BIOPROSPECTS OF MICROALGAL ISOLATES FROM WATER LOGGED AREA OF PUNJAB FOR BIOGAS PRODUCTION

Dissertation

Submitted to the Punjab Agricultural University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY
in
MICROBIOLOGY
(Minor subject: Biotechnology)

By

Rouf Ahmad Dar
(L-2013-BS-80-D)

Department of Microbiology
College of Basic Sciences and Humanities

LUDHIANA-141004

2017

CERTIFICATE I

This is to certify that the dissertation entitled, **"Bioprospects of microalgal isolates from water logged area of Punjab for biogas production"** submitted for the degree of **Doctor of Philosophy**, in the subject of **Microbiology** (Minor subject: **Biotechnology**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Rouf Ahmad Dar (L-2013-BS-80-D)** under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

_____

(Dr. (Mrs.) Urmila Gupta Phutela)
Major Advisor
Senior Scientist (Biogas)
Punjab Agricultural University
Ludhiana-141 004 (India)

CERTIFICATE II

This is to certify that the dissertation entitled, **"Bioprospects of microalgal isolates from water logged area of Punjab for biogas production"** submitted by **Rouf Ahmad Dar (L-2013-BS-80-D)** to the Punjab Agricultural University, Ludhiana, in the partial fulfilment of the requirements for the degree of **Doctor of Philosophy**, in the subject of **Microbiology** (Minor subject: **Biotechnology**) has been approved by the Student's Advisory Committee after an oral examination on the same in collaboration with an External Examiner.

(Dr. (Mrs.) Urmila Gupta Phutela)
Major Advisor

(Dr. K. L. Kalra)
External Examiner
Ex. Senior Microbiologist
Department of Microbiology
PAU, Ludhiana

(Dr. Shammi Kapoor)
Head of Department

(Dr. (Mrs.) Neelam Grewal)
Dean, Postgraduate Studies

ACKNOWLEDGEMENT

At the very outset, with folded hands, I bow my head with reverence and dedicatedly accord my gratitude to the 'Almighty' Allah, the most gracious, the most merciful and compassionate whose grace glory and blessing gave me the courage in odd critical times for the successful completion of this degree. May Allah's blessing be upon his most revered Prophet Mohammad (SAW), his family and all his companions.

Not to fulfill a formality, but to express the feeling of my heart, I put on record my deepest gratitude and profound indebtedness to my esteemed Major Advisor ***Dr. (Mrs.) Urmila Gupta Phutela****, Senior Scientist (Biogas), PAU, Ludhiana, for her invaluable and inspiring guidance, constant encouragement, meticulous suggestions, constructive criticism, which had enabled me to complete my research and degree successfully. Her vast and deep knowledge of the subject, sense of dedication and above all, her parental nature, soft and sober attitude throughout the tenure of this investigation will be a part of memory forever, I consider myself fortunate to be her disciple.*

With the same spirit and respect I pen down my deep sense of gratitude to the respected members of my Advisory Committee, ***Dr. Shammi Kapoor****, Head cum Sr. Mycologist, Department of Microbiology,* ***Dr. Sarabjit Singh Sooch****, Sr. Research Engineer, School of Renewable Energy Engineering,* ***Dr. Jagdeep Singh Sandhu****, Biotechnologist, School of Agricultural Biotechnology and* ***Dr. H. S. Sodhi****, Sr. Mycologist, Department of Microbiology, PAU, Ludhiana, for their encouragement and guidance during the research work and for making improvement while going through the manuscript. I sincerely acknowledge Punjab Agricultural University for providing the necessary facilities regarding course and research work and* ***Indian Council of Medical Research, New Delhi*** *for providing me the fellowship (ICMR-Fellowship) throughout my Ph.D. degree.*

I could not find suitable words, in the present lexicon to convey my sublime obeisance to my reverend ***parents****. Parental blessings and patience were constant sources of encouragement to attain this goal. I express my special thanks to my* ***brother Waseem, sisters Rifat and Tabeena, uncles, my cousins Shakeeb, Moueed, Mehr-un-Nisa and Rooh-un-Nisa,*** *all nears and dears for their support, co-operation and love.*

With due reverence and gratitude, I express my special thanks to my affectionate seniors ***Karamjeet Kaur, Nidhi Sahini and Ajit Kaur,*** *for their unreserved affections, benevolent help and encouragement during the whole research work. I am also indebted to my classmates and my juniors* ***Manisha Parmar, Nishu Sharma,Vishalakshi, Vishwas, Rozy, Milky, Versha, Harpreet, Kailash*** *for rendering their help in one or the other way.*

Pleasant company, ever willing help, regular encouragement and sweet memories of my friends, ***Khalid Gul, Eajaz Ahmad, Shahnawaz Ahmad, Abrar Yousuf, Mudasir Yaqoob, Muzamil Hussain, Naseer Ahmad, Nisar Ahmad Mir, Mohammad Amin, Zahoor Ahmad, Jagveer Singh, Mehraj Ahmad, Todar Mal Poonia, Sumeet Singh Saini, Davinder Singh, Atul Dev Sharma, Arif Ahmad, Najeeb-ul-Tarfeen,Mohd Hussain Malik, Rahul Rana, Pupinder singh, Arshid Ahmad Mir, Tawheed Ahmad, Lucky, Ashok Dhakad and Naresh*** *will always remain as a precious asset to me.*

Last but not the least, I am very thankful to ***Mrs. Mehak Arora*** *(Research Assistant),* ***Mr. Baboo Lal*** *(Junior Lab Assistant),* ***Jagdish*** *(DPL) for their timely help during my research work.*

Place: Ludhiana

ROUF AHMAD DAR

Date:

Title of the Dissertation	**:**	Bioprospects of microalgal isolates from water logged area of Punjab for biogas production
Name of the Student and Admission No.	**:**	Rouf Ahmad Dar (L-2013-BS-80-D)
Major Subject	**:**	Microbiology
Minor Subject	**:**	Biotechnology
Name and Designation of Major Advisor	**:**	Dr. (Mrs.) Urmila Gupta Phutela Senior Scientist (Biogas)
Degree to be Awarded	**:**	Ph.D.
Year of award of Degree	**:**	2017
Total Pages in Dissertation	**:**	170 + VITA
Name of the University	**:**	Punjab Agricultural University, Ludhiana-141 004, Punjab, India

ABSTRACT

The present study was aimed at isolation, identification, screening and characterization of microalgae from the waterlogged area of south-west Punjab, India. Optimization of cultural conditions of potential microalgal strains, their biogas production potential and anaerobic co-digestion studies were also conducted. Nineteen microalgal cultures (BGLR1-BGLR19) were isolated and were screened using different culture media for their growth kinetics. Isolate BGLR6 followed by BGLR5 showed the highest growth and biomass production in Algae culture medium and BG-11 medium respectively. These isolates upon molecular identification were found to be *Asterarcys quadricellulare* BGLR5 (MF661929) and *Spirulina subsalsa* BGLR6 (MF191711). The cultivation conditions for the enhanced production of biomass and other functional components (chlorophyll, carbohydrate, lipid and protein) of BGLR5 and BGLR6 were first screened by Plackett–Burman design and then the significant factors were optimized by Central Composite design (CCD). The optimal cultural conditions for BGLR5 and BGLR6 as per the model were pH of 9.92 & 11.5; temperature of 21.84 & 20°C; light intensity of 80.99 & 81 µmol m^{-2} s^{-1}; growth period of 25 days (both); 15.00 mM NH_4Cl/ $CaCl_2$; 12.28 & 5.00 mM $NaNO_3$ and 7.09 & 2.00 mM K_2HPO_4 respectively. Under these conditions, the response variables generated a desirability index of 84.10 and 94.91% for BGLR5 and BGLR6 respectively. A 0.42 and 1.60-fold increase in dry cell biomass yield was achieved in BGLR5 and BGLR6, compared to the basal condition (0.8858 and 1.0890 g biomass L^{-1}) respectively. The biogas potential of the microalgal biomass under controlled temperature (35 ± 2°C) conditions revealed that in case of BGLR5, the highest biogas yield (51.712 Lkg^{-1}algal biomass) was obtained in the hydrothermally (100°C 30min) pretreated biomass whilst in BGLR6, the highest biogas yield (42.73 Lkg^{-1}algal biomass) was obtained in enzymatically (10% 24hr) pretreated biomass. Further, co-digestion of 50% paddy straw and 50% BGLR5 biomass (replacement of paddy straw by equal amount of microalgal biomass) produced 168.32 L biogas kg^{-1} feedstock with the ultimate biogas yield potential of 361.81 mLg^{-1} VS, reduced lag phase (λ:2.81 d) and increased rate of biogas production (R_m: 8.19 $mLg^{-1}d^{-1}$) which was higher not only in comparison to the anaerobic digestion of paddy straw and algal biomass individually but also to that of co-digestion of BGLR6 biomass with paddy straw. Likewise, 50 and 100% supplementation of BGLR5 biomass led to enhanced biogas production. Utilization of algal biomass cultivated in the open pond for co-digestion study with paddy straw in field scale digesters resulted in an increase of 17.27% biogas compared to control. The enhancement of methane content from 46.4% (control) to 66.5% (digester containing algal biomass) was also achieved. Hence, the present study signifies that the *Asterarcys quadricellulare* BGLR5 and *Spirulina subsalsa* BGLR6 biomass could be utilized as a co-feedstock with paddy straw for biogas production.

Keywords: Microalgae, waterlogged area, Punjab, Chlorophyta, Cyanobacteria, *Asterarcys quadricellulare* BGLR5, *Spirulina subsalsa* BGLR6, Plackett–Burman design, Central Composite design, Anaerobic co-digestion, Biogas

Signature of Major Advisor **Signature of the Student**

ਖੋਜ ਪ੍ਰਬੰਧ ਦਾ ਸਿਰਲੇਖ	:	ਬਾਇਓ ਗੈਸ ਦੇ ਉਤਪਾਦਨ ਲਈ ਪੰਜਾਬ ਦੇ ਸੇਮ ਵਾਲੇ ਖੇਤਰਾਂ ਤੋਂ ਲਏ ਗਏ ਮਾਈਕ੍ਰੋਐਲਗਲ ਨਿਖੇੜਕਾਂ ਜੈਵਿਕ ਸੰਭਾਵਨਾ
ਵਿਦਿਆਰਥੀ ਦਾ ਨਾਮ ਅਤੇ ਦਾਖਲਾ ਨੰ.	:	ਰੌਫ਼ ਅਹਿਮਦ ਡਾਰ (ਐਲ-2013-ਬੀ.ਐਸ.-80-ਡੀ.)
ਮੁੱਖ ਵਿਸ਼ਾ	:	ਸੂਖਮ ਜੀਵ ਵਿਗਿਆਨ
ਨਿਮਨ ਵਿਸ਼ਾ	:	ਬਾਇਓ ਟੈਕਨੋਲੋਜੀ
ਪ੍ਰਮੁੱਖ ਸਲਾਹਕਾਰ ਦਾ ਨਾਮ ਅਤੇ ਅਹੁਦਾ	:	ਡਾ. ਉਰਮੀਲਾ ਗੁਪਤਾ ਫੁਟੇਲਾ ਸੀਨੀਅਰ ਵਿਗਿਆਨੀ (ਬਾਇਓਗੈਸ)
ਡਿਗਰੀ	:	ਪੀ.ਐਚ.ਡੀ.
ਡਿਗਰੀ ਮਿਲਣ ਦਾ ਸਾਲ	:	2017
ਖੋਜ ਪ੍ਰਬੰਧ ਦੇ ਕੁੱਲ ਪੰਨੇ	:	170 + ਵੀਟਾ
ਯੂਨੀਵਰਸਿਟੀ ਦਾ ਨਾਮ	:	ਪੰਜਾਬ ਖੇਤੀਬਾੜੀ ਯੂਨੀਵਰਸਿਟੀ, ਲੁਧਿਆਣਾ - 141004, ਪੰਜਾਬ, ਭਾਰਤ

ਸ਼ਾਰ-ਅੰਸ਼

ਮੌਜੂਦਾ ਅਧਿਐਨ ਦਾ ਮਕਸਦ ਦੱਖਣ-ਪੱਛਮੀ ਪੰਜਾਬ ਦੇ ਸੇਮ ਅਧੀਨ ਖੇਤਰਾਂ ਤੋਂ ਲਈ ਗਈ ਮਾਈਕ੍ਰੋਐਲਗੀ ਦੇ ਨਿਖੇੜਨ, ਪਹਿਚਾਣ, ਜਾਂਚ ਅਤੇ ਚਿੱਤਰ ਚਿੱਤਰਣ ਕਰਨਾ ਸੀ। ਸੰਭਾਵਤ ਮਾਈਕ੍ਰੋਐਲਗੀ ਸਟ੍ਰੇਨਾਂ ਦੇ ਕਲਚਰ ਹਲਾਤਾਂ ਦਾ ਅਨੁਕੂਲਨ ਕੀਤਾ ਗਿਆ, ਉਹਨਾਂ ਦੀ ਬਾਇਓਗੈਸ ਉਤਪਾਦਨ ਦੀ ਸਮਰੱਥਾ ਦਾ ਮੁਲਾਂਕਣ ਅਤੇ ਐਨਆਰੋਬਿਕ ਸਹਿ-ਪਾਚਨ ਅਧਿਐਨ ਵੀ ਕੀਤਾ ਗਿਆ। ਉਨੀ ਮਾਈਕ੍ਰੋਐਲਗੀ ਕਲਚਰ (BGLR1-BGLR19) ਨੂੰ ਨਿਖੇੜਿਆ ਗਿਆ ਅਤੇ ਉਹਨਾਂ ਦੀ ਵਿਕਾਸ ਗਤੀਸ਼ੀਲਤਾ ਲਈ ਵੱਖੋ-ਵੱਖਰੇ ਕਲਚਰ ਮੀਡੀਆ ਦੀ ਵਰਤੋਂ ਕਰਕੇ ਉਹਨਾਂ ਦੀ ਚਾਂਜ ਕੀਤੀ ਗਈ। BGLR6 ਨਿਖੇੜਕ ਨੇ ਅਤੇ ਇਸ ਉਪਰੰਤ BGLR5 ਨਿਖੇੜਕ ਨੇ ਕ੍ਰਮਵਾਰ ਐਲਗੀ ਕਲਚਰ ਅਤੇ BG-11 ਮੀਡੀਅਮ ਵਿੱਚ ਸਭ ਤੋਂ ਵਧੇਰੇ ਵਿਕਾਸ ਦਿਖਾਇਆ ਅਤੇ ਬਾਇਓਗੈਸ ਦਾ ਉਤਪਾਦਨ ਵੀ ਸਭ ਤੋਂ ਵਧੇਰੇ ਕੀਤਾ। ਆਣਵਿਕ ਪਹਿਚਾਣ ਕਰਨ ਤੇ ਪਤਾ ਚੱਲਿਆ ਕਿ ਇਹ ਨਿਖੇੜਕ *Asterarcys quadricellulare* BGLR5 (MF661929) and *Spirulina subsalsa* BGLR6 (MF191711) ਕਿਸਮ ਸਨ। BGLR5 ਅਤੇ BGLR5 ਤੋਂ ਬਾਇਓਗੈਸ ਅਤੇ ਹੋਰ ਕਿਰਿਆਤਮਕ ਸੰਘਟਕਾਂ (ਕਲੋਰੋਫਿਲ, ਕਾਰਬੋਹਾਈਡ੍ਰੇਟ, ਲਿਪਿਡ ਅਤੇ ਪ੍ਰੋਟੀਨ) ਦੇ ਵਧੇਰੇ ਉਤਪਾਦਨ ਲਈ ਕਾਸ਼ਤ ਦੇ ਹਲਾਤਾਂ ਦੀ ਸਭ ਤੋਂ ਪਹਿਲਾਂ ਪਲੇਕੇਟ-ਬੁਰਮੇਨ ਡੀਜ਼ਾਇਨ ਮਾਡਲ ਰਾਹੀਂ ਜਾਂਚ ਕੀਤੀ ਗਈ। ਇਸ ਮਾਡਲ ਦੇ ਅਧਾਰ ਤੇ BGLR5 ਅਤੇ BGLR5 ਲਈ ਕ੍ਰਮਵਾਰ 9.92 ਅਤੇ 11.5 ਪੀ.ਐਚ.; 21.84 ਅਤੇ 20°C ਤਾਪਮਾਨ, 80.99 ਅਤੇ 81 $\mu mol\ m^{-2}\ s^{-1}$ ਰੋਸ਼ਨੀ ਤੀਬਰਤਾ, 25 ਦਿਨਾਂ ਦਾ ਵਿਕਾਸ ਅੰਤਰਾਲ (ਦੋਨਾਂ ਲਈ); 15.00 mM NH_4Cl/ $CaCl_2$; 12.28 & 5.00 mM $NaNO_3$ ਅਤੇ 7.09 & 2.00 mM K_2HPO_4 ਕਲਚਰ ਦੇ ਅਨੁਕੂਲ ਹਲਾਤ ਸਨ। ਇਹਨਾਂ ਹਲਾਤਾਂ ਅਧੀਨ, ਰਿਸਪੋਂਸ ਵੈਰੀਏਬਲਾਂ ਨੇ BGLR5 ਅਤੇ BGLR5 ਲਈ ਕ੍ਰਮਵਾਰ 84.10 ਅਤੇ 94.91% ਅਨੁਕੂਲ ਸੂਚਕਾਂਕ ਦਰਸਾਇਆ। ਮੂਲ ਹਲਾਤਾਂ (0.8858 ਅਤੇ 1.0890 ਗ੍ਰਾਮ ਬਾਇਓਗੈਸ ਪ੍ਰਤੀ ਲਿਟਰ) ਦੇ ਮੁਕਾਬਲੇ BGLR5 ਅਤੇ BGLR6 ਵਿੱਚ, ਸੁੱਕੇ ਸੈਲ ਦੇ ਜੈਵਿਕ ਮਾਦੇ ਦੇ ਝਾੜ ਵਿੱਚ ਕ੍ਰਮਵਾਰ 0.42 ਅਤੇ 1.60-ਫੋਲਡ ਦਾ ਵਾਧਾ ਹੋਇਆ। ਨਿਯੰਤ੍ਰਤ ਤਾਪਮਾਨ (35 ± 2°C) ਅਧੀਨ ਮਾਈਕ੍ਰੋਐਲਗੀ ਦੀ ਜੈਵਿਕ ਮਾਦੇ ਤੋਂ ਬਾਇਓਗੈਸ ਉਤਪਾਦਨ ਦੀ ਸਮਰੱਥਾ ਤੋਂ ਪਤਾ ਚੱਲਿਆ ਕਿ ਨਿਖੇੜਕ BGLR5 ਨਾਲ ਹਾਈਡ੍ਰੋਥਰਮਲ (100°C 30min) ਵਿਧੀ ਰਾਹੀਂ ਉਪਚਾਰਤ ਜੈਵਿਕ ਮਾਦੇ ਤੋਂ ਬਾਇਓਗੈਸ ਦਾ ਉਤਪਾਦਨ ਸਭ ਤੋਂ ਵਧੇਰੇ (51.712 Lkg^{-1}algal biomass) ਹੋਇਆ ਅਤੇ ਜੈਵਿਕ ਮਾਦੇ ਨੂੰ ਇੰਜ਼ਾਈਮੈਟੀਕਲੀ ਸੋਧਨ (10% 24 ਘੰਟੇ) ਨਾਲ ਬਾਇਓਗੈਸ ਦਾ ਉਤਪਾਦਨ ਸਭ ਤੋਂ ਵਧੇਰੇ ((42.73 Lkg^{-1}algal biomass) ਹੋਇਆ। ਇਸ ਮਗਰੋਂ, 50% ਝੋਨੇ ਦੀ ਨਾੜ ਅਤੇ 50% BGLR5 ਜੈਵਿਕ ਮਾਦੇ (ਝੋਨੇ ਦੀ ਨਾੜ ਦੀ ਥਾਂ ਮਾਈਕ੍ਰੋਐਲਗੀ ਦੀ ਜੈਵਿਕ ਮਾਦੇ ਦੀ ਵਰਤੋਂ) ਦੇ ਸਹਿ-ਪਾਚਣ ਨੇ 168.32 ਲਿਟਰ ਬਾਇਓਗੈਸ ਪ੍ਰਤੀ ਕਿ.ਗ੍ਰਾ. ਫੀਡਸਟਾਕ ਦਾ ਉਤਪਾਦਨ ਕੀਤਾ ਅਤੇ ਸਿੱਟੇ ਵਜੋਂ 361.81 mLg^{-1} VS ਬਾਇਓਗੈਸ ਉਤਪਾਦਨ ਦੀ ਸਮਰੱਥਾ ਦਰਸਾਈ, ਲੈਗ ਅੰਤਰਾਲ ਨੂੰ ਘਟਾਇਆ (λ:2.81 d) ਅਤੇ ਬਾਇਓ ਗੈਸ ਦੇ ਉਤਪਾਦਨ ਦੀ ਦਰ ਵਿੱਚ ਵਾਧਾ (R_m: 8.19 $mLg^{-1}d^{-1}$) ਕੀਤਾ, ਜੋਕਿ ਨਾ ਸਿਰ ਝੋਨੇ ਦੀ ਨਾੜ ਦੇ ਅਜੈਵਿਕ ਪਾਚਣ ਅਤੇ ਐਲਗਲ ਜੈਵਿਕ ਮਾਦੇ ਤੋਂ ਇਕੱਲੇ ਤੌਰ ਤੇ ਹੀ ਜ਼ਿਆਦਾ ਸੀ ਸਗੋਂ ਝੋਨੇ ਦੀ ਨਾੜ ਨਾਲ BGLR6 ਦੇ ਜੈਵਿਕ ਮਾਦੇ ਦੇ ਸਹਿ-ਪਾਚਣ ਤੋਂ ਵੀ ਜ਼ਿਆਦਾ ਸੀ। ਇਸੇ ਤਰ੍ਹਾਂ, 50 ਅਤੇ 100% BGLR5 ਦੇ ਜੈਵਿਕ ਮਾਦੇ ਦੀ 50 ਅਤੇ 100% ਸਪਲੀਮੈਂਟੇਸ਼ਨ ਨਾਲ ਬਾਇਓ ਗੈਸ ਦੇ ਉਤਪਾਦਨ ਵਿੱਚ ਵਾਧਾ ਹੋਇਆ। ਕੰਟਰੋਲ ਦੇ ਮੁਕਾਬਲੇ ਖੇਤ ਪੈਮਾਨੇ ਦੇ ਡਾਈਜੈਸਟ੍ਰਾਂ ਵਿੱਚ ਖੁੱਲ੍ਹੇ ਤਲਾਅ ਵਿੱਚ ਕਾਸ਼ਤ ਕੀਤੇ ਐਲਗਲ ਜੈਵਿਕ ਮਾਦੇ ਦੀ ਝੋਨੇ ਨਾੜ ਨਾਲ ਸਹਿ-ਪਾਚਣ ਵਜੋਂ ਵਰਤੋਂ ਕਰਨ ਨਾਲ ਬਾਇਓ ਗੈਸ ਦੇ ਉਤਪਾਦਨ ਵਿੱਚ 17.27% ਦਾ ਵਾਧਾ ਹੋਇਆ। ਮੀਥੇਨ ਦੀ ਮਿਕਦਾਰ ਵਿੱਚ 46.4% (ਕੰਟਰੋਲ) ਤੋਂ 66.5% (ਐਲਗਲ ਜੈਵਿਕ ਮਾਦੇ ਵਾਲਾ ਡਾਈਜੈਸਟਰ) ਦਾ ਵਾਧਾ ਦਰਜ ਕੀਤਾ ਗਿਆ। ਇਸ ਲਈ, ਮੌਜੂਦਾ ਅਧਿਐਨ ਤੋਂ ਇਹ ਤੱਥ ਸਾਹਮਣੇ ਆਏ ਕਿ ਬਾਇਓ ਗੈਸ ਦੇ ਉਤਪਾਦਨ ਲਈ ਝੋਨੇ ਦੀ ਨਾੜ ਨਾਲ *Asterarcys quadricellulare* BGLR5 and *Spirulina subsalsa* BGLR6 ਦੇ ਜੈਵਿਕ ਮਾਦੇ ਦੀ ਵਰਤੋਂ ਕੀਤੀ ਜਾ ਸਕਦੀ ਹੈ।

ਮੁੱਖ ਸ਼ਬਦ: ਮਾਈਕ੍ਰੋਐਲਗੀ, ਸੇਮ ਵਾਲਾ ਖੇਤਰ, ਪੰਜਾਬ, ਕਲੋਰੋਫਾਈਟਾ, *Asterarcys quadricellulare* BGLR5, *Spirulina subsalsa* BGLR6, ਪਲੇਕੇਟ-ਬੁਰਮੇਨ ਡੀਜ਼ਾਇਨ, ਸੈਂਟਰਲ ਕੋਮਪੋਜ਼ਿਗ ਡੀਜ਼ਾਇਨ, ਅਜੈਵਿਕ ਸਹਿ-ਪਾਚਣ, ਬਾਇਓ ਗੈਸ।

ਪ੍ਰਮੁੱਖ ਸਲਾਹਕਾਰ ਦੇ ਹਸਤਾਖਰ **ਵਿਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖਰ**

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER- I

INTRODUCTION

It is the apparent fact that since the beginning of Industrial Revolution there has been a tremendous increase in the demand for global energy. Punishing to this demand we are having the replenishable though easily accessible conventional resources. However, due to their scarcity and incessant demand for energy these resources soar at a rapid pace. Today the industrialized global village is mired with energy demand for itself and the coming generation. Combustion of fossil fuels like petrol, coal or natural gas results in the release of CO_2 and other greenhouse gases, thus debilitating the health of the environment by leading to climatic changes (IPCC 2007). In the quest of searching and developing eco-friendly alternatives, various ideas were put forth. Some voted for wind energy, geothermal energy and others for kinetic energy stored in water (e.g. wave and tidal movements of the oceans or river dams) and the solar energy. Out of these, the huge energy source is undoubtedly the solar irradiation. It is established fact that the energy which reaches earth's surface is around 5600 times the global energy demand (Schenk *et al* 2008). So, therefore, harnessing solar energy by letting photosynthetic organisms to do this job for us is the ray of hope. The resultant biomass is a highly reliable renewable resource of energy by producing fuel from it. This process promises and serves a dual purpose; one is the biological fixation of increasing CO_2 (handy method for mitigation of increasing atmospheric CO_2) and the second is affordable energy production, capable and sufficient to gratify present and the future energy demands.

Biomass includes a range of substrates like bio-wastes (municipal wastes, food wastes, agricultural wastes etc.), bioenergy crops (edible as well as non-edible oilseeds) and several aquatic plants recognized as bio-oil sources. These different kinds of biomass can be converted into several products, like biodiesel, bioethanol or biogas. The "first generation" biofuels derived from plant products like ethanol from sugars derived from plants and biodiesel obtained from lipids have got comparatively unsatisfactory energy balances and thus probably can never assume a noteworthy part in worldwide energy supply, while "second generation" biofuels, which are generated from the whole biomass offer far more significant and noteworthy possibilities (IEA, 2010). But, the utilization of plant biomass for energy production is intricate and vexed as it competes with food or feed production. It is due to this reason that the vast majority of the plants utilized for energy production today (sugar beets, crop plants, sugar cane, canola, etc.) must be cultivated on arable land.

Algae have got various promising benefits compared to higher plants due to their high growth rates and the feasibility and practicability of growing on non-arable lands, in lakes or the ocean, thus alleviating the very intense competition regarding food and feed (Stephens *et al* 2010). Algae can absorb CO_2 gas as the source of carbon for growth and thus extenuate the

global warming problem (Hirano *et al* 1997). The photosynthetic efficiency of microalgae is generally higher than other biomasses like trees. If fuel is recuperated proficiently from them, these can be utilized as fuel rather than fossil fuel (Amin 2009). A propitious strategy thus seems to be the utilization of rapidly growing algae species for anaerobic digestion (AD) to generate biogas, which seems to replace natural gas resources. Some microalgae like the green microalga *Chlamydomonas reinhardtii* contrary to higher plants have the phenomenal capability to generate hydrogen via hydrolysis of water during illumination, which serves as another environment-friendly gaseous fuel (Kruse *et al* 2005). The leftover algal biomass after hydrogen generation can further be used to produce biogas via anaerobic digestion. In spite of the fact that research regarding utilizing of microalgae as feedstock for biogas production is exceptionally constrained, recently done theoretical computations demonstrated their potential (Yen and Brune 2007, Sialve *et al* 2009). Nonetheless, a couple of efficient and methodical investigations have been carried out to date to completely investigate the maximum biodegradability of microalgae and to increase their CH_4 productivity. The biochemical methane potential (BMP) assay represents a valuable method to illustrate both the biodegradability and the methane production capacity of organic raw materials. Some studies have demonstrated that percentage of methane in the biogas and the rate of methane production can be modified by varying initial substrate concentration (Fernandez *et al* 2008). The biomass concentrations of microalgal cultures are low (<0.5–27 g/L) and the concentration of this biomass before digestion process could escalate its production cost, which could ultimately imperil the economics of the whole biofuel generation process. Therefore, to circumvent additional biomass harvesting costs, the optimal concentration of microalgae for anaerobic digestion needs to be determined (Molina *et al* 2003). Consequently, algal biomass seems to be a potential contender in biofuel generation.

Microalgae are unicellular microscopic (2–200 μm), polyphyletic, prokaryotic or eukaryotic photosynthetic microorganisms that can grow expeditiously and sustain in severe conditions due to their unicellular or simple multicellular structure. These are categorized into two prokaryotic divisions which are Cyanophyta and Prochlorophyta and nine eukaryotic divisions namely Glaucophyta, Rhodophyta, Heterokontophyta, Cryptophyta, Dinophyta, Euglenophyta, Haptophyta, Chlorophyta and Chlorarachniophyta. It is assessed that more than 50,000 species exist, and out of this around 30,000, have been studied and explored. These can further be utilized for production of biofuels and other value-added products (Khan *et al* 2009).

Worldwide various organizations and government associations have already evaluated diverse strategies and plans and produced cost estimates for the commercial-scale cultivation of algae. A considerable lot of these examinations prescribe the algae to be utilized in biofuel plants, might be efficaciously cultivated on land adjacent to power stations, for

converting the CO_2 from exhausts into fuel. This reason has ensued appreciable curiosity towards the use of anaerobic digestion or fermentation technology for the production of valuable by-products such as biogas. Biogas comprising a mixture of methane (55–75%) and CO_2 (25–45%) is produced by anaerobic microorganisms during anaerobic digestion. Methane produced from AD process can be utilized as fuel gas or can be further converted to produce electricity (Holm-Nielsen *et al* 2009). Anaerobic digestion (AD) is an advanced and fascinating technology for obtaining bioenergy from waste. In addition to this, it is being exploited for biodegradable waste management globally and is capable of reducing the considerable percentage (70%) of volatile solids in a substrate, while in the meantime producing methane, a source of renewable energy (Shanmugam and Horan 2008). Despite the fact that a lot of research has been done on methanogenesis of industrial waste, agricultural residue, the domestic and livestock residue for the production of biogas (Sakar *et al* 2009, Chanakya *et al* 2012), the literature regarding methanogenesis of algal biomass is scarce. The method of AD of algal biomass for biofuel generation has distinct benefits over other routes or methods. For example, this method does not demand dewatering or additional chemical extraction steps, as required for other liquid biofuels including biodiesel from algal biomass (Malik and Prajapati 2012, Prajapati *et al* 2013). Furthermore, Heerenklage *et al* 2010 have revealed that a large number of algal species belonging to genus *Chlorella*, *Spirulina, Euglena* etc. have substantially higher methane yields (>0.53 m^3 kg^{-1} VS) in comparison to methane yield of feedstocks like maize silage, field grass (<0.35 m^3 kg^{-1} VS). Though it appears that algal biomass has a high potential for biogas production but still there had been extremely less research on the same. However, the situation has been changed and efforts are being made on algal biomass anaerobic digestion for sustainable renewable energy generation. Different algal biomass including *Chlorella vulgaris*, *Dunaliella tertiolecta*, *Chlamydomonas reinhardtii*, *Phaeodactylum triconutum*, *Scenedesmus obliquus* and *Rhizoclonium* have been tried for the production of biogas (Ras *et al* 2011, Zamalloa *et al* 2012, Ehimen *et al* 2013).

Nowadays throughout the globe, there is an inclination towards the development of prospective ways to tackle the rising energy demands. In order to commercialize the biofuel and make it profitable for concerned industries, there is immense need of scientific advances. Lately, the anaerobic digestion of single substrates was used to produce biogas but the result was comparatively low. A remarkable alternative which is by all accounts suitable for meeting the increasing pace of energy requirement is co-digestion of different substrates. It had been demonstrated that better methane yield can be accomplished on co-digestion of various biodegradable wastes, rather than using the single organic material or substrate. Besides this, it has other advantages like perfect digestibility, production of ideal organic fertilizer, reduction of pathogens and odor, cost sharing and environmental safety. It is also

established that the C/N ratio in algal biomass is around 10:1, which is too low for the digestion (Migliore *et al* 2012, Vivekanand *et al* 2012). Due to low C/N ratio, chances of excessive ammonia formation are higher. So as to avoid exorbitant accumulation of ammonia, and to enhance the digestion performance, addition of carbon-rich substrates is required to boost the digestion performance. Paddy straw having high carbon content has high C/N ratio, thus can be co-digested with algal biomass to increase the methane production.

Paddy straw is one of the most abundant lignocellulosic wastes. Paddy straw is the most favorable lignocellulosic biomass to be capitalized as a raw material for bioenergy generation. Generally, structural constituents of paddy straw have been categorized as physical or chemical. The physical structural characteristics or components include accessible surface area, the degree of polymerization, crystallinity, biomass particle size and pore volume etc (Millati *et al* 2011). Whereas, cellulose, hemicelluloses, lignin and silica comprise the chemical structural constituents forming the cellular complexity of the vegetal biomass. Cellulose, a semicrystalline biopolymer with ordered crystalline and disordered amorphous regions, is is a linear homopolymer of repeated units of cellobiose (two anhydrous glucose rings joined via a β-1, 4 glycosidic linkage). Hemicellulose is a hetero-polysaccharide consisting of various subunits like hexoses (mannose, glucose and galactose), pentoses (xylose and arabinose), and sugar acids. Lignin, amorphous heteropolymer, is made of three different phenylpropane units (p-coumaryl, coniferyl and sinapyl alcohol), which form the guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H) subunits, respectively (Martinez *et al* 2005, Wei *et al* 2009). Paddy straw has got low lignin and high silica content in comparison to other cereal straws (Soest 2006). Silicon is mostly found in living plants in three basic forms: insoluble silica (90%), silicate ions (0.5-0.8%) and water-soluble colloidal silicic acid (0-3.3%) (Jan and Alexandra 2006).

In India, annual production of rice is about 136.5 million tones and about 136.50-150.00 million tones of paddy straw are produced annually (www.indiastat.com). Field burning is a major practice adopted so far for the removal of paddy straw which releases gases such as carbon dioxide, carbon monoxide, methane, nitrogen compounds, sulphur dioxide and particulate matter into the atmosphere. Out of these gases, the significant greenhouse gases that contribute to global warming are methane and nitrous oxide (Gadde *et al* 2009). Apart from polluting the environment, paddy straw burning also causes health problems and soil erosion. In order to overcome the problems associated with paddy straw burning, several alternatives to utilize paddy straw has been proposed (Das and Singh 2004) like the production of reducing sugars, ethanol, resins, methane and other miscellaneous products (Demirbas 2008). The specific methane potential of paddy straw ranges from 92 to 280 l kg^{-1} VS added, depending upon the digestion parameters and pretreatment methods (Mussoline *et al* 2013). So this can be utilized along with algal biomass for biogas production. Also, as

reflected in the literature not much attempt has gone into testing co-digestion of paddy straw with algal biomass.

The unconsumed biomass after anaerobic digestion can further be reprocessed to make organic fertilizers which can contribute to better crop yield and soil fertility. Apart from being sustainable and renewable, it would empower viable and worthwhile agricultural practices in contributing considerable efficiencies and lessen costs of algae production. The microalgae demonstrate acceptable stability and high conversion efficiency for anaerobic digestion process because of the lower cellulose and absence of lignin. The anaerobic digestion process depends on certain environmental factors like temperature, pH, inhibitors, C/N ratio, volatile solids, total solids, organic loading rate, hydraulic retention time (HRT), particle size, internal pressure and mixing. Changes in any one of the parameter add difficulty to the process and digesters (Chandra *et al* 2011, Kwietniewska and Tys 2014).

Out of total 50,362 sq km area of Punjab, only 71% of the area is used for agriculture. Southwest zone that is about 34% of total area suffers from the erratic and scanty rainfall problem. There is brackish groundwater with high electrical conductivity (EC) and residual sodium concentration (RSC). In this zone, the groundwater has been found to be alkaline (pH 7.23 to 8.66) and moderate to highly saline (EC 436 to 3510 siemen/cm) in nature. Generally, none of the anions dominate, whereas, among cations, sodium and potassium predominate in 50% of the samples. Southwest area of Punjab is affected by waterlogging which is not suitable for cultivation of crops. But, this type of area could be efficiently utilized for microalgae cultivation. Microalgae are generally cultivated over the standing water and most of the research has been done on algae isolated and grown in the fresh water. Few reports are available on algae grown on standing or saline water.

Keeping the importance of microalgae as future feedstock for biogas production and use of the waterlogged area for microalgae cultivation, in mind, the present the present study was planned to meet the following objectives with a view to utilizing microalgae for mass cultivation and as co-feedstock with paddy straw for prospective biogas production.

1. To isolate, purify, screen and characterize microalgae from waterlogged areas of Punjab
2. To optimize growth parameters of the selected isolate for maximum biomass production
3. To access the effect of microalgae biomass as a co-feedstock on biogas production from paddy straw

CHAPTER – II

REVIEW OF LITERATURE

Algae are principally a huge and heterogeneous group of simple, mostly autotrophic organisms consisting of both unicellular and multicellular organisms. These have the capacity of producing significantly large amounts of biomass and lipids per hectare as compared to any kind of terrestrial biomass. Their biochemical composition comprising of the high quantity of biodegradable constituents like carbohydrates (5-23%), proteins (6-52%), lipids (7-23%) make them suitable substrate for biogas production. It thus becomes pivotal to study the biogas production potential of microalgal biomass which will further accentuate the potential utilization of microalgae. So, the present study focuses on bioprospects of microalgal isolates from waterlogged area of Punjab for biogas production. The literature pertaining to the present research work is reviewed under the following main headings:

2.1 Isolation and purification of microalgae
- 2.1.1 Selection of sources of microalgae
- 2.1.2 Culture media
- 2.1.3 Single-cell isolation
- 2.1.4 Agar plate method
- 2.1.5 Direct isolation by atomizer
- 2.1.6 Dilution techniques
- 2.1.7 Media enrichment
- 2.1.8 Micromanipulation
- 2.1.9 Flow cytometry

2.2 Microalgae cultivation and production process
- 2.2.1 Growth technologies
  - 2.2.1.1 Closed photobioreactors
  - 2.2.1.2 Open ponds
  - 2.2.1.3 Hybrid systems
- 2.2.2 Harvesting and dewatering of microalgal biomass

2.3 Factors affecting microalgal growth
- 2.3.1 Effect of nutrients
- 2.3.2 Effect of temperature, pH and light on the growth

2.4 Optimization of cultural conditions of microalgae using response surface methodology

2.5 Extraction/Conversion technologies for microalgae biomass

2.6 Microalgae as propitious feedstock for biofuel production

2.7 Algae anaerobic digestion
- 2.7.1 Anaerobic digestion of macroalgae
- 2.7.2 Anaerobic digestion microalgae
- 2.7.3 Pretreatment of algal biomass
  - 2.7.3.1 Thermal pretreatments
  - 2.7.3.2 Mechanical pretreatments
  - 2.7.3.3 Chemical pretreatments
  - 2.7.3.4 Biological pretreatment

2.8 Co-digestion

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure. These are primordial photosynthetic organisms having simple cellular structure and a large surface to volume body ratio. This bestows them with the capability to uptake considerable amount of nutrients. Generally, microalgae are more efficacious than plants in converting solar energy due to their simple cellular structure but the mechanism of photosynthesis is exactly similar to that of higher plants (Ghirardi *et al* 2000, Melis 2002, Metzger and Largeau 2005, Singh *et al* 2005, Walter *et al* 2005, Spolaore *et al* 2006). Examples of prokaryotic microalgae are Cyanobacteria (Cyanophyceae) and eukaryotic microalgae are green algae (Chlorophyta) and diatoms (Bacillariophyta) (Li *et al* 2008). Microalgae are found to be present in all ecosystems from water to land, representing a huge number of species existing in a broad range of ecological conditions. More than 50,000 species of microalgae is estimated to be existing, out of which 30,000 have been studied (Richmond 2004). A large number of microalgal species have been described by researchers in the past few decades throughout the world. The freshwater microalgae collection of University of Coimbra (Portugal) is considered as one of the world's largest collection, having more than 4000 strains and 1000 species, bear its witness. This collection of microalgae can be used for diverse applications like food crops for human consumption, as energy source and value-added products for pharmaceutical purposes. Biologists have categorized microalgae in a variety of classes on the basis of their life cycle, basic cellular structure and pigmentation (Sheehan 1998). The four broad groups of algae (at least in provisions of abundance) are shown in Table 2.1.

Table 2.1 Classification of microalgae (adapted from Khan *et al* 2009)

S.No.	Microalgae	Known species	Storage material	Habitat
1	Diatoms (Bacillariophyceae)	100,000 (approximately)	Chyrsolaminarin (polymer of carbohydrates) and TAGs	Fresh, brackish water and oceans
2	Green algae (Chlorophyceae)	8,000 (near about)	Starch and triacylglycerols (TAGs)	Freshwater
3	Blue-green algae (Cyanophyceae)	2,000 (approximately)	Starch and triacylglycerols (TAGs)	Different habitats
4	Golden algae (Chrysophyceae)	1,000 (approximately)	triacylglycerols and carbohydrates	Freshwater

2.1 Isolation and purification of microalgae

2.1.1 Selection of sources of microalgae

Microalgae grow in the natural environments including water, rocks and soil. Their main habitats are brackish, freshwater and marine ecosystems. These can be found in all sorted of environments like general aquatic ecosystems such as lakes, rivers and the oceans,

and also in extreme environments like volcanic waters and salt waters. The indigenous or local microalgae species should be collected because of having a competitive advantage under the local climatic, geographical and ecological conditions. The thermal springs, glacier ice and industrial-waste treatment sites provide ongoing enrichment and selection of organisms adapted to specific conditions and thus yields very different algal isolates.

2.1.2 Culture media

The different culture media have been used and developed for isolation and cultivation of microalgae like AK Medium, COMBO Medium, BG-11, Bolds Basal Medium, Zarrouk's Medium, Conway Medium etc. Some of them are modifications formulated after a detailed study on the nutrient requirement of the organism. Artificial seawater media is common media for marine microalgae but the better growth of algae had been attained by adding small quantities of natural seawater (less than 1–4%) to the artificial seawater (Andersen 2005). A mixture of nitrate and phosphate and soil extracts was used for growing the green dasycladalean *Acetabularia,* unicellular and benthic marine algae. Besides nitrogen and phosphate sources, algal media were formulated to include vitamins, trace metal solutions, antibiotics and metabolically inert chelator like EDTA (Andersen 2005). A vast range of modified media can be devised by meticulously manipulating major nutrients for microalgae growth (Scott *et al* 2010). Antibiotics can be used in the growth medium to inhibit the growth of contaminating cyanobacteria and other bacteria. Bacterial contamination can be prevented by antibiotics or detergent or phenol treatment (Abu *et al* 2007). Thus media composition is of vital importance as it has to be inclusive of indispensable components that support the growth of isolates of interest.

2.1.3 Single-cell isolation

Micropipettes are used to pick up the single cells or filaments under a dissecting microscope. The cells are then transferred to fresh sterile medium or agar medium for isolation (Mutanda *et al* 2011). This is the most common method for single-cell isolation, although automation is more advantageous. The objective of this method is to choose and pick up a single cell from the sample and deposit it, without damage, into a sterile droplet of the medium. These droplets containing the target cell are studied using a multi-test slide. The second method to examine the droplets for the target cell is a tissue-culture inverted microscope which involves the use of welled plates or welled slides. Slides with cover glass-bottoms can be used for critical observations and photographs, and can also be used with 40x and even 100x objective lenses. However, the microalgal droplets can be placed on agar to reduce evaporation but this step depends on the size of the cells. The skill of the technique is important not to shear or damage the cell (Maria *et al* 2016).

2.1.4 Agar plate method

It is one of the common and oldest methods. It involves isolation of microalgal cells on agar plates. This is the preferred method due to the direct establishment of axenic cultures

and simple to use. (Brahamsha1996). The concentration of agar is not considered an important factor. The most likely range of agar used is 0.8% and 1.5–2.0%. The pattern of growth of algae is different; some grow embedded while others grow on the surface of agar medium.

2.1.5 Direct isolation by atomizer

The atomizer is used for the isolation of cells from the liquid suspension (Pfau *et al* 1971). Generally, in this method, a liquid cell suspension is passed through an atomiser wherein it is converted into small droplets or particles with forced sterile air. This leads to the strewing of the cells onto the plate (Andersen 2005). The small droplets which are formed are evenly sprayed on the surface of an agar plate. The plates are incubated, and after colony formation, selected cells are removed and inoculated or transferred to other sterile liquid medium or an agar.

2.1.6 Dilution techniques

The purpose of the dilution method is to pass a single cell in a test tube, flask, thus helping in getting single-cell isolates (Andersen 2005). It becomes easy to determine the necessary dilution for obtaining a single cell in a known volume (through probability) if the approximate concentration of cells is known. This method can be amended in various ways like dilution with distilled water, culture medium, filtered water from the sample site, seawater or some combination of these. Enrichment of isolation tubes or cell wells by elements like selenium, ammonium or others can be done to specifically choose species that require these nutrients (Andersen 2005).

2.1.7 Media enrichment

Enrichment is the process of providing a suitable environment for the growth and reproduction of a special group of microalgae while being inhibitory or lethal for non-target organisms. For single cell isolations, enrichment of cultures has long been used as an initial step. Some of the common enriching substances include soil water extract, nutrients like ammonium, nitrate and phosphate or a trace metal. The algal culture free of bacteria can be obtained by making the medium acidic without harming the alga. Peat moss can be used as a substitute for soil– water extract when enriching for some algae and other desmids from acid habitats. The use of organic substances, like yeast extract, urea or casein, in low concentrations can be done while isolating osmotrophic algae (Mutanda *et al* 2011). The addition of enriching substance is done in small quantities and in phases. Besides this, it can be varied. The most distinctive way for enrichment culturing of hyper-lipid producing microalgae is selective culturing. To isolate the microalgae which can grow at high CO_2 concentration, the culture can be aerated with 1–5% CO_2 and after this, the species with high CO_2 tolerance can be selected (de Morais and Costa 2007, Ramanan *et al* 2010). Although conventional techniques for isolation are adequate, still there are certain limitations. Thus,

there is a persistent and continuous demand for the development of novel techniques for isolation of microalgae.

2.1.8 Micromanipulation

This is an advanced method of isolation. A micromanipulator helps in selection of a single cell from liquid culture. This saves time by 60% over the serial plating technique. This equipment has been described as the ideal tool for algal screening and isolation in the literature (Kacka and Donmez 2008, Moreno-Garrido 2008, Mutanda *et al* 2011). But this technique demands the expertise and experience (Godhe *et al* 2002, Knuckey *et al* 2002).

2.1.9 Flow cytometry

Flow cytometry helps to execute expeditious, strong and quantitative versions of experiments that can be done by fluorescent microscopy. Fluorescence-Activated Cell Sorting (FACS) adds up the beauty of the process. Cells with a particular feature or a combination of features can be isolated from the sample for further investigation or growth. Sinigalliano *et al* (2009) compared electronic cell sorting for isolation of dinoflagellates and other marine eukaryotic phytoplankton to the traditional means like using a micropipette and found it to be the reliable and rapid method of isolation.

2.2 Microalgae cultivation and production process

Extensive studies have been carried out for the cultivation of microalgae as it is the crucial step in the determination of the economic viability of the process. Microalgae can be procured from various sources like existing collection centres of microalgae, Universities or other national and international foundations or from companies specifically devoted to algae growth. These can be obtained from soil and water samples acquired from diverse environments. The best isolations can be done from extreme environments such as thermal springs or industrial wastewaters, as these can sustain and grow in a wide range of environmental conditions, especially of nutrients scarcity and other adverse conditions. The sampling and selection process is well established, although it requires specialized equipment and may be time-consuming (Richmond 2005). The cultivation process of microalgae demands establishment of important factors (Maxwell *et al* 1985) mentioned as:

(i) Growth rate is measured by total amount of biomass accumulated per unit time and unit volume
(ii) Availability of water supply, nutrients and carbon dioxide
(iii) Robustness and tolerance to different environmental stresses and climatic conditions
(iv) The land topography, geology, and ownership
(v) Ease of harvesting and downstream processing of biomass
(vi) Feasibility of obtaining other valuable products.

Therefore, it is very important to interpret how to select the right algae species, create an optimal photo-biological formula for each purpose, and build a cost-effective cultivation

unit. Microalgae are metabolically diverse in nature. They are also capable of changing metabolism in response to changes in the environmental conditions (Chojnacka and Marquez-Rocha 2004). They can grow as:

- Photoautotrophically: Here the sole source of energy is light for microalgae. It is converted to chemical energy during photosynthetic reactions.
- Heterotrophically: This involves the utilization of organic compounds as a carbon and energy source.
- Mixotrophically: Microalgal species growing mixotrophically use photosynthesis as the main source of energy, though both CO_2 and organic compounds are important. Depending on the concentration of organic compounds and light intensity available, the microalgae are capable of living either heterotrophically or autotrophically.
- Photo-heterotrophycally: Photo-heterotrophism involves the usage of light for the utilization of organic compounds as a carbon source. The mixotrophic and photo-heterotrophic metabolisms are not well illustrated and differentiated; so they can be specifically defined and differentiated on the basis of the variation in the energy source they needed to achieve growth and specific metabolite production.

A conventional microalgae growing system consists of (a) growth and cultivation of microalgae, (b) harvesting and dewatering of biomass and (c) extraction and conversion of biomass to the product of interest. The sustainability in terms of energy, economics and environmental aspect, of the whole process, depends on the recycling of the medium. It is quintessential to recover, reprocess and reuse medium (water and fertilizers) in harvest and extraction steps of production.

2.2.1 Growth technologies

Generally, there are mainly two types of microalgae cultivation systems: open ponds & closed photobioreactors (Table 2.2) (Moheimani *et al* 2011, Moheimani 2012).

2.2.1.1 Closed photobioreactors

Closed algal systems often called photobioreactors (PBRs) prevent the cultures from exposing to the atmosphere by covering with a transparent material or containing within the transparent tubing. There are various designs of PBRs available. Some of them include tubular, flat-pannel, annular reactors (Chisti 2006). These PBRs have got tremendous advantages. Prevention of evaporation is one of their visible and recognizable advantages (Dodd 1986, Moheimani *et al* 2011). These systems also alleviate risks of outside contamination; provide controlled and reproducible growth and cultivation environment and pliability in technical design (Jeffery and Wright 1999). Apart from these advantages, the loss of CO_2 is also prevented by sparging it into the photobioreactors. This sparging leads to the generation of larger gas partial pressure in the gas bubbles and PBR headspace, which

Table 2.2 Characteristics of open and closed photobioreactors (adapted from Pulz 2001, Carvalho *et al* 2006, Amin 2009, Harun *et al* 2010, Moheimani *et al* 2013)

Characteristics	Open systems	Closed systems (PBRs)
Cultivation		
Area-to-volume ratio	Large (4–10 times higher)	Small
Algal species/strains	Restricted	Flexible
Risk of contamination	Possible	Unlikely
Control of contamination	Difficult	Easy
Cultivation period	Limited	Extended
Water loss through evaporation	Possible	Prevented
Control of growth conditions	Very difficult	Easy
Efficiency of light utilization	Poor/fair	Fair/excellent
Control of gas transfer	Low	Fair/high
Temperature	Highly variable	Required cooling
Temperature control	None	Excellent
Automatic cooling system	None	Built-in
Automatic heating system	None	Built-in
Cleaning	None	Required due to wall growth and dirt
Harvesting efficiency	Low	High
Biomass production		
Biomass productivity	Low	High
Population density (Algal cell density)	Low	High (3-5 times in PBRs)
Operational mode		
Air pump	Built-in	Built-in
Shear	Low	High
CO2 transfer rate	Poor	Excellent
Mixing efficiency	Poor	Excellent
Water loss	Very high	Low
O2 concentration	Low due to continuous spontaneous outgassing	Exchange device
CO2 loss	High	Low
Economics		
Land required	High	Low
Investment	Small/low	High
Periodical maintenance	Less	More
Operating cost	Lower	Higher
Harvesting cost	High	Lower
Most costly parameters	Mixing	O_2, Temperature control
Scale up technology for commercial level	Easy to scale up	Difficult in most PBR models

ultimately prevents carbon limitation (Pruvost *et al* 2016). Closed and Semi-closed photobioreactors are predominantly utilized for the production of high-value algal products (Becker 1994). Besides these advantages, there are various limitations of these PBRs which are intrinsic to their operating principles. These include confinement of culture in the PBRs resulting in the enhancement in the rate of biofilm formation on the PBR walls, thus leading to oxygen build up in the culture which is having toxic effects on photosynthetic growth. Closed PBRs may also lead to overheating of the culture by absorbing the large quantity of infrared radiation from solar radiation (Torzillo *et al* 1996, Borowitzka 1999, Grobbelaar 2008, Carvalho *et al* 2011). These are also less economical than open ponds (Moheimani and McHenry 2013, Moheimani *et al* 2013). Besides these problems, reducing the light path, sheer complexity, reduction of high oxygen concentration and constant temperature set up are some challenges before the researchers. These limitations can be overcome by appropriate engineering solutions in the reactor field by devising some cheaper and energy efficient design. Although researchers have undertaken to overcome a number of these limitations (Morita *et al* 2001, Kim and Lee 2001, Barbosa *et al* 2003, Miron *et al* 2003).

2.2.1.2 Open ponds

Cultivation of algae in outdoors is mostly done in natural ponds and raceways. These are the most regular and usual open systems for substantial cultivation of microalgae. These have been used for some decades at industrial scale (Oron *et al* 1979, Ugwu *et al* 2008, Morweiser *et al* 2010, Pruvost 2011, Fon Sing *et al* 2013). Open channels (raceway) have lured the attention of cultivation of microalgae at commercial scale as being cost-effective, easier to construct and operate comparatively than closed photobioreactors (Tredici and Materassi 1992, Borowitzka 2013). Furthermore, the growing of microalgae is less challenging in open than closed cultivation systems; but, only a few species of microalgae (e.g. *Dunaliella Salina*, *Chlorella* sp. and *Spirulina* sp.) have been lucratively grown in open ponds (Tredici and Materassi 1992, Moheimani *et al* 2011). The major disadvantages of open systems are intrinsic to their operating principles. First, they are liable to biological contamination by bacteria, other microalgae species and predators due to the direct contact of the microalgal culture with the atmosphere. Thus, only resistant species can be grown for long periods of time. The large surface of contact between the culture and the atmosphere also hinders in controlling the culture environment. The two prominent types of large-scale open cultivation systems utilized on a commercial scale are stirred ponds and unstirred ponds (circular and raceway) (Borowitzka 1993a, b, Borowitzka and Moheimani 2013).

2.2.1.3 Hybrid systems

Although Open ponds are a very efficient and cost-effective method of cultivating algae they get contaminated with unwanted species very quickly. Even though the maintenance of axenic cultures is excellent in PBR, but the setup costs are generally ten times

higher than that of open ponds. It involves the combination of both systems i.e. open ponds and closed photobioreactors. Probably this system is the best logical choice for cost-effective cultivation of productive biofuel strains. Inoculation has always been an integral part of algal aquaculture. A desired strain cultivated in a bioreactor is used for inoculation in open ponds. It may be a simple plastic bag or a high-tech fibre optic bioreactor. The important thing is that the size of inoculum should be large enough to establish in the open system before an unwanted species (Greenwell 2010). But ultimately an open system has to be cleaned and re-inoculated as the contaminating species will end up dominating the system. The hybrid system was used by for the production of astaxanthin from *Haematococcus pluvialis* by Olaizola (2003) and Huntley and Redalje (2007). This approach is also good for production of biofuel, as under low-nutrient conditions algae quickly convert solar energy into chemical energy and store it as lipids for their survival (Gouveia *et al* 2009).

2.2.2 Harvesting and dewatering of microalgal biomass

The major energy consumption processes for microalgae biofuel production are biomass harvesting and drying (Sander and Murthy 2010). The major challenges facing industrial scale microalgae production are energy-efficient and cost-effective microalgae dewatering technique (Wyman and Goodman 1993, Benemann 2013) as the dry biomass production of the algae culture is generally low from a few mgL^{-1} in open ponds to a few gL^{-1} in closed photobioreactors. The objective of harvesting and dewatering is to concentrate the microalgal biomass by several folds. Integration of technologies (flocculation followed by centrifugation) in a two-stage process had proven to be a very potential method of harvesting the microalgae (Vandamme *et al* 2013).

2.3 Factors affecting microalgal growth

2.3.1 Effect of nutrients

Carbon

Among the total cost of nutrients required for algae cultivation, Carbon accounts for 60%. The different carbon sources for microalgae are (i) atmospheric CO_2; (ii) CO_2 from industrial exhaust gases; and (iii) chemically fixed CO_2 in form of soluble carbonates (Kumar *et al* 2010). Utilization of carbonates (viz., Na_2CO_3 and $NaHCO_3$) for cell growth have been found in a number of microalgal species (Hongjin and Guangce 2009, Šoštaric *et al* 2009, Hu and Zhou 2010, Kim *et al* 2010, Romanenko *et al* 2010, Yeh *et al* 2010). Different algae use different mechanisms for the uptake and utilization of carbonates. Some species have got high extracellular carbo-anhydrase (Huertas *et al* 2000), which converts carbonate to free CO_2 to facilitate CO_2 assimilation. Additionally, some use active transport system for the uptake of carbonates (Colman and Rotatore 1995, Merrett *et al* 1996). It has been estimated that for

each kg of algae, about 1.65 kg of carbon is needed based on a mass balance (Bruton *et al* 2009)

Nitrogen

Nitrogen being the main constituent of nucleic acids and proteins, is involved in the primary metabolism of microalgae. Different nitrogen sources such as ammonia, urea and nitrate are used for the mass cultivation of microalgae (Xu *et al* 2001a, Li *et al* 2008a). The choice of the suitable nitrogen source depends on strain under consideration as the metabolic nitrogen pathways are species-specific. Even though ammonium and urea are generally used in mass cultivation being relatively cost-effective, the selection of proper nitrogen source for each algal species separately is important for enhancing biomass and oil productivity (Li *et al* 2008). Urea and nitrate were found to be superior to ammonia for the growth and lipid accumulation in *Chlorella* sp., *Chlorella vulgaris*, *Neochloris oleoabundans* and *Scenedesmus rubescens* (Tam and Wong 1996, Li *et al* 2008b, Liu *et al* 2008, Hsieh and Wu 2009, Pruvost *et al* 2009, Lin and Lin 2011). In contrast, ammonium has been found to improve biomass and lipid content in *Ellipsoidion* sp. than that of urea and nitrate (Xu *et al* 2001b).

Phosphorus

Phosphorus is the primary limiting nutrient in different ecosystems (such as lakes, rivers, and estuaries), and mostly the rate limiting nutrient in the production of phytoplankton. Phosphorus is used by microalgae mainly in the form of phosphates, as in other forms precipitation with metal ions occur. Even if with a lesser magnitude as compared to the larger results on nitrogen, microalgal biomass and lipid productivity can be enhanced by phosphorus starvation as reported in *Monodus subterraneus* (Khozin-Goldberg and Cohen 2006), and produce changes in composition of fatty acids as reported for *Phaeodactylum tricornutum* and *Dunialella tertiolecta* (Siron *et al* 1989).

Other elements

For the growth of microalgae, Iron, sulphur, magnesium, and other elements are also important. Iron is engaged inflow of electrons from H_2O to nicotinamide adenine dinucleotide phosphate (ADP in its oxidized form) (Roden and Zachara 1996). Several studies have been undertaken regarding the effect of iron on microalgae growth. Sung *et al* 1998 found that high concentration of iron concentrations enhances cell growth, while Liu *et al* (2008) found enhanced lipid accumulation in *Chlorella* strains. Cysteine and methionine are two essential amino acids of which sulphur is an essential component. In the absence of sulphur, protein biosynthesis is impeded and the photosynthetic system PSII repair cycle is blocked (Zhang *et al* 2002). Magnesium is required as essential element found largely in the core of the tetrapyrrolic ring of the chlorophyll molecule. Similarly, some trace elements play key roles in photosynthetic electron transport (Raven *et al* 1999). For instance, manganese is essential

for O_2 evolution, and calcium has an indispensable role in facilitating H_2O dehydrogenation and O_2 evolution.

2.3.2 Effect of temperature, pH and light on the growth

Temperature

Temperature is one of the critical factors regulating the cellular, morphological and physiological responses of microalgae (Martinez *et al* 1999, Carvalho and Malcata 2003, de Castro Araujo *et al* 2005, Kitaya *et al* 2005, Shi *et al* 2006, Cho *et al* 2007, Colla *et al* 2007, Converti *et al* 2009). Although several microalgae can tolerate temperatures up to 15 °C lower than the optimal, exceeding the optimum temperature by only 2–4 °C may result in the entire culture loss. Sometimes in closed culture systems, overheating problems may occur as the temperature inside the reactor may reach 55 °C. For such conditions, economical evaporative water cooling systems may be used to reduce the temperature to 20–26 °C. Although algae may be able to grow at a variety of temperatures (Chinnasamy *et al* 2009), but optimal temperatures are specific to each strain (Renaud *et al* 2002, Cho *et al* 2007). For example, the optimum temperature range for *Nannochloropsis, Tetraselmis* and *Isochrysis* species were found to be 19-21, 19-21 and 24-26 °C, respectively (Abu-Rezq *et al* 1999). Seasonal and even daily temperature fluctuations can impact the algae production. As temperatures can rise 30°C higher than the optimum temperature in a closed photobioreactor without temperature control equipment, evaporative cooling or shading techniques are used frequently to prevent the increase of that magnitude (Suh and Lee 2003).

pH

pH is the most important factor regulating the cell metabolism and biomass formation (Goldman *et al* 1982). Though most microalgal species grow best at neutral pH, the optimal pH range for each strain is also narrow (Mayo *et al* 1997, Alyabyev *et al* 2011). However, there are some extremophilic species which survive in environments characterized by extreme pH values (acidophiles or alkalophiles). For example, *Spirulina platensis* grows under alkaline conditions with an optimum pH around 9 (Hu *et al* 1998, Qiang *et al* 1998) while *Chlorococcum littorale* thrive well in acidic pH of 4 (Kodama *et al* 1993, Schnackenberg *et al* 1996). *Galdieria sulphuraria* (Barbier *et al* 2005) and *Chlamydomonas acidophila* (Cuaresma *et al* 2011) have been reported to be resistant to pH 0 and 1.5-2.5, respectively. The pH influences mainly the liquid chemistry of polar compounds and the availability of nutrients such as iron, organic acids and even CO_2 (Coleman and Colman 1981, Lee and Pirt, 1984). A complex relationship exists between CO_2 concentration and pH in microalgal photobioreactor related to the chemical equilibrium among chemical species such as CO_2, H_2CO_3, HCO_3^- and CO_3^{2-} (Livanski and Bartos 1986). The equilibrium between these chemical forms depends on pH with CO_2 the dominant form at lower pH below 7 and CO_3^{2-} the dominant form above pH of 10. Although the increase in CO_2 concentration may lead to higher biomass productivity,

pH may also decrease which may ultimately have a negative effect upon microalgal physiology. The increase in pH can be beneficial in open pond system viz., for neutralization of pathogens in microalgal wastewater treatment, but can also restrict microalgal growth.

Light

Light is also an important factor influencing the growth of microalgae. The photosynthetic cultures utilize the solar energy to be used by the cells either for maintenance purposes or formation of new biomass (Pirt 1986). Subsequently, a direct link exists between the light energy available and the biomass productivity and the cell growth. The effect of light intensity on growth kinetics and biomass accumulation (Chen and Chen, 2006, Yeh *et al* 2010, Pedrosa Bezerra *et al* 2011, Shu *et al* 2011, Amini Khoeyi *et al* 2012, Ruangsomboon 2012) as well as the effect of illumination cycles (hours of light and hours of dark) on biochemical composition (Ratchford and Fallowfield 2003, Meseck *et al* 2005, Umorin and Lind 2005, Jacob-Lopes *et al* 2009, Hodaifa *et al* 2011, Seyfabadi *et al* 2011, Sforza *et al* 2012) have been characterized on different microalgae strains such as *Chlorella vulgaris, Chlorella pyrenoidosa, Nannochloropsis salina, Dunaliella tertiolecta, Monodus subterraneus, Pavlova lutheri, Spirulina platensis, Chaetoceros muelleri, Porphyridium cruentum, Euglena gracilis, Tetraselmis chui, Scenedesmus obliquues and Botryococcus braunii.*

2.4 Optimization of cultural conditions of microalgae using response surface methodology

The most important part of the development of an efficient and economic bioprocess involves the optimization of the process variables. The classical method of optimization i.e., one-factor-at-a-time approach is efficacious in some cases but examining the mutual effect of the factors is important for better optimization. For optimization of various bioprocesses, several statistical approaches like Plackett–Burman design and response surface methodology (RSM) are very logical and productive (Reddy *et al* 2008, Kirrolia *et al* 2014). The Plackett–Burman design is being used for screening the significant parameters from a large number of variables, and this information can be utilized for further optimization (Haddar *et al* 2010). RSM is a compilation of statistical techniques that are useful for designing experiments, establishing models, assessing the effects of various factors and searching for optimal conditions of studied parameters for worthwhile responses (Haddar *et al* 2010, Matsudo *et al* 2015). It helps in identifying the interactions between various factors with less number of experimental runs (Anvari and Khayati 2014). In short, these statistical approaches help in rapid and authentic short listing of process conditions.

This technique provides the means of attaining optimum operating conditions of complete systems. It is a powerful and an efficient mathematical tool applied for optimization

of any process, e.g. media components on fermentation (Adinarayana and Elliaiah 2002, Park *et al* 2002). It gives information about the interactions between various variables, process optimization and gives multiple responses at the same time. Statistical modeling is done to develop an appropriate model for the responses y (performance function) and independent variables x (decision variables).

In general, the relationship is:

$$y = \Phi(x_1, x_2 \text{ ----------}, x_n) + \varepsilon$$

Where the form of the true response function Ø is unknown and perhaps very complicated one, another mathematical simpler function f must be found to approximate Ø and describe the system and ε is the term that represents another source of variability or error on the response. Often assuming it to have a normal distribution with mean zero and variance σ^2, then the new function $y' = f(x_1, x_2 \text{ ----------}, x_n)$. This estimates y´ rather than true value of y (Cox and Cocharan 1964). The most commonly used expression is polynomial of first or second degree is given as:

$$y' = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \varepsilon$$

$$y' = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_i x_i^2 + \sum_{i=1}^{n-1} \sum_{j=1}^{n-1} \beta_{ij} x_i x_j + \varepsilon$$

Where β_o, β_i, β_{ij} are the constant coefficients usually determined by least square method and ε is the error involved in estimating the coefficient β from the experimental data. After the coefficients have been determined by taking the first partial derivatives of above equations equating them to zero and solving the system of n equations.

$$\partial y'/\partial x_i = 0 \ (i = 1, 2, \ldots . \ n)$$

n = total number of variables

2.5 Extraction/conversion technologies for microalgae biomass

Post-dewatering, the microalgae biomass can be used directly as a source of animal feed or human food. Microalgae provide the promising material for producing a high protein (*Spirulina*), high carbohydrate (*Chlorella*) and high essential oil similar to fish oil. Additionally, conversion of microalgae biomass to renewable fuels is a win-win initiative. For the conversion of microalgal biomass into bioenergy, different processes and/or technologies (Fig. 2.1) are employed viz., biochemical conversion, chemical reaction, direct combustion and thermochemical conversion (Chiaramonti *et al* 2007, Medipally *et al* 2015). However, a lot of work needs to be done to determine the most energy saving and economically viable process for extraction of biomass and its conversion to renewable energy.

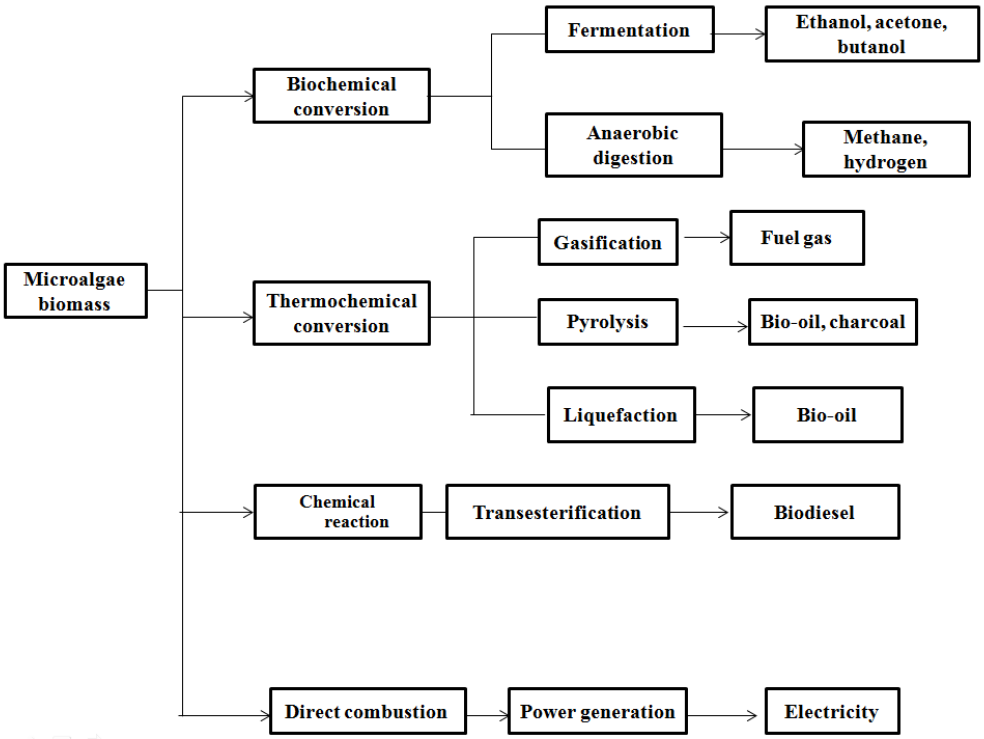


Fig. 2.1 Microalgae biomass conversion process

2.6 Microalgae as propitious feedstock for biofuel production

Microalgae seem to serve as the only recent renewable way to produce biofuels (Chisti 2007, Schenk *et al* 2008). The production of biofuels from microalgae do not affect the environment and food supply chain for the population, unlike other traditionally used bioenergy crops. These are more preferred sources for biofuel production rather than lignocellulosic substrates due to their specific characteristics like having low viscosity, high caloric value and low density, also by virtue of their intrinsically high lipid content, semi-steady state production, and aptness in a wide range of climates (Miao *et al* 2004).

Apart from above-mentioned characteristics, the special feature which makes it unique than other available advanced substrates is the wide range of species available for biofuel production. Various species may be selected to optimize the production of different biofuels. Algae besides providing suitable solutions to pollution in the form of being used as energy sources (like jet fuel, aviation gas, biodiesel, bioethanol and gasoline) offers a wide range of other indispensable co-products like food, nutritional compounds, omega-3 fatty acids, animal feed, recombinant proteins, pigments, medicines, pharmaceuticals, and vaccines organic fertilizers and biodegradable plastics (Pulz 2004, Pienkos and Darzins 2009).

Microalgae have numerous advantages over higher plants as a source of transportation biofuels and some of them are enlisted below (Chisti 2007, Huntley and Redalje 2007, Li *et al* 2008, Schenk *et al* 2008, Rodolfi *et al* 2008, Khan *et al* 2009):

1. Microalgae can be grown in brackish/saline water /coastal seawater, on non-arable land, and do not compete for resources with conventional agriculture.

2. Microalgae can grow in trivial lands (e.g., arid/semiarid lands and desert) that are not appropriate for conventional agriculture.
3. The growth rate of microalgae is high (1-3 doublings/day) and capacity of production and accumulation of neutral lipids/oil is also large (20–50% dry cell weight (DCW)). Oil production per area by microalgae cultures could exceed the production of best oilseed crops.
4. These sequester CO_2 from flue gases produced from fossil fuel-fired power plants and other sources, and thereby decreasing emissions of the main greenhouse gas. One kg of algal biomass requires about 1.8 kg of CO_2 (Rodolfi *et al* 2008).
5. Microalgae consume phosphorus and nitrogen from a range of wastewater sources (e.g. concentrated animal feed operations, agricultural run-off, and municipal and industrial wastewaters), thus helping in the wastewater bioremediation.
6. Microalgae have the potential to produce value-added co-products or by-products (e.g., proteins, biopolymers, pigments, polysaccharides, fertilizer and animal feed) and do not demand pesticide and herbicide.
7. Microalgae can be grown throughout the year in proper culture vessels (photobioreactors) with high annual biomass yield on an area basis.

2.7 Algae anaerobic digestion

Anaerobic digestion (AD) is a scientific process whereby anaerobic microorganisms in an anaerobic environment decompose biodegradable matter (Dickerson *et al* 2009) into biogas, nutrients and additional cell matter. The consortium of microbes works synergistically to deconstruct recalcitrant biomass structures (like lignocelluloses) into their fundamental constituents. The AD is suitable for transforming non- sterile, complex raw material into energy-rich biogas. Biogas is a mixture of CH_4 (50-60%), CO_2 (30-40%), H_2 (1-5%), N_2 (0.5%), CO, H_2S and water vapors (traces).

Biogas has been considered as a substantial source of energy and can be utilized for various applications: heat or electricity generation from burned biogas, liquefaction of biogas into methanol, compression of biogas to be served as a source of car fuel similar to that of compressed natural gas (CNG), and purification of biogas to be fed into gas distribution grids (Roubaud and Favrat 2005). Biogas plant provided a much-needed lighting source to a rural home in addition to multiple-end uses. Digested biomass from biogas plants can be used in different ways like aquaculture, vermicompost, pest repellant, mushroom cultivation, N_2 rich organic manure and rooting medium, etc.

All types of biomass can be utilized as raw material for biogas production. Historically, anaerobic digestion has predominantly been associated with the treatment of

animal manure and sewage sludge from aerobic wastewater treatment. Nowadays, most of the agricultural biogas plants digest manure from pigs, cows, and chicken with the addition of co-substrates e.g., algal biomass, lignocellulosic wastes (wheat straw, sorghum stalk, rice straw, water hyacinth, oat straw and fodder beet etc.), organic wastes from agriculture-related industries, and food waste, collected municipal biowaste from households and energy crops to increase the content of organic material for attaining a higher gas yield (Chayanon *et al* 2014). The composition of biogas and the methane yield depends on the feedstock type, digestion system, and retention time (Braun 2007).

2.7.1 Anaerobic digestion of macroalgae

Macroalgae, or seaweeds, because of their abundant growth in eutrophic coastal waters, fouling beaches and waterways, have gained wider attention as a biofuel substrate. The process of anaerobic digestion has been used widely for biogas production from macroalgal biomass (Habig *et al* 1984, Vergara-Fernandez *et al* 2008). The macroalgal biomass is easily hydrolyzed to sugars and proteins in comparison to the biomass of higher plants, probably due to the reason that algal biomass has low lignin content (Nkemka and Murto 2010) and a high proportion of hemicelluloses.

Methane potential of macroalgal species such as *Laminaria* sp., *Macrocystis* sp., *Sargassum* sp., *Durvillea antarctica* and *Macrocystis pyrifera* has been reported (Bird *et al* 1990, Chynoweth *et al* 2005, Vergara-Fernandez *et al* 2008, Singh and Gu 2010). The methane yield is species specific and also depends on the operational and functional conditions. The yield levels of up to 400 L/kg volatile solids (VS) have been reported.

The effect of nitrogen (NH_4Cl) supplementation of the biomass of macroalgae *Gracilaria tikvahiae* and *Ulva* sp. on biogas production has been discussed by Habig *et al* (1984). It was reported that decrease in nitrogen concentration leads to increase in biogas yield from 410 L kg^{-1} VS (33.7 % CH_4) to 560 L kg^{-1} VS (59.5 % CH_4), implying that nitrogen deficient conditions improve the methane production. The operating temperature (25, 35 and 55 °C) and the inoculum used have also been found to influence the net biogas production (Migliore *et al* 2012). The best operating temperature was 35 °C. Similarly, the exploitation of anaerobic sediments as inoculum significantly enhanced the biogas yield as per the temperature. The yield increased from 53 to 283 L kg^{-1} VS at 25 °C, 175 to 375 L kg^{-1} VS at 35 °C, and from 2 to 104 L kg^{-1} VS at 55 °C. The effect of pre-treatment of algal biomass on biogas production has also been studied. For example, Grala *et al* (2012) studied the effect of hydrothermal and enzymatic pretreatment on a mixture of *Ectocarpus, Pilayella* and *Enteromarpha* biomass and found that yield of biogas increased from 33 to 54 L kg^{-1} VS. The pretreatment improves the biogas production by biodegrading the recalcitrant substances

like polyphenols, cellulosic fibers and lignin type components found in some species acting as the barriers to anaerobic digestion. These recalcitrant compounds decrease the biodegradability of the biomass and hence limiting biogas production (Bird *et al* 1990, Briand and Morand 1997).

2.7.2 Anaerobic digestion microalgae

Microalgae, both freshwater and marine have drawn considerable attention as substrates for anaerobic digestion. A wide array of microalgal species, digester configurations and functional parameters has been tested for methane production through anaerobic digestion (González-Fernández *et al* 2012a). Several factors like the composition of the substrate (contents of protein, lipids and carbohydrates), the design of digester, temperature and hydraulic retention time effect the methane biogas production (Sialve *et al* 2009). The biomass composition is in turn influenced by the cultural conditions.

Nearly a half-century ago, the anaerobic co-digestion of *Chlorella* sp. and *Scenedesmus* sp. was studied by Golueke *et al* (1957). The biogas quality and productivity from mixed culture were found to be comparable to that from sewage sludge. Higher biogas production (1020 L kg^{-1} VS) was noticed at a higher temperature (55 °C) compared to a mesophilic range (986 L kg^{-1} VS at 35 °C) and the methane content ranged from 61 to 63%. The biogas production varies from species to species because of different structure and composition of the cell wall and is independent of the taxonomic group of a species, or its habitat (marine or freshwater) (Mussgnug *et al* 2010, Zamalloa *et al* 2012). *Chlamydomonas reinhardtii* has been found to produce up to 390 L CH_4 kg^{-1} VS which is higher compared to methane obtained (100 L CH_4 kg^{-1} VS) from the biomass residue of *Scenedesmus* left after lipid extraction. The susceptibility of individual species to anaerobic digestion is related to the structure of their cell walls. Species such as *Arthrospira platensis, Chlamydomonas reinhardtii* and *Epicrates gracilis* constitute protein-based cell walls free of cellulose and hemicellulose (Mussgnug *et al* 2010) and are easy to hydrolyze than that of species like *Chlorella kessleri* and *Scenedesmus obliquus*, constituting carbohydrate-based cell walls containing hemicellulose that are arduous to hydrolyze. Similarly, the rigidity of the cell wall of *S. Obliquus* is due to the presence of a sporopollenin-like biopolymer (Burczyk and Dworzanskib 1988). The presence of complex silica-based cell wall in diatoms main has been proposed to be the main cause for their low biogas production (Hildebrand *et al* 2012). The easily degradable cell wall or the total absence of cell wall does not guarantee that an alga is a promising feedstock for anaerobic digestion. The other factors like presence of inhibitory substances also affect degradability of the substrate (Hildebrand *et al* 2012). The possibility of digestion of residual algal biomass left after extraction of lipids for biodiesel has been

elucidated and was confirmed that anaerobic digestion is a good alternative for its disposal as this residue accounts to about 65 % of the initial biomass. The biogas can be produced from the carbohydrates and proteins in the residual biomass. For example, methane yield of 390 L CH_4 kg^{-1} VS was obtained by Yang *et al* (2011) by digesting residual *Scenedesmus* biomass. Ramos-Suárez *et al* (2014) also observed a methane yield of 177 L kg^{-1} VS (79.1 % CH_4) from whole biomass of *Scenedesmus* sp. However, the yield from the residual biomass after amino acids extraction was 364 L kg^{-1} VS (58.3 % CH_4) and it was 401 L kg^{-1} VS (68.0 % CH_4) after lipid extraction. The reason for the significant increase in biogas yield from the biomass residue remaining after extraction of the amino acid may be credited to the hydrolysis of the microalgal cell walls during the extraction process and an increase in the C/N ratio of the algal biomass through loss of protein. Similarly, the slightly lower methane yield after extraction of lipids may be ascribed to the loss of carbon in the lipids which otherwise would impart the most to methane production.

2.7.3 Pretreatment of algal biomass

The biogas production by anaerobic digestion could be restrained by the resistant nature of both macroalgae and microalgae. Therefore, to enhance the methane yield, the biomass is required to be pretreated to increase its digestibility. Various pretreatment approaches have proven promising in enhancing the digestibility of organic feedstocks such as sewage sludge and lignocellulosic biomass (Hendriks and Zeeman 2009, Carrère *et al* 2010). All these methods can be tried with algal biomass also. The efficacy of a pretreatment depends mainly on the features of the alga, like recalcitrant nature of the cell wall and biochemical composition of cells.

The pretreatment methods can be broadly categorized into three types: physical, chemical and biological. Physical treatment may be wholly mechanical or it may consist of a thermal process. In case of macroalgae, physical pretreatments involve washing, drying and maceration or chopping. The physical pretreatments are effective in hydrolyzing or disrupting microalgal cells but these in combination with heat are very much efficient and effectual. Thermochemical pretreatments have been strongly utilized in case of microalgae among chemical pretreatments.

2.7.3.1 Thermal pretreatments

This involves treating the algal biomass with heat. The biomass is generally exposed at a temperature ranging from 50 to 270 °C to improve the disruption of organic matter before being utilized for anaerobic digestion process (Hendriks and Zeeman 2009, Carrère *et al* 2010). The nature of the substrate determines the optimal treatment temperature and the time of exposure. Thermal pretreatments can be purely thermal or may consist the action of water

and heat (i.e., hydrothermal treatment) or the action of heat along with with other physical forces like, in steam explosion of biomass. A purely thermal pretreatment is generally considered as one executed at a temperature of <100 °C. This sort of pretreatment has improved the biogas yield in both mesophilic and thermophilic anaerobic digestion processes. Compared to treatments involving higher temperatures, low temperature based thermal pretreatment is advantageous in being less energy demanding. The gain in net energy recovery shifts from neutral to positive has been witnessed in laboratory scale digesters having 20 days of hydraulic retention time after exposing the biomass to a thermal pretreatment at 75 °C (Passos and Ferrer 2014). Thus it was concluded that the net energy recovery from an anaerobic digestion process can be enhanced or improved by a convenient thermal pretreatment.

A hydrothermal pretreatment by definition involves the use of a temperature higher than 100 °C and that too under pressure. The consequences of both hydrothermal pretreatment and lower temperature pretreatment on biogas production are comparable. The hydrothermal pretreatment involves shorter time compared to a thermal pretreatment. For example, in one case after pretreating the algal biomass at 70–95 °C for 3–10 h, the methane yield was enhanced by 60 % (González-Fernández *et al* 2012, Passos *et al* 2013a). In comparison to this, after pretreating the algal biomass at 120–140 °C for 15–30 min, the yield was found to have improved by 60– 120% (Alzate *et al* 2012, Cho *et al* 2013). Whilst, for *Chlorella* sp. and *Scenedesmus* sp., maximum methane yield was obtained after a pretreatment at 120 °C. It increased methane yield from 0.34 to 0.40 L CH_4 g^{-1} VS (Cho *et al* 2013). The cells are further affected by releasing the pressure rapidly after pretreating at high temperature. The effects of hydrothermal and steam explosion treatments are similar to each other. The steam explosion involves the heating of biomass held under pressure in a pressure vessel (like 160 °C at around 6 bars for a few minutes10–30 min) and then releasing the pressure rapidly to flash off steam (Keymar *et al* 2013). It has been commercially utilized in pretreating lignocellulosic biomass prior to enzymatic digestion and is available for pretreatment of sewage sludge (Kepp *et al* 2000).

Steam explosion pretreatment has also experimented for biogas production from microalgae in anaerobic batch tests. It was observed to be very much efficient than the ultrasonic pretreatment (Alzate *et al* 2012). The methane yield was enhanced by 41– 55 % in case of algal biomass treated by steam explosion relative to control (Alzate *et al* 2012). Keymar *et al* 2013 too observed the increase in biogas production from microalgae biomass and the residual biomass left after lipid extraction pretreated with a steam explosion by 81 and 58 %, respectively. The extent of substrate solubilization was improved by pretreatment more than the rate of anaerobic digestion (Keymar *et al* 2013).

2.7.3.2 Mechanical pretreatments

Mechanical pretreatments involve the use of a physical force to directly break cells. The mechanical methods are mostly applied for the extraction of lipid from microalgae because these are independent of the algal species and do not contaminate the extracted lipid with chemicals (Lee *et al* 2012). The main disadvantage of mechanical pretreatments is that they are highly energy demanding. Generally, the physical pretreatments utilized for microalgae are sonication with ultrasound, irradiation with microwaves, grinding in bead mills, and disruption in high-pressure homogenizers.

Ultrasonication, a pretreatment method wherein the cells are exposed to sonic waves undergoing compression and decompression cycles very fastly. These waves create high turbulence in the cell slurry. This actually generates cavitation bubbles which on imploding damage cells directly, or indirectly (Kim *et al* 2013). It is an efficient technique of cell disruption (Kim *et al* 2013). The consequences of this method like other pretreatment methods depend on depend on the algal species and the conditions under which the pretreatment has been carried out. The parameters which are changed during sonication are mostly the sonication power and the time of exposure rather than sonication frequency which is usually fixed. To identify the optimal treatment conditions, various combinations of sonication power input (e.g., 600–1000 W) and exposure time (e.g., 20–100 min) may be screened out. In one case, the highest amount of glucose (0.37 g glucose/g dry weight) released from the biomass was achieved when it was treated at 800 W for 80 min (Zhao *et al* 2013). It was seen by researchers that increase in the methane yield did not exceed 30% if the sonication energy input was <75 MJ/kg TS (Alzate *et al* 2012, Gonzalez-Fernandez *et al* 2012b). The yield enhanced by 75–90 % if the energy levels or sonication power of 100–200 MJ/kg TS was utilized (Gonzalez-Fernandez *et al* 2012b, Park *et al* 2013). The results clearly illustrate that high sonication energy input (>100 MJ kg^{-1} TS) is important for enhancing methane yield from microalgal biomass. Ultrasonication pretreatment appears to affect the methane yield more than it affects the rate of hydrolysis (Passos *et al* 2014a, b).

Microwaves comprise electromagnetic waves falling in the frequency range of 300 MHz to 300 GHz. The working principle of microwaves is that these increase the vibrational frequency of water molecules as soon as they are absorbed. This, in turn, results in the increase in kinetic energy of the molecules resulting in the heating effect which ultimately causes the temperature to rise. The microwave energy is incompetent of breaking covalent bonds, but hydrogen bonds can be broken. The heat generated produced by the vibration of water molecules denatures proteins (Kaatze 1995). Similar to sonication, the most important controllable parameters of microwave pretreatment are the output power and the duration period (Passos *et al* 2013b). These waves actually penetrate the biomass and thus the

treatment can be expeditious (Kim *et al* 2013), but it is energy intensive and this in turn depends on the concentration of biomass. Optimal pretreatment conditions are those with an energy input to the biomass of about 65.4 MJ/kg TS, regardless of the output power and the duration of exposure (Passos *et al* 2013b). It was revealed that at optimal irradiation input, an eight-fold increase in biomass solubilization was observed (Passos *et al* 2013b). For macroalgae, mechanical pretreatments such as maceration and grinding are very effective in enhancing the methane yield by as much as 70 % compared to the untreated biomass.

2.7.3.3 Chemical pretreatments

Chemical pretreatments in comparison to mechanical and thermal pretreatments are comparatively less utilized. Out of the various potential chemical methods, only the acid and alkali pretreatments seem to have been tried with microalgae. These two pretreatments solubilize polymers to increase the accessibility of quickly digestible small molecules (Bohutskyi and Bouwer 2013). The leftover small quantity of alkali in the pretreated biomass might be helpful in combating the pH decline during the succeeding acidogenesis step. Despite being efficient, several acid and alkali treatments lead to the formation of by-products which can be inhibitory to the microorganisms involved in anaerobic digestion.

Alkaline pretreatments involving NaOH, $Ca(OH)_2$, and NH_4OH have been comprehensively studied for use with solid organic wastes. Mendez *et al* 2014 studied the effect of thermochemical pretreatment of microalgae with both alkali and acids. The green alga *Chlorella vulgaris* was first given thermal treatment at 120 °C for 40 min and then NaOH or H_2SO4 was added to it. The researchers concluded that the biodegradability of algal biomass was enhanced in all cases; however, the highest increase in methane yield of 93 % was attained in case of thermal pretreatment without the addition of any chemicals. Nevertheless, the thermo-alkaline pretreatment resulted in higher solubilization of proteins and carbohydrates in comparison to the thermal pretreatment. The effect of pretreatment on macroalgae *Palmaria palmate* (Jard *et al* 2013) and *Gracilaria vermiculophylla* (Oliveira *et al* 2014), resulted in the enhanced biomass solubilization, but not the biogas yield. This was explained as being an outcome of the unidentified inhibitory byproducts of the pretreatment processes restricting the anaerobic digestion (Mendez *et al* 2014). In general, chemical pretreatments have not been comprehensively investigated and the outcomes are often contradictory. However, a combination of thermal and chemical pretreatments can be promising.

2.7.3.4 Biological pretreatment

It involves the utilization of enzymes to disrupt the algal cell wall and hydrolyze biopolymers. Biological pretreatment is one of the promising alternatives to the other

pretreatments (Bohutskyi and Bouwer 2013). The enzymatic pretreatment does not usually generate inhibitory compounds when carried out in mild conditions. Hydrolytic enzymes hydrolyze the polymers constituting the algal cell wall to make the biomass more suitable to anaerobic digestion. There are various factors affecting the enzymatic pretreatment like temperature, pH, enzyme dose, and exposure time of the treatment. The temperature and pH are fixed to the optimal activity range of the specific enzyme used. Although this is a very potential method but still there are some drawbacks like the high cost of the enzymes and the fact that there is no such enzyme which can degrade the cell walls of different microalgae.

The literature on enzymatic pretreatment is quite limited. The enzyme endo-β-1,4-glucanase from *Cellulomonas* sp. YJ5 could hydrolyze the cell walls of *Chlorella sorokiniana* to cause cell lysis after 60–180 min of treatment (Fu *et al* 2010). A combined treatment involving mechanical blending and an enzyme cocktail (α-amylase, protease, lipase, xylanase) has been applied to *Rhizoclonium* sp. biomass. This pretreatment enhanced the methane yield by >20% than using the mechanical pretreatment alone. Pretreating the biomass with enzyme cocktail showed the best results while pretreating the biomass with single enzyme, cellulase exhibited the best results (Ehimen *et al* 2010). The efficiency of the enzyme cocktail is due to the fact that in this system the hydrolytic action of one enzyme makes substrate available for the other one. This explains the efficiency of the enzyme cocktail as compared to one enzyme system.

2.8 Co-digestion

Co-digestion involves digestion of more than one feedstock together. It is carried out to maintain the C/N ratio of the mixture close to optimal. The execution of anaerobic digestion is strongly determined by the C/N ratio of the substrate. Generally, the nitrogen content is high in algal biomass so its C/N ratio is around 6-10 (Yen and Brune 2007). This is quite lower than the required range of 25–32. The biomass with high protein content may lead to the production of high levels of ammonia and further accretion of the toxic volatile fatty acids. Therefore, such sort of feedstocks having high N content must be supplemented with carbon-rich co-feedstocks to corroborate good conversion to methane.

Co-digestion of substrates was earlier thought of providing synergistic interactions during digestion, but now it is established that the methane production is mainly dependent on total organic rate preferably than of synergistic effect of different substrates (Mata-Alvarez *et al* 2014). Hence, a large number of carbon-rich substrates are appropriate for co-digestion with the algal biomass. Generally, the biogas yield enhanced significantly (e.g., from 50 to 200 %) as a result of co-digestion. This was primarily by virtue of the contribution of carbon by the cosubstrate that led to the increase of C/N ratio to 20 (Yen and Brune 2007, Zhong *et*

al 2012, Ramos-Suárez *et al* 2014). Mostly the sewage sludge consisting of primary sludge, waste activated sludge, or a mixture of both, has mainly been utilized as the co-feedstock. The microalgal biomass is produced as a by-product of wastewater treatment processes, and mixing of this biomass with sewage sludge produced in the same process for utilization in the anaerobic digester may efficaciously optimize waste management (Solé *et al* 2014).

Other carbon-rich cosubstrates that have been used for biogas production are paper waste (Yen and Brune 2007), crop and plant waste (Zhong *et al* 2012, Ramos-Suárez *et al* 2014) and waste lipids (Park and Li 2012). Yen and Brune 2007 stated that the addition of paper waste to a mixture of *Scenedesmus* sp. and *Chlorella* sp. approximately doubled methane production from 140 to 230 L CH_4 kg^{-1} VS. Similarly, the methane yield was improved by 8–74 % on codigesting microalgal biomass with different amounts of swine manure (González-Fernández *et al* 2011). Co-digestion of these substrates made the process to operate at higher organic loading rates which would have not been possible rather carrying out digestion of feed stocks singly. Co-digestion besides enhancing the methane yield, it also increases the kinetics of the anaerobic digestion process (Costa *et al* 2012, Ramos-Suárez *et al* 2014, Solé *et al* 2014). Co-digestion of the microalgae biomass residue of a lipid extraction process, with lipid-rich waste (fat, oil, and grease) increased the methane yield to 540 L CH_4 kg^{-1} VS compared to a yield of 150 L CH_4 kg^{-1} VS when the algal residual biomass was digested alone (Park and Li 2012). Similarly, co-digestion of the oil-extracted Chlorella biomass residue with waste glycerol from a biodiesel production process resulted in the methane yield increasing by 4–7 % (Ehimen and Connaughton 2009). It has been pointed out that some solvents used in extracting the oil from the algal biomass can adversely affect subsequent methane production by anaerobic digestion (Ehimen and Connaughton 2009). Chloroform is one such solvent.

CHAPTER- III

MATERIALS AND METHODS

The present study was carried out in the Biogas Laboratory, School of Renewable Energy Engineering, Punjab Agricultural University, Ludhiana (unless mentioned otherwise). The materials and methods used during this study are described under the following headings:

3.1 Procurement and physicochemical analysis of water samples
3.2 Isolation, purification and maintenance of microalgal strains
3.3 Morphological identification
3.4 Screening of the microalgal isolates
  3.4.1 Estimation of dry cell biomass or weight
  3.4.2 Estimation of chlorophyll
  3.4.3 Estimation of carbohydrates
  3.4.4 Estimation of lipids
  3.4.5 Estimation of proteins
  3.4.6 Estimation of mineral content of algal biomass
3.5 Molecular Identification
  3.5.1 18S rRNA sequence analysis
  3.5.2 16S rRNA sequence analysis
3.6 Optimization of cultural conditions of the selected isolates
  3.6.1 Screening and selection of significant factors by Placket-Burman design
  3.6.2 Optimization by Central Composite design of response surface methodology
3.7 Assessment of biogas potential of selected microalgal isolates
  3.7.1 Pretreatment of algal biomass (a) Enzymatic pretreatment (b) Hydrothermal pretreatment
  3.7.2 Microscopy of the untreated and pretreated samples
    3.7.2.1 Optical microscopy
    3.7.2.2 Scanning Electron Microscopic (SEM) analysis
  3.7.3 Fourier transform infrared (FT-IR) spectroscopy
  3.7.4 Determination of biogas production potential through BMP test
3.8 Co-digestion of algal biomass with paddy straw for biogas production
  3.8.1 Replacement with algal biomass
  3.8.2 Supplementation of algal biomass
  3.8.3 Computation of daily and cumulative biogas yields
3.9 Multipopulation microalgal species cultivation and biogas production at field scale
3.10 Analytical procedures
  3.10.1 Determination of salinity of water samples
  3.10.2 Proximate and chemical composition of feedstock fed into the digesters
3.11 Statistical analysis
3.12 Media composition
3.13 Standard curves of carbohydrates, proteins and lipids

3.1 Procurement and physicochemical analysis of water samples

The water samples were collected from the waterlogged areas of Punjab (Table 4.1), India in sterile bottles and brought to the Biogas Laboratory, School of Renewable Energy Engineering, Punjab Agricultural University, India. The collected samples were filtered and analysed in terms of pH, electrical conductivity and salinity (CO_3^{2-}, HCO_3^-, Ca^{2+}, Mg^{2+}). The pH of all the collected water samples was determined by the Mettler Toledo^{TM} (SevenEasy 8603) pH meter and electrical conductivity of these samples was measured with the digital conductivity meter (Sanco, CC 603-1). The salinity described in section 3.11.1 was determined according to Richards (1954).

3.2 Isolation, purification and maintenance of microalgal strains

The collected water samples were observed microscopically (Olympus microscope, 528293 Magnus Icon Freedom Model) for the presence of the microalgal species. After this, the algal water samples were enriched in different media like ACM, BBM, BG-11, Conway and GMF/2. The compositions of media used are given in section 3.13. Enrichment was carried out in 250 ml Erlenmeyer conical flasks containing 100 ml of medium. For enrichment, ten ml of each algal water sample was inoculated to the flasks separately in triplicates. Isolation of microalgae was done after 20-30 days of incubation when visible growth was observed in the flasks kept in the growth chamber maintained at temperature 25±2 °C. In order to isolate single and pure microalgal species from the water samples, standard dilution, plating and streaking methods using different culture media: Algae culture medium (Lembi and Waaland 1988), Guillard's F/2 medium (Guillard and Ryther 1962), Bold's basal medium (Bischoff and Bold 1963), BG-11 medium (Stainer *et al* 1971) and Conway medium (Walne 1966), were used to separate algal populations under critical and continuous microscopic observations. After isolation, the strains were labelled based on the laboratory name as BGLR1-19. Isolated microalgae were maintained as stock cultures and were stored on a low light, cool shelf. The stock cultures were maintained by re-plating onto new nutrient medium at least once a month or culturing into fresh culture broth.

3.3 Morphological identification

The identification of the microalgal strains was done by observing morphology. Isolated microalgal cultures were identified to the genus level by observing the morphology of the individual cells (Wehr and Sheath 2003). The cultures were photographed at (40×) using the Olympus 528293 microscope (Magnus Icon Freedom Model) with the Debro 5.1 Megapixel digital camera and the Toup view software program.

3.4 Screening of the microalgal isolates

The nineteen microalgal isolates (listed in Table 4.2) were subjected to a time course study in five different media (Algae culture medium, Guillard's F/2 medium, Bold's basal

medium, BG-11 medium and Conway medium) for evaluating the growth kinetics. The experiment was carried out in Erlenmeyer flasks of 250 ml capacity, containing 200 ml medium, under the standard laboratory conditions (25±°C temperature, 40 μmolm^{-2}s^{-1} light intensity and 12 h:12 h light:dark photoperiod). Before inoculation, the media in the flasks were sterilized in an autoclave for 20 min at 121 °C. Fresh inoculum (10% v/v) was utilized for all experiments. Growth rate was monitored by measuring the changes of turbidity in the culture medium (Absorbance at 750 nm) by using a UV–Vis spectrophotometer (Hitachi UV-VIS U-2800) at 3 days interval up to 35 days. All the experiments were carried out using triplicate samples. These strains were also screened in terms of their dry cell biomass, chlorophyll, carbohydrates, lipids and proteins.

The growth processes of most of the microorganism can be well elucidated by Logistic model. There are many growth kinetic models available like Richards, Schnute, Logistic and Chapman Richards; among all these, the Logistic model uses simple calculation for studying microbial growth as it is independent of substrate consumption (Yang *et al* 2011). So, a non-linear modified Logistic equation (Zwietering *et al* 1990, Celekli *et al* 2009) was fitted to experimental data for biomass production by microalgal isolates BGLR1-19 cultivated in the media (in which they showed the highest biomass production during screening) for this study. The modified Logistic equation used for this study can be expressed as:

$$\mathbf{Y} = \frac{\mathbf{A}}{[\mathbf{1} + \mathbf{exp}\left\{\mathbf{4}\left(\frac{\boldsymbol{\mu}}{\mathbf{A}}\right)(\boldsymbol{\lambda} - \mathbf{t}) + \mathbf{2}\right\}]}$$

Where, A is asymptote value (biomass g L^{-1}); μ is growth rate (day^{-1}) and λ is lag time (days). In order to analyse the goodness of fitting, the predicted data obtained from the model equation for all the cultures was plotted against experimental data and coefficient of determination (R^2) and sum of squared deviations were calculated. The fitting of the data in the model was done by using the MS Solver of Excel 2007.

3.4.1 Estimation of dry cell biomass or weight

The dried biomass was determined gravimetrically (Mitra *et al* 2012). The dry cell weight (DCW) or biomass was used to characterize the growth of the microalgal isolates. A known volume of microalgal culture was centrifuged at 7,000 rpm for 10 min. The cell pellet obtained was then dried in oven at 60 °C for overnight and then weighed.

3.4.2 Estimation of chlorophyll

The chlorophyll was estimated by modified method of El-Baky *et al* (2008) wherein twenty ml of microalgal culture was centrifuged at 6,000 rpm for 10 minutes and the pellet obtained was dissolved in acetone (20 ml, 100 %) and kept overnight in dark at 4˚C for

complete extraction. After this, it was centrifuged at 10,000 rpm for 5 min and the contents of total chlorophyll (T-Chl) (chlorophyll a (Chl-a) and chlorophyll b (Chl-b)) in the supernatant were spectrophotometrically (Hitachi UV-Vis Spectrophotometer U-2800) determined by applying standard equations (Lichtenthaler 1987) as:

$$\mathbf{Chla = 11.24 * A_{661.6} - 2.04 * A_{644.8}}$$

$$\mathbf{Chlb = 20.13 * A_{664.8} - 4.19 * A_{661.6}}$$

$$\mathbf{Chl\ a+b = 7.05 * A_{661.6} - 18.09 * A_{644.8}}$$

Where, $A_{661.6}$ = Absorbance taken at wavelength 661.6

$A_{644.8}$ = Absorbance taken at wavelength 644.8

3.4.3 Estimation of carbohydrates

Carbohydrates were estimated by the method of DuBois et al (1956). Two ml homogenized sample was diluted with 1 ml of distilled water and mixed with 5 ml of 5% phenol and vortex it on vortex mixture. Thereafter, 5 ml of concentrated H_2SO_4 was added to the test tube. The addition of sulphuric acid was done directly on the solution so that the contents were mixed and heated due to exothermic reaction. After this it was again swirled on the vortex mixture. The test tube was cooled in the running tap water and then absorbance was measured at 490 nm. Carbohydrate content was estimated using a standard calibration curve (given in section 3.14 as Fig.3.1) prepared from standard glucose solution (500 µg/ml). For preparation of standard curve, stock solution (500 µg/mL) of glucose was prepared by dissolving 0.05g glucose in 100 ml distilled water. Different concentrations of glucose ranging from 50-500 µg/ml were taken by taking 0.1, 0.2 and 0.3 ml the standard glucose solution and adding 0.9, 0.8, 0.7 ml and so on distilled water was, respectively followed by 5 ml 5% phenol and 5 ml concentrated H_2SO_4.

3.4.4 Estimation of lipids

The lipids were estimated by sulfo-phospho-vanillin (SPV) assay (Mishra *et al* 2014). Phosphovanillin reagent was prepared by initially dissolving 0.6 g vanillin in 10 ml absolute ethanol; 90 ml deionized water and stirred continuously. Subsequently, 400 ml of concentrated phosphoric acid was added to the mixture, and the resulting reagent was stored in the dark until use. To ensure high activity, fresh phospho-vanillin reagent was prepared shortly before every experiment run. For SPV reaction of the algal culture for lipid quantification, a known amount of biomass which are either suspended in a known volume of liquid culture or harvested via centrifugation at 4000 rpm for 5 min, or volume (5 ml) in 100 µl water, was used. Two mL of concentrated (98%) sulfuric acid was added to the sample and was heated for 10 min at 100 °C and was cooled for 5 min in ice bath. Five mL of freshly prepared phospho-vanillin reagent was then added and the sample was incubated for 15 min at 37 °C in incubator shaker at 200 rpm. Absorbance reading at 530 nm was taken in order to quantify the lipid within the sample using the standard curve (given in section 3.14 as Fig.3.2).

For the preparation of standard curve, the standard lipid stock was prepared using commercial canola oil at 20 mg in 10 ml chloroform (final concentration, 2 mg/ml), which was subsequently stored at −20 °C before use. Different amount of lipid in microliters of standard oil solution was added in the triplicate test tubes (0, 40, 80, 120, 160, 200, 240 µg/ml). The tubes were kept at 60 °C for 10 min to evaporate the solvent and 100 µl of water was added to the lipid standard. Further sample was prepared by following SPV reaction methods.

3.4.5 Estimation of proteins

Proteins were estimated according to Lowry *et al* (1951). The culture solution was first homogenized and 2 ml of homogenized sample was mixed with 1 ml of 1 N NaOH. The mixture was then heated at 100 °C for 15 min and cooled under running tap water. After cooling, it was mixed with 5 ml of 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartarate solution (also known as alkaline copper sulphate reagent). The contents were mixed thoroughly and after 10 min, 0.5 ml of 1:1 Folin Ciocalteu's reagent was added and the contents were mixed immediately. Allowed the colour to develop for 30 min. Then the absorbance was recorded at 620 nm after setting the instrument with reagent blank. The protein content was estimated using a standard calibration curve prepared from standard Bovine Serum Albumin (BSA) of concentration 100 µg/ ml (given in section 3.14 as Fig.3.3).

3.4.6 Estimation of mineral content of algal biomass

The mineral components (As, B, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Ni, P, Pb, S and Zn) of the dried microalgal biomass were determined by the inductively coupled plasma-atomic emission spectroscopy (ICP-AES), at the Department of Soil Science, Punjab Agricultural University, Punjab (India) according to the method of Mitra *et al* (2015). The dried algal biomass was first digested by concentrated HNO_3 and then put in a muffle furnace at 400 ºC for 4 h. After this, the furnace was switched off and the sample was left to cool down. The ash obtained was then again mixed with HNO_3, diluted with distilled water and filtered with a 0.45 µm membrane filter. Then the mineralogical analysis was performed through inductively coupled plasma-atomic emission spectroscopy (ICP-AES).

3.5 Molecular Identification

The molecular identification of the two potential isolates was carried out. The culture BGLR5 being eukaryotic in nature was identified by 18S rRNA sequence analysis and BGLR6 being prokaryotic in nature was identified by 16S rRNA sequence analysis.

3.5.1 18S rRNA sequence analysis

For molecular identification of BGLR5, culture was harvested in the exponential growth phase and genomic DNA was extracted by using the Chromous Genomic DNA isolation kit (Chromous Biotech Pvt. Ltd., Bangalore, India). The 18S rRNA gene (~1.8-2 kb)

of isolate was amplified by polymerase chain reaction (PCR) using the following pair of 18S rRNA specific primers: 18s forward primer 5'-GTAGTCATAKGCTNGTCTS-3' and 18s reverse primer 5'- GARACCTDGTTAVGACTY -3'. PCR reactions were carried out in 100 µl reaction mixture containing DNA template (1µl of 100 ng/µl), 400 ng of each primers, 4 µl of 10 mM of each dNTPs mixture, 1 µl of Taq DNA Polymerase (3U/µl). The PCR amplification was performed in the Thermal Cylcer ABI2720 under conditions: initial denaturation at 94°C for 5 min, 35 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min and final extension at 72°C for 7 min. The amplified PCR products were evaluated by comparision with a 500 bp DNA ladder. All PCR reagents were of Chromous Biotech Pvt. Ltd. (Bangalore, India) make. PCR products were then sequenced by an ABI 3500XL Genetic Analyzer. Data analysis was performed by Seq Scape_v 5.2 software. The phylogenetic analysis and dendrogram construction was done by using Weighbor (a weighted version of Neighbor Joining that gives significantly less weight to the longer distances in the distance matrix) and 100 rounds of bootstrap resampling (Bruno *et al* 2000). The BLAST program at the NCBI server was used for comparative analyses and homologous sequence search. The 18s rDNA sequence was submitted to GenBank database.

3.5.2 16S rRNA sequence analysis

For molecular identification of BGLR6, culture was harvested in the exponential growth phase and genomic DNA was extracted by using the Chromous Genomic DNA isolation kit (Chromous Biotech Pvt. Ltd., Bangalore, India). The 16S rRNA gene (1.3 kb) of isolate was amplified by polymerase chain reaction (PCR) using the following pair of 16S rRNA specific primers: 16s forward primer 5'-CMGSCVTDACACAWGCHAGYC-3' and 16s reverse primer 5'- GGCGSMTGWGTNCAAGSV -3'. PCR reactions were carried out in 100 µl reaction mixture containing DNA template (1µl of 50 ng/µl), 400 ng of each primers, 2.5 mM of each dNTPs mixture, 1 µl of Taq DNA Polymerase (3U/µl). The PCR amplification was performed in the Thermal Cylcer ABI2720 under conditions: initial denaturation at 95°C for 5 min, 35 cycles at 94°C for 30 sec, 50°C for 30 sec, 72°C for 1.30 min and final extension at 72°C for 7 min. The amplified PCR products were evaluated by comparision with a 500 bp DNA ladder (LAD02). All PCR reagents were of Chromous Biotech Pvt. Ltd. (Bangalore, India) make. PCR products were then sequenced by an ABI 3130 Genetic Analyzer. Data analysis was performed by Seq Scape_v 5.2 software. The phylogenetic analysis and dendrogram construction was done by using Weighbor (a weighted version of Neighbor Joining that gives significantly less weight to the longer distances in the distance matrix) and 100 rounds of bootstrap resampling (Bruno *et al* 2000). The BLAST program at the NCBI server was used for comparative analyses and homologous sequence search. The 16s rDNA sequence was submitted to GenBank database.

3.6 Optimization of cultural conditions of the selected isolates

The optimization was carried out in two steps, firstly the screening of cultural factors was done and then the significant factors obtained through screening were further optimized through response surface methodology.

3.6.1 Screening and selection of significant factors by Placket-Burman design

Placket-Burman design was applied to assess the significance of various physicochemical factors like pH, Temperature, Light intensity, Growth period, $CaCl_2$, $NaNO_3$, K_2HPO_4 on responses like biomass, chlorophyll, carbohydrate, lipid and protein for BGLR6. Similarly, for BGLR5, all the factors to be screened were same to that of BGLR6 except one NH_4Cl which was used instead of $CaCl_2$. The Statgraphics Centurion XVII software was used to analyze the data. Each factor was tested at two levels: -1 for a low level and +1 for a high level, according to the Placket-Burman design (Table 4.7 and 4.16). The experimental design together with the coded factor levels is given in Table 4.7 and 4.16.

3.6.2 Optimization by Central Composite design of response surface methodology

Response surface methodology (RSM), based on Central Composite design (CCD), was utilized to identify the optimum levels of variables having significant effects on various responses as mentioned in above section. Optimization experiment was designed by using Statgraphics Centurian XVI.I software.

The seven significant factors selected by Placket-Burman approach, used in designing the experiment included pH, Temperature, Light intensity, Growth period, $CaCl_2$/ NH_4Cl, $NaNO_3$ and K_2HPO_4, in which each was evaluated at three coded levels (Table 4.8 and 4.17). A total of 39 runs were conducted. The central value of all factors was coded as zero. The full experimental design with various combinations obtained, under which experiments were performed. Biomass, chlorophyll, carbohydrate, lipid and protein production were studied under varied pH (7.5, 9.5, 11.5), temperature (20, 27.5, 35°C), light intensity (40.5, 60.75, 81.0 $\mu molm^{-2}s^{-1}$), growth period (10, 18, 25 days), $CaCl_2$/ NH_4Cl (15, 25, 35 mM), $NaNO_3$ (5, 12, 20 mM) and K_2HPO_4 (2, 5, 8 mM) with the intention to find the actual potential of the organism under study.

The relationship between the response and the independent factors was elucidated by using the second order polynomial equation and the regression coefficients were obtained by multiple linear regression

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} + x_i x_j$$

Where, y is the predicted response, β_0 is the interception coefficient, β_i is the linear

coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient. The response values (y) in each trial were the mean of the triplicates.

For optimization, the analysis of variance (ANOVA) for the overall effect of seven variables on the response variable according to fitted model was done using software Statgraphics Centurian XVI.I. CCD was utilized for multiple regression analysis of the experiment and F test was applied to analyse the statistical significance of quadratic polynomial equation. Coefficient of correlation (r) and determination coefficient of correlation (R^2) were used to analyse the performance of regression equation. The combination of different optimized variables, which produced the maximum yield of response, was utilized to produce the response under study to verify and validate the model.

3.7 Assessment of biogas potential of selected microalgal isolates

The biogas potential of the algal biomass of the selected isolates was studied as:

3.7.1 Pretreatment of algal biomass

The algal biomass of the two isolates were subjected to two pretreaments: (a) Enzymatic pretreatment and (b) Hydrothermal pretreatment

(a) Enzymatic pretreatment

The pretreatment was carried out with the enzyme mix consisting of cellulose (sp. activity 1.3 U/mg), hemicellulase (sp. activity 0.3−3.0 U/mg) and protease (sp. activity ≥500 U/g). For evaluating the best pretreatment conditions, two enzyme doses were compared (10 and 20% w/w) over an exposure time of 12 and 24 h (i.e., 10% 12 h, 10% 24 h, 20% 12 h and 20% 24 h). Here both the enzyme doses and exposure time were varied. The untreated one was run in parallel to these sets. The microalgal biomass was placed in Erlenmeyer flasks (250 mL) where the corresponding dose of enzyme for corresponding exposure time was added (10 and 20% w/w). Both doses were assayed in triplicate for the three studied enzymes. Trials were set in a BOD incubator with controlled temperature at 37 °C, under continuous mixing. This temperature was set as the optimal for enzymatic activity.

(b) Hydrothermal pretreatment

Hydrothermal pretreatment was carried out in an autoclave. The pretreatment conditions were 100°C and 120°C for 30 min. The exposure time was kept same for both the temperatures. Pretreatment was performed in Erlenmeyer flasks with a useful volume of 200 mL. The flasks were plugged with cotton. During hydrothermal pretreatment biomass was placed in the autoclave and temperature was raised to the target value. In this moment, biomass was maintained under the target temperature for the whole exposure time. Then pressure was gradually released to reach atmosphere conditions. Finally, biomass was cooled to room temperature and stored at 4°C until use.

Samples obtained from these pretreatments and without pretreatment were analysed for total solids and volatile solids by the standard methods of AOAC (2009) given in the analytical procedures section below.

3.7.2 Microscopy of the untreated and pretreated samples

The optical microscopy and scanning electron microscopy were carried out to evaluate the effect of the various pretreatments.

3.7.2.1 Optical microscopy

The untreated and treated samples were observed microscopically (Olympus 528293 microscope) to assess the qualitative information on the effect of enzymatic and alkaline hydrothermal pretreatment on microalgae cells. The optical microscope used was equipped with the Debro 5.1 Megapixel digital camera and the Toup view software program.

3.7.2.2 Scanning Electron Microscopic (SEM) analysis

The surface structure of the selected potential microlagal isolates (BGLR5 and BGLR6) before and after pretreatments (Enzymatic and alkaline hydrothermal pretreatment) were also analyzed by Scanning Electron Microscope Imaging according to Bozzola and Russell (1999) at Electron Microscopy and Nanoscience Laboratory (EMNL), Punjab Agricultural University, Punjab (India). The sample was processed by dehydrating it with the increasing alcohol series (50%, 70%, 80%, 90%, 95% and 100%) followed by mounting it on the carbon tape. Thereafter, sputter coating was carried out with the gold particles and then viewed under the Scanning Electron Microscope (Hitachi S-3400N, Germany) at the accelerating voltage of 15000V.

3.7.3 Fourier transform infrared (FT-IR) spectroscopy

Changes in the composition of the structural components of untreated and treated biomass were analysed by Fourier transform infrared spectrometer (Thermo Scientific Nicolet 6700 spectrometer) using an attenuated total reflection accessory and deuterated triglycine sulphate detector. FT-IR was done at Electron Microscopy and Nanoscience Laboratory (EMNL), PAU, Ludhiana. One mg algal samples (both untreated and pre-treated) were mixed with 10 mg activated KBr. KBr was activated by heating in oven at 60°C for 12 hours to get rid of moisture. The mixture of algal sample and KBr was then made a fine intimate powder by crushing in crystal mortar with a crystal pestle. The fine powder was then pressed in a Hydraulic Pellet Press to make a uniform thin pellet. The pellet was then put inside the FT-IR spectrometer. Spectra were collected in the 4000– 400 cm^{-1} range, with a 4 cm^{-1} interval and 32 scans at room temperature.

3.7.4 Determination of biogas production potential through BMP test

The biogas production potential of selected algal biomass after different pretreatments for both BGLR5 and BGLR6 were determined through biochemical methane potential (BMP)

test protocol (Alzate *et al* 2012). The experiments were conducted in 300 mL capacity digesters (Borosil). The anaerobic digester set up consists of a digester, a gas collecting chamber and a liquid collecting chamber (Fig.3.4). The digester was sealed with rubber cork and araldite (adhesive). The working volume was kept up to 150 mL. The inoculum (digested biogas slurry) was aseptically transferred to experimental bottles from an actively running cattle dung based biogas plant. The inoculum was first degassified by incubating it at thermophilic temperature (45-50°C) for 7-10 days. This incubating the inoculum also increases the microflora responsible for anaerobic digestion in it. The substrate to inoculum ratio of 0.5 was used for the experiments. Distilled water was used to make-up the working volume up to 150 mL whenever needed. Digester containing only untreated biomass was used as the control. After inoculation, the prepared digesters were kept under stationary conditions at 35 ± 2 °C for incubation and the volume of biogas produced was measured after every 24 h for 30 days through acidic (HCl) water (pH <3) displacement method. Biogas production from the control bottle was also studied simultaneously. A total of seven digesters (A-G) for each culture (BGLR5 and BGLR6) were established and the biogas production studies were carried out for these.

Fig. 3.4 Anaerobic digester setup for determination of biogas production potential through BMP test

The samples at the time of digester setting up (initial stage) and after anaerobic digestion period of 30 days were processed for the determination of volatile solids by the standard methods of AOAC (2000). The volatile solids reduction (VSR%) was determined by using the following equation:

$$\mathbf{VSR}(\%) = \frac{(\mathbf{VSbd} - \mathbf{VSad})}{\mathbf{VSbd}} \times \mathbf{100}$$

Where VS_{bd} and VS_{ad} signifies volatile solids before and after anaerobic digestion.

3.8 Co-digestion of algal biomass with paddy straw for biogas production

The co-digestion study of algal biomass with paddy straw was carried out in two ways. In the first method, replacement of paddy straw by equal amount of algal biomass was done for both cultures (BGLR5, BGLR6), and in the second the algal biomass which produced highest biogas in replacement experiment, was used as supplement (in different amounts) to the paddy straw.

3.8.1 Replacement with algal biomass

In order to see the effect of microalgae on biogas production from the paddy straw, the straw was co-digested with BGLR6 and BGLR5 biomass separately in different combinations. The microalga was used, as imbalanced C/N ratio is one of the crucial hurdles in anaerobic digestion process. The algae has low C/N ratio whereas paddy straw has high C/N ratio, and using them collectively will balance the C/N ratio. The paddy straw was collected from the farms of Punjab Agricultural University (PAU), Ludhiana, India and the algae was grown in the Biogas Laboratory, School of Renewable Energy Engineering, PAU, Ludhiana. The inoculum cattle dung slurry (CDS) was procured from the working biogas plant, PAU, Ludhiana. The inoculum was degassified by incubating it at the high temperature of 45°C for a week prior to its use and also the paddy straw was soaked overnight before setting up of digesters. The batch system composed of a 2L glass reactor, 2L glass bottle for gas collection which was filled with dilute hydrochloric acid solution (pH <3 to avoid CO_2 dissolution) and 2L liquid collection beaker. The experimental design for the anaerobic digestion of paddy straw and algae was carried out at ambient temperature that ranged between 35°C to 38°C in five batch digesters labelled A–E in triplicates for each culture BGLR5 and BGLR6 as:

Digester A: comprised 100% Soaked paddy straw (SPS) in 250 ml of cattle dung slurry (CDS) (i.e. 250 g of SPS),

Digester B: comprised 80% SPS and 20% algal biomass in 250 ml of CDS (i.e. 200 g of SPS and 50g algal biomass),

Digester C: comprised 70% SPS and 30% algae biomass in 250 ml of CDS (i.e. 175 g of SPS and 75g algal biomass),

Digester D: comprised 50% SPS and 50% algae biomass in 250 ml of CDS (i.e. 125 g of SPS and 125g algal biomass),

Digester E: comprised 30% SPS and 70% algae biomass in 250 ml of CDS (i.e. 75 g of SPS and 175g algal biomass),

The samples at the time of digester setting up (initial stage) and after anaerobic digestion period of 46 days were processed for the determination of volatile solids by the standard methods of AOAC (2000). The volatile solids reduction (VSR%) was determined by using the equation mentioned in section 3.7.4 above.

Apart from this, proximate and chemical composition analysis (described in section 3.11.2) was also done for the samples before and after digestion by the standard methods of AOAC (2000). The biogas production was measured by water displacement method i.e. by measuring the amount of acidic water (pH <3 to avoid CO_2 dissolution) displaced by the gas produced.

3.8.2 Supplementation of algal biomass

The supplementation of algal biomass (BGLR5) with paddy straw was done because it resulted in the higher biogas production in replacement experiment of co-digestion. Here the experimental conditions were same to that of the replacement co-digestion experiment; the only difference is that the amount of paddy straw was kept same in all the digesters. The batch system was also same to that of the replacement co-digestion experiment. The experimental design for the anaerobic digestion of paddy straw and algae was carried out at ambient temperature that ranged between 35°C to 38°C in five batch digesters labelled A–G in triplicates as:

Digester A: comprised 100% Soaked paddy straw (SPS) in 250 ml of cattle dung slurry (CDS) (i.e. 250 g of SPS),

Digester B: comprised 100% SPS and 20% algal biomass in 250 ml of CDS (i.e. 250 g of SPS and 50g algal biomass),

Digester C: comprised 100% SPS and 30% algae biomass in 250 ml of CDS (i.e. 250 g of SPS and 75g algal biomass),

Digester D: comprised 100% SPS and 50% algae biomass in 250 ml of CDS (i.e. 250 g of SPS and 125g algal biomass),

Digester E: comprised 100% SPS and 70% algae biomass in 250 ml of CDS (i.e. 250 g of SPS and 175g algal biomass),

Digester F: contained SPS and algae biomass in 1:1 ratio and 250 ml of CDS (i.e. 250 g of SPS and 250g algal biomass),

Digester G: contained SPS and algae biomass in 1:2 ratio and 250 ml of CDS (i.e. 250 g of SPS and 500g algal biomass)

Similarly to that of the replacement co-digestion experiment, the samples at the time of digester setting up (initial stage) and after anaerobic digestion period of 46 days were processed for the determination of volatile solids by the standard methods of AOAC (2000). The volatile solids reduction (VSR%) was determined by using the equation mentioned in section 3.7.4 above.

3.8.3 Computation of daily and cumulative biogas yields

The kinetics of biogas production in all the biogas experiments was studied by using the modified Gompertz equation and also a modified first order kinetic equation. Biogas production from the different digesters was noted daily. From this the daily and cumulative biogas production in terms of mL biogas g^{-1} VS was calculated. The cumulative biogas data was then fitted with the Gompertz equation for calculating the enhancement in ultimate biogas yield (P), maximum rate of biogas production (R_m) & lag phase (λ) in the gas production profile. The used Gompertz equation, adopted from Prajapati *et al* (2015) is given as

$$\mathbf{M = P * exp[-exp\left\{\left(\frac{Rm*e}{P}\right) * (\lambda - t) + 1\right\}]}$$

Where, M is the cumulative biogas yield (mL biogas g^{-1} VS added), t = time and e = 2.718.

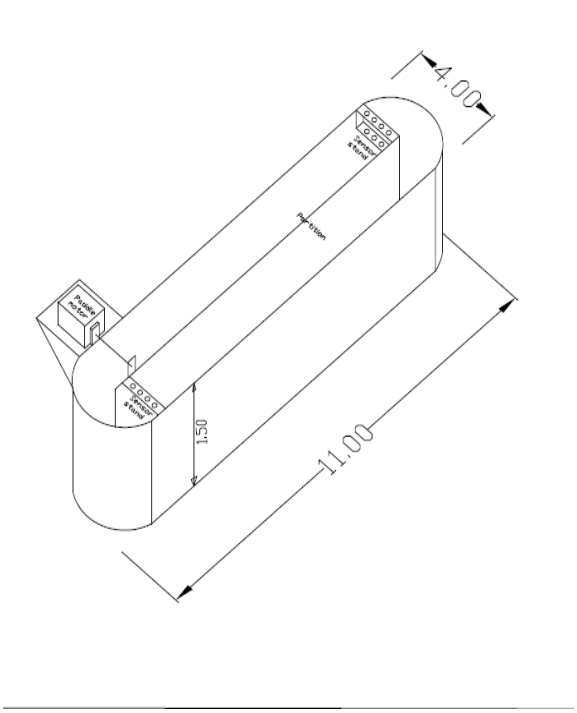
Moreover, first order kinetic equation of anaerobic digestion was also used to the cumulative biogas data so as to predict the improvement in the hydrolysis constant (k_h). This hydrolysis constant gives the assessment of the substrate degradability. It was presumed that the hydrolysis in the substrate (SPS and algal biomass) digestion follows first order kinetics and k_h was then determined by fitting the cumulative biogas data to the first order hydrolysis kinetics model adopted from Angelidaki *et al* (2009).

$$\mathbf{M = P\{1 - exp(-K_h t)\}}$$

MS Solver of Excel 2007 was utilized as the platform to fit the experimental data in the models.

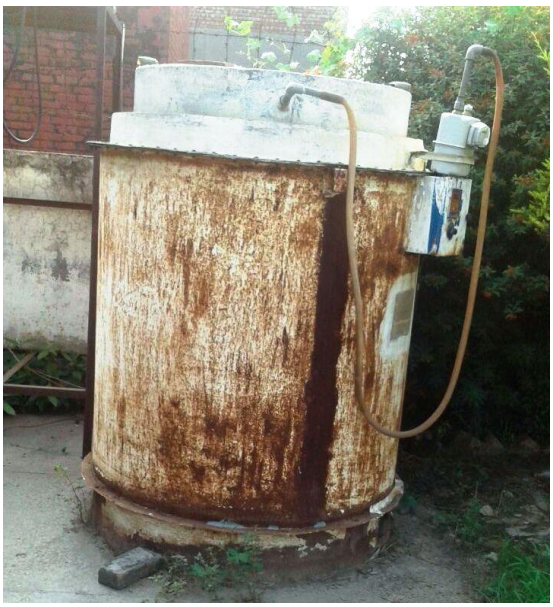
3.9 Multipopulation microalgal species cultivation and biogas production at field scale

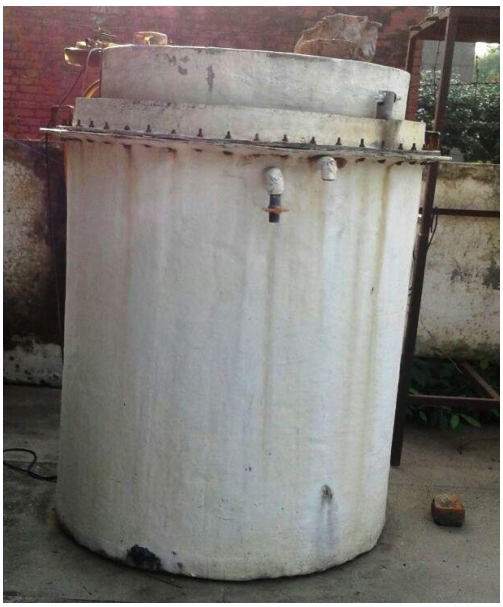
A rectangular shaped experimental open air pond of dimensions 11×4 ft (l×b) and 1.5 ft depth was constructed (Fig. 3.5 a,b). The floor and walls were painted with Wall Putty (a white cement based product) to give interior rough plasters a smooth finish. The pond was designed to enhance/promote algal growth. It was kept shallow in order to allow maximum light penetration. The pond was filled with simple tap water in which only sodium bicarbonate was added @ 10 g L^{-1} of the tap water. The cultivation was carried out from January 2017 and it is still being carried out. Although, the biomass is procured from time to time from the pond. Once the biomass is removed from the pond, it is again refilled with the sodium bicarbonate dissolved tap water. The pond was initially inoculated by using the mixed inoculums containing multialgal population like cyanobacteria, diatoms and green algae. The identification of the microalgal species in the pond was done by observing the morphology of the individual cells. Identification to the genus level was done by observing the morphology of the individual cells (Wehr and Sheath 2003). The culture was photographed at (40×) using the Olympus 528293 microscope (Magnus Icon Freedom Model) with the Debro 5.1 Megapixel digital camera and the Toup view software program. The pH of the cultivation pond was estimated throughout the study by using the pH metre (Mettler Toledo™ SevenEasy 8603).



(a) In line diagram of algal pond **(b) Rectangular open air algal pond**

Fig. 3.5 Open air algal pond for mass cultivation of multipopulation algal biomass





(a) Control **(b) Experimental (Algal suspension+ paddy straw)**

Fig. 3.6 a,b Field scale double walled biogas digesters (1460 mm × 1040 mm) of 1 m^3 capacity

As the flow in an algal pond needs to be turbulent to keep the cells in suspension, to enhance vertical mixing, prevent thermal stratification and facilitate removal of the oxygen generated by photosynthesis, the culture in the pond was first mixed by manual mixing and later on, two impellers (agitators) both having two blades moving by the help of suitable single phase electric motor were installed. The impellers move the culture in the pond in clockwise direction. The pond was covered by muslin cloth in order to avoid the contamination of the pond by various contaminants like leaves, dust etc. The biomass was estimated after harvesting it through filtration and gravity settling and the wet weight of biomass per litre was determined.

The algal suspension (5.3 mg VS L^{-1}) obtained after three months was used with the paddy straw (70 kg) for biogas production. This algal suspension served in two ways-firstly the soaking of paddy straw was done with this and secondly, it provided the microalgal biomass as a co-feedstock for carrying out the anaerobic digestion process. The biodigested slurry (100 L) was used as inoculums in each. The anaerobic digestion was carried out in the 1 m^3 capacity double walled field scale biogas digester (1460 mm × 1040 mm) (Fig. 3.6 a,b) made up of Fibre Reinforced Plastic (FRP) which provides complete insulation to the digester. Digester is composed of two shells: outer and inner. The digester is designed in such a way that it maintains the temperature. It is provided with a heater at the bottom to heat up the water which circulates between the two shells and thus maintains the digester temperature. The digester temperature can be adjusted between 10-100 °C. The moisture content inside the digester is maintained by an ultrasonic nebuliser which sprays the water mist inside the digester via ultrasonic waves to have a uniform humidity inside the digester. The lid of the digester is kept in a water seal to avoid leakage of the biogas produced. In this biogas digester, biogas produced is automatically measured by the gas meter (m^3/h). The composition of gas was measured by using a portable gas analyser (GFM series 11068). A gas utility valve is provided on the gas meter itself through which biogas produced can be used for various purposes like lighting up of mantles, running engines etc.

3.10 Analytical procedures

3.10.1 Determination of salinity of water samples

The salinity was measured as described by Richards (1954) in terms of cations and anions like CO_3^{2-}, HCO_3^-, Ca^{2+}, Mg^{2+}.

Determination of carbonates, bicarbonates and chlorides in water samples

Carbonates and bicarbonates in given samples were determined by titrating a known volume of water samples (25 ml) against standard sulphuric acid (N/10) using phenolphthalein and methyl red as indicators respectively. For the determination of carbonates, known volume of sample (25 ml) was taken in a conical flask (100 ml) and a drop of phenolphthalein indicator was added to it. Pink colour appeared in the sample. Then the

sample was titrated with N/10 H_2SO_4 till it becomes colourless. The amount of H_2SO_4 used for titration was recorded for carbonate estimation. Bicarbonates were analyzed in continuation with carbonates. After titration, a drop of methyl red indicator was added the conical flask which gave yellow colour. It was then titrated against N/10 H_2SO_4 till pink colour appeared. For chloride determination, potassium indicator was added which gave the yellow colour. These were the titrated against N/40 $AgNO_3$ till red brick colour appeared.

Calculations:

Amount of N/10 H_2SO_4 required for 25 ml water in presence of phenolphthalein = X ml

Amount of N/10 H_2SO_4 required for the neutralization of bicarbonate solution in presence of methyl red = Y ml.

Total volume of N/10 H_2SO_4 required for the complete neutralization of carbonates = 2X

Total volume of N/10 H_2SO_4 required for the complete neutralization of bicarbonates originally present in water = Y − X ml.

$$\text{Amount of carbonates mEq/L} = \frac{\text{Volume of acid used (2X ml)} \times \text{Normality of acid} \times 1000}{\text{Vol. of water used}}$$

$$\text{Amount of bicarbonates mEq/L} = \frac{\text{Volume of acid used (Y} - \text{X ml)} \times \text{Normality of acid} \times 1000}{\text{Vol. of water used}}$$

Volume of N/40 $AgNO_3$ required for 25 ml of the water after subtracting blank reading=Z ml.

$$\text{Amount of chlorides mEq/L} = \frac{\text{Volume of acid used (Z ml)} \times \text{Normality of acid} \times 1000}{\text{Vol. of water used}}$$

Determination of Calcium and Magnesium in algal water samples

For the determination of calcium and magnesium, Ericom black tea indicator was added to known volume (1 ml) of algal water samples. The ammonium buffer was added which gave pink colour to the samples. The samples were then titraed against 0.01 N EDTA solution till pink color changed to blue. The amount of EDTA used for titration was recorded to estimate the calcium and magnesium in milliequivalents. Calculations for Calcium:

Volume of 0.01 N EDTA solution used = X ml.

$$\text{Milliequivalents per litre of calcium} = \frac{\text{X ml} \times \text{Normality of EDTA solution} \times 1000}{\text{ml of aliquot}}$$

Calculations for Magnesium:

Volume of 0.01 N EDTA solution used = Y ml.

$$\text{Milliequivalents per litre of calcium and magnesium} = \frac{\text{Yml} \times \text{Normality of EDTA solution} \times 1000}{\text{Millilitres of water taken}}$$

Milliequivalents of magnesium (Mg) = $(Ca^{2+} + Mg^{2+}) - Ca^{2+}$ (both in meq/L)

3.10.2 Determination of proximate and chemical composition of feedstock fed into the digesters

Standard methods of AOAC (2000) were followed for the determination of proximate and chemical composition (*i.e.*, Total Solids (TS), Volatile Solids (VS), ash, cellulose, hemi-cellulose, lignin and silica) of feedstock fed into the various digesters for biogas production.

3.10.2.1 Determination of Total Solids (TS)

An oven dried silica crucible was weighed. The weight of oven dried crucible was tared and 2 g sample (untreated/pretreated paddy straw) was weighed. The crucible containing sample was placed in hot air oven at 100°C (for overnight). The sample was dried till a constant weight was achieved. The crucible containing sample was cooled in a desiccator and weighed. TS of the sample was determined as follow:

$$TS\ \% = W_2\text{-}W_0 / W_1\text{-}W_0 \times 100$$

Where,

W_0 = Weight of oven dried empty silica crucible

W_1 = Weight of fresh sample and crucible

W_2 = Weight of oven dried sample and crucible

3.10.2.2 Determination of ash

The crucible containing 1 g of oven dried sample was ignited in a muffle furnace at 600°C for 3 h (or until the sample became carbon-free). The crucible while still hot was placed in oven at 100°C for 1 h after removing it from the furnace. The crucible containing ash was cooled in a desiccator and weighed. Ash was calculated by the formula given below:

$$Ash\ \% = W_3\text{-}W_0 / W_2\text{-}W_0 \times 100$$

Where,

W_0 = Weight of oven dried empty silica crucible

W_2 = Weight of oven dried sample and crucible

W_3 = Weight of furnace burnt sample and crucible

3.10.2.3 Determination of Volatile Solids (VS)

Volatile solids were obtained by subtracting the ash content from 100.

$$VS\ \% = 100\text{- Ash}\%$$

3.10.2.4 Determination of hemi-cellulose

Hemicellulose was calculated after determining Neutral Detergent Fibre (NDF) and Acid Detergent Fibre (ADF).

3.10.2.4.1 Determination of Neutral Detergent Fibre (NDF)

Dried and ground paddy straw sample (0.5 g) was weighed in a spoutless beaker. Fifty ml of neutral detergent solution (Annexure I) was added to the sample. The mixture was

heated to boil on a hot plate. The boiling of mixture was adjusted to an even level and refluxed for 60 min from the onset of boiling by keeping the round bottomed flasks over the spoutless beakers. The vacuum was allowed from a suction pump (oil free vacuum pump, MACRO Scientific Works Pvt. Ltd.) and the liquid content was filtered through sintered glass crucible (G-1) mounted on suction flask. The washing of the sample with hot distilled water was repeated till the foam stops coming into the flask. The vacuum was removed and the residue was washed twice with acetone. The crucible was kept at 100°C in hot air oven for overnight. The crucible was weighed after cooling in a desiccator. The neutral detergent fibre (NDF) of the sample was calculated by the given formula.

$$NDF\ \% = W_1 - W_0 / S \times 100$$

Where,

W_0 = Weight of oven dried crucible

S = Initial weight of sample (dried, ground paddy straw)

W_1 = Weight of oven dried sample and crucible

3.10.2.4.2 Determination of Acid Detergent Fibre (ADF)

Dried and ground paddy straw sample (0.5 g) was weighed in a spoutless beaker. Fifty ml of acid detergent solution (Annexure I) was added to the sample. The mixture was heated to boil on a hot plate. The boiling of mixture was adjusted to an even level and refluxed for 60 min from the onset of boiling by keeping the round bottomed flasks over the spoutless beakers. The vacuum was allowed from a suction pump and the liquid content was filtered through sintered glass crucible (G-1) mounted on suction flask. The washing of the sample with hot distilled water was repeated till the foam stops coming into the flask. The vacuum was removed and the residue was washed twice with acetone. The crucible was kept at 100°C in hot air oven for overnight. The crucible was weighed after cooling in a desiccator. The acid detergent fibre (ADF) of the sample was calculated by the given formula.

$$ADF\ \% = W_1 - W_0 / S \times 100$$

Where,

W_0 = Weight of oven dried crucible

S = Initial weight of sample (dried, ground paddy straw)

W_1 = Weight of oven dried sample and crucible

3.10.2.4.3 Calculation of hemi-cellulose

Hemi-cellulose was obtained by subtracting ADF from NDF

NDF = cellulose+hemi-cellulose+lignin+silica

ADF = cellulose+lignin+silica

$$\text{Hemi-cellulose \%} = NDF\% - ADF\%$$

3.10.2.5 Determination of cellulose, lignin and silica

Cellulose, lignin and silica were calculated after determining acid detergent lignin (ADL).

3.10.2.5.1 Determination of Acid Detergent Lignin (ADL)

The acid detergent fibre (ADF) residue (from Section 3.15.4.2) was covered with cold solution of 72% H_2SO_4 (w/w) (Annexure I). The crucible was filled about half way with the acid (10 ml) and stirred. The lumps were broken with a glass rod. The crucible was refilled with 72% H_2SO_4 and stirred as the acid drains. After 3 h, suction was applied to wash the contents of the crucible with hot distilled water until the washings were acid free to pH paper. The crucible was heated at 100°C in hot air oven. Dried sample was cooled and weighed.

3.10.2.5.2 Calculation of cellulose

The cellulose content of the sample was calculated by the following formula:

$$\text{Cellulose \%} = W_1\text{-}W_2 / S \times 100$$

Where,

S = Initial weight of sample (dried, ground paddy straw)

W_1 = Weight of oven dried fibre (ADF) and crucible

W_2 = Weight of 72% H_2SO_4 treated sample and silica crucible

3.10.5.2.3 Determination of lignin

The crucible containing 72% H_2SO_4 treated sample (from Section 3.15.5.1) was ignited at 600°C in muffle furnace for 3 h (or until carbon-free). The crucible while still hot was placed in oven at 100°C for 1 h after removing it from furnace. The crucible was cooled in a desiccator and weighed. Lignin was determined as given below:

$$\text{Lignin \%} = W_3\text{-}W_2 / S \times 100$$

Where,

S = Initial weight of sample (dried, ground paddy straw)

W_2 = Weight of 72% H_2SO_4 treated sample and crucible

W_3 = Weight of furnace burnt sample and crucible

3.10.5.2.4 Determination of silica

Three-four drops of hydrobromic acid were added into the crucible containing ignited (burnt) sample (from Section 3.15.5.3). After about 30 min, the crucible was washed with distilled water 2-3 times. The crucible was then dried in oven at 100°C. Dried sample was cooled and weighed.

$$\text{Silica \%} = W_4\text{-}W_0 / S \times 100$$

Where, W_0 = Weight of empty oven dried crucible

S = Initial weight of sample (dried, ground paddy straw)

W_4 = Weight of hydrobromic acid treated sample and crucible.

3.11 Statistical analysis

All experimentation was completed in triplicate. Statistical analyses were done by using Statistical analysis system software (SAS software). Different analysis were carried out

like LSD (p=0.05), Duncan's multiple range test (DMRT), Students t-test for different experiments as stated in the footnote of the tables in the results and discussion section.

3.13 Media composition

Algae culture medium

Ingredients	g/L
$NaNO_3$	1.000
K_2HPO_4	0.500
$MgSO_4.7H_2O$	0.513
NH_4Cl	0.050
$CaCl_2.2H_2O$	0.058
$FeCl_3$	0.003
$NaHCO_3$	16.800
$MnSO_4$	4.4×10^{-4}
Mo^{6+}	4.8×10^{-4}
Cu^{2+}	7.2×10^{-5}
EDTA	8×10^{-3}
Zn^{2+}	2.3×10^{-4}
SO_4^{2-}	3.6×10^{-2}
Final pH (at 25°C)	7.0±0.2

BG-11 Medium

Stocks	Components	Concentration
1	$NaNO_3$	15.0 g/L
		Concentration per 500 ml
2	K_2HPO_4	2.0 g
3	$MgSO_4.7H_2O$	3.75 g
4	$CaCl_2.2H_2O$	1.80 g
5	$C_6H_8O_7$ *	0.30 g
6	$(NH_4)_5[Fe(C_6H_4O_7)_2]$ **	0.30 g
7	$EDTANa_2$	0.05 g
8	Na_2CO_3	1.00 g
		Concentration per litre
9	Trace metal solution	
	H_3BO_3	2.86 g
	$MnCl_2.4H_2O$	1.81 g
	$ZnSO_4.7H_2O$	0.22 g
	$Na_2MoO_4.2H_2O$	0.39 g
	$CuSO_4.5H_2O$	0.08 g
	$Co(NO_3)2.6H_2O$	0.05 g

*Citric acid

** Ammonium ferric citrate green

Medium	per litre
Stock solution 1	100.0 ml
Stock solutions 2-8	10.0 ml each
Stock solution 9	1.0 ml

Make up to 1 litre with deionized water. Adjust pH to 7.1 with 1M NaOH or HCl. For agar add 15.0 g per litre of Bacteriological Agar. Autoclave at 15 psi for 15 minutes.

Bold's Basal Medium (BBM)

Stocks	Components	Concentration (g/100ml)
1	$NaNO_3$ Sodium nitrate	2.5
2	$CaCl_2.2H_2O$	0.25
3	$MgSO_4.7H_2O$	0.75
4	K_2HPO_4	0.75
5	KH_2PO_4	1.75
6	NaCl	0.25
7	EDTA-KOH Solution	
	EDTA. Na_2	5.00
	KOH	3.10
8	Ferric solution	
	$FeSO_4.7H_2O$	0.498
	H_2SO_4 (Conc.)	0.100
9	H_3BO_3	1.142
10	Trace elements solution	
	$ZnSO_4.7H_2O$	0.882
	$MnCl_2.4H_2O$	0.144
	MoO_3	0.071
	$CuSO_4.5H_2O$	0.157
	$Co(NO_3).6H_2O$	0.049

Store all stock solutions in the refrigerator

To prepare BB medium –standard method

- Add 10 ml of each stock (1-6) to 940 ml distilled water
- Add 1 ml of stock solutions (7-10)
  Autoclave at 121°C (15 psi for 15 min)

Conway Medium

Medium Components	Concentration (g/L)
Macronutrients	
$NaNO_3$	0.075
NaH_2PO_4	0.005
Micronutrients	
$Na_2H_2EDTA.2H_2O$	0.00436
$FeCl_3.6H_2O$	0.00315
$MnCl_2.4H2O$	0.18
$ZnSO_4$	0.022
$CoCl2.6H_2O$	0.010
$CuSO_4.5H_2O$	0.0098
$Na_2MoO_4.2H_2O$	0.0063
Na_2SiO_3	0.030
Vitamins	
Vitamin B1	2×10^{-4}
Vitamin B12	1×10^{-5}

Guillard's F/2 medium

Medium Components	Concentration (g/L)
Macronutrients	
KNO_3	0.1
Na_3PO_4	0.02
Micronutrients	
$FeCl_3.6H_2O$	0.0013
$CuSO_4.5H_2O$	0.004
$CoCl_2.6H_2O$	0.004
$ZnCl_2$	0.0042
$CuSO_4.5H_2O$	0.004
$MnCl_2.4H2O$	0.00036
$(NH_4)6Mo_7O_{24}.4H_2O$	0.0018
$Na_2H_2EDTA.2H_2O$	0.045
H_3BO_3	0.0334
Vitamins	
Vit B1	2×10^{-4}
Vit B12	1×10^{-5}

3.14 Standard curves of carbohydrates, proteins and lipids

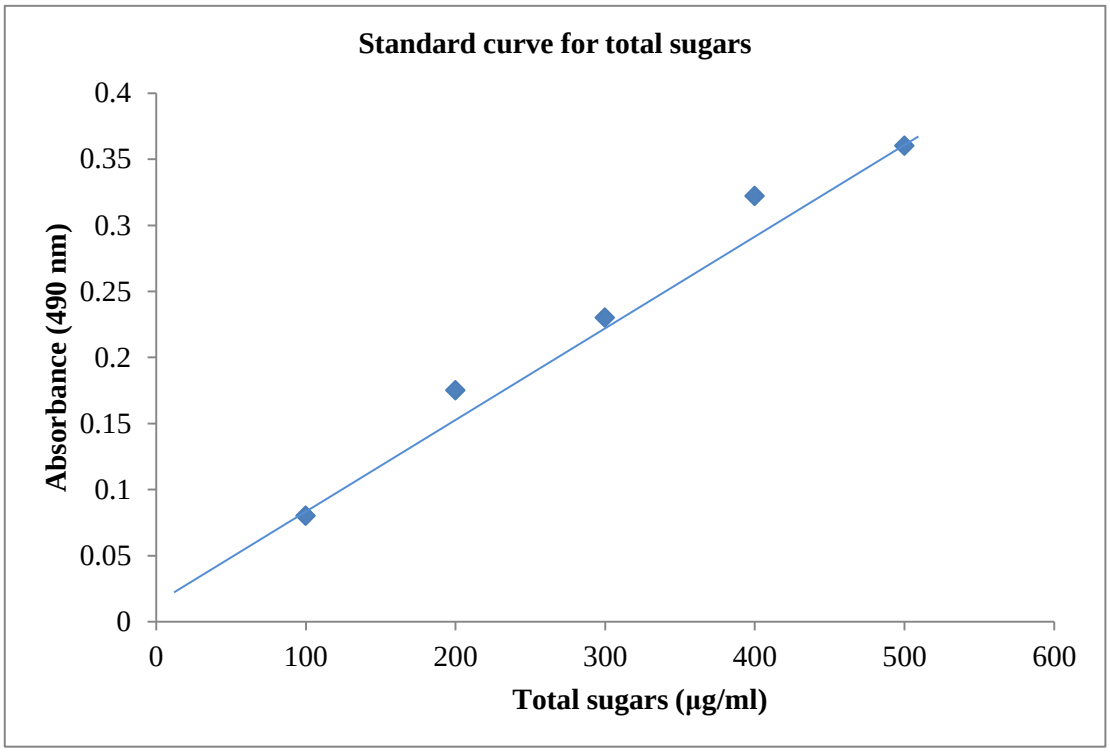


Fig. 3.1 Standard curve for carbohydrate estimation

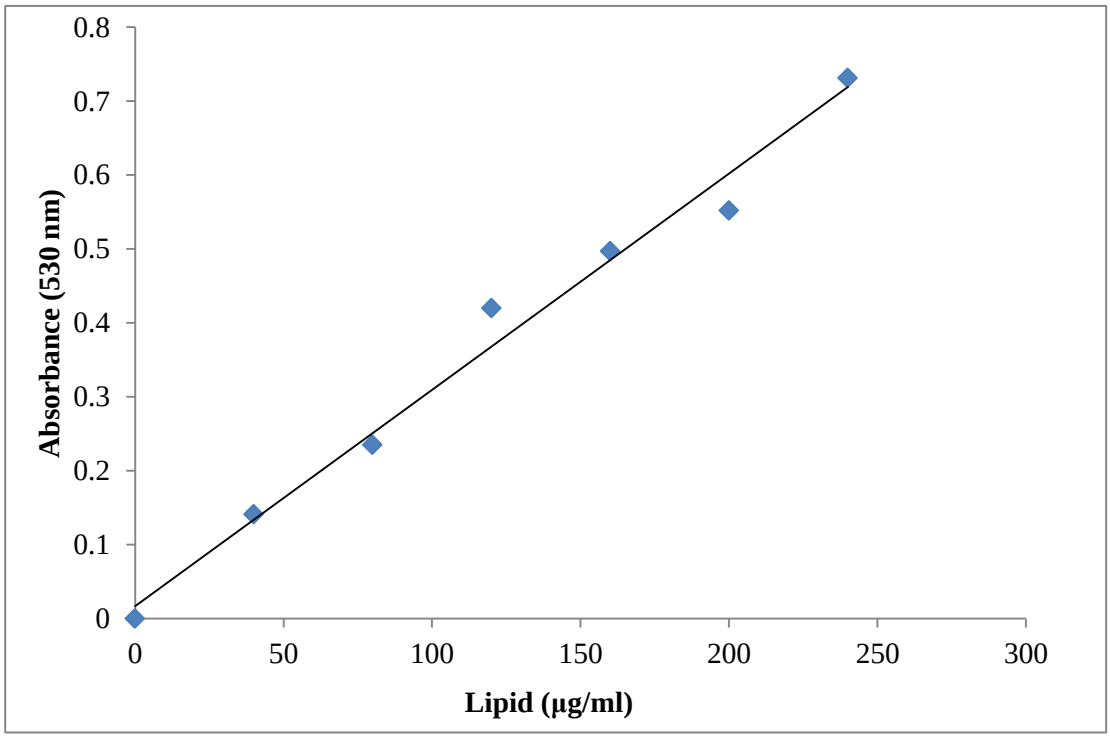


Fig.3.2 Standard curve for lipid estimation

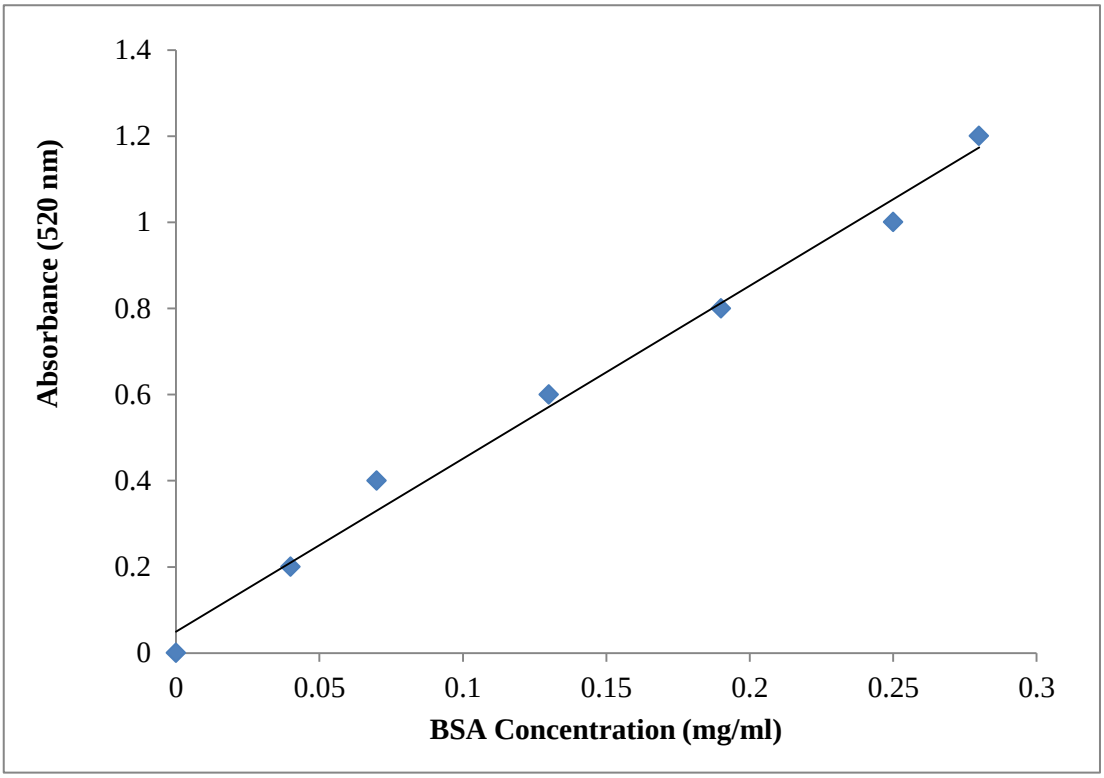


Fig. 3.3 Standard curve for protein estimation

CHAPTER- IV

RESULTS AND DISCUSSION

The south-west zone of Punjab suffers from the water logging problem which makes the land unsuitable for cultivation of crops but this area can be efficaciously utilized to cultivate microalgae. Microalgae (eukaryotes and prokaryotes) have lured special global attention as biofuel substrates due to their expeditious biomass production in comparison to oleaginous plants. The biomass productivity has been found to be approximately 10-fold higher than that of conventional crops and can be cultivated on non-arable land areas or in lakes or the ocean, and thus extenuating food and feed competition. Therefore, owing to this, microalgae are considered to play a remarkable role in the biofuels sector as substrate for several biomass energy conversion technologies. These technologies consist of anaerobic digestion to produce bio-methane, lipid esterification to produce bio-diesel and anaerobic fermentation to produce bio-ethanol. So therefore, keeping the importance of microalgae as future feedstock for biogas production and use of the waterlogged area for microalgae cultivation, in mind, the present study focused primarily on the isolation, purification and screening of the microalagal isolates and molecular characterization of potential isolates. Secondly, it involves assessment of the combined effects of various physico-chemical parameters by the application of Placket-Burman design and Central Composite design of RSM, mainly on biomass production, and also on other functional components of potential microalgal isolates. Finally, the co-digestion studies with paddy straw to assess and evaluate biogas production, were carried out, with an aim of protecting environment by utilizing agricultural waste in a productive way and producing biogas, a prospective form of bioenergy. This is of its first kind of study carried out to explore the microalgal diversity of the unexplored waterlogged area of Punjab, India. The results pertaining to this study have been presented and discussed below:

4.1 Procurement and analysis of water samples

Samples for isolation of microalgae were collected from different places of water logged area of Punjab, India, whose details are given in Table 3. The physicochemical analysis of water samples collected from different sites were carried out and the data is presented in Table 4.1. It was revealed from the data that the pH varied from sample to sample, and ranged between 7.27-8.37. The maximum pH (8.37) was observed in sample collected from Baja Marar area of Muktsar, Punjab, whereas the least pH (7.27) was observed in sample procured from Marar (Muktsar), Punjab. The electrical conductivity of the samples ranged between 0.65-2.02 S/cm. The carbonate ions were not found in the samples. The

bicarbonate ions in the range 7.6-14.0 mEq/L were present in the samples. Similarly Cl^- and $Ca^{2+}+Mg^{2+}$ were calculated and were found to be in the range 5-22 and 2.5-10.1 mEq/L.

4.2 Isolation and morphological identification of microalgae

A total of 19 microlagal isolates were isolated and purified from the water samples collected from waterlogged areas of Punjab (India) through direct enrichment in nutrient media and standard methods of isolation like repeated dilution, plating etc. Upon examination of cultures at microscopic level, the strains were found to be belonging to different groups and of diverse morphology like some were found to be unicellular, some filamentous and some colonial in nature. The isolates mainly belonged to chlorophyta and cyanobacteria. The microscopic images or microphotographs, isolate name, tentative identification based on using taxonomic keys are given in Fig. 4.1 and Table 4.2.

4.3 Screening of microalgal isolates

Screening was done mainly on the basis of the biomass production. The microalgal isolates were were grown on five different media (ACM, BBM, BG-11, Conway and Guillard's F/2 medium) for aperiod of 35 days. The growth profile was studied after 2 days of interval for a period of 35 days by measuring the absorbance at 750 nm. On studying the growth profile it was observed that the all the microalgal species followed the conventional growth kinetics (all three phases lag, log and stationary phase) in all the culture media (Fig. 4.2 a-s). However, these growth phases were not prominent for some of the isolates in some media. The highest absorbances were attained in ACM (0.835) for BGLR6 followed by BG11 (0.633) for BGLR5 while the lowest ones were observed for Conway (0.078) in case of BGLR17 and GM F/2 (0.090) for the same BGLR17. All the nineteen isolates revealed a wide range of values in terms of dry cell biomass ranging from 0.3063 to 1.0890 g L^{-1} (Table 4.3). Each isolate individually showed the highest dry weight of biomass in different medium. Microalgal isolates BGLR1-19 showed highest biomass production in ACM, BG11, BBM, BG11, BG11, ACM, ACM, BG11, BG11, BG11, BBM, ACM, ACM, ACM, BG11, ACM, BG11, BG11, BG11, BG11. ACM and BG11 were found to be most favorable as maximum isolates showed maximum biomass production in these two media. The lowest biomass production was noticed in Conway medium. The possible reason is that the ACM and BG11 are nutrient rich as they contain higher iniatial concentrations of nitrate (N) and phosphate (P) than other media particularly from that of Conway and GM F/2. Due to this reason the growth was enhanced in these media. Our results are similar to those of the Jazzar *et al* (2016) who too observed higher growth in Algal medium having higher N and P initial concentration. Apart from this, Algal culture medium contains appropriate and suitable concentrations of

micronutrients like iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), sulphur (S) and molybdenum (Mo) (given in materials amd methods section) which most likely enhance the biomass production, whilst in Conway medium the trace elements like Co, Cu, Mn, Mo and Zn are in very low quantities and also the sulphur is absent, which ultimately contribute to the reduction in biomass production. These micronutrients are supposed to play very important roles in the algal cell metabolism. Fe is thought to be important in biological-oxidation and reduction and synthesis of chlorophyll, S has important role in cell division, Zn is needed in RNA synthesis, Cu in photosynthesis, Mn has role in chlorophyll production and O_2/CO_2 utilization in photosynthesis and Mo allows nitrate assimilation and reduction (Carvalhao *et al* 2006). Thus, these elements affect the growth of microalgal cultures. That is why less growth was observed in Conway medium with respect to other media as other media are having these trace elements in optimum concentration. Also, San Pedro *et al* (2013) observed the similar growth patterns for different microalgal strains (*Tetraselmis suecica*, *Phaeodactylum tricornutum*, *Tetraselmis chuii* and *Nannochloropsis gaditana*) in various culture media like Algal, Arnon, M&M and f/2. In between the ACM and BG11 higher biomass was achieved in ACM although N content is more in BG11. So this can be concluded that more biomass was produced in ACM as more phosphate is available in ACM than BG11. Over all the isolates BGLR5 and BGLR6 were found to have produced highest dry cell biomass. The other functional parameters like chlorophyll, carbohydrates, lipids and proteins were also calculated (Table 4.3). The highest carbohydrates were obtained for BGLR5 (43.71%) in BG11 media followed by BGLR5 (40.36%) in ACM and BGLR6 (39.576%) in BG11, whereas the lowest was obtained for BGLR10 (10.80%) in Conway medium. Similarly the highest lipids were observed for BGLR8 (22.57%) in GM F/2 medium whereas the lowest was noticed for BGLR12 (2.49%) in BG11 medium. Also the highest protein content (44.59%) was observed for BGLR6 in ACM followed by BGLR13 (43.65%) in BG11 and lowest (21.19%) was achieved for BGLR14 in Conway medium followed by BGLR5 (21.303%) in Conway medium and again BGLR5 (21.99%) but in GM F/2 medium. The lower protein content obtained in Conway and GM F/2 media may be due to the limitation or less amount of nitrate present in these media compared to other media considered in the study (Go *et al* 2012). The limitation of nitrate in Conway and GM F/2 compared to other media has a tremendous effect on the growth of microalgae. The nitrate present in the culture medium is converted to nitrite through nitrate reduction pathway. This nitrite is then reduced to ammonium; this in turn produces glutamine which is responsible for protein production inside the algal cells. So, nitrate limitation in the medium is believed to reduce or decrease

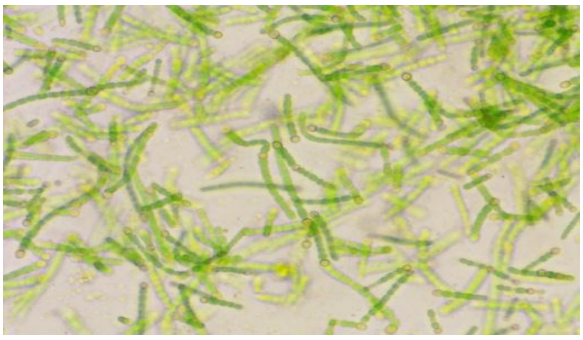
proteins, such as Rubisco proteins which is liable for CO_2 fixation in the Calvin cycle. This not only is thought to decrease proteins but also decreases chlorophylls needed for photosynthesis. Consequently, the photosynthetic performance of algal cells will reduce, thus triggering the inhibition of cell growth (Aravantinou *et al* 2013). The highest chlorophyll was observed for BGLR6 and BGLR5. The chlorophyll content was significantly higher than other isolates (Table 4.4). The chlorophyll was measured for the isolates grown in the medium in which they showed highest biomass production previously.

The kinetics of growth and biomass production of microalgal isolates in their respective medium in which they showed highest biomass production during screening, were studied by the Logistc model. Logistic equation was found to fit the growth kinetics. The high coefficient of determination (R^2) and low sum of squared deviations (SSD) values (Table 4.5) for all the isolates indicated that the Logistic model was fitted well to the experimental data and is highly appropriate for microalgal growth. Thus could be regarded as adequate to explain biomass production of microalgal isolates BGLR1-19. The kinetic modelling includes the evaluation of biological or kinetic parameters like A (asymptote value); μ (growth rate); λ (lag time). These variables were calculated by using the modified Logistic model (Table 4.5). The asymptote values for the algal isolates BGLR1-19 varied from 1.9444 g L^{-1} to 4.6444 g L^{-1}. This parameter determines the highest potential of biomass production and is important for biotechnological purposes. The highest A is for BGLR6 followed by BGLR5. The growth rate (μ) observed for these cultures ranged between 0.0064 to 0.0313 day^{-1}. The highest growth rate was noticed in BGLR6 (0.0313 day^{-1}) followed by BGLR5 (0.0230 day^{-1}) (Table 4.5). Lag time (λ) varied between 0.1300 to 6.6059 days. As can be noticed from the table 4.5 that the lag time for most of the isolates is around one day i.e., the cultures assumed quickly after inoculation, so that is why the characteristic three conventional phases of growth was not observed in growth curves of these microalgal species (Fig.4.2 a-s). These isolates were found to have higher μ, A and λ than reported in some studies (Andrade and Costa 2007, Colla *et al* 2007, Celekli *et al* 2009).

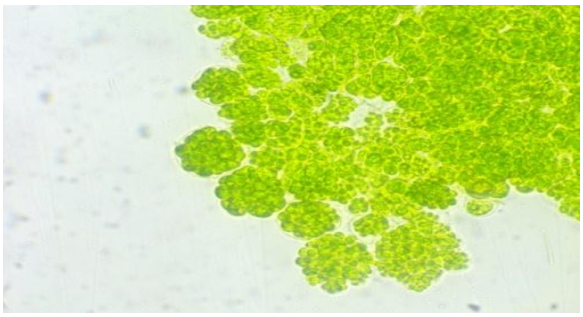
Hence, BGLR5 and BGLR6 were found to have high potential of biomass production. So, these species can be used to produce further higher biomass by optimizing their cultural conditions and this biomass can be utilized in various biotechnological purposes.

Table 4.1 Details of collection sites from waterlogged areas of Punjab and compositional analysis of water

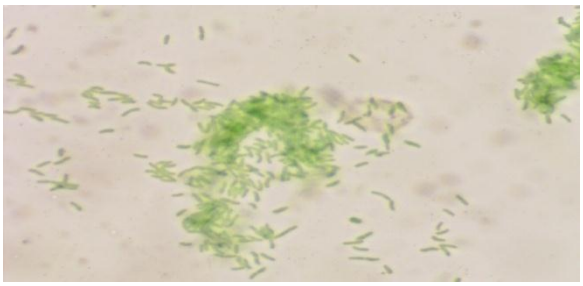
S.No.	Place of Collection	Latitude	Longitude	pH	Electrical Conductivity (S/cm)	CO_3^- (mEq/L)	HCO_3^- (mEq/L)	Cl^- (mEq/L)	$Ca^{2+}+Mg^{2+}$ (mEq/L)	RSC (mEq/L)
1	Marar	N 30° 32ˈ477"	E 074° 40ˈ903"	7.27	1.59	0	12	20	7	5
2	Marar	N 30° 32ˈ199"	E 074° 40ˈ182"	7.63	1.41	0	14	14	4.9	9.1
3	Baja Marar	N 30° 32ˈ691"	E 074° 38ˈ975"	8.29	1.6	0	8	22	5.1	2.9
4	Baja Marar	N 30° 33ˈ712"	E 074° 38ˈ535"	8.37	2.02	0	8	21.5	10.1	2.1
5	Sarainaga	N 30° 31ˈ065"	E 074° 39ˈ772"	7.86	2.01	0	11.6	12.3	9.5	2.1
6	Rupana	N 30° 24ˈ996"	E 074° 31ˈ346"	8.1	0.95	0	9.6	13.3	4.5	5.1
7	Bhondhur	N 30° 19ˈ472"	E 074° 31ˈ059"	8.07	1.27	0	10	11.3	4	6
8	Smag	N 30° 17ˈ347"	E 074° 35ˈ780"	8.08	0.67	0	9.2	4.9	2.5	6.7
9	Bhullar	N 30° 25ˈ233"	E 074° 35ˈ720"	8.27	0.65	0	7.6	5	2.6	5



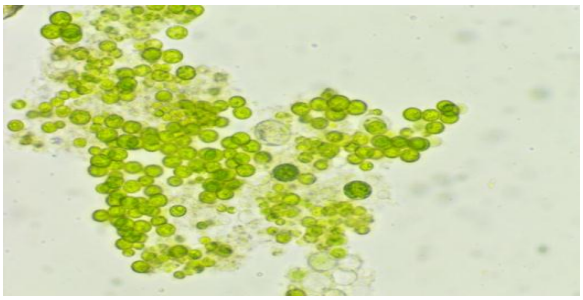
BGLR1



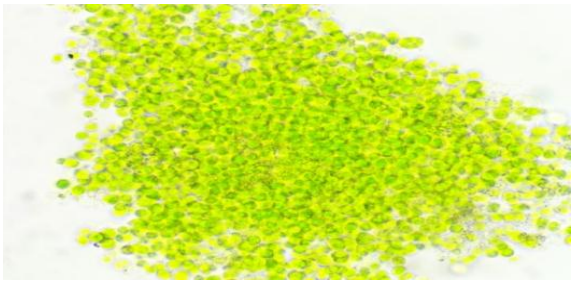
BGLR2



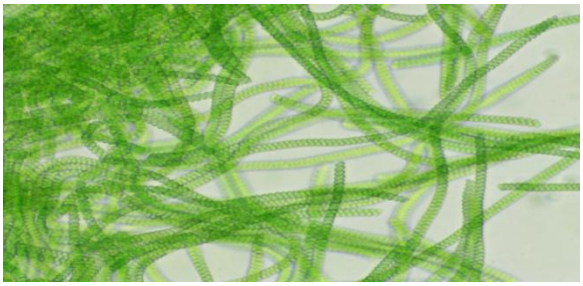
BGLR3



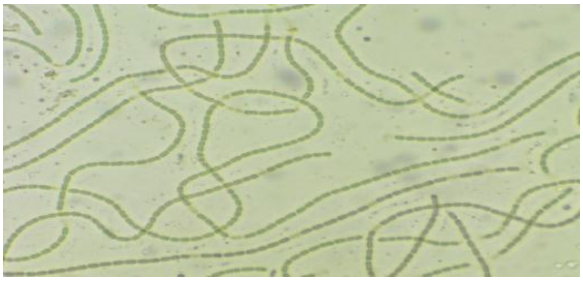
BGLR4



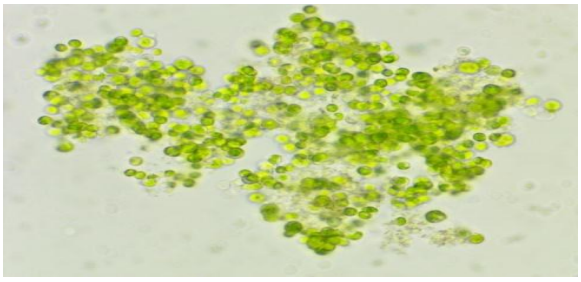
BGLR5



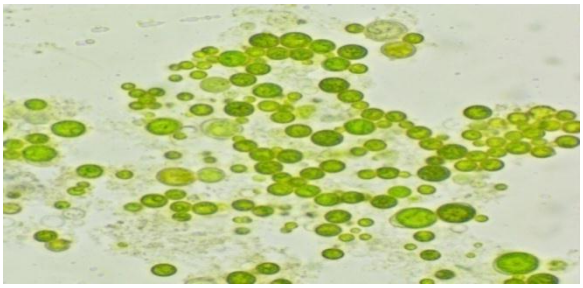
BGLR6



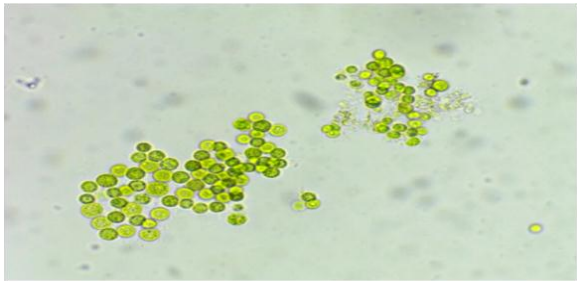
BGLR7



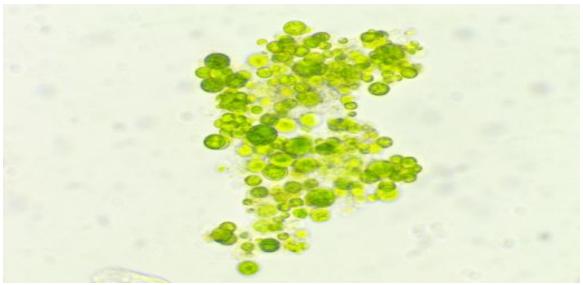
BGLR8



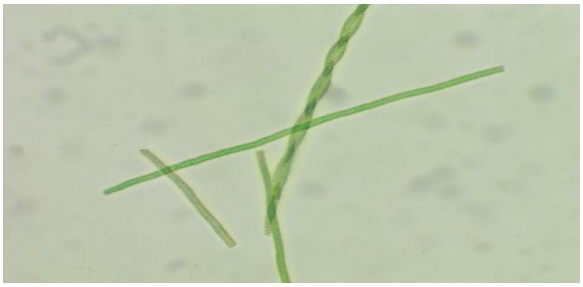
BGLR9



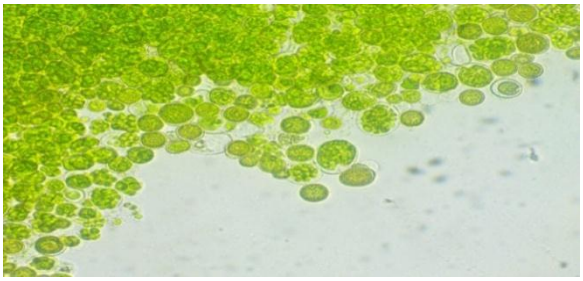
BGLR10



BGLR11

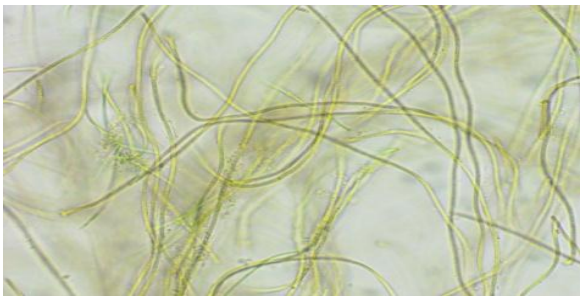


BGLR12

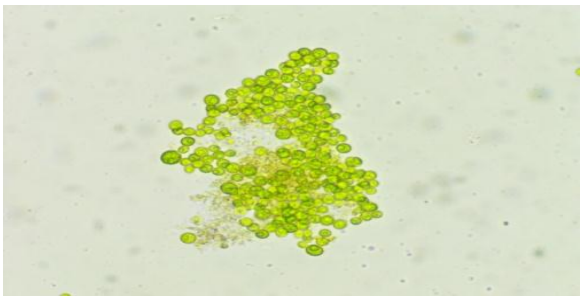


BGLR13

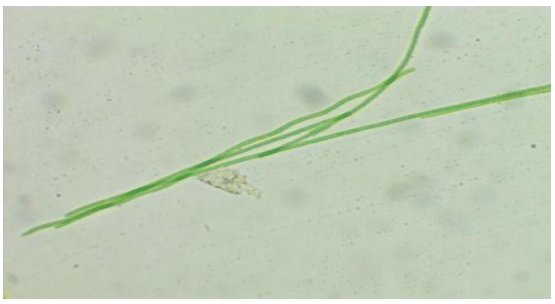
BGLR14



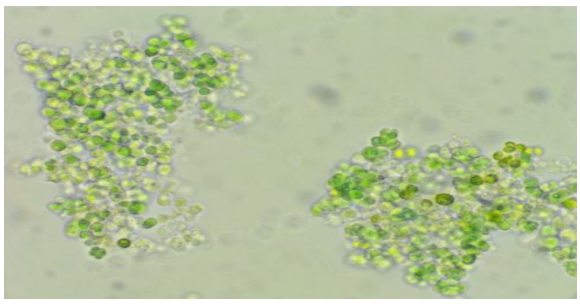
BGLR15



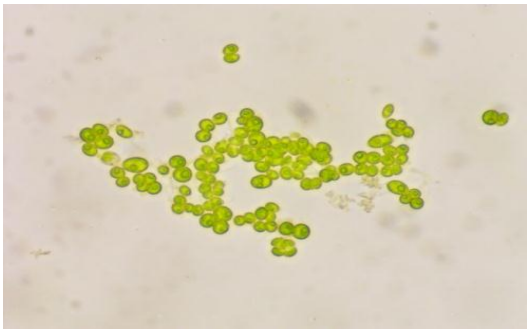
BGLR16



BGLR17



BGLR18



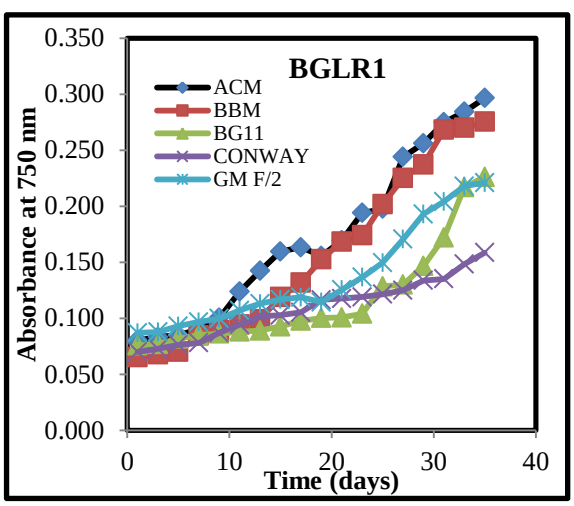
BGLR19

Fig. 4.1 Microphotographs of isolated microalgal isolates taken at 40X

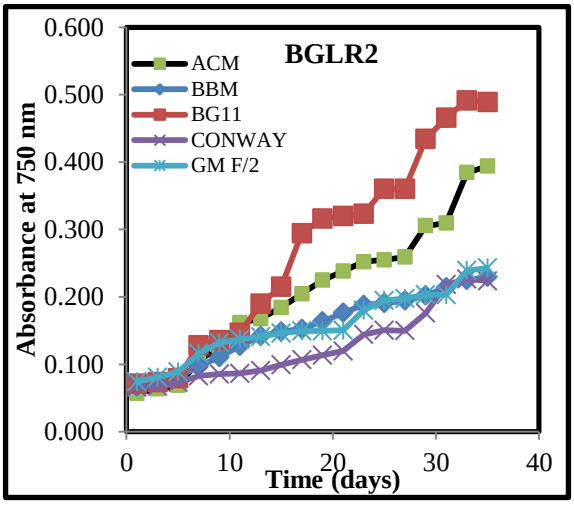
Table 4.2 Morphological features and tentative identification of isolated microalgal isolates

Isolate	Shape	Size	Other morphological characters	Genus	Family
BGLR1	Filamentous and moniliform	Medium	Heterocyst present, Nostoc balls are greenish in colour, multicellular, Cells are spherical or cylindrical in nature	*Nostoc*	Nostocaceae
BGLR2	Globular with irregular margin	Medium	Colonies are usually spherical or globular in nature. The individual cells vary in number. Cells are held together which makes its margin irregular or wrinkled	*Coelastrum*	Scenedesmaceae
BGLR3	Rod and curved	Small	Unicellular small cells somewhat curved, forming groups of 2-3 cells	*Scenedesmus*	Scenedesmaceae
BGLR4	Spherical or slightly oblong	Medium	Unicellular, cells may be solitary or in irregular clumps, single pyrenoid in each cell present	*Chlorococcum*	Chlorococcaceae
BGLR5	Spherical	Medium	Solitary cells with distinct chloroplast	*Chlorella*	Chlorellaceae
BGLR6	Filamentous	Large	Long unicellular thread, filamentous that grow in the form of a tight coiled right or left handed helix, No sheath is visible under light microscopy, trichomes are in constant motion	*Spirulina*	Spirulinaceae
BGLR7	Filamentous	Large	Uniform trichomes, heterocysts generally intercalary, heterocysts are larger than vegetative cells	*Anabaena*	Nostocaceae
BGLR8	Spherical	Medium	Solitary cells with distinct chloroplast	*Chlorella*	Chlorellaceae
BGLR9	Spherical	Large	Unicellular, cells may be solitary or in irregular clumps, single pyrenoid in each cell present	*Chlorococcum*	Chlorococcaceae

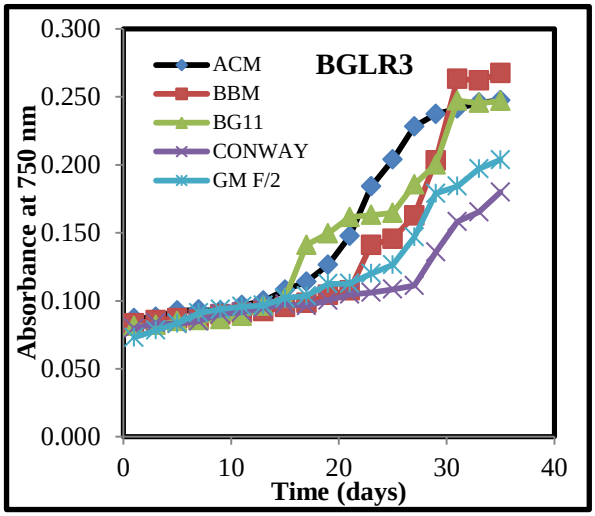
Isolate	Shape	Size	Other morphological characters	Genus	Family
BGLR10	Spherical	Small	Cells seem to solid throughout with visible transparent sheath	*Dictyococcus*	Dictyococcaceae
BGLR11	Spherical	Medium	Vegetative cells with thin walls, multinucleate cells, chloroplast massive with a single pyrenoid	*Neochloris*	Chlorococcaceae
BGLR12	Filamentous	Large	Long unicellular thread, helical in nature	*Spirulina*	Spirulinaceae
BGLR13	Oval	Small to large	Unicellular, cells are solitary as well as in irregular clumps, single pyrenoid present	*Chlorococcum*	Chlorococcaceae
BGLR14	Filamentous	Medium	Unbranched filamentous structure showing gliding moment, no heterocyst present, green in colour, within filament cells are in discoid form	*Oscillatoria*	Oscillatoriaceae
BGLR15	Filamentous	Large	Uniseriate trichomes composed of cylindrical or barrel shaped cells, filaments form wooly mats or tufts which are yellowish or brownish in colour	*Tolypothrix*	Tolypothrichaceae
BGLR16	Spherical	Small	Solitary cells with distinct chloroplast	*Chlorella*	Chlorellaceae
BGLR17	Filamentous	Large	Unbranched filaments showing gliding moment, heterocyst absent	*Oscillatoria*	Oscillatoriaceae
BGLR18	Oval	Small	Unicells, spherical to ovoid, no pyrenoid	*Nanochloropsis*	Eustigmataceae
BGLR19	Oval	Medium	Solitary cells with distinct chloroplast	*Chlorella*	Chlorellaceae


BGLR1
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Time (days)
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
10
20
30
40

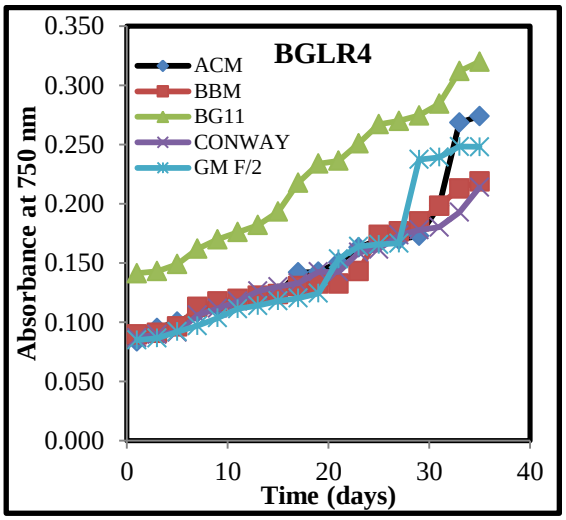
(a)


BGLR2
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Time (days)
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0
10
20
30
40

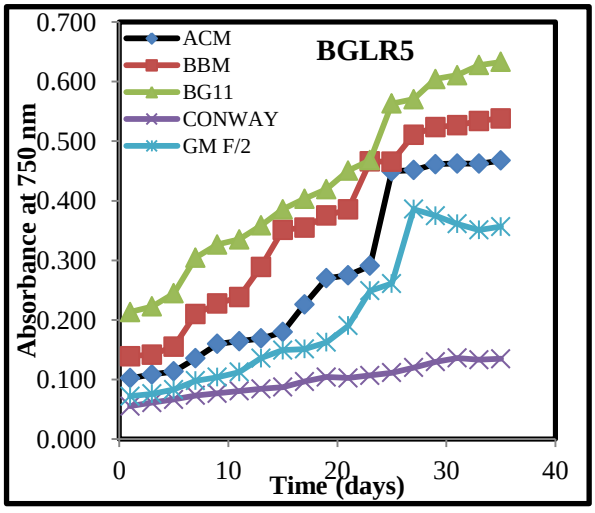
(b)


BGLR3
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Time (days)
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
10
20
30
40

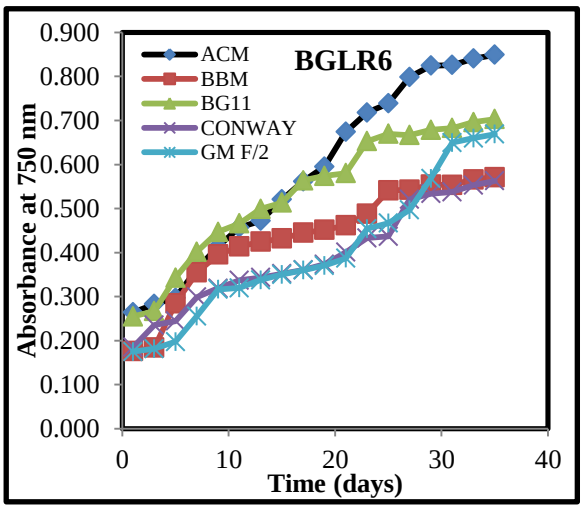
(c)


BGLR4
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Time (days)
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
10
20
30
40

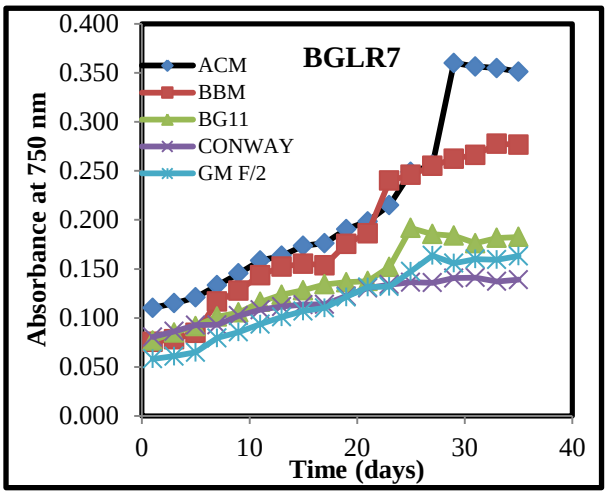
(d)


BGLR5
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Time (days)
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0
10
20
30
40

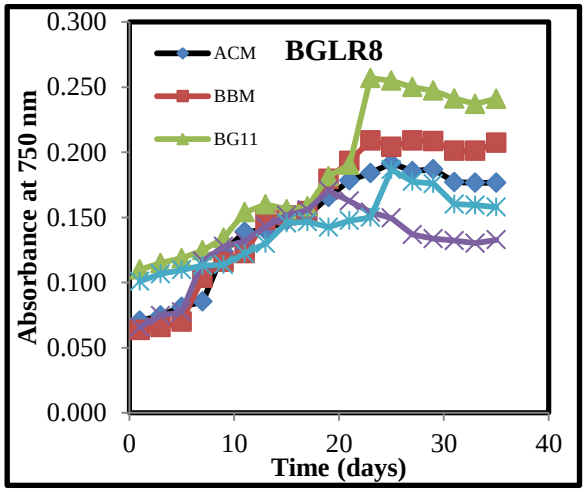
(e)


BGLR6
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Time (days)
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0
10
20
30
40

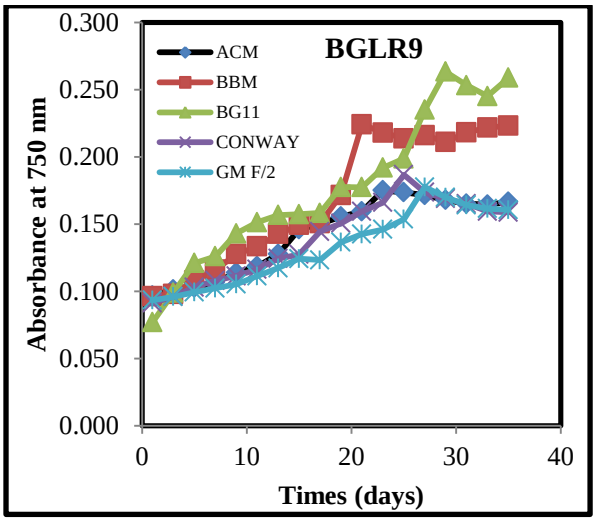
(f)


BGLR7
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Time (days)
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
10
20
30
40

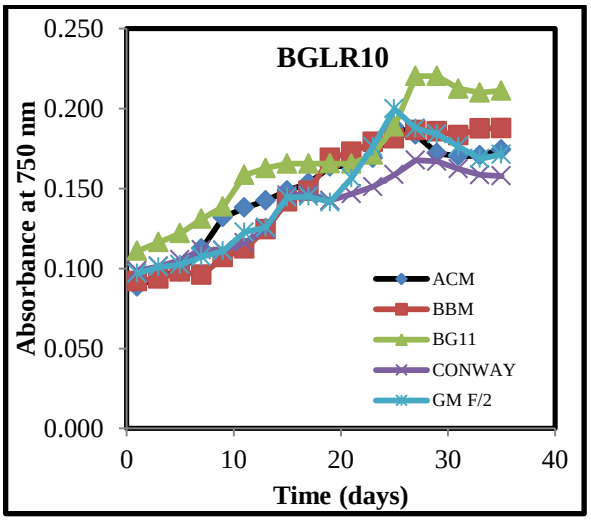
(g)


BGLR8
ACM
BBM
BG11
Absorbance at 750 nm
Time (days)
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
10
20
30
40

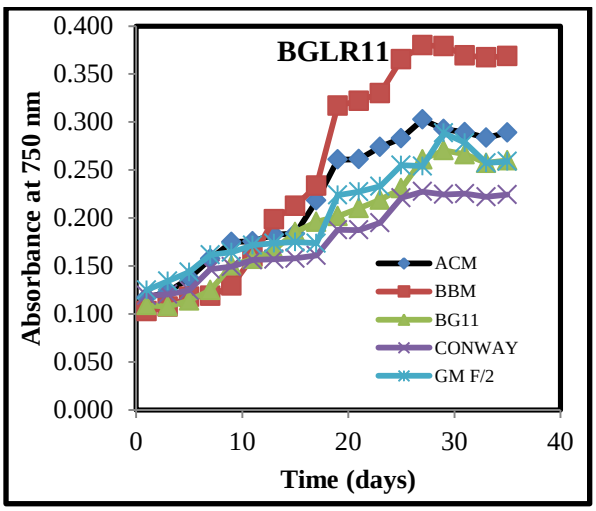
(h)


BGLR9
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Times (days)
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
10
20
30
40

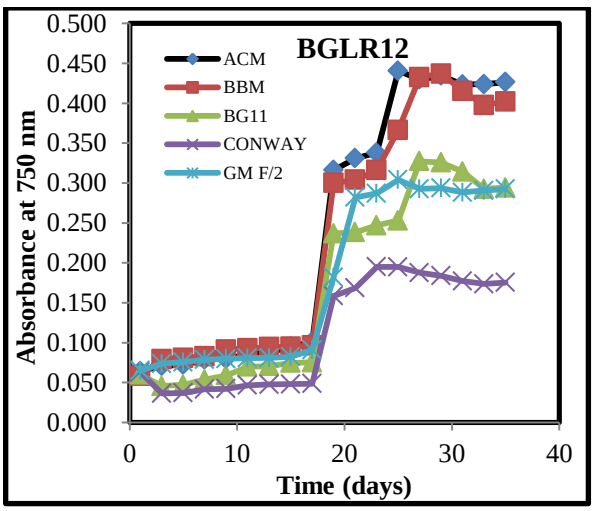
(i)


BGLR10
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Time (days)
0.250
0.200
0.150
0.100
0.050
0.000
0
10
20
30
40

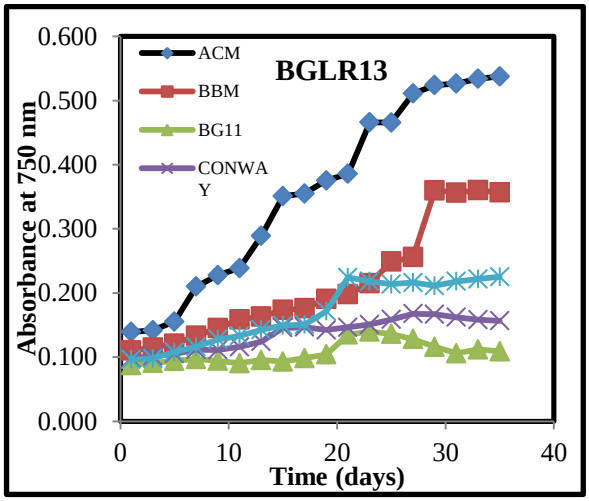
(j)


BGLR11
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Time (days)
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
10
20
30
40

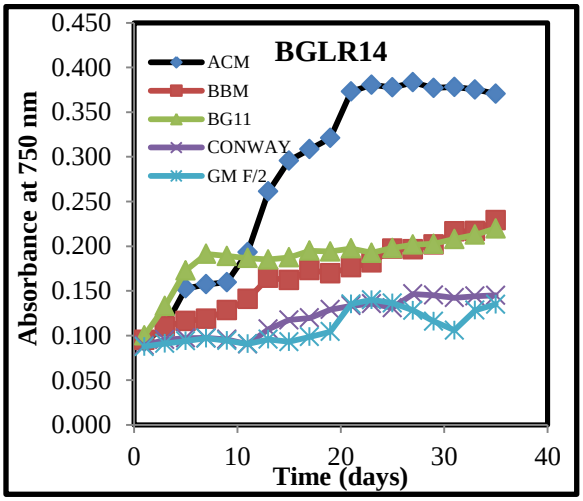
(k)


BGLR12
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Time (days)
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
10
20
30
40

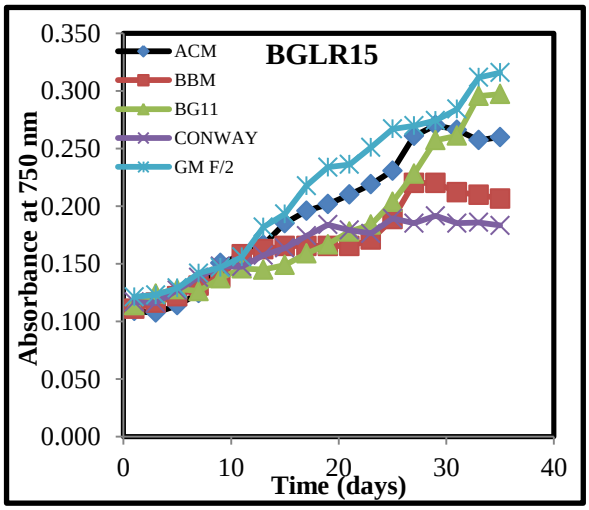
(l)


BGLR13
ACM
BBM
BG11
CONWAY
Absorbance at 750 nm
Time (days)
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0
10
20
30
40

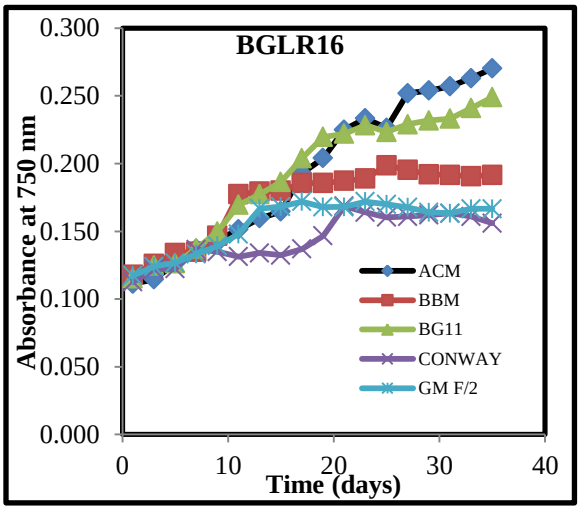
(m)


BGLR14
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Time (days)
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
10
20
30
40

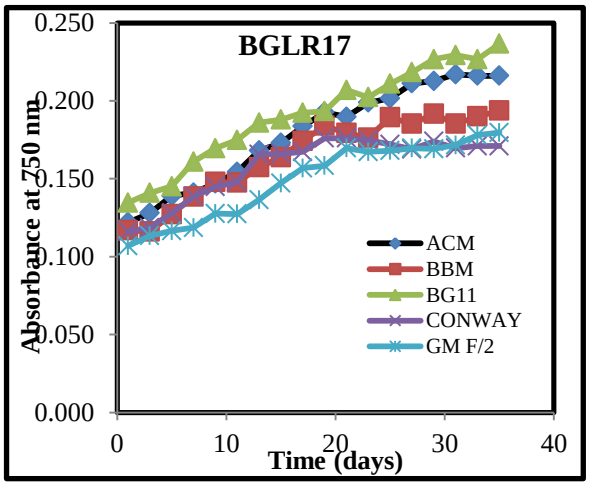
(n)


BGLR15
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Time (days)
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
10
20
30
40

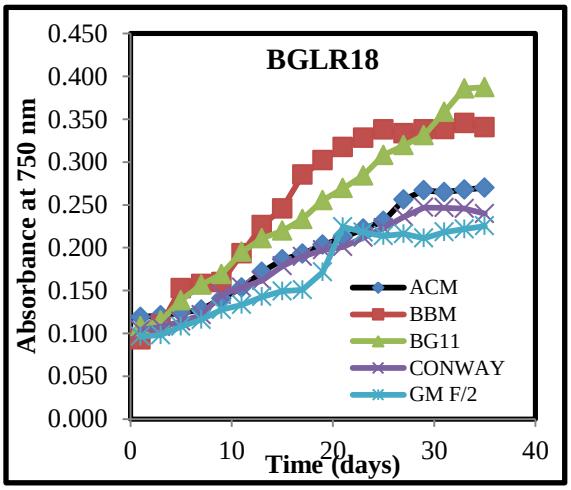
(o)


BGLR16
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Time (days)
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
10
20
30
40

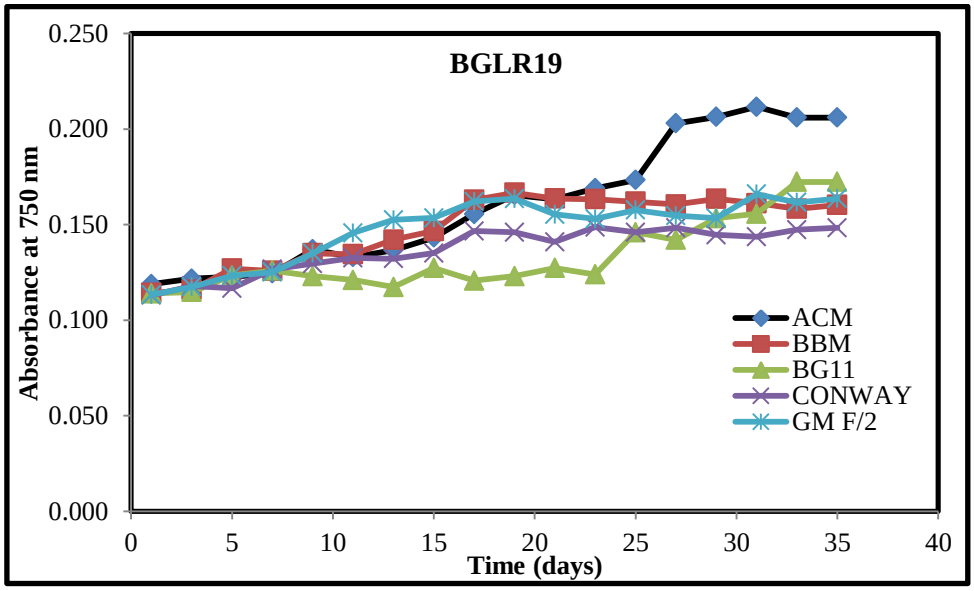
(p)


BGLR17
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Time (days)
0.250
0.200
0.150
0.100
0.050
0.000
0
10
20
30
40

(q)


BGLR18
ACM
BBM
BG11
CONWAY
GM F/2
Absorbance at 750 nm
Time (days)
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
10
20
30
40

(r)



(s)

Fig. 4.2 (a-s) Growth curves of microalgal isolates BGLR1-BGLR19 in five different culture media (ACM, BBM, BG11, Conway and GM F/2) for a period of 35 days

Table 4.3 Screening of different microalgal isolates on different media

Culture	Medium	Dry Biomass ($g\ L^{-1}$)	Carbohydrates ($g\ L^{-1}$)	Lipid ($g\ L^{-1}$)	Protein ($g\ L^{-1}$)
BGLR1					
	ACM	0.4884	0.1294	0.0443	0.1658
	BBM	0.4311	0.0990	0.0224	0.1555
	BG11	0.4169	0.0916	0.0413	0.1445
	CONWAY	0.3564	0.0596	0.0438	0.1019
	GM F/2	0.4130	0.0895	0.0416	0.1670
BGLR2					
	ACM	0.6484	0.2139	0.0699	0.1731
	BBM	0.5729	0.1740	0.0204	0.1691
	BG11	0.7450	0.2650	0.0576	0.1782
	CONWAY	0.5488	0.1613	0.0883	0.1879
	GM F/2	0.5931	0.1847	0.0506	0.1702
BGLR3					
	ACM	0.4241	0.0953	0.0461	0.1046
	BBM	0.4492	0.1086	0.0532	0.1704
	BG11	0.4432	0.1054	0.0678	0.1680
	CONWAY	0.3979	0.0815	0.0493	0.1113
	GM F/2	0.4069	0.0863	0.0777	0.1228

Table 4.3 contd…

Culture	Medium	Dry Biomass ($g\ L^{-1}$)	Carbohydrates ($g\ L^{-1}$)	Lipid ($g\ L^{-1}$)	Protein ($g\ L^{-1}$)
BGLR4					
	ACM	0.5518	0.1629	0.0667	0.1778
	BBM	0.5005	0.1357	0.0276	0.1653
	BG11	0.6424	0.2107	0.0342	0.1626
	CONWAY	0.3828	0.0735	0.0517	0.1014
	GM F/2	0.5216	0.1469	0.045	0.1637
BGLR5					
	ACM	0.7815	0.3194	0.0942	0.2200
	BBM	0.8597	0.3256	0.0848	0.2079
	BG11	0.8858	0.3872	0.0976	0.2019
	CONWAY	0.5830	0.1794	0.0887	0.1242
	GM F/2	0.7158	0.2496	0.0690	0.1574
BGLR6					
	ACM	1.0890	0.3745	0.1131	0.4157
	BBM	0.8245	0.3070	0.0591	0.3437
	BG11	0.9522	0.3768	0.0878	0.4301
	CONWAY	0.8164	0.3027	0.0880	0.3292
	GM F/2	0.9190	0.3570	0.0789	0.3696
BGLR7					
	ACM	0.5679	0.1714	0.0839	0.1689
	BBM	0.5297	0.1512	0.0394	0.1668
	BG11	0.4341	0.1006	0.0551	0.1618
	CONWAY	0.3828	0.0735	0.0788	0.1606
	GM F/2	0.4120	0.0890	0.0528	0.1591
BGLR8					
	ACM	0.4180	0.0921	0.0536	0.1610
	BBM	0.4200	0.0932	0.0423	0.1585
	BG11	0.4935	0.1320	0.0354	0.1649
	CONWAY	0.3727	0.0682	0.0483	0.1311
	GM F/2	0.4049	0.0852	0.0913	0.1603
BGLR9					
	ACM	0.6937	0.2379	0.0414	0.1608
	BBM	0.7047	0.2437	0.0368	0.1616
	BG11	0.8637	0.3277	0.0533	0.2180
	CONWAY	0.5116	0.1416	0.0938	0.1647
	GM F/2	0.5317	0.1522	0.0871	0.1606

Table 4.3 contd…

Culture	Medium	Dry Biomass ($g\ L^{-1}$)	Carbohydrates ($g\ L^{-1}$)	Lipid ($g\ L^{-1}$)	Protein ($g\ L^{-1}$)
BGLR10					
	ACM	0.4140	0.0900	0.0521	0.1605
	BBM	0.4552	0.1118	0.0178	0.1629
	BG11	0.5216	0.1469	0.0356	0.1607
	CONWAY	0.3063	0.0331	0.0366	0.1064
	GM F/2	0.4090	0.0874	0.0393	0.1603
BGLR11					
	ACM	0.4200	0.0932	0.0355	0.1611
	BBM	0.4512	0.1097	0.0236	0.1595
	BG11	0.4059	0.0858	0.0321	0.1603
	CONWAY	0.3898	0.0773	0.0761	0.1362
	GM F/2	0.3942	0.0795	0.0483	0.1496
BGLR12					
	ACM	0.7379	0.2613	0.0425	0.2750
	BBM	0.6776	0.2293	0.0301	0.2599
	BG11	0.6142	0.1958	0.0153	0.2620
	CONWAY	0.4713	0.1203	0.0389	0.1622
	GM F/2	0.5971	0.1868	0.0517	0.1591
BGLR13					
	ACM	0.8560	0.3237	0.057	0.2196
	BBM	0.6259	0.0846	0.089	0.1592
	BG11	0.3565	0.0596	0.061	0.1556
	CONWAY	0.4038	0.1320	0.078	0.1600
	GM F/2	0.4842	0.1272	0.080	0.1586
BGLR14					
	ACM	0.9653	0.3814	0.0780	0.2420
	BBM	0.7480	0.2666	0.0451	0.1666
	BG11	0.5518	0.1629	0.0500	0.1680
	CONWAY	0.3858	0.0751	0.0711	0.0818
	GM F/2	0.3758	0.0698	0.0476	0.0842
BGLR15					
	ACM	0.5505	0.1011	0.0851	0.1843
	BBM	0.4565	0.1125	0.0406	0.1639
	BG11	0.5786	0.1622	0.0556	0.1657
	CONWAY	0.4349	0.1370	0.0485	0.1270
	GM F/2	0.5965	0.1865	0.0598	0.1768

Table 4.3 contd…

Culture	Medium	Dry Biomass ($g\ L^{-1}$)	Carbohydrates ($g\ L^{-1}$)	Lipid ($g\ L^{-1}$)	Protein ($g\ L^{-1}$)
BGLR16					
	ACM	0.5562	0.1656	0.0504	0.1888
	BBM	0.3666	0.0646	0.0190	0.1323
	BG11	0.3774	0.0706	0.0374	0.1308
	CONWAY	0.3406	0.0507	0.0418	0.0974
	GM F/2	0.3420	0.0517	0.0171	0.1382
BGLR17					
	ACM	0.3531	0.0574	0.0381	0.0942
	BBM	0.3477	0.0396	0.0114	0.0947
	BG11	0.4119	0.0912	0.0318	0.0985
	CONWAY	0.3210	0.0538	0.0560	0.1190
	GM F/2	0.3298	0.0437	0.0199	0.0946
BGLR18					
	ACM	0.5214	0.1347	0.0517	0.1521
	BBM	0.6374	0.1517	0.0182	0.1642
	BG11	0.7067	0.2043	0.0515	0.2375
	CONWAY	0.5116	0.1858	0.0426	0.1396
	GM F/2	0.5086	0.1416	0.0461	0.1266
BGLR19					
	ACM	0.4579	0.1146	0.0844	0.1763
	BBM	0.4096	0.0626	0.0461	0.1375
	BG11	0.4237	0.0933	0.0781	0.1416
	CONWAY	0.3643	0.0886	0.0518	0.1144
	GM F/2	0.4187	0.0920	0.0568	0.1266
LSD ($p \leq 0.05$)		0.0111	0.0056	0.001	0.0036

Values are means; cultural conditions;Temperature (25 °C), light intensity (40 µmol m^{-2} s^{-1}) and photoperiod (12:12 light:dark); LSD ($p \leq 0.05$) was calculated for all the mean values

Table 4.4 Estimation of Chlorophyll after 35 days of growth period

Microalgal Isolates	Chl a (mgL^{-1})	Chl b (mgL^{-1})	Total Chlorophyll (mgL^{-1})	Carotenoids (mgL^{-1})
BGLR1	2.495^{ef}	12.450^{f}	14.123^{g}	0.175^{def}
BGLR2	$2.668d^{ef}$	5.681^{k}	7.465^{k}	0.326^{cdef}
BGLR3	3.884^{cdef}	13.798^{de}	16.395^{f}	0.084^{ef}
BGLR4	2.911^{def}	7.201^{j}	9.154^{j}	0.335^{cdef}
BGLR5	9.235^{a}	10.297^{g}	22.508^{c}	0.518^{c}
BGLR6	9.539^{a}	15.116^{c}	24.656^{b}	0.224^{def}
BGLR7	3.361^{cdef}	9.088^{i}	11.315^{i}	0.214^{cdef}
BGLR8	3.019^{cdef}	10.612^{gh}	12.644^{h}	0.255^{cdef}
BGLR9	4.322^{cde}	10.588^{h}	13.474^{gh}	0.246^{cdef}
BGLR10	1.785^{f}	9.989^{h}	11.172^{i}	0.381^{cde}
BGLR11	2.547^{ef}	5.037^{k}	6.730^{k}	0.097^{ef}
BGLR12	9.854^{a}	20.056^{a}	26.596^{a}	0.409^{cd}
BGLR13	5.233^{bc}	14.747^{d}	18.229e	0.141def
BGLR14	2.617^{ef}	5.531^{k}	7.284^{k}	0.363^{cdef}
BGLR15	3.348^{cdef}	8.670^{i}	10.900^{i}	0.256^{cdef}
BGLR16	4.970^{bcd}	13.578^{ef}	16.902^{f}	0.771^{b}
BGLR17	4.746^{bcde}	3.564^{l}	7.722^{k}	1.790^{a}
BGLR18	6.548^{b}	18.125^{b}	19.532^{d}	0.392^{cd}
BGLR19	2.949^{cdef}	5.743^{k}	7.718^{k}	0.155^{def}

Values are means;cultural conditions;Temperature (25 °C), light intensity (40 µmol m^{-2} s^{-1}) and photoperiod (12:12 light:dark);Values (Means) superscripted by different alphabets in the column differ significantly ($P<0.05$) from each other (Duncan's multiple range test)

Table 4.5 Biological parameters obtained from modified Logistic model; A is asymptote value; μ is growth rate; λ is lag time; R^2 is coefficient of determination; SSD is sum of squared deviations

Cultures	Biological paramters			Statistical parameters	
	A (g L^{-1})	μ (day^{-1})	λ (days)	R^2	SSD
BGLR1	2.6444	0.0129	1.1274	0.9640	0.0023
BGLR2	2.6652	0.0219	1.0000	0.9917	0.0093
BGLR3	2.7085	0.0120	6.6059	0.9966	0.0122
BGLR4	3.2677	0.0112	2.1911	0.9980	0.0007
BGLR5	3.8535	0.0222	0.1300	0.9952	0.0179
BGLR6	4.6444	0.0313	1.1274	0.9937	0.0292
BGLR7	2.8969	0.0123	0.5274	0.9971	0.0198
BGLR8	3.0102	0.0098	1.1107	0.9982	0.0069
BGLR9	2.9012	0.0103	1.1160	0.9979	0.0027
BGLR10	3.0785	0.0069	1.1127	0.9991	0.0019
BGLR11	2.9544	0.0179	1.0190	0.9947	0.0212
BGLR12	1.9444	0.0229	0.0574	0.9886	0.0780
BGLR13	3.6444	0.0213	1.5994	0.9949	0.0216
BGLR14	3.0584	0.0230	3.1213	0.9942	0.0736
BGLR15	2.9444	0.0129	1.2574	0.9969	0.0036
BGLR16	2.9944	0.0109	1.2174	0.9978	0.0017
BGLR17	3.3205	0.0064	1.5107	0.9993	0.0006
BGLR18	3.1519	0.0160	2.4464	0.9959	0.0008
BGLR19	3.0563	0.0067	2.1263	0.9991	0.0010

Cultural conditions;Temperature (25 °C), light intensity (40 μmol m^{-2} s^{-1}) and photoperiod (12:12 light:dark); Values are calculated for the media in which these cultures showed highest biomass production in screening experiment

4.4 Micronutrient analysis of potential isolates by ICP-AES

Some of the isolates like BGLR4, BGLR5, BGLR6, BGLR9, BGLR11, BGLR13, BGLR18 and BGLR19 were processed by the method of Mitra *et al* (2015) for the determination of the micronutrients like copper, calcium, iron, potassium, magnesium, phosphorus, manganese, lead, arsenic etc. This was done as these mineral elements have potential impact on the anaerobic digestion process besides to check the nutritive value of these isolates. It was observed that the heavy metals were not found in these and other elements like potassium, magnesium etc which affect the anaerobic process are within the limits (Table 4.6).

4.5 Molecular Identification of the potential isolates

Based on the screening experiment, it was found that the isolates BGLR5 and BGLR6 produced highest dry biomass and are having relatively higher content of other functional ingredients. So these two isolates were selected for further studies. The isolates were the identified at molecular level. The isolate BGLR5 on tentative morphological identification was found to be *Chlorella* sp. whereas BGLR6 was found to be *Spirulina* sp. So BGLR5 being eukaryotic in nature was identified through 18S rRNA sequencing while as BGLR6 was identified by 16s rRNA sequencing.

The identification of the isolate BGLR6 was confirmed through 16s rDNA sequencing. At a molecular level, the amplification, sequencing and bioinformatic analysis of the ~1.3kb 16s-rDNA fragment allowed to confirm the microscopic identification of the isolate. The 16s rRNA sequence analysis of this isolate and the phylogenetic tree construction (Fig. 4.3) showed 99% similarity with two *Spirulina* sequences (*S. Subsalsa* FACHB351 and *S. subsalsa*) available in the NCBI database. *Spirulina subsalsa* FACHB351 was found to be the most similar to the isolate. Hence, the BGLR6 isolate was identified as *Spirulina subsalsa* BGLR6. The 16s rDNA sequence was submitted to GenBank database under the accession number MF191711.

Similarly through 18s rDNA sequencing, the amplification sequencing and bioinformatic analysis of the ~2 kb 18s-rDNA fragment allowed to confirm the microscopic identification of the isolate. The 18s rRNA sequence analysis of this isolate and the phylogenetic tree construction (Fig. 4.4) showed 99% similarity with the sequences of *Scenedesmus* sp. and *Asterarcys quadricellulare* available in the NCBI database. *Asterarcys quadricellulare* was found to be the most similar to the isolate. Hence, the BGLR5 isolate was identified as *Asterarcys quadricellulare* BGLR5. The 18s rDNA sequence was submitted to GenBank database under the accession number MF661929. The gel images of extraction of genomic DNA from microalgal isolates (BGLR5, BGLR6) and further PCR amplification of 18s rDNA region from algal sample BGLR5 and 16s rDNA region from algal sample BGLR6 are shown in Figure 4.5 (a-d).

Table 4.6 Micronutrient analysis of potential isolates by ICP-AES

Micronutrients (%)	BGLR4	BGLR5	BGLR6	BGLR8	BGLR9	BGLR11	BGLR13	BGLR18	BGLR19
Arsenic	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Boron	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.08	0.02
Calcium	0.50	1.10	0.64	0.66	0.12	0.22	0.10	1.05	0.30
Cadmium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Chromium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.02	0.02
Iron	0.67	0.13	0.08	0.54	0.06	0.08	0.01	0.02	0.26
Potassium	2.16	1.00	0.59	0.41	0.22	0.45	0.65	0.24	0.65
Magnesium	0.35	0.30	0.98	0.00	0.00	0.12	1.56	0.33	0.00
Manganese	0.02	0.01	0.00	0.02	0.02	0.02	0.02	0.02	0.11
Nickel	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phosphorus	1.20	1.00	0.68	1.16	0.48	0.92	1.85	0.43	0.90
Lead	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sulphur	0.71	0.44	0.27	0.25	0.07	0.21	0.35	0.24	0.36
Zinc	0.01	0.01	0.01	0.10	0.00	0.01	0.01	0.01	0.01

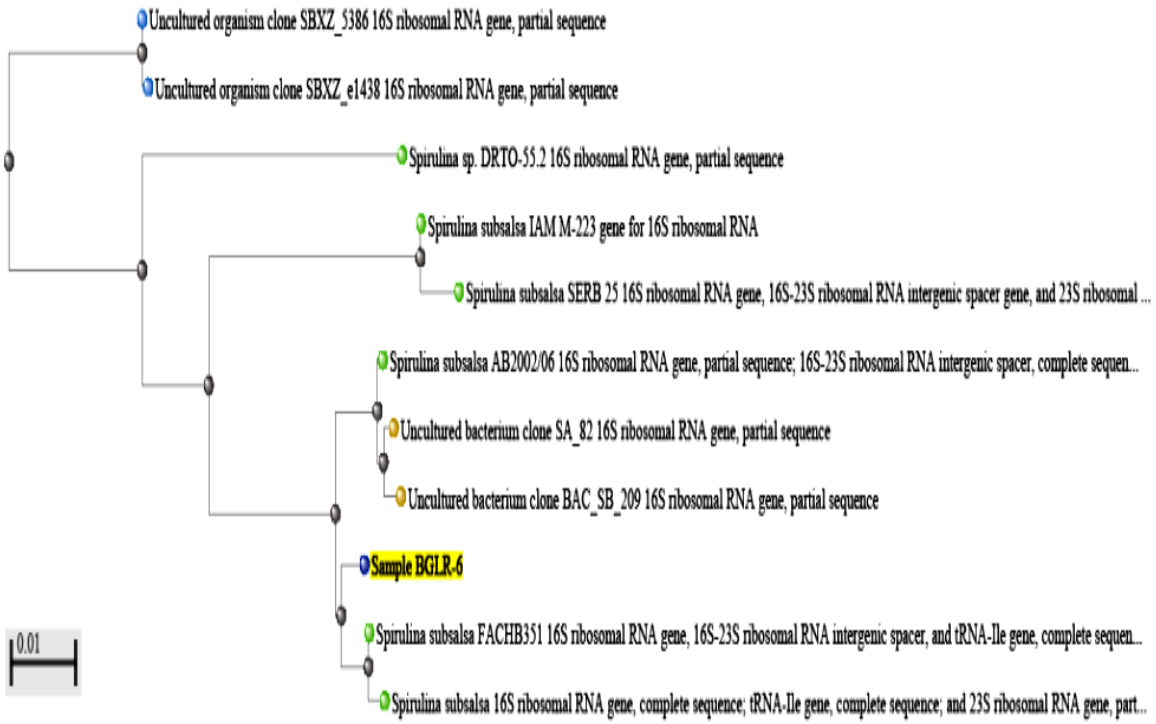


Fig. 4.3 Phylogenetic analysis of the 16S rDNA sequences (approximately 1190 bp) of BGLR6. The tree was constructed by Weighbor (a weighted version of Neighbor Joining that gives significantly less weight to the longer distances in the distance matrix) and 100 rounds of bootstrap resampling (Bruno *et al* 2000).

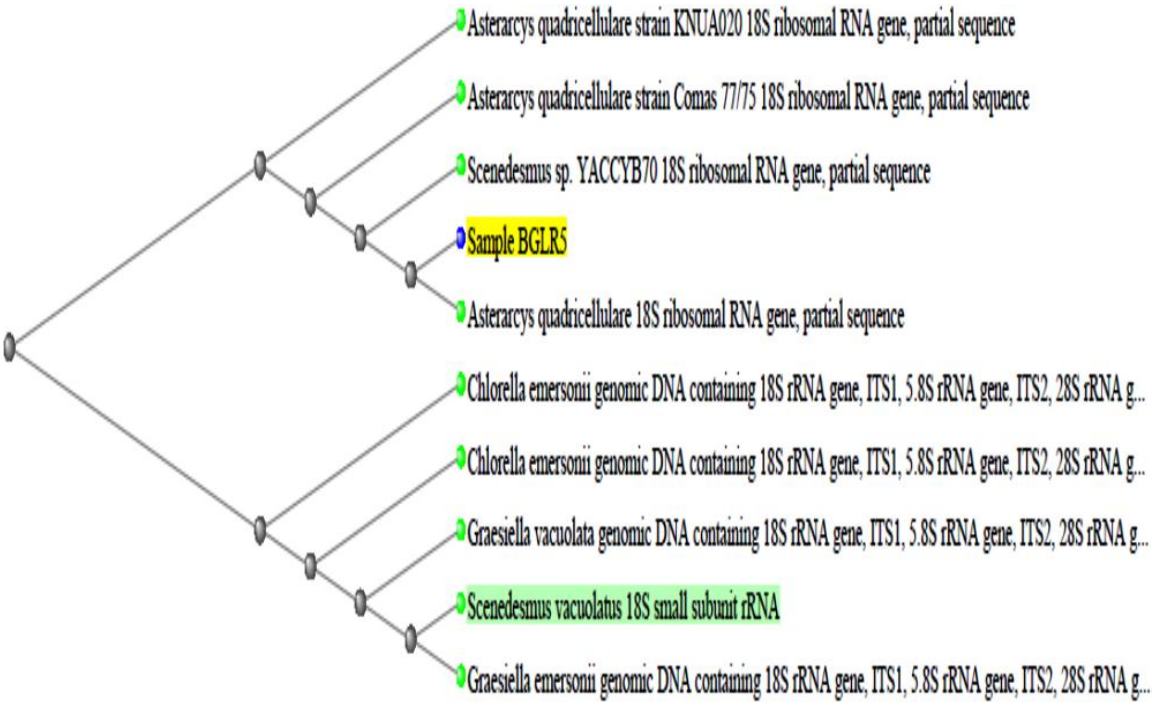
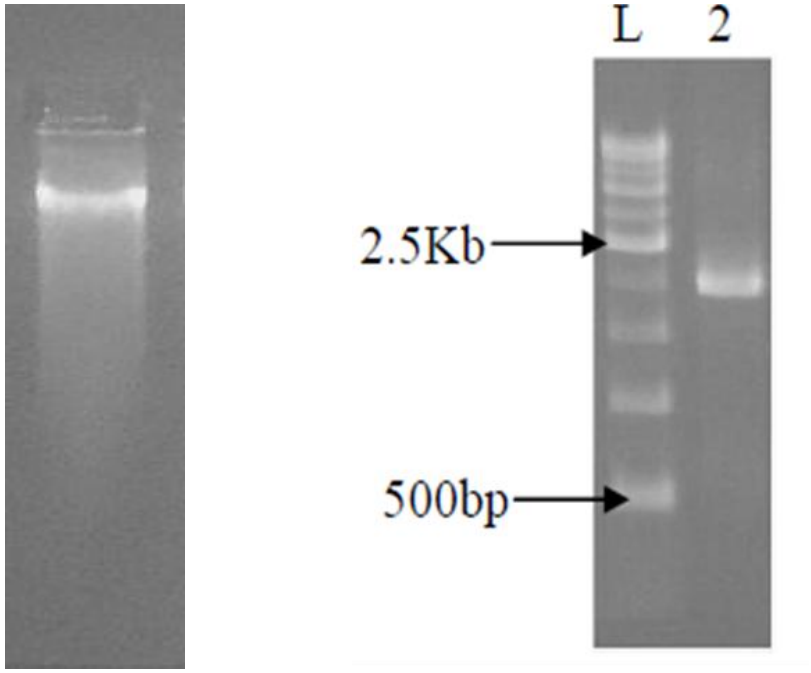
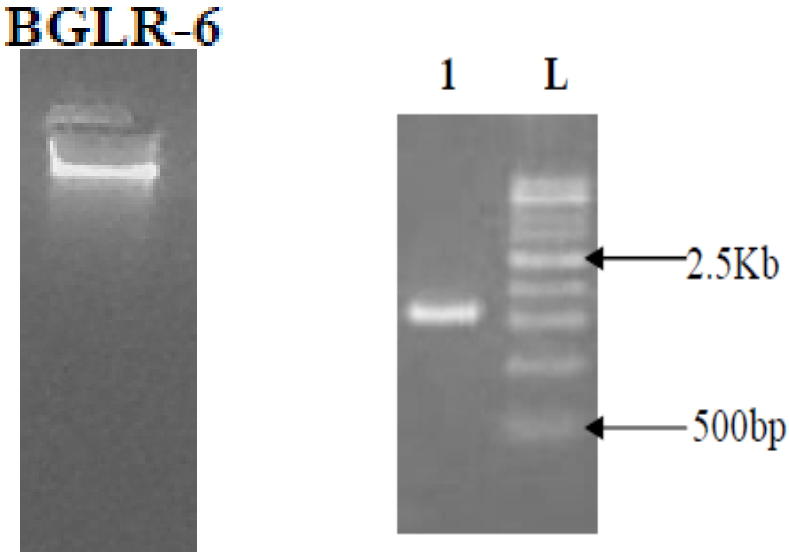


Fig.4.4 Phylogenetic analysis of the 18S rDNA sequences (approximately 1637 bp) of BGLR5. The tree was constructed by Weighbor (a weighted version of Neighbor Joining that gives significantly less weight to the longer distances in the distance matrix) and 100 rounds of bootstrap resampling (Bruno *et al* 2000).



(a) (b)



(c) (d)

Fig. 4.5 Gel images of extraction of genomic DNA from microalgal samples (a) BGLR5 and (c) BGLR6 using the Algal Genomic DNA Isolation Kit and (b) PCR amplification of 18s rDNA region from Algal sample BGLR5 and (d) PCR amplification of 16s rDNA region from Algal sample BGLR6. The size of PCR amplified product is ~ 2kb in case of BGLR5 and ~ 1.3 kb for BGLR6.

4.6 Optimization of cultural factors of *Spirulina subsalsa* BGLR6 and *Asterarcys quadricellulare* BGLR5

4.6.1 Evaluation of significant factors by Placket-Burman design for *Spirulina subsalsa* BGLR6

The Placket-Burman design was applied to comprehensively analyze the impact of seven different factors on the various responses (Table 4.8) in *Spirulina subsalsa* BGLR6. The experimental data given in Table 4.7, were calculated by Statgraphics Centurion XVII software. Each response variable was the average of the triplicates. The results of variance analysis and estimation of parameters are given in Table 4.8. The evaluation of significance of factors was determined on the basis of p-value. As per the statistical rule, if the p-value of the factor was less than 5%, it means that the factor is having significant effect on the response value. The factors with p-value <0.05 were selected for further optimization studies. The variables having larger F-ratio and smaller p value are regarded more significant ($p<0.05$) (Montgomery 2005). It is quite evident from the Table 4.8 that all the factors have low p value <0.05, thus are all important and significant but the pH is having larger F value, smaller p value followed by light intensity in all the responses, making these two factors very important in determining the responses. Hence, all these factors were selected for optimization studies by CCD of RSM. The factors could further be assessed by coefficient estimate. The factors having positive coefficient of estimate will have positive effect on the response variable and those having negative are negatively correlated with the response (Jiang *et al* 2013). As shown in Table 4.8, temperature, $CaCl_2$ and K_2HPO_4 had a negative effect on all the responses whereas others are having positive impact on the responses considered in the current study. There negative impact varies from response to response as is evident from their p value (Table 4.8). Lower value of these will favour the increased production of the responses. Our results are in line to that of the Yang *et al* (2014) who too observed that the higher value of the phosphate source reduced the growth i.e. biomass and lipid production in *Scenedesmus* sp. Similarly it was observed that the nitrogen source $NaNO_3$ had a positive effect on the responses like biomass, lipid, carbohydrate, protein. This is also in harmony to that of the Yang *et al* 2014 who too observed it having positive effect on the lipid production. Though usually it is found that the nitrogen stress leads to the accumulation of lipids (Mandal and Mallick 2009, Welter *et al* 2013). The possible reason for this could be that the concentration of $NaNO_3$ did not reach the limiting level for *Spirulina Subsalsa* BGLR6 as it may not have got depleted during this period of time. The Paretro charts obtained also describe the order of significance of the variables affecting the various response variables in the Plackett-Burman design (Fig. 4.6 a-e).

The adequacy of the statistical model was checked by calculating the R^2, Adjusted R^2 and P-value. The R^2, adjusted R^2 and P-value for all the responses were given in the Table 4.9. In all cases, the R^2 and adjusted R^2 are greater than 95% indicating that the model explained the data well as it was fit to it and reasonable. In short, all the factors were having significant impact on the response variables. Therefore, these all were selected to make further optimization by the Central Composite design.

4.6.2 Optimization of physicochemical factors by RSM using CCD

The CCD of the RSM was aimed at identifying the optimum levels of the selected seven factors obtained from Placket-Burman design viz. pH, temperature, light intensity, growth period, $CaCl_2$, $NaNO_3$ and K_2HPO_4 at different levels (Table 4.10) on the response variables, "biomass, chlorophyll, carbohydrate, lipid and protein". The 39 experimental combinations were generated using the CCD for above seven factors. The experimental design, experimental values, observed desirability and predicted desirability are presented in the Table 4.11.

The results achieved in CCD were examined by standard analysis of variance (ANOVA), and the relationship between the response and the independent factors was described by the second order polynomial equation. Analysis of variance was carried out in order to make sure that the model is rational and logical as explained in Table 4.12. The analysis of variance (ANOVA) and analysis of the experimental results (Table 4.12) indicated that the developed model for each measured response had a good lack of fit. The ANOVA table segregates the variation in each response variable into distinct sections for each of the effects. It then compares the mean square against an estimate of the experimental error to analyse the statistical significance of each effect. The number of effects as shown in Table 4.12, having p-value less than 0.05 in biomass, chlorophyll, carbohydrate, lipid and protein are 12, 12, 19, 11 and 13 respectively, indicating that they are significantly different from zero at the 95.0% confidence level. ANOVA for the seven variables indicated that the production of five responses under study could be described well by CCD model with relatively high coefficient of determination. The effect of independent variables pH, temperature, light intensity, growth period, $CaCl_2$, $NaNO_3$ and K_2HPO_4 described in Table 4.12 were significant as their p value were less than 0.05. The significance of the interactions varied from response to response variable. The linear terms were significant model terms in all the cases. For biomass interactive (AB, AF, DE and FG) and quadratic (C^2) were significant model terms. The AA, BC, BE, BF and DE interactive terms are significant model terms for chlorophyll. In lipid the interactive (BF and BG) and quadratic terms (C^2 and G^2)

acted as significant effects whereas for carbohydrate, the interactive (AC, AG, BC, BE, CD, CE, CF, CG, DE and DG) and quadratic (C^2) were significant model terms. Similarly for protein the significant model terms were linear (AE, BE, CE and FG) and quadratic (A^2 and C^2).

The R-Squared statistic indicated that the model as fitted explained 80.14, 85.09, 94.59, 76.82 and 84.83% of the variability in the response variables biomass, chlorophyll, carbohydrate, lipid and protein respectively. This gives very good correlation between input variables and their responses, indicating that the model is correlated well with the measured data and was statistically significant. The closer the R^2 value to 1, the better the prediction of the model (Kim *et al* 2012). The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, was 70.98, 78.20, 89.18, 67.38 and 76.94% respectively as clearly presented in Table 4.13. Since the P-value is greater than 5.0%, there is no indication of serial autocorrelation in the residuals at the 5.0% significance level. Further, the observed and predicted values were utilized to examine the homogeneous variance assumption by plotting the predicted values against the observed values. The data was observed to be homogeneously spreaded around the line of fit, indicating the suitability of the model in our study (Fig. 4.7a-e).

The final regression equations for the biomass production, chlorophyll, carbohydrate, lipid and protein, which have been fitted to the data, are as follows:

Biomass = 1.593 + 0.121*A + 0.041*B - 0.0576*C + 0.070*D + 0.019*E- 0.073*F - 0.074*G - 0.006*A*B + 0.007*A*F + 0.001*C^2 - 0.002*D*E + 0.004*F*G

Chlorophyll = 1.698 - 0.052*A - 0.037*B + 0.051*C + 0.135*D - 0.024*E-0.183*F - 0.059*G + 0.011*A*F - 0.002*B*C + 0.002*B*E + 0.004*B*F - 0.004*D*E

Carbohydrates = 0.310 + 0.083*A - 0.009*B - 0.024*C + 0.011*D -0.001*E + 0.007*F + 0.230*G - 0.001*A*C - 0.007*A*G-0.0002*B*C + 0.001*B*E + 0.0004*C^2+0.0002*C*D-0.0001*C*E-0.0002*C*F- 0.0004*C*G - 0.001*D*E + 0.001*D*G - 0.017*G^2

Lipid = 0.415 - 0.014*A - 0.012*B + 0.022*C - 0.002*D -0.003*E -0.017*F- 0.133*G +0.001*B*F + 0.001*B*G - 0.0002*C^2 + 0.010*G^2

Protein = -0.458 + 0.641*A - 0.022*B - 0.039*C + 0.010*D-0.041*E-0.007*F-0.038*G-0.036*A^2+0.003*A*E + 0.001*B*E + 0.0004*C^2 - 0.0002*C*E + 0.002*F*G

Where, A: pH, C: Light intensity, D: Growth period, E: $CaCl_2$, F: $NaNO_3$ and G: K_2HPO_4

Thus according to the obtained results and the equations (5-9), pH of 10.57, temperature of 20°C, the light intensity of 80.99 μmol m^{-2} s^{-1}, 24.99 days of the growth period, 15.00 mM $CaCl_2$, 5.00 mM $NaNO_3$ and 2.00 mM of K_2HPO_4 were the optimal and most desired conditions as per the model (Table 4.14), for different responses considered for this study. At these settings, the response variables generated a desirability index of 84.10%. These optimal values were very much close to the run 24 of CCD, which on the basis of observed and predicted desirability from the fitted model, is the most desirable run in terms of results obtained. Particularly, the biomass as per run 24 (2.559 g L^{-1}) and optimal values obtained from model (2.319 g L^{-1}) were found to have increased by 1.35 and 1.13 folds respectively, than the basal medium conditions (1.089 g L^{-1}). Similarly the content of carbohydrates was also found to have increased. Interestingly, this is of quite significance as more the substrate rich in carbohydrates, more it is favourable for anaerobic digestion and fermentation (John *et al* 2011).

4.6.3 3D Response surface plots for various interactions

The response surface graphs were plotted to demonstrate the interaction between different factors and their impact on the desired response. These plots are used to determine the sensitivity of two interacting variables by keeping the other factors at central values. So these graphs are quite interesting and helpful in understanding the interaction effects of the two factors. The response surface 3D plots with contours are shown in Fig. 4.8(a-d). The interactions among different factors can be easily described from these plots because, generally, the more elliptical or ovate the shape of the contour, the better is the interaction among the factors whilst circular shape specifies least interaction between the factors. The various significant interactions between different factors for biomass are elucidated below because for anaerobic digestion biomass is needed. So therefore, only the significant interactions of biomass are discussed here.

4.6.3.1 pH versus temperature interaction

The interaction between pH and temperature (AB) was found significant as is obvious from Table 4.12 and from Fig. 4.8(a). It is clearly depicted in the response plot and contours below that high pH and low temperature has positive effect on biomass production provided other factors are kept at central level. The contour lines plotted below on the base of surface plots in Fig. 4.8(a), are curvilinear revealing good interaction between the variables. The F value and the p value for this interaction are 5.58 and 0.0260 respectively in our case, thus proving that this interaction is quite significant as per Montgomery (2005).

4.6.3.2 pH versus $NaNO_3$ interaction

pH and $NaNO_3$ interaction was significant as it has p value 0.0079. The maximum biomass can be produced as the $NaNO_3$ concentration is increased while decrease or increase

in pH is not found to have any positive or negative effect on the biomass production as shown in Fig. 4.8(b). The biomass increase is from 1.3 to 1.8 g L^{-1} as the $NaNO_3$ level rises from 11 to 20 mM. This shows more the nitrogen source available in the medium more will be growth of *Spirulina sabsalsa* BGLR6.

4.6.3.3 Growth period versus $CaCl_2$

The 3D surface plot with contours at the base for growth period versus $CaCl_2$ (Fig. 4.8(c)) clearly illustrates that $CaCl_2$ at low level increases the biomass genesis while increasing the growth period has positive impact on biomass genesis. This means that increased growth period and lower $CaCl_2$ concentrations contribute to high yields of biomass collectively. The greater impact can be found at 30-40 days growth period and $CaCl_2$ concentration ranging from 23-27 mM $CaCl_2$.

4.6.3.4 $NaNO_3$ versus K_2HPO_4

Fig. 4.8(d) illustrates the interaction between these two factors. Singly, $NaNO_3$ factor is having positive impact whereas K_2HPO_4 is having negative impact but collectively they are having positive effect on the biomass production. The p value for this interaction is 0.029 signifying that it is quite significant. Biomass increased from 1-1.4 g L^{-1} when $NaNO_3$ concentration raises from 0-20 mM, thereafter remains same and also when concentration of K_2HPO_4 is increased from 3-8 mM K_2HPO_4 concentration.

4.6.4 Validation or confirmation of the model

The validity of the model needed to be checked to establish its significance. So, we conducted validation experiment of the statistical model by cultivation of *Spirulina subsalsa* BGLR6 under optimal conditions (pH of 11.5, 20°C of temperature, the light intensity of 81 μmol m^{-2} s^{-1}, 25 days of the growth period, 15 mM $CaCl_2$, $NaNO_3$ of 5 mM and 2 mM of K_2HPO_4) as suggested by the model in the ACM. The experimental value for different responses (biomass, chlorophyll, carbohydrate, lipid and protein) was found to be 2.309 gL^{-1}, 4.087 mgL^{-1}, 0.743 gL^{-1}, 0.279 gL^{-1} and 0.883 gL^{-1} respectively. These values are close to that obtained from CCD of RSM. This close relationship of the values obtained from validation experiment to that of the RSM values and model predicted response values at optimum, revealed the confirmation, validity and acceptability of the model for the optimization of different physico-chemical factors discussed in this study.

Table 4.7 Experimental design and results of *Spirulina Sabsalsa* BGLR6 in Placket Burman Design

Run	pH	Temp. (°C)	Light Intensity ($\mu molm^{-2}s^{-1}$)	Growth Period (days)	$CaCl_2$ (mM)	$NaNO_3$ (mM)	K_2HPO_4 (mM)	Biomass* (gL^{-1})	Chlorophyll (mgL^{-1})	Carbohydrate (gL^{-1})	Lipid (gL^{-1})	Protein (gL^{-1})	Observed Desirability	Predicted Desirability
1	10.5 (+1)	35 (+1)	40.5 (-1)	25 (+1)	20 (-1)	5 (-1)	2 (-1)	1.7957	3.8	0.5763	0.3720	0.7530	0.5873	0.5862
2	10.5 (+1)	25 (-1)	81 (+1)	15 (-1)	20 (-1)	5 (-1)	8 (+1)	2.2226	4.5	0.6303	0.5970	0.9730	0.6839	0.6922
3	10.5 (+1)	35 (+1)	40.5 (-1)	25 (+1)	35 (+1)	5 (-1)	8 (+1)	0.7699	1.5	0.3267	0.1050	0.2880	0.3767	0.3777
4	7.5 (-1)	25 (-1)	40.5 (-1)	15 (-1)	20 (-1)	5 (-1)	2 (-1)	0.5558	1.9	0.2175	0.1720	0.1760	0.3170	0.3243
5	7.5 (-1)	35 (+1)	40.5 (-1)	15 (-1)	20 (-1)	20 (+1)	8 (+1)	0.3102	1.3	0.1275	0.0330	0.1100	0.2679	0.2419
6	7.5 (-1)	25 (-1)	81 (+1)	25 (+1)	35 (+1)	5 (-1)	8 (+1)	1.0823	2.5	0.354	0.1980	0.4790	0.3492	0.3413
7	7.5 (-1)	35 (+1)	81 (+1)	25 (+1)	20 (-1)	20 (+1)	8 (+1)	1.3266	2.9	0.3933	0.3760	0.5460	0.4498	0.4703
8	10.5 (+1)	25 (-1)	40.5 (-1)	15 (-1)	35 (+1)	20 (+1)	8 (+1)	1.1096	2.8	0.3585	0.1970	0.460	0.4769	0.4889
9	10.5 (+1)	35 (+1)	81 (+1)	15 (-1)	35 (+1)	20 (+1)	2 (-1)	2.1117	4.6	0.5865	0.5510	0.8540	0.7079	0.7003
10	7.5 (-1)	35 (+1)	81 (+1)	15 (+1)	35 (+1)	5 (-1)	2 (-1)	0.4721	1.1	0.1869	0.1180	0.1160	0.3188	0.3277
11	10.5 (+1)	25 (-1)	81 (+1)	25 (+1)	20 (-1)	20 (+1)	2 (-1)	3.7431	7.6	0.8853	0.9310	1.5160	0.9422	0.9288
12	7.5 (-1)	25 (-1)	40.5 (-1)	25 (+1)	35 (+1)	20 (+1)	2 (-1)	0.7739	2.4	0.3102	0.0690	0.3340	0.3547	0.3633

* It represents dry biomass

Table 4.8 Statistical analysis of Placket Burman Design

Source	Levels		Sum of Squares	DF[a]	Mean Square	Coefficient of Estimate	F-Ratio	[b]P-Value
	-1 level	+1 level						
Biomass								
A:pH	7.5	10.5	4.3581	1	4.3581	1.20528	137.12	0.0003
B:Temperature	25	35	0.6080	1	0.6080	-0.450183	19.13	0.0119
C:Light Intensity	40.5	81	2.6539	1	2.6539	0.94055	83.5	0.0008
D:Growth Period	15	25	0.6118	1	0.6118	0.451583	19.25	0.0118
E:$CaCl_2$	20	35	1.1008	1	1.1008	-0.60575	34.63	0.0042
F:$NaNO_3$	5	20	0.5112	1	0.5112	0.412783	16.08	0.0160
G:$K2HPO_4$	2	8	0.5769	1	0.5769	-0.438517	18.15	0.0131
Total error			0.1271	4	0.0318			
Total (corr.)			10.5478	11				
Chlorophyll								
A:pH	7.5	10.5	1.3441E-05	1	0.0000	0.00211667	92.17	0.0007
B:Temperature	25	35	3.5208E-06	1	0.0000	-0.00108333	24.14	0.0080
C:Light Intensity	40.5	81	7.5208E-06	1	0.0000	0.00158333	51.57	0.0020
D:Growth Period	15	25	1.6875E-06	1	0.0000	0.00075	11.57	0.0272
E:$CaCl_2$	20	35	4.2008E-06	1	0.0000	-0.00118333	28.81	0.0058
F:$NaNO_3$	5	20	3.3075E-06	1	0.0000	0.00105	22.68	0.0089
G:$K2HPO_4$	2	8	2.9008E-06	1	0.0000	-0.000983333	19.89	0.0112
Total error			5.8333E-07	4	0.0000			
Total (corr.)			3.7163E-05	11				
Carbohydrate								
A:pH	7.5	10.5	0.2623	1	0.2623	0.2957	253.73	0.0001
B:Temperature	25	35	0.0260	1	0.0260	-0.0931	25.15	0.0074
C:Light Intensity	40.5	81	0.1045	1	0.1045	0.1866	101.04	0.0006
D:Growth Period	15	25	0.0455	1	0.0455	0.1231	43.97	0.0027
E:$CaCl_2$	20	35	0.0417	1	0.0417	-0.1179	40.34	0.0031
F:$NaNO_3$	5	20	0.0114	1	0.0114	0.0616	11.01	0.0294
G:$K2HPO_4$	2	8	0.0273	1	0.0273	-0.0954	26.41	0.0068
Total error			0.0041	4	0.0010			
Total (corr.)			0.5228	11				

Source	Levels		Sum of Squares	DF[a]	Mean Square	Coefficient of Estimate	F-Ratio	[b]P-Value
	-1 level	+1 level						
Lipid								
A:Ph	7.5	10.5	0.2661	1	0.2661	0.297833	170.38	0.0002
B:Temperature	25	35	0.0309	1	0.0309	-0.1015	19.79	0.0113
C:Light Intensity	40.5	81	0.2769	1	0.2769	0.303833	177.31	0.0002
D:Growth Period	15	25	0.0122	1	0.0122	0.0638333	7.83	0.0489
E:$CaCl_2$	20	35	0.1288	1	0.1288	-0.207167	82.43	0.0008
F:$NaNO_3$	5	20	0.0295	1	0.0295	0.0991667	18.89	0.0122
G:$K2HPO_4$	2	8	0.0417	1	0.0417	-0.117833	26.67	0.0067
Total error			0.0062	4	0.0016			
Total (corr.)			0.7923	11				
Protein								
A:pH	7.5	10.5	0.7921	1	0.7921	0.513833	129.84	0.0003
B:Temperature	25	35	0.1346	1	0.1346	-0.211833	22.07	0.0093
C:Light Intensity	40.5	81	0.4653	1	0.4653	0.393833	76.28	0.0009
D:Growth Period	15	25	0.1255	1	0.1255	0.2045	20.57	0.0105
E:$CaCl_2$	20	35	0.1984	1	0.1984	-0.257167	32.52	0.0047
F:$NaNO_3$	5	20	0.0893	1	0.0893	0.1725	14.63	0.0187
G:$K2HPO_4$	2	8	0.0665	1	0.0665	-0.148833	10.89	0.0299
Total error			0.0244	4	0.0061			
Total (corr.)			1.8960	11				

[a] **DF is Degrees of Freedom;** [b] Significance level at a p-value ≤ 0.05

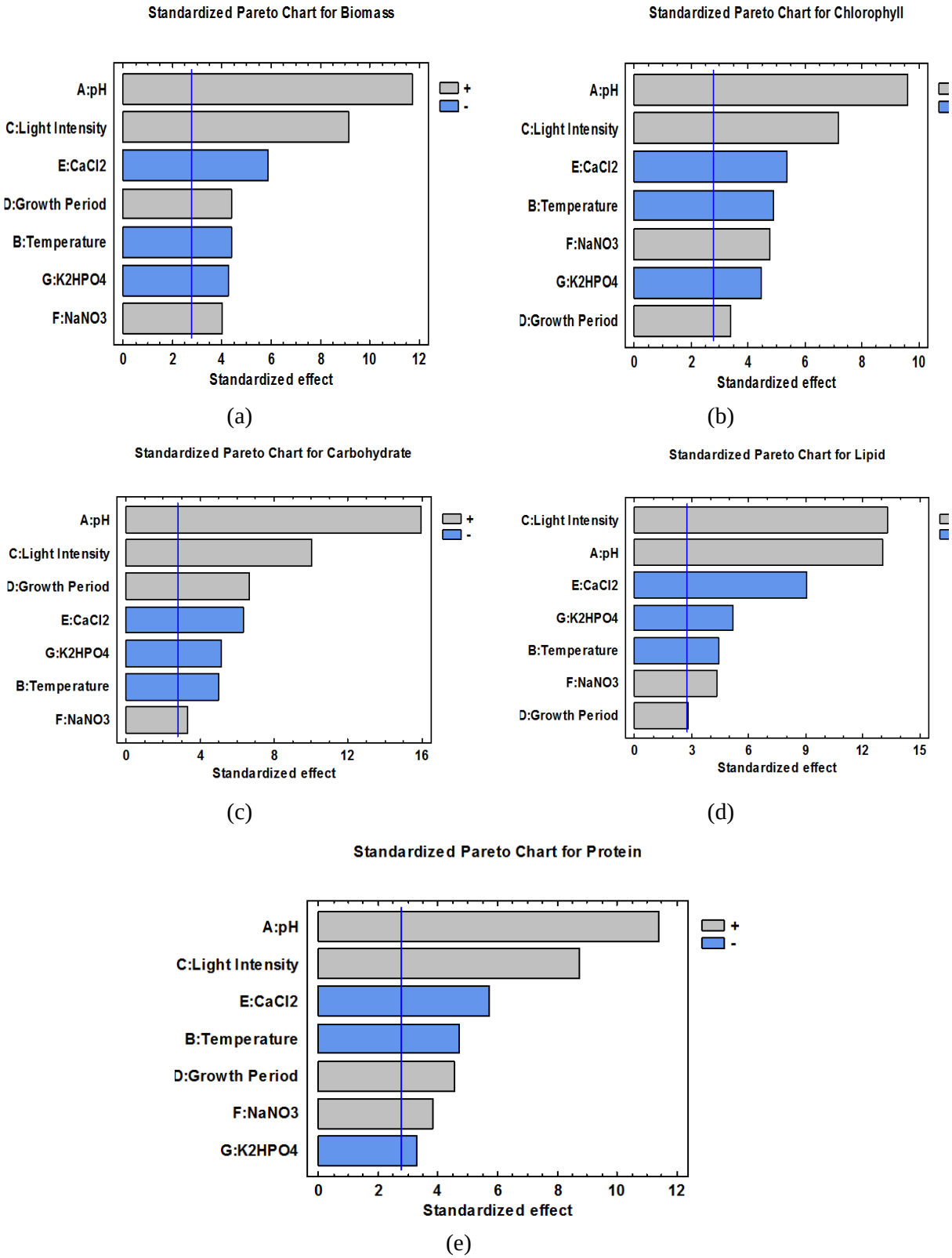


Fig.4.6 (a-e) Paretro charts of the various response variables for BGLR6 (a) Biomass, (b) Chlorophyll, (c) Carobhydrate, (d) Lipid and (e) Protein

Table 4.9 Analysis of the experimental results of Placket Burman Design

Model	Biomass	Chlorophyll	Carbohydrate	Lipid	Protein
Model DF	7	7	7	7	7
P-value	0.0011	0.0019	0.0005	0.0005	0.0013
Error DF	4	4	4	4	4
Stnd. Error	0.1783	0.0004	0.0322	0.0395	0.0781
R-squared	0.9879	0.9843	0.9921	0.9921	0.9871
Adj. R-squared	0.9669	0.9568	0.9782	0.9783	.0.9646

DF is degrees of freedom

Table 4.10 Different factors used in the study with their levels

Name	Units	Low	High	Levels
A:pH		7.5	11.5	-1, 0, +1
B:Temperature	°C	20.0	35.0	-1,0,+1
C:Light intensity	$\mu molm^{-2}s^{-1}$	40.5	81.0	-1,0,+1
D:Growth period	Days	10.0	25.0	-1,0,+1
E:$CaCl_2$	mM	15.0	35.0	-1,0,+1
F:$NaNO_3$	mM	5.0	20.0	-1,0,+1
G:K_2HPO_4	mM	2.0	8.0	-1,0,+1

Where -1, represents lowest level; 0, represents middle level and +1 represents highest level

Table 4.11 Central Composite Design (CCD) and response values for different responses

Run	pH	Temperature (°C)	Light intensity ($\mu molm^{-2}s^{-1}$)	Growth period (days)	$CaCl_2$ (mM)	$NaNO_3$ (mM)	K_2HPO_4 (mM)	Biomass (gL^{-1})	Chlorophyll (mgL^{-1})	Carbohydrates (gL^{-1})	Lipid (gL^{-1})	Protein (gL^{-1})	Observed Desirability	Predicted Desirability
1	7.5	20.0	81.0	10.0	35.0	20.0	2.0	1.637	1.836	0.352	0.275	0.492	0.355	0.320
2	11.5	20.0	40.5	10.0	35.0	20.0	2.0	1.484	2.362	0.419	0.16	0.611	0.379	0.385
3	7.5	20.0	40.5	10.0	15.0	20.0	2.0	1.183	1.384	0.282	0.175	0.553	0.268	0.369
4	9.5	27.5	81.0	18.0	25.0	12.5	5.0	1.375	2.202	0.597	0.134	0.831	0.386	0.440
5	7.5	35.0	40.5	10.0	35.0	20.0	2.0	1.281	2.087	0.372	0.162	0.422	0.259	0.290
6	7.5	35.0	40.5	25.0	35.0	20.0	8.0	1.211	1.573	0.340	0.073	0.589	0.234	0.258
7	9.5	20.0	60.75	18.0	25.0	12.5	5.0	1.382	2.03	0.418	0.182	0.639	0.346	0.423
8	11.5	20.0	81.0	25.0	15.0	20.0	8.0	2.843	4.172	0.541	0.168	1.087	0.756	0.642
9	11.5	35.0	40.5	10.0	15.0	20.0	2.0	1.256	2.168	0.360	0.201	0.523	0.368	0.362
10	11.5	35.0	81.0	10.0	35.0	20.0	2.0	1.657	2.297	0.298	0.186	0.670	0.393	0.379
11	11.5	35.0	40.5	10.0	35.0	5.0	2.0	1.295	2.056	0.442	0.173	0.691	0.366	0.315
12	7.5	35.0	40.5	10.0	15.0	20.0	8.0	1.336	1.458	0.219	0.31	0.501	0.263	0.246
13	7.5	35.0	81.0	25.0	35.0	20.0	2.0	1.631	1.849	0.349	0.173	0.546	0.346	0.350
14	7.5	35.0	40.5	10.0	15.0	5.0	2.0	1.391	1.75	0.234	0.275	0.653	0.305	0.271
15	9.5	27.5	60.75	18.0	35.0	12.5	5.0	1.344	2.165	0.333	0.160	0.600	0.354	0.292
16	7.5	35.0	81.0	10.0	15.0	5.0	8.0	1.225	1.35	0.309	0.234	0.563	0.254	0.000
17	7.5	20.0	40.5	25.0	35.0	20.0	2.0	1.196	1.469	0.242	0.209	0.580	0.282	0.230
18	11.5	35.0	81.0	25.0	15.0	5.0	8.0	1.338	1.521	0.352	0.150	0.572	0.275	0.392
19	11.5	20.0	81.0	25.0	35.0	5.0	8.0	1.399	2.269	0.380	0.181	0.594	0.384	0.375
20	7.5	20.0	81.0	25.0	35.0	20.0	8.0	1.155	1.382	0.314	0.125	0.557	0.249	0.327
21	11.5	35.0	40.5	25.0	35.0	20.0	2.0	1.448	2.863	0.361	0.101	0.910	0.408	0.382
22	11.5	27.5	60.75	18.0	25.0	12.5	5.0	1.427	1.718	0.347	0.146	0.553	0.324	0.362
23	7.5	35.0	40.5	25.0	35.0	5.0	2.0	1.213	1.293	0.280	0.141	0.632	0.222	0.280
24	11.5	20.0	81.0	25.0	15.0	5.0	2.0	2.559	4.546	0.822	0.328	0.973	0.876	0.823
25	7.5	35.0	40.5	10.0	35.0	5.0	8.0	1.106	1.015	0.292	0.227	0.420	0.175	0.154
26	7.5	35.0	81.0	10.0	35.0	5.0	2.0	1.351	1.501	0.358	0.259	0.507	0.281	0.274
27	9.5	27.5	60.75	10.0	25.0	12.5	5.0	1.228	1.52	0.347	0.190	0.572	0.292	0.307
28	11.5	35.0	40.5	10.0	35.0	20.0	8.0	1.595	2.595	0.256	0.055	0.656	0.280	0.327
29	11.5	20.0	81.0	10.0	35.0	5.0	2.0	1.623	2.598	0.436	0.363	0.626	0.459	0.473
30	11.5	20.0	40.5	25.0	15.0	5.0	8.0	1.856	2.997	0.543	0.215	0.696	0.465	0.478
31	7.5	35.0	81.0	10.0	35.0	20.0	8.0	1.164	1.443	0.202	0.209	0.523	0.231	0.225
32	7.5	35.0	81.0	10.0	15.0	20.0	2.0	1.438	2.021	0.290	0.290	0.533	0.333	0.317
33	9.5	27.5	60.75	18.0	25.0	20.0	5.0	1.509	2.676	0.325	0.190	0.776	0.377	0.380
34	7.5	20.0	81.0	25.0	15.0	5.0	8.0	2.05	3.96	0.947	0.298	0.925	0.758	0.678
35	7.5	20.0	40.5	10.0	35.0	20.0	8.0	1.181	1.46	0.326	0.108	0.504	0.207	0.149
36	11.5	20.0	81.0	10.0	15.0	5.0	8.0	1.52	2.413	0.310	0.206	0.616	0.375	0.438
37	7.5	35.0	40.5	25.0	15.0	20.0	2.0	1.544	2.69	0.359	0.244	0.698	0.406	0.432
38	7.5	20.0	40.5	10.0	35.0	5.0	2.0	1.461	1.316	0.294	0.338	0.405	0.257	0.274
39	9.5	27.5	60.75	18.0	25.0	12.5	8.0	1.1	1.915	0.226	0.244	0.588	0.307	0.318

Table 4.12 Analysis of variance (ANOVA) for the quadratic model of various physicochemical factors of *Spirulina Sabsala* BGLR6 for different responses

Source	Sum of Squares	DF	Mean Square	F-Ratio	P-Value
Biomass					
A:pH	0.25174	1	0.25174	6.79	0.0150
B:Temperature	0.366367	1	0.366367	9.88	0.0041
C:Light intensity	0.276509	1	0.276509	7.46	0.0112
D:Growth period	0.573297	1	0.573297	15.46	0.0006
E:$CaCl_2$	0.696907	1	0.696907	18.79	0.0002
F:$NaNO_3$	0.29786	1	0.29786	8.03	0.0088
G:K_2HPO_4	0.189761	1	0.189761	5.12	0.0323
AB	0.206852	1	0.206852	5.58	0.0260
AF	0.307277	1	0.307277	8.29	0.0079
CC	0.210689	1	0.210689	5.68	0.0247
DE	0.609592	1	0.609592	16.44	0.0004
FG	0.19774	1	0.19774	5.33	0.0291
Total error	0.964158	26	0.037083		
Total (corr.)	4.85501	38			
Chlorophyll					
A:pH	0.868126	1	0.868126	6.31	0.0186
B:Temperature	1.63965	1	1.63965	11.91	0.0019
C:Light intensity	0.660298	1	0.660298	4.8	0.0377
D:Growth period	1.81114	1	1.81114	13.16	0.0012
E:$CaCl_2$	3.2633	1	3.2633	23.71	<0.0001
F:$NaNO_3$	0.781569	1	0.781569	5.68	0.0248
G:K_2HPO_4	0.925143	1	0.925143	6.72	0.0154
AF	0.757523	1	0.757523	5.5	0.0269
BC	1.56849	1	1.56849	11.4	0.0023
BE	0.701452	1	0.701452	5.1	0.0326
BF	1.01443	1	1.01443	7.37	0.0116
DE	2.33469	1	2.33469	16.96	0.0003
Total error	3.57808	26	0.137619		
Total (corr.)	23.9908	38			
Lipid					
A:pH	0.021851	1	0.021851	13	0.0012
B:Temperature	0.011018	1	0.011018	6.56	0.0164
C:Light intensity	0.0114095	1	0.0114095	6.79	0.0147
D:Growth period	0.00880222	1	0.00880222	5.24	0.0301
E:$CaCl_2$	0.0211281	1	0.0211281	12.57	0.0015
F:$NaNO_3$	0.0298075	1	0.0298075	17.74	0.0003
G:K_2HPO_4	0.0311517	1	0.0311517	18.54	0.0002
BF	0.0181048	1	0.0181048	10.77	0.0028
BG	0.00705996	1	0.00705996	4.2	0.0502
CC	0.00873061	1	0.00873061	5.2	0.0308
GG	0.0146423	1	0.0146423	8.71	0.0065
Total error	0.0453672	27	0.00168027		
Total (corr.)	0.195756	38			

Table 4.12 contd...

Source	Sum of Squares	DF	Mean Square	F-Ratio	P-Value
Carbohydrate					
A:pH	0.0132207	1	0.0132207	5.52	0.0297
B:Temperature	0.0652533	1	0.0652533	27.27	0
C:Light intensity	0.0149598	1	0.0149598	6.25	0.0217
D:Growth period	0.114588	1	0.114588	47.88	0
E:$CaCl_2$	0.0944953	1	0.0944953	39.48	0
F:$NaNO_3$	0.0259501	1	0.0259501	10.84	0.0038
G:K_2HPO_4	0.0502223	1	0.0502223	20.98	0.0002
AC	0.037098	1	0.037098	15.5	0.0009
AG	0.0505136	1	0.0505136	21.11	0.0002
BC	0.0200574	1	0.0200574	8.38	0.0093
BE	0.0386135	1	0.0386135	16.13	0.0007
CC	0.0373428	1	0.0373428	15.6	0.0009
CD	0.0156827	1	0.0156827	6.55	0.0192
CE	0.0146975	1	0.0146975	6.14	0.0228
CF	0.0207225	1	0.0207225	8.66	0.0084
CG	0.01216	1	0.01216	5.08	0.0362
DE	0.0634655	1	0.0634655	26.52	0.0001
DG	0.0117058	1	0.0117058	4.89	0.0394
GG	0.040036	1	0.040036	16.73	0.0006
Total error	0.0454722	19	0.00239328		
Total (corr.)	0.840436	38			
Protein					
A:pH	0.0547621	1	0.0547621	10.62	0.0032
B:Temperature	0.0236135	1	0.0236135	4.58	0.0423
C:Light intensity	0.0221255	1	0.0221255	4.29	0.0488
D:Growth period	0.170607	1	0.170607	33.1	0
E:$CaCl_2$	0.0887725	1	0.0887725	17.22	0.0003
F:$NaNO_3$	0.0239618	1	0.0239618	4.65	0.0409
G:K_2HPO_4	0.0239326	1	0.0239326	4.64	0.041
AA	0.0363646	1	0.0363646	7.05	0.0136
AE	0.0751313	1	0.0751313	14.57	0.0008
BE	0.0788703	1	0.0788703	15.3	0.0006
CC	0.0388405	1	0.0388405	7.53	0.011
CE	0.0313694	1	0.0313694	6.09	0.0208
FG	0.0694453	1	0.0694453	13.47	0.0011
Total error	0.128874	25	0.00515495		
Total (corr.)	0.849532	38			

Table 4.13 Analysis of the experimental results of Central Composite Design

Model	Biomass	Chlorophyll	Carbohydrates	Lipid	Protein
Transformation	None	none	none	none	none
Model d.f.	12	12	19	11	13
P-value	0.0000	0.0000	0.0000	0.0000	0.0000
Error d.f.	26	26	19	27	25
Stnd. error	0.192569	0.37097	0.0489211	0.0409911	0.071798
R-squared	80.14	85.09	94.59	76.82	84.83
Adj. R-squared	70.98	78.20	89.18	67.38	76.94

Table 4.14 Factor settings at optimum and response values at optimum conditions as per model

Cultural factors							
	pH	**Temp. (°C)**	**Light intensity ($\mu molm^{-2}s^{-1}$)**	**Growth period (Days)**	**$CaCl_2$ (mM)**	**$NaNO_3$ (mM)**	**K_2HPO_4 (mM)**
Setting at optimum	10.57	20.0	80.99	24.99	15.00	5.00	2.00

Response	**Optimized**	**Prediction**	**Lower 95.0% Limit**	**Upper 95.0% Limit**	**Desirability (%)**
Biomass (gL^{-1})	yes	2.319	2.084	2.554	74.53
Chlorophyll (mgL^{-1})	yes	4.071	3.630	4.511	87.07
Carbohydrates (gL^{-1})	yes	0.848	0.767	0.929	89.83
Lipid (gL^{-1})	yes	0.345	0.295	0.396	80.24
Protein (gL^{-1})	yes	1.087	0.969	1.205	99.99
Optimized desirability			84.10%		

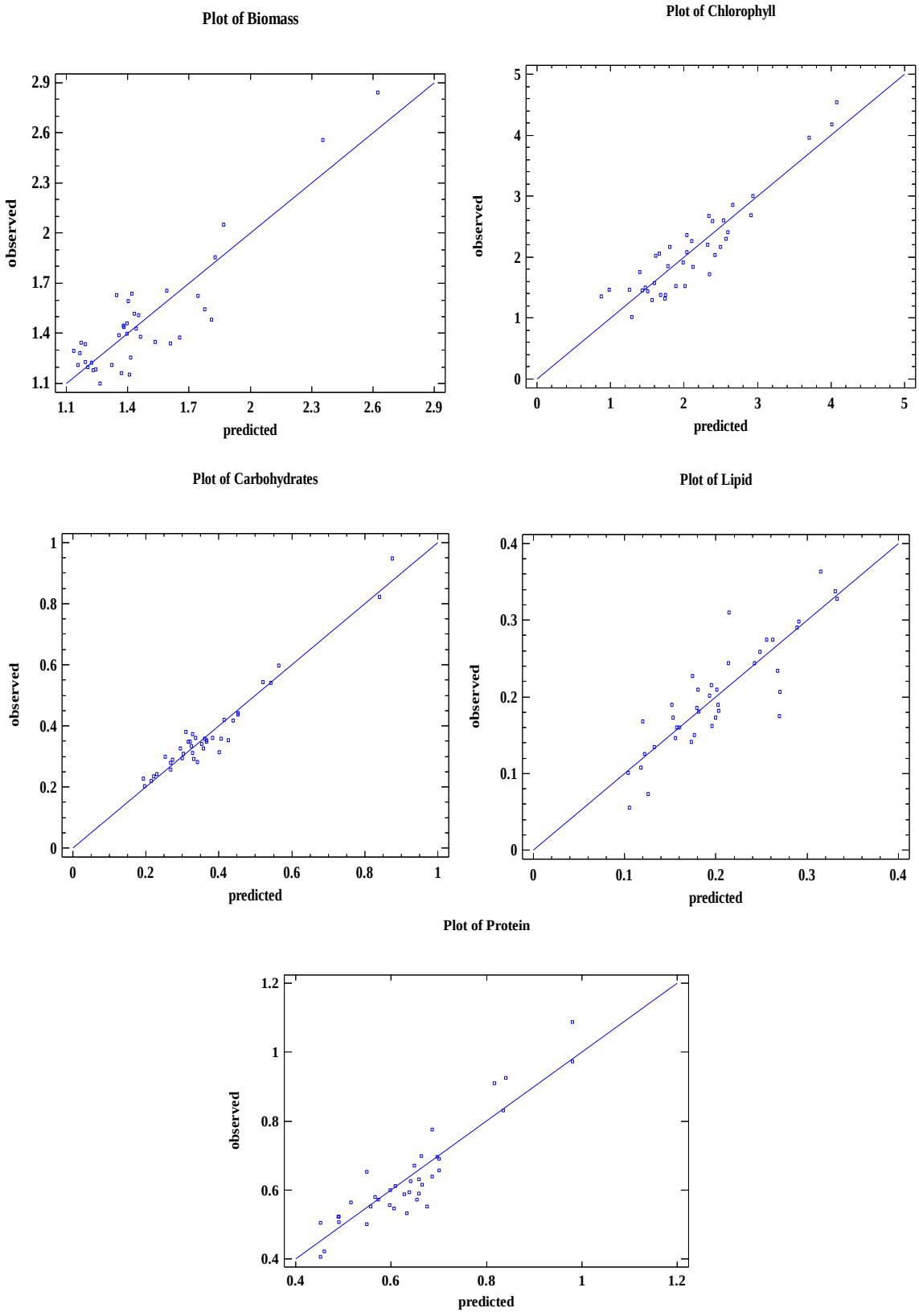


Fig. 4.7 Observed vs predicted results of different responses: (a) biomass, (b) chlorophyll, (c) carbohydrate, (d) lipid and (e) protein

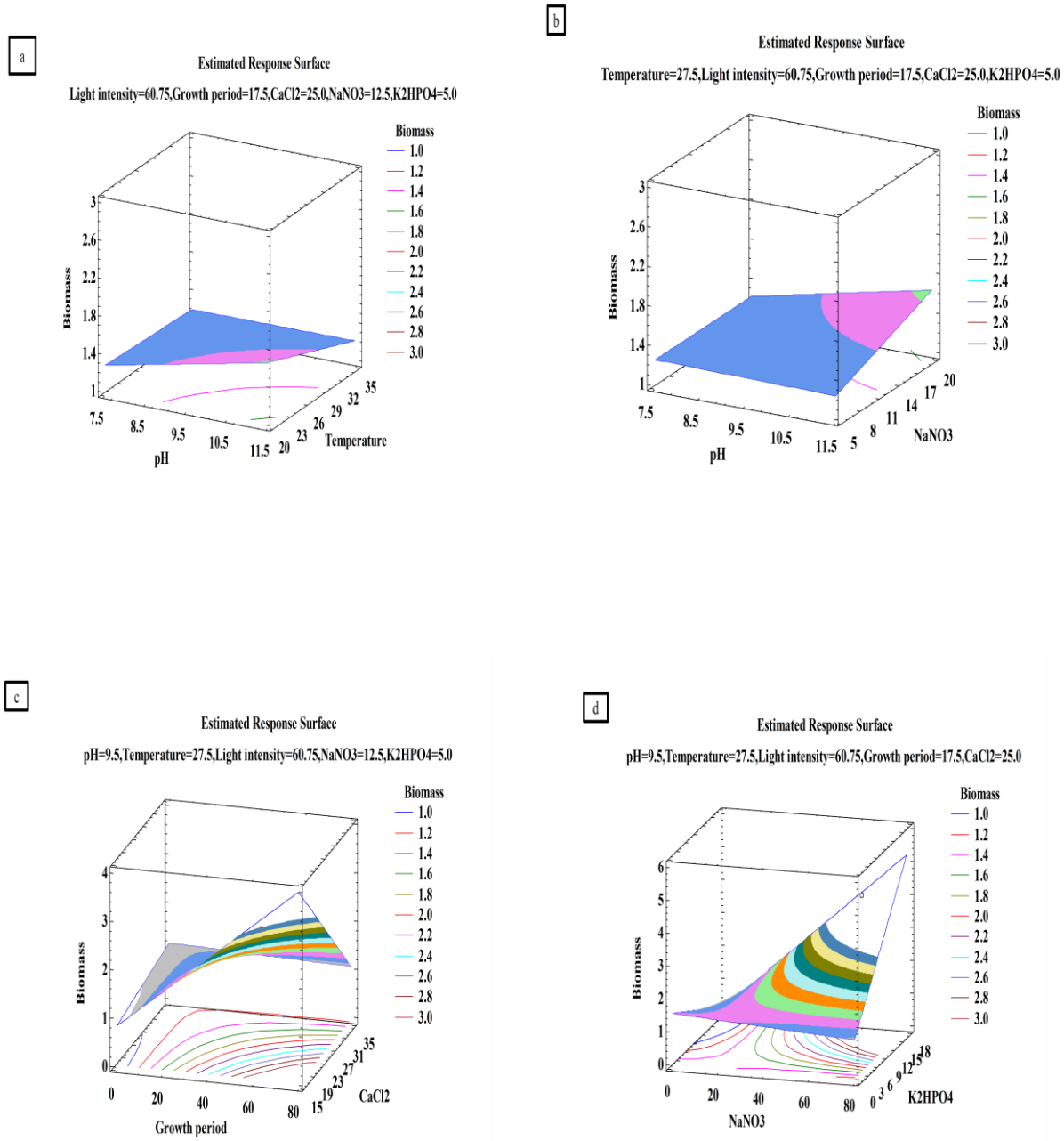


Fig. 4.8 Response surface 3D plots for various interactions: (a) pH versus temperature interaction, (b) pH versus $NaNO_3$ interaction, (c) Growth period versus $CaCl_2$ and (d) $NaNO_3$ versus K_2HPO_4

4.6.5 Identifying the significant variables affecting biomass production and other functional components of *Asterarcys quadricellulare* BGLR5 using the Placket-Burman design

This experiment was conducted in 12 runs, with each run screening one of 12 independent variables (pH, temperature, light intensity, growth period, NH_4Cl, $NaNO_3$ and K_2HPO_4) in the Plackett–Burman experimental design (Table 4.15) in order to study the effect of each selected variable on the production of biomass, chlorophyll, carbohydrates, lipids and proteins. The design matrix selected for the screening of significant variables for the various responses under study are shown in Table 4.15. All trials were performed in triplicate.

The relationship between a set of independent variables and the response variables was determined by variance analysis and multiple regression model. Statistical analysis of the response variables was performed and the results are presented in Table 4.16. In the current experiment, variables with P values of <0.05 (confidence levels of >95 %) were considered to have significant effects on response variables. With respect to the main effect of each variable, all variables taken in this study, namely, pH, temperature, light intensity, growth period, NH_4Cl, $NaNO_3$ and K_2HPO_4, significantly affected the responses under study. The significance of each coefficient was determined by P values, which are listed in Table 4.16. The variables having larger F-ratio and smaller P value are regarded more significant ($p<0.05$) (Montgomery 2005). From the Table 4.16, it is clearly demonstrated that all the factors have low p value <0.05, thus are all important and significant. Temperature, with a probability value (P) of <.0001, F value of 1753.30 and coefficient of estimate of -0.3455, was decided to be the most significant factor for biomass production, followed by K_2HPO_4 (P value <.0001, F value 584.58, coefficient of estimate of 0.1995), light intensity (P value <.0001, F value 394.24, coefficient of estimate of 0.1638), $NaNO_3$ (P value 0.0001, F value 236.28, coefficient of estimate of -0.1268), growth period (P value 0.0188, F value 14.57, coefficient of estimate of 0.0315) and pH (P value 0.0233, F value 12.78, coefficient of estimate of 0.0295) and NH_4Cl (P value 0.0572, F value 7.00, coefficient of estimate of -0.0218). Similarly, for other responses the values are given in Table 4.16. The model P value for all responses were found to be <0.05 (<.0001 for biomass, 0.0001 for chlorophyll, 0.0002 carbohydrate, lipid <.0001 and protein 0.0003) (Table 4.17) implies that the model and model terms are significant. For further optimization by the CCD, we selected the significant variables to determine the optimal range of these variables. The Paretro charts (Fig. 4.9 a-e) for various responses easily determine the order of significance of various factors under study.

The model's goodness of fit was checked using the coefficient of determination (R^2). In this study, the values of the determination coefficient for various responses were found to be close to 1 as can be seen in Table 4.17. For biomass, R^2 is 0.9987, which means that 99.87 % of the variability in the response was attributed to the given independent variables and that only 0.13 % of the total variation is not explained by the independent variables. Similarly for others, coefficient of determination (R^2) was found to be 99.70, 99.49, 99.90 and 99.41 for chlorophyll, carbohydrate, lipid and protein respectively. In addition, the adjusted coefficient of determination (Adj. R^2 = 0.9963) was also very high for biomass and other response variables (chlorophyll 0.9919, carbohydrate 0.9861, lipid 0.9972 and protein 0.9837), which indicates a high significance of the model.

4.6.6 Optimization of cultural factors of *Asterarcys quadricellulare* BGLR5 by central composite design (CCD)

The CCD was applied to study the interactions among the significant factors and also to determine their optimal levels. The most significant independent variables affecting biomass production and other responses (in the Plackett–Burman experiment), namely, pH, temperature, light intensity, growth period, NH_4Cl, $NaNO_3$ and K_2HPO_4 were selected (Table 4.18) and further investigated using CCD. A total of 39 experiments with different combinations of the seven independent variables were performed; the observed values for all response variables together with the experimental design matrix and observed and predicted desirability are given in Table 4.19. The results were analyzed by ANOVA and show considerable variation in biomass production and for other responses. The minimum biomass production (0.366 gL^{-1}) was observed in run number 37, while maximum biomass production (1.260 gL^{-1}) was achieved in run number 34. The observed and predicted desirability for this run was found to be highest among all the runs.

Variance and multiple regression analysis were employed to analyze the data. The results of the ANOVA, which is needed to test the significance and adequacy of the model and model coefficients, are presented in Table (4.20-4.24). The ANOVA table segregates the variation in each response variable into distinct sections for each of the effects. It then compares the mean square against an estimate of the experimental error to analyse the statistical significance of each effect. The number of effects as shown in Table (4.20-4.24), having p-value less than 0.05 in biomass, chlorophyll, carbohydrate, lipid and protein are 33, 26, 33, 31 and 33 respectively, indicating that they are significantly different from zero at the 95.0% confidence level. ANOVA for the seven variables indicated that the production of five responses under study could be described well by CCD model with relatively high coefficient

of determination. The effect of independent variables pH, temperature, light intensity, growth period, NH_4Cl, $NaNO_3$ and K_2HPO_4 described in Table 4.20-4.24 were significant as their p value were less than 0.05. The significance of the interactions varied from response to response variable. The linear terms were significant model terms in all the cases. For biomass interactive (AB, AC, AD, AF, AG, BC, BD, BE, BG, CD, CE, CF, DE, DF, DG, EF, EG and FG) and quadratic (A^2, B^2, C^2, D^2, E^2 and F^2) were significant model terms. The AB,AC, AF, AG, BC, BD, BE, CD, CF, DE, EF, EG, FG interactive terms and A^2, B^2, C^2, E^2 and F^2 quadratic terms are significant model terms for chlorophyll. In lipid the interactive (AB, AC, AD, AE, AF, AG, BC,BD, BE, BF, BG, CD, CG, DE, DG, EF, EG and FG) and quadratic terms (A^2, B^2, D^2, E^2, F^2 and G^2) acted as significant effects whereas for carbohydrate, the interactive (AB, AC, AD, AE, AF, BC, BD, BE, BF, BG, CD, CE, CF, CG, DE, DF, DG, EF, EG and FG) and quadratic (A^2, B^2, C^2, E^2, F^2 and G^2) were significant model terms. Similarly for protein the significant model terms were linear (AB, AC, AD, AE, BC, BD, BE, BF, BG, CD, CE, CF, CG, DE, DF, DG, EF, EG, and FG) and quadratic (A^2, C^2, D^2, E^2, F^2 and G^2). The coefficient of determination (R^2) values, which are always between 0 and 1, explains us how much deviation or variability in the observed response values can be explained by the experimental variables and their interactions. The closer the R^2 value is to 1, the stronger the model is and the better it predicts the response (Kaushik *et al* 2006). The R^2 of the model for biomass, chlorophyll, carbohydrates, lipids and proteins were 0.9999, 0.9983, 0.9999, 0.9997 and 1.0000 respectively (Table 4.25). This can be interpreted as, that 99.99%, 99.83%, 99.99%, 99.97% and 100% of variability in the responses (biomass, chlorophyll, carbohydrates, lipids and proteins) respectively could be explained by the model and that only 0.01, 0.17, 0.01, 0.03%of the total variance for the ist four responses could not be explained by the model. The regression model having an R^2 value of >0.9 is considered to be having a very high correlation (Chen *et al* 2009). The R^2 values found in our study are >0.9, thus reflect a very good fit between the observed and predicted responses and implies that the model is reliable for the production of the various responses under study. The predicted and observed values of various responses in our study are very much close as can be seen in Fig. 4.10(a-e). The values almost fall on the line of fit, therefore depicting the significance and accuracy of the model. In addition, the value of the adjusted coefficient of determination (Adj. R^2) was also very high (99.96 for biomass, chlorophyll 99.47 %, 99.90 % for carbohydrates, lipid 99.84 % and 99.99 % for protein) in our study (Table 4.25), which indicates a high significance of the model (Akhnazarova and Afarov 1982). Thus, we considered the analysis of the response trend using the model to be reasonable.

The final regression equations for the biomass production, chlorophyll, carbohydrate, lipid and protein, which have been fitted to the data, are as follows:

Biomass = -1.00328 + 0.530948*A + 0.017628*B - 0.052074*C + 0.032903*D- 0.039770*E + 0.116465*F + 0.194392*G - 0.026576*A^2 + 0.001014*A*B - 0.000206*A*C - 0.002206*A*D + 0.000549*A*E-0.001074*A*F-0.002103*A*G- 0.000642*B^2- 0.000148*B*C- 0.000297*B*D + 0.000601*B*E - 0.000252*B*G + 0.000442*C^2 + 0.000114*C*D + 0.000037*C*E + 0.000072*C*F - 0.000525*D^2 + 0.000227*D*E - 0.000123*D*F + 0.000424*D*G + 0.000122*E^2 + 0.000506*E*F - 0.001138*E*G - 0.004686*F^2 - 0.003854*F*G - 0.006535*G^2

Chlorophyll = -90.5223 + 26.1943*A + 3.99601*B - 1.16359*C - 0.240588*D - 2.15533*E + 1.84562*F - 0.78312*G - 1.33055*A^2 - 0.026960*A*C + 0.015297*A*D - 0.025411*A*F - 0.114345*A*G - 0.060175*B^2 - 0.008399*B*C - 0.018301*B*D + 0.007232*B*E + 0.008961*C^2 + 0.008712*C*D + 0.001945*C*F + 0.007026*D*E + 0.016989*E^2 + 0.041944*E*F + 0.025677*E*G - 0.123716*F^2 - 0.059047*F*G + 0.164976*G^2

Carbohydrates = -0.339147 + 0.197815*A + 0.016948*B - 0.022591*C + 0.016618*D - 0.022102*E + 0.048614*F + 0.038648*G - 0.009180*A^2 + 0.000289*A*B - 0.000050*A*C - 0.001692*A*D + 0.000142*A*E - 0.000277*A*F - 0.000435*B^2 - 0.000015*B*C - 0.000221*B*D + 0.00019*B*E - 0.000170*B*F + 0.000592*B*G + 0.000170*C^2 + 0.000059*C*D + 0.000046*C*E + 0.000068*C*F - 0.000061*C*G + 0.000092*D*E - 0.000130*D*F + 0.000213*D*G + 0.000195*E^2 + 0.000151*E*F - 0.000230*E*G - 0.001749*F^2 - 0.001059*F*G - 0.004332*G^2

Lipid = 0.0761248 + 0.025285*A - 0.014617*B - 0.000488*C - 0.000959*D - 0.006103*E + 0.034334*F + 0.008245*G - 0.001752*A^2 + 0.000071*A*B + 0.000047*A*C + 0.000206*A*D + 0.000189*A*E - 0.000226*A*F - 0.000304*A*G + 0.000289*B^2 - 0.000021*B*C - 0.000027*B*D + 0.000039*B*E - 0.000090*B*F + 0.000116*B*G + 0.000033*C*D + 0.000045*C*G - 0.000072*D^2 + 0.000023*D*E - 0.000058*D*G + 0.0000582*E^2 - 0.000027*E*F + 0.000029*E*G - 0.001253*F^2 - 0.000262*F*G - 0.000774*G^2

Protein = -0.717817 + 0.199305*A + 0.001526*B - 0.018011*C - 0.008146*D + 0.018671*E + 0.058244*F + 0.073727*G - 0.009189*A^2 - 0.000164*A*B - 0.000160*A*C - 0.000689*A*D + 0.000226*A*E - 0.000385*A*F - 0.000076*B*C - 0.000080*B*D + 0.000139*B*E - 0.000043*B*F - 0.000118*B*G + 0.000169*C^2 + 0.000048*C*D

$+ 0.000013*C*E + 0.000027*C*F - 0.000224*C*G + 0.000338*D^2 + 0.000090*D*E - 0.000058*D*F+ 0.000295D*G - 0.000548*E^2 + 0.000211*E*F - 0.000430*E*G - 0.002229*F^2 - 0.001632*F*G - 0.001742*G^2$

Where, A: pH, C: Light intensity, D: Growth period, E: NH_4Cl, F: $NaNO_3$ and G: K_2HPO_4

Thus according to the obtained results and the above mentioned regression equations, pH of 9.92, temperature of 21.84°C, the light intensity of 80.99 μmol m^{-2} s^{-1}, 25 days of the growth period, 15.00 mM NH_4Cl, 12.28 mM $NaNO_3$ and 7.09 mM of K_2HPO_4 were the optimal and most desired conditions as per the model (Table 4.26), for different responses considered for this study. At these settings, the response variables generated a desirability index of 94.91%.

4.6.7 Validation or confirmation of the model

The validity of the model needed to be checked to establish its significance. So, a validation experiment of the statistical model was conducted by cultivating *Asterarcys quadricellulare* BGLR5 under optimal conditions (pH of 9.92, 21.84°C of temperature, the light intensity of 80.99 μmol m^{-2} s^{-1}, 25 days of the growth period, 15 mM NH_4Cl, $NaNO_3$ of 12.28 and 7.09 of K_2HPO_4) as suggested by the model in the BG-11 medium. The experimental value for different responses (biomass, chlorophyll, carbcarbohydrate, lipid and protein) was found to be 1.229 gL^{-1}, 31.019 mgL^{-1}, 0.329 gL^{-1}, 0.117 gL^{-1} and 0.409 gL^{-1} respectively. The values of responses obtained are close to that obtained from CCD of RSM and model predicted values at optimum. So, this close relationship of the values obtained from validation experiment to that of the RSM values and model predicted response values at optimum, revealed the confirmation, validity and acceptability of the model for the optimization of different physico-chemical factors discussed in this study.

Table 4.15 Experimental design and results of *Asterarcys quadricellulare* BGLR5 in Placket Burman Design

Run	pH	Temperature (°C)	Light Intensity ($\mu molm^{-2}s$)	Growth Period (days)	NH_4Cl (Mm)	$NaNO_3$ (mM)	K_2HPO_4 (mM)	Biomass (g/L)	Chlorophyll (mg/L	Carbohydrate (g/L)	Lipid (g/L)	Protein (g/L)	Observed Desirability	Predicted Desirability
1	10.5	35	40.5	25	20	5	2	0.25	10.31	0.09	0.03	0.09	0.34	0.33
2	10.5	25	81	15	20	5	8	0.92	32.72	0.36	0.08	0.19	0.87	0.88
3	10.5	35	40.5	25	35	5	8	0.41	9.72	0.13	0.04	0.05	0.37	0.38
4	7.5	25	40.5	15	20	5	2	0.52	23.77	0.18	0.05	0.14	0.53	0.53
5	7.5	35	40.5	15	20	20	8	0.26	4.72	0.10	0.02	0.05	0.25	0.24
6	7.5	25	81	25	35	5	8	0.91	26.39	0.34	0.07	0.19	0.63	0.63
7	7.5	35	81	25	20	20	8	0.44	8.77	0.18	0.03	0.10	0.37	0.38
8	10.5	25	40.5	15	35	20	8	0.60	14.75	0.25	0.04	0.16	0.59	0.60
9	10.5	35	81	15	35	20	2	0.24	5.35	0.10	0.02	0.09	0.30	0.29
10	7.5	35	81	15	35	5	2	0.31	10.88	0.07	0.04	0.06	0.33	0.34
11	10.5	25	81	25	20	20	2	0.61	18.96	0.27	0.04	0.25	0.66	0.67
12	7.5	25	40.5	25	35	20	2	0.412	9.35	0.17	0.03	0.17	0.38	0.38

Values are means of triplicate; Biomass is dry cell biomass

Table 4.16 Statistical analysis of Placket Burman Design (BGLR5)

Source	Levels		Sum of Squares	DF[a]	Mean Square	Coefficient of Estimate	F-Ratio	[b]P-Value
	-1 level	+1 level						
Biomass								
A:pH	7.5	10.5	0.00261075	1	0.00261075	0.0295	12.78	0.0233
B:Temperature	25	35	0.358111	1	0.358111	-0.3455	1753.30	0.0000
C:Light Intensity	40.5	81	0.0805241	1	0.0805241	0.163833	394.24	0.0000
D:Growth Period	15	25	0.00297675	1	0.00297675	0.0315	14.57	0.0188
E:NH_4Cl	20	35	0.00143008	1	0.00143008	-0.0218333	7.00	0.0572
F:$NaNO_3$	5	20	0.0482601	1	0.0482601	-0.126833	236.28	0.0001
G:$K2HPO_4$	2	8	0.119401	1	0.119401	0.1995	584.58	0.0000
Total error			0.000817	4	0.00020425			
Total (corr.)			0.61413	11				
Chlorophyll								
A:pH	7.5	10.5	5.24195	1	5.24195	1.32186	8.15	0.0462
B:Temperature	25	35	483.771	1	483.771	-12.6987	751.87	0.0000
C:Light Intensity	40.5	81	77.283	1	77.283	5.07553	120.11	0.0004
D:Growth Period	15	25	6.29711	1	6.29711	-1.4488	9.79	0.0352
E:NH_4Cl	20	35	43.403	1	43.403	-3.80364	67.46	0.0012
F:$NaNO_3$	5	20	224.512	1	224.512	-8.65086	348.93	0.0000
G:$K2HPO_4$	2	8	28.4279	1	28.4279	3.07831	44.18	0.0027
Total error			2.5737	4	0.643425			
Total (corr.)			871.509	11				
Carbohydrate								
A:pH	7.5	10.5	0.001728	1	0.001728	0.024	13.24	0.0220
B:Temperature	25	35	0.0654163	1	0.0654163	-0.147667	501.27	0.0000
C:Light Intensity	40.5	81	0.0124163	1	0.0124163	0.0643333	95.14	0.0006
D:Growth Period	15	25	0.001083	1	0.001083	0.019	8.30	0.0450
E:NH_4Cl	20	35	0.000936333	1	0.000936333	-0.0176667	7.17	0.0553
F:$NaNO_3$	5	20	0.001083	1	0.001083	-0.019	8.30	0.0450
G:$K2HPO_4$	2	8	0.020172	1	0.020172	0.082	154.57	0.0002
Total error			0.000522	4	0.0001305			
Total (corr.)			0.103357	11				
Lipid								
A:pH	7.5	10.5	0.00000890907	1	0.0000089091	-0.00172328	8.69	0.0421
B:Temperature	25	35	0.00133092	1	0.00133092	-0.0210628	1297.73	0.0000
C:Light Intensity	40.5	81	0.000300847	1	0.000300847	0.0100141	293.34	0.0001
D:Growth Period	15	25	0.0000173032	1	0.0000173032	-0.00240161	16.87	0.0148
E:NH_4Cl	20	35	0.0000109209	1	0.0000109209	-0.00190796	10.65	0.0310
F:$NaNO_3$	5	20	0.00185628	1	0.00185628	-0.0248749	1809.99	0.0000
G:$K2HPO_4$	2	8	0.000563457	1	0.000563457	0.0137047	549.40	0.0000
Total error			0.00000410231	4		0.00000102558		
Total (corr.)			0.00409274	11				
Protein								
A:pH	7.5	10.5	0.00124225	1	0.00124225	0.020349	18.54	0.0126
B:Temperature	25	35	0.0356876	1	0.0356876	-0.109068	532.76	0.0000
C:Light Intensity	40.5	81	0.00369051	1	0.00369051	0.0350738	55.09	0.0018
D:Growth Period	15	25	0.0022704	1	0.0022704	0.02751	33.89	0.0043
E:NH_4Cl	20	35	0.000905481	1	0.000905481	-0.0173732	13.52	0.0213
F:$NaNO_3$	5	20	0.000530469	1	0.000530469	0.0132975	7.92	0.0481
G:$K2HPO_4$	2	8	0.000532214	1	0.000532214	-0.0133193	7.95	0.0479
Total error			0.000267946	4	0.0000669864			
Total (corr.)			0.0451268	11				

[a] DF is Degrees of Freedom; [b] Significance level at a p-value ≤ 0.05

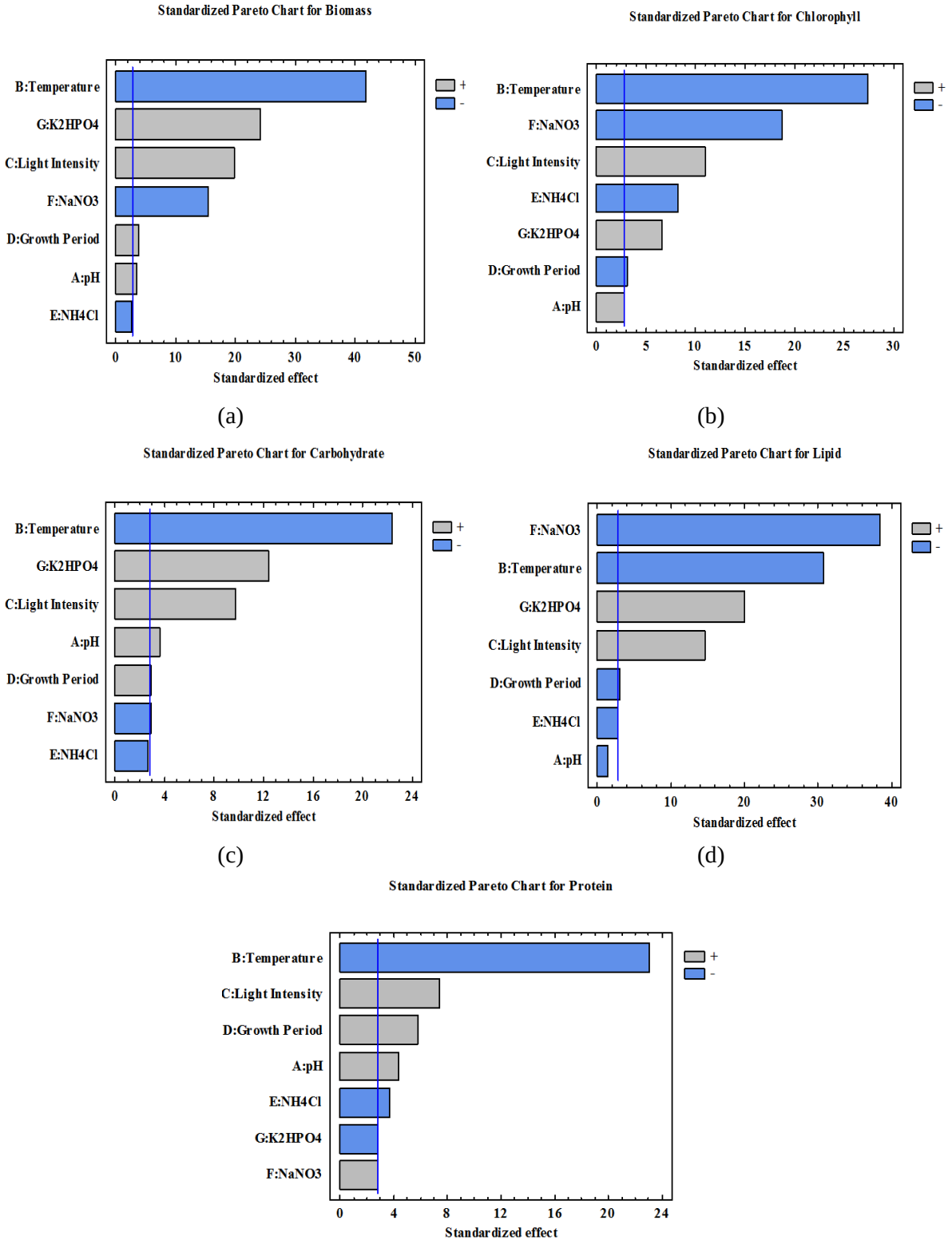


Fig. 4.9 (a-e) Paretro charts of the various response variables for BGLR5 (a) Biomass, (b) Chlorophyll, (c) Carobhydrate, (d) Lipid and (e) Protein

Table 4.17 Analysis of the experimental results of Placket Burman Design for BGLR5

Model	Biomass	Chlorophyll	Carbohydrate	Lipid	Protein
Model DF	7	7	7	7	7
P-value	0.0000	0.0001	0.0002	0.0000	0.0003
Error DF	4	4	4	4	4
Stndard error of estimate	0.0142916	0.802138	0.0114237	0.00101271	0.00818452
Mean absolute error	0.00741667	0.353815	0.006	0.000482597	0.00455604
R-squared (%)	99.87	99.70	99.49	99.90	99.41
Adj. R-squared (%)	99.63	99.19	98.61	99.72	98.37

DF is degrees of freedom

Table 4.18 Different factors used in the CCD study with their levels

Name	Units	Low	High	Levels
A:pH		7.5	11.5	-1, 0, +1
B:Temperature	°C	20.0	35.0	-1,0,+1
C:Light intensity	$\mu molm^{-2}s^{-1}$	40.5	81.0	-1,0,+1
D:Growth period	Days	10.0	25.0	-1,0,+1
E:NH_4Cl	mM	15.0	35.0	-1,0,+1
F:$NaNO_3$	mM	5.0	20.0	-1,0,+1
G:K_2HPO_4	mM	2.0	8.0	-1,0,+1

Table 4.19 Central Composite Design (CCD) and response values for different responses

Run	pH	Temp. (°C)	Light intensity ($\mu molm^{-2}s^{-1}$)	Growth period (days)	NH_4Cl (mM)	$NaNO_3$ (mM)	K_2HPO_4 (mM)	Biomass (gL^{-1})	Chlorophyll (mgL^{-1})	Carbohydrates (gL^{-1})	Lipid (gL^{-1})	Protein (gL^{-1})	Observed Desirability	Predicted Desirability
1	7.5	20	81	10	35	20	2	0.538	10.659	0.282	0.026	0.212	0.325	0.311
2	11.5	20	40.5	10	35	20	2	0.530	10.962	0.286	0.028	0.213	0.304	0.310
3	7.5	20	40.5	10	15	20	2	0.612	14.299	0.290	0.057	0.170	0.362	0.369
4	9.5	27.5	81	18	25	12.5	5	1.098	27.605	0.390	0.130	0.405	0.747	0.745
5	7.5	35	40.5	10	35	20	2	0.519	12.968	0.198	0.040	0.182	0.301	0.312
6	7.5	35	40.5	25	35	20	8	0.488	7.257	0.132	0.018	0.202	0.217	0.205
7	9.5	20	60.75	18	25	12.5	5	0.955	21.841	0.323	0.136	0.379	0.656	0.655
8	11.5	20	81	25	15	20	8	0.598	12.885	0.155	0.038	0.188	0.377	0.375
9	11.5	35	40.5	10	15	20	2	0.512	14.684	0.231	0.037	0.135	0.345	0.338
10	11.5	35	81	10	35	20	2	0.536	9.745	0.273	0.027	0.166	0.309	0.314
11	11.5	35	40.5	10	35	5	2	0.635	13.983	0.251	0.102	0.187	0.447	0.434
12	7.5	35	40.5	10	15	20	8	0.544	10.847	0.132	0.045	0.172	0.267	0.270
13	7.5	35	81	25	35	20	2	0.521	9.195	0.255	0.027	0.201	0.311	0.305
14	7.5	35	40.5	10	15	5	2	0.562	27.674	0.208	0.093	0.153	0.388	0.376
15	9.5	27.5	60.75	18	35	12.5	5	0.889	21.932	0.334	0.129	0.296	0.662	0.661
16	7.5	35	81	10	15	5	8	0.876	22.196	0.183	0.102	0.245	0.471	0.457
17	7.5	20	40.5	25	35	20	2	0.540	10.097	0.290	0.026	0.201	0.326	0.319
18	11.5	35	81	25	15	5	8	0.821	21.773	0.185	0.108	0.253	0.488	0.500
19	11.5	20	81	25	35	5	8	0.837	20.109	0.201	0.105	0.317	0.567	0.553
20	7.5	20	81	25	35	20	8	0.662	12.876	0.231	0.030	0.236	0.364	0.391
21	11.5	35	40.5	25	35	20	2	0.463	8.346	0.148	0.039	0.168	0.276	0.280
22	11.5	27.5	60.75	18	25	12.5	5	0.803	18.614	0.280	0.119	0.311	0.602	0.601
23	7.5	35	40.5	25	35	5	2	0.477	9.390	0.200	0.075	0.158	0.292	0.298
24	11.5	20	81	25	15	5	2	0.657	34.096	0.249	0.093	0.195	0.603	0.602
25	7.5	35	40.5	10	35	5	8	0.770	17.278	0.191	0.107	0.278	0.462	0.475
26	7.5	35	81	10	35	5	2	0.380	6.722	0.161	0.071	0.111	0.221	0.227
27	9.5	27.5	60.75	10	25	12.5	5	0.908	22.977	0.320	0.120	0.362	0.649	0.653
28	11.5	35	40.5	10	35	20	8	0.519	8.583	0.171	0.027	0.196	0.255	0.258
29	11.5	20	81	10	35	5	2	0.520	13.975	0.227	0.071	0.175	0.373	0.378
30	11.5	20	40.5	25	15	5	8	1.098	24.956	0.271	0.062	0.412	0.590	0.600
31	7.5	35	81	10	35	20	8	0.418	6.888	0.129	0.026	0.104	0.176	0.170
32	7.5	35	81	10	15	20	2	0.379	9.587	0.173	0.035	0.107	0.213	0.218
33	9.5	27.5	60.75	18	25	20	5	0.548	11.924	0.206	0.027	0.190	0.289	0.289
34	**7.5**	**20**	**81**	**25**	**15**	**5**	**8**	1.260	28.983	0.307	0.090	0.396	0.782	0.775
35	7.5	20	40.5	10	35	20	8	0.470	7.982	0.113	0.018	0.180	0.187	0.177
36	11.5	20	81	10	15	5	8	1.120	27.995	0.241	0.083	0.345	0.614	0.623
37	7.5	35	40.5	25	15	20	2	0.366	8.340	0.152	0.033	0.107	0.197	0.200
38	7.5	20	40.5	10	35	5	2	0.455	10.231	0.186	0.061	0.128	0.275	0.276
39	9.5	27.5	60.75	18	25	12.5	8	0.967	24.049	0.260	0.117	0.375	0.655	0.654

Table 4.20 Analysis of variance (ANOVA) and regression coefficients for the quadratic model of various physicochemical factors of *Asteracycs quadricellulare* BGLR5 for Biomass

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value	Regression coeffs
A:pH	0.0049	1	0.0049	224.84	<.0001	0.5309
B:Temperature	0.0888	1	0.0888	4086.59	<.0001	0.0176
C:Light intensity	0.0027	1	0.0027	123.19	0.0001	-0.0521
D:Growth period	0.0023	1	0.0023	106.35	0.0001	0.0329
E:NH4Cl	0.0558	1	0.0558	2567.74	<.0001	-0.0398
F:NaNO3	0.2871	1	0.2871	13215.38	<.0001	0.1165
G:K2PHO4	0.2121	1	0.2121	9766.23	<.0001	0.1944
AA	0.0126	1	0.0126	578.61	<.0001	-0.0266
AB	0.0051	1	0.0051	235.87	<.0001	0.0010
AC	0.0015	1	0.0015	69.81	0.0004	-0.0002
AD	0.0240	1	0.0240	1107.08	<.0001	-0.0022
AE	0.0026	1	0.0026	121.99	0.0001	0.0005
AF	0.0057	1	0.0057	264.42	<.0001	-0.0011
AG	0.0036	1	0.0036	164.32	0.0001	-0.0021
BB	0.0014	1	0.0014	66.49	0.0005	-0.0006
BC	0.0110	1	0.0110	508.66	<.0001	-0.0001
BD	0.0060	1	0.0060	274.75	<.0001	-0.0003
BE	0.0470	1	0.0470	2163.03	<.0001	0.0006
BG	0.0007	1	0.0007	32.84	0.0023	-0.0003
CC	0.0364	1	0.0364	1674.14	<.0001	0.0004
CD	0.0068	1	0.0068	311.43	<.0001	0.0001
CE	0.0013	1	0.0013	58.07	0.0006	0.0000
CF	0.0026	1	0.0026	120.61	0.0001	0.0001
DD	0.0010	1	0.0010	43.92	0.0012	-0.0005
DE	0.0065	1	0.0065	298.42	<.0001	0.0002
DF	0.0011	1	0.0011	50.6	0.0009	-0.0001
DG	0.0020	1	0.0020	90.52	0.0002	0.0004
EE	0.0002	1	0.0002	7.52	0.0407	0.0001
EF	0.0309	1	0.0309	1422.22	<.0001	0.0005
EG	0.0252	1	0.0252	1158.29	<.0001	-0.0011
FF	0.0771	1	0.0771	3550.26	<.0001	-0.0047
FG	0.1664	1	0.1664	7661.43	<.0001	-0.0039
GG	0.0038	1	0.0038	176.69	<.0001	-0.0065
Total error	0.0001	5	0.0000			
Total (corr.)	2.0734	38				
Constant						-1.0033

Table 4.21 Analysis of variance (ANOVA) and regression coefficients for the quadratic model of various physicochemical factors of *Asteracycs quadricellulare* BGLR5 for chlorophyll

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value	Regression coeffs
A:pH	4.2948	1	4.2948	14.3600	0.0026	26.1943
B:Temperature	61.9224	1	61.9224	207.0100	<.0001	3.9960
C:Light intensity	2.5599	1	2.5599	8.5600	0.0127	-1.1636
D:Growth period	2.1185	1	2.1185	7.0800	0.0207	-0.2406
E:NH4Cl	252.5140	1	252.5140	844.1800	<.0001	-2.1553
F:NaNO3	496.0530	1	496.0530	1658.3600	<.0001	1.8456
G:K2PHO4	20.9526	1	20.9526	70.0500	<.0001	-0.7831
AA	32.4541	1	32.4541	108.5000	<.0001	-1.3306
AB	3.7521	1	3.7521	12.5400	0.0041	-0.0270
AC	8.6783	1	8.6783	29.0100	0.0002	0.0153
AF	3.4404	1	3.4404	11.5000	0.0054	-0.0254
AG	11.4155	1	11.4155	38.1600	<.0001	-0.1143
BB	12.9929	1	12.9929	43.4400	<.0001	-0.0602
BC	38.4678	1	38.4678	128.6000	<.0001	-0.0084
BD	23.8971	1	23.8971	79.8900	<.0001	-0.0183
BE	7.3395	1	7.3395	24.5400	0.0003	0.0072
CC	15.3232	1	15.3232	51.2300	<.0001	0.0090
CD	41.3210	1	41.3210	138.1400	<.0001	0.0087
CF	2.1046	1	2.1046	7.0400	0.0211	0.0019
DE	6.7298	1	6.7298	22.5000	0.0005	0.0070
EE	3.2855	1	3.2855	10.9800	0.0062	0.0170
EF	226.2890	1	226.2890	756.5100	<.0001	0.0419
EG	13.3533	1	13.3533	44.6400	<.0001	0.0257
FF	55.3773	1	55.3773	185.1300	<.0001	-0.1237
FG	40.8309	1	40.8309	136.5000	<.0001	-0.0590
GG	2.5231	1	2.5231	8.4300	0.0132	0.1650
Total error	3.5895	12	0.2991			
Total (corr.)	2127.6300	38				
Constant						-90.5223

Table 4.22 Analysis of variance (ANOVA) and regression coefficients for the quadratic model of various physicochemical factors of *Asteracycs quadricellulare* BGLR5for carbohydrate

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value	Regression coeffs
A:pH	0.0001	1	0.0001	27.6000	0.0033	0.1978
B:Temperature	0.0145	1	0.0145	3370.5500	<.0001	0.0169
C:Light intensity	0.0000	1	0.0000	7.3600	0.0422	-0.0226
D:Growth period	0.0000	1	0.0000	10.8500	0.0216	0.0166
E:NH4Cl	0.0010	1	0.0010	224.4000	<.0001	-0.0221
F:NaNO3	0.0055	1	0.0055	1270.7500	<.0001	0.0486
G:K2PHO4	0.0108	1	0.0108	2509.8700	<.0001	0.0386
AA	0.0015	1	0.0015	356.1800	<.0001	-0.0092
AB	0.0004	1	0.0004	94.0800	0.0002	0.0003
AC	0.0001	1	0.0001	20.7200	0.0061	0.0000
AD	0.0143	1	0.0143	3314.5800	<.0001	-0.0017
AE	0.0002	1	0.0002	41.6700	0.0013	0.0001
AF	0.0004	1	0.0004	86.5100	0.0002	-0.0003
BB	0.0007	1	0.0007	156.8500	0.0001	-0.0004
BC	0.0001	1	0.0001	27.5700	0.0033	0.0000
BD	0.0033	1	0.0033	772.9500	<.0001	-0.0002
BE	0.0045	1	0.0045	1036.1000	<.0001	0.0002
BF	0.0020	1	0.0020	459.5600	<.0001	-0.0002
BG	0.0039	1	0.0039	914.5300	<.0001	0.0006
CC	0.0055	1	0.0055	1279.4200	<.0001	0.0002
CD	0.0018	1	0.0018	408.4200	<.0001	0.0001
CE	0.0019	1	0.0019	444.2700	<.0001	0.0000
CF	0.0023	1	0.0023	531.0100	<.0001	0.0001
CG	0.0003	1	0.0003	69.3700	0.0004	-0.0001
DE	0.0010	1	0.0010	238.9000	<.0001	0.0001
DF	0.0012	1	0.0012	278.3300	<.0001	-0.0001
DG	0.0005	1	0.0005	114.9000	0.0001	0.0002
EE	0.0004	1	0.0004	99.4700	0.0002	0.0002
EF	0.0028	1	0.0028	644.7900	<.0001	0.0002
EG	0.0010	1	0.0010	237.2400	<.0001	-0.0002
FF	0.0110	1	0.0110	2557.5500	<.0001	-0.0017
FG	0.0126	1	0.0126	2920.4500	<.0001	-0.0011
GG	0.0017	1	0.0017	400.3800	<.0001	-0.0043
Total error	0.0000	5	0.0000			
Total (corr.)	0.1645	38				
Constant						-0.3391

Table 4.23 Analysis of variance (ANOVA) and regression coefficients for the quadratic model of various physicochemical factors of *Asteracycs quadricellulare* BGLR5for lipid

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value	Regression coeffs
A:pH	5.141E-05	1	5.141E-05	21.6	0.0023	0.0252855
B:Temperature	0.0004751	1	0.0004751	199.59	<.0001	-0.0146169
C:Light intensity	0.0002884	1	0.0002884	121.15	<.0001	-0.0004876
D:Growth period	1.287E-06	1	1.287E-06	0.54	0.4861	-0.0009588
E:NH4Cl	3.196E-05	1	3.196E-05	13.42	0.0080	-0.0061035
F:NaNO3	0.0161073	1	0.0161073	6766.1	<.0001	0.0343343
G:K2PHO4	1.076E-06	1	1.076E-06	0.45	0.5229	0.0082452
AA	5.612E-05	1	5.612E-05	23.57	0.0018	-0.0017522
AB	2.436E-05	1	2.436E-05	10.23	0.0151	7.063E-05
AC	7.912E-05	1	7.912E-05	33.24	<.0001	4.653E-05
AD	0.0002131	1	0.0002131	89.5	<.0001	0.0002064
AE	0.000322	1	0.000322	135.26	<.0001	0.0001895
AF	0.0002555	1	0.0002555	107.34	<.0001	-0.0002263
AG	7.51E-05	1	7.51E-05	31.54	0.0008	-0.0003041
BB	0.0003012	1	0.0003012	126.52	<.0001	0.0002894
BC	0.0002261	1	0.0002261	94.96	<.0001	-2.124E-05
BD	5.08E-05	1	5.08E-05	21.34	0.0024	-2.71E-05
BE	0.0001913	1	0.0001913	80.37	<.0001	3.853E-05
BF	0.0005842	1	0.0005842	245.42	<.0001	-9.007E-05
BG	0.0001531	1	0.0001531	64.33	0.0001	0.0001156
CD	0.0005648	1	0.0005648	237.26	0.0000	3.306E-05
CG	0.0001622	1	0.0001622	68.14	0.0001	4.459E-05
DD	1.822E-05	1	1.822E-05	7.65	0.0278	-7.172E-05
DE	6.491E-05	1	6.491E-05	27.27	0.0012	2.273E-05
DG	3.847E-05	1	3.847E-05	16.16	0.0051	-5.815E-05
EE	3.837E-05	1	3.837E-05	16.12	0.0051	5.816E-05
EF	8.416E-05	1	8.416E-05	35.35	0.0006	-2.608E-05
EG	1.62E-05	1	1.62E-05	6.81	0.0350	2.861E-05
FF	0.0056538	1	0.0056538	2374.96	<.0001	-0.0012528
FG	0.000784	1	0.000784	329.32	<.0001	-0.0002617
GG	5.544E-05	1	5.544E-05	23.29	0.0019	-0.0007744
Total error	1.666E-05	7	2.381E-06			
Total (corr.)	0.0551693	38				
Constant						0.0761248

Table 4.24 Analysis of variance (ANOVA) and regression coefficients for the quadratic model of various physicochemical factors of *Asteracycs quadricellulare* BGLR5 for protein

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value	Regression coeffs
A:pH	0.000047	1	0.000048	89.92	0.0002	0.199305
B:Temperature	0.017558	1	0.017559	32938.76	<.0001	0.001526
C:Light intensity	0.004851	1	0.004852	9101.80	<.0001	-0.018011
D:Growth period	0.000903	1	0.000903	1693.01	<.0001	-0.008146
E:NH4Cl	0.000004	1	0.000004	8.43	0.0337	0.018670
F:NaNO3	0.025663	1	0.025663	48141.73	<.0001	0.058244
G:K2PHO4	0.036183	1	0.036183	67876.33	<.0001	0.073727
AA	0.001544	1	0.001544	2897.11	<.0001	-0.009189
AB	0.000131	1	0.000131	245.03	<.0001	-0.000164
AC	0.000933	1	0.000933	1751.25	<.0001	-0.000160
AD	0.002369	1	0.002369	4444.32	<.0001	-0.000689
AE	0.000452	1	0.000453	849.41	<.0001	0.000226
AF	0.000719	1	0.000719	1349.48	<.0001	-0.000385
BC	0.002920	1	0.002920	5477.14	<.0001	-0.000076
BD	0.000437	1	0.000437	820.00	<.0001	-0.000080
BE	0.002438	1	0.002437	4573.18	<.0001	0.000139
BF	0.000127	1	0.000127	238.68	<.0001	-0.000043
BG	0.000157	1	0.000157	294.39	<.0001	-0.000119
CC	0.005457	1	0.005457	10237.65	<.0001	0.000169
CD	0.001153	1	0.001153	2162.48	<.0001	0.000048
CE	0.000142	1	0.000142	266.50	<.0001	0.000013
CF	0.000373	1	0.000373	699.33	<.0001	0.000027
CG	0.003999	1	0.004000	7501.66	<.0001	-0.000224
DD	0.000405	1	0.000405	760.39	<.0001	0.000339
DE	0.000983	1	0.000983	1844.18	<.0001	0.000090
DF	0.000240	1	0.000240	450.58	<.0001	-0.000058
DG	0.000949	1	0.000949	1780.26	<.0001	0.000295
EE	0.003403	1	0.003403	6383.63	<.0001	-0.000548
EF	0.005399	1	0.005399	10128.09	<.0001	0.000211
EG	0.003592	1	0.003592	6737.93	<.0001	-0.000430
FF	0.017886	1	0.017886	33552.40	<.0001	-0.002229
FG	0.029923	1	0.029923	56132.48	<.0001	-0.001632
GG	0.000280	1	0.000280	525.72	<.0001	-0.001742
Total error	0.0000027	5	5.33075E-7			
Total (corr.)	0.310191	38				
Constant						-0.717817

Table 4.25 Analysis of the experimental results of Central Composite Design

Model	Biomass	Chlorophyll	Carbohydrates	Lipid	Protein
Transformation	None	None	None	none	none
Model d.f.	33	26	33	31	33
P-value	0.0000	0.0000	0.0000	0.0000	0.0000
Error d.f.	5	12	5	7	5
Stnd. error	0.00466061	0.546921	0.00207756	0.00154292	0.00073012
R-squared (%)	99.99	99.83	99.99	99.97	100.00
Adj. R-squared (%)	99.96	99.47	99.90	99.84	99.99

Table 4.26 Factor settings at optimum and response values at optimum conditions as per model

	Cultural factors						
	pH	**Temp. (°C)**	**Light intensity ($\mu molm^{-2}s^{-1}$)**	**Growth period (Days)**	**NH_4Cl (mM)**	**$NaNO_3$ (mM)**	**K_2HPO_4 (mM)**
Setting at optimum	9.91588	21.8411	80.9997	25.0	15.000	12.2766	7.09275

Response	**Optimized**	**Prediction**	**Lower 95.0% Limit**	**Upper 95.0% Limit**	**Desirability**
Biomass (gL^{-1})	Yes	1.26155	1.24649	1.27661	1.0
Chlorophyll (mgL^{-1})	Yes	34.0964	32.7324	35.4604	1.0
Carbohydrates (gL^{-1})	Yes	0.390004	0.383796	0.396212	1.0
Lipid (gL^{-1})	Yes	0.142147	0.137854	0.146441	1.0
Protein (gL^{-1})	Yes	0.435603	0.433244	0.437961	1.0
Optimized desirability	0.949137				

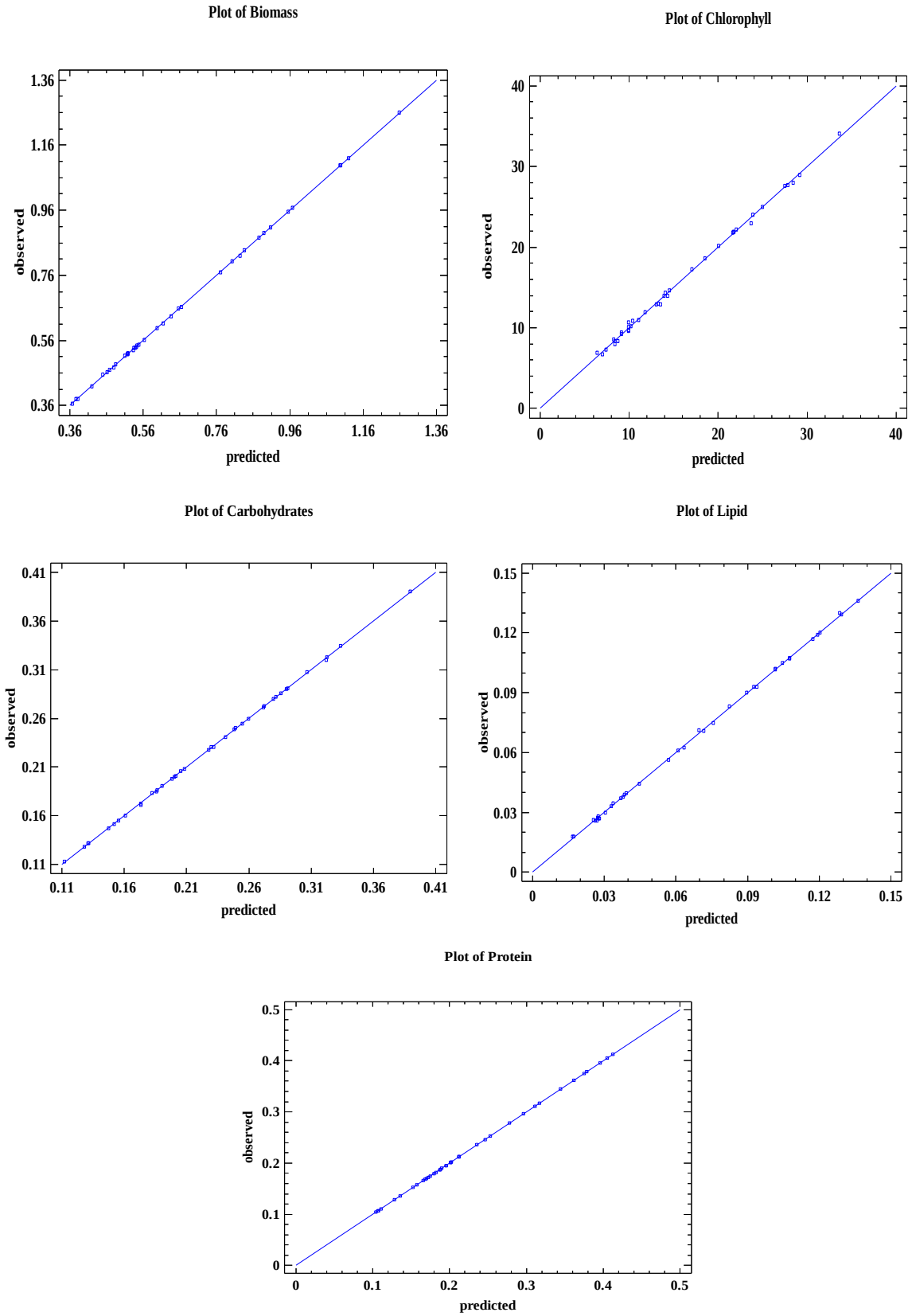
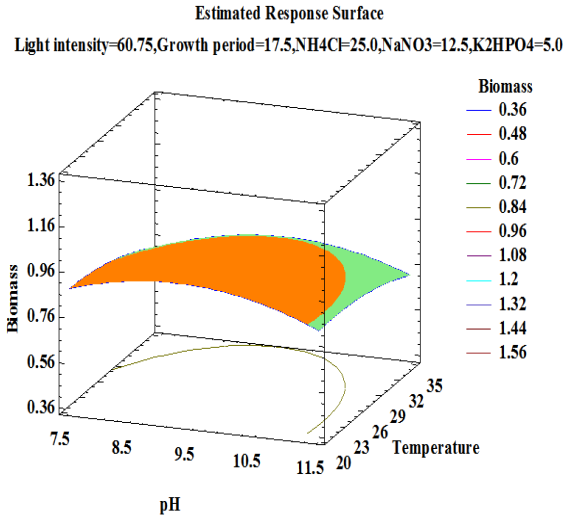


Fig. 4.10 (a-e) Observed vs predicted results of different responses: (a) biomass, (b) chlorophyll, (c) carbohydrate, (d) lipid and (e) protein

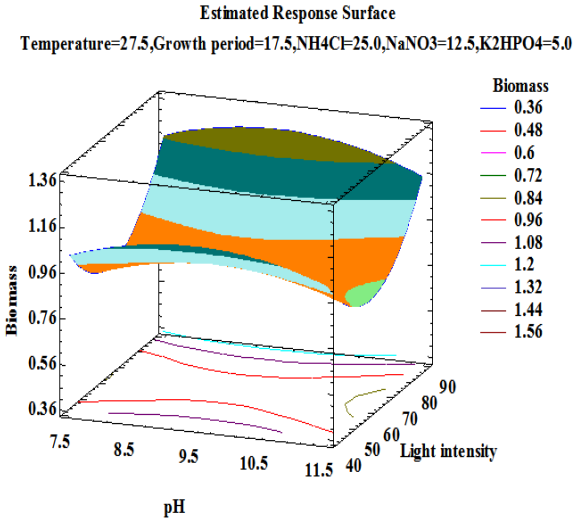
4.6.8 3D Response surface plots for various interactions of cultural factors for biomass production

The response surface plots illustrate the interaction effects and optimum levels of the factors. These are generated by varying the two variables whereas keeping others fixed. Figure 4.11a depicts biomass production as a function of pH and temperature. The plot shows that maximum biomass production reached at the middle value of pH range chosen in the study and at the lower temperature. In case of pH and light intensity interaction (Fig. 4.11b), the the highest biomass was obtained at the moderate range of pH and the higher light intensity range. Figure 4.11c represents the interaction of pH and growth period and it was noticed from the surface plot that the highest biomass production was obtained at the moderate range of both variables and similarly for pH and NH_4Cl interaction, the highest response was noted in the moderate range of both the variables (Fig. 4.11d). The interaction between pH and $NaNO_3$ while keeping other factors fixed at the optimum level resulted in the higher biomass production at middle range of both the variables and further it was seen that the biomass production decreased abruptly on increasing the concentration of $NaNO_3$ (Fig. 4.11e). Higher concentration and higher pH were found to have negative effect on biomass yield while keeping others fixed at the optimum value. Figure 4.11f shows that the highest biomass yield was obtained at middle range of pH and at the higher levels of K_2HPO_4. The lower temperature and higher light intensity were found to be favourable for the higher biomass production (Fig. 4.11g). The biomass was observed to abruptly increase with the increase in light intensity. Similarly the figure 4.6 h depicts that the lower temperature results in higher biomass and increase in growth period was not found to have prominent effect on yield but a small decrease was noticed on increasing the growth period after 22 days. The middle range is optimum as per the plot for biomass production. Figure 4.11i represents the interaction between temperature and NH_4Cl and it shows that lower temperature is favourable while NH_4Cl was not found to have some prominent effect on biomass, though the middle range was found to produce slightly increased biomass production. The interaction of K_2HPO_4 and temperature revealed that the lower range 20 -26 °C and higher K_2HPO_4 levels were found to have positive effect on biomass production. The biomass increases with increase in the K_2HPO_4 concentration (Fig. 4.11j). The interaction between light intensity and growth period (Fig. 4.11k) predicted that a slight increase was noticed on increasing the growth period while as biomass increases abruptly on increasing the light intensity, although here a decrease was noticed in the moderate range of light intensity (50-60 $\mu molm^{-2}s^{-1}$**)**, but after that it increased the biomass on increasing light intensity. The effect of light interaction between

light intensity and NH_4Cl (Fig. 4.11l) is similar to that of the light intensity and growth period, lower and higher intensity of light were found to be favourable, although higher intensity resulted in more biomass production but incase of NH_4Cl a slight decrease at the higher concentration (35 Mm) was noticed. The interaction between light intensity and $NaNO_3$ was found significant as is obvious from Table 4.20. It is clearly depicted in the response surface plot and contours below (Fig. 4.11m) that the high light intensity and moderate $NaNO_3$ has positive effect in biomass production provided other factors are kept at central or optimum level. The contour lines plotted on the base of surface plots below are curvilinear and thus depict that the interaction is quite significant. Growth period and NH_4Cl interaction was significant as it has p value <.0001. The maximum biomass can be produced at the middle range of growth period while decrease or increase in NH_4Cl is not found to have any positive or negative effect on the biomass production as shown in Fig. 4.11n. The 3D surface plot with contours at the base for growth period versus $NaNO_3$ (Fig. 4.11o) clearly illustrates that $NaNO_3$ and growth period at moderate range increases the biomass genesis while the growth period was found to have slight positive impact on biomass genesis. The greater impact can be found at 13-22 days growth period and $NaNO_3$ concentration ranging from 18-17 mM $NaNO_3$. Fig 4.11p illustrates the interaction between the two factors growth period and K_2HPO_4. The p value for this interaction is 0.0002 signifying that it is quite significant. Biomass increased from 0.72-1.2 gL^{-1} when K_2HPO_4 concentration raises from 2-8 mM, but maximum production of biomass was found at 7-8 mM K_2HPO_4 concentration. Fig. 4.11q depicts the significant ineration between NH_4Cl and $NaNO_3$ as p value is 0.0407. The surface plot with contours below shows that the higher biomass can be achieved at low levels (15-19 mM) of NH_4Cl and middle levels (11-17 mM) of $NaNO_3$. The interaction between NH_4Cl and K_2HPO_4 represented by Figure 4.11r was found to be significant as p value is <.0001. The NH_4Cl was found to affect the biomass production slighltly whereas on increasing K_2HPO_4 concentration, the biomass was found to be increasing. The highest biomass while varying the concentration of these two variables keeping other variables fixed was obtained at 15-19 mM NH_4Cl and 6-8 mM K_2HPO_4. The response surface plot (Fig. 4.11s) illustrates the interaction between $NaNO_3$ and K_2HPO_4 while keeping other factors constant at optimal level. The interaction is statistically significant as p value is <.0001. Moderate levels of both the variables were found to be suitable for highest biomass production.

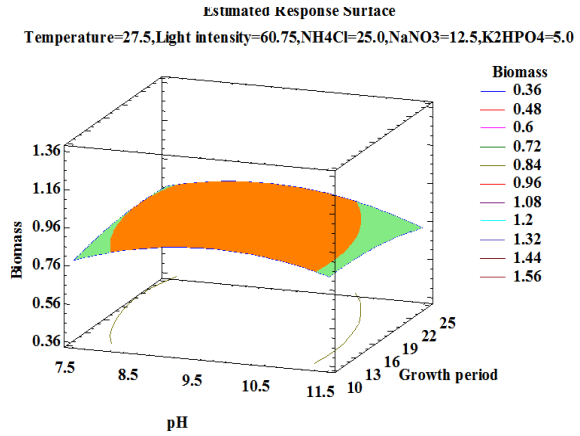


(a) pH versus temperature interaction

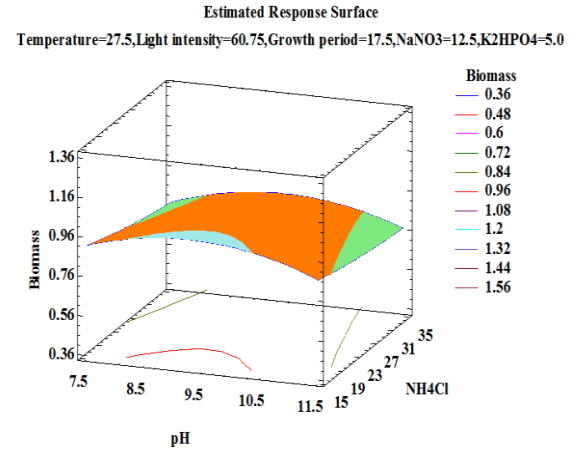


(b) pH versus light intensity interaction

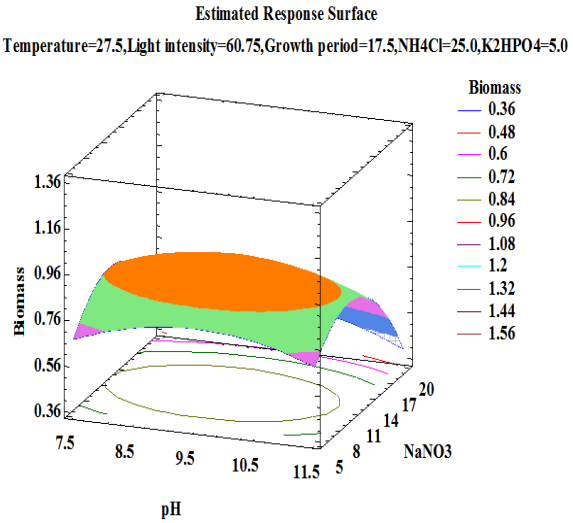
Highest biomass was



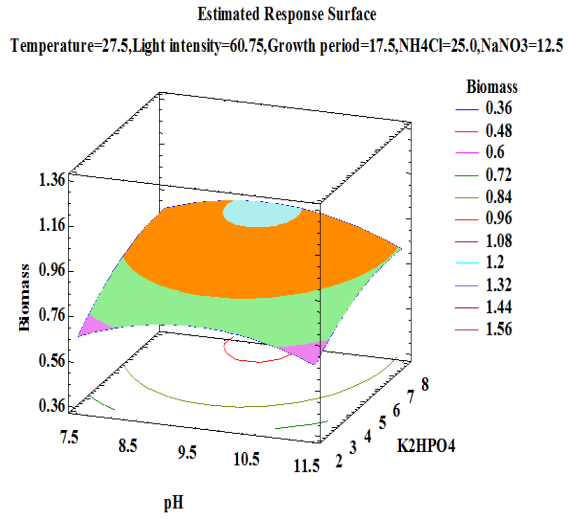
(c) pH versus growth period interaction



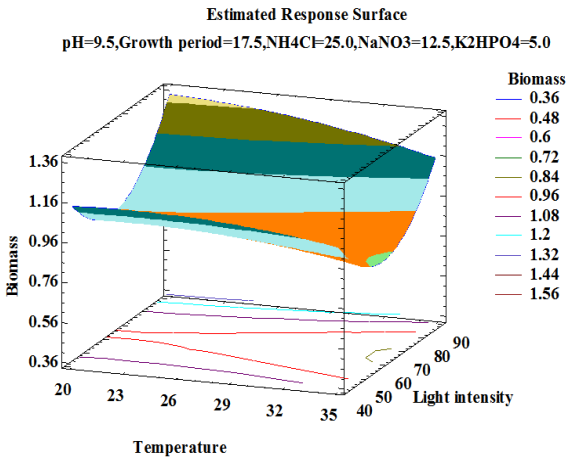
(d) pH versus NH_4Cl interaction



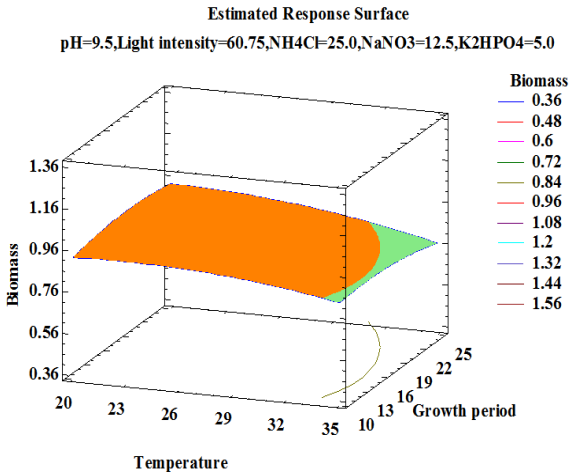
(e) pH versus $NaNO_3$ interaction



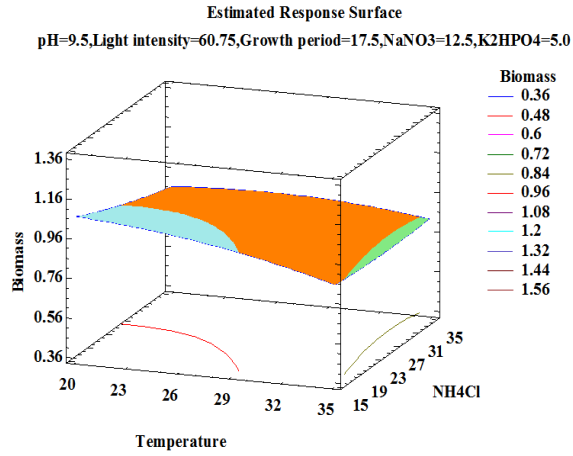
(f) pH versus K_2HPO_4 interaction



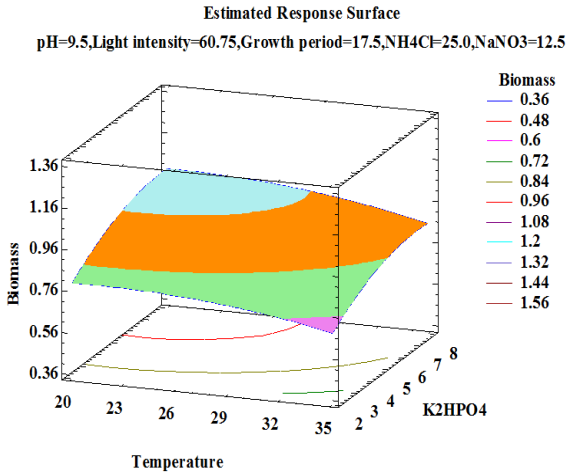
(g) temperature versus light intensity



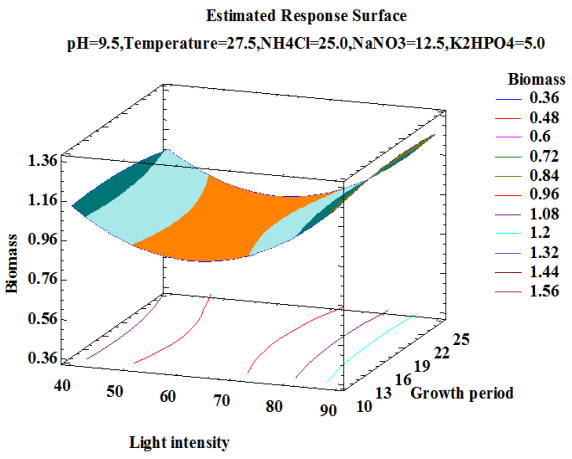
(h) temperature versus growth period



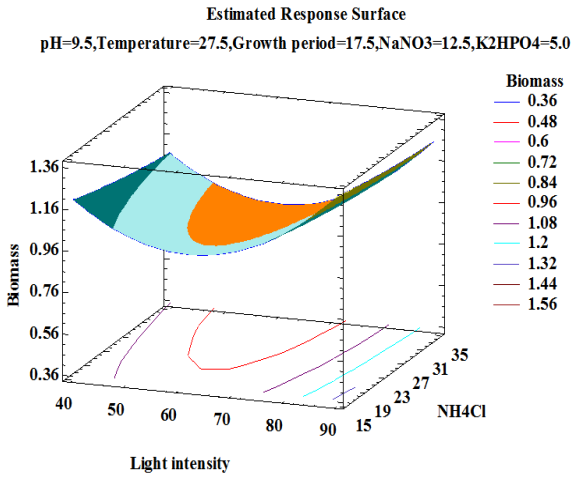
(i) temperature versus NH_4Cl



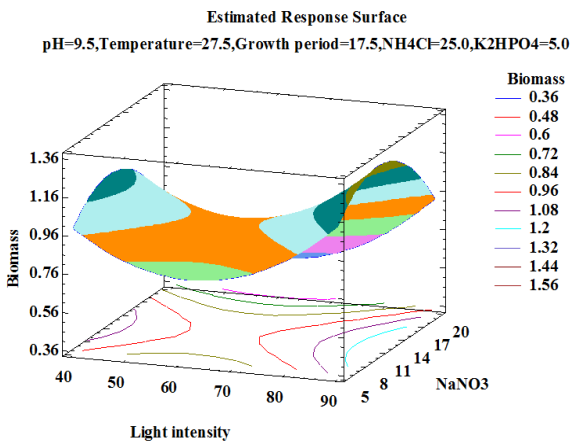
(j) temperature versus K_2HPO_4



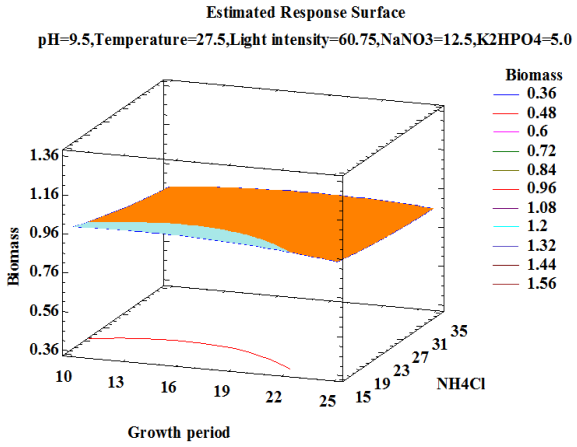
(k) light intensity versus growth period



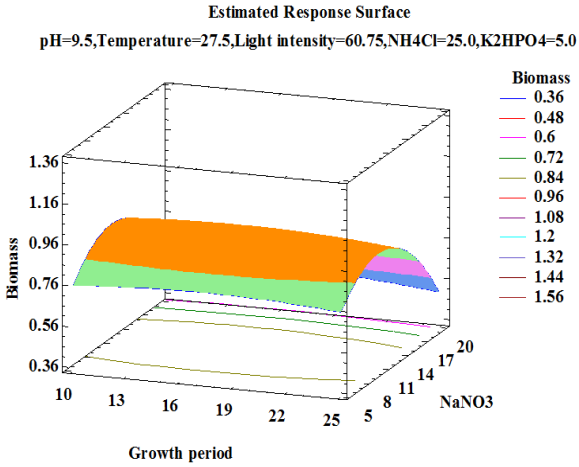
(l) light intensity versus NH_4Cl



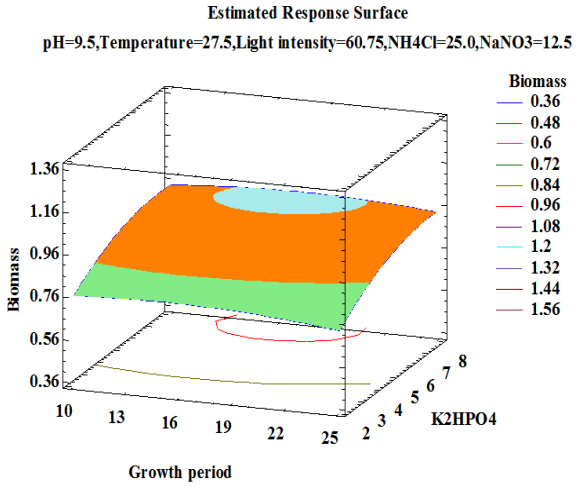
(m) light intensity versus $NaNO_3$



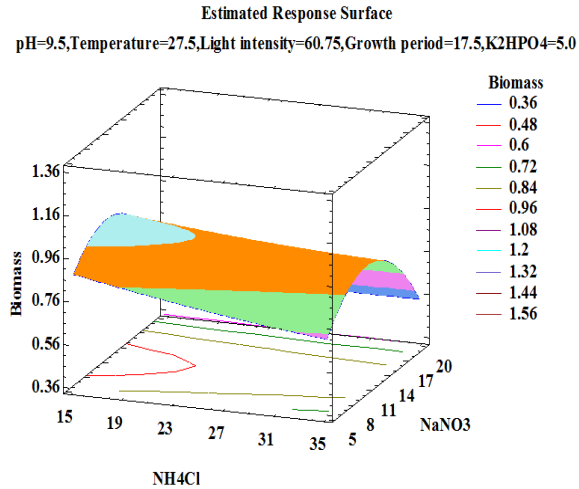
(n) growth period versus NH_4Cl



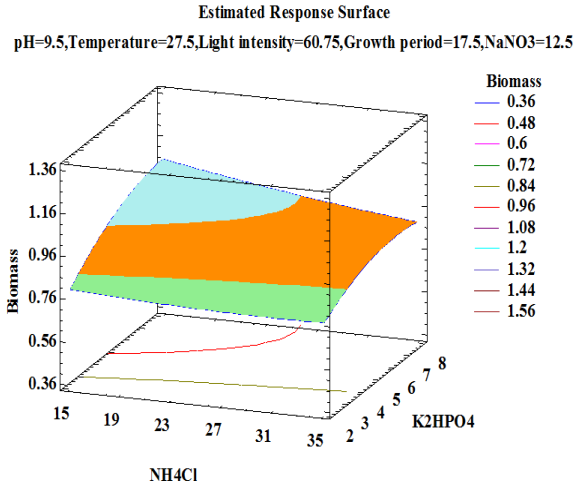
(o) growth period versus $NaNO_3$



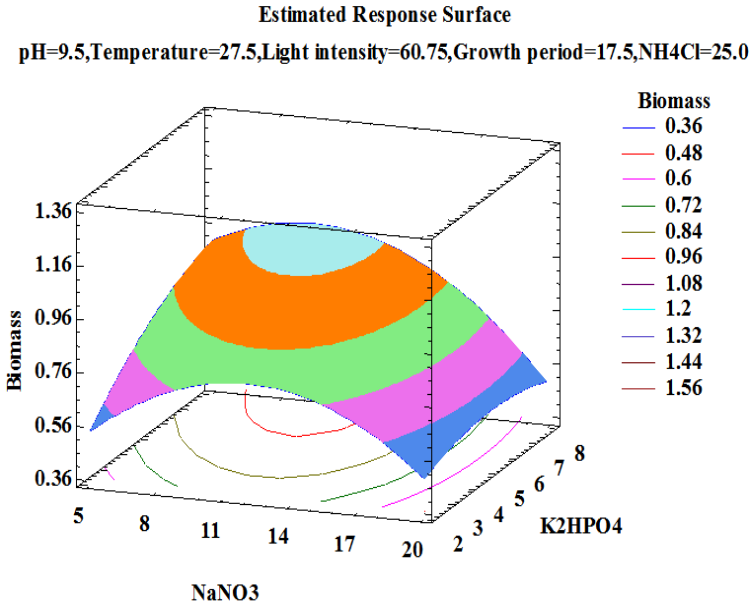
(p) growth period versus K_2HPO_4



(q) NH_4Cl versus NaNO3



(r) NH_4Cl versus K_2HPO_4



(s) $NaNO_3$ versus K_2HPO_4

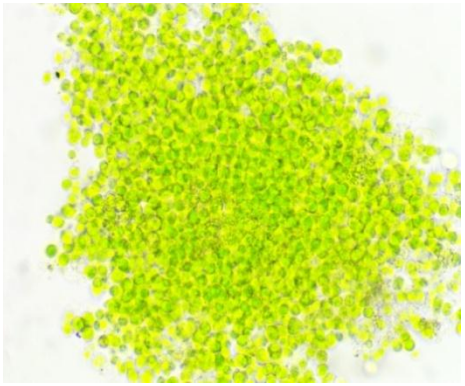
Fig. 4.11(a-s) Response surface 3D plots for various interactions of cultural factors for biomass

4.7 Biogas potential of algal biomass

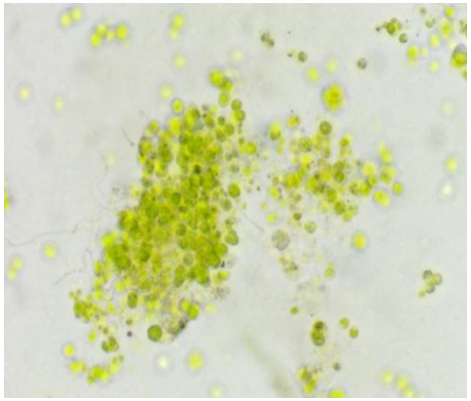
Biogas potential of the microalgal biomass of both strains was determined by biochemical methane potential test (BMP) protocol (Alzate *et al* 2012). An important issue when dealing with microalgae anaerobic digestion is its cell wall. It is mostly composed of organic compounds with low biodegradability and/or bioavailability, such as cellulose and hemicellulose. This tough cell wall hinders the methane production, since organic matter retained in the cytoplasm is not easily accessible to anaerobic bacteria. This is not an isolated case for microalgae, many other organic substrates, such as waste activated sludge and lignocellulosic biomass consist of a complex structure, which hampers the hydrolysis rate in the anaerobic digestion process (Hendriks and Zeeman 2009, Carrère *et al* 2010, Carlsson *et al* 2012, De la Rubia *et al* 2013). For this reason, pretreatment techniques have been used to solubilize particulate biomass and improve the anaerobic digestion rate and extent. The algal biomass of both the strains was given enzymatic and hydrothermal pretretment. Pretreatment techniques were pointed out as a necessary step for microalgae cell disruption and biogas production by Chen and Oswald (1998). The effectiveness of pretreatment methods on biogas production depends on the characteristics of microalgae, i.e., the toughness and structure of the cell wall, and the macromolecular composition of cells. After pretreatment, the biomass was subjected to anaerobic digestion for biogas production. The untreated biomass was run in parallel to act as control.

4.7.1 Effect of enzymatic and hydrothermal pretreatment on algal biomass

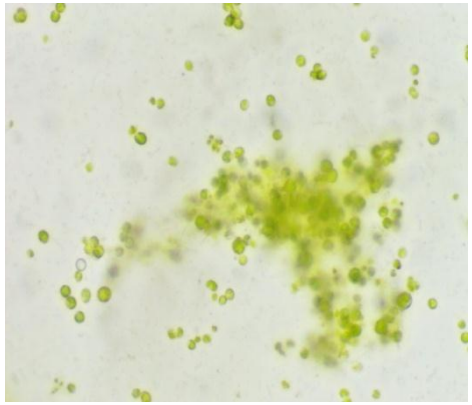
The enzyme pretreatment was carried out by using an enzyme mix (cellulose, hemicellulose and protease). Two enzyme concentrations viz., 10 and 20 % (w/w) were each applied for 12 h and 24 h to the biomass of the both algal strains. Similarly, hydrothermal was carried out at 100 °C and 120 °C for the same exposure time of 30 min. After both the pretreaments, depigmentation was noticed in all the sets. The algal biomass of both the strains *Aterarcycs quadricellulare* BGLR5 and *Spirulina sabsalsa* BGLR6 turned yellow in colour and sort of aggregation was also observed. In case of enzymatic pretreatment, the depigmentation was found more as both the exposure time and dose were increased. However over all, the highest depigmentation occurred in hydrothermal pretreatement. The cells of BGLR5 got constricted, shrinked on hydrothermal treatment and formed more prominent aggregates than enzymatcally pretreated cells in both the microalgal strains. The depigmentation and aggregation are the indications of the damage of the cell wall. The probable reason of this aggregation of microalgal cells might be the release of the cellular content through enzymatic action on cell wall. Our results are in line to those of the various researchers who too observed the aggregation of algal cells on enzymatic pretreatment (Gerken *et al* 2013, Prajapati *et al* 2015). In case of *Spirulina sabsalsa* BGLR6, uncoiling started from the 20 % 24 h enzymatic pretreatment and coiled structure got disintegrated. In 10 % 24h pretreatment, the rod and coiled structure was intact, but got grouped together. However, hydrothermal pretreatment damaged the cells in both, the structure of algal cells was lost, as if it appeared that the cell wall was disrupted. Also more of the aggregation was noticed in *Spirulina sabsalsa* BGLR6 biomass. These things can be better seen from optical microscopy images (Fig. 4.12 a-g and 4.14 a-g) as well as the SEM images (Fig. 4.13 a-e, 4.15 a-e). These images supported well the visual findings. The other researchers too found that hydrothermal pretreatment led to the disruption of the cell wall of algal cells (Schwede *et al* 2013, Passos *and* Ferrer 2014).



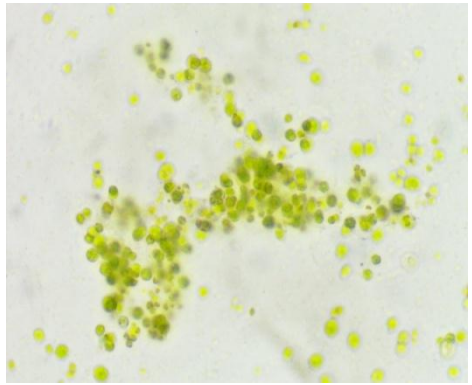
a) Untreated BGLR5



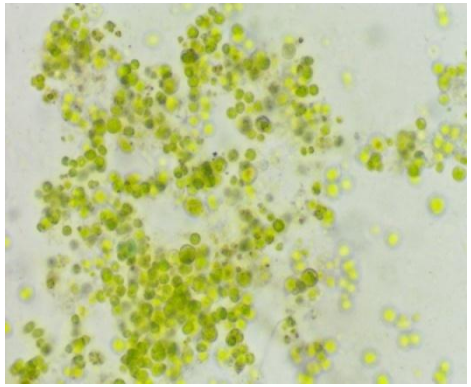
b) 10 % 12h



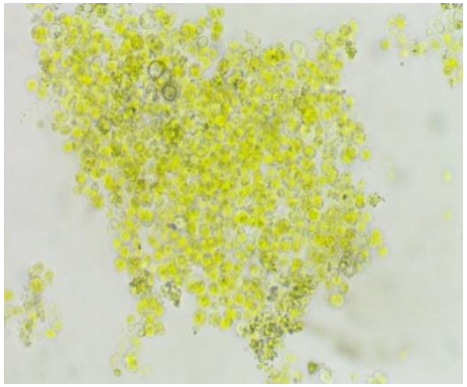
c) 20 % 12h



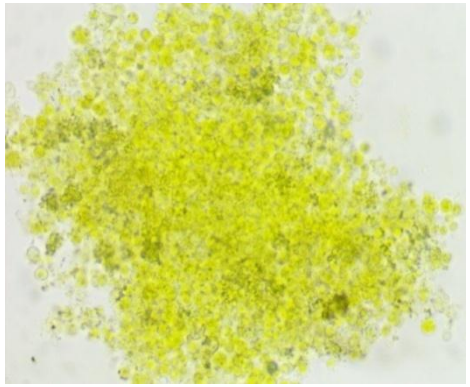
d) 10 % 24h



e) 20 % 24h

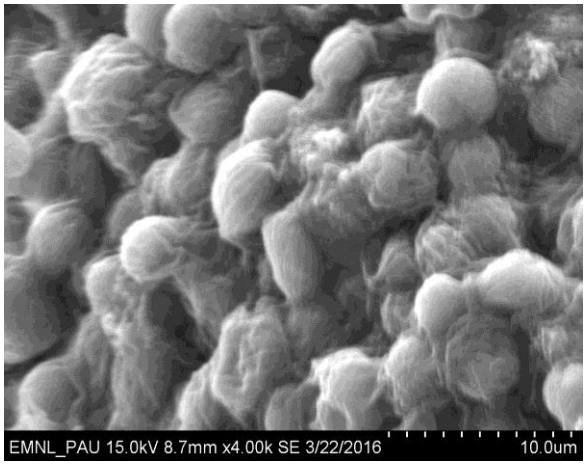


f) 100°C 30min

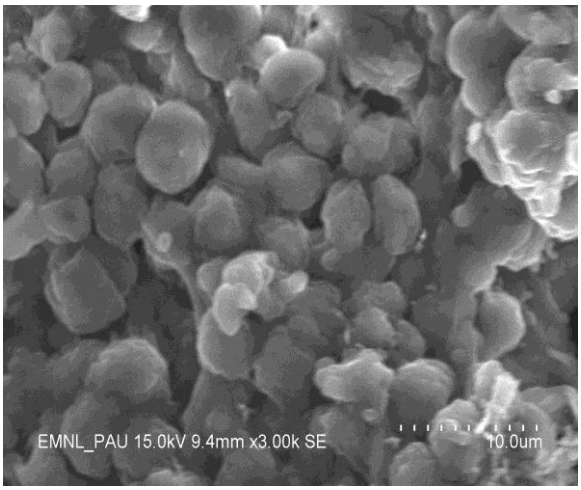


g) 120°C 30min

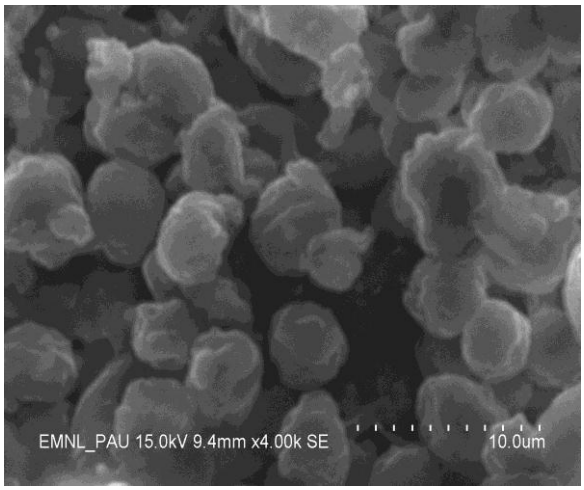
Fig. 4.12 (a-g) Optical microscopy images of BGLR5 biomass pretreatment (Enzymatic (b,c,d e) and hydrothermal pretreatmen (f, g))



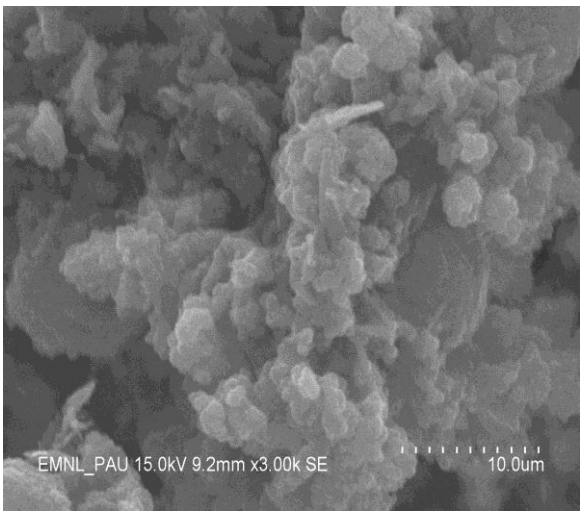
a) Untreated BGLR5



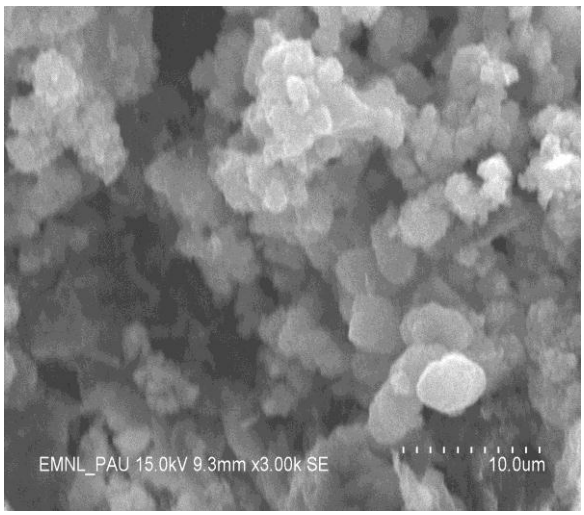
b) 10 % 24h



c) 20 % 24h

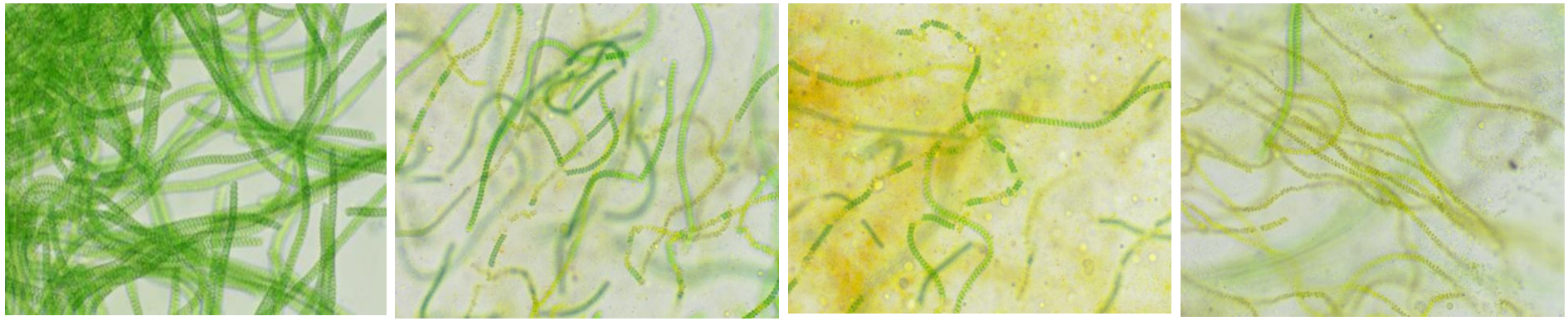


d) 100°C 30min

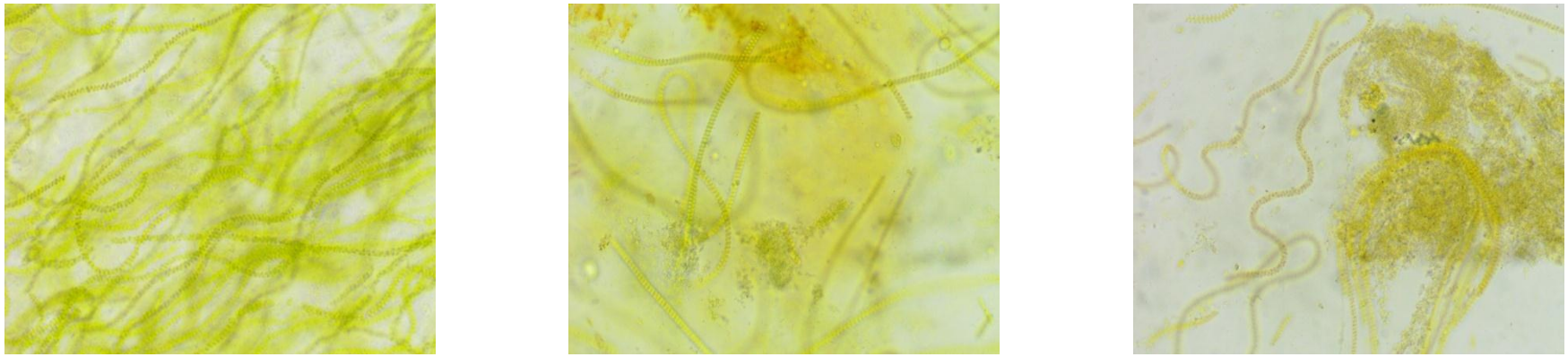


e) 120°C 30min

Fig. 4.13 (a-e) SEM images of BGLR5 biomass pretreatment (Enzymatic (b, c) and hydrothermal pretreatment (d,e))

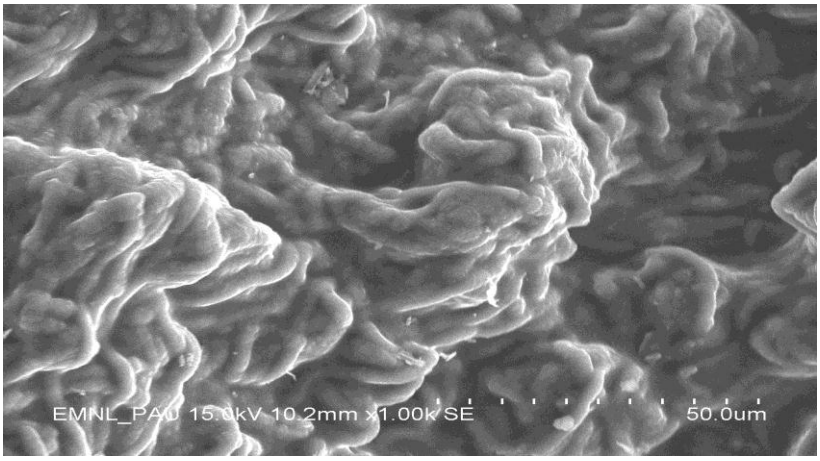


a) Untreated BGLR5 **b) 10 % 12h** **c) 20 % 12h** d) **10 % 24h**

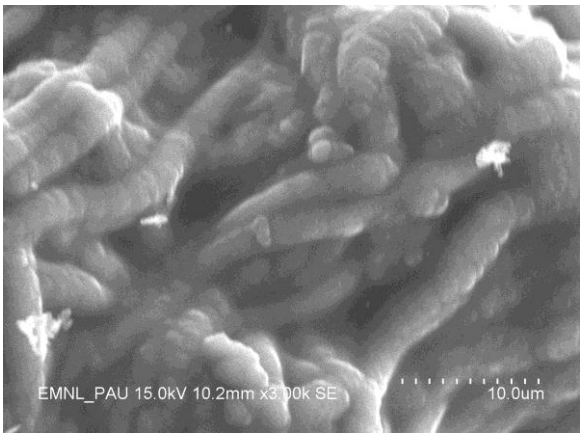


e) 20 % 24h **f) 100°C 30min** **g) 120°C 30min**

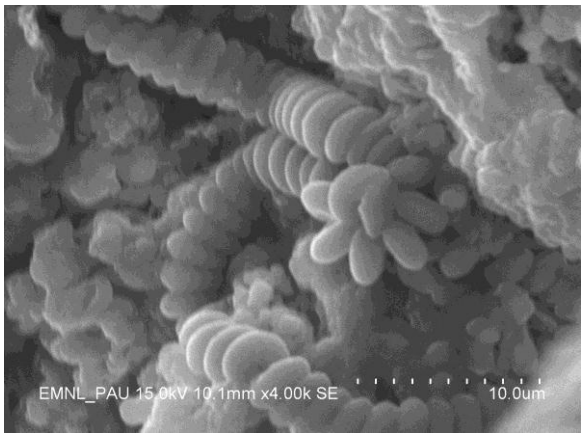
Fig. 4.14 (a-g) Optical microscopy images of BGLR6 biomass pretreatment (Enzymatic (b, c, d, e) and hydrothermal pretreatment (f, g))



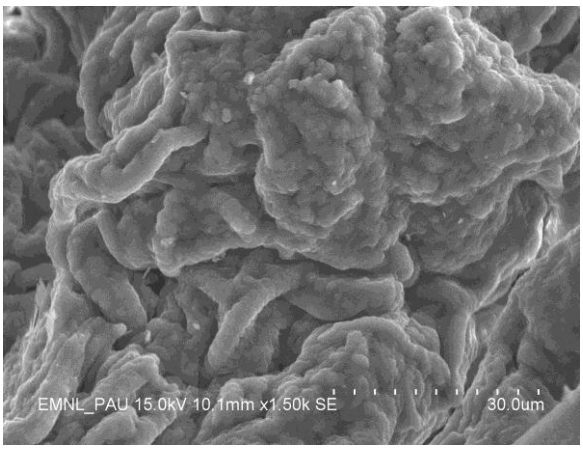
a) Untreated BGLR6



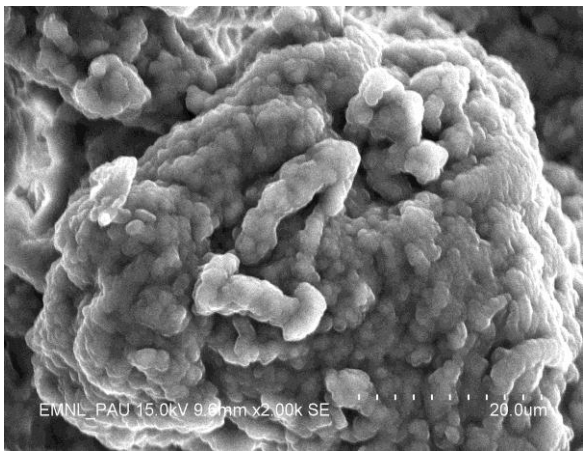
b) 10 % 24h



c) 20 % 24h



d) 100°C 30min



e) 120°C 30min

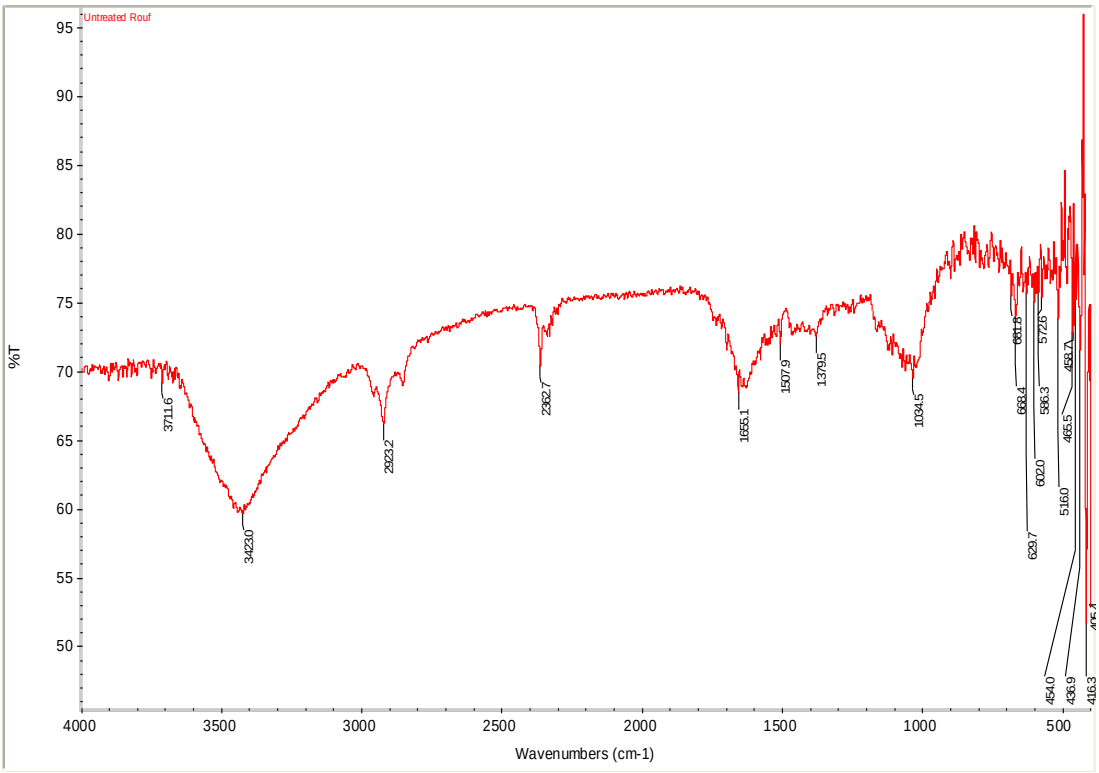
Fig. 4.15 (a-e) SEM images of BGLR5 biomass pretreatment (Enzymatic (b.c) and hydrothermal pretreatment (d.e))

4.7.2 Characterization of pretraeted microalgae biomass (BGLR5 and BGLR6) by FT-IR

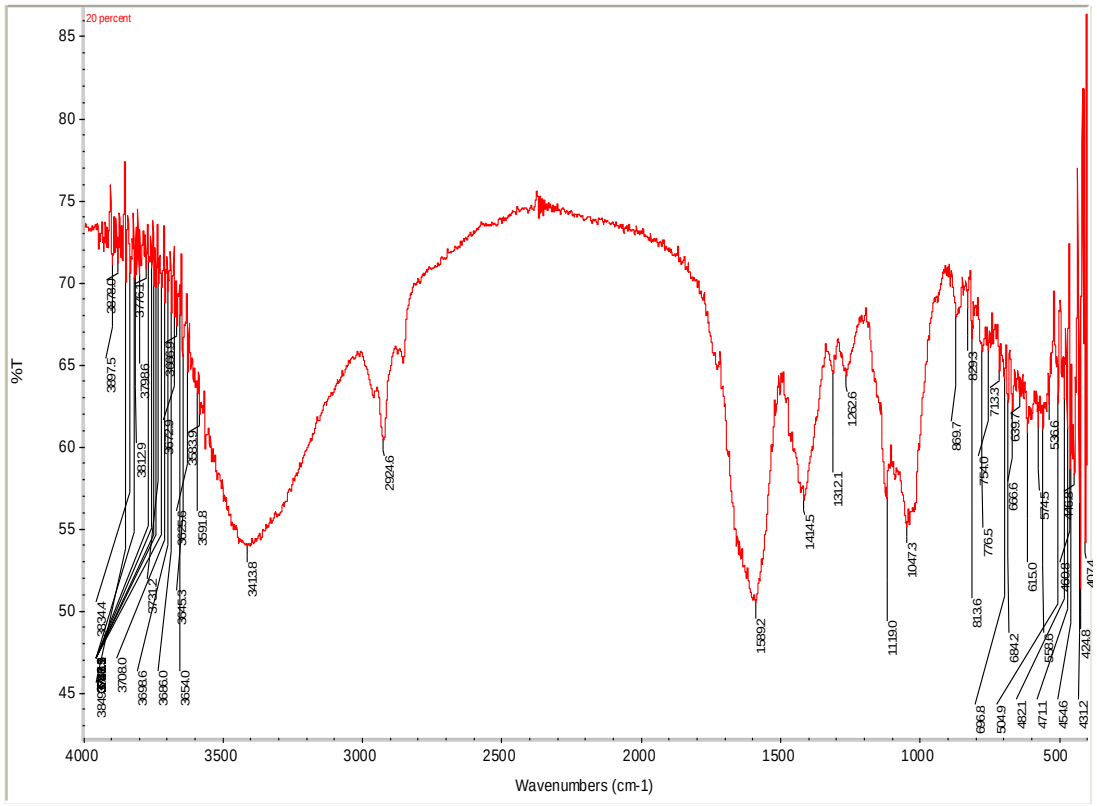
FT-IR is one of the easiest methods with simple method of sample preparation. It has been suggested as one of the advanced tools for obtaining information about biomass structure and chemical changes taking place during pretreatments (Fan *et al* 2012, Monlau *et al* 2012, Salehian *et al* 2013). It provides only relative values and not the absolute values, as FT-IR spectra involves contributions from both amorphous and crystalline regions (Fan *et al* 2012). The fingerprint regions of the FT-IR spectra of raw and pretreated microalgal biomass of BGLR5 and BGLR6 cultures are presented in Fig. 4.16 (a-d) and 4.17 (a-e). This FT-IR analysis of the samples was carried out in order to illustrate whether the pretreatments have affected proteins, carbohydrates and cellulose present in the algal cells, as cellulose has been found to be the main component creating trouble in degrading the microalgae cells. The spectra obtained for pretreated microalgal biomass of BGLR5 (Fig. 4.16 a-d) showed different peak intensities at particular wave numbers depending on the pretreatment. The majority of the variations were observed in the spectral range of 4000-3400 cm^{-1} and 1700-500 cm^{-1}. The spectral region between 3000-1700 cm^{-1} also showed variation between the samples. Here in this region an upshift was observed with respect to control (untreated one). Strong absorption bands at approximately 3400-3200, 2923.2, 1651, 1484.4, 1409.9, 1050.9 and 872.0 cm^{-1} was observed in the spectra of pretreated microalgal samples. The region between correspond to the symmetric stretching of O-H and N-H bonds of proteins; whereas other peaks correspond to assymmetric stretching of CH_2 of lipids and symmetric stretching of CH_2 of carbohydrates; symmetric stretching of C=O of protein amide I; symmetric stretching of C-N and deformation of N-H bonds of protein amide II; symmetric deformation (bend) of protein CH_2, symmetric deformation (bend) of CH_3 of carboxylic acids, symmetric stretching of C-O of COO^- of carboxylates and symmetric deformation (bending of methyl) of ($N(CH_3)_3$) of lipids; symmetric stretching of C-O-C of polysaccharides respectively (Sigee *et al* 2002, Benning *et al* 2004, Movasaghi *et al* 2008, Murdock and Wetzel 2009, Duygu *et al* 2012, Ramos *et al* 2013). The downshifts were observed in the spectral regions 3423-3407, 29232, 23627, 1655.1-800 cm^{-1}. The downshifts were more in case of 20% 24 h and 120 °C 30min pretreated samples than 100 °C 30 min and untreated one. As FT-IR spectras clearly determined that the untreated sample showed small bands in the 3423-3407, 29232, 23627, 1655.1-800 cm^{-1} whereas the intensity of the downward shift was high in case of pretreated samples, thus clearly predicted that the pretreatments affected the carbohydrate and protein structures of the cells. Also it was found that the bands observed within the region 980-1119.0 were having

good intensity compared to untreated one, thus establishing that the cellulose structure too was affected by the pretreaments given. The BGLR5 being the *Aterarcycs quadricellulare*, a green algae, has cell wall made up of primarily cellulose and pectin (Murdock and Wetzel 2009). Therefore, it can be concluded that the cell wall of BGLR5 was affected by the given pretreatments.

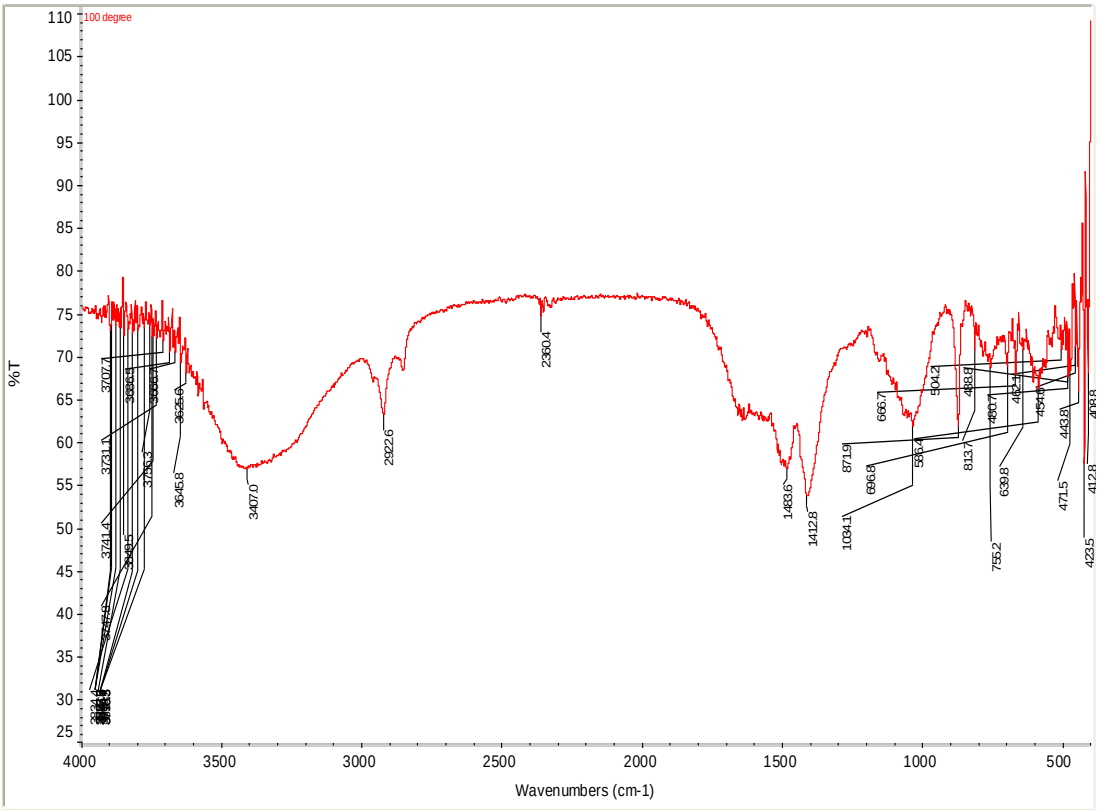
The unique appearances of absorption spectra from untreated and pretreated BGLR6 biomass samples shown in Figure 4.17 had 5-7 clear bands over the wave number range 4000-500 cm^{-1}. These bands were identified tentatively in accordance to the available literature (Sigee *et al* 2002, Benning *et al* 2004, Movasaghi *et al* 2008, Murdock and Wetzel 2009, Duygu *et al* 2012, Ramos *et al* 2013). A functional group was allotted to each peak. Mostly a downshift was observed at all peaks in all the samples except the enzymatically pretreated samples (10% 24 h and 20% 24 h) wherein an upshift was noted approximately at the peak 1408.1 cm^{-1}. The absorption bands at approximately 3406.0, 2924.2, 1668.6, 1408.1, 1056.7 and 871.3 cm^{-1} was observed in the spectra of pretreated microalgal samples. These peaks correspond to stretching of O-H and N-H bonds (amide I) of proteins; assymmetric stretching of CH_2 of lipids and symmetric stretching of CH_2 of carbohydrates; mainly stretching of C=O of protein amide I; symmetric deformation (bend) of CH_2 of protein, symmetric deformation (bend) of CH_3 of carboxylic acids, symmetric stretching of C-O of COO^- of carboxylates and symmetric deformation (bending of methyl) of $(N(CH_3)_3)$ of lipids; symmetric stretching of C-O-C of polysaccharides respectively. Also a general downshift was observed in all the samples with respect to untreated one in the region 2850-1700 cm^{-1} which mainly represents lpids, carbohydrates and proteins (Duygu *et al* 2012). So, from these spectras it was determined that the treatment affected mostly the protein and carbohydrate structures of the microalgal cells. The intensity of bands at approximately 1408.1 and 1056.7 cm^{-1} in all the pretreated samples was high compared to the control, thus depicting that the proteins and carbohydrates were greatly affected. We know the fact that the cell wall of cyanobacteria is composed of peptidoglycan (polymer of sugars and amino acids) (Murdock and Wetzel 2009), thus we can say that the cell wall of BGLR6 was affected by the preteatments.



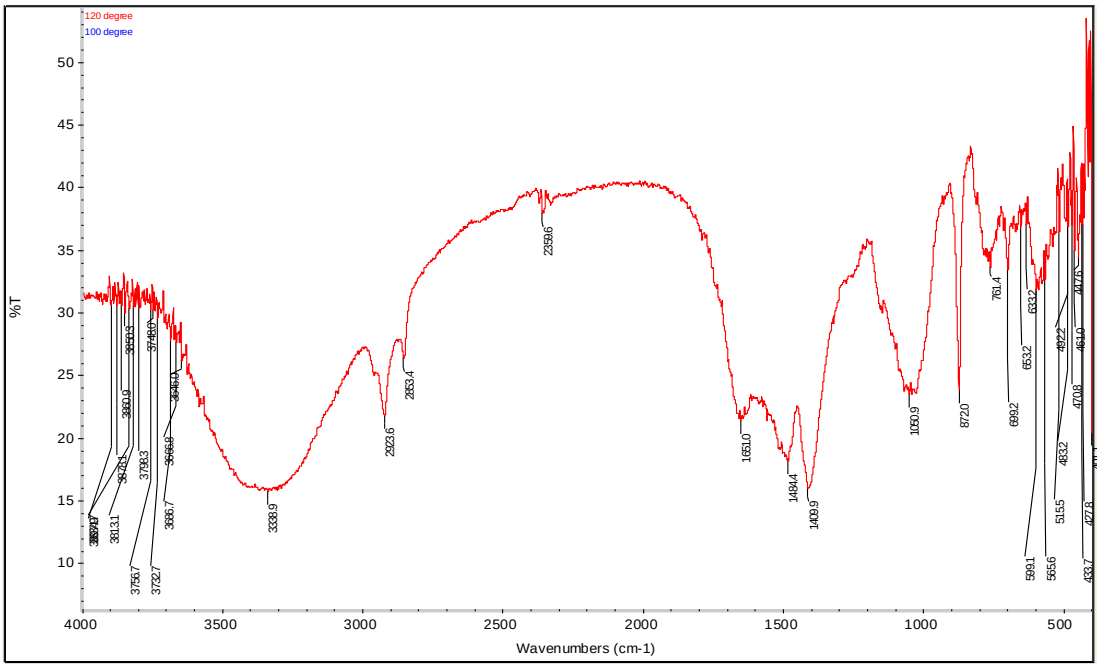
(a) FTIR Spectra of Untreated BGLR5



(b) 5BGLR5 20% 24 h

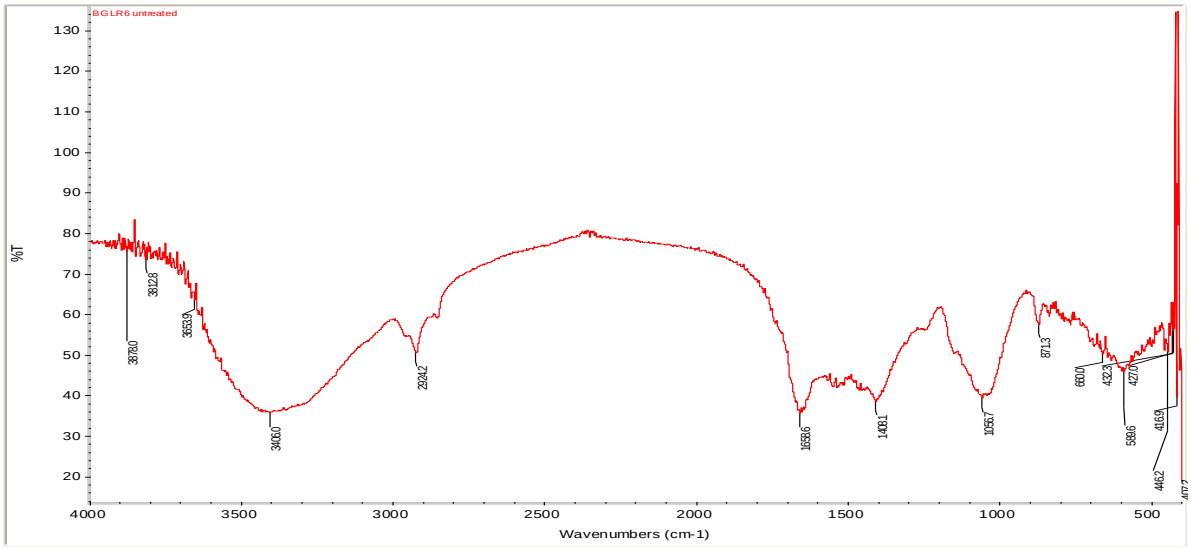


(c) BGLR5 100°C 30min

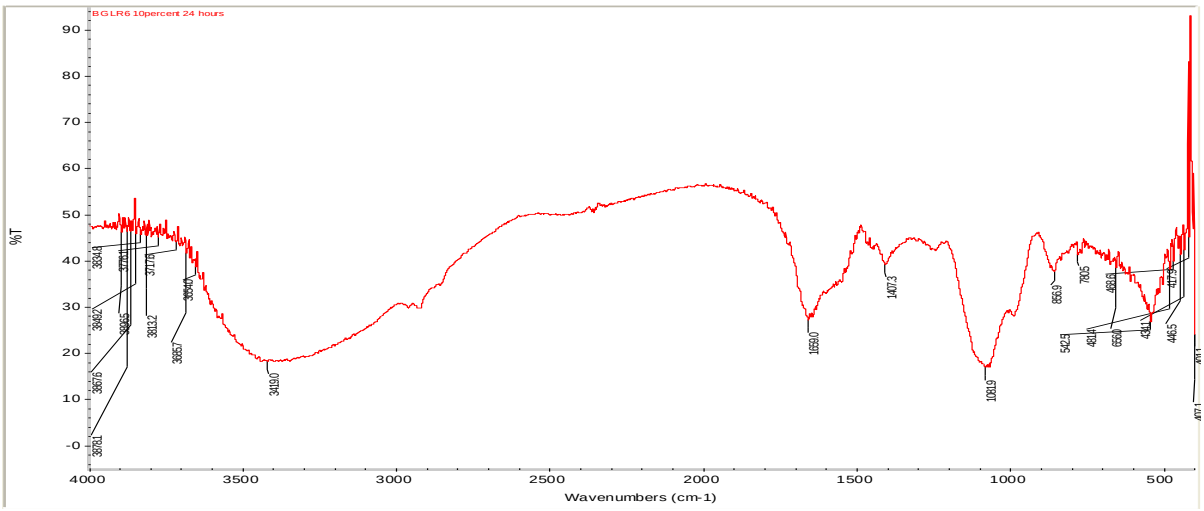


(d) BGLR5 120°C 30min

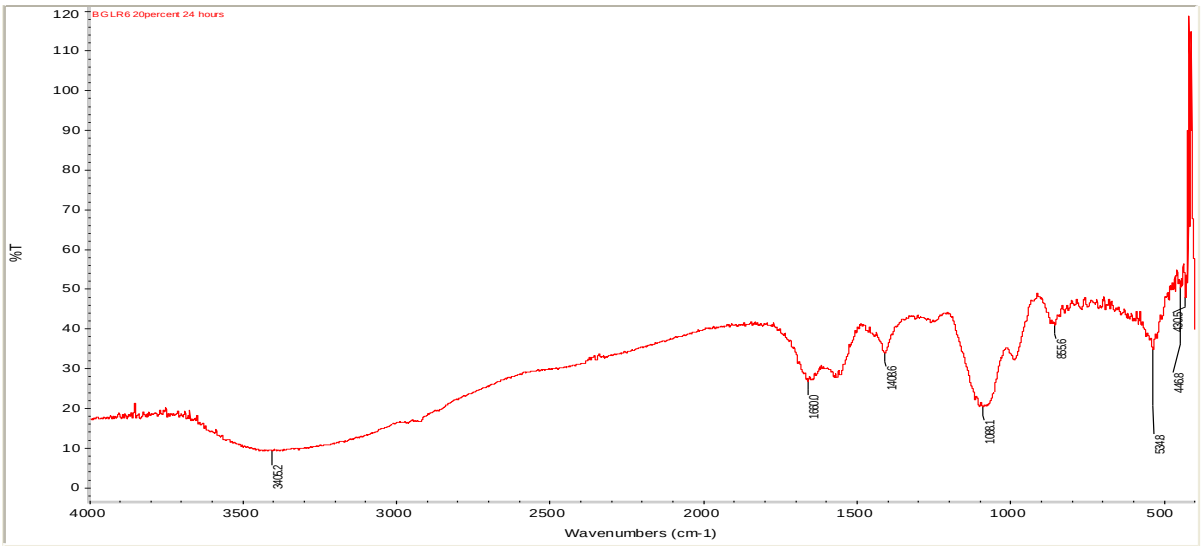
Fig. 4.16 (a-d) Infrared absorption spectra (cm^{-1}) of pretreated BGLR5 biomass, (a) Untreated one, (b) Enzymatically pretreated biomass (20% 24 h), (c, d) hydrothermally pretreated biomass (100°C 30min and 120°C 30min).



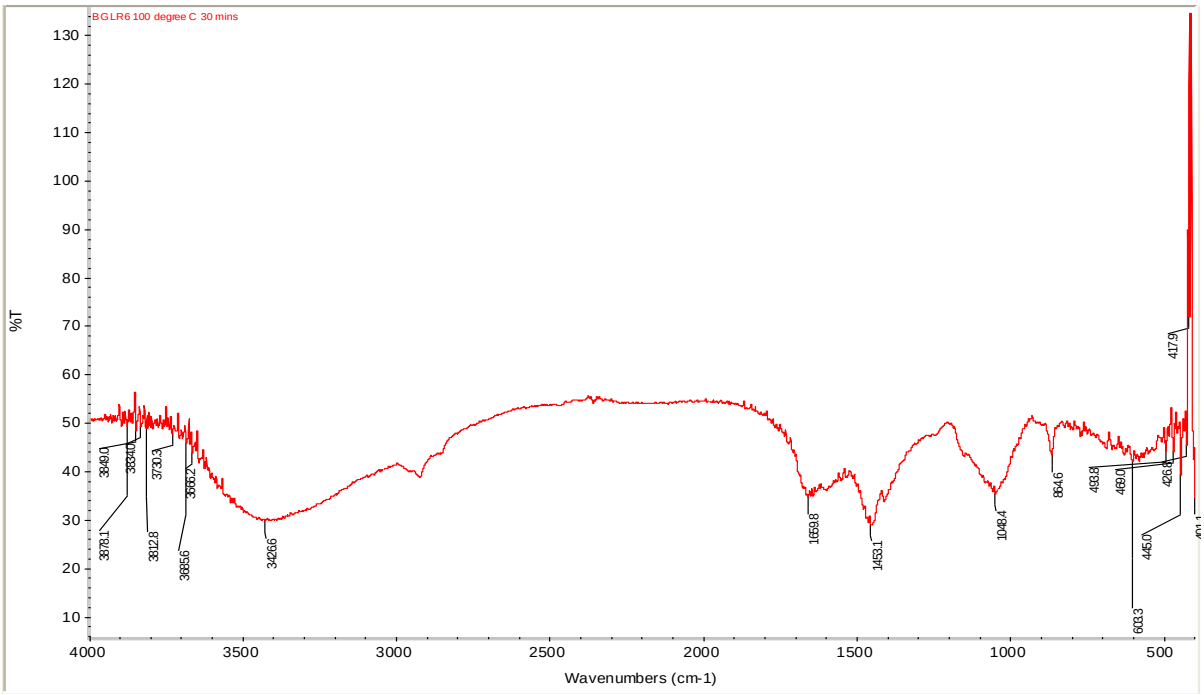
(a) FTIR Spectra of Untreated BGLR6



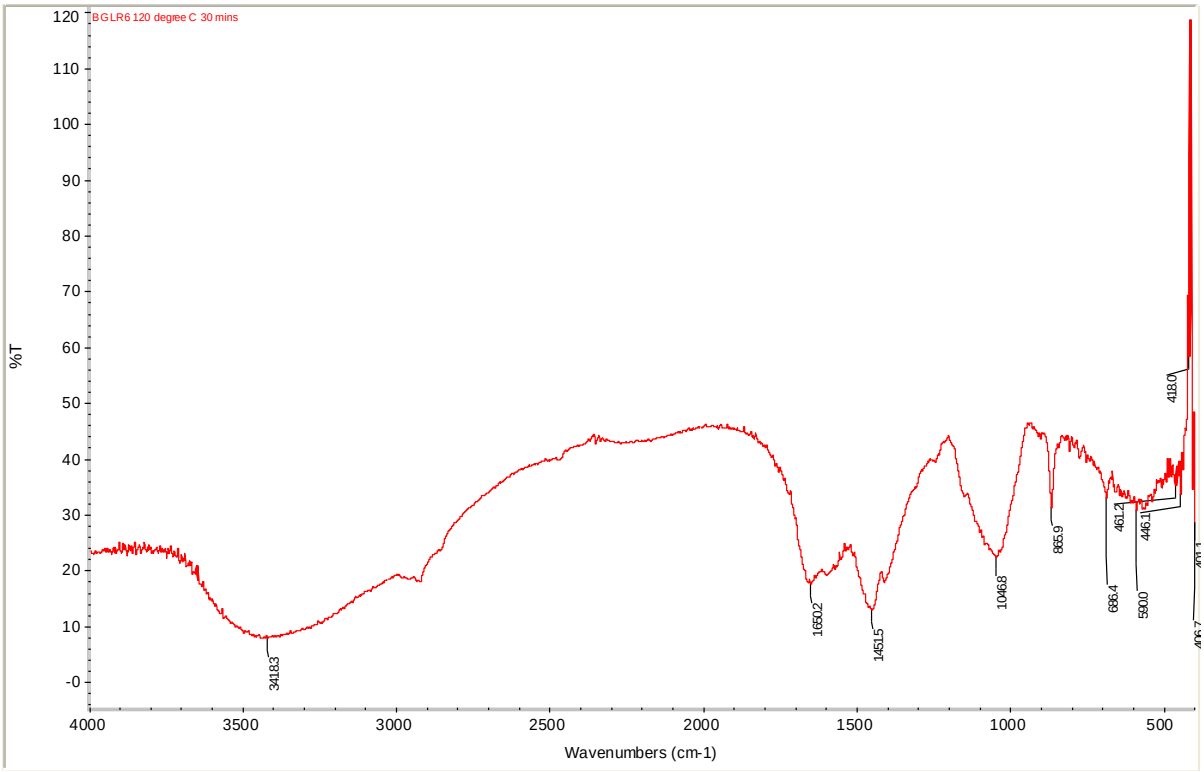
(b) BGLR6 10% 24 h



(c) BGLR6 20% 24 h



(d) BGLR6 100°C 30min



(e) BGLR6 100°C 30min

Fig.4.17 (a-e) Infrared absorption spectra (cm^{-1}) of pretreated BGLR6 biomass, (a) Untreated one, (b) Enzymatically pretreated biomass (10% 24 h), (c) Enzymatically pretreated biomass (20% 24 h), (d, e) hydrothermally pretreated biomass (100°C 30min and 120°C 30min)

4.7.3 Biogas production potential of microalgae biomass

The biogas production potential of algal biomass (*Aterarcycs quadricellulare* BGLR5 and *Spirulina sabsalsa* BGLR6) was measured under controlled temperature (35 ± 2°C) conditions for 30 days. A total of seven digesters (A-G) as shown in Table 4.27 and 4.28 were established for each strain. The modified Gompertz equation was utilized to fit the cumulative daily biogas yield and to calculate various kinetic constants. Further, a modified first order kinetic equation was applied to calculate the hydrolysis constant. The biogas measured (in L biogas kg^{-1} Feedstock) ranged from 27.370 - 51.712 for *Asterarcycs quadricellulare* BGLR5. While as for *Spirulina sabsalsa* BGLR6, it ranged from 23.770 – 42.730. Overall highest biogas production was observed in *Aterarcycs quadricellulare* BGLR5. It was observed in both the strains, that the biogas production started without any lag phase (λ) in all the experimental sets except the untreated one i.e., control as can be seen in Table 4.27 and 4.28. In case of BGLR5 biomass, the biogas produced from microalgal biomass pretreated with hydrothermal treatment of 100°C for 30 min (F) was significantly higher than others, while as biogas produced from biomass pretreated with enzyme mix of 10 % dose for 24 h (D) was not significantly different from algal biomass pretreated with 20 % enzyme mix for 24 h (E) and hydrothermally pretreated biomass at 120 °C for an exposure time of 30 min i.e., digester G (Table 4.27). The lowest biogas production was found in untreated algal biomass digester (A). Similarly, for BGLR6 biomass, the biogas produced from digester D containing biomass pretreated with enzyme mix of dose 10 % for 24 h (D) of exposure time was significantly higher than all others. Whereas, biomass pretreated with 20 % enzyme dose for 12 h (C), 20 % enzyme dose for 24 h (E) and 100 °C for 30 min (F) did not differ significantly from each other. Also, biogas from 10 % 12 h (B), 20 % 12 h (C) enzymatically pretraeted biomass did not differ significantly from each other. Further, biogas from hydrothermally pretreated at 120°C for 30 min was not found significantly different from 10 % 12 h (B), 20 % 12 h (C). The lowest biogas here was also observed from untreated biomass.

The kinetics of biogas production from the untreated and pre-treated biomass of both the strains of microalgae (BGLR5 and BGLR6) for all the sets of experiments produced as mentioned above was studied. In case of BGLR5, it was found that digester F produced the highest biogas production potential (P) of 975.09 mLg^{-1} VS at a maximum biogas production rate (R_m) of 29.78 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 0.07 days (Table 4.27) at the end of 30 days. Digester D follows digester F in biogas production potential estimated to be 865.02 mLg^{-1}VS at a maximum biogas production rate of 37.10 mLg^{-1} VS with a lag phase of 0.10 days. Digester E follows digester D in biogas production. The least biogas production was noticed in A i.e., control. It is clear from the Table 4.27, 1.36 and 1.93-fold increase in R_m for

digester F and D respectively was observed, to that of the control (untreated microalgal biomass). Further it was observed that with pretreatment, the maximum biogas production rate (R_m) increased in all pretreatments. It was observed that the lag phase (λ) decreased from that of the control. The volatile solid reduction (VSR) was also seen to have increased on pretreatment of microalgae biomass. The volatile solid reduction was also found to be highest in digester F. The VSR elucidates the digestibility more effectively. VSR for control was 51.45 % and that of the set F and D were 77.65 and 66.44 % respectively. This determines and confirms that pretreatment increases the digestibility of the substrates. The modified Gompertz equation suitably and satisfactorily described biogas production with a goodness of fit (R^2) of 0.9974, 0.9971, 0.9998, 0.9970, 0.9970, 0.9971 and 0.9906 for digesters F, D, E, C, B, G and A, respectively as can been observed from Fig. 4.18. The effect of pretreatment can be easily observed in terms of the hydrolysis constant also. In this study, it was observed that on co-digestion the hydrolysis constant also got increased (0.0568-0.0704 per d). The maximum hydrolysis constant was observed in digester G, but the biogas production was significantly less than the digester F, C and A. This is due to the fact that K_h constitutes the part of ultimate biogas yield being converted to the actual biogas yield.

Similarly, while studying the kinetics of biogas production from BGLR6, it was noticed that digester D produced the highest biogas production potential (P) of 768.92 mLg^{-1} VS at a maximum biogas production rate (R_m) of 32.16 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 0.09 days (Table 4.28) at the end of 30 days. Digester E follows digester D in biogas production potential estimated to be 589.46 mLg^{-1}VS at a maximum biogas production rate of 22.82 mLg^{-1} VS with a lag phase of 0.07 days. Digester C follows digester E in biogas production. The least biogas production was noticed in A i.e., control. It is clear from the Table 4.28, that the maximum biogas production rate (R_m) increased in all sets containing biomass of algae pretreated with different treatment methods. It was observed in all the sets that the lag phase (λ) decreased from that of the control. An increase in the volatile solid reduction (VSR) was observed on pretreatment of microalgae biomass. The volatile solid reduction was found to be highest in digester G. The VSR elucidates the digestibility more effectively. VSR for control was 49.88 % and that of the set D and E were 67.88 and 68.98 % respectively. This determines and confirms that pretreatment increases the digestibility of the substrates. The modified Gompertz equation suitably and satisfactorily described biogas production with a goodness of fit (R^2) of 0.9969, 0.9968, 0.9983, 0.9989, 0.9985, 0.9982 and 0.9973 for digesters A, B, C, D, E, F and G respectively, as can been observed from Fig. 4.19. The effect of pretreatment can be easily observed in terms of the hydrolysis constant also. In this study, it was observed that on co-digestion the hydrolysis constant also got increased (0.0331-0.0861 per d). The maximum hydrolysis constant was observed in digester F, but the biogas

production was significantly less than the digester D. This is because of the fact that K_h comprises the part of ultimate biogas yield being converted to the actual biogas yield. Further, the cumulative biogas yield curves obtained from the digesters under study for both the strains were found to be sigmoidal in nature (Fig. 4.18 and 4.19). These type of curves have been described in anaerobic batch digestion experiments by various researchers (Sung and Liu 2003; Cuetos *et al* 2011).

Table 4.27 Value of different parameters estimated from Gompertz model and volatile solid reduction (VSR) obtained for BGLR5 biomass

Digester	Pretreatment	Total Biogas ($Lkg^{-1}S$)	P ($mLg^{-1}VS$)	R_m ($mLg^{-1}d^{-1}$)	λ (d)	k_h (per d)	R^2	VSR (%)
A	Untreated MA	27.37^{d}	218.72	12.64	3.47	0.0568	0.9906	51.45
B	10%12hr	40.896^{c}	712.14	28.91	0.14	0.0621	0.9970	64.82
C	20%12h	44.288^{c}	829.92	32.59	0.11	0.0602	0.9970	65.40
D	10%24hr	45.856^{b}	865.02	37.10	0.10	0.0663	0.9971	66.44
E	20%24hr	47.776^{b}	837.12	33.06	0.10	0.0603	0.9998	70.08
F	100°C30min	51.712^{a}	975.09	29.78	0.07	0.0443	0.9974	77.65
G	120°C30min	46.368^{b}	732.97	33.70	0.27	0.0704	0.9971	68.17

#MA represents microalga *Asterarcys quadricellulare* BGLR5 biomass; S: substrate; P: ultimate biogas yield; R_m: maximum rate of biogas production; λ: lag phase; k_h: hydrolysis constant; R^2: Coefficient of determination; VSR: volatile solid reduction; Values (Means) superscripted by different alphabets in the column differ significantly ($P \leq 0.05$) from each other (Duncan's multiple range test).

Table 4.28 Value of different parameters estimated from Gompertz model and volatile solid reduction (VSR) obtained for BGLR6 biomass

Digester	Pretreatment	Total Biogas ($Lkg^{-1}S$)	P ($mLg^{-1}VS$)	R_m ($mLg^{-1}d^{-1}$)	λ (d)	k_h (per d)	R^2	VSR (%)
A	Untreated MA	23.77^{e}	74.80	4.07	7.71	0.0331	0.9969	49.88
B	10%12hr	32.77^{cd}	554.07	20.41	0.33	0.0550	0.9968	63.90
C	20%12h	31.13^{bcd}	560.48	23.32	0.11	0.0646	0.9983	63.34
D	10%24hr	42.73^{a}	768.92	32.16	0.09	0.0654	0.9989	67.88
E	20%24hr	34.73^{b}	589.46	22.82	0.07	0.0595	0.9985	68.98
F	100°C30min	33.30^{bc}	519.03	28.17	0.05	0.0861	0.9982	72.59
G	120°C30min	27.57^{d}	399.00	13.32	0.07	0.0494	0.9973	76.10

#MA represents microalga *Spirulina subsalsa* BGLR6 biomass; S: substrate; P: ultimate biogas yield; R_m: maximum rate of biogas production; λ: lag phase; k_h: hydrolysis constant; R^2: Coefficient of determination; VSR: volatile solid reduction; Values (Means) superscripted by the same alphabets in the column are not significantly ($P \leq 0.05$) different from each other (Duncan's multiple range test).

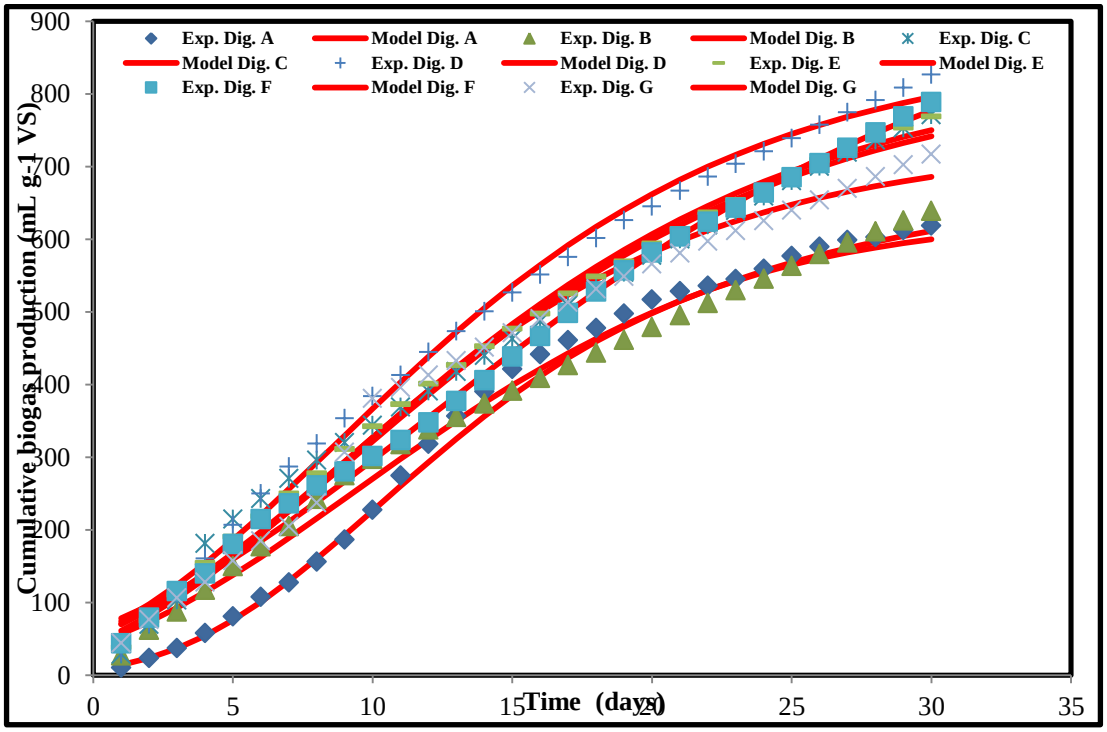


Fig 4.18 Variation and fitting of the cumulative biogas data of Biogas potential of BGLR5 with the Gompertz model for the different digesters (A–G) with time

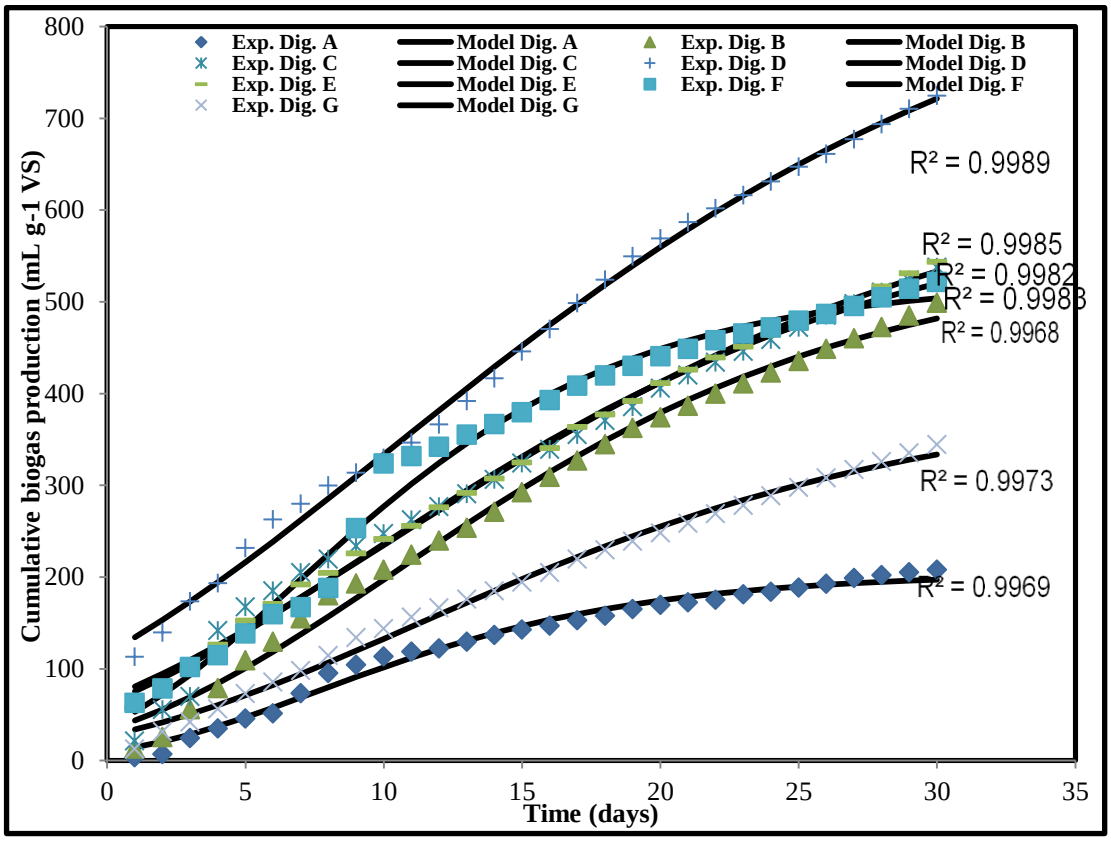


Fig. 4.19 Variation and fitting of the cumulative biogas data of Biogas potential of BGLR6 with the Gompertz model for the different digesters (A–G) with time

4.8 Anaerobic co-digestion of algal biomass with paddy straw for biogas production

The anaerobic co-digestion of algal biomass with paddy straw was carried out for both the microalgal strains *Spirulina subsalsa* BGLR6 and *Asterarcys quadricellulare* BGLR5. The strategy followed as mentioned in materials and methods section, was first these were co-digested with paddy straw by adding the same amount of these to that of the straw replaced. Secondly, the algal biomass which showed highest biogas production was then supplemented to the straw while keeping the content of paddy straw same.

4.8.1 Co-digestion of *Spirulina subsalsa* BGLR6 biomass with paddy straw for biogas production

The study of biogas production from paddy straw and microalgal biomass and their mixtures was conducted in digesters labelled A–E. Biogas production was monitored for a period of 46 days. The strategy followed was that the paddy straw content was decreased in successive digesters and the same amount of algal biomass was added. The modified Gompertz equation was then applied to fit the cumulative daily biogas yield. This adequately described the biogas production from these feedstocks (Fig. 4.20). The various kinetic constants using non-linear regression and other characteristics of the digesters A–E were calculated and are shown in Table 4.29.

At the end of 46-days period, it was noticed that digester C produced the highest biogas production potential (P) of 260.03 mLg^{-1} VS at a maximum biogas production rate (R_m) of 8.16 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 3.84 days (Table 4.29). Digester D follows digester C in biogas production potential estimated to be 250.45 mLg^{-1}VS at a maximum biogas production rate of 10.73 mLg^{-1} VS with a lag phase of 1.94 days. Digester B is at third number in biogas production. The least biogas production was noticed in A. The cumulative biogas yield curves obtained from the digesters under study were found to be sigmoidal in nature (Fig. 4.20). This type of curves has been described in anaerobic batch digestion experiments by various researchers (Sung and Liu 2003; Cuetos *et al* 2011). It is clear from the Table 4.29, that R_m got increased by 49.72 and 96.88% for digester C and D respectively, to that of the soaked paddy straw. Further it was observed that on increasing the algal biomass content, the maximum biogas production rate (R_m) increased in all co-digestion sets. Also, the lag phase (λ) decreased till replacing of 50% of paddy straw with 50% of microalgal biomass and afterwards it started increasing. Moreover the enhancement in the volatile solid reduction (VSR) was also seen on co-digestion of microalgae with paddy straw. The volatile solid reduction was also found to be highest in digester C. The VSR defines the digestibility more comprehensively. VSR for soaked paddy straw was 32.48% and that of the set C and D was 65.50 and 58.17% respectively. This also elucidated and confirmed that co-digestion increases or improves the digestibility of the substrates. The maximum biogas yield, ultimate biogas

yield (P), more R_m, less λ (d) and more VSR in digesters C followed by D and B could be attributed to the better C/N ratio as co-digestion of paddy straw with algal biomass improves the C/N ratio as shown in Table 4.29. The more production of biogas in the C/N ratio range of 20-30 in our case exhibits similarity to that of the results of Zhong *et al* (2012) who observed highest biogas yield on the co-digestion of of corn straw (C/N =70) with algal biomass at C/N ratio of 20 and Yen and Brune (2007) who obtained highest gas yield in the C/N ratio range of 20-25. The less biogas production in digester A and E could be attributed to the more and sub-optimum C/N ratio present. This imbalanced C/N ratio mostly leads to ammonia toxicity. There are various reasons for ammonia toxicity like nature of feed, operating conditions (pH and concentration of toxic ions like Na^+ and Ca^{2+}), inoculums and ammonium released from protein hydrolysis (Chen *et al* 2008, Sialve *et al* 2009). In our case the most probable reason seems to be the hydrolysis of proteins, since *Spirulina* spp. are rich in proteins. The hydrolysis of proteins increases both alkalinity and pH of the anaerobic digesters. The alkaline pH and high concentration of ammonia then results in the conversion of acetate, main substrate for the methanogens, into ammonium acetate or ammonium bicarbonate and thus leads to the depletion of the available substrate to methanogens (Shanmugam and Horan 2009). This reduction of acetate and reduced growth of methanogens due to high ammonia release during digestion of algal biomass could be a major reason of less biogas production. The modified Gompertz equation was found to reasonably and adequately describe biogas production with a goodness of fit (R^2) of 0.992, 0.996 and 0.993 for digesters C, D and B, respectively as can been observed from Fig. 4.20. The effect of co-digestion can be easily observed in terms of the hydrolysis constant. In this study, it was observed that on co-digestion the hydrolysis constant also got increased (0.0216-0.0880 per d). The maximum hydrolysis constant was observed in digester E, but the biogas production was significantly less than the digester B and C. This is due to the fact that K_h constitutes the part of ultimate biogas yield being converted to the actual biogas yield. Our results are in line to those of the Prajapati *et al* (2015), who too observed that co-digestion of algae with different substrates like cattle dung and sugar cane bagasse increased the methane yield and VSR. Co-digestion besides enhancing the biogas yield, it also increases the kinetics of the anaerobic digestion process (Costa *et al* 2012; Ramos-Suárez *et al* 2014; Solé *et al* 2014) which too was noticed in our case.

The increase in biogas production upon co-digestion of microalgal biomass with paddy straw can also be attributed to the mineral content of the microalgal biomass. The mineral element contents of the microalgal biomass (mg/100 g) as determined by ICP-OES (Table 4.6) were as follows: K (410), Ca (660), Fe (540), Mn (20), Mg (980), P (1160) and S (440). The overall increase in biogas in digesters B, C, D and E that of A, is due to the

presence of elements like potassium, calcium and iron which have been found to speed up the metabolism of biogas formation (Bożym *et al* 2015), and the absence of the toxic elements like Cu, As, Pb, Cd, and Cr, which are having toxic or inhibitory effect on the anaerobic digestion process, thus on biogas production and biomethane content (Mudhoo and Kumar 2013). Actually the impact of the inhibitory effect of these heavy metals depends on their concentration (Şengör *et al* 2009). At low concentrations, these have been found to be favourable and at higher concentrations, these are inhibitory for biogas and biomethane production. In our case, the concentration of the metals within the algal biomass was not high enough to inhibit the AD process (Soares *et al* 2012). However, on increasing the algal biomass content beyond 30%, it was observed that the biogas production did not increased, this may be due to the increase in P content in the digester, as P has been reported to reduce the efficiency and amount of biogas and methane formation (Chen *et al* 2008), since phosphorus is present in the algal biomass used in this study.

The feedstock fed into the digesters A-E was evaluated by determining the proximate and chemical composition before and after anaerobic digestion. Results from Table 4.30 indicate that there was a smooth decrease in total solids and volatile solids on anaerobic digestion. The total solids decreased significantly on anaerobic digestion for a period of 46 days. Volatile solids also decreased significantly on anaerobic digestion. However, ash content was found to be increasing significantly on increasing the algal biomass content and also significant change was observed in it also on anaerobic digestion. The ash content was found to have increased after digestion as compared to before digestion. This increase is obvious as the volatile solids got decreased because of anaerobic digestion process. Also the silica content increased significantly on increasing the microalgal biomass percentage and lignin content decreased on increasing the algal biomass. There was found significant change in every response may it be cellulose, hemicellulose, hemicellulose, lignin and silica before and after anaerobic digestion of the feedstock in all cases. The highest significant percent change in total (52.15%) and volatile solids (27.90%) were noticed in digester containing 70% paddy straw (PS) and 30% microalgal biomass (MA). The highest percent change in volatile solids of set C consisting of 70% paddy straw (PS) and 30% microalgal biomass (MA) before and after anaerobic digestion is responsible for highest biogas production among all the sets. The decrease in cellulose and hemicellulose content might be the result of breakdown or hydrolysis of cellulose and hemicellulose into fermentable sugars (Ding *et al* 2012; Jalc *et al* 1998). The significantly higher percent change in volatile solids on co-digestion may be due the fact that co-digestion process provides synergistic interactions during digestion (Mata-Alvarez *et al* 2014) and ultimately leads to the higher biogas production than digesting the substrates singly.

Table 4.29 Estimated kinetic constants using non-linear regression models and other characteristics of the digesters A–E

Digester	Substrate	C/N	Total Biogas ($Lkg^{-1}FS$)	P ($mLg^{-1}VS$)	R_m ($mLg^{-1}d^{-1}$)	λ (d)	k_h (per d)	R^2	VSR (%)
A	PS	61.13	48.56^{b}	231.52	5.45	9.60	0.0216	0.992	32.48
B	80%PS+20 %MA#	37.34	104.45^{d}	246.15	6.67	3.99	0.0357	0.993	59.53
C	70%PS+30 % MA	29.98	128.86^{f}	260.03	8.16	3.84	0.0375	0.992	65.50
D	50%PS+50 % MA	19.76	116.48^{e}	250.45	10.73	1.94	0.0595	0.996	58.17
E	30%PS+70 % MA	12.98	79.26^{c}	189.91	12.57	2.05	0.0880	0.980	57.75

#MA represents microalgae *Spirulina subsalsa* BGLR6; PS: Paddy straw; FS: feedstock; P: ultimate biogas yield; R_m: maximum rate of biogas production; λ: lag phase; k_h: hydrolysis constant; R^2: Coefficient of determination; VSR: volatile solid reduction; values followed by different superscripts in the column are significantly different at p=0.05.

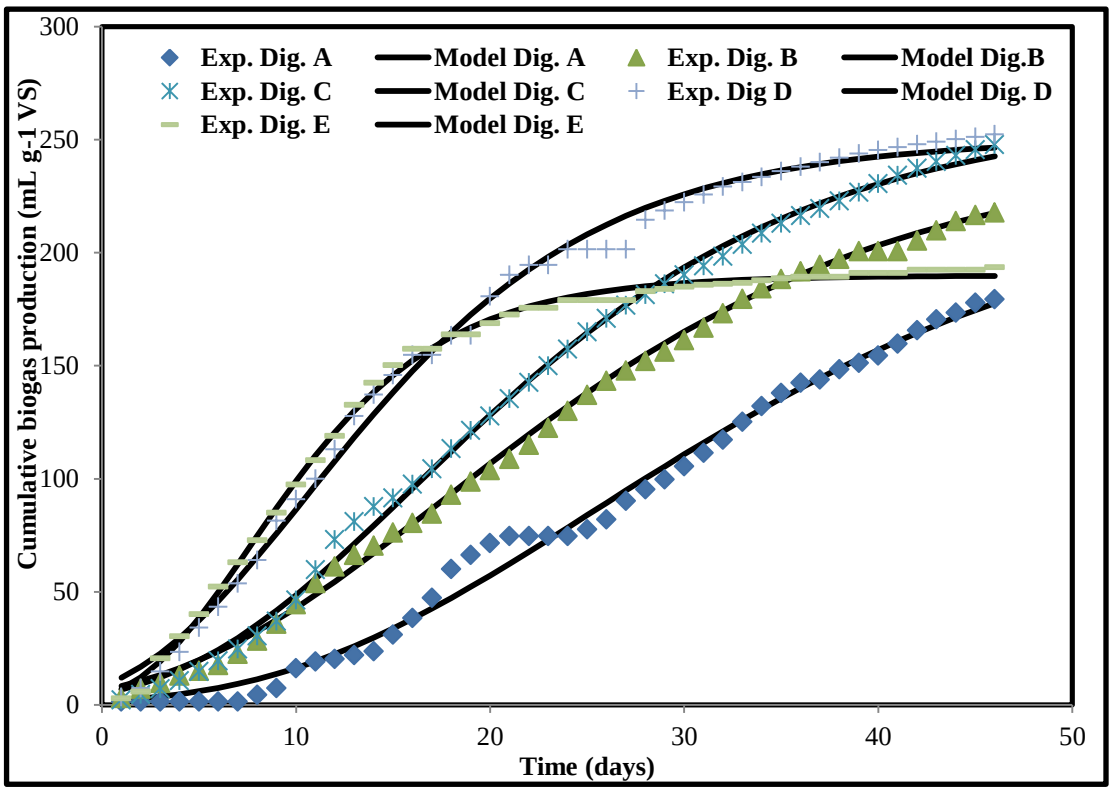


Fig. 4.20 Variation and fitting of the cumulative biogas data of *Spirulina subsalsa* BGLR6 biomass co-digested with paddy straw with the Gompertz model for the different digesters (A–E) with time. A, B, C, D and E have been discussed in text and above table

Table 4.30 Comparative profile of proximate and chemical composition of feedstock before and after anaerobic digestion

Samples	Proximate Composition (%)			Chemical Composition (%)			
	Total Solids (TS)	Volatile Solids (VS)	Ash	Cellulose	Hemicellulose	Lignin	Silica
Before Anaerobic Digestion							
PS	95.57^{Aa}	87.20^{Aa}	12.80^{Fa}	45.60^{Aa}	22.80^{Aa}	12.00 ^{Ba}	8.00^{Da}
80%PS+20% MA	95.88 ^{Aa}	84.00 ^{Ba}	16.00^{Ea}	42.00^{Bb}	18.00^{Ba}	12.00^{Ba}	10.00^{ABa}
70%PS+30% MA	95.05 ^{Aa}	83.50 ^{Ba}	16.50^{Ea}	37.00^{Ca}	18.50^{Ba}	12.00^{Bb}	9.20 ^{BCb}
50%PS+50% MA	95.75 ^{Aa}	77.70 ^{Ca}	22.30^{Db}	22.00^{Eb}	19.32^{Ba}	10.00^{Ca}	10.00A^{Ba}
30%PS+70% MA	94.85 ^{Aa}	74.73 ^{Da}	25.27^{Cb}	22.00^{Ea}	11.80^{Ea}	8.20 ^{Db}	10.00^{ABb}
After Anaerobic Digestion							
SPS	74.00 ^{Bb}	76.04 ^{CDb}	11.85^{Fb}	40.00^{Bb}	18.00^{Bb}	10.00^{Cb}	8.00^{Da}
80%PS+20% MA	50.23 ^{CDb}	64.89 ^{Eb}	26.07^{Cb}	28.00^{Da}	16.43^{Cb}	8.00^{Db}	8.50^{CDb}
70%PS+30% MA	45.48 ^{Eb}	60.20 ^{Fb}	28.13^{Bb}	30.00^{Db}	13.67^{Db}	13.00^{ABa}	9.50^{BCa}
50%PS+50% MA	48.01 ^{Db}	58.80 ^{Fb}	26.38^{Ba}	30.00^{Da}	9.78^{Fb}	10.00^{Ca}	8.00^{Db}
30%PS+70% MA	50.48 ^{Cb}	59.32 ^{Fb}	32.59^{Aa}	16.00^{Fb}	10.00^{Fb}	14.00^{Aa}	11.00^{Aa}

PS: Soaked paddy straw (control); MA: represents microalgal biomass; Values are means. Within the same column, different capital letters indicate a significant difference between different samples for each before and after anaerobic digestion and different small letters indicate a significant difference between before and after anaerobic digestion under the same sample based on Duncan's Multiple range test ($p<0.05$)

4.8.2 Co-digestion of *Asterarcys quadricellulare* BGLR5 biomass with paddy straw for biogas production

The study of biogas production from paddy straw and *Asterarcys quadricellulare* BGLR5 biomass and their mixtures was conducted in digesters labelled A–E. Here, the paddy straw content was decreased in successive digesters and the same amount of algal biomass was added. Biogas production was monitored for a period of 46 days. The modified Gompertz equation was then applied to fit the cumulative daily biogas yield. This adequately described the biogas production from these feedstocks (Fig. 4.21). The various kinetic constants using non-linear regression and other characteristics of the digesters A–E were calculated and are shown in Table 4.31.

At the end of 46-days period, it was noticed that digester D produced the highest biogas production potential (P) of 361.81 mLg^{-1} VS at a maximum biogas production rate (R_m) of 8.19 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 2.81 days (Table 4.31). Digester C follows digester D in biogas production potential estimated to be 349.50 mLg^{-1}VS at a maximum biogas production rate of 7.37 mLg^{-1} VS with a lag phase of 3.40 days. Digester E is at third number in biogas production. The least biogas production was noticed in digester A followed by digester B. The cumulative biogas yield curves obtained from the digesters under study were found to be sigmoidal in nature (Fig. 4.21). This type of curves has been described in anaerobic batch digestion experiments by various researchers (Sung and Liu 2003, Cuetos *et al* 2011). It is clear from the Table 4.31, that R_m got increased by 50.27 and 35.22% for digester D and C respectively, to that of the soaked paddy straw. Further it was observed that on increasing the algal biomass content, the maximum biogas production rate (R_m) increased in all co-digestion sets but beyond 50 %, it got decreased. The lag phase (λ) also decreased continuously on increasing the microalgal biomass. Moreover the enhancement in the volatile solid reduction (VSR) was also seen on co-digestion of microalgae with paddy straw. The volatile solid reduction was also found to be highest in digester D. The VSR explains the digestibility more comprehensively. VSR for soaked paddy straw was 32.48% and that of the set D and C was 68.08 and 65.72 % respectively. This explained and confirmed that co-digestion increases or improves the digestibility of the substrates. The maximum biogas yield, ultimate biogas yield (P), more R_m, less λ (d) and more VSR in digesters D followed by C and E could be due to the better C/N ratio as co-digestion of paddy straw with algal biomass improves the C/N ratio. Similarly, the less biogas production in digester A, B and E could be attributed to the more and sub-optimum C/N ratio present. This imbalanced C/N ratio mostly leads to ammonia toxicity as discussed in the above section. The modified Gompertz equation was found to reasonably and adequately describe biogas production with a goodness of fit (R^2) of 0.994, 0.996 and 0.996 for digesters D, C and E, respectively as can been observed

from Fig. 4.21 and Table 4.31. The hydrolysis constant is also a measure of the effect of co-digestion. In this study, it was observed that over all on co-digestion the hydrolysis constant also got increased (0.02-0.04 per d). The maximum hydrolysis constant was observed in digester B, but the biogas production was significantly less than the digester D, C and E. This is due to the fact that K_h constitutes the part of ultimate biogas yield being converted to the actual biogas yield.

The increase in biogas production upon co-digestion of microalgal biomass with paddy straw can also be attributed to the mineral content of the microalgal biomass. The mineral element contents of the microalgal biomass (mg/100 g) as determined by ICP-OES (Table 4.6) were as follows: K (1000), Ca (1100), Fe (130), Mg (300), Mn (10), P (1000) and S (440). The overall increase in biogas in digesters D, C, E and B that of A, is due to the presence of elements like potassium, calcium and iron which have been found to speed up the metabolism of biogas formation (Bożym *et al* 2015), and the absence of the toxic elements like Cu, As, Pb, Cd, and Cr, which are having toxic or inhibitory effect on the anaerobic digestion process, thus on biogas production and biomethane content (Mudhoo and Kumar 2013). Actually the impact of the inhibitory effect of these heavy metals depends on their concentration (Şengör *et al* 2009). At low concentrations, these have been found to be favourable and at higher concentrations, these are inhibitory for biogas and biomethane production. In our case, the concentration of the metals within the algal biomass was not high enough to inhibit the anaerobic digestion (AD) process (Soares *et al* 2012). However, on increasing the algal biomass content beyond 50%, it was observed that the biogas production did not increased, this may be due to the increase in P content in the digester, as P has been reported to reduce the efficiency and amount of biogas and methane formation (Chen *et al* 2008), since phosphorus is present in the algal biomass used in this study.

The feedstock fed into the digesters A-E was evaluated by determining the proximate and chemical composition before and after anaerobic digestion. Results from Table 4.32 indicate that there was a smooth decrease in total solids and volatile solids on anaerobic digestion. The total and volatile solids decreased significantly on anaerobic digestion for a period of 46 days. However, ash content increased significantly on increasing the algal biomass content. The ash content significantly increased after digestion as compared to before digestion. This can be explained by the fact that volatile solids decreased because of anaerobic digestion process and this decrease in volatile solids led to the increase in ash content. The silica content increased significantly on increasing the microalgal biomass percentage. It was noticed that as the ash content increased, the silica content also increased. Significant change was observed in each response, be it cellulose, hemicellulose,

hemicellulose, lignin and silica before and after anaerobic digestion of the feedstock in all cases. The highest significant percent change in total (51.01%) and volatile solids (34.84%) were noticed in digester containing 50% paddy straw (PS) and 50% microalgal biomass (MA). The highest percent change in volatile solids of set D consisting of 50% paddy straw (PS) and 50% microalgal biomass (MA) before and after anaerobic digestion is responsible for highest biogas production among all the sets. The decrease in cellulose and hemicellulose content might be the result of breakdown or hydrolysis of cellulose and hemicellulose into fermentable sugars (Kuijik *et al* 2015). The significantly higher percent change in volatile solids on co-digestion may be due the fact that co-digestion process provides synergistic interactions during digestion (Mata-Alvarez *et al* 2014). So, it was concluded that co-digestion improves the digestibility of the substrates as well as biogas production. Our results are in corroboration with many researchers. Yen and Brune 2007 stated that the addition of paper waste to a mixture of *Scenedesmus* sp. and *Chlorella* sp. approximately doubled methane production from 140 to 230 L CH_4 kg^{-1} VS. Similarly, the methane yield was improved by 8–74 % on codigesting microalgal biomass with different amounts of swine manure (González-Fernández *et al* 2011). Co-digestion of these substrates made the process to operate at higher organic loading rates which would have not been possible rather carrying out digestion of feed stocks singly.

Table 4.31 Estimated kinetic constants using non-linear regression models and other characteristics of the digesters A–E of *Asterarcys quadricellulare* BGLR5 co-digested with paddy straw

Digester	Substrate	Total Biogas ($Lkg^{-1}FS$)	P ($mLg^{-1}VS$)	R_m ($mLg^{-1}d^{-1}$)	λ (d)	k_h (per d)	R^2	VSR (%)
A	PS	48.56[a]	231.52	5.45	9.60	0.02	0.992	32.48
B	80%PS+20%MA[#]	110.64[b]	245.14	7.82	3.77	0.04	0.972	59.17
C	70%PS+30% MA	147.48[d]	349.50	7.37	3.40	0.03	0.996	65.72
D	50%PS+50% MA	168.32[e]	361.81	8.19	2.81	0.02	0.994	68.08
E	30%PS+70% MA	127.9[c]	318.26	6.62	2.16	0.03	0.996	64.04

#MA represents microalgae *Asterarcys quadricellulare* BGLR5; PS: Paddy straw; FS: feedstock; P: ultimate biogas yield; R_m: maximum rate of biogas production; λ: lag phase; k_h: hydrolysis constant; R^2: Coefficient of determination; VSR: volatile solid reduction; values followed by different superscripts in the column are significantly different at p=0.05.

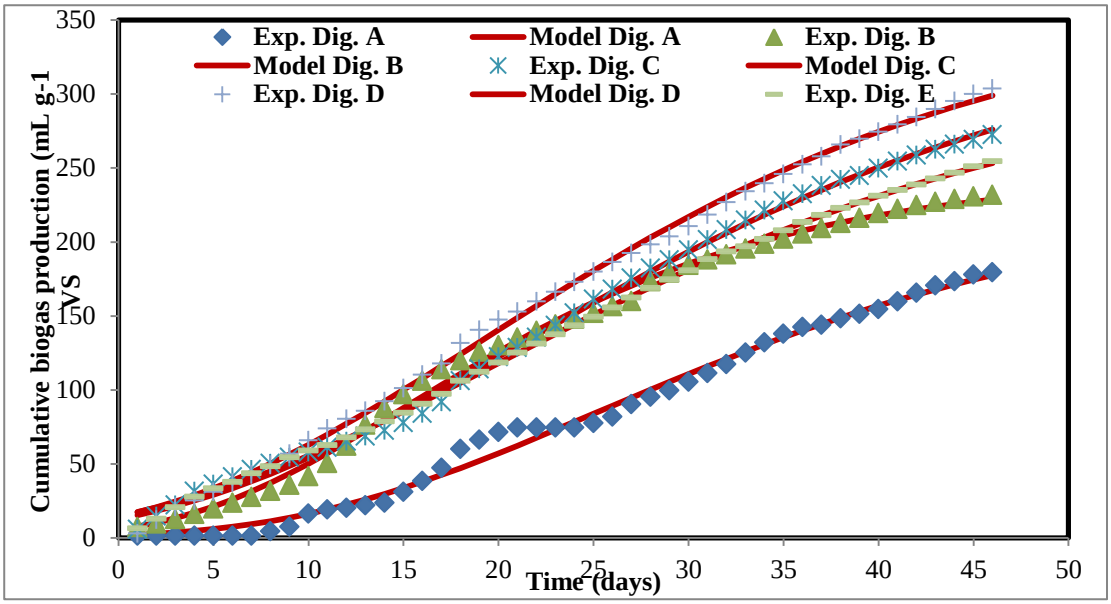


Fig. 4.21 Variation and fitting of the cumulative biogas data of *Asterarcys quadricellulare* BGLR5 co-digested with paddy straw with the Gompertz model for the different digesters (A–E) with time. A, B, C, D and E have been discussed in text and above table

Table 4.32 Comparative profile of proximate and chemical composition of feedstock before and after anaerobic digestion

Samples	Proximate Composition (%)			Chemical Composition (%)			
	Total Solids (TS)	Volatile Solids (VS)	Ash	Cellulose	Hemi cellulose	Lignin	Silica
Before Anaerobic Digestion							
PS	95.57^{Aa}	87.20^{Aa}	12.80^{GHa}	45.60^{Aa}	22.80^{Aa}	$8.20C^{Db}$	9.20^{Ca}
80%PS+20% MA	94.67^{Aa}	87.16^{Aa}	12.84^{GHb}	41.67^{Ba}	22.56^{Aa}	$9.10B^{Cb}$	8.56^{CDb}
70%PS+30% MA	95.15^{Aa}	86.56^{Aa}	13.44^{Fb}	38.56^{CDa}	21.45^{Ba}	8.34^{CDb}	8.10^{Db}
50%PS+50% MA	95.87^{Aa}	84.96^{ABa}	15.04^{Eb}	40.45^{BCa}	23.17^{Aa}	7.85^{Db}	9.15^{Cb}
30%PS+70% MA	94.34^{Aa}	83.16^{Ba}	16.84^{Db}	36.67^{Da}	20.17^{Ca}	8.13^{CDb}	9.24^{Cb}
After Anaerobic Digestion							
PS	74.00^{Bb}	76.04^{Cb}	11.85^{Hb}	40.00^{BCb}	18.00^{Db}	10.00^{ABa}	8.00^{Db}
80%PS+20% MA	54.23^{Cb}	62.12^{Db}	23.1^{Ca}	33.17^{Eb}	16.50^{Eb}	9.70^{ABa}	9.40^{BCa}
70%PS+30% MA	48.12^{Deb}	58.67^{Eb}	26.18^{Ba}	31.72^{EFb}	17.65^{Db}	8.70^{CDa}	10.5^{Aa}
50%PS+50% MA	46.97^{Eb}	55.36^{Fb}	28.49^{Aa}	29.31^{Gb}	15.00^{Fb}	9.80^{ABa}	10.32^{ABa}
30%PS+70% MA	49.54^{Db}	56.94^{EFb}	27.15^{Ba}	30.63^{FGb}	14.33^{Fb}	10.45^{Aa}	10.73^{Aa}

PS: Soaked paddy straw (control); MA: represents microalgal biomass; Values are means. Within the same column, different capital letters indicate a significant difference between different samples for each before and after anaerobic digestion and different small letters indicate a significant difference between before and after anaerobic digestion under the same sample based Duncan's Multiple range test ($p<0.05$)

4.8.3 Supplementation of algal biomass to the paddy straw for anaerobic digestion

The *Asterarcys quadricellulare* BGLR5 biomass was supplemented to the paddy straw as it was found to be producing more biogas than that of the *Spirulina sabsalsa* BGLR6 from the previous experiments discussed above. A total of seven digesters labelled A–G as shown in Table 4.33. Digster A contains only paddy straw (250 g) and not algal biomass, so this acts as control. In other digesters B-G, the paddy straw content was kept constant as taht of digester A but at the same time, the amount of algal biomass was added in increasing amount as 50, 75, 125, 175, 250 and 500 g in the digesters B, C, D, E, F and G respectively. Biogas production was observed for a period of 46 days. The kinetics of biogas production in all the digesters was studied by using the modified Gompertz equation and also a modified first order kinetic equation. These mathematical models adequately described the biogas production from these feedstocks (Fig. 4.22). The various kinetic constants using non-linear regression and other characteristics of the digesters A–G were calculated and are shown in Table 4.33.

It was observed that digester D produced the highest biogas production potential (P) of 265.14 mLg^{-1} VS at a maximum biogas production rate (R_m) of 8.13 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 3.32 days (Table 4.33) at the end of 46-days period,. Digester C and E follows digester D in biogas production potential estimated to be 240.65 mLg^{-1}VS at a maximum biogas production rate of 8.01 mLg^{-1} VS with a lag phase of 3.99 days and 235.06 mLg^{-1}VS at a maximum biogas production rate of 7.93 mLg^{-1} VS with a lag phase of 2.78 days. Digester B was found to be at fourth number in biogas production. The least biogas production was noticed in digester A followed by digester G. The cumulative biogas yield curves obtained from the digesters under study were found to be sigmoidal in nature (Fig. 4.22). This type of curves has been described in anaerobic batch digestion experiments by various researchers (Sung and Liu 2003, Cuetos *et al* 2011). It is clear from the Table 4.33, that R_m got increased by 49.17, 46.97 and 45.50 % for digester D, C and E respectively, to that of the soaked paddy straw i.e., digester A. Further it was observed that on increasing the algal biomass content, the maximum biogas production rate (R_m) increased upto digester D (50 % supplementation of algal biomass) and then started decreasing onwards, while a sharp decrease in the digester G was noticed. The lag phase (λ) also decreased continuously on increasing the microalgal biomass up to digester E and afterwards it started increasing. Moreover the enhancement in the volatile solid reduction (VSR) was also seen on co-digestion of microalgae with paddy straw. The volatile solid reduction was found to be highest in digester G followed by E, B, C, F and D. The VSR explains the digestibility more comprehensively. This explained and confirmed that co-digestion increases or improves the digestibility of the substrates. The maximum biogas yield, ultimate biogas yield (P), more R_m, less λ (d) and more VSR in

digesters D followed by C and E could be due to the better C/N ratio as co-digestion of paddy straw with algal biomass improves the C/N ratio. Similarly, the less biogas production in digester A, F and G could be attributed to the more and sub-optimum C/N ratio present. This imbalanced C/N ratio mostly leads to ammonia toxicity as discussed in the above section. The modified Gompertz equation was found to reasonably and adequately describe biogas production with a goodness of fit (R^2) of 0.993, 0.992 and 0.994 for digesters D, C and E, respectively as can been observed from Fig. 4.22 and Table 4.33. The hydrolysis constant is also a measure of the effect of co-digestion. In this study, it was observed that over all on co-digestion the hydrolysis constant also got increased (0.02-0.0456 per d). The maximum hydrolysis constant was observed in digester F, but the biogas production was significantly less than the digester D. This is due to the fact that K_h constitutes the part of ultimate biogas yield being converted to the actual biogas yield. The total biogas production of digester D and E were not significantly different and also digester B and C were statistically same. The biogas was more in digesters D and F because more of the volatile solids were reduced.

Table 4.33 Estimated kinetic constants using non-linear regression models and other characteristics of the digesters A–G of Paddy straw supplemented with BGLR5 biomass

Digester	Substrate	Total Biogas (L)	P ($mLg^{-1}VS$)	R_m ($mLg^{-1}d^{-1}$)	λ (d)	k_h (per d)	R^2	VSR (%)
A	PS	12.14^{a}	231.52	5.45	9.60	0.02	0.992	32.48
B	250g PS + 50 g MA	41.94^{c}	237.55	7.82	4.06	0.0412	0.992	86.29
C	250g PS + 75 g MA	42.49^{c}	240.65	8.01	3.99	0.0417	0.9921	85.41
D	250g PS + 125 g MA	54.15^{e}	265.14	8.13	3.32	0.0400	0.993	79.68
E	250g PS + 175 g MA	55.20^{e}	235.06	7.93	2.78	0.0454	0.9942	86.36
F	250g PS + 250 g MA	47.95^{d}	206.78	7.33	3.57	0.0456	0.9928	84.16
G	250g PS + 500 g MA	31.20^{b}	86.62	3.16	4.29	0.0448	0.9913	88.81

#MA represents microalgae *Asterarcys quadricellulare* BGLR5; PS: Paddy straw; P: ultimate biogas yield; R_m: maximum rate of biogas production; λ: lag phase; k_h: hydrolysis constant; R^2: Coefficient of determination; VSR: volatile solid reduction; values followed by different superscripts in the column are significantly different at p=0.05.

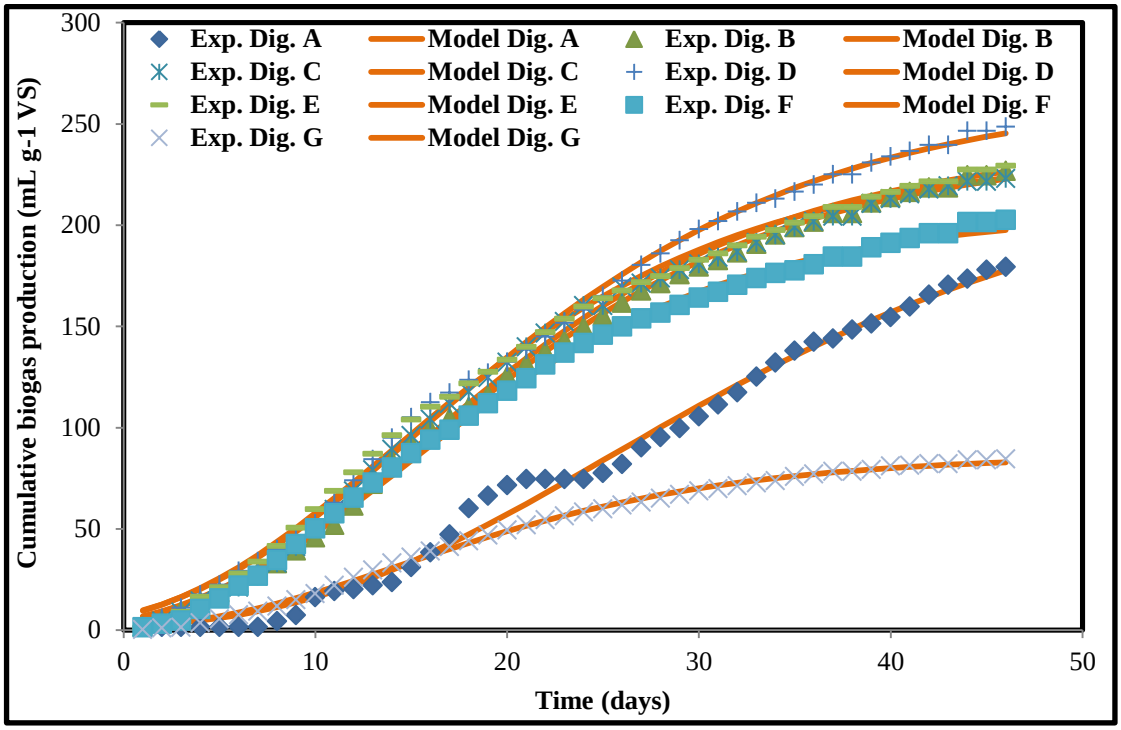


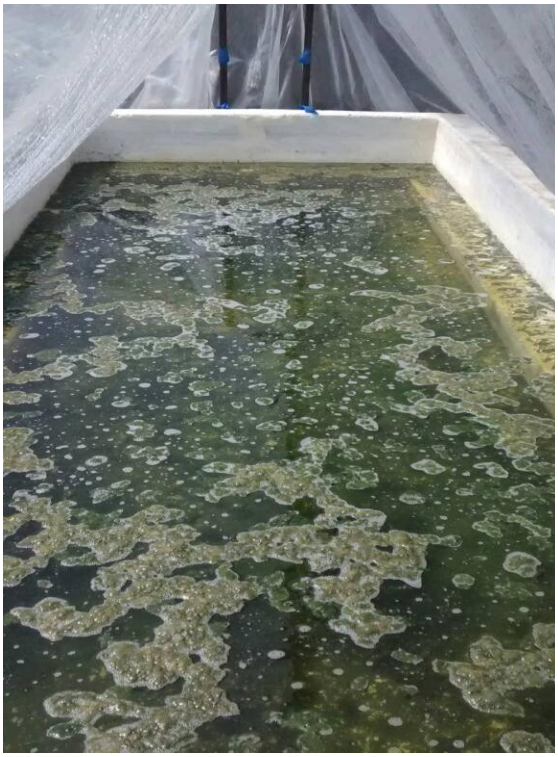
Fig. 4.22 Variation and fitting of the cumulative biogas data with the Gompertz model for the different digesters (A–G) with time

4.9 Cultivation of multipopulation microalgal species in open air algal pond and biogas production

Figure 4.23 shows the open air algal pond. The study was focused mainly on growing mixed algal species in low cost medium, as only tap water added with sodium bicarbonate was utilized. The sodium bicarbonate was added to the culture medium to provide it a carbon source. Every time with the addition of water to the pond sodium carbonate was added to it. The physico-chemical parameters of the algal pond during the culture period were as follows: air temperature 10-35 °C (from January to April, 2017), pH 8.7-11.4, photoperiod 12:12 (light:dark). The algae population of the pond were identified tentatively by microscopic examination. The population consisted of blue green algae (*Spirulina* sp.), diatom (*Nitzschia* sp.) and green algae (*Chlorococcum* sp.) mainly. Initially, the population of *Spirulina* sp. was found to be dominating but with the passage of time, green algae (*Chlorococcum* sp.) dominated the microalgal population in the pond (Fig. 4.24). So, a shift in the algal community was noted in the algal pond and this shift may be possibly by the increased pH, as pH was found to have increased from initial 8.8 to 11.4 and different nutrient requirements of microalgal species. Besides this, the environmental factors, mixing of culture in the pond and nutrients also affect the composition of microalgal community of the pond (Veldhuis et al 2005, Hassler et al 2011). The diatom *Nitzschia* sp. and green algae were found till the end of the culture period but at the end *Spirulina* sp. was not observed.

(a) Microalgae cultivation

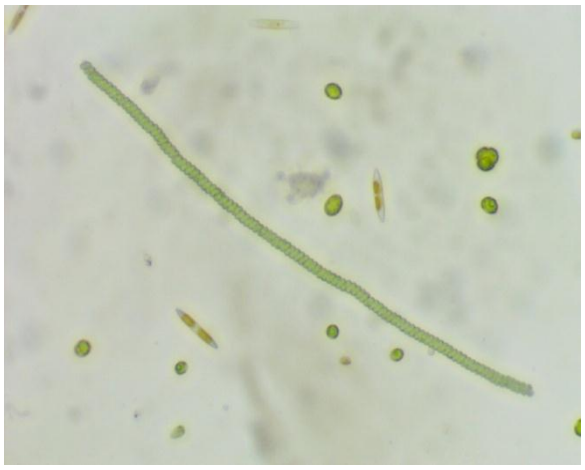
(b) Mixing of culture manually



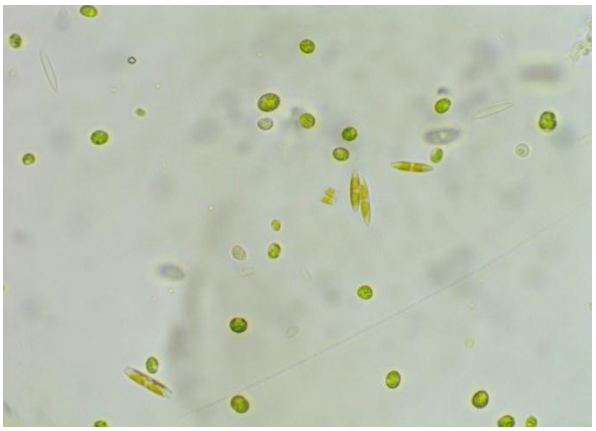
(c) Growth of mixed culture after 2 months

(d) Pond with impellers for agitation

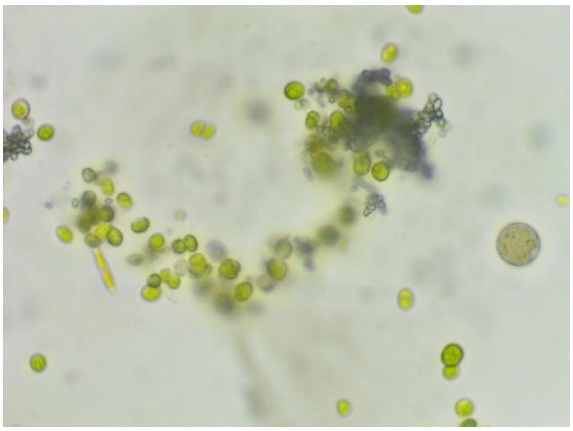
Fig. 4.23 Cultivation of multipopulation in open air algal pond



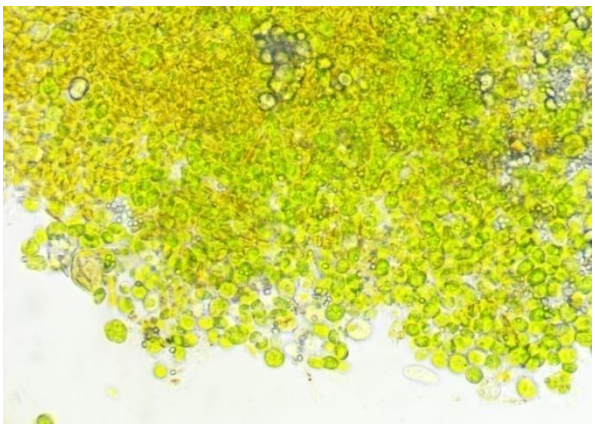
(a) *Spirulina* sp., *Nitzschia* sp., *Chlorococcum* sp. (After 5 days)



(b) *Nitzschia* sp., *Chlorococcum* sp. (15 days)



(c) *Nitzschia* sp., *Chlorococcum* sp. *and* other diatoms (After 25-35 days)



(d) *Nitzschia* sp., *Chlorococcum* sp. (After 45 days)

Fig. 4.24 Microalgal population in open air algal pond (*Spirulina* sp., *Nitzschia* sp., *Chlorococcum* sp.). Variuos pictures depict shift of microalgal community

After attaining the promising growth and biomass of algal species in the pond (wet weight 15 g L^{-1}), which was studied by taking the known volume of microalgae suspension from the pond, harvested through filtration and gravity settling. The algal suspension was then utilized for biogas production along with paddy straw. The paddy straw was mixed with this algal suspension in the field scale digester (Digester B). The bio-digested slurry was used as inoculum for this anaerobic digestion study. A control containing only paddy straw mixed with bio-digested slurry (inoculum) was run in parallel (Digester A) to evaluate the biogas production from co-digestion of paddy straw and algal suspension. The anaerobic digestion was carried out for a period of 46 days and it was observed that the biogas production in digester B was significantly higher than the digester A (Table 4.34). An increase of 17.27% was achieved in case of digester B (0.129 m^3 biogas kg^{-1} feedstock fed) than that of the control i.e., digester A (0.110 m^3 biogas kg^{-1} feedstock fed). These values were closer to the previous results of the supplementation experiment. As, the lid of field scale biogas digester was fitted with a fixed gas meter, one has to just read the meter to know the amount of biogas produced. So, the chances of error in biogas measurement are negligible as compared to water displacement method in lab scale digesters. Besides the increase in biogas production, the quality of biogas also got improved as the methane content increased from 46.4% (control) to 66.5% (digester containing algal biomass) and CO_2, H_2S, CO got decreased as can be seen in Table 4.34. The content of methane (CH_4), carbon dioxide (CO_2), hydrogen sulphide (H_2S), carbon monoxide (CO) and hydrogen (H_2) in biogas produced from Digester B significantly differed from that of the Digester A. As biogas produced from digester B containing algal biomass along with paddy straw has low CO_2 content than digester A (control) will be having high calorific value compared to biogas of digester A because CO_2 lowers the calorific value (Ramaraj and Dussadee 2015). The low H_2S in biogas is also advantageous as it will minimize the damage caused by H_2S through corrosion in biogas plants (compressors, gas storage tanks and engines). The decrease in CO_2 and H_2S may be due to the fact that algae utilize these leaving behind a purified biogas containing almost pure methane (Yan and Zheng 2013). The removal of CO_2 and H_2S from biogas is the very crucial points to the technological and economic feasibility of upgrading process of the gas (Iovane *et al* 2014). Therefore, utilizing microalgal biomass will be helpful in upgradation biogas process technology (involving purification methods and techniques). This study also revealed that on using directly the algal suspension with paddy straw resulted in higher biogas production than the control. This method is advantageous as it saves our time and energy needed for harvesting the microalgal biomass.

Table 4.34 Biogas production from co-digestion of paddy straw and algal suspension with respect to only paddy straw (Control) at field scale

Digester	Biogas Production		Biogas Composition				
	Biogas (m^3/kg feedstock)	Biogas (m^3/kg VS)	CH_4 (%)	CO_2 (%)	H_2S ppm	CO ppm	H_2 ppm
A (Control)	0.110	0.132	46.4	53.9	1490	132	168.7
B	0.129 (17.27)	0.154 (16.67)	66.5	34.6	133.3	95	122.7
LSD (P=0.05)	0.017	0.013	2.575	5.190	92.132	31.359	19.590

VS: Volatile solids; Digester A (Control): paddy straw+ bio-digested slurry (biogas slurry); Digester B; paddy straw+ algal suspension+bio-digested slurry; Value in the parenthesis determines percent increase; LSD: Least significant difference at 5% level

CHAPTER - V

SUMMARY

Microalgae (eukaryotes and prokaryotes) have lured special global attention as biofuel substrates due to their expeditious biomass production in comparison to oleaginous plants. The biomass productivity has been found to be approximately 10-fold higher than that of conventional crops and can be cultivated on non-arable land areas or in lakes or the ocean, and thus extenuating food and feed competition. Therefore, microalgae are considered to play a remarkable role in the biofuels sector as substrate for several biomass energy conversion technologies. These technologies consist of anaerobic digestion to produce bio-methane, lipid esterification to produce bio-diesel and anaerobic fermentation to produce bio-ethanol. Each of these conversion techniques is appropriate for a particular feedstock, e.g. biodiesel production involves biomass rich in lipids and anaerobic fermentation surmises biomass rich in carbohydrates. Microalgae can assimilate CO_2 gas as the carbon source for growth and thus mitigate the problem of global warming.

Punjab, known as the bread basket of India, where out of total 50,362 sq km area, only 71% of area is used for agriculture. South-west zone that is about 34% of total area suffers from the erratic and scanty rainfall problem and is hugely affected by waterlogging which is not suitable for cultivation of crops. But, this type of area could be efficiently utilized for microalgae cultivation.

Keeping the importance of microalgae as future feed stock for biogas production and use of waterlogged area for microalgae cultivation, in mind, the present study was conducted to isolate, purify, screen and characterize microalgae from waterlogged areas of Punjab. It also aimed to optimize growth parameters of the selected isolate for maximum biomass production and subsequently assess the effect of microalgae biomass as a co-feedstock on biogas production from paddy straw.

To carry out this study, the water samples from different locations of waterlogged area of Punjab were collected and were analyzed for various parameters like electrical conuctivity, carbonates, bicarbonates, chlorides, calcium and magnesium ions. These samples were used to isolate and purify microalgal cultures by enrichment technique and other standard methods of isolation and purification. After isolation, the isolates obtained were further screened for the biomass production and other functional ingredients like chlorophyll, carbohydrates, lipids and proteins. The potential isolates which produced highest biomass or dry cell weight were selected and further identified by molecular methods, so as to confirm the tentative identification. After this, the selected potential isolates were optimized for their physio-chemical cultural factors (pH, light intensity, growth period, temperature, salt source concentration ($NH_4Cl/CaCl_2$), nitrate source ($NaNO_3$) and phosphate source (K_2HPO_4)). The

isolates were further cultivated under these optimal conditions for enhanced biomass production. Thereafter, for both the isolates, biogas potential was determined by BMP protocol. The algal biomass of the both isolates was subjected to two different pretreatments (enzymatic and hydrothermal) under different doses, exposure time and temperature to evaluate the effect on biogas potential of the isolates. Lastly the algal biomass of the selected isolates was co-digested with paddy straw in order to see the effect on biogas production. Algal biomass was co-digested with paddy straw in two ways, firstly the algal biomass was co-digested with paddy straw by adding the same amount of biomass to that of the straw replaced. Secondly, the algal biomass which showed highest biogas production was then supplemented to the straw while keeping the content of paddy straw same.

Analyses of water samples from various locations depicts that there is brackish ground water with high electrical conductivity (EC) and residual sodium concentration (RSC). The water samples were found to be alkaline (pH 7.27 to 8.37) and moderate to highly saline (EC 0.65 to 2.02 siemen/cm) in nature. The carbonate ions were not found in the samples. The bicarbonate ions in the range 7.6-14.0 mEq/L were present in the samples. Similarly Cl^- and $Ca^{2+}+Mg^{2+}$ were calculated and were found to be in the range 5-22 and 2.5-10.1 mEq/L. A total of 19 microlagal cultures were isolated and purified from the water samples collected from waterlogged areas of Punjab (India). Upon examination of cultures at microscopic level, the strains were found to be belonging to different groups and of diverse morphology like some were found to be unicellular, some filamentous and some colonial in nature. Screening was done mainly on the basis of the biomass production. The microalgal isolates were grown on five different media (ACM, BBM, BG-11, Conway and Guillard's F/2 medium) for a period of 35 days. The growth profile was studied after 2 days of interval for a period of 35 days by measuring the absorbance at 750 nm. It was observed that the all the microalgal species followed the conventional growth kinetics (all three phases lag, log and stationary phase) in all the culture media. However, these growth phases were not prominent for some of the isolates in some media. The highest absorbances were attained in ACM (0.835) for BGLR6 followed by BG11 (0.633) for BGLR5 while the lowest ones were observed for Conway (0.078) in case of BGLR17 and GM F/2 (0.090) for the same BGLR17. All the nineteen isolates revealed a wide range of values in terms of dry cell biomass ranging from 0.3063 to 1.0890 g L^{-1} but overall the isolates BGLR5 and BGLR6 were found to have produced significantly highest dry cell biomas. The kinetics of growth and biomass production of microalgal isolates in their respective medium in which they showed highest biomass during screening, were studied by the Logistic model. The kinetic modelling includes the evaluation of biological or kinetic parameters like A (asymptote value); μ (growth rate); λ (lag time). These variables were calculated by using the modified Logistic model. The asymptote values for the algal isolates BGLR1-19 varied from 1.9444 g L^{-1} to 4.6444 g L^{-1}.

This parameter determines the highest potential of biomass production and is important for biotechnological purposes. The highest A is for BGLR6 followed by BGLR5. The highest growth rate (μ) was noticed in BGLR6 (0.0313 day^{-1}) followed by BGLR5 (0.0230 day^{-1}). Some of the isolates like BGLR4, BGLR5, BGLR6, BGLR9, BGLR11, BGLR13, BGLR18 and BGLR19 were processed for the determination of the micronutrients like copper, calcium, iron, potassium, magnesium, phosphorus, manganese, lead, arsenic etc. This was done as these mineral elements have potential impact on the anaerobic digestion process besides to check to the nutritive value of these isolates. It was observed that the heavy metals were not found in these and other elements like potassium, magnesium etc. which affect the anaerobic process are within the limits.

Based on the screening experiment, it was found that the isolates BGLR5 and BGLR6 produced highest dry biomass and are having relatively higher content of other functional ingredients. So these two isolates were selected for further study. The isolates were then identified at molecular level. The isolate BGLR5 on tentative morphological identification was found to be *Chlorella* sp. whereas BGLR6 was found to be *Spirulina* sp. So BGLR5 being eukaryotic in nature was identified through 18S rRNA sequencing while BGLR6 was identified by 16S rRNA sequencing. The 16S rRNA sequence analysis of this isolate and the phylogenetic tree construction showed 99% similarity with two *Spirulina* sequences (*S. Subsalsa* FACHB351 and *S. subsalsa*) available in the NCBI database. *Spirulina subsalsa* FACHB351 was found to be the most similar to the isolate. Hence, the BGLR6 isolate was identified as *Spirulina subsalsa* BGLR6. The 16S rDNA sequence was submitted to GenBank database under the accession number MF191711. The 18S rRNA sequence analysis of this isolate and the phylogenetic tree construction showed 99% similarity with the sequences of *Scenedesmus* sp. and *Asterarcys quadricellulare* available in the NCBI database. *Asterarcys quadricellulare* was found to be the most similar to the isolate. Hence, the BGLR5 isolate was identified as *Asterarcys quadricellulare* BGLR5. The 18S rDNA sequence was submitted to GenBank database under the accession number MF661929.

The optimization was carried out in two steps, firstly the screening of cultural factors was done by Placket-Burman design and then the significant factors obtained through screening were further optimized through response surface methodology. The Placket-Burman design was applied to comprehensively analyze the impact of seven different factors on the various responses in *Spirulina subsalsa* BGLR6 and *Asterarcys quadricellulare* BGLR5. All the factors mentioned above were found to be significant and were selected for optimization studies by CCD of RSM. In case of *Spirulina subsalsa* BGLR6, pH of 10.57, temperature of 20°C, the light intensity of 80.99 μmol m^{-2} s^{-1}, 24.99 days of the growth period, 15.00 mM $CaCl_2$, 5.00 mM $NaNO_3$ and 2.00 mM of K_2HPO_4 were the optimal and

most desired conditions as per the model, for different responses considered for this study. At these settings, the response variables generated a desirability index of 84.10%. These optimal values were very much close to the run 24 of CCD, which on the basis of observed and predicted desirability from the fitted model, is the most desirable run in terms of results obtained. Particularly, the biomass as per run 24 (2.559 gL^{-1}) and optimal values obtained from model (2.319 gL-1) were found to have increased by 1.60 and 1.34 folds respectively, than the basal medium conditions (0.987 gL^{-1}). Similarly, *Asterarcys quadricellulare* BGLR5, the obtained results and regression equations, pH of 9.92, temperature of 21.84°C, the light intensity of 80.99 µmol m^{-2} s^{-1}, 25 days of the growth period, 15.00 mM $CaCl_2$, 12.28 mM $NaNO_3$ and 7.09 mM of K_2HPO_4 were the optimal and most desired conditions as per the model, for different responses considered for this study. At these settings, the response variables generated a desirability index of 94.91%. The models were validated for both the isolates and it was observed that the values of responses obtained were close to that obtained from CCD of RSM and model predicted values at optimum. So, this close relationship of the values obtained from validation experiment to that of the RSM values and model predicted response values at optimum, revealed the confirmation, validity and acceptability of the model for the optimization of different physico-chemical factors discussed in this study for the two isolates BGLR6 and BGLR5.

From the biogas potential studies of algal biomass pretreated with different methods revealed that in case of BGLR5 biomass, the biogas produced from microalgal biomass pretreated with hydrothermal treatment of 100°C for 30 min (F) was significantly higher than others, while as biogas produced from biomass pretreated with Enzyme mix of 10 % dose for 24 h (D) was not significantly different from algal biomass pretreated with 20 % enzyme mix for 24 h (E) and hydrothermally pretreated biomass at 120 °C for an exposure time of 30 min i.e., digester G. Similarly, for BGLR6 biomass, the biogas produced from digester D containing biomass pretreated with enzyme mix of dose 10 % for 24 h (D) of exposure time was significantly higher than all others. Whereas, biomass pretreated with 20 % enzyme dose for 12 h (C), 20 % enzyme dose for 24 h (E) and 100 °C for 30 min (F) did not differ significantly from each other. Also, biogas from 10 % 12 h (B), 20 % 12 h (C) enzymatically pretraeted biomass did not differ significantly from each other. Further, biogas from hydrothermally pretreated at 120°C for 30 min was not found significantly different from 10 % 12 h (B), 20 % 12 h (C). The lowest biogas here was also observed from untreated biomass.

From the co-digestion studies, it was observed that in case of replacement of paddy straw by algal biomass, it was noticed for BGLR6, that digester C (70% PS+30% algal biomass) produced the highest biogas production potential (P) of 260.03 mLg^{-1} VS at a

maximum biogas production rate (R_m) of 8.16 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 3.84 days. Digester D (50% PS+50% algal biomass) follows digester C in biogas production potential estimated to be 250.45 mLg^{-1}VS at a maximum biogas production rate of 10.73 mLg^{-1} VS with a lag phase of 1.94 days. Digester B is at third number in biogas production. The least biogas production was noticed in A (only soaked paddy straw i.e., control). Also for BGLR5, it was noticed that digester D (50% PS+50% algal biomass) produced the highest biogas production potential (P) of 361.81 mLg^{-1} VS at a maximum biogas production rate (R_m) of 8.19 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 2.81 days. Digester C (70% PS+30% algal biomass) follows digester D in biogas production potential estimated to be 349.50 mLg^{-1}VS at a maximum biogas production rate of 7.37 mLg^{-1} VS with a lag phase of 3.40 days. Digester E (30% PS+70% algal biomass) is at third number in biogas production. The least biogas production was noticed in digester A (only paddy straw) followed by digester B (80% PS+20% algal biomass). Likewise on supplementation of algal biomass while keeping the amount of paddy straw same, it was observed that the digester D (250g PS + 125 g algal biomass) produced the highest biogas production potential (P) of 265.14 mLg^{-1} VS at a maximum biogas production rate (R_m) of 8.13 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 3.32 days at the end of 46-days period,. Digester C (250g PS + 75 g algal biomass) and E (250g PS + 175 g algal biomass) follows digester D (250g PS + 125 g algal biomass) in biogas production potential estimated to be 240.65 mLg^{-1}VS at a maximum biogas production rate of 8.01 mLg^{-1} VS with a lag phase of 3.99 days and 235.06 mLg^{-1}VS at a maximum biogas production rate of 7.93 mLg^{-1} VS with a lag phase of 2.78 days. Digester B (250g PS + 50 g algal biomass) was found to be at fourth number in biogas production. The least biogas production was noticed in digester A (control containing only paddy straw followed by digester G (250g PS + 500 g algal biomass).

In addition to this, cultivation of multipopulation microalgal species in open air algal pond was carried out and the algae population of the pond were identified tentatively by microscopic examination as blue green algae (*Spirulina* sp.), diatom (*Nitzschia* sp.) and green algae (*Chlorococcum* sp.) mainly. Initially, the population of *Spirulina* sp. was found to be dominating but with the passage of time, green algae (*Chlorococcum* sp.) dominated the microalgal population in the pond biogas production. After attaining the promising growth and biomass of algal species in the pond (wet weight 15 g L^{-1}), which was studied by taking the known volume of microalgae suspension from the pond, harvested through filtration and gravity settling. The algal suspension was then utilized for biogas production along with paddy straw. The paddy straw was mixed with this algal suspension in the field scale digester (Digester B). The bio-digested slurry was used as inoculum for this anaerobic digestion study. A control containing only paddy straw mixed with bio-digested slurry (inoculum) was run in

parallel (Digester A) to evaluate the biogas production from co-digestion of paddy straw and algal suspension. The anaerobic digestion was carried out for a period of 46 days and it was observed that the biogas production in digester B was significantly higher than the digester A An increase of 17.27% was achieved in case of digester B (0.129 m^3 biogas kg^{-1} feedstock fed) than that of the control i.e., digester A (0.110 m^3 biogas kg^{-1} feedstock fed). These values were closer to the results of the supplementation experiment conducted during this study. The enhancement of methane content from 46.4% (control) to 66.5% (digester containing algal biomass) was also achieved. The direct utilization of algal slurry saved time, energy as well as minimized harvesting cost, a part from meeting the requirement of soaking of paddy straw and above all providing the microalgal biomass in large amount for biogas production.

Conclusions

1. A total of nineteen isolates (BGLR1-19) were isolated and purified.
2. Upon screening BGLR5 and BGLR6 were found to be potential in terms of biomass production and higher growth kinetic parameters like A (asymptote value determining highest potential of biomass production) and growth rate (μ). Thus, these were thus selected for further study.
3. Optimal conditions for BGLR5 were found to be the pH of 9.92, the temperature of 21.84°C, the light intensity of 80.99 μmol m^{-2} s^{-1}, the growth period of 25 days, the NH_4Cl of 15.00 mM, the $NaNO_3$ of 12.28 mM and the K_2HPO_4 of 7.09 mM and for BGLR6 were the pH of 10.57, temperature of 20°C, the light intensity of 80.99 μmol m^{-2} s^{-1}, the growth period of 24.99 days, the $CaCl_2$ of 15.00 mM, the $NaNO_3$ of 5.00 mM and the K_2HPO_4 of 2.00 mM.
4. Biogas potential of BGLR5 was found to be more than that of BGLR6. Over all pretreatment (enzymatic or hydrothermal) of algal biomass was found to have enhancing effect on biogas production compared to untreated biomass.
5. From the co-digestion studies of algal biomass with paddy straw, it was revealed that the biogas production was enhanced both in replacement as well as supplementation experiments. The enhanced biogas production may be due to the stabilization of the C/N ratio and the synergistic interactions provided by co-digestion process. The co-digestion of BGLR5 with paddy straw resulted in more biogas than the BGLR6. Supplementation of algal biomass to paddy straw rather than replacement of paddy straw with equal amount of algal biomass was found to be more propitious and substantial in terms of biogas production.
6. It was demonstrated that multipopulation microalgal species in open air algal pond can be economically cultured on large scale in simple tap water added with sodium

bicarbonate @ 10 g L^{-1}. The microalgal population in open air algal being exposed to wide range temperature and high biomass growth makes this multipopulation as a promising source of biofuel (biogas) production.

7. Co-digestion of algal biomass with paddy straw improved the methane content and decreased significantly the CO_2, CO, H_2S and H_2 content in biogas.

Future prospective

1. Screening of prospective algae (single or consortium) having the required traits regarding biogas production.
2. Comprehensive analysis of digestion process of algal biomass so as to reveal the critical inhibitory factors for establishment of approaches to surmount the inhibitions by pretreatments or co-digestion.
3. Upgradation of lab scale studies to pilot scale level ascertaining the significance of biogas generation through algal biomass digestion**.**

REFERENCES

Abu G O, Ogbonda K H and Aminigo R E (2007) Optimization studies of biomass production and protein biosynthesis in a Spirulina sp. Isolated from an oil polluted flame pit in the Niger delta. *Afr J Biotechnol* **6**: 2550-54.

Abu-Rezq T S, Al-Musallam L, Al-Shimmari J and Dias P (1999) Optimum production conditions for different high-quality marine algae. *Hydrobiologia* **403**: 97-107.

Adinarayana K and Ellaiah P (2002) Response surface optimization of the critical medium components for production of alkaline protease by a newly isolated *Bacillus* sp. *J Pharm Pharmaceut Sci* **5**: 1152-58.

Akhnazarova S and Afarov K V (1982) *Experiment optimization in chemistry and chemical engineering*. Pp. 312. Mir Publishers, Moscow.

Alyabyev A, Andreyeva I and Rachimova G (2011) Influence of pH shift and concentration on growth rate and fatty acid composition of *Ellipsoidion* sp. (Eustigmatophyta). *J Appl Phycol* **13**: 463-69.

Alzate M E, Muñoz R, Rogalla F, Fdz-Polanco F and Pérez-Elvira S I (2012) Biochemical methane potential of microalgae: Influence of substrate to inoculum ratio, biomass concentration and pretreatment. *Bioresource Technol* **123**: 488-94.

Amin S (2009) Review on biofuel oil and gas production processes from microalgae. *Energ Conver Manag* **50**: 1834-40.

Amini Khoeyi Z, Seyfabadi J and Ramezanpour Z (2011) Effect of light intensity and photoperiod on biomass and fatty acid composition of the microalgae *Chlorella vulgaris*. *Aquacult Int* **20**: 41-49.

Andersen R A (2005) *Algal Culturing Techniques*. Pp. 596. Elsevier Academic Press, Burlington, Massachusetts, U.S.A.

Andrade M R and Costa J A V (2007) Mixotrophic cultivation of microalga *Spirulina platensis* using molasses as organic substrate. *Aquaculture* **264**: 130-34.

Angelidaki I, Alves M, Bolzonella D, Borzacconi L, Campos J L, Guwy A J, Kalyuzhnyi S, Jenicek P and van Lier J B (2009) Defining the biomethane potential (BMP) of solid organic wastes and energy crops: a proposed protocol for batch assays. *Water Sci Technol* **59**: 927-34.

Anvari M and Khayati G (2104) The effect of citrus pulp type on pectinase production in solid-state fermentation: process evaluation and optimization by Taguchi design of experimental (DOE) methodology. *J Biosci Biotech* **3**: 227-33.

AOAC (2000) Association of Official Analytical Chemists, *Official Methods of Analysis*, 17th Edition, Maryland, USA.

Aravantinou A F, Theodorakopoulos M A and Manariotis I D (2013) Selection of microalgae for wastewater treatment and potential lipids production. *Bioresource Technol* **147**: 130-134.

Banerjee A, Sharma R, Chisti Y and Banerjee U C (2002) *Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. *Crit Rev Biotechnol* **22**: 245-79.

Barbier G G, Zimmermann M and Weber A P M (2005) Genomics of the thermoacidophilic red alga *Galdieria sulphuraria*. I: Hoover R B, Levin G V, Rozanov A Y and Gladstone G R (ed), *Proc Astrobiology and Planetary Missions*. Vol 5906, pp. 67-78, San Diego, CA, USA.

Barbosa M J, Janssen M, Ham N, Tramper J, Wijffels R H (2003) Microalgae cultivation in air-lift reactors: modelling biomass yield and growth rate as a function of mixing frequency. *Biotechnol Bioeng* **82(2)**: 170-79.

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

Benemann J (2013) Microalgae for biofuels and animal feeds. *Energies* **6**: 5869-86.

Benning L G, Phoenix V R, Yee N and Tobin M J (2004) Molecular characterization of cyanobacterial silification using synchrotron infrared micro-spectroscopy. *Geocimica et Cosmochimica Acta* **68**: 729-41.

Bird K T, Chynoweth D P and Jerger D E (1990) Effects of marine algal proximate composition on methane yields. *J Appl Phycol* **2**: 207-13.

Bischoff H W and Bold H C (1963) *Phycological studies (IV) some soil algae from enchanted rock and related algal species.* Pp. 1-95. University of Texas Publications (6318).

Bohutskyi P and Bouwer E (2013) Biogas production from algae and cyanobacteria through anaerobic digestion: A review, analysis, and research needs. In: *Advanced biofuels and bioproducts.* Pp. 873-975. New York: Springer.

Borowitzka M A (1993a) Large-scale algal culture systems: the next generation. I: Sargeant J, Washer S, Jones M and Borowitzka M A (ed) *11th Australian biotechnology conference*, pp 61, Perth, WA.

Borowitzka M A (1993b) Products from microalgae. *Infofish* 5(93): 21-26

Borowitzka M A (1999) Commercial production of microalgae: ponds, tanks, and fermenters. *J Biotechnol* **35**: 313-21.

Borowitzka M A (2013) High-value products from microalgae their development and commercialisation. *J Appl Phycol* **25**: 743-56

Bożym M, Floozak I, Zalanowska, Wojdalski J and Klimkiewicz M (2015) An analysis of metal concentrations in food wastes for biogas production. *Renew Energ* **77**: 467-72.

Bozzola J J and Russell L D (1999) *Electron microscopy: Principles and techniques for biologists*. Pp. 48-69. Jones and Bartlett Publishers Inc, Sudbury.

Brahamsha B (1996) A genetic manipulation system for oceanic cyanobacteria of the genus *Synechococcus. Appl Environ Microb* **62**: 1747-51.

Braun R (2007) Anaerobic digestion: a multi-facetted process for energy, environmental management and rural development. In: Paolo R (ed) *Improvement of crop plants for industrial uses*. Pp. 335-416, Springer, Dordrecht, Netherland.

Briand X and Morand P (1997) Anaerobic digestion of *Ulva* sp 1 relationship between *Ulva* composition and methanisation. *J Appl Phycol* **9**: 511-24.

Bruno W J, Socci N D and Halpern A L (2000) Weighted Neighbor Joining: A likelihood-based approach to distance-based phylogeny reconstruction. *Mol Biol Evol* **17(1)**: 189-97.

Bruton T, Lyons H, Lerat Y, Stanley M and Rasmussen M B (2009) *A review of the potential of marine algae as a source of biofuel in Ireland*. Pp.1-88. Dublin: Sustainable Energy Ireland.

Burczyk J and Dworzanskib J (1988) Comparison of sporopollenin like algal resistant polymer from cell wall of *Botryococcus, Scenedesmus* and *Lycopodium clavatum* by GC pyrolysis. *Phytochemistry* **27**: 2151-53.

Carlsson M, Lagerkvist A and Morgan-Sagastume F (2012) The effects of substrate pre-treatment on anaerobic digestion systems: A review. *Waste Manage* **32(9)**: 1634-50.

Carrère H, Dumas C, Battimelli A, Batstone D J, Delgenes J P, Steyer J P and Ferrer I (2010) Pretreatment methods to improve sludge anaerobic degradability: A review. *J Hazard Mater* **183**: 1-15.

Carvalho A P and Malcata F X (2003) Kinetic modeling of the autotrophic growth of *Pavlova lutheri*: study of the combined influence of light and temperature. *Biotechnol Progr* **19**: 1128-35.

Carvalho A P, Meireles L A and Malcata F X (2006) Microalgal reactors: a review of enclosed system designs and performances. *Biotechnol Progr* **22**: 1490-506.

Carvalho A P, Silva S O, Baptista J M and Malcata F X (2011) Light requirements in microalgal photobioreactors: An overview of biophotonic aspects. *Appl Microbiol Biot* **89**:1275-88.

Celekli A and Yavuzatmaca M (2009) Predictive modeling of biomass production by *Spirulina platensis* as function of nitrate and NaCl concentrations. *Bioresource Technol* **100**: 1847-51.

Chanakya H N, Malayil S and Reddy B V V (2012) Anaerobic digestion for bioenergy from agroresidues and other solid wastes-an overview of science, technology and sustainability. *J Indian Inst Sci* **92**:111-43.

Chandra R, Takeuchi H and Hasegawa T (2011) Methane production from lingo cellulosic agricultural crop wastes: a review in context to second generation of biofuel production. *Renew Sust Energ Rev* **16(3)**: 1462-76.

Chayanon S, Surendra K C, Devin T, Hans O and Samir K K (2014) Anaerobic digestion of lignocellulosic biomass: Challenges and opportunities *Bioresource Technol* 36: 104-07.

Chen G Q and Chen F (2006) Growing phototrophic cells without light. *Biotechnol Lett* **28**: 607-16.

Chen P H and Oswald W J (1998) Thermochemical treatment for algal fermentation. *Environ Int* 24: 889-97.

Chen X C, Bai J X, Cao J M, Li Z J, Xiong J, Zhang L, Hong Y and Ying H J (2009) Medium optimization for the production of cyclic adenosine 3′, 5′-monophosphate by *Microbacterium* sp. no. 205 using response surface methodology. *Bioresource Technol* **100**: 919-924.

Chen Y R, Cheng J J and Creamer K S (2008) Inhibition of anaerobic digestion process: a review. *Bioresource Technol* 99(10): 4044-64.

Chiaramonti D, Oasmaa A and Solantausta Y (2007) Power generation using fast pyrolysis liquids from biomass. *Renew Sust Energ Rev* 11(6): 1056-86.

Chinnasamy S, Ramakrishnan B, Bhatnagar A and Das K C (2009) Biomass production potential of wastewater alga *Chlorella vulgaris* ARC1 under elevated levels of CO_2 and temperature. *Int J Mol Sci* **10**: 518-32.

Chisti Y (2006) Microalgae as sustainable cell factories. *Environ Eng Manag J* **5(3)**: 261-74.

Chisti Y (2007) Biodiesel from microalgae. *Biotechnol Adv* **25(3)**: 294-306.

Cho S H, Ji S C, Hur S B, Bae J, Park I S and Song Y C (2007) Optimum temperature and salinity conditions for growth of green algae *Chlorella ellipsoidea* and *Nannochloris oculata*. *Fisheries Sci* **73**: 1050-56.

Cho S, Park S, Seon J, Yu J and Lee T (2013) Evaluation of thermal, ultrasonic and alkali pretreatments on mixed-microalgal biomass to enhance anaerobic methane production. *Bioresource Technol* **143**: 330-36.

Chojnacka K and Marquez-Rocha F J (2004) Kinetic and stoichiometric relationships of the energy and carbon metabolism in the culture of microalgae. *Biotechnol* **3**: 21-34.

Chynoweth D P (2005) Renewable biomethane from land and ocean energy crops and organic wastes. *Hort Sci* **40**: 283-86.

Coleman J R and Colman B (1981) Inorganic carbon accumulation and photosynthesis in a blue-green alga as a function of external pH. *J Phycol* **27**: 2-8.

Colla L M, Reinehr C O, Reichert C and Costa J A V (2007) Production of biomass and nutraceutical compounds by *Spirulina platensis* under different temperature and nitrogen regimes. *Bioresource Technol* **98**: 1489-93.

Colman B and Rotatore C (1995) Photosynthetic inorganic carbon uptake and accumulation in two marine diatoms. *Plant Cell Environ* **18**: 919-24.

Converti A, Casazza A A, Ortiz E Y, Perego P and Del Borghi M (2009) Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. *Chem Eng Process* **48**: 1146-51.

Costa J C, Gonçalves P R, Nobre A and Alves M M (2012) Biomethanation potential of macroalgae *Ulva* spp. and *Gracilaria* spp. and in co-digestion with waste activated sludge. *Bioresource Technol* **114**: 320-26.

Cox G W and Cochran W G (1964) *Experimental designs.* John Wiley & Sons Inc, New York.

Cuaresma M, Casal C, Forján E and Vílchez C (2011) Productivity and selective accumulation of carotenoids of the novel extremophile microalga *Chlamydomonas acidophila* grown with different carbon sources in batch systems. *J Ind Microbiol Biotechnol* **38**: 167-77.

Cuetos M J, Fernandez C, Gomez X and Moran A (2011) Anaerobic co-digestion of swine manure with energy crop residues. *Biotechnol Bioprocess Eng* **16**: 1044-52.

Das H and Singh S (2004) Useful by-products from cellulosic wastes of agriculture and food industry-A critical appraisal. *Crit Rev Food Sci* **44 (2)**: 77-89.

De Castro Araujo S and Tavano-Garcia V M (2005) Growth and biochemical composition of the diatom *Chaetoceros cf. wighamii brightwell* under different temperature, salinity and carbon dioxide levels. I. Protein, carbohydrates and lipids. *Aquaculture* **246**: 405-12.

De la Rubia M A, Riau V, Raposo F and Borja R (2013) Thermophilic anaerobic digestion of sewage sludge: focus on the influence of the start-up. A review. *Crit Rev Biotechnol* **33(4)**: 448-60.

De Morais M G, Costa J A V (2007) Carbon dioxide fixation by *Chlorella kessleri, C.Vulgaris, Scenedesmus obliquus* and *Spirulina* sp. cultivated in flasks and vertical tubular photobioreactors. *Biotechnol Lett* **29**: 1349-52.

Demirbas A (2008) Products from lignocellulosic materials via degradation processes. *Energy Sources, Part A: Recovery Utiliz Environ Effects* **30 (1):** 27-37.

Dickerson, Jennifer, Jillian H, Stephanie M, April M, Jared S, Sihong Z (2009) Recovering energy waste while cutting costs: sustainable energy conversion of bio-waste. Miami University of Ohio. (Retrieved January 20, 2011 www.cas.muohio.edu/~stevenmh/MUITDCReport.pdf.)

Ding S Y, Liu Y S, Zeng Y N, Himmel M E, Baker J O and Bayer E A (2012) How does plant cell wall nanoscale architecture correlate with enzymatic digestibility? *Science* **338**: 1055-060.

Dodd J C (1986) Elements of pond design and construction. In A. Richmond (ed) *Handbook of microalgal mass culture*. Pp. 265-283, CRC Press, Boca Raton, Florida.

DuBois M, Gilles K A, Hamilton J K, Rebers P A and Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* **28(3)**: 350-56.

Duygu D Y, Udoh A U, Ozer T B, Akbulut A, Erkaya I A, Yildiz K and Guler D (2012) Fourier transform infrared (FTIR) spectroscopy for identification of *Chlorella vulgaris* Beijerinck 1890 and *Scenedesmus obliquus* (Turpin) Kützing 1833. *African J Biotechnol* **11**: 3817-24.

Ehimen E A, Connaughton S, Sun Z and Carrington G C (2009) Energy recovery from lipid extracted, transesterified and glycerol codigested microalgae biomass. *GCB Bioenergy* **1**: 371-81.

Ehimen E A, Holm-Nielsen J B, Poulsen M, Boelsmand J E(2013) Influence of different pretreatment routes on the anaerobic digestion of a filamentous algae. *Renew Energ* **50**: 476-80.

Ehimen E, Sun Z and Carrington C (2010) Variables affecting the in situ transesterification of microalgae lipids. *Fuel* **89**: 677-84.

El-Baky H H A, El-Baz F K, El-Baroty G S (2008) Characterization of nutraceutical compounds in blue green alga *Spirulina maxima. J Med Plants Res* **2(10)**: 292-300.

Fan J, Yan C, Andre C, Shanklin J, Schwender J and Xu C (2012) Oil accumulation is controlled by carbon precursor supply for fatty acid synthesis in *Chlamydomonas reinhardtii*. *Plant Cell Physiol* **53**: 1380-90.

Fernandez J, Perez M and Romero L (2008) Effect of substrate concentration on dry mesophilic anaerobic digestion of organic fraction of municipal solid waste (OFMSW). *Bioresource Technol* **99**: 6075-80.

Fon Sing S, Isdepsky A, Borowitzka M and Moheimani N (2013) Production of biofuels from microalgae. *Mitig Adapt Strat Glob Change* **18(1)**: 47-72

Fu C C, Hung T C, Chen J Y, Su C H and Wu W T (2010) Hydrolysis of microalgae cell walls for production of reducing sugar and lipid extraction. *Bioresource Technol* **101**: 8750-54.

Gadde B, Bonnet S, Menke C and Garivait S (2009) Air pollutant emissions from rice straw open field burning in India, Thailand and Phillipines. *Environ Pollut* **157**: 1554-58.

Gerken H G, Donohoe B and Knoshaug E P (2013) Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production. *Planta* **237**: 239-53.

Ghirardi M L, Zhang L, Lee J W, Flynn T, Seibert M, Greenbaum E and Melis A (2000) Microalgae: A green source of renewable H_2. *Trends Biotechnol* **18**: 506-11.

Go S, Lee S J, Jeong G T and Kim S K (2012) Factors affecting the growth and the oil accumulation of marine microalgae, *Tetraselmis suecica*. *Bioproc Biosyst Eng* **35**: 145-50.

Godhe A, Anderson D M and Rehnstam-Holm A S (2002) PCR amplification of microalgal DNA for sequencing and species identification: studies on fixatives and algal growth stages. *Harmful Algae* **27**: 1-8.

Goldman J C, Azov J, Riley C B and Dennet M R (1982) The effect of pH in intensive microalgal cultures. I. Biomass regulation. *J Exp Marine Biol Ecol* **57**: 1-13.

Golueke C G, Oswald W J and Gotaas H B (1957) Anaerobic digestion of algae. *Appl Microbiol* **5**: 47-55.

González-Fernández C, Molinuevo-Salces B and García-González M C (2011) Evaluation of anaerobic codigestion of microalgal biomass and swine manure via response surface methodology. *Appl Energ* **88**: 3448-53.

González-Fernández C, Sialve B, Bernet N and Steyer J P (2012) Impact of microalgae characteristics on their conversion to biofuel. Part II: Focus on biomethane production. *Biofuel, Bioprod Biorefin* **6**: 205-18.

Gouveia L, Marques A E, da Silva T L and Reis A (2009) *Neochloris oleabundans* UTEX 1185: a suitable renewable lipid source for biofuel production. *J Ind Microbiol Biotechnol* **36(6)**: 821-26.

Grala A, Zieliński M, Dębowski M and Dudek M (2012) Effects of Hydrothermal depolymerization and enzymatic hydrolysis of algae biomass on yield of methane fermentation process. *Pol J Environ Stud* **21(2)**: 361-66.

Greenwell H C, Laurens L M L, Shields R J, Lovitt R W and Flynn K J (2010) Placing microalgae on the biofuels priority list: a review of the technological challenges. *J Royal Soc Interf* **7(46)**: 703-26.

Grobbelaar J U (2008) Factors governing algal growth in photobioreactors: the "open" versus "closed" debate. *J Appl Phycol* **21**: 489-92.

Guillard R R L and Ryther J H (1962) Studies of marine planktonic diatoms: i. *Cyclotella nana* Hustedt and *Detonula confervacea* (cleve) gran. *Can J Microbiol* **8**: 229-39.

Habig C, De Busk A T and Ryther J H (1984) The effect of nitrogen content on methane production by the marine algae *Gracilaria tikvahiae* and *Ulva* sp. *Biomass* **4**: 239-51.

Haddar A, Fakhfakh-Zouari N, Hmidet N, Frikha F, Nasri M and Kamoun AS (2010) Low-cost fermentation medium for alkaline protease production by *Bacillus mojavensis* A21 using hulled grain of wheat and sardinella peptone. *J Biosci Bioeng* **110**: 288-294.

Harun R, Singh M, Forde G M and Danquah M K (2010) Bioprocess engineering of microalgae to produce a variety of consumer products. *Renew Sust Energ Rev* **14(3)**: 1037-47.

Hassler C S, Djajadikarta J R, Doblin M A, Everett J D and Thompson PA (2011) Characterisation of water masses and phytoplankton nutrient limitation in the East Australian Current separation zone during spring 2008. *Deep-Sea Res II* **58(5)**: 664-67

Heerenklage J, Maxfield T, Zapf A, Adwiraah H, Wieczorek N and Koerner I (2010) Anaerobic digestion of microalgae—possibilities and limits. *3rd International Symposium on Energy from Biomass and Waste*, 8-11 November, Venice, Italy.

Hendriks A T W M and Zeeman G (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technol* **100**: 10-18.

Hildebrand M, Davis A K, Smith S R, Traller J C and Abbriano R (2012) The place of diatoms in the biofuels industry. *Biofuels* **3(2)**: 221-40.

Hirano A, Ueda R and Hirayama S (1997) CO_2 fixation and ethanol production with microalgal photosynthesis and intracellular anaerobic fermentation. *Energy* **22(2/3)**: 137-42.

Hodaifa G, Martinez M E and Sánchez S (2011) Daily doses of light in relation to the growth of *Scenedesmus obliquus* in diluted three-phase olive mill wastewater. *J Chem Tech Biot* **84**: 1550-58.

Holm-Nielsen J B, Al Seadi T and Oleskowicz-Popiel P (2009) The future of anaerobic digestion and biogas utilization. *Bioresource Technol* **100**: 5478-84.

Hongjin Q and Guangce W (2009) Effect of carbon source on growth and lipid accumulation in *Chlorella sorokiniana* GXNN01. *Chin J Oceanol Limn* **27**: 762-68.

Hsieh C H and Wu W T (2009) A novel photobioreactor with transparent rectangular chambers for cultivation of microalgae. *Biochem Eng J* **46**: 300-05.

Hsieh C H and Wu W T (2009) Cultivation of microalgae for oil production with a cultivation strategy for urea limitation. *Bioresource Technol* **100**: 3921-26.

Hu H and Zhou Q (2010) Regulation of inorganic carbon acquisition by nitrogen and phosphorus levels in the *Nannochloropsis* sp. *World Journal of Microb Biot* **26**: 957-61.

Hu Q, Zarmi Y and Richmond A (1998) Combined effects of light intensity, light path and culture density on output rate of *Spirulina platensis* (Cyanobacteria). *Eur J Phycol* **33**: 165-71.

Huertas E I, Colman B, Espie G S and Lubian L M (2000) Active transport of CO_2 by three species of marine microalgae. *J Phycol* **36**: 314-20.

Huntley M E and Redalje D G (2007) CO_2 Mitigation and renewable oil from photosynthetic microbes: A new appraisal. *Mitig Adapt Strat Gl* **12**: 573- 608.

IEA (2010) *Sustainable Production of Second-Generation Biofuels (Report)*. (http: //www.iea.org/papers/2010/second generation biofuels.pdf.)

Iovane P, Nanna F, Ding Y, Bikson B and Molino A (2014) Experimental test with polymeric membrane for the biogas purification from CO_2 and H_2S. *Fuel* **135**: 352-58.

IPCC (2007) *Intergovernmental panel on climate change: fourth assessment report (AR4)*. (http: //www.ipcc.ch/pdf/assessment-report/ar4/syr/ar4 syr.pdf.)

Jacob-Lopes E, Scoparo C H G, Lacerda L M C F and Franco T T (2009) Effect of light cycles (night/day) on CO_2 fixation and biomass production by microalgae in photobioreactors. *Chem Eng Process*: *Process Intensification* **48**: 306-10.

Jalc D, Nerud F and Siroka P (1998) The effectiveness of biological treatment of wheat straw by white-rot fungi. *Folia Microbiol* **43**: 687-89.

Jan P and Alexandra P (2006) Process of removing silica from cellulosic material. U.S. Patent, 20060225852.

Jard G, Dumas C, Delgenes J P, Marfaing H, Sialve B, Steyer J P and Carrère H (2013) Effect of thermochemical pretreatment on the solubilization and anaerobic biodegradability of the red macroalga *Palmaria palmate*. *Biochem Eng J* **79**: 253-58.

Jazzar S, Berrejeb N, Messaoud C, Marzouki M N and Smaali I (2016) Growth parameters, photosynthetic performance, and biochemical characterization of newly isolated green microalgae in response to culture condition variations. *Appl Biochem Biotechnol* **179**: 1290-1308.

Jeffery S W and Wright S W (1999) Photosynthetic pigments in the Haptophyta. In: Cohen Z (ed) *Chemical from microalgae*. Pp 111-32. Taylor and Francis, Philadelphia.

Jiang P, Zhang Y, Shan Z X and Zheng Q H (2013) Optimizing the extraction yield of polyprenols from needles of *Cunninghamia Ianceolata* (Lamb.) hook using response surface methodology and its antioxidative activities. *Bioresources* 1: 545-56.

John R P, Anisha G S, Nampoothiri K M and Pandey A (2011) Micro and macroalgal biomass: a renewable source for bioethanol. *Bioresource Technol* **102(1)**: 186-93.

Kaatze U D O (1995) Fundamentals of microwaves. *Radiat Phys Chem* **45**: 539-48.

Kacka A and Donmez G (2008) Isolation of *Dunaliella* sp. from a hypersaline lake and their ability to accumulate glycerol. *Bioresource Technol* **99**: 8348-52.

Kaushik R, Saran S, Isar J and Saxena R (2006) Statistical optimization of medium components and growth conditions by response surface methodology to enhance lipase production by *Aspergillus carneus*. *J Mol Catal B Enzyme* **40**: 121-26.

kemka V N and Murto M (2010) Evaluation of biogas production from seaweed in batch tests and in UASB reactors combined with the removal of heavy metals. *J Environl Manage* **91**: 1573-79.

Kepp U, Machenbach I, Weisz N and Solheim O E (2000) Enhanced stabilisation of sewage sludge through thermal hydrolysis—Three years of experience with full-scale plant. *Water Sci Technol* **42**: 89-96.

Keymar P, Ruffell I, Pratt S and Lant P (2013) High pressure thermal hydrolysis as pre-treatment to increase the methane yield during anaerobic digestion of microalgae. *Bioresource Technol* **131**: 128-33.

Khan S A, Rashmi Hussain M Z, Prasad S and Banerjee U C (2009) Prospects of biodiesel production from microalgae in India. *Renew Sust Energ Rev* **13**: 2361-72.

Khan S A, Rashmi, Mir Z H, Prasad S and Banerjee U C (2009) Prospects of biodiesel production from microalgae in India. *Renew Sust Energ Rev* **13**: 2361-72.

Khozin-Goldberg I and Cohen Z (2006) The effect of phosphate starvation on the lipid and fatty acid composition of the fresh water eustigmatophyte *Monodus subterraneus*. *Phytochemistry* **67**: 696-701.

Kim J, Lee J and Siddiqui K F (2010) Effects of total inorganic carbon on growth of *Chlorella vulgaris*. *10th AIChE Annual Meeting*. Salt Lake City, UT.

Kim J, Yoo G, Lee H, Lim J, Kim K, Kim C H, Park M S and Yang J W (2013) Methods of downstream processing for the production of biodiesel from microalgae. *Biotechnol Adv* **31**: 862-76.

Kim N and Lee C (2001) A theoretical consideration on oxygen production rate in microalgal cultures. *Biotechnol Bioprocess Eng* **6**: 352-58.

Kim W, Park J M, Gim G H, Jeong S H, Kang C M, Kim D K and Kim S W (2012) Optimization of culture conditions and comparison of biomass productivity of three green algae. *Bioprocess Biosyst Eng* **35**: 19-27.

Kirrolia A, Bishnoi N R and Singh R (2014) Response surface methodology as a decision-making tool for optimization of culture conditions of green microalgae *Chlorella* spp. for biodiesel production. *Ann Microbiol* 64: 1133-47.

Kitaya Y, Azuma H and Kiyota M (2005) Effects of temperature, CO_2/O_2 concentrations and light intensity on cellular multiplication of microalgae, *Euglena gracilis*. *Adv Space Res* **35**: 1584-88.

Knuckey R M, Brown M R, Barrett S M and Hallegraeff G M (2002) Isolation of new nanoplanktonic diatom strains and their evaluation as diets for juvenile Pacific oysters (*Crassostrea gigas*). *Aquaculture* **211**: 253-74.

Kodama M, Ikemoto H and Miyachi S (1993) A new species of highly CO_2- tolerant fast growing marine microalga suitable for high-density culture. *J Mar Biotechnol* **1**: 21-25.

Kruse O, Rupprecht J, Mussgnug J H, Dismukes G C and Hankamer B (2005) Photosynthesis: a blueprint for solar energy capture and biohydrogen production technologies. *Photochem and Photobiol Sci* **4**: 957-70.

Kumar A, Ergas S, Yuan X, Sahu A, Zhang Q, Dewulf J, Malcata F X and van Langenhove H (2010) Enhanced CO_2 fixation and biofuel production via microalgae: recent developments and future directions. *Trends Biotechnol* **28**: 371-80.

Kwietniewska E and Tys J (2014) Process characteristics, inhibition factors and methane yields of anaerobic digestion process, with particular focus on microalgal biomass fermentation. *Renew Sust Energ Rev* **34**: 491-500.

Lakaniemi A M, Hulatt C J, Thomas D N and Puhakka J A (2011) Biogenic hydrogen and methane production from *Chlorella vulgaris* and *Dunaliella tertiolecta* biomass. *Biotechnol Biofuels* **4**: 1-12.

Lee A K, Lewis D A and Ashman P J (2012) Disruption of microalgal cells for the extraction of lipids for biofuels: Processes and specific energy requirements. *Biomass Bioenergy* **46**: 89-101.

Lee Y K and Pirt S J (1984) CO_2 absorption rate in an algal culture: Effect of pH. *Journal of Chemical Technology and Biotechnology* **34**: 28-32.

Lembi C A and Waaland J R (ed) (1988) *Algae and human Affairs*. Cambridge University Press.

Li Q, Du W and Liu D (2008a) Perspectives of microbial oils for biodiesel production. *Appl Microbiol Biot* **80**: 749-56.

Li Y, Horsman M, Wang B, Wu N and Lan C Q (2008b) Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. *Appl Microbiol Biot* **81**: 629-36.

Lichtenthaler H K (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Meth Enzymol.* **148**: 350-82.

Lin Q and Lin J (2011) Effect of nitrogen source and concentration on biomass and oil production of a *Scenedesmus rubescens* like microalga. *Bioresource Technol* **102**: 1615-21.

Liu Z Y, Wang G C and Zhou B C (2008) Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. *Bioresource Technol* **99**: 4717-22.

Livansky K and Bartos J (1986) Relationship between pCO2 and pH in a medium for algal culture. *Archiv für Hydrobiologie - Supplement* 73: 425-31.

Lowry O H, Rosebrough N J, Farr A L and Randall R J (1951) Protein measurement with folin-phenol reagent. *J Biol Chem* **193**: 265-75.

Malik A and Prajapati S K (2012) Algae—a substrate for fermentative biogas production. *National Convention on "Current and Emerging Trends in Indian Biogas and Bio-fertilizers Development"*. Indian Institute of Technology Delhi, New Delhi, India.

Mandal S and Mallick N (2009) Microalga *Scenedesmus obliquus* as a potential source for biodiesel production. *Appl Microbiol Biotechnol* **84**: 281-91.

Maria G S Jutson, Richard K Pipe and Carmelo R. Tomas (2016) The Cultivation of Marine Phytoplankton. In Tsaloglou M N (ed) *Microalgae*: *Current Research and Applications.* Pp11-26. Caister Academic Press, U.K.

Martinez AT, Speranza M, Ruiz-Duenas F J, Ferreira P, Camarero S, Guillen F, Martinez M J, Gutierrez A and del Rio J C (2005) Biodegradation of lignocellulosics: Microbial, chemical and enzymatic aspects of the fungal attack of lignin. *Int Microbiol* **8**: 195-204.

Martínez M E, Jiménez J M and El Yousfi F (1999) Influence of phosphorus concentration and temperature on growth and phosphorus uptake by the microalga *Scendesmus obliquus*. *Bioresource Technol* **67**: 233-40.

Martínez M E, Sánchez S, Jiménez J M, El Yousfi F and Muñoz L (2000) Nitrogen and phosphorus removal from urban wastewater by the microalga *Scenedesmus obliquus*. *Bioresource Technol* **73**: 263-72.

Mata-Alvarez J, Dosta J, Romero-Güiza M S, Fonoll X, Peces M and Astals S (2014) A critical review on anaerobic co-digestion achievements between 2010 and 2013. *Renew Sustainable Energy Rev* **36**: 412-27.

Matsudo M C, Moraes F A, Bezerra R P, Arashiro R E, Sato S and Carvalho J C M (2015) Use of acetate in fed-batch mixotrophic cultivation of *Arthrospira platensis. Ann Microbiol* **65**: 1721-28.

Maxwell E L, Folger A G, Hogg S E (1985) *Resource evaluation and site selection for microalgae production systems.* Pp.100. SERI/TR-215-2484, Solar Energy Research Institute, Colarado.

Mayo A W (1997) Effects of temperature and pH on the kinetic growth of unialga *Chlorella vulgaris* cultures containing bacteria. *Water Environ Res* **69**: 64-72.

Medipally S R, Yusoff F M, Banerjee S and Shariff M (2014) Microalgae as sustainable renewable energy feedstock for biofuel production. *BioMed Res Int* **2015**: 1-13 (Article ID 519513).

Melis A (2002) Green alga hydrogen production: progress, challenges and prospects. *Int J Hydrogen Energy* **27**: 1217-28.

Mendez L, Mahdy A, Demuez M, Ballesteros M and González-Fernández C (2014) Effect of high pressure thermal pretreatment on *Chlorella vulgaris* biomass: Organic matter solubilisation and biochemical methane potential. *Fuel* **117**: 674-79.

Merrett M J, Nimer N A and Dong L F (1996) The utilization of bicarbonate ions by the marine microalga *Nannochloropsis oculata* (Droop) Hibberd. *Plant Cell Environt* **19**: 478-84.

Meseck S L, Alix J H and Wikfors G H (2005) Photoperiod and light intensity effects on growth and utilization of nutrients by the aquaculturer feed microlaga, *Tetraselmis chui* (PLY429). *Aquaculture* **246**: 393-404.

Metzger P and Largeau C (2005) *Botryococcus braunii*: a rich source for hydrocarbons and related ether lipids. *Appl Microbiol Biotechnol* **66**: 486-96.

Miao X and Wu Q (2006) Biodiesel production from heterotrophic microalgal oil. *Bioresource Technol* **97(6)**: 841-6.

Migliore G, Alisi C, Sprocati A R, Massi E, Ciccoli R, Lenzi M, Wang A and Cremisini C (2012) Anaerobic digestion of macroalgal biomass and sediments sourced from the Orbetello Lagoon, Italy. *Biomass Bioenerg* **42**: 69-77.

Millati I R, Syamsiah S, Niklasson C, Cahyanto M N, Lundquist K and Taherzadeh MJ (2011) Biological pretreatment of lignocelluloses with white-rot fungi and its applications: A review. *Bioresources* **6 (4)**: 5224-59.

Miron A S, Garcia M C C, Gomez A C, Camacho F G, Grima E M and Chisti Y (2003) Shear stress tolerance and biochemical characterization of *Phaeodactylum tricornutum* in quasi steady state continuous culture in outdoor photobioreactors. *Biochem Eng J* **16**: 287-97.

Mishra S K, Suh W I, Farooq W, Moon M, Shrivastav A, Park-Min S and Yang J W (2014) Rapid quantification of microalgal lipids in aqueous medium by a simple colorimetric method. *Bioresour Technol* **155**: 330-33.

Mitra M, Patidar SK, George B, Shah F, Mishra S (2015) A euryhaline *Nannochloropsis gaditana* with potential for nutraceutical (EPA) and biodiesel production. *Algal Res* **8**: 161-67.

Moheimani N (2012) Inorganic carbon and pH effect on growth and lipid productivity of *Tetraselmis suecica* and *Chlorella* sp (Chlorophyta) grown outdoors in bag photobioreactors. *J Appl Phycol* 1-12.

Moheimani N R and McHenry M P (2013) Developments of five selected microalgae companies developing "closed" bioreactor biofuel production systems. *Int J Innov Sustain Dev* **7**: 367-86

Moheimani N R, Cord-Ruwisch R, Raes E and Borowitzka M (2013) Non-destructive oil extraction from *Botryococcus braunii* (Chlorophyta). *J Appl Phycol* **25(6)**: 1653-61.

Moheimani N R, McHenry M P and de Boer K (2013) The forefront of low-cost and high-volume open microalgae biofuel production. In: Gupta V K, Schmoll M, Maki M, Tuohy M and Mazutti M A (ed) *Applications of microbial engineering.* Pp 426-49. CRC Press, Boca Raton.

Moheimani N R, Lewis D, Borowitzka M A and Pahl S (2011) Harvesting, thickening and dewatering microalgae. In: Carioca J O B (ed) *International microalgae and biofuels workshop*. pp 227. Fortaleza, Brasil.

Molina E, Belarbi E, Acien F, Robles A and Chisti Y (2003) Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnol Adv* **20**: 491-515.

Monlau F, Barakat A, Steyer J P and Carrere H (2012) Comparison of seven types of thermo-chemical pretreatments on the structural features and anaerobic digestion of sunflower stalks. *Bioresource Technol* **120**: 241-47.

Montgomery D C (2005) *Design and Analysis of Experiments: Response Surface Method and Designs*. Wiley, New York.

Moreno-Garrido I (2008) Microalgae immobilization: current techniques and uses. *Bioresource Technol* **99**: 3949-64.

Morita M, Watanabe Y, Saiki H (2001) Evaluation of photobioreactor heat balance predicting changes in culture medium temperature due to light irradiation. *Biotechnol Bioeng* **74(6)**: 465-75

Movasaghi Z, Rehman S and Rehman I (2008) Fourier Transform Infrared (FTIR) Spectroscopy of Biological Tissues. *Appl Spectrosc Rev* **43(2)**: **1**34-79.

Mudhoo A and Kumar S (2013) Effects of heavy metals as stress factors on anaerobic digestion processes and biogas production from biomass. *Int J Environ Sci Technol* **10(6)**: 1383-98.

Murdock J N and Wetzel D L (2009) FT-IR Microspectroscopy enhances biological and ecological analysis of algae. *Appl Spectroscopy Rev* **44**: 335-61.

Mussgnug J H, Klassen V, Schlüter A and Kruse O (2010) Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. *J Biotechnol* **150**: 51-60.

Mussoline W, Esposito G, Giordano A and Lens P (2013) The anaerobic digestion of rice straw: A review. *Crtic Rev Env Sci Tech* **43**: 895: 915.

Mutanda T, Ramesh D, Karthikeyan S, Kumari S, Anandraj A and Bux F (2011) Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production. *Bioresource Technol* **102**: 57-70.

Olaizola M (2003) Commercial development of microalgal biotechnology: from the test tube to the marketplace. *Biomol Eng* **20**: 459-66.

Oliveira J V, Alves M M and Costa J C (2014) Design of experiments to assess pre-treatment and co-digestion strategies that optimize biogas production from macroalgae *Gracilaria vermiculophylla*. *Bioresource Technol* **162**: 323-30.

Oron G, Shelef G and Levi A (1979) Growth of *Spirulina maxima* on cow manure wastes. *Biotechnol Bioeng* **21**: 2165-73.

Oswald J A (1988) Large-Scale Algal Culture Systems (Engineering Aspects), In: L. J. Borowitzka and M. A. Borowitzka (ed) *Microalgal biotechnology*. Pp. 357-95. Cambridge University Press, Cambridge.

Park K Y, Kweon J, Chantrasakdakul P, Lee K and Cha H Y (2013) Anaerobic digestion of microalgal biomass with ultrasonic disintegration. *Int Biodeter Biodegr* **85**: 598-602.

Park S and Li Y (2012) Evaluation of methane production and macronutrient degradation in the anaerobic co-digestion of algae biomass residue and lipid waste. *Bioresource Technol* **111**: 42-48.

Park Y S, Kang S W, Lee J S, Hong S I and Kim S W (2002) Xylanase production in solid state fermentation by *Aspergillus niger* KK2 mutant using statistical designs. *App Microbiol Biotechnol* **58**: 761-66.

Passos F and Ferrer I (2014) Microalgae conversion to biogas: Thermal pretreatment contribution on net energy production. *Environ Sci Techno* **48**: 7171-78.

Passos F and Ferrer I (2015) Influence of hydrothermal pretreatment on microalgal biomass anaerobic digestion and bioenergy production. *Water Res* **68**: 364-73.

Passos F, Astals S and Ferrer I (2014a) Anaerobic digestion of microalgal biomass after ultrasound pretreatment. *Waste Manag* **34(11)**: 2098-103.

Passos F, Garcia J and Ferrer I (2013a) Impact of low temperature on the anaerobic digestion of microalgal biomass. *Bioresource Technol* **138**: 79-86.

Passos F, Hernandez-Marine M, Garcia J and Ferrer I (2014b) Long-term anaerobic digestion of microalgae grown in HRAP for wastewater treatment. Effect of microwave pretreatment. *Water Res* **49**: 351-59.

Passos F, Sole M, Garcia J and Ferrer I (2013b) Biogas production from microalgae grown in wastewater: Effect of microwave pretreatment. *Appl Energ* **108**: 168-75.

Pedrosa Bezerra R, Ortiz Montoya E Y, Sato S, Perego P, Monteiro de Carvalho J C and Converti A (2011) Effects of light intensity and dilution rate on the semicontinuous cultivation of *Artrosphira (Spirulina) platensis*. A kinetic Monodtype approach. *Bioresource Technol* **102**: 3215-19.

Pfau J, Werthmiller K and Senger H (1971) Permanent automatic synchronization of microalgae achieved by photoelectrically controlled dilution. *Arch Microbiol* **75**: 338.

Pienkos P T and Darzins A (2009) The promise and challenges of microalgal-derived biofuels. *Biofuels Bioprod Bioref* **3**: 431-40.

Pirt S J, Lee Y K, Walach M R, Pirt M W, Balyuzi H H M and Bazin M J(1983) A tubular photobioreactor for photosynthetic production of biomass from carbon dioxide: design and performance. J Chem Technol Biot **33(B)**: 35-38.

Prajapati S K, Kaushik P, Malik A and Vijay V K (2013) Phycoremediation and biogas potential of native algal isolates from soil and wastewater. *Bioresource Technol* **8**: 135: 232.

Prajapati S K, Malik A, Vijay V K and Sreekrishnand T R (2015) Enhanced methane production from algal biomass through short duration enzymatic pretreatment and codigestion with carbon rich waste. *RSC Adv* **5**: 67175.

Pruvost J, Van Vooren G, Cogne G and Legrand J (2009) Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. *Bioresource Technol* **100**: 5988-95.

Pruvost J, Van Vooren G, Le Gouic B, Couzinet-Mossion A and Legrand J (2011) Systematic investigation of biomass and lipid productivity by microalgae in photobioreactors for biodiesel application. *Bioresource Technol* **102**: 150-58.

Pulz O (2001) Photobioreactors: production systems for phototrophic microorganisms. Appl Microbiol Biot **57**: 287-93.

Qiang H, Zarmi Y and Richmond A (1998) Combined effects of light intensity, light path and culture density on output rate of *Spirulina platensis* (Cyanobacteria). *Eur J Phycol* **33**: 165-71.

Ramanan R, Kanan K, Deshkar A, Yadav R and Chakrabarti T (2010) Enhanced algal CO_2 sequestration through calcite deposition by *Chlorella* sp. and *Spirulina platensis* in a mini-raceway pond. *Bioresource Technol* **101**: 2616-22.

Ramaraj R and Dussadee N (2015) Biological purification processes for biogas using algae cultures: A review. *Int J Sust Green Energ* **4**(1-1): 20-32

Ramos Ó L, Reinas I, Silva S I, Fernandes J C, Cerqueira M A, Pereira R N, Vincente A A, Pintado M E and Malcat F X (2013) Effect of whey protein purity and glycerol content upon physical properties of edible films manufactured therefrom. *Food Hydrocolloid* **30**: 110-22.

Ramos-Suárez J, Martínez A and Carreras N (2014) Optimization of the digestion process of *Scenedesmus* sp. and *Opuntia maxima* for biogas production. *Energ Convers Manage* **88**: 1263-70.

Ras M, Lardon L, Sialve B, Bernet N and Steyer J P (2011) Experimental study on a coupled process of production and anaerobic digestion of *Chlorella vulgaris*. *Bioresource Technol* **102**: 200-06.

Ratchford I A and Fallowfield H J (2003) The effect of light: dark cycles of medium frequency on photosynthesis by *Chlorella vulgaris* and the implications for waste stabilisation pond design and performance. *Water Sci Technol* **48**: 69-74.

Ratha S K, Prasanna R, Gupta V, Dhar D W and Saxena A K (2012) Bioprospecting and indexing the microalgal diversity of different ecological habitats of India. *World J Microbiol Biotechnol* **28**: 1657- 67.

Raven J A, Evans M C W and Korb R E (1999) The role of trace metals in photosynthetic electron transport in O_2-evolving organisms. *Photosynth Res* **60**: 111-49.

Reddy LVA, Wee Y J, Yun J S, Ryu H W (2008) Optimization of alkaline protease production by batch culture of *Bacillus* sp. RKY3 through Plackett-Burman and response surface methodological approaches. *Bioresource Technol* 99: 2242-49

Renaud S M, Thinh L V, Lambrinidis J and Parry D L (2002) Effect of temperature on growth, chemical composition and fatty acid composition of tropical Australian microalgae grown in batch cultures. *Aquaculture* **211**: 195-214.

Richards L A (1954) *Diagnosis and Improvement of Saline and Alkaline Soils.* United States Salinity Laboratory Staff. Agricultural Handbook No. 60. Pp. 160. United States Department of Agriculture.

Richmond A (2004) *Handbook of microalgal culture: biotechnology and applied phycology.* Blackwell Science Ltd.

Roden E E and Zachara J M (1996) Microbial reduction of crystalline iron (III) oxides: influence of oxide surface area and potential for cell growth. *Environ Sci Technol* **30**: 1618-28.

Rodolfi L, Zittelli G C, Bassi N, Padovani G, Biondi N, Bonini G and Tredici M R (2008) Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnol Bioeng* **102**: 100-12.

Romanenko N I and Krot Y G (2010) Species-specific peculiarities of the green algae growth at additional carbon nutrition. Report 1. Growth rate of the green algae at maximal saturation of medium by CO_2 in open cultivation system. *Hydrobiol J* **46**: 60-72.

Roubaud A and Favrat D (2005) Improving performances of a lean burn cogeneration biogas engine equipped with combustion prechambers. *Fuel* **84**: 20-17.

Ruangsomboon S (2012) Effect of light, nutrient, cultivation time and salinity on lipid production of newly isolated strain of the green microalga, *Botryococcus braunii* KMITL 2. *Bioresource Technol* **109**: 261-65.

Sakar S, Yetilmezsoy K and Kocak E (2009) Anaerobic digestion technology in poultry and livestock waste treatment-a literature review. *Waste Manage Res* **27**: 3-18.

Salehian P, Karimi K, Zilouei H and Jeihanipour A (2013) Improvement of biogas production from pine wood by alkali pretreatment. Fuel **106**: 484-89.

San Pedro A, González-López C V, Acién F G and Molina-Grima E (2013) Marine microalgae selection and culture conditions optimization for biodiesel production. *Bioresource Technol* **134**: 353-361.

Sander K and Murthy G S (2010) Life cycle analysis of algae biodiesel. *Int J Life Cycle Ass* **15(7)**: 704-17

Schenk P M, Hall S R T, Stephens E, Marx U C, Mussgnug JH, Posten C, Kruse O and Hankamer B (2008) Second generation biofuels: high-efficiency microalgae for biodiesel production. *Bioenerg Res* **1(1)**: 20-43.

Schenk P M, Thomas-Hall S R, Stephens E, Marx U C, Mussgnug J H, Posten C, Kruse O and Hankamer B (2008) Second generation biofuels: high-efficiency microalgae for biodiesel production. *BioEnerg Res* **1**: 20-43.

Schnackenberg J, Ikemoto H and Miyachi S (1996) Photosynthesis and hydrogen evolution under stress conditions in a CO_2-tolerant marine green alga *Chlorococcum littorale*. *Journal of Photoch Photobio B* **34**: 59-62.

Schwede S, Rehman Z U, Gerber M, Theiss C and Span R (2013) Effects of thermal pretreatment on anaerobic digestion of *Nannocloropsis salina* biomass. *Bioresource Technol* **143**: 505-11.

Scott S A, Davey M P, Dennis J S, Horst I, Howe C J, Lea-Smith D J and Smith A (2010) Biodiesel from algae: challenges and prospects. *Curr Opin Biotech* **21**: 1-10.

Şengör S S, Barua S, Gikas P, Ginn T R, Peyton B, Sani R K, Spycher N F (2009) Influence of heavy metals on microbial growth kinetics including lag time: mathematical modelling and experimental verification. *Environ Toxicol Chem* **28(10)**: 2020-29.

Seyfabadi J, Ramezanpour Z and Amini K Z (2011) Protein, fatty acid, and pigment content of *Chlorella vulgaris* under different light regimes. *J Appl Phycol* **23**: 721-26.

Sforza E, Bertucco A, Morosinotto T and Giacometti G M (2012) Photobioreactors for microalgal growth and oil production with *Nannocholoropsis salina*: from lab-scale experiments to large-scale design. *Chem Eng Res Design* **90(9)**: 1151-58.

Shanmugam P and Horan N J (2009) Optimising the biogas production from leather fleshing waste by co-digestion with MSW. *Bioresource Technol* **100(18)**: 4117-20.

Sheehan J, Dunahay T, Benemann J and Roessler P (1998) A look back at the U.S. Department of Energy's aquatic species program: biodiesel from algae. *NREL/TP-580-24190*, National Renewable Energy Laboratory, USA.

Shi X, Wu Z and Chen F (2006) Kinetic modeling of lutein production by heterotrophic *Chlorella* at various pH and temperatures. *Mol Nutr Food Res* **50**: 763-68.

Sialve B, Bernet N and Bernard O (2009) Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. *Biotechnol Adv* **27**: 409-16.

Sigee D C, Dean A, Levado E and Tobin M J (2002) Fourier-transform infrared spectroscopy of Pediastrum duplex: characterization of a micro-population isolated from a eutrophic lake. *Eur J Phycol* **37**: 19-26.

Singh J and Gu S (2010) Commercialization potential of microalgae for biofuel production. *Renew Sustain Energ* **14**: 2596-610.

Singh S, Kate B N and Banerjee U C (2005) Bioactive compounds from cyanobacteria and microalgae: an overview. *Crit Rev Biotechnol* **25**: 73-95.

Sinigalliano C D, Winshell J, Guerrero M A, Scorzetti G, Fell J W, Eaton R W, Brand L and Rein K S (2009) Viable cell sorting of dinoflagellates by multiparametric flow cytometry. *Phycologia* **48**: 249-57.

Siron R, Giusti G and Berland B (1989) Changes in the fatty acid composition of *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* during growth and under phosphorous deficiency. *Mar Ecol Prog Ser* **55**: 95-100.

Soares B M, Vieira A A, Lemões J S, Santos C M M, Mesko M F, Primel E G, Montes D'Oca M G and Duarte F A (2012) Investigation of major and trace element distribution in the extraction-transesterification process of fatty acid methyl esters from microalgae *Chlorella* sp. *Bioresource Technol* **110**: 730-34.

Solé M, Passos F, Sicart A and Ferrer I (2014) Codigestion of (pretreated) microalgae and primar sludge: Closing the loop in wastewater treatment systems. *Proc Young Alganeers Symposium 2014*. Montpellier-Narbonne, France.

Šoštaric M, Golob J, Bricelj M, Klinar D and Pivec A (2009) Studies on the growth of *Chlorella vulgaris* in culture media with different carbon sources.*Chem Biochem Eng Q* **23**: 471-477.

Spolaore P, Joannis-Cassan C, Duran E and Isambert A (2006) Commercial applications of microalgae. *J Biosci Bioeng* **101**: 87-96.

Stanier R Y, Kunisawa R, Mandel M and Cohen-Bazire G (1971) Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriol Rev* **35**: 171-205.

Stephens E, Ross I L, King Z, Mussgnug J H, Kruse O, Posten C, Borowitzka M A and Hankamer B (2010). An economic and technical evaluation of microalgal biofuels. *Nat Biotechnol* **28**: 126-28.

Suh I S and Lee C G (2003) Photobioreactor engineering: Design and performance. *Biotechnol Bioproc E* **8**: 313-321.

Sung K D, Lee J S, Shin C S and Park S C (1998) Enhanced cell growth of *Chlorella* sp. KR-1 by the addition of iron and EDTA. *J Microbiol Biot*echnol **8**: 409-11.

Sung S and Liu T (2003) Ammonia inhibition on thermophilic anaerobic digestion. *Chemosphere* **53**: 43-52.

Tam N F Y and Wong Y S (1996) Effect of ammonia concentrations on growth of *Chlorella vulgaris* and nitrogen removal from media. *Bioresource Technol* **57**: 45-50.

Torzillo G, Accolla P, Pinzani E and Masojidek J (1996) In situ monitoring of chlorophyll fluorescence to assess the synergistic effect of low temperature and high irradiance stresses in *Spirulina* cultures grown outdoors in photobioreactors. *J Appl Phyco* **8**: 283-91.

Tredici M R and Materassi R (1992) From open ponds to vertical alveolar panels: the Italian experience in the development of reactors for the mass cultivation of phototrophic microorganisms. *J Appl Phycol* **4**: 221-31.

Ugwu C U, Aoyagia H and Uchiyamaa H (2008) Photobioreactors for mass cultivation of algae. *Bioresource Technol* **99**: 4021-28.

Umorin M and Lind O (2005) Cell volume distribution dynamics of *Chlorella vulgaris* Beij. in batch cultures under continuous light. *Biotechnol Lett* **27**: 347-54.

Vallee B L and Ulner D D (1972) Biochemical effects of mercury, cadmium, and lead. *Annu Rev Biochem* **41**: 91-128.

Van Kuijk S J A, Sonnenberg A S M, Baars J J P, Hendriks W H and Cone J W (2015) Fungal treated lignocellulosic biomass as ruminant feed ingredient: A review. *Biotechnol Adv* **33**: 191-02

Vandamme D, Foubert I and Muylaert K (2013) Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. *Trends Biotechnol* **31**: 233-39.

Veldhuis M J W, Timmermans K R, Croot P and Vander Wagt B (2005) Picoplankton: a comparative study of their biochemical composition and photosynthetic properties. *J Sea Res* **53(1-2)**: 7-24.

Vergara-Fernandez A, Vargas G, Alarcón N and Velasco A (2008) Evaluation of marine algae as a source of biogas in a two-stage anaerobic reactor system. *Biomass Bioenergy* **32**: 338-44.

Vivekanand V, Eijsink V G and Horn S J (2012) Biogas production from the brown seaweed *Saccharina latissima*: thermal pre treatment and codigestion with wheat straw. *J Appl Phycol* **24(5)**: 1295-301.

Walne P R (1966) Experiments in the large-scale culture of the larvae of *Ostrea edulis* L. *Fish Invest Ser* **2(25)**: 1-53.

Walter T L, Purton S, Becker D K and Collet C (2005) Microalgae as bioreactor. *Plant Cell Rep* 24: 629-41.

Wehr J D and Sheath R G (2003) *Freshwater algae of North America: Ecology and classification*. Pp. 935. Academic press, USA.

Wei H, Xu Q, Taylor L E 2nd, Baker J O, Tucker M P and Ding S Y (2009) Natural paradigms of plant cell wall degradation. *Curr Opin Biotechnol* **20**: 330-38.

Welter C, Schwenk J, Kanani B, Blargan J V and Belovicha J M (2013) Minimal medium for optimal growth and lipid production of the microalgae *Scenedesmus dimorphus*. *Environ Prog Sustain Energ* **4**: 937-45.

Wyman C E and Goodman B J (1993a) Biotechnology for production of fuels, chemicals, and materials from biomass. *Appl Biochem Biotechnol* **39(1)**: 41-59.

Xu N, Zhang X, Fan X, Han L and Zeng C (2001a) Effects of nitrogen source salting on the energetics of microalgae *Chlorella vulgaris* and *Dunaliella maritima*. *J Therm Anal Calorim* **104**: 201-07.

Xu N, Zhang X, Fan X, Han L and Zeng C (2001b) Effects of nitrogen source and concentration on growth rate and fatty acid composition of *Ellipsoidion* sp. (Eustigmatophyta). *J Appl Phycol* **13**: 463-69.

Yan C and Zheng Z (2013) Performance of photoperiod and light intensity on biogas upgrade and biogas effluent nutrient reduction by the microalgae *Chlorella* sp.. *Bioresource Technol* **139**: 292-99.

Yang F, Long L, Sun X, Wu H, Li T and Xiang W (2014) Optimization of medium using response surface methodology for lipid production by *Scenedesmus* sp. *Mar Drugs* **12**: 1245-57.

Yang J S, Rasa E, Tantayotai P, Scow K M, Yuan H L and Hristova K R (2011) Mathematical model of *Chlorella minutissima* UTEX2341 growth and lipid production under photoheterotrophic fermentation conditions. *Bioresource Technol* 102: 3077-82.

Yang Z, Guo R, Xu X, Fan X and Luo S (2011) Hydrogen and methane production from lipid-extracted microalgal biomass residues. *Int J Hydrogen Energ* **36**: 3465-70.

Yeh K L, Chang J S and Chen W M (2010) Effect of light supply and carbon source on cell growth and cellular composition of a newly isolated microalga *Chlorella vulgaris* ESP-31. *Eng Life Sci* **3**: 201-08.

Yen H and Brune D E (2007) Anaerobic co-digestion of algal sludge and waste paper to produce methane. *Bioresource Technol* **98**: 130-34.

Zamalloa C, Boon N and Verstraete W (2012) Anaerobic digestibility of *Scenedesmus obliquus* and *Phaeodactylum tricornutum* under mesophilic and thermophilic conditions. *Appl Energ* **92**: 733-38.

Zhang L, Happe T and Melis A (2002) Biochemical and morphological characterization of sulfur-deprived and H_2-producing *Chlamydomonas reinhardtii* (green alga). *Planta* **214**: 552-61.

Zhao G, Chen X, Wang L, Zhou S, Feng H, Chen W N and Lau R (2013) Ultrasound assisted extraction of carbohydrates from microalgae as feedstock for yeast fermentation. *Bioresource Technol* **128**: 337-44.

Zhong W, Zhang Z, Luo Y, Qiao W, Xiao M and Zhang M (2012) Biogas productivity by co-digesting Taihu blue algae with corn straw as an external carbon source. *Bioresource Technol* **114**: 281-86.

Zwietering M H, Jongenburger I, Rombouts FM and Van't Riet K (1990) Modeling of bacterial growth curve. *Appl Environ Microbiol* **56**: 1875-81.

ANNEXURE I

Preparation of solutions for proximate analysis

The following solutions were required during proximate analysis.

Neutral Detergent Fibre (NDF) solution

Ingredients	**Quantity (g l^{-1})**
Sodium Lauryl Sulphate	30
Disodium ethylene diamine tetra-acetate(EDTA)	18.6
Sodium borate decahydrate (Borax)	6.8
Disodium hydrogen phosphate(Na_2HPO_4)	4.56

Preparation

Disodium ethylene diamine tetra-acetate (EDTA) (18.6g) and sodium borate decahydrate (Borax) (6.8g) were dissolved in hot distilled water. Thirty gram of sodium lauryl sulphate was added to this mixture. Disodium hydrogen phosphate (Na_2HPO_4) (4.56g) was dissolved separately, in distilled water by heating. Afterwards, Na_2HPO_4 solution was added to the solution containing other ingredients. The pH range was between 6.9-7.1. The final volume was made one litre.

Acid Detergent Fibre (ADF) solution

Ingredients	**Quantity (g l^{-1})**
Concentrated H_2SO_4	28 ml
Cetyl trimethyl ammonium bromide (CTAB)	20

Preparation

Twenty gram of cetyl trimethyl ammonium bromide (CTAB) was dissolved in distilled water and 28 ml concentrated H_2SO_4 was added to it along the walls of the beaker. The mixture was cooled and the final volume was made one litre.

Preparation of 72% H_2SO_4 (sulphuric acid)

72% Sulphuric acid (H_2SO_4) solution was prepared as follows:

Specific gravity of H_2SO_4 = 1.84

Purity = 98%

Density = Mass/ Volume

1.84 =72/ Volume

Volume = 72/ 1.84 =39.1 ml

If purity is 98% then, 39.1/ 98%

39.1/ 98 x 100 = 39.9 i.e. 40 ml

But here, 72% H_2SO_4 was prepared by dissolving 40 ml H_2SO_4 in 28 ml distilled water

VITA

Name	**:**	Rouf Ahmad Dar
Father's Name	**:**	Late Sh. Mohammad Ramzan Dar
Mother's Name	**:**	Smt. Halima Bano
Nationality	**:**	Indian
Date of Birth	**:**	04.11.1988
Permanent home address	**:**	H.No. 86, Near Jamia Masjid Sehpora, Ganderbal, Jammu and Kashmir (191131)
Email Address	**:**	roufulramzan086@gmail.com

EDUCATIONAL QUALIFICATIONS

Bachelor's degree	**:**	B.Sc. (Industrial Microbiology)
University and year of award	**:**	Punjabi University, Patiala 2011
%age of marks	**:**	71.16%
Master's degree	**:**	M.Sc. (Microbiology)
University and year of award	**:**	Punjab Agricultural University, Ludhiana 2013
OCPA	**:**	8.11 /10.00
Title of Master's Thesis	**:**	Production of lignolytic enzymes from *Thermoascus aurantiacus* MTCC 375 for enhancing biogas production from paddy straw
Ph.D. degree	**:**	Ph.D. (Microbiology)
University and year of award	**:**	Punjab Agricultural University, Ludhiana 2017
OCPA	**:**	7.66 /10.00
Title of Dissertation	**:**	Bioprospects of microalgal isolates from water logged area of Punjab for biogas production

Awards/Distinction/Scholarship/Fellowship

1. University medal in B.Sc.
2. Merit certificate in B.Sc. and M.Sc.
3. Received University fellowship in M.Sc. from Punjab Agricultural University.
4. Qualified ASRB-NET 2014, 2017
5. Qualified ICAR-SRF 2014
6. Qualified GATE 2013, 2014
7. Qualified CSIR-UGC-NET (LS) 2014
8. Qualified ICMR-JRF Examination 2015 and received fellowship in Ph.D. program from Indian Council of Medical Research, New Delhi.
9. Got selected for the Maulana Azad National Fellowship (MANF) for the year 2016-17.