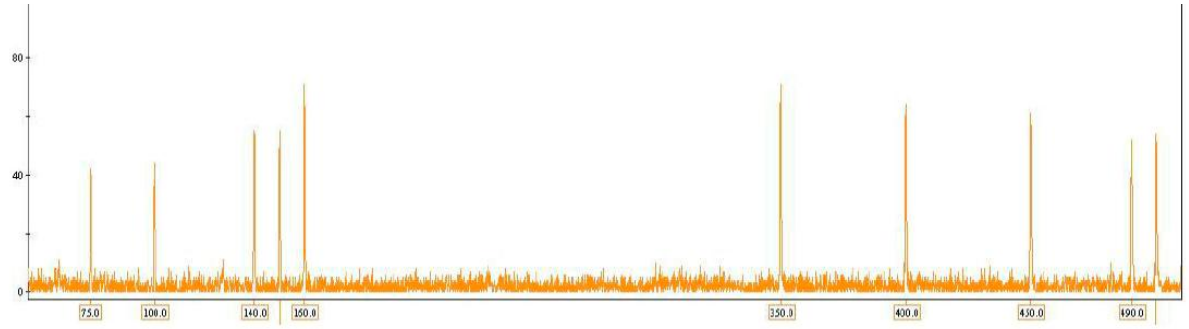
Lane Description: 1 – PCR amplicon, L – 500bp DNA Ladder

RESULT:

Minimum number of Bacteria found in the given sample is 4.

OTHER RELAVENT INFORMATION:

• 6-carboxyfluorescein (FAM)-labeled primers gives blue color in Genescan analysis. (Please note that Blue colored peaks which corresponds to tRF's are sizedby internal size standard)
• Orange peaks are internal size standard (LIZ 500, Chromous)



The GeneScan™ 500 LIZ® Size Standard is a fifth dye-labeled size standard for the reproducible sizing of fragment analysis data. Use this size standard for fragments between 35 and 500 bp. The standard contains 16 LIZ® dye-labeled, single-stranded DNA fragments. Since the standard is labeled with the fifth dye, users can genotype a greater number of markers in a given lane, compared to the four-dye system.
Size Fragments in the 35-500 Nucleotides Range GeneScan™ 500 LIZ® Size Standard is designed for sizing DNA fragments in the 35-500 nucleotides range and provides 16 single-stranded labeled fragment of: 35, 50, 75, 100,139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 nucleotides. The sizing curve generated from these fragments make the GeneScan™ 500 LIZ® Size Standard ideal for a variety of fragment analysis applications such as Microsatellites, Fragment Length Polymorphisms and Relative Fluorescent Quantitation. Each of the DNA fragments is labeled with the LIZ® fluorophore which results in a single peak when run under denaturing conditions. With the 5th dye LIZ® your marker fragments can be labeled with the dyes FAM™, VIC™, NED™ or PET®. Each kit contains enough standard for 800 analyses.

Restriction Digestion of the fluorescently labeled PCR product:

- The purified PCR products were taken up for 4 base cutter digestions.
- Digestion mix was incubated at 37deg for 30 minutes, checked for complete
- digestion.
- In case of partial digestion, the enzyme (5U) was added again and digestion
- continued for another 30minutes.
- The digested product was checked on gel for complete digestion and same
- was taken up for fragment analysis.

Digestion Reaction mix:

PCR product (100ng/µl) 1000ng
10X Assay Buffer 5.0 µl
MboI (10U/ µl) 1.0 µl
dH2O: X µl
Total 50.0 µl

Population Analysis of given sample (Identification of Algae). To identify algae from the given samples to its nearest species based on 18s rDNA sequence data.

STEPS FOLLOWED:

1. Identified the minimum number of organism by t-RFLP.
2. The 1.8kb 18s rDNA fragment was amplified using high –fidelity PCR polymerase.
3. The PCR product was cloned at Not I site in pBlueScript vector.
4. Positive clones are screened by colony PCR.
5. The clones are sequenced bi-directionally using the forward and reverse primer.
6. The sequence data was aligned and analyzed to identify the algae.

RESULT:

Yellow Highlight: Not I Restriction Site

Blue Highlight: Vector Backbone
Black: 18S rRNA sequence data
18S Forward Primer:
5'- GTAAGCTCGGCGGCCGCGTAGTCATATGCTTGTCTC -3'
18S Reverse Primer:
5'-GTAAGCTCGGCGGCCGCGAAACCTTGTTACGACTT -3'

Pic: gDNA loaded on 1% Agarose Gel

Lane description:

1. Sample BPGC

PCR amplification using consensus 18s rDNA primers:

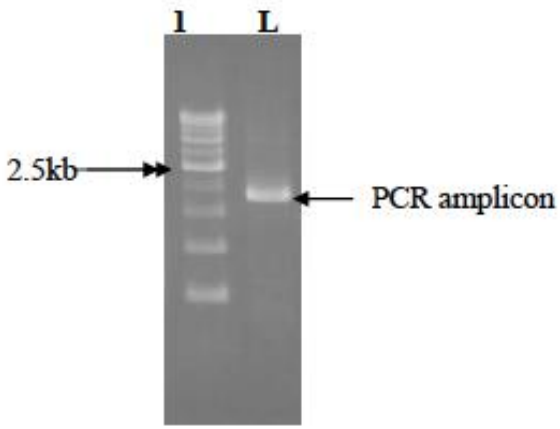
PCR Amplification conditions:
DNA: 1 µl
18S Forward Primer 400ng
18S Reverse Primer 400ng
dNTPs (2.5mM each) 4 µl
10X Taq DNA polymerase Assay Buffer 10 µl
Taq DNA Polymerase Enzyme (3U/ µl) 1 µl

Water X µl
Total reaction volume: 100 µl

PCR Cycle conditions:

94 ºC	94 ºC	50 ºC	72 ºC	72 ºC
5 min	30 sec	30 sec	120 sec	7 min
	35 cycles			

PCR amplification of 18S rDNA fragment from genomic DNA of the samples:

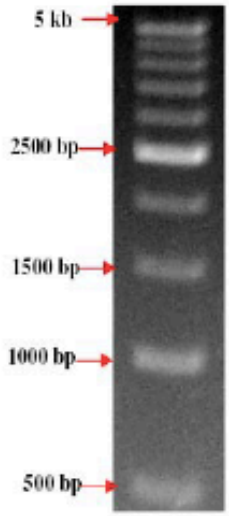


Pic: PCR Amplicon loaded on 1% Agarose Gel

Lane description:

1. Sample BPGC
L. 500bp DNA Ladder

500bp DNA Ladder (LAD02):



500 bp ladder contains 10 DNA fragments of size 500 bp, 1000 bp, 1500 bp, 2000 bp, 2500 bp, 3000 bp, 3500 bp, 4000 bp, 4500 bp and 5000 bp

3. Name of the Thermal Cycler ABI2720

4. Name of the sequencer with model No: ABI 3500 XL Genetic Analyzer

Sequencing Reaction:

The Sequencing mix Composition and PCR Conditions are as follows:

10µl Sequencing Reaction
- Big Dye Terminator Ready Reaction Mix : 4µl
- Template (100ng/ul) :1µl
- Primer (10pmol/λ) :2µl
- Milli Q Water :3µl

PCR Conditions: (25 cycles)

Initial Denaturation : 96°C for 1min
Denaturation : 96°C for 10 sec
Hybridization : 50 °C for 5 sec
Elongation : 60 °C for 4 min

Instrument and Chemistry Details

Sequencing Machine : ABI 3500 XL Genetic Analyzer
Chemistry : Big Dye Terminator version 3.1"Cycle sequencing kit.
Polymer &Capillary Array : POP_7 polymer 50 cm Capillary Array.
Analysis protocol : BDTv3-KB-Denovo_v 5.2
Data Analysis : Seq Scape_ v 5.2Software
Reaction Plate : Applied Biosystem Micro Amp Optical 96-Well Reaction plate

Identification software details:

Phylogenetic Tree Builder uses sequences aligned with System Software aligner. Adistance matrix is generated using the Jukes-Cantor corrected distance model. Whengenerating the distance matrix, only alignment model positions are used, alignment inserts are ignored and the minimum comparable position is 200. The tree is created using Weighbor with alphabet size 4 and length size 1000.

Weighbor Tree: Weighbor is a weighted version of Neighbor Joining that gives significantly less weight to the longer distances in the distance matrix. The weights are based on variances and covariances expected in a simple Jukes-Cantor model. Jukes-Cantor Correction: The Jukes-Cantor distance correction is a model which considers that as two sequences diverge, the probability of a second substitution at any nucleotide site increases. For distance-based trees such as Weighbor, the difference in nucleotides is considered for the distance, therefore, second substitutions will not be counted and the distance will be underestimated. Jukes and Cantor createad a formula that calculates the distance taking into account more than just the individual differences (1969; Evol.of Protein Molecules, Academic Press) Bootstrap: Bootstrapping is a statistical method for estimating the sampling distribution by resampling with replacement from the original sample. In making phylogenetic trees, the approach is to create a pseudoalignment by taking random positions of the original alignment. Some columns of the alignment could be selected more than once or not selected at all. The pseudoalignment will be as long as the original alignment and will be used to create a distance matrix and a tree. The process is repeated 100 times and amajority consensus tree is displayed showing the number (or percentage) of times aparticular group was on each side of a branch without concerning the subgrouping.

Sample: Clone 01

Aligned Data – Clone1: 2117bp

CTAGACTACTATAGGGGCGATTGGAGCTCCACCGCGGTGGCGGCCGCGAAACCTTG
TTACGACTTCTCCTTCCTCTAGGTGGGAGGGTTTAATGAACTTCTCGGCGGCTGAGA
GCGGAGACCGCACCCGGTCGCCAATCCGAACACTTCACCAGCACACCCAATCGGTA
GGAGCGACGGGCGGTGTGTACAAAGGGCAGGGACGTAATCAACGCAAGCTGATGA
CTTGCGCTTACTAGGCATTCCTCGTTGAAGATTAATAATTGCAATAATCTATCCCCAT
CACGATGCAGTTTCAAAGATTACCCGGGCCTCTCGGCCAAGGCTAGGCTCGTTGAAT
GCATCAGTGTAGCGCGCGTGCGGCCCAGAACATCTAAGGGCATCACAGACCTGTTA
TTGCCTCATGCTTCCATTGGCTAGTCGCCAATAGTCCCTCTAAGAAGTCCGCCGGCT
GGCGAGCCAACCGTGACTATTTAGCAGGCTGAGGTCTCGTTCGTTACCGGAATCAAC
CTGACAAGGCAACCCACCAACTAAGAACGGCCATGCACCACCACCCATAGAATCAA
GGAAGAGCTCTCAATCTGTCAATCCTCACTATGTCTGGACCTGGTAAGTTTTCCCGT
GTTGAGTCAAATTAAGCCGCAGGCTCCACGCCTGGTGGTGCCCTTCCGTCAATTCCT
TTAAGTTTCAGCCTTGCGACCATACTCCCCCCGGAACCCAAAAACTTTGATTTCTCAT
AAGGTGCCGGCGGAGTCATCGAAGAAACATCCGCCGATCCCTAGTCGGCATCGTTT
ATGGTTGAGACTAGGACGGTATCTAATCGTCTTCGAGCCCCCAACTTTCGTTCTTGAT
TAATGAAAACATCCTTGGCAAATGCTTTCGCAGTAGTTCGTCTTTCATAAATCCAAG
AATTTCACCTCTGACAATGAAATACGAATGCCCCCGACTGTCCCTCTTAATCATTACT
CCGGTCCTACAGACCAACAGGATAGGCCAGAGTCCTATCGTGTTATTCCATGCTAAT
GTATTCAGAGCGTAGGCCTGCTTTGAACACTCTAATTTACTCAAAGTAACAGCGCCG
ACTCCGAGTCCCGGACAGTGAAGCCCAGGAGCCCGTCCCCGGAAACAAGGTGGGCC

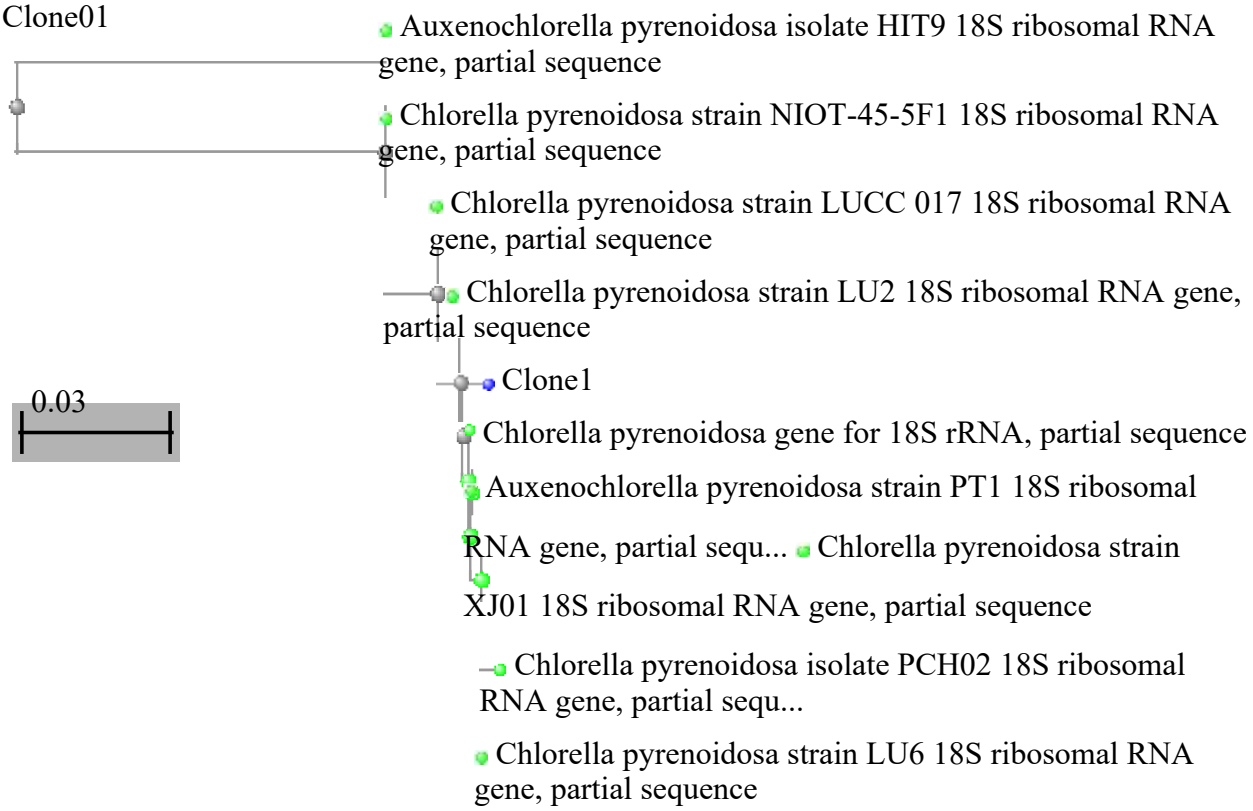
CTGCCAGTGCACACCGAAACGGCGGACCGGCAGGCCCCACCCGAAATCCAACTACG
AGACATGCAACTGCAGCAACTTAAATATACGCTATTGGAGCTGGAATTACCGCGGCT
GCTGGCCAATTCATACCGGGCAGTGCATGGCATTTCCTCCATGCACTTTGGCCCCTC
CGTGCCTTTGCTGATCGGGAGCTATTGCACAGAGCACACTTTGTCATCGTGTGGGTT
TAGCACGTCCCTTGAGGCGGCTCAATGAGTCACCCGGTGCCTTTGCGTGCGTTACGA
CGGCCCAGAGAAGATCGCTCTTCAAGGGCTCGCCACGGGATTAACCTACTAACAAA
GTGAAGGCCTCCCCCGTACCAGGTCACCTCGCCGGCAGTCTACCCGATCCATGTTGT
GACTGCTCGACTAGCAGGCATAGCATCGACTGAGCGATGCCCTACTATCTCTGGCGT
CAACAGCAAAAAAAACACTGATGAGCGTTCACCAGACTTGCCCTCCAATTGATCCTC
GTTAAGGGGTTTAGATTGTACTCATTCCAATTACCAGACCTGAAAAGGCCCAGTATT
GTTATTTATTGTCACTACCTCCCTGTGTCAGGATTGGGTAATTTGCGCGCCTGCTGCC
TTCCTTGGATGTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTAATCCT
CCGTCACCCGTTACCACCATGGTAGGCCTCTATCCTACCATCGAAAGTTGATAGGGC
AGAAATTTGAATGAAACATCGCCGGCACAAGGCCATGCGATTCGTGAAGTTATCAT
GATTCACCGCGAGTCGGGCAGAGCCCGGTCGGCCTTTTATCTAATAAATACGTCCCT
TCCAGAAGTCGGGATTTACGCACGTATTAGCTCTAGATTTACTACGGGTATCCGAGT
AGTAGGTACCATCAAATAAACTATAACTGATTTAATGAGCCATTCGCAGTTTCACAG
TATAAAGCAGTTTATACTTAGACATGCATGGCTTAATCTTTGAGACAAGCATATGAC
TACGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATATCAAG
CTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAGCTTTTGTTCCCTTTA
GTGAGGGTTAATGCGCGCTTGGCGAATTATTGAACACTC

• The microbe was found to be most similar to Chlorella pyrenoidosa gene for 18S rRNA, partial sequence

Sequence ID: AB240151.1

Sl. No.	Organism Name	Accession No.	Percentage Match
1	*Chlorella pyrenoidosa* gene for 18SrRNA	AB240151.1	99%
2	*Auxenochlorella pyrenoidosa* strain PT1 18S ribosomal RNA gene	KX752082.1	99%
3	*Chlorella pyrenoidosa* strain XJ01 18S ribosomal RNA gene	KC416209.1	99%
4	*Chlorella pyrenoidosa* isolate PCH02 18S ribosomal RNA gene	KT250598.1	97%
5	*Chlorella pyrenoidosa* strain LU6 18S ribosomal RNA gene	JQ360516.1	99%
6	*Chlorella pyrenoidosa* strain LUCC017 18S ribosomal RNA gene	KC794704.1	99%
7	*Chlorella pyrenoidosa* strain LU2 18S ribosomal RNA gene	JN794534.1	99%
8	*Auxenochlorella pyrenoidosa* isolate HIT9 18S ribosomal RNA gene	MF040792.1	81%
9	*Chlorella pyrenoidosa* strain PS 18S ribosomal RNA gene	HQ834484.1	100%
10	*Chlorella pyrenoidosa* strain NIOT-45-5F1 18S ribosomal RNA gene	KM403396.1	99%

Phylogenetic tree-
Clone01



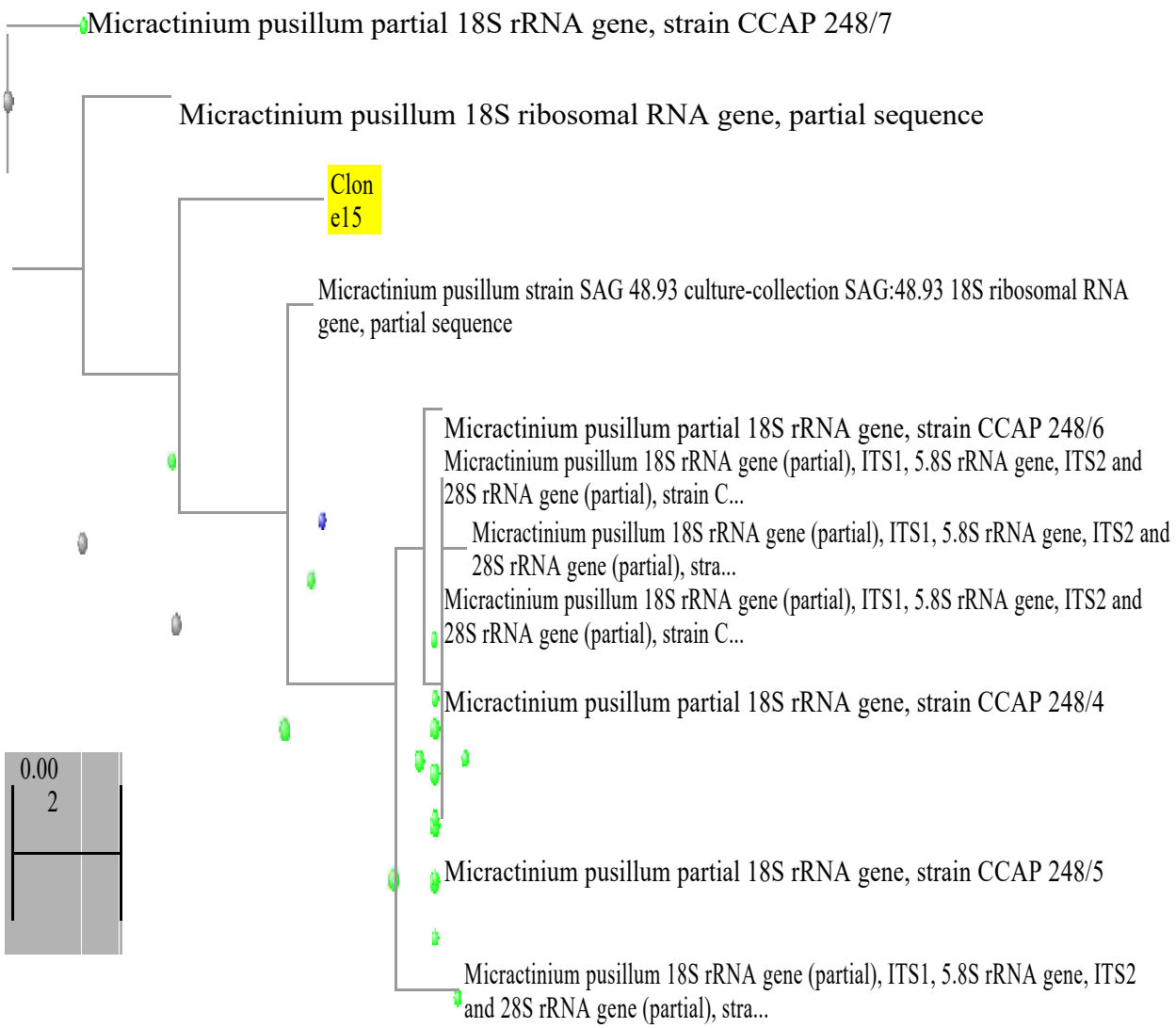
Clone: 15

Aligned Data – Clone 15: 2119bp

CGAACCTATATAGGGCGATTGGAGCTCCACCGCGGTGGCGGCCGCGAAACCTTGTT
ACGACTTCTCCTTCCTCTAGGTGGGAGGGTTTAATGAACTTCTCGGCGGCTGAGAGC
GGAGACCGCACCCGGTCGCCAATCCGAACACTTCACCAGCACACCCAATCGGTAGG
AGCGACGGGCGGTGTGTACAAAGGGCAGGGACGTAATCAACGCAAGCTGATGACTT
GCGCTTACTAGGCATTCCTCGTTGAAGATTAATAATTGCAATAATCTATCCCCATCA
CGATGCAGTTTCAAAGATTACCCGGGCCTCTCGGCCAAGGCTAGGCTCGTTGAATGC
ATCAGTGTAGCGCGCGTGCGGCCCAGAACATCTAAGGGCATCACAGACCTGTTATTG
CCTCATGCTTCCATTGGCTAGTCGCCAATAGTCCCTCTAAGAAGTCCGCCGACTGGC
GAGCCAACCGTGACTATTTAGCAGGCTGAGGTCTCGTTCGTTACCGGAATCAACCTG
ACAAGGCAACCCACCAACTAAGAACGGCCATGCACCACCACCCATAGAATCAAGAA
AGAGCTCTCAATCTGTCAATCCTCACTATGTCTGGACCTGGTAAGTTTTCCCGTGTTG
AGTCAAATTAAGCCGCAGGCTCCACGCCTGGTGGTGCCCTTCCGTCAATTCCTTTAA
GTTTCAGCCTTGCGACCATACTCCCCCCGGAACCCAAAAACTTTGATTTCTCATAAG
GTGCCGGCGGAGTCATCGAAGAAACATCCGCCGATCCCTAGTCGGCATCGTTTATGG
TTGAGACTAGGACGGTATCTAATCGTCTTCGAGCCCCCAACTTTCGTTCTTGATTAAT
GAAAACATCCTTGGCAAATGCTTTCGCAGTAGTTCGTCTTTCATAAATCCAAGAATT
TCACCTCTGACAATGAAATACGAATGCCCCCGACTGTCCCTCTTAATCATTACTCCG
GTCCTACAGACCAACAGGATAGGCCAGAGTCCTATCGTGTTATTCCATGCTAATGTA
TTCAGAGCGTAGGCCTGCTTTGAACACTCTAATTTACTCAAAGTAACGGCGCCGACT
CCGAGTCCCGGACAGTGAAGCCCAGGAGCCCGTCCCCGGCAAACAAGGTGGGCCCT
GCCAGTGCACACCGAAACGGCGGACCGGCAGGCCCCACCCGAAATCCAACTACGAG

CTTTTTAACTGCAGCAACTTAAATATACGCTATTGGAGCTGGAATTACCGCGGCTGC
TGGCCAATTCATACCGGGCAGTGTGAAGCATTTCCTCAACACACTATGGCCCCTCCG
TGCCTTTGCTGATCGGGAGCTATTCTCTAGAGCACACTTTGTCATCGTGTGGGTTTAG
CACGTCCCTTAAGCCAACTCAGAGAGTCACCCGGTGCCTTTGCGTGCGTTACGACGG
CCCAGAGAAGATCGCTCTTCAAGGGCTCGCCACGGGATTAACCTACTAACAAAGTG
AAGGCCTCCCCCGTACCAGGTCGTCTCGCCGGCAGTCTACCCGATCCATGTTGTGAC
TGCTCGACTAGCAGGCATAACACTGTTCGCACAATGCCCTACTATCTCTGGCGTCAA
CAGCAAAAAAAACACTGATGAGCGTTCACCAGACTTGCCCTCCAATTGATCCTCGTT
AAGGGGTTTAGATTGTACTCATTCCAATTACCAGACCTGAAAAGGCCCAGTATTGTT
ATTTATTGTCACTACCTCCCTGTGTCAGGATTGGGTAATTTGCGCGCCTGCTGCCTTC
CTTGGATGTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTAATCCTCC
GTCACCCGTTACCACCATGGTAGGCCTCTATCCTACCATCGAAAGTTGATAGGGCAG
AAATTTGAATGAAACATCGCCGGCACAAGGCCATGCGATTCGTGAAGTTATCATGAT
TCACCGCGAGTCGGGCAGAGCCCGGTCGGCCTTTTATCTAATAAATACGTCCCTTCC
AGAAGTCGGGATTTACGCACGTATTAGCTCTAGATTTACTACGGGTATCCGAGTAGT
AGGTACCATCAAATAAACTATAACTGATTTAATGAGCCATTCGCAGTTTCACAGTAT
AAAGCAGTTTATACTTAGACATGCATGGCTTAATCTTTGAGACAAGCATATGACTAC
GCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTT
TCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAGCTTTTGTTCCCTTTAGTGA
GGGTTAATTGCGCGCTTGGCGT

• The microbe was found to be most similar to Micractinium pusillum partial 18S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 211/92
Sequence ID: FM205863.1


Micractinium pusillum partial 18S rRNA gene, strain CCAP 248/7
Micractinium pusillum 18S ribosomal RNA gene, partial sequence
Clon
e15
Micractinium pusillum strain SAG 48.93 culture-collection SAG:48.93 18S ribosomal RNA gene, partial sequence
Micractinium pusillum partial 18S rRNA gene, strain CCAP 248/6
Micractinium pusillum 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain C...
Micractinium pusillum 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), stra...
Micractinium pusillum 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain C...
Micractinium pusillum partial 18S rRNA gene, strain CCAP 248/4
0.00
2
Micractinium pusillum partial 18S rRNA gene, strain CCAP 248/5
Micractinium pusillum 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), stra...

Sl. No.	Organism Name	Accession No.	Percentage Match
1	*Micractinium pussillum* 18S ribosomal RNA gene	AF237662.1	99%%
2	*Micractinium pussillum* strain SAG 48.93 culture-collection	AF364102.1	99%
3	*Micractinium pussillum* partial 18S ribosomal RNA gene	AM231740.1	99%%
4	*Micractinium pussillum* partial 18S ribosomal RNA gene, strain CCAP248/6	AM231739.1	99%%
5	*Micractinium pussillum* partial 18S ribosomal RNA gene, ITS1, 5.8S rRNA gens	FM205874.1	99%
6	*Micractinium pussillum* 18S ribosomal RNA gene, ITS1,5.8S rRNA gene,ITS2 and 28S rRNA gene	FM205872.1	99%
7	*Micractinium pussillum* 18S ribosomal RNA gene	FM205836.1	99%
8	*Micractinium pussillum* partial 18S ribosomal RNA gene, strain CCAP248/4	AMAM231737.1	99%
9	*Micractinium pussillum* partial 18S ribosomal RNA gene	AM231738.1	99%
10	*Micractinium pussillum* partial 18S ribosomal RNA gene, ITS1,5.8S rRNA gene	FM205867.1	99%

Sample: Clone 16

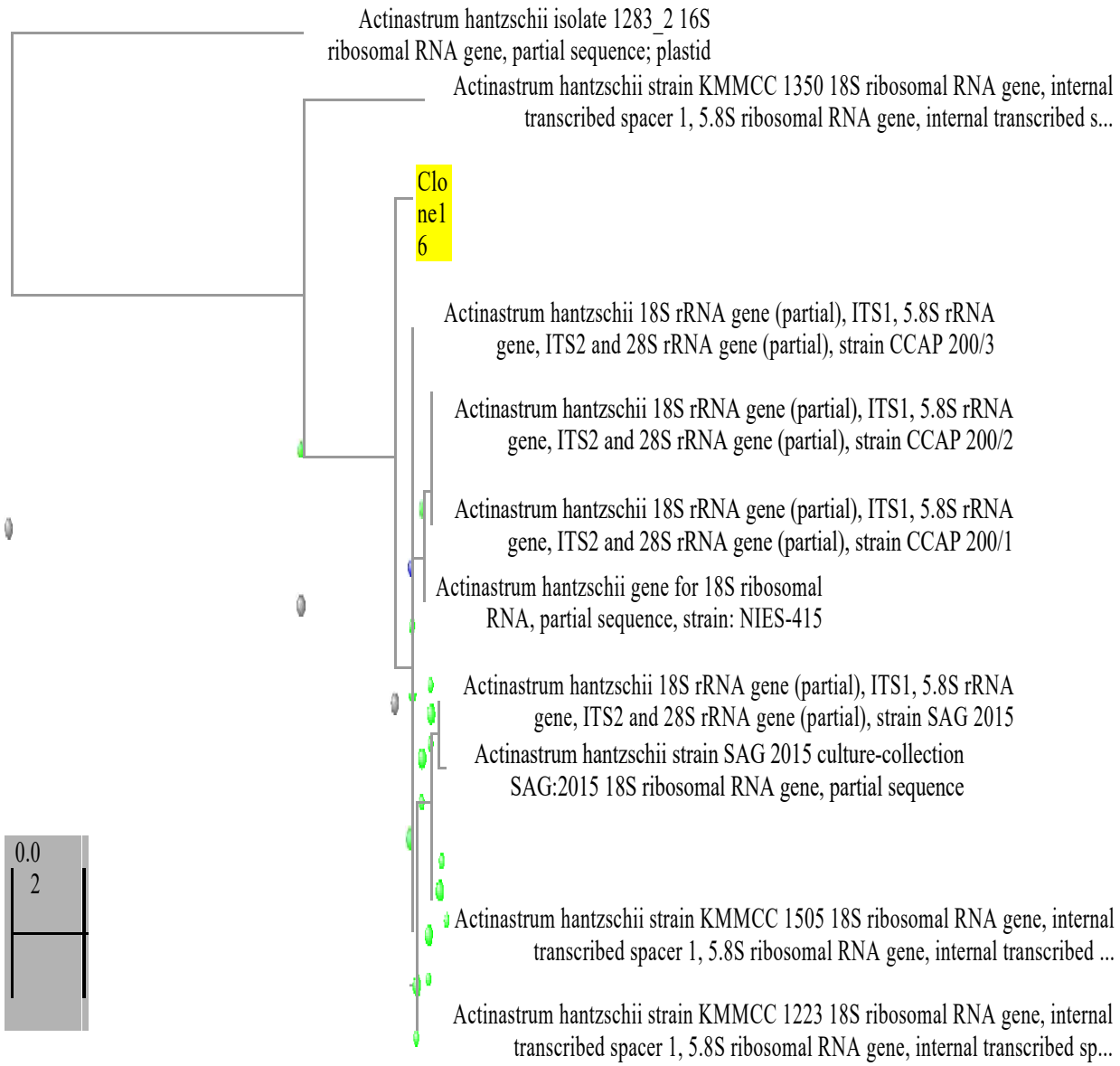
Aligned Data – Clone 16: 2164bp

CGAACTTCATATAGGGCGATTGGAGCTCCACCGCGGTGGCGGCCGCGAAACCTTGTT
ACGACTTCTCCTTCCTCTAGGTGGGAGGGTTTAATGAACTTCTCGGCGGCTGAGAGC
GGAGACCGCACCCGGTCGCCAATCCGAACACTTCACCAGCACACCCAATCGGTAGG
AGCGACGGGCGGTGTGTACAAAGGGCAGGGACGTAATCAACGCAAGCTGATGACTT
GCGCTTACTAGGCATTCCTCGTTGAAGATTAATAATTGCAATAATCTATCCCCATCA
CGATGCAGTTTCAAAGATTACCCGGGCCTCTCGGCCAAGGCTAGGCTCGTTGAATGC
ATCAGTGTAGCGCGCGTGCGGCCCAGAACATCTAAGGGCATCACAGACCTGTTATTG

CCTCATGCTTCCATTGGCTAGTCGCCAATAGTCCCTCTAAGAAGTCCGCCGGCTGGC
GAGCCAACCGTGACTATTTAGCAGGCTGAGGTCTCGTTCGTTACCGGAATCAACCTG
ACAAGGCAACCCACCAACTAAGAACGGCCATGCACCACCACCCATAGAATCAAGAA
AGAGCTCTCAATCTGTCAATCCTCACTATGTCTGGACCTGGTAAGTTTTCCCGTGTTG
AGTCAAATTAAGCCGCAGGCTCCACGCCTGGTGGTGCCCTTCCGTCAATTCCTTTAA
GTTTCAGCCTTGCGACCATACTCCCCCCGGAACCCAAAAACTTTGATTTCTCATAAG
GTGCCGGCGGAGTCATCGAAGAAACATCCGCCGATCCCTAGTCGGCATCGTTTATGG
TTGAGACTAGGACGGTATCTAATCGTCTTCGAGTCCCCAACTTTCGTTCTTGATTAAT
GAAAACATCCTTGGCAAATGCTTTCGCAGTAGTTCGTCTTTCATAAATCCAAGAATT
TCACCTCTGACAATGAAATACGAATGCCCCCGACTGTCCCTCTTAATCATTACTCCG
GTCCTACAGACCAACAGGATAGGCCAGAGTCCTATCGTGTTATTCCATGCTAATGTA
TTCAGAGCGTAGGCCTGCTTTGAACACTCTAATTTACTCAAAGTAACAGCGCCGACT
CCGAGTCCCGGACAGTGAAGCCCAGGAGCCCGTCCCCGGCAAACAAGGTGGGCCCT
GCCAGTGCACACCGAAACGGCGGACCGGCAGGCCCCACCCGAAATCCAACTACGAG
CTTTTTAACTGCAGCAACTTAAATATACGCTATTGGAGCTGGAATTACCGCGGCTGC
TGGCCAATTCATACCGGGCAGTGCATGGCATTTCCTCCATGCACTTTGGCCCCTCCG
TGCCTTTGCTGATCGGGAGCTATTGCACAGAGCACACTTTGTCATCGTGTGGGTTTA
GCACGTCCCTTGAGGCGGCTCAATGAGTCACCCGGTGCCTTTGCGTGCGTTACGACG
GCCCAGAGAAGATCGCTCTTCAAGGGCTCGCCACGGGATTAACCTACTAACAAAGT
GAAGGCCTCCCCCGTACCAGGTCACCTCGCCGGCAGTCTACCCGATCCATGTTGTGA
CTGCTCGACTAGCAGGCATAGCATCGACTGAGCGATGCCCTACTATCTCTGGCGTCA
ACAGCAAAAAAAACACTGATGAGCGTTCACCAGACTTGCCCTCCAATTGATCCTCGT
TAAGGGGTTTAGATTGTACTCATTCCAATTACCAGACCTGAAAAGGCCCAGTATTGT
TATTTATTGTCACTACCTCCCTGTGTCAGGATTGGGTAATTTGCGCGCCTGCTGCCTT
CCTTGGATGTGGTAGCCGTTTCTCTGGCTCCCTCTCCGGAATCGAACCCTAATCCTCC
GTCACCCGTTACCACCATGGTAGGCCTCTATCCTACCATCGAAAGTTGATAGGGCAG
AAATTTGAATGAAACATCGCCGGCACAAGGCCATGCGATTCGTGAAGTTATCATGAT
TCACCGCGAGTCGGGCAGAGCCCGGTCGGCCTTTTATCTAATAAATACGTCCCTTCC
AGAAGTCGGGATTTACGCACGTATTAGCTCTAGATTTACTACGGGTATCCGAGTAGT
AGGTACCATCAAATAAACTATAACTGATTTAATGAGCCATTCGCAGTTTCACAGTAT
AAAGCAGTTTATACTTAGACATGCATGGCTTAATCTTTGAGACAAGCATATGACTAC
GCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTA
TCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAGCTTTTGTTCCCTTTAGTGA
GGGTTAATTGCGCGCTTGGCGTAATGATT

• The microbe was found to be most similar to Actinastrum hantzschii 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain SAG 2015
Sequence ID: FM205841.1

Sl. No.	Organism Name	Accession No.	Percentage Match
1	*Actinastrum hantzschii* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene	FM205841.1	95%
2	*Actinastrum hantzschii* strain SAG 2015 culture collection SAG:2015	AF288365.1	95
3	*Actinastrum hantzschii* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene , ITS2, and 28S rRNA	FM205883.1	99%
4	*Actinastrum hantzschii* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene	FM205882.1	99%
5	*Actinastrum hantzschii* strain KMMCC 1505 18S rRNA gene (partial), ITS1, 5.8S rRNA gene , internal transcribed spacer1, 5.8S rRNA gene, internal transcribed spacer 2	JQ315762.1	99%
6	*Actinastrum hantzschii* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene	FM205884.1	99%
7	*Actinastrum hantzschii* strain KMMCC 1223 18S rRNA gene (partial)	JQ315761/1	99%
8	*Actinastrum hantzschii* 18S rRNA gene (partial)	LC192144.1	99%
9	*Actinastrum hantzschii* strain KMMCC 1350 18S rRNA gene	JQ315763.1	95%
10	*Actinastrum hantzschii* isolate 1283_2 16S rRNA gene	KM514784.1	100%



Sample: Clone 17

GTACTATATAGGGCGATTGGAGCTCCACCGCGGTGGCGGCCGCGTAGTCATATGCTT
GTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAACTGCTTTATACTGTGAAACTG
CGAATGGCTCATTAAATCAGTTATAGTTTATTTGATGGTACCTACTACTCGGATACC
CGTAGTAAATCTAGAGCTAATACGTGCGTAAATCCCGACTTCTGGAAGGGACGTATT
TATTAGATAAAAGGCCGACCGGGCTCTGCCCGACTCGCGGTGAATCATGATAACTTC
ACGAATCGCATGGCCTTGTGCCGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTT
TCGATGGTAGGATAGAGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAGGGTT
CGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTGACAATAAATAACAATACTG
GGCCTTTTCAGGTCTGGTAATTGGAATGAGTACAATCTAAACCCCTTAACGAGGATC
AATTGGAGGGCAAGTCTGGTGAACGCTCATCAGTGTTTTTTTTGCTGTTGACGCCAG
AGATAGTAGGGCACTGTGCGAACGGTGTTATGCCTGCTAGTCGAGCAGCCACAACA
TGGATCGGGTAGACTGCCGGCGAGGTGACCTGGTACGGGGGAGGCCTTCACCCTCG

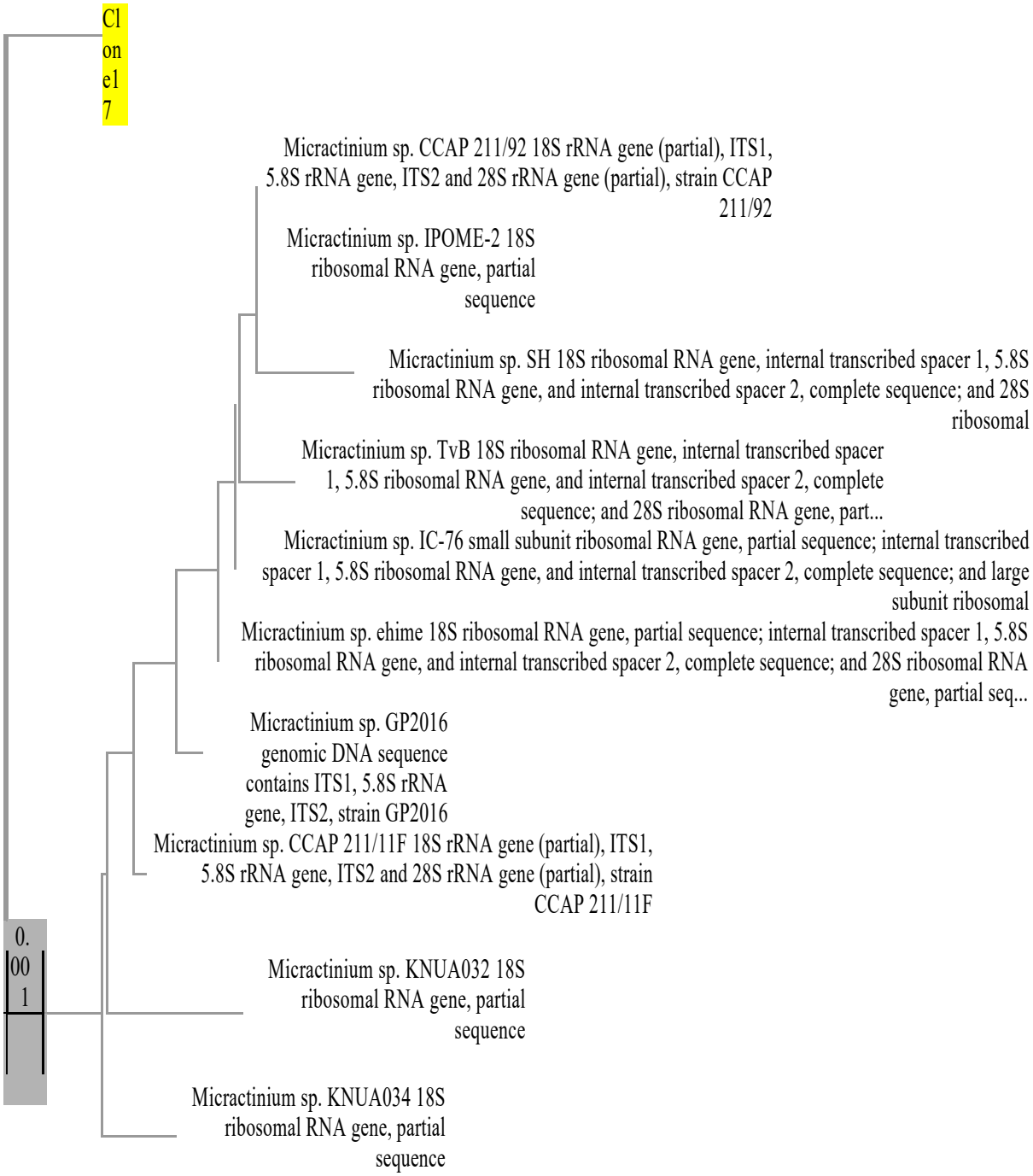
TTAGTAGGTTAATCCCGTGGCGAGCCCTTGAAGAGCGATCTTCTCTGGGGCCGTCGT
AACGCACGCAAAAGGCACCGGGTGACTCATTGAGCCGCCTCAAGGGACGTGCTAAA
CCCACACGATGACAAAGTGTGCTCTGTGCAATAGCTCCCGATCAGCAAAGGCACGG
AGGGGCCAAAGTGCATGGAGGAAATGCCATGCACTGCCCGGTATGAATTGGCCAGC
AGCCGCGGTAATTCCAGCTCCAATAGCGTATATTTAAGTTGCTGCAGTTAAAAAGCT
CGTAGTTGGATTTCGGGTGGGGCCTGCCGGTCCGCCGTTTCGGTGTGCACTGGCAGG
GCCCACCTTGTTGCCGGGGACGGGCTCCTGGGCTTCACTGTCCGGGACTCGGAGTCG
GCGCTGTTACTTTGAGTAAATTAGAGTGTTCAAAGCAGGCCTACGCTCTGAATACAT
TAGCATGGAATAACACGATAGGACTCTGGCCTATCCTGTTGGTCTGTAGGACCGGA
GTAATGATTAAGAGGGACAGTCGGGGGCATTCGTATTTCATTGTCAGAGGTGAAAT
TCTTGGATTTATGAAAGACGAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATT
AATCAAGAACGAAAGTTGGGGGCTCGAAGACGATTAGATACCGTCCTAGTCTCAAC
CATAAACGATGCCGACTAGGGATCGGCGGATGTTTCTTCGATGACTCCGCCGGCACC
TTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGGAGTATGGGTCGCAAGGGCTGA
AACTTAAAGGAATTGACGGAAAGGCACCACCCAGGAGTGGAGTATGCCGGCCTTAA
TTTCGACTCAACCACGGGGAAACCTTAACCAGGGTCCAGACCATAGTTACCAGGTC
CAGACATAGTTAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGT
GCATGGCCGTTCTTAGTTGGTGGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGA
GACCTCAGCCTGCTAAATAGTCACGGTTGGCTCGCCAGCCGGCGGACTTCTTAGAGG
GACTATTGGCGACTAGCCAATGGAAGCATGAGGCAATAACAGGTCTGTGATGCCCT
TAGATGTTCTGGGCCGCACGCGCGCTACACTGATGCATTCAACGAGCCTAGCCTTGG
CCGAGAGGCCCGGGTAATCTTTGAAACTGCATCGTGATGGGGATAGATTATTGCAA
TTATTAATCTTCAACGAGGAATGCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATT
ACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGGGTGTGCTGGTG
AAGTGTTCGGATTGGCGACCGGGTGCGGTCTCCGCTCTCAGCCGCCGAGAAGTTCAT
TAAACCCTCCCACCTAGAGGAAGGAGAAGTCGTAACAAGGTTTCGCGGCCGCTCTA
GAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCG
ACCTCGAGGGGGGGCCCGGTACCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCG
CGCT TGGCGTAAGCTTTGACCTTC

The microbe was found to be most similar to Micractinium sp. SH 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: KM820919.1

Sl. No.	Organism Name	Accession No.	Percentage Match
1	*Micractinium* sp. CCAP 211/92 18S rRNA gene (partial)	FM205863.1	94%
2	*Micractinium* sp. SH 18S rRNA gene	KM820919.1	94%
3	*Micractinium* sp. TvB 18S rRNA gene	KM820917.1	94%
4	*Micractinium* sp. IPOME-2 18S rRNA gene	KR936170.1	94%
5	*Micractinium* sp. GP 2016 genomic DNA sequence contains ITS1, 5.8S rRNA gene	LT605003.1	97%
6	*Micractinium* sp. ehime 18S rRNA gene	JX889639.1	97%
7	*Micractinium* sp. CCAP 211/11F 18S rRNA gene (partial), ITS1, 5.8S rRNA gene	FM205877.1	97%
8	*Micractinium* sp. IC-76 small subunit rRNA gene	MF629793.1	96.00%
9	*Micractinium* sp. KNUA034 18S rRNA gene	KM243325.1	96%

Phylogenetic tree

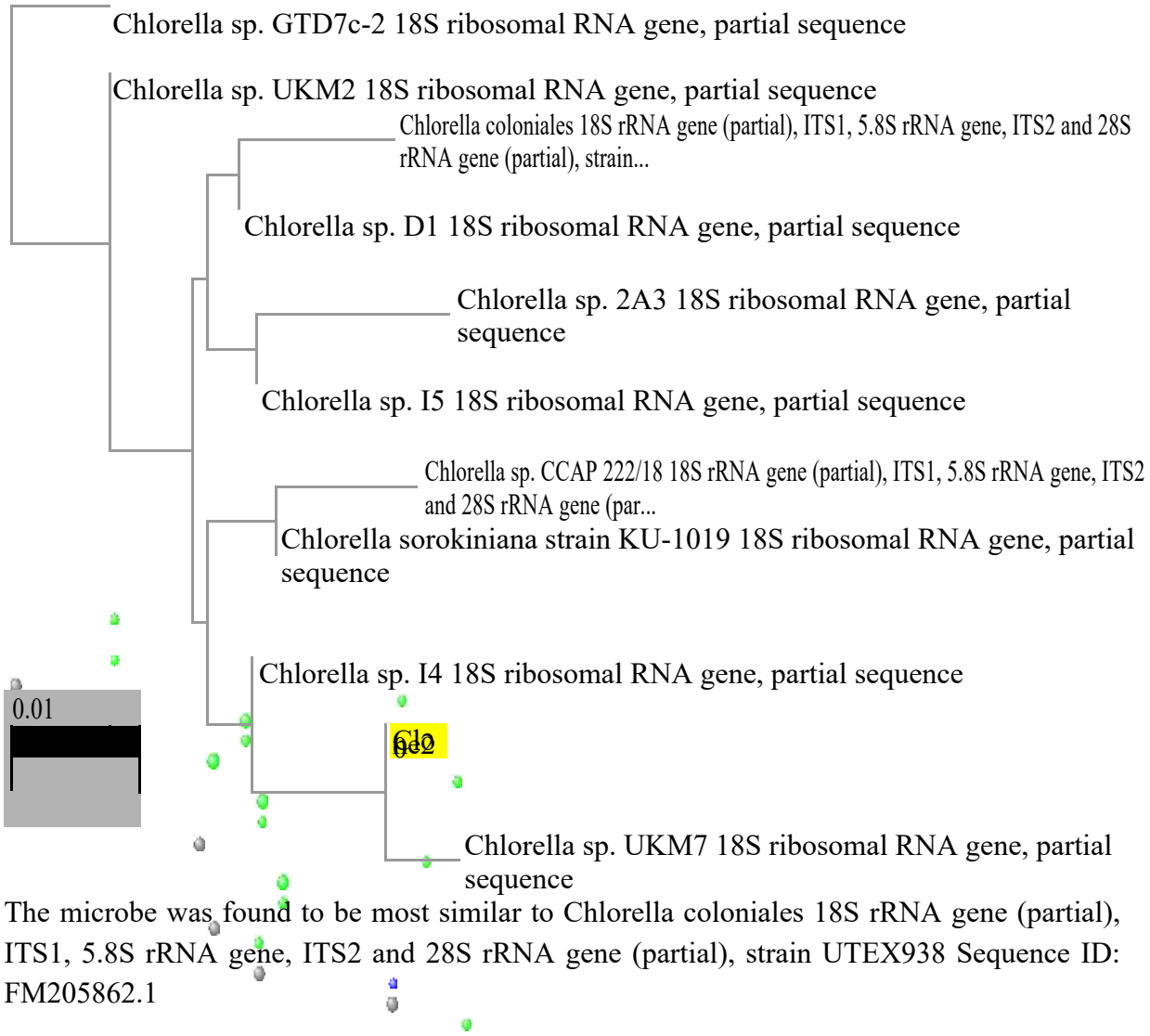


Sample: 20

Aligned Data – Clone 20: 2117bp

CTAACTTATATAGGGCGATTGGAGCTCCACCGCGGTGGCGGCCGCGAAACCTTGTT
ACGACTTCTCCTTCCTCTAGGTGGGAGGGTTTAATGAACTTCTCGGCGGCTGAGAGC
GGAGACCGCACCCGGTCGCCAATCCGAACACTTCACCAGCACACCCAATCGGTAGG
AGCGACGGGCGGTGTGTACAAAGGGCAGGGACGTAATCAACGCAAGCTGATGACTT
GCGCTTACTAGGCATTCCTCGTTGAAGATTAATAATTGCAATAATCTATCCCCATCA
CGATGCAGTTTCAAAGATTACCCGGGCCTCTCGGCCAAGGCTAGGCTCGTTGAATGC
ATCAGTGTAGCGCGCGTGCGGCCCAGAACATCTAAGGGCATCACAGACCTGTTATTG
CCTCATGCTTCCATTGGCTAGTCGCCAATAGTCCCTCTAAGAAGTCCGCCGGCTGGC
GAGCCAACCGTGACTATTTAGCAGGCTGAGGTCTCGTTCGTTACCGGAATCAACCTG
ACAAGGCAACCCACCAACTAAGAACGGCCATGCACCACCACCCATAGAATCAAGAA
AGAGCTCTCAATCTGTCAATCCTCACTATGTCTGGACCTGGTAAGTTTTCCCGTGTTG
AGTCAAATTAAGCCGCAGGCTCCACGCCTGGTGGTGCCCTTCCGTCAATTCCTTTAA
GTTTCAGCCTTGCGACCATACTCCCCCCGGAACCCAAAAACTTTGATTTCTCATAAG
GTGCCGGCGGAGTCATCGAAGAAACATCCGCCGATCCCTAGTCGGCATCGTTTATGG
TTGAGACTAGGACGGTATCTAATCGTCTTCGAGCCCCCAACTTTCGTTCTTGATTAAT
GAAAACATCCTTGGCAAATGCTTTCGCAGTAGTTCGTCTTTCATAAATCCAAGAATT
TCACCTCTGACAATGAAATACGAATGCCCCCGACTGTCCCTCTTAATCATTACTCCG
GTCCTACAGACCAACAGAATAGGCCAGAGTCCTATCGTGTTATTCCATGCTAATGTA
TTCAGAGCGTAGGCCTGCTTTGAACACTCTAATTTACTCAAAGTAACAGCGCCGACT
CCGAGTCCCGGACAGTGAAGCCCAGGAGCCCGTCCCCGGAAACAAGGTGGGCCCTG
CCAGTGCACACCGAAACGGCGGACCGGCAGGCCCCACCCGAAATCCAACTACGAGC
TTTTTAACTGCAGCAACTTAAATATACGCTATTGGAGCTGGAATTACCGCGGCTGCT
GGCCAATTCATACCGGGCAGTGCATGGCATTTCCTCCATGCACTTTGGCCCCTCCGT
GCCTTTGCTGATCGGGAGCTATTGCACAGAGCACACTTTGTCATCGTGTGGGTTTAG
CACGTCCCTTGAGGCGGCTCAATGAGTCACCCGGTGCCTTTGCGTGCGTTACGACGG
CCCAGAGAAGATCGCTCTTCAAGGGCTCGCCACGGGATTAACCTACTAACAAAGTG
AAGGCCTCCCCCGTACCAGGTCACCTCGCCGGCAGTCTACCCGATCCATGTTGTGAC
TGCTCGACTAGCAGGCATAGCATCGACTGAGCGATGCCCTACTATCTCTGGCGTCAA
CAGCAAAAAAAACACTGATGAGCGTTCACCAGACTTGCCCTCCAATTGATCCTCGTT
AAGGGGTTTAGATTGTACTCATTCCAATTACCAGACCTGAAAAGGCCCAGTATTGTT
ATTTATTGTCACTACCTCCCTGTGTCAGGATTGGGTAATTTGCGCGCCTGCTGCCTTC
CTTGGATGTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTAATCCTCC
GTCACCCGTTACCACCATGGTAGGCCTCTATCCTACCATCGAAAGTTGATAGGGCAG
AAATTTGAATGAAACATCGCCGGCACAAGGCCATGCGATTCGTGAAGTTATCATGAT
TCACCGCGAGTCGGGCAGAGCCCGGTCGGCCTTTTATCTAATAAATACGTCCCTTCC
AGAAGTCGGGATTTACGCACGTATTAGCTCTAGATTTACTACGGGTATCCGAGTAGT
AGGTACCATCAAATAAACTATAACTGATTTAATGAGCCATTCGCAGTTTCACAGTAT
AAAGCAGTTTATACTTAGACATGCATGGCTTAATCTTTGAGACAAGCATATGACTAC
GCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTA
TCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAGCTTTTGTTCCCTTTAGTGA
GGGTTAATTGCGCGCTTGGCGTAATCATT

Sl. No.	Organism Name	Accession No.	Percentage Match
1	*Chlorella coloniales* 18S rRNA gene (partial)	FM205862.1	94%
2	*Chlorella* sp. 2A3	AF357146.1	93%
3	*Chlorella* sp. UKM7	KP898731.1	99%
4	*Chlorella* sp GTD7c-2	JQ411025.1	94%
5	*Chlorella* sp. UKM2	KP262476.1	99%
6	*Chlorella* sp. CCAP222/18	FM205858.1	99%
7	*Chlorella sorokiniana* strain KU-1019	KF444207.1	99%
8	*Chlorella* sp. 15	KF879580.1	99%
9	*Chlorella* sp. 14	KF879579.1	99%
10	*Chlorella* sp. D1	KF879578.1	99%



The microbe was found to be most similar to Chlorella coloniales 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain UTEX938 Sequence ID: FM205862.1

Appendix III

List of publications

1. **Ram Chavan** & Srikanth Mutnuri (2019) Tertiary treatment of domestic wastewater by *Spirulina platensis* integrated with microalgal biorefinery. *Biofuels*, 10:1, 33-44.

2. **Ram Chavan** and Srikanth Mutnuri. (2019) Phycoremediation and biogas production potential of *Chlorella vulgaris* grown in secondarily treated wastewater. *International Journal of Recent Technology and Engineering* (IJRTE), 8:3,1939-1945.

3. **Ram Chavan** and Srikanth Mutnuri. (2020) Demonstration of pilot scale integrative treatment of nitrogenous industrial effluent for struvite and algal biomass production. *Journal of Applied Phycology*, 32, 1215–1229.

4. Ram Chavan and Srikanth Mutnuri. (2020) Domestic wastewater treatment by constructed wetland and microalgal treatment system for the production of value-added products. *Environmental Technology,* 1-14.

Appendix IV

List of conferences and Workshops attended

International Conference:

Ram Chavan and Srikanth Mutnuri (2017). Development and demonstration of pilot scale integrative treatment of nitrogenous industrial effluent for struvite production and microalgae cultivation. (Poster Presentation) at ABO summit-October,2017, Salt Lake City-USA.

National Conferences:

- Ram Chavan and Srikanth Mutnuri (2019). Value added products from sea-Macrolagal biorefinery concept with special focus on Ulva sp. and Porphyra sp. (Poster presentation at India International Seaweed Expo and Summit-January,2019.
- Participated in Novel Sanitation Approaches and wastewater treatment systems (November 2017) at BITS Pilani, KK Birla Goa Campus.
- Participated in Terra-preta sanitation and decentralized wastewater systems (November 2015) at BITS Pilani, KK Birla Goa Campus.
- Participated in Decentralized biogas digesters and their slurry management (DBDSM-November 2014) at BITS Pilani, KK Birla Goa Campus.

Workshops attended

- BIRAC workshop on Bio-entrepreneurship Grant-writing and Intellectual Property Management (February,2016) at BITS Pilani, KK Birla Goa Campus.
- Workshop on Scientific manuscript writing (March 2018) at BITS Pilani, KK Birla Goa Campus.

Appendix V

Brief biography of candidate:

Name	Mr. Chavan Ram Indrajit
Education	MTech (Biotechnology,2014)-LPU ,Punjab
	M.Sc. (Biotechnology,2009)-University of Mumbai
	B.Sc. (Biotechnolgy,2006)-SRTMU, Nanded

Work Experience:

Worked as an Assistant Professor at SP College of Agriculture, Chiplun (February-May 2014).

Research Experience:

- Worked as Research Scholar on consultancy project by German Technical Cooperation on GIZ-BMU – Waste to Energy project (November-December 2017) under the supervision of Dr. M. Srikanth at BITS Pilani, KK Birla Goa Campus.
- Worked as JRF in project entitled " treatment of nitrogenous wastewater by using microalgae and its utilization for the production of value-added products" (May 2014-April 2017) funded by BITS Pilani-CORE-WWE under the under the supervision of Dr. M. Srikanth at BITS Pilani, KK Birla Goa Campus.
- Worked as Water Advanced Research and Innovation (WARI) Intern (May 2017-November 2017) at University of Nebraska-Lincoln-USA under the supervision of Prof. Concetta DiRusso. The internship was funded by DST-INDIA, DWFI-USA, IUSSTF-India and University of Nebraska-Lincoln.

Appendix VI

Brief biography of supervisor:

Dr. Srikanth Mutnuri was a recipient of DAAD-UGC Scholarship to complete his Doctoral Research at UFZ – Centre for Environmental Research, Germany and obtained his degree from Anna University Chennai in the year 2004. He joined BITS Pilani K.K Birla Goa Campus as a full-time faculty by 2005. He was a Recipient of American Society for Microbiology & Indo US Science and Technology Forum (ASM IUSSTF) Indo US Research Professorship for October 2010. Prof. Srikanth Mutnuri is currently Professor and Head of the Department of Biological Sciences. The focus of his laboratory is on Sustainable Development Goals with major emphasis on Clean water Sanitation, Zero hunger, Clean Energy, Climate Action and Responsible consumption and production. Through his research projects he has demonstrated single household vertical wetlands based domestic wastewater treatment, single household empowered septic tank for domestic wastewater treatment, 100 people equivalent empowered septic tank for domestic wastewater treatment and various capacity anaerobic digestors starting from 60 m^3 to 600 m^3. The advantage of these treatment systems is their cost effectiveness / affordability. For example, our 100 people equivalent treatment system operating costs is Rs 176/day (total operating costs) whereas just the chlorination cost in a conventional treatment system is Rs. 300/day. His other project focuses on Terra preta sanitation, Phosphate rich organic manure and Struvite production for improving soil fertility. He had installed 5 ton per day Anaerobic digestor for Mormugao Municipal corporation which handles organic waste as well as septage solids. He had also installed 500 m^2 constructed wetland for treating polluted river water.