CHARACTERIZATION OF STRESS TOLERANT MICROALGAE STRAINS 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: Biochemistry)

By

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CERTIFICATE-I

This is to certify that the dissertation entitled **"Characterization of stress tolerant microalgae strains for biogas production"** submitted for the degree of **Doctor of Philosophy** in the subject of **Microbiology** (Minor subject: **Biochemistry**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Ms. Nishu Sharma (L-2015-BS-82-D)** under my supervision and that no part of this thesis has been submitted for any other degree.

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

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CERTIFICATE-II

This is to certify that the dissertation entitled, **"Characterization of stress tolerant microalgae strains for biogas production"** submitted by **Ms. Nishu Sharma** (Admn No. **L-2015-BS-82-D**) to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**, in the subject of **Microbiology** (Minor subject: **Biochemistry**) has been approved by the Student's Advisory Committee along with Head of the Department after an oral examination on the same, in collaboration with an external examiner.

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ABSTRACT

The present research work was aimed at characterization of stress tolerant microalgae strains for biogas production. A total of six strains, five stress tolerant microalgae strains viz. BGLR4, BGLR7, BGLR10, BGLR18, BGLRS and one standard strain *Spirulina* sp. NCIM 5143 were used in the present study. These strains were screened for maximum biomass production, chlorophyll content, protein, carbohydrate and lipids on four different media viz. Blue green-11(BG-11), Bold's basal medium (BBM), Algal culture medium (ACM) and Zarrouk's medium (ZM) and on different concentrations of unsterilized dairy wastewater (20%, 40%, 60%, 80% and 100%). Among all the six strains, BGLR18 and BGLRS were found to produce maximum biomass BBM and ACM respectively while *Spirulina* sp. NCIM 5143 produced maximum biomass on ZM. BGLR18 and BGLRS showed maximum growth on 60% concentration of dairy wastewater whereas *Spirulina* sp. NCIM 5143 on 100% concentration of dairy wastewater. Maximum percent reduction in various physico-chemical parameters of dairy wastewater was shown by BGLR18 and BGLRS at 60% dairy wastewater while *Spirulina* sp. NCIM 5143 at 100%. Further, studies were carried out with strains BGLR18 and BGLRS and compared with standard strain *Spirulina* sp. NCIM 5143. Cultural conditions of BGLR18, BGLRS and *Spirulina* sp. NCIM 5143 were optimized using response surface methodology (RSM) based on central composite design. The optimized and most desirable cultural conditions of pH, temperature, light intensity, growth period and inoculum concentration for BGRL18 were 10.02, 20.00°C, 7599.41 Lux, 21.75 days and 1.00% ; for BGLRS were 11.17, 26.03 °C, 4000.02 Lux, 39.37 days and 10.00% and for *Spirulina* sp. NCIM 5143 were 10.35, 24.38 °C, 8000.00 Lux, 25.13 days and 1.00% respectively. On molecular identification, BGLR18 showed 96% resemblance to *Chlorosarcinopsis eremi* and BGLRS showed 99% resemblance with *Scenedesmus* sp. *MKB*. These three strains showed wide variation in their total nitrogen, total protein,total phenols, antioxidant activity, DPPH radical scavenging activity. ICP-AES analysis showed that heavy metals were negligible in all the three strains while essential elements like Ca, Fe, P and Mg were present in higher concentrations than *Spirulina* sp. NCIM 5143. GC-MS analysis of methanolic extract showed that many bioactive compounds were present in all the strains. These strains were evaluated for their biogas production potential alone and in co-digestion with paddy straw. The highest biogas was produced by BGLRS (*Scenedesmus* sp. *MKB*) (28.80 Litres) with highest biogas production potential (P) of 30.85 mLg^{-1} VS showing maximum biogas production rate (R_m) of 0.58 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 1.30 days. While in co-digestion experiment, the highest biogas production was recorded by BGLR18 (*Chlorosarcinopsis eremi*) (168.46 Litres) with maximum biogas production potential (P) of 214.30 mLg^{-1} VS showing maximum biogas production rate (R_m) of 8.76 mLg^{-1} d^{-1} with a lag phase (λ) of 12.10 days. Hence, the stress tolerant microalgae strains can be co-digested with paddy straw for enhancing biogas production.

Keywords: BGLR18, BGLRS, Dairy Wastewater, Response Surface Methodology, *Chlorosarcinopsis eremi*, *Scenedesmus* sp. *MKB*, *Spirulina* sp. NCIM 5143.

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

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ਸਾਰ-ਅੰਸ਼

ਇਸ ਅਧਿਐਨ ਦਾ ਮੁੱਖ ਉਦੇਸ਼ ਬਾਇਓਗੈਸ ਦੇ ਉਤਪਾਦਨ ਲਈ ਤਣਾਅ ਸਹਿਣ ਵਾਲੇ ਸੂਖਮ ਸ਼ੈਵਾਲ ਦੀ ਗੁਣਵੱਤਾ ਨੂੰ ਜਾਂਚਣਾ ਹੈ। ਸੂਖਮ ਸ਼ੈਵਾਲ ਦੀਆਂ ਕੁੱਲ 6 ਨਸਲਾਂ ਲਈਆਂ ਗਈਆਂ। ਜਿਹਨਾਂ ਵਿੱਚੋ ਪੰਜ (BGLR4, BGLR7, BGLR10, BGLR18, BGLRS) ਤਣਾਅ ਸਹਿਣ ਵਾਲੇ ਅਤੇ ਇੱਕ *ਸਪਿਰੂਲਿਨਾ* ਕਿਸਮ NCIM 5143 ਪ੍ਰਮਾਣਿਤ ਕਿਸਮ ਹੈ। ਇਹਨਾਂ ਕਿਸਮਾਂ ਨੂੰ ਸੱਭ ਤੋਂ ਵੱਧ ਜੈਵਿਕ ਮਾਸ, ਕਲੋਰੋਫਿਲ ਦੀ ਮਾਤਰਾ, ਪ੍ਰੋਟੀਨ, ਕਾਰਬੋਹਾਈਡਰੇਟ ਅਤੇ ਲਿਪਿਡ ਦੀ ਜਾਂਚ ਕਰਨ ਲਈ 4 ਅਲੱਗ-ਅਲੱਗ ਮਾਧਿਅਮ (BG-11, BBM, ACM, ਤੇ ZM) ਅਤੇ ਡੇਅਰੀ ਦੇ ਗੰਦੇ ਨਿਰਵਿਘਨ ਪਾਣੀ ਦੀ ਭਿੰਨ-ਭਿੰਨ ਮਾਤਰਾ (20%, 40%, 60%, 80% ਅਤੇ 100%) ਵਿੱਚ ਉਗਾਇਆ ਗਿਆ। ਸਾਰੀਆਂ ਕਿਸਮਾਂ ਵਿੱਚ, BGLR18 ਨੇ BBM ਮਾਧਿਅਮ ਅਤੇ BLGRS ਨੇ ACM ਮਾਧਿਅਮ ਵਿੱਚ ਜਦਕਿ *ਸਪਿਰੂਲਿਨਾ* ਕਿਸਮ NCIM 5143 ਤੇ ZM ਵਿੱਚ ਸਭ ਤੋਂ ਵੱਧ ਜੈਵਿਕ ਮਾਸ ਪੈਦਾ ਕੀਤਾ। BGLR18 ਅਤੇ BGLRS ਵਿੱਚ ਡੇਅਰੀ ਦੇ ਗੰਧਲੇ ਪਾਣੀ ਦੀ 60% ਮਾਤਰਾ ਜਦਕਿ *ਸਪਿਰੂਲਿਨਾ* ਕਿਸਮ ਵਿੱਚ 100% ਮਾਤਰਾ ਤੇ ਸਭ ਤੋਂ ਵੱਧ ਵਿਕਾਸ ਦਰ ਪਾਈ ਗਈ। ਸਾਰੀਆਂ ਕਿਸਮਾਂ ਵਿੱਚੋਂ ਸਭ ਤੋਂ ਘੱਟ ਭੌਤਿਕ ਰਸਾਇਣ ਮਾਪਦੰਡ ਡੇਅਰੀ ਦੇ 60% ਗੰਧਲੇ ਪਾਣੀ ਵਿੱਚ ਜਦਕਿ *ਸਪਿਰੂਲਿਨਾ* ਕਿਸਮ ਵਿੱਚ ਇਹ 100% ਗੰਧਲੇ ਪਾਣੀ ਨਾਲ ਪਾਇਆ ਗਿਆ। ਅੱਗੇ ਦਾ ਅਧਿਐਨ BGLR18 ਅਤੇ BGLRS ਨਾਲ ਕੀਤਾ ਅਤੇ ਇਹਨਾਂ ਦੀ *ਸਪਿਰੂਲਿਨਾ* ਕਿਸਮ NCIM 5143 ਨਾਲ ਤੁਲਨਾ ਕੀਤੀ ਗਈ। BGLR18, BGLRS ਅਤੇ *ਸਪਿਰੂਲਿਨਾ* ਕਿਸਮ NCIM 5143 ਨੂੰ ਉਗਾਉਣ ਲਈ ਆਰ.ਐੱਸ.ਐੱਮ. ਰਾਹੀਂ ਸਹੀ ਹਾਲਾਤ ਜਾਂਚੇ ਗਏ ਜੋ ਕਿ ਸੀ.ਸੀ.ਡੀ. ਤੇ ਅਧਾਰਿਤ ਸੀ। ਸਭ ਤੋਂ ਅਨੁਕੂਲ ਅਤੇ ਲੋੜੀਂਦਾ ਪੀਐੱਚ, ਤਾਪਮਾਨ, ਰੋਸ਼ਨੀ ਦੀ ਤੀਬਰਤਾ, ਵਿਕਾਸ ਦੀ ਮਿਆਦ ਅਤੇ ਇੰਨਆਕੂਲਮ ਦੀ ਮਾਤਰਾ ਦੇ ਹਾਲਾਤ BGLR18 ਵਾਸਤੇ 10.02, 20.00°C, 7599.41 Lux, 21.75 ਦਿਨ ਅਤੇ 1.00%; BGLRS ਵਾਸਤੇ 11.17, 26.03°C, 4000.02 Lux, 39.37 ਦਿਨ ਅਤੇ 1.00% ਅਤੇ *ਸਪਿਰੂਲਿਨਾ* ਕਿਸਮ NCIM 5143 ਵਾਸਤੇ 10.35, 24.38°C, 8000.00 Lux, 25.13 ਦਿਨ ਅਤੇ 1.00% ਕ੍ਰਮਵਾਰ ਸਨ। ਪਛਾਣ ਕਰਨ ਤੇ ਸਿੱਟਾ ਨਿਕਲਿਆ ਕਿ BGLR18 *ਕਲੋਰੋਸਾਰਲੀਨੋਪਸਿਸ ਇਰਿਮੀ* ਨਾਲ 96% ਮਿਲਦਾ ਜੁਲਦਾ ਹੈ ਅਤੇ BGLRS *ਸੇਨੇਡੇਸਮਸ* ਕਿਸਮ *MKB.* ਨਾਲ 99% ਮਿਲਦਾ ਜੁਲਦਾ ਹੈ। ਇਹਨਾਂ ਨਸਲਾਂ ਵਿੱਚ ਕੁੱਲ ਨਾਈਟਰੋਜਨ, ਪ੍ਰੋਟੀਨ, ਫਿਨੋਲ ਅਤੇ ਐਂਟੀਆਕਸੀਡੈਂਟਸ ਦੀ ਮਾਤਰਾ ਵਿੱਚ ਕਾਫੀ ਫਰਕ ਦਿਖਾਈ ਦਿੱਤਾ। ਆਈਸੀਪੀ-ਏ.ਸੀ.ਐੱਸ. ਵਿਸ਼ਲੇਸ਼ਣ ਨੇ ਦਿਖਾਇਆ ਕਿ ਤਿੰਨੋਂ ਤਣਾਅ ਸਹਿਣ ਵਾਲੇ ਸੂਖਮ ਸ਼ੈਵਾਲਾਂ ਵਿੱਚ ਭਾਰੀਆਂ ਧਾਤਾਂ ਨਾ ਬਰਾਬਰ ਸਨ ਜਦਕਿ ਇਹਨਾਂ ਵਿੱਚ *ਸਪਿਰੂਲਿਨਾ* ਕਿਸਮ NCIM 5143 ਦੇ ਮੁਕਾਬਲੇ Ca, Fe, P ਅਤੇ Mg ਕਾਫੀ ਮਾਤਰਾ ਵਿੱਚ ਪਾਈਆਂ ਗਈਆਂ। ਜੀ.ਸੀ.ਐੱਮ.ਐੱਮ. ਰਾਹੀਂ ਜਾਂਚ ਕਰਨ ਤੇ ਪਤਾ ਚਲਿਆ ਕਿ ਇਹਨਾਂ ਸਾਰੀਆਂ ਕਿਸਮਾਂ ਵਿੱਚ ਬਹੁਤ ਸਾਰੇ ਬਾਇਓਐਕਟਿਵ ਪਦਾਰਥ ਸਨ।ਇਹਨਾਂ ਕਿਸਮਾਂ ਨੂੰ ਇਕੱਲੇ ਅਤੇ ਝੋਨੇ ਦੀ ਪਰਾਲੀ ਨਾਲ ਬਾਇਓਗੈਸ ਬਣਾਉਣ ਲਈ ਜਾਂਚਿਆ ਗਿਆ।ਸਭ ਤੋਂ ਵੱਧ ਬਾਇਓਗੈਸ ਉਤਪਾਦਨ BGLRS (*ਸੇਨੇਡੇਸਮਸ* ਕਿਸਮ *MKB.*) (28.80 ਲੀਟਰ) ਜਿਸਦੀ ਬਾਇਓਗੈਸ ਉਤਪਾਦਨ ਸਮਰੱਥਾ 30.85 mLg^{-1} VS, ਵੱਧ ਤੋਂ ਵੱਧ ਬਾਇਓਟੈਸ ਉਤਪਾਦਨ ਰੇਟ 0.58 mLg^{-1} ਅਤੇ ਪਛੜ ਪੜਾਅ 1.30 ਦਿਨ ਸਨ। ਜਦਕਿ ਪਰਾਲੀ ਨਾਲ ਸਭ ਤੋਂ ਵੱਧ ਉਤਪਾਦਨ BGLR18 (*ਕਲੋਰੋਸਾਰਲੀਨੋਪਸਿਸ* ਇਰਿਮੀ) (168.46 ਲੀਟਰ) ਵਿੱਚ ਪਾਇਆ ਗਿਆ ਜਿਸਦੀ ਬਾਇਓਗੈਸ ਉਤਪਾਦਨ ਸਮਰੱਥਾ 214.30 mLg^{-1} VS, ਵੱਧ ਤੋਂ ਵੱਧ ਬਾਇਓਗੈਸ ਉਤਪਾਦਨ ਰੇਟ 8.76 mLg^{-1} ਅਤੇ ਪਛੜ ਪੜਾਅ 12.10 ਦਿਨ ਸਨ। ਇਸ ਲਈ ਤਣਾਅ ਸਹਿਣ ਵਾਲੇ ਸੂਖਮ ਸ਼ੈਵਾਲ ਦਾ ਪਰਾਲੀ ਨਾਲ ਸਹਿ-ਨਿਦਾਨ ਕਰਨ ਤੇ ਉਤਪਾਦਨ ਵਿੱਚ ਵਾਧਾ ਹੁੰਦਾ ਹੈ।

ਮੁੱਖ ਸ਼ਬਦ:- BGLR18, BGLRS, ਡੇਅਰੀ ਦਾ ਗੰਧਲਾ ਪਾਣੀ, ਰਿਸਪੌਂਸ ਸਰਫੇਸ ਮੈਥੋਡੋਲੋਜੀ, *ਕਲੋਰੋਸਾਰਸੀਨੋਪਸਿਸ ਇਰਿਮੀ, ਸੇਨੇਡੇਸਮਸ* ਕਸਮ *MKB.*,, *ਸਪਿਰੂਲਿਨਾ* ਕਿਸਮ NCIM 5143

ਮੁੱਖ ਸਲਾਹਕਾਰ ਦੇ ਹਸਤਾਖਰ **ਵਿੱਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖਰ**

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CHAPTER I

INTRODUCTION

Energy is of utmost importance for the existence of human race. Fossil fuels such as petrol, coal and natural gas contribute to the 88% of the total energy consumption (Fernandes *et al* 2007). In the last 50 years, due to population explosion and rapid industrialization, primary energy consumption rate has increased substantially which led the world to face two challenging situations including energy crisis and environmental pollution (Gupta and Tuohy 2013). Also the invention of newer sophisticated technologies involving higher energy inputs has caused increased demand for fossil fuels such as coal, petroleum etc (Bhagea *et al* 2019). Various types of harmful greenhouse gases such as carbon dioxide (CO_2), carbon monoxide (CO), nitrogen oxide (NO_x) and sulfur dioxide (SO_2) are emitted by the burning of these fossil fuels) (Hosseini *et al* 2013). It has been estimated that by 2035, worldwide energy related carbon dioxide emissions are likely to escalate by 1.6% a year (Milano *et al* 2016). The negative impact of carbon dioxide generated from burning of these fossil fuels include rise in temperature which results in water scarcity in dry areas, torrential rainfall in wet areas, melting of glaciers, increase in sea water level and intense heat waves. The increasing demand for fossil fuels and its adverse environmental impacts has prompted the scientists to think about alternate renewable energy sources.

Energy security and environmental issues can be handled efficiently by renewable energy sources at universal level by providing cleaner fuel and reducing green house gases emission. A few alternative renewable energy sources having less influence on environment include geothermal energy, hydropower, ocean energy, wind energy, solar energy and bioenergy (Shunmugam 2009). Energy crops for oilseeds (edible as well as non-edible), various aquatic plants (bio-oil sources) and biowastes like food wastes, municipal wastes, agricultural wastes etc. have been recognized as energy fuels (Kodihalli *et al* 2018). The production of biogas from microalgae as co-feed stock in anaerobic digestion process has been receiving much attention now a days.

Microalgae are unicellular or simple multicellular, microscopic (2-200 µm), prokaryotic or eukaryotic photosynthetic microorganisms that has the ability to thrive in harsh conditions (Greenwell *et al* 2010). Richmond (2004) reported that out of 50,000 existings species of microalgae, only around 30,000 have been studied and analyzed. Microalgae are versatile microorganisms and can be found thriving in freshwater and in marine water environments. These are the most important biological resources that have the ability to adapt to various environments (Kamarudin *et al* 2015). Microalgae require water, nutrients, light and CO_2 for growth both autotrophically as well as mixotrophically (Daneshwar *et al* 2019).

Microalgal cultivation depends on various environmental factors (physical and

chemical) including temperature, light intensity, nutrient content, salt concentration and pH (Hu 2004, Guschina and Harwood 2006, Hu *et al* 2008, Singh and Dhar 2011). Microalgae have been grown on variety of synthetic media but costs exceeding the final products under large scale cultivation is a major limitation associated with the use of these commercial media (Li *et al* 2007). In practical sales, cultivation costs largely effect biofuel production from microalgal biomass. Inputs to algal cultivation must be inexpensive for attaining economic feasibility (Singh *et al* 2017). Therefore, there is urgency to search for alternative low cost substrate for cultivation of microalgae.

Use of wastewater generated from various activities for microalgal cultivation has been studied for more than half a century. Dairy industry is one among the top 10 environment polluting industries (Monroy *et al* 1995). It was reported that dairy wastewater had BOD, COD (Kushwaha *et al* 2011), nutrients like nitrogen and phosphorus (Gavala *et al* 1999) in the range of 40-48,000 mg/ L, 80-95,000 mg/L, 14-830 mg/L and 9-280 mg/L respectively. Without any kind of pretreatment, these effluents are dumped to the nearby stream or land where their decomposition occurs and anaerobic conditions are created and many types of dangerous diseases like malaria, dengue, yellow fever and chicken guniya spread (Shete and Shinkar 2013). Many conventional physical and chemical treatments are used for wastewater treatment but they are associated with high cost and more sludge production (Yuan *et al* 2011). Microalgae use nutrients from wastewater and accumulate heavy metals present in them and thus provide a cheap alternative low cost wastewater treatment (Pittman *et al* 2011). Some species of microalgae *Chlorella* sp. *C. sorokiniana* and *C. vulgaris* are efficiently used for the biological treatment of the wastewater (Sriram and Seenivasan 2012).

Wastewater for microalgal cultivation has several advantages including low operation cost and energy requirements, removes heavy metals and variety of xenobiotic compounds, decrease concentration of other pollutants present in wastewater, uptakes CO_2 and increases concentration of oxygen (Ling *et al* 2014), recycles assimilated nitrogen and phosphorus in the form of algal biomass and is safe and ecofriendly due to natural ecosystems (Converti *et al* 2006, Sydney *et al* 2011, Aslan and Kapdan 2006). Moreover, harvested microalgal biomass from wastewater treatment can be used as a energy feedstock for anaerobic digestion to produce biogas. This will serve the dual purpose of treatment of waste water as well as low cost substrate for cultivation of microalgae.

Biogas is composed of 40-70% methane (CH_4), 20-30% carbon dioxide (CO_2), 100-3000 ppm hydrogen sulfide (H_2S) and water, trace gases and other impurities (Ramaraj *et al* 2016). The biogas generated from such a process can be used as biofuel, thus providing an alternative, ecofriendly fuel replacing fossil fuels and also for various other purposes like

generation of electricity, heating etc.

India is an agricultural country and about 600 Mt of crop residues are generated (Jain *et al* 2014). Among which paddy crop is the major contributor (about 34%) to the total residues generated (Bhattacharyya *et al* 2015). Crop residue burning has serious effects on environment by releasing polluting substances. These include methane (CH_4), Nitrous oxide (N_2O), carbon dioxide (CO_2), other toxic gases, nitrogen oxide (NO), carbon monoxide (CO), aerosols, particulates, smoke, emission of greenhouse gases which causes many types of respiratory diseases and other dangerous effects like loss of nutrients (Carbon, Nitrogen, Phosphorus , Potassium and Sulphur) resulting in erosion of soil, effect on nutrient cycling and soil quality, depletion of the organic carbon pool from soil and energy rich residues (Mittal *et al* 2009). In states like Gujarat, Jammu & Kashmir, Maharashtra, Bihar, Assam, West Bengal and Tamil Nadu, paddy straw is used as cattle feed, cooking fuel, thatching for houses in rural areas, mulching material, compost making, electricity generation, biofuels, and in boilers for parboiling paddy whereas in Punjab, Haryana and Uttar Pradesh, management of paddy straw is done by burning (Koopman and Koppejan 1997, Singh and Panigrahy 2011, Badarinath *et al* 2008, Roy and Kaur 2016). However, paddy straw can be treated by anaerobic digestion thereby producing clean fuel like biogas.

Optimal C:N ratio reported for anaerobic digestion is 25 to 30 (Yen and Brune 2007) but paddy straw has C:N ratio of 90:1 which can be brought down by supplying an exogenous source of nitrogen (Sharma *et al* 2014). Therefore, co-digestion of paddy straw with substrates having low C:N ratio like microalgae is favourable for efficient anaerobic digestion process.

In Punjab only 71% of total area (50,362 sq km) is used for agriculture. South west zone of Punjab (34% of total area) suffers from the erratic and scanty rainfall problem. Brackish ground water found here has high electrical conductivity (EC) and residual sodium concentration (RSC). This area of Punjab is affected by water logging which is not suitable for cultivation of crops. But, this type of area could be efficiently utilized for microalgae cultivation and for isolation of stress tolerant microalgae strains. Twenty stress tolerant microalgae strains previously isolated from the water logged areas of Punjab (Dar 2017) were already available at Biogas Laboratory, Department of Renewable Energy Engineering, College of Agricultural Engineering and Technology, Punjab Agricultural University, Ludhiana. Out of these twenty stress tolerant strains, five native strains and one standard strain was selected for evaluation in the present study.

Keeping these points in mind, present work was proposed to cultivate and optimize stress tolerant microalgae strains on different media and anaerobic digestion of harvested microalgal biomass with paddy straw to enhance biogas production with the following

objectives:

- To screen and optimize the growth of stress tolerant microalgae strains for biomass productivity
- To characterize microalgae biomass for biogas production

CHAPTER II

REVIEW OF LITERATURE

Microalgae are organisms with simple structure possessing ability for higher growth rate and photosynthetic efficiency for providing biofuels (Chellamboli and Perumalsamy 2014). As many types of biomolecules like polysaccharides, proteins and lipids are produced by microalgae, in future, it has applications in the area of food, medicine, genetic engineering and biodiesel (Lavens and Sorgeloos 1996). During the last few years, microalgae is attracting great interest for the production of many valuable products ranging from therapeutic proteins to biofuels (Ilavarasi *et al* 2011). Many favourable attributes of microalgae for biogas production include : 1). ability to grow on non-aerable land, 2). can use fresh, saline or wastewater 3). production of lipids, proteins and carbohydrates in large quantities for biofuel generation like biodiesel, bioethanol etc. and other valuable co-products (Brennan and Owende 2010, Kruse and Hankamer 2010, Georgianna and Mayfeld 2012, Grewe *et al* 2014, Formighieri 2015).

The literature pertaining to the present research work is reviewed under the following main headings:

2.1 Microalgal growth on commercial media

2.2 Microalgal growth on wastewater/Industrial effluent

2.3 Environmental factors affecting microalgal growth

- 2.3.1 Light
- 2.3.2 Temperature
- 2.3.3 pH
- 2.3.4 Nutrients

2.4 Optimization of Microalgal growth conditions using Response Surface Methodology (RSM)

2.5 Anaerobic digestion of microalgal biomass

2.1 Microalgal growth on commercial media

To grow and multiply, algae requires certain nutrients in appropriate quantities and for different classes of algae requirements of nutrients also vary (Praba *et al* 2016). For example, *Isochrysis* sp. needs minor nutrients like cobalamine B12 and thiamine B1 for rapid growth and multiplication (Provasoli *et al* 1958). Elements like N, P, K, Mg, Ca, S, Fe, Cu, Mn and Zn supplied in the form of salts are mainly required by green algae (Kaplan *et al* 1986). Many researchers have reported that *Chlorella* sp. showed different growth rate on different media (Jayasankar and Vasala 2008, Sostaric *et al* 2009). Elemental composition of *Chlorella* also have been reported under different conditions and stages of growth (Oh-Hama and Miyachi 1988, Harrison *et al* 1990).)

Kodihalli *et al* (2018) studied the effect of different media (Bold's Basal Medium

(BBM), Blue Green-11 medium (BG-11) and Tris-Acetate Phosphate medium (TAP)) on growth of microalgal strains which were collected from different regions of India and further purified and identified by 18S rRNA sequencing . The identified strains were *Desmodesmus abundans*, *Scendesmus pectinatus*, *Tetranephris brasiliensis*, *Desmodesmus armatus*, *Scendesmus costato-granulatus*, *Neocystis brevis*, *Chlorella sorokiniana*, *Scendesmus acutus*, *Pseudomuriella* species and *Chlorella minutissima.* Quantitative and qualitative lipid analysis were also done for these microalgal strains for evaluation of their biodiesel production potential. It was reported that highest growth rate was observed from *Desmodesmus abundans* on 19th day in TAP medium. Compared to BBM and BG11, biomass weight (wet and dry) was found maximum in *Chlorella minutissima*, *Desmodesmus abundans* and *Scendesmus costato-granulatus* in TAP. They further observed that maximum lipid was in *Desmodesmus armatus* (43.7%) grown in TAP media.

Madkour *et al* (2012) compared the growth characteristics and biochemical composition of *Spirulina platensis* on Zarrouk's medium with that of Zarrouk's medium in which all the nutrients were substituted with cheap fertilizers and chemicals. Four different concentrations of nitrogen viz. 10%, 20%, 30% and 40% of ammonium nitrate concentration used in Zarrouk's medium and urea were taken. The results showed that growth of *S. platensis* was increased with increasing concentrations of ammonium nitrate and decreased with increasing concentration of urea. The maximum biomass, chlorophyll and protein yield were recorded using ammonium nitrate which were comparable with that of Zarrouk's medium.

Ramaraj *et al* (2016) evaluated the growth, biomass production and biochemical composition of *Chlorella vulgaris* on artificial medium named Rameshprabu medium (rice fertilizer, fish meal, lime, urea, rice bran). Results showed that algae was efficient in reduction of nitrogen, phosphorus, and chemical oxygen demand (COD) from the medium and maximum biomass was obtained in terms of high total carbohydrates, protein and lipids.

Radmann *et al* (2015) studied the biosurfactant production by evaluation of microalgal growth both autotrophically and mixotrophically. Microalgal species involved were *Arthrospira* sp. LEB 18, *Synechococcus nidulans* LEB 25, *Chlorella minutissima* LEB 108, *Chlorella vulgaris* LEB 106 and *Chlorella homosphaera*. Erlenmeyer photobioreactors with medium containing $NaHCO_3$ as the autotrophic carbon source and glucose or molasses for mixotrophic growth were used for the cultivation of these strains. They reported that growth of microalgae as well as biosurfactant production stimulated with organic carbon sources. They also draw attention for use of molasses which is an organic substrate for cultivation of microalgae**.**

2.2 Microalgal growth on wastewater/Industrial effluents

Microalgal cultivation in wastewaters from various industries for removal of nutrients

is very efficient for the treatment of wastewater and also manages freshwater ecosystem. Moreover, algal biomass harvested from such wastewater can be used as feedstock for anaerobic digestion for biofuel production, reduces cost of wastewater treatment and can also be used for the production of high-value added products (Clarens *et al* 2010). In open pond systems, large amount of water is needed for algal cultivation but their property to grow efficiently in industrial, farm, municipal and agricultural wastewater (Umdu *et al* 2009) can not only solve this problem but also bioremediate wastewater which can further be used for other purposes (Hena *et al* 2015). The potential of microalgae for the bioremediation of dairy wastewater has been reported by various researchers.

Vignesh *et al* (2016) studied the growth of *Nostoc* sp. (Algae) and *Abelmoschus esculentus* (plant) on milk industry effluent and reported that milk industry effluent contains the essential nutrients required for the growth of the organisms and hence, reduces the cost of treating the milk industry effluent.

Shekhawat *et al* (2012) treated different concentrations of dairy wastewater (10%, 30%, 50%, 70% and 100%) with algae to study its bioremediation potential. It was concluded that optimal concentration for microalgal growth was 30% and all the physico-chemical parameters (ammonia, nitrate, phosphorus and BOD) decreased but DO (Dissolved oxygen) increased by 10^{th} day .

Kothari *et al* (2013) studied microalgae *Chlamydomonas polypyrenoideum* for the phycoremediation of dairy industry wastewater in a batch experiment. After the completion of growth period of 10 days, nitrate was reduced to 90%, nitrite to 74%, phosphate to 70%, chloride to 61%, fluoride to 58% and ammonia to 90% as compared to control. Lipids were also higher than standard medium BG-11 and oil extracted through transesterification reaction was analyzed by FTIR showed that it was comparable with oil from other sources.

Wang *et al* (2010) reported the bioremediation potential of *Chlorella vulgaris* grown on undigested dairy manure and wastewater. Results showed that the ammonium nitrogen, total nitrogen, total phosphorus and COD were reduced to 99.7%, 89.5%, 92.0% and 75.5% respectively within five days and were further reduced to 100%, 93.6%, 89.2% and 55.4%, respectively within twenty days.

Ahmed (2014) evaluated the ability of *Spirulina* sp. for the removal of pollutants from dairy wastewater. It was reported that there were 80%, 72%, 61%, 56%, 71%, 56%, 77%, 54% and 59% reduction in nitrate, active phosphorus, sulphate, total hardness, alkalinity, chloride, COD, TDS, calcium and magnesium hardness, respectively during the tenth day of treatment.

Chiu *et al* (2015) summarized the effects of different kinds of wastewater on the biomass and lipid production by algae *Chlorella*. They further reported the effect of ammonium ion present in wastewater on growth of *Chlorella* and limitations of wastewater-

based of microalgal culture.

Hena *et al* (2015) isolated microalgal strains from collection tank of dairy wastewaters and holding tank and consortium were selected on the basis of fluorescence after treatment with nile red reagent. About 98% of nutrients were removed from treated wastewater by these native strains. The biomass production of 153.54 t ha^{-1} $year^{-1}$ and lipid content of 16.89% was obtained. They also reported that about 70% of lipids from algae could be converted to biodiesel.

Joshi *et al* (2014) evaluated the growth of *Spirulina sp.* (NCIM No. 5143) at lab scale by using different concentration (10^{-1} to 10^{-6}) of various substrates like cow urine, rain water, cheese whey, and tube well water with Zarrouk' s medium at a temperature of 30 ± 2°C, pH of 9.5 ± 2 and photo-period of 10-12 hours. It was reported that *Spirulina* can be successfully cultivated on all the four substrates with maximum chlorophyll and protein content as they all provided nutrients to algae and reduced its cost and thus provide economical and cheap medium for cultivation of *Spirulina*.

Issa *et al* (2015) reported that wastewater can be treated with cultivation of cloacal algae and bioactive compounds can also be produced from harvested biomass. They found that nitrogen was reduced to 90% (by *Scenedesmus*, *Ankistrodesmus, Oscillatoria*) and phosphorus to 91% (by *Scenedesmus*, *Ankistrodesmus* and *Lyngbya*) in Al-Garega wastewater and Al-Garega and /or Shahat wastewater respectively. Total inorganic carbon was reduced to 75% (by *Nitzschia, Oscillatoria* and *Lyngbya*) and 65% (by *Oscillatoria* and *Euglena*) in Shahat and Al-Garega wastes respectively. They further reported reduction in heavy metals like Cd (88%), Zn (78%) and Pb (75%) by algae *Oscillatoria* and *Lyngbya* in Al-Garega wastewater while in Shahat waste water, the maximum reduction in Cd, Pb and Zn was 72%, 80% and 87% respectively by *Scenedesmus* and *Euglena, Lyngbya* and *Ankistrodesmus* respectively. Bioactivity of aqueous and ethanolic extracts of most algae showed significant activity against *Bacillus cereus* and *Escherichia coli* and against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, low zone of inhibition was reported.

In another study conducted by Guruvaiah *et al* (2014), two selected microalgae namely SBC39 (*Scenedesmus*) and SBC212 (*Chlorella* sp.) were grown on different proportions of wastewater (1%, 5%, 10%, 20%, 40%, 50% and 80%) by replacing BBM and BG-11 medium. It was found that the combination of 80% wastewater and 20% BG-11 medium supported the growth of SBC 39 and SBC 212 followed by 50% wastewater and 50% BG-11.

Girard *et al* (2014) reported that *Scenedesmus* can be grown using whey permeate (WP). Under mixotrophic, heterotrophic and phototrophic conditions, μ_{max} of 1.083± 0.030 day^{-1}, 0.702± 0.025 day^{-1} , 0.267± 0.083 day^{-1} respectively was obtained by substituting 40% (v/v) of the culture medium with WP. On analysis, it was found that extracellular nitrogen

sources present in culture medium were completely depleted while neutral lipid (NL) accumulation occurred intarcellularly, under high pH. They also found that cultures supplemented with WP accumulated less lipids than control cultures (photoautotrophic cultures).

Ding *et al* (2015) investigated the growth of microalgae on dairy wastewater. They reported that dry weight and relative growth rate of 0.86 g/L and 0.28 d^{-1} at 5 times dilution respectively, 0.74 g/L and 0.26 d^{-1} at 10 times dilution respectively, 0.59 g/L and 0.23 d^{-1} at 20 times dilution respectively. The greatest dilution (20×) showed that removal rates for ammonia was 99.26%, P was 89.92%, COD was 84.18% for 10× dilution, removal rate for ammonia was 93%, P was 91% and COD was 88% and finally for 5× dilution removal, removal rate for ammonia was 83%, P was 92%, COD was 90%.

Sahana and Shirnalli (2018) in their study determined the effect of growth of five microalgae strains viz. *Chlorella* MA-6, *Chlorella* MA-14, *Botryococcus* MA-5, *Botryococcus* sp., *Scenedesmus* sp. and different dilutions i.e. 20%, 40%, 60%, 80% and 100% on various physico-chemical properties such as pH, total solids, chemical oxygen demand, nitrate, phosphate of untreated and treated dairy industry effluent. The results revealed that maximum reduction in various physico-chemical parameters take place in 40 per cent of untreated effluent and in 100 per cent of treated effluent. They further reported that out of five strains used in the study *Chorella* MA-6 was the most efficient as it reduced all the parameters significantly compared to all other strains.

Lu *et al* (2016) reported that treatment of mixed wastewater with algae can significantly reduce nutrients present. Much higher ammonia nitrogen in slaughter house wastewater is a major limiting factor for growth of algae. However, algae grown on dairy waste water mixed with slaughter house waste water contained high protein and oil content in the range of 55.98-66.91% and 19.10-20.81% respectively which can be exploited to produce biofuel and animal feed.

Sreekanth *et al* (2014) studied *Chlorella vulgaris, Botryococcus braunii* and a mixed algal culture in treatment of dairy waste water. *C. vulgaris* showed biomass productivity, chlorophyll and lipid yield of 0.51g/l, 0.039mg/l and 0.030g/l respectively in indoor cultures whereas 0.59g/l, 0.045mg/l and 0.035g/l respectively in outdoor cultures. Analysis of lipids by Gas Chromatography Mass Spectrophotometer (GC MS) showed that *C. vulgaris* contained palmitic acid, stearic acid and oleic acid as major components where as *B. braunii* contained oleic acid as major component.

Jitha and Madhu (2016) used a two stage photobioreactor with immobilized microalgal cells for bioremediation of dairy wastewater and biomass production. Reduction of 94-99.5% in phosphate content after 48 h of treatment in the primary and secondary photobioreactors was reported. The level of phosphate, total hardness, ammonical nitrogen in

the medium strength effluent (MSE) were reduced by 97%, 93%, 81% respectively. BOD was reduced to 370 mg l^{-1} from 1500 mg l^{-1} after 48 hrs of treatment in the primary reactor. COD was reduced to 85 mg l^{-1} from an initial value of 1500 mg l^{-1} in medium strength effluent (MSE) and 90-95% removal of COD was also obtained in high strength effluent (HSE) during the study period. Biomass harvested after every 15 days was analyzed through Gas chromatography indicated the presence of C14:0, C16:0, C18:0, C18:1 and C18:2 fatty acids revealed its potential for biodiesel production.

Stemmler *et al* (2016) isolated nineteen microalgal strains from two activated sludge and one aerated-stabilization basin systems. They reported that *Chlorella* and *Dictyochloris* grew faster under mixotrophic conditions and glucose as compared to two culture collection strains. Under photoautotrophic and mixotrophic conditions, *Chlorella* and *Scenedesmus* produced high lipid content. They further found that composition of fatty acids varies considerably with commensal bacteria depending on the axenic and non-axenic strains of wastewaters. An equal proportion of saturated and unsaturated fatty acids were found in strains grown with bacteria tended.

Ajayan *et al* (2015) studied the phycoremediation of tannery wastewater by *Scenedesmus* sp. Different concentrations of tannery wastewater viz. 10%, 25%, 50%, 75% and 100% inoculated with *Scenedesmus* reduced the heavy metal content as given below : Cr by 81.2-96%, Cu by 73.2-98%, Pb by 75-98% and Zn by 65-98% and nutrients content NO_3^- by 44.3% and PO_4^{3-} by 95%.

Asmare *et al* (2014) studied two microalgal strains *Chlorella vulgaris* and *Scenedesmus dimorphus* and their co-culture from dairy facility for their growth parameters and nutrient sequestration profiles at two different dilutions i.e. 10% and 25%. They observed that at 10% manure, *S. dimorphus* and *C. vulgaris*, and their coculture showed average specific growth rates and biomass concentration of 0.263 d^{-1} and 0.290±0.059 g/L respectively, 0.063 d^{-1} and 0.145±0.011 g/L respectively, 0.250 d^{-1} and 0.400±0.060 g/L respectively. While at 25% manure *S. dimorphus* and *C. vulgaris*, and their coculture showed average specific growth rates and biomass concentration of 0.232 d^{-1} and 0.543±0.149 g/L respectively, 0.234 d^{-1} and 0.364±0.113 g/L respectively, 0.289 d^{-1} and 0.612±0.255 g/L respectively. Biomass, lipid accumulation capacities and volatile suspended solids of *S. dimorphus*is were higher than *C. vulgaris* whereas chlorophyll content and total suspended solids of *C. vulgaris* were higher than *S. dimorphus*. However, higher values of biomass, specific growth rate and reduction in nitrogen, phosphorus and TSS were higher for mixed coculture at 25% dairy wastewater.

Ichsan *et al* (2014) reported that bioenergy like biogas, hydrogen, bioethanol, biodiesel and also many value added products like proteins, anti-oxidants, olefin, glycerol, pigment, plastic, paraffin, etc can be produced by microalgae. Microalgae cultivation can also

be integrated with biogas system from agricultural wastewaters.

2.3 Environmental factors affecting microalgal growth

Environmental factors like temperature and light intensity and availability of nutrients have great impact on biochemical composition of microalga (Morris *et al* 1974, Gordillo *et al* 1998, Nakamura and Miyachi 1982, Kalacheva *et al* 2002, Fábregas *et al* 2004). These environmental factors (light, pH, temperature, concentration of various nutrients) greatly impact processes like photosynthesis and biosynthesis of lipids, carbohydrates, proteins, pigments and various other compounds in microalgae (Juneja *et al* 2013).

2.3.1 Light

Microalgae cultivated under the influence of different intensities of light showed various changes in their chemical composition. Under low light conditions, amount of structural lipids of microalgae i.e. polyunsaturated fatty acids increase whereas higher light conditions promote the accumulation of storage lipids i.e. saturated and mono-unsaturated fatty acids (Orcutt and Patterson 1974, Walsh *et al* 1997).

2.3.2 Temperature

Temperature also have pronounced effect on the lipid content in microalgae. In *Ochromonas danica* and *Nannochloropsis oculata*, there was increase in lipid content by elevating temperature (Aaronson 1973, Converti *et al* 2009). So, suitable high temperature boosts the production of high quantity and high quality of diesels. Temperature also has effect on the level of carbohydrate content. It was reported that temperature increase from 25 to 40°C resulted in 50% increase in carbohydrate content of *Spirulina* sp (Ogbonda *et al* 2007). Temperature is a very important factor for determining the activity and rate of reaction of many intracellular enzymes affecting various processes in algae like photosynthesis, respiration intensity, growth (Tan *et al* 2009).

2.3.3 pH

Algal growth is mainly affected by the pH of the environment in which algae is growing since the solubility and availability of CO_2 and essential nutrients required for algal growth is determined by pH (Chen and Durbin 1994, Goldman 1973). Uptake of inorganic carbon sources by algae markedly results in rise in pH (Hansen 2002). At higher values of pH i.e. at alkaline conditions, microalgae can easily capture CO_2 from the environment and produce more biomass (Melack 1981, Zang *et al* 2011).

2.3.4 Nutrients

Under optimum temperature and pH, rate of uptake of the most limiting nutrient is in direct proportion to growth rate of algae which is explained by Michaelis-Menten equation (Titman 1976).

2.3.4.1 Nitrogen

It is also very important factor for protein biosynthesis. Under nitrogen limiting

conditions, most of the photosynthetically fixed carbon is used for the synthesis of lipids or carbohydrates. It was reported that after nitrogen limiting conditions, lipid content in *Neochlorisoleo abundans* and *Nannochloropsis* sp. F&M-M24 increased about two fold and one fold, respectively (Rodolfi *et al* 2009). Increase in concentration of nitrogen cause increased microalgal growth. Growth of microalgae also depends on type or form of nitrogen for example, microalgae can easily absorb NH^{+}_{4}- N than NH_3- N (McCarthy and Wynne 1982, Dortch 1990).

2.3.4.2 Phosphorous

Many cellular metabolic processes in microalgae is affected by phosphorus and its limitation causes higher lipid content. For instance, *Scenedesmus* sp. LX1 contained 25-28% and 53% lipids under phosphorus sufficient and phosphorus-limiting conditions, respectively (Xin *et al* 2010). For microalgal growth optimum concentration of phosphorus is beneficial. Microalgal growth is inhibited at TP $\leq$ 0.045mg/L and at TP $\geq$ 1.65mg/L microalgae growth rate is also not significantly promoted (Dortch 1990).

2.3.4.3 Sulfur

It was reported that activity of enzyme hydrogenase and hence hydrogen production increases by limitation of element sulphur inside the algal cells as its limitation creates an anaerobic environment (Esquível *et al* 2011, Dutta *et al* 2005). Fatty acid content in *Chlamydomonas reinhardtii* doubled with limitation of sulfur (Matthew *et al* 2009).

2.3.4.4 Carbon

Carbon content affects the activity of nitrogenase enzyme and nitrogenase-dependent hydrogen production (Dutta *et al* 2005). Saturated fatty acids are accumulated under high carbon dioxide concentration whereas unsaturated fatty acids are produced under low concentration (Riebesell *et al* 2000, Hu and Gao 2003).

2.3.4.5 Trace mineral nutrients.

It has been reported that in *Agmenellum quadruplicatum* due to iron limitation, glucose content increased from 5 to 45% (Hardie *et al* 1983).

2.3.4.6 Salinity

Carrieri *et al* (2010) found that in cyanobacterium *Arthrospira maxima*, under high salt concentration, ethanol production increased by 121-fold as compared to low concentration of salt. Algal growth rate and composition can be altered by exposure of algae to altered salinity levels than their natural levels. For instance, In *Dunaliella*, by changing NaCl concentration from 0.4 to 4 M, increase in saturated and monounsaturated fatty acids was observed (Xu and Beradall 1997). Microalage has the ability to alter the salinity on its own. It was reported that seawater microalgae can tolerate higher salinity rather than fresh water microalgae (Zhu *et al* 2003).

2.4 Optimization of microalgal growth conditions using Response Surface Methodology (RSM)

Response Surface Methodology (RSM) is a technique for optimization process, consisting of complex calculations and develops an experimental design which combines all the independent variables and uses the data input from the experiment and finally give a set of equations that can give theoretical value of output. Then new values of independent variables can be used to predict the value of dependent variables (Said and Amin 2015).

Spolaore *et al* (2006) studied the effects of light, temperature, pH and aeration rate on the microalga *Nannochloropsis oculata* using central composite design of response surface method. The optimum growth conditions for the microalgae was estimated as 52 μmol photons m^{-2} s^{-1}, 21 °C, 8.4 and 14.7 VVH. These factors obtained were experimentally validated by testing again and maximum growth rate achieved with these parameters was 0.0359 h^{-1}.

Skorupskaite *et al* (2015) investigated several potential inexpensive waste materials (technical glycerol and liquid waste i.e. the liquid fraction of the digestate after biogas production) for *Chlorella* sp. biomass production rate and optimization was done by response surface methodology. The highest biomass (2.41 g L^{-1}) was reported in medium containing 0.114 g L^{-1} N and 2.70 g L^{-1} technical glycerol.

Similarly, Azma *et al* (2010) studied the effect of glucose and sodium acetate (carbon sources) and peptone, yeast extract, meat extract, malt extract, urea, sodium nitrate and ammonium nitrate (nitrogen sources) in Walne medium on growth of green microalgae, *Tetraselmis suecica*. Secondly, the optimization of most influencing medium component was done by response surface methodology (RSM). The most optimal conditions were media consisting glucose, peptone, yeast extract and meat extract in the concentration of 5.78 g/L, 9 g/L, 4.48 g/L and 3.01 g/L respectively and from these optimum values cell concentration of 28.88 g/L was obtained in heterotrophic cultivation which was about 3 times higher than photoautotrophic culture (8.40 g/L) and 2 times higher than non-optimized medium for heterotrophic cultivation (13.81 g/L).

Response surface methodology was used to study the combined effect of nutrient (i.e. nitrogen, phosphorous and iron) stresses on the lipid productivity of an oleaginous microalgal strain, *Ankistrodesmus falcatus* KJ671624 by Singh *et al* (2015). Under concentration of nitrogen 750 mg L^{-1}, phosphorus 0 mg L^{-1} and iron 9 mg L^{-1} (nutrient stress conditions), lipid content and lipid productivity of 59.6% and 74.07 mg L^{-1} d^{-1} respectively were obtained and 38.49% increase in saturated fatty acid content as compared to BG-11 medium was also found. Biodiesel conversion of 91.54 ± 1.43% was reported.

In a similar study carried out by Yang *et al* (2014) RSM was used to assess the effects of medium composition on lipid production by *Scenedesmus* sp. and revealed that $NaHCO_3$,

$NaH_2PO_4 \cdot 2H_2O$ and $NaNO_3$ were found to be three significant factors by Plackett-Burman design which were further optimized by a Box-Behnken design of RSM. The results showed that optimum values for $NaHCO_3$, $NaH_2PO_4 \cdot 2H_2O$ and $NaNO_3$ were 3.07 g L^{-1}, 15.49 mg L^{-1} and 803.21 mg L^{-1} respectively and lipid content of 304.02 mg·L^{-1} was obtained by using these optimal conditions which agreed with the predicted value of 309.50 mg L^{-1}.

Dar *et al* (2019) optimized the cultural factors of a microalgal strain *Spirulina subsalsa* (MF191711) isolated from the waterlogged area of Punjab. Screening of significant cultural factors (pH, light, temperature, incubation period, and concentration of nutrients viz. $CaCl_2$, $NaNO_3$ and K_2HPO_4) was done by Placket-Burman design and optimization done by central composite design (CCD) of Response Surface Methodology (RSM). Results showed that the optimal conditions for biomass production were pH of 11.5, temperature of 20 °C, Light intensity of 81 µmolm^{-2} s^{-1}, growth period of 25 days, $CaCl_2$ of 15 mM, $NaNO_3$ of 5 mM and K_2HPO_4 of 2 mM concentration. As compared to basal conditions (0.987gL^{-1}), these optimal conditions resulted in 1.60-fold higher biomass. It was also reported that in co-digestion studies, 70% paddy straw with 30% microalgal biomass resulted in maximum biogas production with P (highest ultimate biogas yield) of 260.03 mLg^{-1}VS, R_m (rate of biogas production) of 8.16 mL$g^{-1}d^{-1}$ and lag phase (λ) of 3.84 days as compared to paddy straw and microalgal biomass individually.

Nazir *et al* (2018) studied the interactive effects of the cultural factors (fructose, agitation speed and concentration of monosodium glutamate) of *Aurantiochytrium* SW1 on response variables (growth, lipids and DHA production) using CCD of RSM. The optimal conditions obtained for fructose, agitation speed and monosodium glutamate were 70g/L, 250 rpm and 10g/L respectively. At the end, model validation was done by applying the estimated optimum conditions, which confirmed the model validity with values of biomass, lipid and DHA as 19.0g/L, 9.13g/L and 4.75g/L DHA respectively which were 28, 36 and 35% respectively higher than in the original medium before optimization.

Abirami *et al* (2017) tested a screening technique for selection of oleaginous microbes and optimization for several culture factors was also done. They reported that f/2 medium produced maximum algal growth at 25° C. With urea as a nitrogen source, biomass yield of 8.6 g/L and lipid content of 0.17 g/dry wt (17%) were obtained and EPA yield of 0.9% was found in *N. gaditana* cultivated in laboratory conditions.

Fung *et al* (2013) optimized the biomass productivity of microalgae *Chlorella vulgaris* by variation of potassium bicarbonate and sodium nitrate concentration in culture medium. *Chlorella vulgaris* was cultivated in flat panel photobioreactor with air supply rate of 3.5 L/min and light intensity of 4500 Lux for 6 hours daily. Optimization was performed with the design expert software using Central Composite Design (CCD). The optimum biomass productivity of 36 mg/L/day was obtained at 6 g/L of potassium bicarbonate and 0.36

g/L sodium nitrate concentration.

Malek *et al* (2016) cultivated microalgae in an outdoor open pond and its optimization study was done. A non-linear mathematical model was developed and data for local climatic conditions was incorporated into the model and also algal culture supplied with dissolved CO_2 from a CO_2 rich gas was also modeled. Based on minimization of the cultivation cost, optimal monthly operating profiles for the dilution rate, CO_2 gas flow rate, and makeup water flow rate were determined. The case study included in the analysis was for cultivating *Nannochloropsis Salina* over an annual production cycle in California United States of America.

Delrue *et al* (2017) rationalized different concentration of modified Zarrouk's medium to reduce production cost of *Spirulina* cultivation. They reported that five times dilution of the modified Zarrouk's medium do not affect microbial growth and at 20% concentration of modified Zarrouk's medium higher dry biomass weights was observed compared to Zarrouk's medium. Replacement of iron sulfate by iron EDTA resulted increase in iron content of *Spirulina* and also maximum specific growth rate was observed at 430 $\mu mol/m^2/s$. Experiment conducted in a PBR (1000 L) for a period of 40 days gave areal productivity of 58.4 $g/m^2/day$. Production cost for PBR was 2 to 20 times higher as compared to open ponds was revealed by techno-economic analysis.

Optimization of growth of freshwater isolated *Chlorella* sp. was done on various growth media (Bold's basal medium, CFTRI medium, Bangladesh M3 and Zarrouk's medium) (Vishnu and Sumathi 2014). They further evaluated the isolates for chlorophyll a and b content, phytochemicals and finally for bacterial susceptibility testing by AWD assay.

Al-Shatri *et al* (2014) optimized the growth of *Scendesmus dimorphus* in different media viz. BBM, M_4N, BG-11, N-8 under various environmental factors. Highest biomass productivity of 0.1406 g/l with specific growth rate of 0.10483/day were reported in Bold's Basal medium.

2.5 Anaerobic digestion of microalgal biomass

Although many researchers have investigated microalgae as potential source of biogas but low C/N ratio of microalgae is not conducive for the anaerobic digestion process. Carbon to nitrogen ratio of 20 to 30 is optimum for anaerobic digestion (Mital 1996, Chandra *et al* 2012). Lignocellulosic material like paddy straw has high C/N ratio which is mixed with microalgae (low C/N ratio) to maintain the required C/N ratio for the anerobic digestion process.

Golueke *et al* (1957) first reported the process of anaerobic digestion of microalgae by processing of microalgae biomass grown in waste water treatment ponds. Several studies thereafter have confirmed that algal biomass can be anaerobically digested to produce biomethane (Brennan and Owende 2010).

In another study, algae and cow dung (CD) were mixed with organic wastes in three different proportions, viz. 80:20% (S1), 40:60% (S2) and 20:80% (S3) on mass basis and evaluated for important parameters such as the pH, temperature, hydraulic retention time (HRT) and carbon/nitrogen ratio (C/N). Among three samples, S2 gave highest methane (CH_4) and carbon dioxide (CO_2) content in the biogas (Kumar *et al* 2016).

Ezekoye *et al* (2014) characterized biogas generated from rice husks and algae in a metal fixed-dome biodigester. Batch type anaerobic digestion of 35 kg of slurry was carried out in the biodigester at mesophilic temperatures range. The slurry was made by mixing rice husks (5 kg) and algae (30 kg) (mixed with water in the ratio of 1:6) and anaerobic digestion was carried out in the biodigester at mesophilic temperatures in the range of 29 $^{\circ}$C - 33.45 $^{\circ}$C. After a period of 75 days, the cumulative biogas produced was 156.25 litres and percentage of the methane component of produced biogas was 52.3%.

Passos *et al* (2015) enzymatically pretreated microalgal biomass under different conditions for enhancing its biochemical methane potential (BMP). On the basis of microalgal cell wall composition, cellulase, glucohydrolase and an enzyme mix consisting of cellulase, glucohydrolase and xylanase were selected. High value of organic matter solubilisation was obtained after 6 h of pretreatment with an enzyme dose of 1% cellulase and enzyme mix. They further reported that pretreatment of microalgae increased the methane content by 8 and 15% for cellulase and the enzyme mix respectively.

Perazzoli *et al* (2016) reported that microalgae polyculture dominated by *Scenedesmus* spp. showed increased carbohydrate, protein and lipid content when grown on N and P free medium as compared to nutrient rich medium. They also reported that microalgae grown on nutrient deficient medium contained more volatile solids and consequently gave higher biogas yield compared to nutrient rich medium.

Budiyono and Kusworo (2012) studied biogas production from cassava starch effluent by adding microalgae as nitrogen source and yeast as substrate activator with the treatment of gelled and ungelled feed using 5 Litre anaerobic digester. They reported that after addition of cassava starch effluent and yeast, biogas production of 726.43 ml/g TS was obtained with addition of microalgae, cassava starch effluent, yeast, ruminant bacteria, urea and without microalgae was 189 ml/g TS.

CHAPTER III

MATERIALS AND METHODS

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

3.1 Maintenance of pure cultures of microalgal strains

3.2 Morphological identification of the microalgal strains

3.3 Procurement and physico-chemical analysis of dairy wastewater

- 3.3.1 Determination of Dissolved oxygen (DO)
- 3.3.2 Determination of Biochemical Oxyen Demand (BOD)
- 3.3.3. Determination of Chemical Oxyen Demand (COD)
- 3.3.4. Determination of Chloride concentration (Cl^-)
- 3.3.5. Determination of Total Kjeldahl Nitrogen (TKN)
- 3.3.6. Determination of Phosphorus content
- 3.3.7. Determination of Total solids (TS), Total dissolved solids (TDS), Total suspended solids (TSS)

3.4 Screening of the microalgal strains on dairy wastewater and commercial media

- 3.4.1 Estimation of dry biomass 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

3.5 Optimization of culture conditions of selected microalgae strains by response surface methodology (RSM)

3.6 Molecular Identification of the selected microalgal strains

3.7 Biochemical characterization of selected microalgal strains

- 3.7.1 Estimation of total phenolic content (TPC)
- 3.7.2. Estimation of total antioxidant activity
- 3.7.3. Estimation of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity
- 3.7.4. Estimation of total kjeldahl nitrogen (TKN)
- 3.7.5 Evaluation of microalgae strains for phytochemical compounds
- 3.7.5.1. Quinones (Borntrager's test)
- 3.7.5.2. Tannins (Brameyer's test)
- 3.7.5.3. Saponins (Foam test)

3.1 Maintenance of pure cultures of microalgal strains

Five stress tolerant microalgal strains viz. BGLR4, BGLR7, BGLR10, BGLR18, BGLRS (Dar 2017) already available at Biogas Laboratory, Department of Renewable Energy Engineering, College of Agricultural Engineering and Technology, PAU, Ludhiana, Punjab India and one standard strain *Spirulina* sp. NCIM 5143 procured from National Collection of Industrial Microorganisms (NCIM) laboratory, Pune, India were used in the present study. These strains were maintained as pure cultures at refrigeration temperature.

3.2 Morphological identification of the microalgal strains

The microalgae strains were observed under microscope to determine their morphology. The cultures were photographed using the Olympus 528293 microscope (Magnus Icon Freedom Model) at 40× using Debro 5.1 Megapixel digital camera and Toup view software program.

3.3 Procurement and physico-chemical analysis of dairy wastewater

Dairy wastewater used in the present study was routinely collected from a local dairy located at Ferozepur road in Ludhiana. The sample collection was carried out in a clean and dry 25-liter plastic can. The effluent was collected immediately after milk processing and then brought to the laboratory and kept in refrigerator at 4°C until use. Physico-chemical analysis

of dairy wastewater sample was done according to the standard procedures given by APHA (2005).

3.3.1 Determination of Dissolved Oxygen (DO)

Modified Winkler method was used for determining the dissolved oxygen in dairy wastewater sample (APHA 2005).

Principle : The Mn^{++} ions released from $MnSO_4$ react with OH^- ions to form white precipitate if no DO is present and brown precipitate if DO is present in water as shown below:

$MnSO_4 + 2NaOH = Mn(OH)_2\downarrow + Na_2SO_4$ (white ppt.) (in the absence of DO)

$2\ Mn(OH)_2 + O_2 = MnO(OH)_2\downarrow$ (brown ppt.) (in the presence of DO)

or

$2\ Mn(OH)_2 + O_2 = MnO_2\downarrow + H_2O$ (brown ppt.) in the presence of DO

The MnO_2 and H^+ ions formed due to addition of concentrated H_2SO_4, oxidise iodide ions (I^-) released by the alkali-iodide (NaOH +KI) to iodine (I_2) as shown below:

$MnO_2 + 2KI + 2H_2SO_4 = MnSO_4 + K_2SO_4 + 2H_2O + I_2$

The amount of I_2 can finally be computed by titrating the solution against the standard sodium thiosulphate (0.025 N) solution.

The interference by nitrite ion is prevented by adding sodium azide to the water sample

$NaN_3 + H^+ = HN_3 + Na^+$

$HN_3 + NO_2^- + H^+ = N_2 + N_2O + H_2O$

Reagents : 1). Standard manganese sulphate solution: 36.4 g of $MnSO_4.H_2O$ was dissolved in distilled water, filtered and diluted to 100 ml with distilled water, 2). Alkali-iodide-azide reagent: 500 g of NaOH (sodium hydroxide) and 135 g of NaI (sodium iodide) was dissolved in distilled water, total volume was made 1 litre with distilled water and 10 g of sodium azide (NaN_3) in 40 mL distilled water was also added, 4). Concentrated sulphuric acid, 5). Standard sodium thiosulphate (0.025 N): Dissolved 6.205 g sodium thiosulphate ($Na_2S_2O_3.5H_2O$) in boiled and cooled distilled water and diluted to 1 litre and 0.4 g of sodium hydroxide was added to preserve it , 6). Starch Indicator : Added 2.0 g of starch to 100 ml of hot distilled water and stirred continuously.

Procedure : BOD bottles (300ml capacity) in triplicates were filled with the waste sample to be analyzed and any contact of the sample with air or any kind of bubbling and trapping of air was avoided. Manganese sulphate solution (1 mL) and 1 ml alkali-iodide azide reagent was added to 300 mL sample bottle below the surface of the liquid. The bottle was closed with the glass stopper. The contents were mixed by inverting and shaking the bottle vigorously. Brown precipitate would be formed if dissolved oxygen is present in water. If dissolved oxygen is not present in water, white precipitate would be formed. After 2 minutes of settling, stopper was removed and concentrated sulphuric acid (2 ml) was added. The glass stopper

was inserted again and contents were mixed by gentle inversion until dissolution was completed. Then the sample was taken for titration which was equivalent of 200ml of original sample. For it 201 ml (after making correction for spilled water $= \frac{200}{300} \times 302$) of this solution was taken in a 500 ml Erlenmeyer flask and titrated against N/40 sodium thiosulphate solution until yellow colour became very light. Now 1 mL of starch solution (end point indicator) was added which gave blue colour to the solution and titration was continued with N/40 sodium thiosulphate solution till the blue colour just disappeared and solution became colourless. The volume of sodium thiosulphate solution used was noted. Repeated the titration three times for concordant values. D.O. was calculated by the following formula :

$$\text{DO}\left(\frac{\text{mg}}{\text{L}}\right) = \frac{\text{mL of sodium thiosulphate used} \times \text{N} \times 1000 \times 8}{\text{ml of sample taken}}$$

where

N=Normality of sodium thiosulphate solution

3.3.2 Determination of Biological oxygen demand (BOD)

BOD was determined by standard method of APHA (2005)

Principle : High organic content in wastewater cause more oxygen consumption by micro-organisms for degradation of the substrate resulting in high BOD. BOD is the measure of amount of dissolved oxygen used by micro-organisms present in the wastewater to degrade organic matter of wastewater completely to CO_2 and H_2O (Zheng *et al* 2013). This test measures the concentration of Dissolved oxygen (DO) at the beginning and end of the five day incubation period (also called BOD_5). The change in DO content over the period of five day period represents the oxygen demand for respiration by the aerobic micro-organisms in the sample.

Reagents : 1). Phosphate buffer solution : Dissolved 8.5g of KH_2PO_4, 21.75g of K_2HPO_4, 33.4 g $Na_2HPO_4.7H_2O$, 1.7g NH_4Cl in distilled water and diluted to 1 litre. The pH of the solution was maintained around 7.2, 2). Magnesium sulphate solution : Dissolved 22.5g of anhydrous $MgSO_4.7H_2O$ in distilled water and diluted to 1000 ml, 3). Calcium chloride solution: Dissolved 27.5g of anhydrous $CaCl_2$ in distilled water and diluted to 1000 ml, 4). Ferric chloride solution : Dissolved 0.25g of $FeCl_3.6H_2O$ in distilled water and diluted to 1000 ml.

Procedure : To prepare dilution water, 2 ml each of phosphate buffer, magnesium sulphate solution, calcium chloride solution and ferric chloride solution were added to two litre of distilled water. To the above prepared dilution water, 1-2 ml of test sample was added. Then the samples were shaken vigorously for few minutes to aerate the sample. Three BOD bottles were filled with the above diluted samples and two bottles with dilution water (without sample). Dissolved oxygen (DO) in one of the bottle filled with the diluted sample (X mg/l) and bottle filled with dilution water (P mg/l) was determined by modified winkler method

(described in section 3.3.1). Incubated the other two bottles filled with sample and 2nd bottle filled with distilled water at 20°C for 5 days. Then, the DO of diluted sample (Y mg/l) and dilution water (Q mg/l) was determined. For other concentrations and for all samples, BOD bottles were prepared in the same manner. Calculated the BOD as per the given formula:

BOD (mg/l)=((DO in sample bottle at the beginning- DO in sample bottle after 5th day)-(DO in dilution water at the beginning- DO in dilution water after 5th day)×Dilution factor (DF)

$$\text{BOD}\left(\frac{\text{mg}}{\text{L}}\right) = ((\text{X} - \text{Y}) - (\text{P} - \text{Q}))\frac{\text{B}}{\text{A}}$$

where

X= DO in sample bottle at the beginning

Y= DO in sample bottle after 5th day

P= DO in dilution water at the beginning

Q= DO in dilution water after 5th day

DF=B/A=Total volume made/initial volume used

3.3.3 Determination of Chemical oxygen demand (COD)

Chemical oxygen demand was determined by using COD digestion apparatus (APHA 2005).

Principle : Potassium dichromate in the presence of sulphuric acid, silver sulphate and mercury sulphate oxidize the organic matter present in wastewater to produce carbon dioxide and water. The quantity of potassium dichromate used is calculated by the difference in the volumes of ferrous ammonium sulphate consumed in blank and sample titrations which is equivalent to the oxygen used to oxidize the organic matter present in water.

Reagents: 1). Standard ferrous ammonium sulphate (0.25 M) : Ninety eight grams of $Fe(NH_4)_2(SO_4)_2.6H_2O$ was dissolved in distilled water and 20 ml of concentrated H_2SO_4 was added and final volume was made to 1 litre, 2). Standard potassium dichromate solution (0.0417 M): Dissolved 12.259 g of $K_2Cr_2O_7$ and 33.3 g of mercuric sulphate in a clean dry beaker and added 167 ml of concentrated sulphuric acid. The contents were mixed, cooled and total volume was made 1 litre with distilled water, 3). Sulphuric acid reagent: Weighed exactly 5.06 g of silver sulphate and added 500 ml of concentrated sulphuric acid to it. Allowed to stand for 24 hours to get it dissolved completely, 4). Ferroin indicator : Dissolved 1.485g of 1,10-phenanthroline monohydrate and 695 mg of ferrous sulphate in distilled water and final volume was made 100 ml.

Procedure: Two digestion tubes for sample and one for the blank were taken. Each of the sample tube contained 20 ml of diluted sample, 10 ml of potassium dichromate solution and 30 mL of concentrated sulfuric acid reagent while blank contained all the reagents but 20 ml distilled water in place of diluted sample. Digestion tubes were placed in the holes in the instrument and digested for 2 hours at 150°C. After that tubes were removed and cooled at room temperature. Then the contents were diluted to about 100 ml with distilled water and

transferred to a conical flask and 2-3 drops of ferroin indicator was added. The colour of the solution became green/bluish in colour and the excess dichromate was titrated with standard ferrous ammonium sulfate solution. Change in colour of the solution to reddish brown colour indicated the end point. Initial and final reading in the burette were noted. Chemical oxygen demand was calculated as per the following formula :

$$\mathrm{COD}\left(\frac{\mathrm{mg}}{\mathrm{L}}\right) = \frac{\mathrm{A-B\times M\times 8\times 1000}}{\text{ml ofsample taken}} \times \mathrm{DF}$$

where

A= ml of ferrous ammonium sulfate solution used for blank

B= ml of ferrous ammonium sulfate solution used for sample

M= Molarity of ferrous ammonium sulfate solution used

8= milliequivalent weight of oxygen

DF= Dilution Factor

3.3.4 Determination of chloride concentration (Cl^-)

Chloride concentration in dairy wastewater sample was determined by commonly used Argentometric method (APHA 2005).

Principle: The silver nitrate solution is used as a titrant and potassium chromate used as indicator which supplies chromate ions (CrO_4^-). During titration silver ions combine with chloride ions as :

$Ag^+ + Cl^- = AgCl$ (White ppt.)

As the concentration of chloride ions in water approaches zero the silver ions combines with chromate ions to form reddish-brown precipitate of silver chromate as follows:

$Ag^+ + CrO_4^- = AgCrO_4$ (Reddish brown ppt.)

This is taken as evidence of all the chlorides having been precipitated.

Reagents : 1). Standard silver nitrate solution (1/35.5 N): Dissolved 4.79g of $AgNO_3$ in distilled water and diluted to 1 litre. Store in a brown bottle, 2). Potassium chromate indicator (5%): Dissolved 50 g potassium dichromate ($K_2Cr_2O_7$) in a little distilled water. Added silver nitrate solution till red precipitate is formed, kept overnight, filtered and diluted to 1 litre with distilled water.

Procedure : Titration apparatus was set and burette was filled with the N/35.5 silver nitrate solution. Sample pH was adjusted between 7.00 to 10.00. Taken 25.00 mL of sample in a 250.00 ml Ehlenmeyer flask and 0.25 mL of potassium chromate indicator was added to the flask and mixed thoroughly. Sample became light yellow in colour. Titrated the sample with silver nitrate solution till brick red colour appeared and a blank was run simultaneously.

Chloride concentration can be obtained by the following formula :

$$\mathrm{Cl^-}\left(\frac{\mathrm{mg}}{\mathrm{L}}\right) = \frac{\mathrm{(X-Y)\times D\times 1000}}{\text{ml ofsample taken}}$$

where X = Amount of silver nitrate solution used for titration of sample

Y = Amount of silver nitrate solution used for titration of blank

D =Dilution factor

3.3.5 Determination of Total Kjeldahl Nitrogen (TKN)

Total nitrogen was estimated by standard Total Kjeldahl Method (TKN) in semi automatic N-analyzer (Pelican Kelplus-Classic DX) (Plate I) according to the methods given by AOAC (2002).

Principle : In the digestion step, organic nitrogen present in the test sample is converted to ammonium ions by the agency of a catalyst at 300°C in the digestion block. In the second step (distillation step), the sample is made alkaline with NaOH and nitrogen is distilled off as NH_3 which is then trapped in boric acid solution. The amount of nitrogen trapped as ammonia is quantified by titrating with standard HCl solution (Saez-Plaza *et al* 2013).

Reagents: 1). Preparation of N/10 HCl

Equivalent weight of HCl= 36.00 g (app.)

Specific gravity = 1.18

36.00 g of HCl in 1000 ml=1N

1180.00g of HCl in 1000 ml=32.78N

Assay of HCl =36%

Actual Normality (N)= $\frac{36\times32.78}{100}$=11.79 N

For 1000 ml of 0.1N HCl from 11.79 N stock solution .

Using equation : $N_1V_1=N_2V_2$

11.79*V1=0.1*1000.00=8.48 ml

Dissolved 8.48 ml of HCl in distilled water to make final volume 1000ml, 3 g of the digestion mixture (K_2SO_4 : CuSO4 :: 9:1), 2). concentrated sulphuric acid, 4). 4% boric acid = Dissolve 4g in 100 ml of distilled water.

Procedure :. The procedure was carried out in three steps:

1). Digestion : A known quantity of the sample (1ml) was taken in a digestion tube. Added 3 g of the digestion mixture and 10 ml of H_2SO_4. The mixture was digested at 300°C in the digestion block.

2). Distillation : This was done by raising the pH with NaOH which converts the ammonical radical to ammonia and distills out which is then collected in 4% boric acid (trapping medium).

3). Titration : Determination of amount of N_2 in the condensate flask was done by titrating with 0.1 N HCl in the presence of the mixed indicator. The colour of the mixture changes from brick red to bluish colour. The amount of HCl used for titration was noted. A blank consisting of distilled water was run simultaneously and the amount of N_2 present in the sample can be calculated using the formula :

$$\text{Nitrogen}\left(\frac{\text{mg}}{\text{L}}\right) = \frac{14.01 \times (\text{mL titrant} - \text{mL blank}) \times \text{N} \times 100}{\text{ml of sample taken}}$$

where

N= Normality of titrant

14.01= Atomic weight of nitrogen

Crude protein content :

The crude protein content is calculated by the following formula:

Crude protein content (%) = micro-Kjeldahl nitrogen content (%) × 6.25 (based on the assumption that nitrogen constitutes 16% of protein).

3.3.6 Determination of Phosphorus content

Phosphorus content was estimated by the method of vanadomolybdophosphoric method (Jackson 1967).

Principle : It is based on the principle that in dilute orthophosphate solution, ammonium molybdate reacts under acidic conditions to form a heteropoly acid, molybdophosphoric acid. In the presence of vanadium, yellow vanadomolybdophosphoric acid is formed. The intensity of the yellow colour is proportional to phosphate concentration.

Reagents : 1). Triple acid : HNO_3: $HClO_4$: H_2SO_4 :: 9:3:1, 2). Vanadomolybdate reagent: Dissolved 25g ammonium molybdate and 1.25g of ammonium metavanadate in 600 ml distilled water. 330 ml concentrated hydrochloric acid was added and total volume was made 1000 ml, Standard P solution potassium dihydrogen phosphate (100 ppm): Dissolve 0.4387 g KH_2PO_4 in 1 litre of distilled water.

Procedure : Sample (0.5 ml) was taken in a digestion and 10 ml of triple acid was added and samples were digested in the digestion apparatus. After cooling, the total volume of 50 ml was made with distilled water. Pipetted out 5 ml of extract and 5 ml of vanadomolybdate reagent was added and total volume was made 25 ml with distilled water. Absorbance was measured at 470 nm using UV-vis spectrophotometer. The phosphorus content was estimated using a standard calibration curve prepared from standard potassium dihydrogen orthophosphate (KH_2PO_4) of concentration in the range of 2-10 mg/l (as given in section 3.13, Fig.3.1). Total phosphorus was calculated using the formula :

$$\text{TP}\left(\frac{\text{mg}}{\text{L}}\right) = \frac{\text{mg P}}{\text{ml of sample taken}} \times 1000$$

3.3.7 Determination of Total Solids (TS), Total Dissolved Solids (TDS) and Total suspended Solids (TSS)

Total Solids (TS), Total Dissolved Solids (TDS) and Total suspended Solids (TSS) were determined according to the standard methods (APHA 2005).

3.3.7.1. Total solids (TS)

Apparatus : Clean dry crucible, analytical balance, hot air oven, graduated cylinder.

Plate I Semi automatic N-analyzer (Pelican Kelplus-Classic DX)

Procedure : Taken a silica crucible and cleaned it thoroughly and dry it in hot air oven at 105°C. Weighed the empty crucible in analytical balance and pour 50 ml of sample into it. Placed the crucible in hot air oven carefully to avoid any splashing during its placement. Dried the sample at 105°C for 24 hours to get constant mass. Removed the crucible from the oven and kept it in the dessicator for about 20-30 minutes to attain room temperature. Removed the crucible from the dessicator and its weight was determined. Subtracted the initial weight from the final weight to find the weight of the residue left. Calculated the total solids in the sample using the formula :

$$\text{Total solids}\left(\frac{\text{mg}}{\text{L}}\right) = \frac{\text{Weight of the residue left in the crucible in mg}}{\text{ml of well mixed sample taken}} \times 1000$$

3.3.7.2 Total dissolved solids (TDS)

Apparatus : Clean dry crucible, analytical balance, hot air oven, filter paper, graduated cylinder, filtration apparatus

Procedure : Taken a clean dry silica crucible and weighed the empty crucible in analytical balance. Taken about 100 ml of the sample and filtered it using Whatmann filter paper no. 42 placed on the funnel. Taken 50 ml of filtered sample into the crucible and placed carefully in hot air oven carefully. Dried the sample at 105°C for 24 hours to get constant mass. Removed the crucible from the oven and kept in the dessicator for about 20-30 minutes to attain room temperature. Removed the crucible from the dessicator and weight was noted. Subtracted the initial weight from the final weight to find the weight of the residue left. Calculated the total dissolved solids in the sample using the formula :

$$\text{Total dissolved solids}\left(\frac{\text{mg}}{\text{L}}\right) = \frac{\text{Weight of the residue left in the crucible in mg}}{\text{ml of well mixed sample taken}} \times 1000$$

3.3.7.3 Total suspended solids (TSS)

Apparatus : Clean dry crucible, analytical balance, hot air oven, filter paper, graduated cylinder, filtration apparatus

Procedure : Taken a evaporating dish and filter paper and their initial weight was noted. Placed the filtration apparatus with the weighed filter in filter flask and suction was applied to the filtration flask and filter paper was seated with a small amount of distilled water. Poured 50 ml of well mixed sample into filtration apparatus. Sample was drawn through filter into the filter flask. Rinsed graduated cylinder into filtration apparatus with three successive 10 ml portions of distilled water and allowed complete filtration between each rinsing. Continued suction for three minutes after filtration of final rinse is completed. Filter paper was put on evaporating dish and kept the dish in an oven at 105°C for 1 hour. Removed the crucible from the oven and kept it in the dessicator for about 20-30 minutes to attain room temperature. Weighed the cooled dish along with filter paper and without filter paper. The weight of the filtered material was found by subtracting the initial weight from the final weight. Calculated

the total suspended solids in the sample using the formula :

$$\text{Total suspended solids } \left(\frac{\text{mg}}{\text{L}}\right) = \frac{\text{Weight of the filtered material in mg}}{\text{ml of well mixed sample taken}} \times 1000$$

3.4 Screening of microalgae strains on dairy wastewater and commercial media

Five stress tolerant microalgae strains viz. BGLR4, BGLR7, BGLR10, BGLR18, BGLRS and one standard strain i.e. *Spirulina* sp. NCIM 5143 were screened for growth on four different commercial media (BG11, ACM, BBM and ZM) along with different concentrations of unsterilized dairy wastewater (20%, 40%, 60%, 80% and 100%). Triplicate erlenmeyer flasks (250 ml) containing 100 ml of growth media were inoculated with microalgal culture (1%) and incubated at temperature of 28±2° C, pH of 8.50-10.00, light intensity of 4000 Lux, photoperiod of 16:8 hours for a period of 30 days. After the completion of incubation period, flasks were taken out and tested for various parameters.

The growth kinetics of these strains were studied by a non-linear logistic model. Therefore, experimental data for biomass production by these microalgae isolates in the growth media in which they showed the highest biomass production during screening experiment was fitted with a non-linear modified Logistic equation (Celekli *et al* 2009, Zwietering *et al* 1990) as given below:

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

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 data fitting in the model was done by using the MS Solver of Excel 2007.

3.4.1 Estimation of dry biomass weight

The dried biomass weight was determined by weighing dried samples of the culture suspensions according to the method described by Guruvaiah *et al* (2014). Five ml of algal culture suspension was filtered through pre-weighed filter paper kept at room temperature. The filter paper was dried overnight at 110° C and then weighed again at room temperature. Then dry biomass weight was calculated by subtracting weight of filter paper from the weight of dried filter paper.

3.4.2. Estimation of chlorophyll

Chlorophyll content was determined by the method of El-Baky *et al* (2008) with little modifications.

Principle : All chlorophylls are fat soluble molecules and possess a phytyl chain (esterified to carboxyl group of the ring and provides lipid characteristics) bound to a porphyrin ring system. These hydrophobic compounds can be extracted from living tissues (containing

water) with various organic solvents such as acetone, methanol or ethanol which can take up water from these tissues. Less polar pigments like chlorophyll a does not completely extract with 80% aqueous acetone. However, extraction with 100% acetone ensures complete extraction (Lichtenthaler 1987).

Procedure : Twenty ml of microalgal culture was centrifuged at 6,000 rpm for 10 minutes and the pellet obtained was dissolved in 20 ml of 100% acetone. The sample was kept overnight in dark at 4˚C for complete extraction. After extraction, sample was subjected to centrifugation at 10,000 rpm for 5 min. Then the supernatant obtained was used for determination of chlorophyll content spectrophotometrically at two different wavelengths i.e. 661.6 nm and 644.8 nm by using Hitachi UV-Vis Spectrophotometer U-2800. Total chlorophyll (T-Chl), chlorophyll a (Chl-a) and chlorophyll b (Chl-b)) was determined by applying standard equations (Lichtenthaler 1987) as:

$$\text{Chla} = 11.24 * \text{A661.6} - 2.04 * \text{A644.8}$$

$$\text{Chlb} = 20.13 * \text{A644.8} - 4.19 * \text{A661.6}$$

$$\text{Chl a} + \text{b} = 7.05 * \text{A661.6} + 18.09 * \text{A644.8}$$

3.4.3 Estimation of carbohydrates

The method of Dubios *et al* (1956) was used to determine carbohydrate content in the samples.

Principle: In the hot acidic medium, glucose present in sample is dehydrated to hydroxymethyl furfural which formed a green coloured product with phenol and showed absorption maxima at 490 nm.

Reagents : 1). 70% and 80% ethanol : 70 ml and 80 ml ethanol respectively to make final volume 100 ml with distilled water, 2). 5% phenol : 5g in 100 ml distilled water, 3). Concentrated sulphuric acid,

Procedure : For preparation of extract, 1 ml of sample was added to 5.00 ml of 70% ethanol and heated in boiling water bath till ethanol evaporates. Above step was repeated with 80% ethanol. Remaining extract was cooled down and total volume was made 1 ml with distilled water. From this 0.1 ml was taken and diluted to 1 ml by adding 0.9 ml of distilled water. Five ml of 5% phenol was added and vortexed properly. Then 5 ml of concentrated H_2SO_4 was added. The addition of sulphuric acid was done directly on the solution so that the contents were mixed and heated due to exothermic reaction. The test tubes were cooled under the running tap water and then absorbance was measured at 490 nm. Carbohydrate content was estimated using a standard calibration curve prepared from standard glucose solution (200 µg/ml) (as given in section 3.13, Fig.3.2). Stock solution of glucose (200 µg/ml) was prepared dissolving 0.02 g glucose in 100 ml distilled water. Stock was diluted by taking 0.1, 0.2 and 0.3 ml and adding 0.9, 0.8, 0.7 ml and so on distilled water, respectively for preparation of different dilutions. This was followed by addition of 5 ml of 5% phenol solution and 5 ml of

concentrated sulphuric acid.

3.4.4 Estimation of lipids

Principle: Unsaturated compounds reacts with sulphuric acid to form carbonium ions and vanillin reacts with phosphoric acid to form aromatic phosphate ester. Then the carbonium ion formed reacts with the activated carbonyl group of phosphovanillin and a colored complex is formed that is stabilized by resonance and shows absorption maxima at 525 nm (Knight *et al* 1972).

Reagents : 1). Concentrated sulphuric acid, 2). Phospho-vanillin reagent : 0.6 g of vanillin was dissolved in 10 ml absolute ethanol and 90 ml deionized water and stirred continuously. Then 400 ml of concentrated phosphoric acid was added to the mixture. The reagent prepared was stored in dark until use.

Procedure : The lipids were estimated by sulfo-phospho-vanillin (SPV) assay given by Mishra *et al* 2014. Initially biomass of known amount suspended in a known volume of liquid culture (0.5 ml diluted to 1ml with distilled water) was taken and 2 mL of concentrated (98%) sulfuric acid was added to it. Then the sample was heated for 10 min at 100 ˚C, and was cooled for 5 min in ice bath. After this 5 mL of freshly prepared phospho-vanillin reagent was added and the sample was incubated for 15 min at 37 °C in incubator shaker at 200 rpm. Absorbance was taken at 530 nm and lipid content was determined from the standard curve. A standard curve for lipids was prepared using commercial canola oil at a concentration of 2 mg/ml under similar conditions (as given in section 3.13, Fig.3.3).

3.4.5 Estimation of proteins

Proteins were estimated according to Lowry *et al* (1951).

Principle : 1). Formation of complex between Cu^{+2} and peptide bonds in an alkaline solution causing reduction of copper to Cu^{+} ions

Cu^{+2}+ protein = (Cu^{+2}-protein complex)

Cu^{+2} + (polar amino acids)$_{red}$ = Cu^{+} (amino acids)$_{ox}$

2). Cu^{+2} and radical group of amino acids reduce a yellow phosphomolybdate-phosphotungstate complex (Folin-Ciocalteau reagent(F-C reagent))

Na_2MoO_4+ Na_2WoO_4 +H_3PO_4 to a deep blue color

Cu^{+} + (F-C)$_{ox}$= Cu^{2+} +(F-C)$_{red}$

Reagents: 1). A: 2% Na_2CO_3 in 0.1 N, 2). B: 1% Sodium potassium Tartrate in H_2O, 3). C: 0.5% CuSO4.5 H_2O in H_2O 4). Reagent A: Mix 48 ml of A, 1 ml of B and 1 of ml C, 5). Reagent B- 1 part Folin Ciocalteu's phenol reagent: 1 part water, 6). BSA (Bovine Serum Albumin) Standard - 1 mg/ ml

Procedure : Initially, 2 ml of homogenized sample mixed with 1 ml of 1 N NaOH was heated at 100 °C for 15 min. After cooling, 0.9 ml of distilled water was added to 0.1 ml of culture. Then Lowry reagent A (5 ml) (alkaline $CuSO_4$) was added, vortexed and incubated for 10

minutes at room temperature. Then reagent B (0.5ml) was added, mixed properly and kept for 20 minutes. Blank contained only distilled water (1 ml) instead of sample solution. After 20 minutes, absorbance was taken spectrophotometrically at 520 nm using UV-Vis spectrophotometer. Bovine serum albumin was used for preparation of standard curve in the range of 20 to 100 µg/ml A (as given in section 3.13, Fig.3.4).

3.5 Optimization of culture conditions of selected microalgae strains by response surface methodology (RSM)

The interactive effect of five significant factors (independent variables) light intensity, pH, temperature, incubation period, inoculum concentration on five responses (dependent variables) viz. biomass, chlorophyll, carbohydrates, lipids, proteins were determined statistically using response surface methodology (RSM) based on central composite design. Statgraphics Centurian XVI.I. was adopted for the optimization experiment. Each independent variable was taken at a central coded value considered as zero and evaluated at three different values. Biomass, chlorophyll, carbohydrate, lipid and protein production were studied under varied pH (7.5, 9.5 and 11.5), temperature (20, 30 and 40°C), light intensity (4000, 6000 and 8000 Lux) and growth period (20, 30 and 40 days).

Optimization experiment was carried out in 250 ml erlenmeyer flasks containing 100 ml of the media at different pH levels. The flasks were sterilized by autoclaving at 120° C for 20 minutes at 15 psi pressure and inoculated with the microalgal culture (different concentrations) under aseptic conditions. These flasks were incubated at different temperatures for different incubation period (Table 4.31, 4.40, 4.47) (Plate II a, b and c). At the end their respective growth period flasks were taken out and studied for various response variables. All the experiments were carried out in duplicates and the data represent the mean. The following second order polynomial equation describes the relationship between dependent variables and independent variables

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

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.

Finally, statistical analysis of the model was carried out. Analysis of variance (ANOVA) was done involving Fischer's F test to predict the overall significance of the model, coefficient of correlation (r) and correlation coefficient (R^2) were used to analyse regression equation. The fitted polynomial equation was expressed in the form of contour plots and at the end model was also validated by using the combination of different optimized variables to produce maximum yield of the response under study.

3.6 Molecular Identification of the selected microalgal strains

Out of six microalgae strains, two strains producing maximum biomass on unsterilized dairy wastewater and commercial media were selected for identification by molecular tool methodology at Chromous Biotech Pvt. Ltd., Bangalore, India. The protocol involves the following four steps:

1. Firstly, genomic DNA was isolated from the sample.
2. High-fidelity PCR polymerase was used for the amplification of the ITS fragment (~650bp).
3. PCR product was sequenced bi-directionally.
4. The sequence data so obtained was aligned and analyzed for identification of the microalgae to its closest neighbours.

Microalgal biomass harvested at exponential phase were grinded in a pestle and mortar and treated with lysis buffer to release the genomic DNA using genomic DNA isolation kit. Specifications of amplification by PCR reaction carried out with a total reaction volume of 100 µl are as follows :

Genomic DNA - 1 µl (100 ng), ITS-Forward Primer - 400ng (5' - GRAAGNAHADGTVGKAAYAWSG - 3'), ITS-Reverse Primer - 400ng (5' **-** TCCTNCGYTKATKGVTADGH **-** 3'), dNTPs (10mM each)- 4 µl, 10X Chrom Taq DNA Polymerase Assay Buffer - 10 µl, Chrom Taq DNA Polymerase Enzyme (3U/µl)-1 µl and water- X µl. PCR amplification of the genomic DNA was carried out in following four steps : Initial denaturation of genomic DNA was carried out at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec each and annealing at 52°C for 30 secs, extension of DNA was done at 72° for 45 sec and then a final extension at 72°C for 7 min. The amplified product obtained from PCR reaction was electrophorized in 1.2% agarose gel (with ethidium bromide) to know the size of bp. PCR product was sequenced using Cycle Sequencing kit and Big Dye Terminator Version 3.1" by ABI 3500 Genetic Analyzer of Applied Biosystem Micro Amp, USA. Sequencing was done by denaturation at 96°C (5 min), followed by 25 cycles of denaturation at 96°C (30 sec), hybridization at 50°C (30 sec) and elongation at 60°C (1.30 min). The resultant nucleotide amplicons was analyzed with Seq Scape_ v 5.2 software, using BDTv3-KB-Denovo_v 5.2 protocol. Weighbor (alphabet size 4 and length size 1000) was used to build the phylogenetic tree. Distance matrix was generated by using Jukes-Cantor corrected distance model.

3.7 Biochemical characterization of selected microalgal strains

3.7.1 Estimation of total phenolic content (TPC)

Total phenolic content of crude methanolic extracts of microalgae was quantified using Folin Ciocalteu's method as described by Taga *et al* (1984).

Principle: Determination of total phenols by F-C method is based on the basic oxidation-

a)

b)

c)

Plate II a), b) and c) Optimization studies of microalgae strains incubated at different temperatures (°C) and different light intensities (LUX)

reduction mechanism. Phenolic compounds present in the sample reacts with Folin-Ciocalteu reagent to form a blue complex constituted by a phosphotungstic-phosphomolybdenum complex which can be quantified by using UV-Vis spectrophotometry where the maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds (Schofield *et al* 2001 and Gülçin *et al* 2004).

Reagents: 1). 2% Na_2CO_3=2g Na_2CO_3 in 100 ml distilled water, 2). 50% Folin Ciocalteu's phenol reagent :1 part Folin Ciocalteu's phenol reagent and 1 part water.

Procedure: Methanolic extracts (100 µL aliquot) of microalgal sample was taken mixed and 2% Na_2CO_3 (2 ml) was added and kept at room temperature for 2 min. Then, Folin Ciocalteu's phenol reagent (100 µL , 50%) was added, vortexed properly and incubated at room temperature in the dark for 30 minutes. Then the absorbance was measured at 720 nm using UV- vis spectrophotometer. The total phenolic content was calculated using gallic acid standard curve and expressed as gallic acid equivalent (GAE) per gram dry weight of extract. A standard curve of gallic acid was prepared in the range of 10 to 100 ug/ml under same conditions (as given in section 3.13, Fig.3.5).

3.7.2 Estimation of total antioxidant activity

Total antioxidant activity of microalgae was measured by the method of Prieto *et al* (1999)**.**

Principle: Sodium sulphide causes reduction of phosphomolybdic acid to phosphomolybdenum blue complex which is further oxidized by the addition of nitrite and this results in reduction in intensity of the blue colour.

Reagents: TAC reagent: Mixed 7.45 ml of sulphuric acid (0.6 M), 0.9942 g of sodium phosphate (28 mM) and 1.23596 g of ammonium molybdate in 250 ml distilled water.

Procedure: Three ml of TAC reagent (total antioxidant reagent) was dissolved in 300µl of methanolic extract of microalgae. Blank was maintained with distilled water replacing the TAC reagent. Absorbance of all the samples were taken at 695 nm using UV-Vis spectrophotometer. Total antioxidant activity is expressed as the number of equivalents of ascorbic acid. A standard curve of ascorbic acid was prepared in the range of 10 to 100 ug/ml under same conditions (as given in section 3.13, Fig.3.6).

3.7.3. Estimation of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

DPPH radical scavenging assay were done by the method of Yen and Chen (1995).

Reagents : 0.16mM DPPH methanolic solution= Dissolved 63.04 mg in 1000ml of distilled water

Principle: DPPH is source of free radicals and shows absorbance due to free radicals. When sample with antioxidant activity is added, it donates hydrogen electron and DPPH radical is neutralized. Due to neutralization reaction, DPPH changes to non-radical form and its dark purple colour is changed to yellow due to formation of reduced compound diphenylpicrylhydrazine (Gangwar *et al* 2014). The scavenging reaction between (DPPH) and

an antioxidant (HA) can be written as, DPPH-H + (A) = (DPPH) + (H-A) Reduction of DPPH to DPPH-H causes absorbance to decrease.

Procedure: Two ml of methanolic extracts was mixed with 2 ml of 0.16 mM DPPH methanolic solution. Then the mixture was vortexed for 1 minute and left to stand at room temperature for 30 minutes in dark. The absorbance of all the samples were measured at 517 nm. The scavenging effect (%) was calculated by the formula given by Duan *et al* (2006).

Scavenging effect (%) = $(1-(A_{sample}-A_{blank})/ A_{blank})\times100$

3.7.4. Estimation of total kjeldahl nitrogen

Same as in section 3.3.5

3.7.5 Evaluation of microalgae strains for phytochemical compounds

Microalgal strains (Methanolic extracts) were also screened for the presence of five phytochemicals viz. quinines, tannins, saponins, terpenoids and steroids (Widowati *et al* 2017, Tyagi 2017).

3.7.5.1. Quinones (Borntrager's test)

Principle: When quinones reacted with alkaline aqueous medium dark red colour is formed due to the conversion of quinines to derivative of quinone.

Procedure : Five mL microalgae extract was taken in a test tube and added few drops of 1 N NaOH. Red colour formed indicated the class of quinone compound.

3.7.5.2. Tannins (Brameyer's test):

Principle: Tannin is a polyphenolic compound which form a complex with $FeCl_3$ i.e. Fe (III)+ which is intense in colour.

Procedure: Taken microalgal extracts in a test tube and 2-3 drops of 1% $FeCl_3$ solution was added. Appearance of greenish-black or dark blue colour indicated positive test.

3.7.5.3. Saponins (Foam test):

Principle: Saponin molecules are made up of polycyclic aglycones (hydrophobic part) also called as sapogenin which is either steroid (C27) or a triterpenoid (C30) attached to one or more sugar side chains (hydrophilic part). The combination of these two parts attribute foaming properties to the saponins.

Procedure: Microalgal extract diluted with warm distilled water was taken in a test tube, vortexed and incubated for 10 minutes at room temperature. Samples showed positive results when the foam was formed and did not disappear until 15 min after the drops of HCl were added.

3.7.5.4. Terpenoids and Steroids (Lieberman-Burchard test):

Principle: Due to the reaction of hydroxyl group (-OH) of cholesterol (steroids and triterpenoids) with reagents, conjugation of the unsaturation in the adjacent fused ring increases resulting in color formation.

Procedure: Methanolic extract was added with three drops of acetic anhydride and one drop

of sulfuric acid (H_2SO_4). The red color indicated terpenoids, while blue color indicated steroids.

3.7.6 Inductively coupled plasma-atomic emission spectroscopy of microalgal biomass (ICP-AES)

The mineral components (As, B, Ca, Cd, Cr, Cu, Fe, 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). Processing of the sample for analysis was done using wet digestion method of Hseu (2004) with little modifications. Dried algal biomass (0.5g) was first mixed with diacid HNO_3:$HClO_4$ (3:1) and kept overnight. Thereafter, samples were digested at hot plate at 150°C. Initially red fumes were formed then white fumes revolved in flasks. The tubes were cooled and total volume was made 50 ml with distilled water. The solution was then filtered through whatmann filter paper and kept in refrigerator. The mineralogical analysis was performed through inductively coupled plasma-atomic emission spectroscopy (ICP-AES).

3.7.7 GC-MS (Gas Chromatography coupled with Mass Spectroscopy) analysis of selected microalgal strains

Microalgae strains were analyzed by GC-MS at Central Instrument Laboratory/ Sophisticated Analytical Instrumentation Facility (CIL/SAIF) at Panjab University Chandigarh, India. Samples were processed initially at Biogas Laboratory, Department of Renewable Energy Engineering, Punjab Agricultural University, Ludhiana using method of Krishnakumar *et al* (2013) with little modifications. Extraction was performed by using 80% methanol as solvent. One gram of dried powder of microalgal biomass was extracted with 50 ml of 80% methanol with continuous stirring of 50 rpm for 7 days at room temperature. Then the samples were filtered through Whatmann No. 1 filter paper and filtrate was dried at 40° C for 24 hours. Then the samples were dissolved in the 80% methanol in the ratio of 1:10 and stored in a refrigerator till use. The extracts were analyzed using gas chromatography-mass spectrometry (GC-MS) (Thermo Trace 1300 GC coupled with Thermo TSQ 800 Triple Quadrupole MS) equipped with a splitless injector. A BP 5MS column of dimensions 30m ×0.25mm×0.25µm was used with helium as a carrier gas. The oven temperature of the column was initially programmed at 50 °C for 4.0 min. The temperature was then programmed from 50 to 250°C at the rate of 5°C/min for 1.0 minute, then programmed from 250 to 280°C (rate=15°C/ min) and held at 280°C for 18 min and injector temperature was 260°C. The flow rate of the helium carrier was 1.0 mL/ min. 1.0 µL of sample was injected and mass spectra were collected over the range of m/z 40-650. National Institute of Standards and Technology (NIST) library (2.0) was used for identification of the each component by comparison of mass spectra of different compounds.

3.8 Proximate and chemical composition of selected microalgal strains

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.

3.8.1 Determination of Total Solids (TS)

Two grams of sample was weighed in a pre-weighed and oven dried silica crucible and the crucible was kept in hot air oven at 100°C till a constant weight was achieved. Then crucible containing sample was taken out, cooled in a desiccator and weighed again. TS of the sample was determined as follow:

$$TS\% = \frac{W2-W0}{W1-W0} \times 100$$

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

3.8.2 Determination of ash

Oven dried sample (1g) kept in a crucible was ignited in a muffle furnace at temperature of 600°C for a period of 3 hours or until the sample became carbon-free. After removing from furnace, crucible containing sample (still hot) was kept in a hot air oven at 100°C for 1 h and then the crucible was placed in a desiccator for cooling and weight was taken. Ash was calculated by the formula given below:

$$Ash\% = \frac{W3-W0}{W2-W0} \times 100$$

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

3.8.3 Determination of Volatile Solids (VS)

Volatile solids (%) can be calculated by subtracting the ash content from 100.

VS% = 100-Ash%

3.8.4 Determination of hemi-cellulose

Neutral Detergent Fibre (NDF) and Acid Detergent Fibre (ADF) was first determined and then hemicelluloses content was calculated.

3.8.4.1 Determination of Neutral Detergent Fibre (NDF)

Taken 0.5 g of dried and finely grinded test sample and 50 ml of neutral detergent solution (Annexure I) in a spoutless beaker and kept 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. Sample was washed with hot distilled water repeatedly until the foam stops coming. After that vacuum was removed , residue was washed twice with acetone and the crucible containing residue was kept overnight in a hot air oven at 100°C. Next day, after cooling the crucible in a dessicator, weight was taken and neutral detergent fibre (NDF) of the sample was calculated by the formula given below.

$$NDF\% = \frac{W1-W0}{S} \times 100$$

where

W_0 = Weight of oven dried crucible

S = Initial weight of sample

W_1 = Weight of oven dried sample and crucible

3.8.4.2 Determination of Acid Detergent Fibre (ADF)

Taken 0.5 g of dried and finely grinded test sample and 50 ml of acid detergent solution (Annexure I) in a spoutless beaker and kept 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. Sample was washed with hot distilled water repeatedly until the foam stops coming. After that vacuum was removed, residue was washed twice with acetone and the crucible containing residue was kept overnight in a hot air oven at 100°C. Next day, after cooling the crucible in a dessicator, weight was taken and neutral detergent fibre (ADF) of the sample was calculated by the formula given below.

$$ADF\% = \frac{W1-W0}{S} \times 100$$

where

W_0 = Weight of oven dried crucible

S = Initial weight of sample

W_1 = Weight of oven dried sample and crucible

3.8.4.3 Calculation of hemi-cellulose

Hemi-cellulose was calculated by subtracting ADF from NDF

NDF = cellulose+hemi-cellulose+lignin+silica

ADF = cellulose+lignin+silica

Hemi-cellulose% = NDF% - ADF%

3.8.5 Determination of cellulose, lignin and silica

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

3.8.5.1 Determination of Acid Detergent Lignin (ADL)

The residue obtained from section 3.8.4.2 (acid detergent fibre (ADF) residue) was covered with 10ml of cold solution of 72% H_2SO_4 (w/w) (Annexure I). The mixture was stirred continuously and lumps were broken with a glass rod. As the acid drains, the crucible was refilled with 72% H_2SO_4 and stirred. The process was continued for three hours. After that, suction was applied and contents inside the crucibele were washed with hot distilled till the washings were acid free to pH paper. Then the crucible was kept in a hot air oven at 100°C. The samples were then dried, cooled in a dessicator and weight was taken.

3.8.5.2 Calculation of cellulose

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

$$\text{Cellulose}\% = \frac{W1-W2}{S}\times 100$$

where

S = Initial weight of sample

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

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

3.8.5.3 Determination of lignin

The crucible containing acid (72% H_2SO_4) treated sample (from Section 3.8.5.1) was ignited in a muffle furnace at temperature of 600°C for a period of 3 hours or until the sample became carbon-free. After removing from furnace, crucible containing sample (still hot) was kept in a hot air oven at 100°C for 1 h and then the crucible was placed in a desiccator for cooling and weight was taken.

$$\text{Lignin}\% = \frac{W3-W2}{S}\times 100$$

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

3.8.5.4 Determination of silica

The ignited sample thus obtained from above was then taken and three to four drops of hydrobromic acid were added to it and kept for 30 minutes. Then the crucible was washed with distilled water 2-3 times, dried in oven at 100°C, cooled and weighed.

$$\text{Silica}\% = \frac{W4-W0}{S}\times 100$$

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.

Plate III Biogas production from microalgae and paddy straw at lab scale

3.9 Assessment of biogas production potential of selected microalgae strains at lab scale

The biogas production potential of selected microalgal biomass was done as under (Plate III):

3.9.1 Biogas production potential of three selected microalgal strains

This experimental set up consisted of anaerobic bioreactor of 2 litre capacity made up of glass, a gas collecting chamber and a liquid collecting chamber. The anaerobic digester was sealed with rubber cork and araldite as adhesive. The bioreactor worked in a batch mode without stirring. The working volume was kept up to 1800 mL. The bio-digested slurry (inoculum) taken from an actively running cattle dung based biogas plant was transferred to experimental glass bottles. All the digesters were kept under stationary conditions and the volume of biogas produced was measured after every 24 h for 4-5 months by water displacement method. A total of three digesters (in duplicate) labelled as A, C and D for each of the three strains of microalgae (BGLRS, BGLR18, *Spirulina* sp. NCIM 5143) were established and the biogas production studies were done. A control consisting of microalgal biomass was run simultaneously.

Digester A: 1500 ml microalgae (biomass only)

Digester C: 1500 ml microalgae (biomass only) + 10% biodigested slurry + 20% cow dung

Digester D: 1500 ml microalgae (Extract water only) + 10% biodigested slurry+ 20% cow dung

3.9.2 Co-digestion of microalgal biomass with paddy straw for biogas production

Anaerobic digestion of microalgae alongwith paddy straw was carried out for the purpose of evaluating the effect of microalgae on anaerobic digestion of paddy straw for biogas production. Microalgae was grown in the Biogas Laboratory, Department of Renewable Energy Engineering, PAU, Ludhiana. The experimental set up consisted of anaerobic bioreactor of 2 Litre capacity glass bottles comprising of a digester (glass digestor), a gas collecting chamber and a liquid collecting chamber. The anaerobic digester was sealed with rubber cork and araldite as adhesive. The bioreactor worked in a batch mode without stirring. The working volume was kept up to 1800 mL. A total of three digesters (in duplicate) labelled as shown below for each of the three strains of microalgae (BGLRS, BGLR18, *Spirulina* sp. NCIM 5143) were established and the biogas production studies were done.

Following sets of experiments were conducted for biogas production:

Digester B : 250g paddy straw (soaked)

Digester E: 1500 ml microalgae (biomass only) + 10% bio-digested slurry + 20% cow dung + 250g paddy straw

Digester F: 1500 ml microalgae (Extract water only)+10% cow dung slurry+ 20% cow dung+250g paddy straw

3.10 Assessment of biogas production potential of BGLRS at field level

3.10.1 Mass cultivation of BGLRS strain in outdoor open algal raceway pond

3.10.1.1 Preparation of inoculum for outdoor open algal raceway pond

Microalgae strain BGLRS producing maximum biomass was used for mass cultivation in an open raceway pond. The inoculum of BGLRS to be used for the outdoor mass cultivation was prepared under laboratory conditions. BGLRS was grown in Algal culture medium (ACM) at 28±2°C in glass ponds illuminated with LED light source (Philips) at an intensity of 4000 lux in a 16:8 hours light dark regime ((Plate IV a and b).

3.10.1.2 Outdoor cultivation of microalgae in open raceway pond

Pilot scale evaluation of BGLRS strain was carried out at the demonstration area of Department of Renewable Energy Engineering, Punjab Agricultural University, Ludhiana. An outdoor algal raceway pond used for cultivation of microalgae was designed and fabricated from Ghaziabad. The pond was double walled made of fibre sheet with the wall width of 7.62 cm. The inner dimensions of the pond were such that the length was 152.4 cm. One large paddle wheel was installed within the raceway pond for mixing and were driven by speed adjustable motors. The paddle wheel was turned on in day time and turned off in night. The speed of the paddle wheel (revolutions per minute) was controlled through a knob. The pond also had the provision for the injection of CO_2 through a microporous polymer tube (gas diffuser) placed at the side of the pond. A temperature adjustment knob was also provided. A heater was also placed inside the pond for attaining the desired temperature during winters. pH was checked manually by pH meter. The working culture depth was (light path) was 2.032 cm providing an effective culture volume of 150 L. The depth of the pond was kept at 2.54 cm keeping in mind the penetration of sufficient light for microalgal growth. A partition wall was also constructed in the middle of the pond. Illumination was provided by natural light for 12 hour and shading was done by covering the pond with green sheet. The pond was filled with 150 L of simple tap water and initially pH was adjusted with carbonate-bicarbonate buffer @ 2.06 g Na_2CO_3 and 16.8g $NaHCO_3$ per litre and only three chemicals viz. $NaNO_3$ (1.00 g/L), K_2HPO_4 (0.500 g/L) and $MgSO_4.7H_2O$ (0.513 g/L) were added. The pond was inoculated in the mid-July. Once the pH was set at 11 (optimum pH for BGLRS as determined from RSM) then inoculum @ 10% per litre was added (Plate V).

3.10.2 Co-digestion of paddy straw with BGLRS biomass for biogas production

Co-digestion of paddy straw with microalgae BGLRS was carried out at farm of Department of Renewable Energy Engineering, Punjab Agricultural University, Ludhiana. The process of anaerobic digestion was carried out at field scale in the digesters of 1 m^3 capacity made up of fibre reinforced plastic (FRP). The digester has the dimensions of 1460 mm × 1040 mm and was double walled. Two separate systems were provided inside the digester for temperature and uniform humidity maintenance. A heater was provided at the

bottom of the digestor to heat up the water which circulates between two walls for the purpose of maintaining the temperature. The water mist was sprayed inside by ultrasonic nebulizer through ultrasonic waves for maintaining humidity. To prevent the leakage of the gas produced, the digester lid was kept in a water seal. The volume of biogas formed was measured by the gas meter (m^3/h) automatically attached at the side of the digester (Plate VI). After three months of growth, algal suspension obtained was evaluated for biogas production by co-digestion with paddy straw at field scale. The microalgal biomass was harvested by filtrating though a clean muslin cloth. Paddy straw was soaked in water overnight. The feedstock added to the digester comprised of 50 kg paddy straw, 10% biodigested slurry (5 kg) (act as inoculum) and 40% cow dung (20 kg) and 6.5 Kg microalgal biomass. Mixing of lignocellulosic biomass like paddy straw with microalgae biomass maintains the required C/N ratio for anaerobic digestion process (25 to 30).

3.10.3 Proximate and chemical composition of initial and final feedstock fed into the digesters

The feedstock fed initially into the digesters and after processing period of 128 days for the biogas production at lab and field scale were evaluated for their proximate and chemical composition according to the standard methods of AOAC (2000). Percent reduction in volatile solids reduction (VSR%) was calculated by equation given below:

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

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

3.10.4 Computation of daily and cumulative biogas yields

The kinetics of biogas produced was studied by modified Gompertz equation. Biogas production from different digesters was noted daily and calculation of cumulative biogas production (mL biogas g^{-1} VS) was done. The cumulative biogas data thus obtained was then fitted with the Gompertz equation for calculating various parameters P (the enhancement in ultimate biogas yield), R_m (maximum rate of biogas production) and λ (lag phase).The Gompertz equation was adopted from Prajapati *et al* (2015) is given as

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

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

Experimental data was fitted in the model using MS Solver of Excel 2007.

3.11 Statistical analysis

All experimentation were completed in triplicates. Values superscripted by different alphabets in tables represent the significant difference between the values based on Tukey's HSD Multiple Range test using IBM SPSS Statistics 22. Critical difference (C.D.) (0.05%) was calculated using software CPCS1.

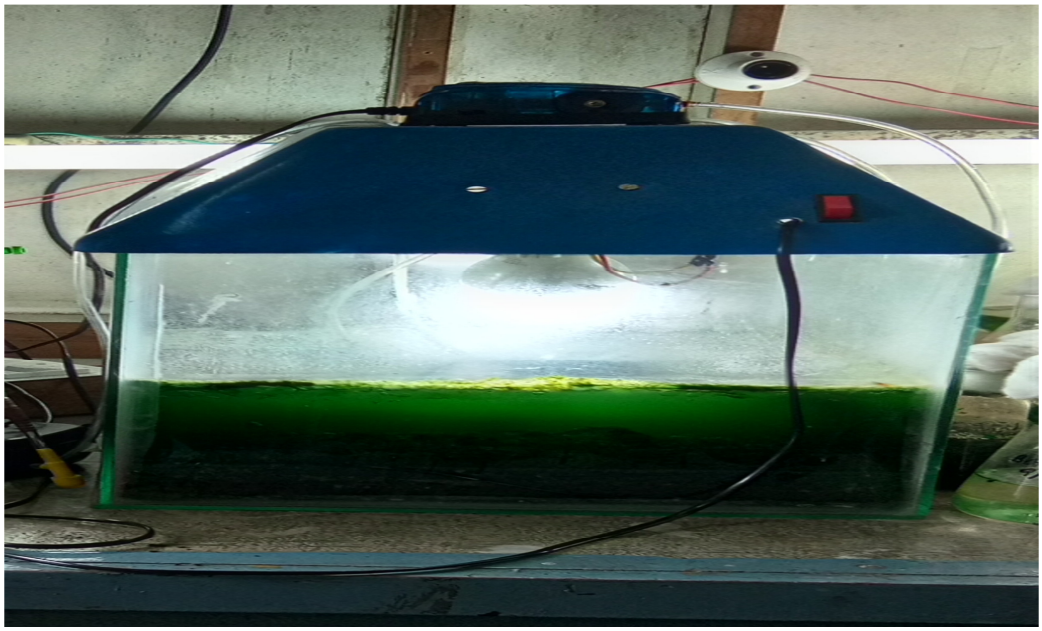
3.12 Media composition

Algae culture medium

	Ingredients	Chemical Name	Composition (g/L)
1.	$NaNO_3$	Sodium nitrate	1.000
2.	K_2HPO_4	Di-potassium hydrogen phosphate	0.500
3.	$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate	0.513
4.	NH_4Cl	Ammonium chloride	0.050
5.	$CaCl_2.2H_2O$	Calcium chloride dehydrate	0.058
6.	$FeCl_3$	Ferric chloride	0.003
7.	$NaHCO_3$	Sodium hydrogen carbonate	16.800
	Final pH (at 25°C)		7.0±0.2

BG-11 Medium

Stocks	Components	Chemical name	Concentration (g/l)
1	$NaNO_3$	Sodium nitrate	15.0
			Concentration g/ 500 ml
2	K_2HPO_4	Di-potassium hydrogen phosphate	2.0
3	$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate	3.75
4	$CaCl_2.2H_2O$	Calcium chloride Dehydrate	1.80
5	$C_6H_8O_7$	Citric acid	0.30
6	$(NH_4)_5[Fe(C_6H_4O_7)_2]$	Ammonium ferric citrate green	0.30
7	$EDTANa_2$	Ethylenediaminetetraacetic acid disodium salt	0.05
8	Na_2CO_3	Sodium carbonate	1.00
9	**Trace metal solution**		**Concentration g/ litre**
	H_3BO_3	Boric acid	2.86
	$MnCl_2.4H_2O$	Manganese chloride tetrahydrate	1.81
	$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate	0.22
	$Na_2MoO_4.2H2O$	Sodium molybdate dihydrate	0.39
	$CuSO_4.5H_2O$	Copper sulphate pentahydrate	0.08
	$Co(NO_3)_2.6H_2O$	Cobalt nitrate hexahydrate	0.05
	Medium		**per litre**
	Stock solution 1		100.0 ml
	Stock solutions 2-8		10.0 ml each
	Stock solution 9		1.0 ml



a)

b)

Plate IV a) and b) Cultivation of microalgae in glass ponds for inoculum preparation

Plate V Mass cultivation of BGLRS BGLRS (*Scenedesmus* sp. *MKB.*) **in outdoor algal raceway pond**

Plate VI Biogas production from co-digestion of BGLRS (*Scenedesmus* sp. *MKB.*) **and paddy straw at field scale.**

Total volume was made 1 litre with deionized water and pH was adjusted to 7.1 with 1M NaOH or HCl. For solid medium agar was added @ 15.0 g per litre. Medium was sterilized by autoclaving at 15 psi for 15 minutes.

Bold's Basal Medium (BBM)

Stocks	**Components**	**Chemical name**	**Concentration (g/100ml)**
1	$NaNO_3$	Sodium nitrate	2.5
2	$CaCl_2.2H_2O$	Calcium chloride dihydrate	0.25
3	$MgSO_4.7H_2O$	Magnesium sulphate Heptahydrate	0.75
4	K_2HPO_4	Di-potassium hydrogen phosphate	0.75
5	KH_2PO_4	Potassium dihydrogen phosphate	1.75
6	NaCl	Sodium chloride	0.25
7	EDTA-KOH Solution		
	EDTA. Na_2	Ethylenediaminetetraacetic acid disodium salt	5.00
	KOH	Potassium hydroxide	3.10
8	Ferric solution		
	$FeSO_4.7H_2O$	Ferrous sulphate heptahydrate	0.498
	H_2SO_4 (Conc.)	Sulphuric acid	0.100
9	H_3BO_3	Boric acid	1.142
10	Trace elements solution		
	$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate	0.882
	$MnCl_2.4H_2O$	Manganese chloride tetrahydrate	0.144
	MoO_3	Molybdenum trioxide	0.071
	$CuSO_4.5H_2O$	Copper sulphate pentahydrate	0.157
	$Co(NO_3).6H_2O$	Cobalt nitrate hexahydrate	0.049

All the stocks were stored in the refrigerator. Ten ml each of stocks 1 to 6 and one ml of stocks 7 to 10 were added to distilled water to make final volume of 1 litre was autoclaved at 121°C (15 psi for 15 min)

Zarrouk's media

Stocks	**Components**	**Chemical name**	**Concentrations (g/L)**
1.	$NaNO_3$	Sodium nitrate	2.50
2.	K_2HPO_4	Di-potassium hydrogen phosphate	0.50
3.	K_2SO_4	Potassium sulphate	1.00
4.	NaCl	Sodium chloride	1.00
5.	$MgSO_4$.7H2O	Magnesium sulphate heptahydrate	0.20
6.	$CaCl_2$.2H2O	Calcium chloride dihydrate	0.04 ml
7.	EDTA	Ethylenediaminetetraacetic acid	0.08
8.	$NaHCO_3$	Sodium hydrogen carbonate	16.80
9.	Micronutrient solution		1.0 ml
	Micronutrient		**Concentration (g/l)**
1.	H_3BO_3	Boric acid	2.86
2.	$MnCl_2.4H_2O$	Manganese chloride	1.81
3.	$ZnSO_4$	Zinc sulphate	0.22
4.	$Na_2MoO_4.2H_2O$	Sodium molybdate dihydrate	0.017
5.	$CuSO_4.5H_2O$	Copper sulphate pentahydrate	0.07
6.	$Co(NO_3)_2.6H_2O$	Cobalt nitrate hexahydrate	0.05

3.13 Standard curves

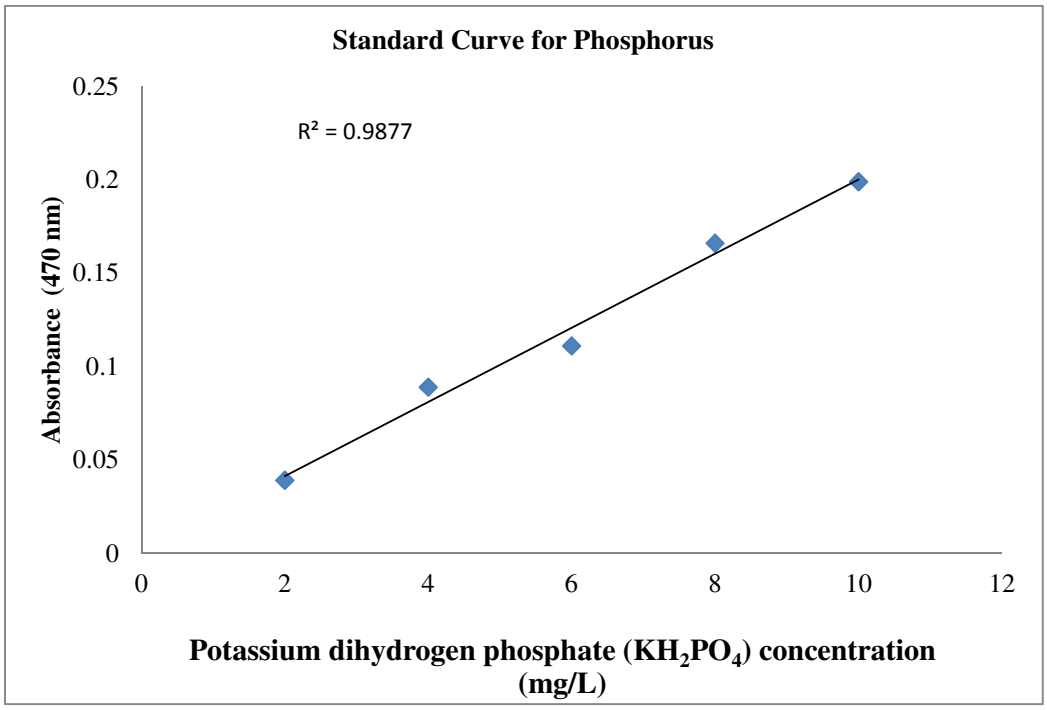


Fig 3.1 Standard curve for phosphorus estimation

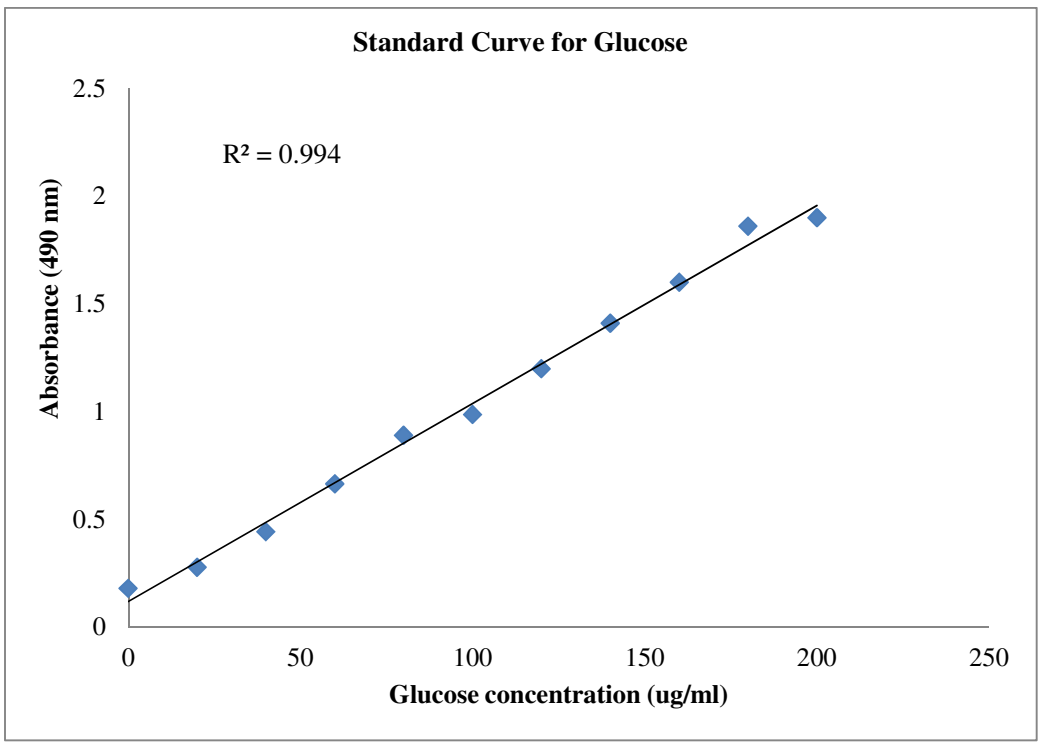


Fig 3.2 Standard curve for carbohydrate estimation

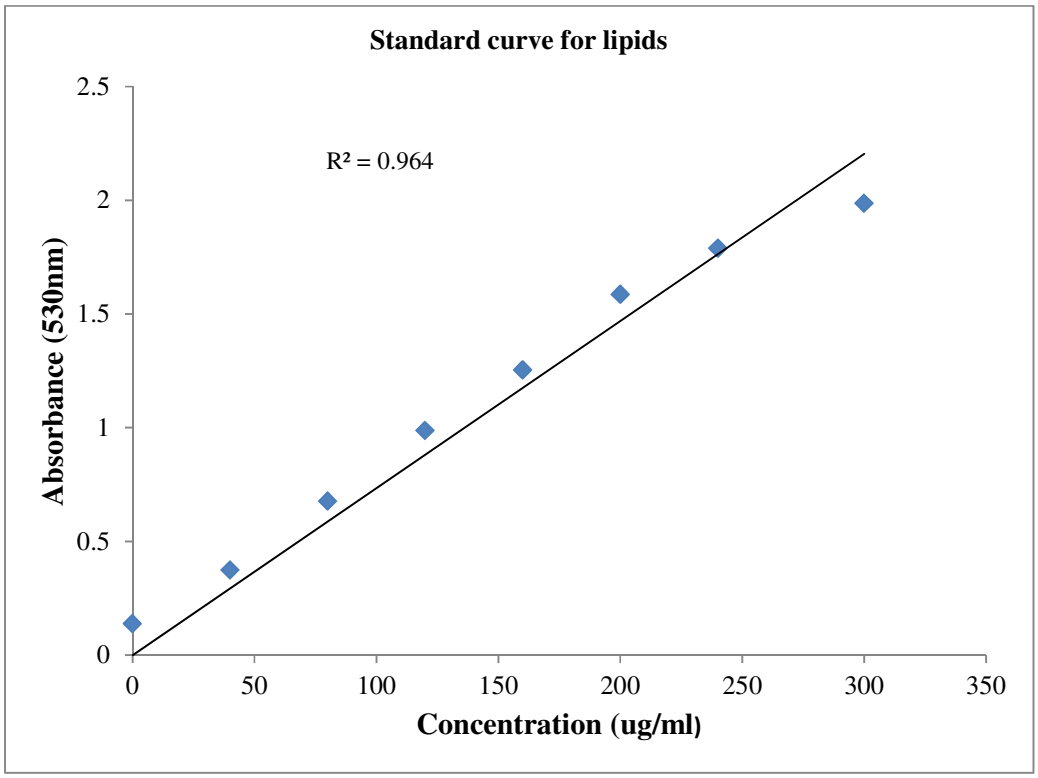


Fig 3.3 Standard curve for Lipid estimation

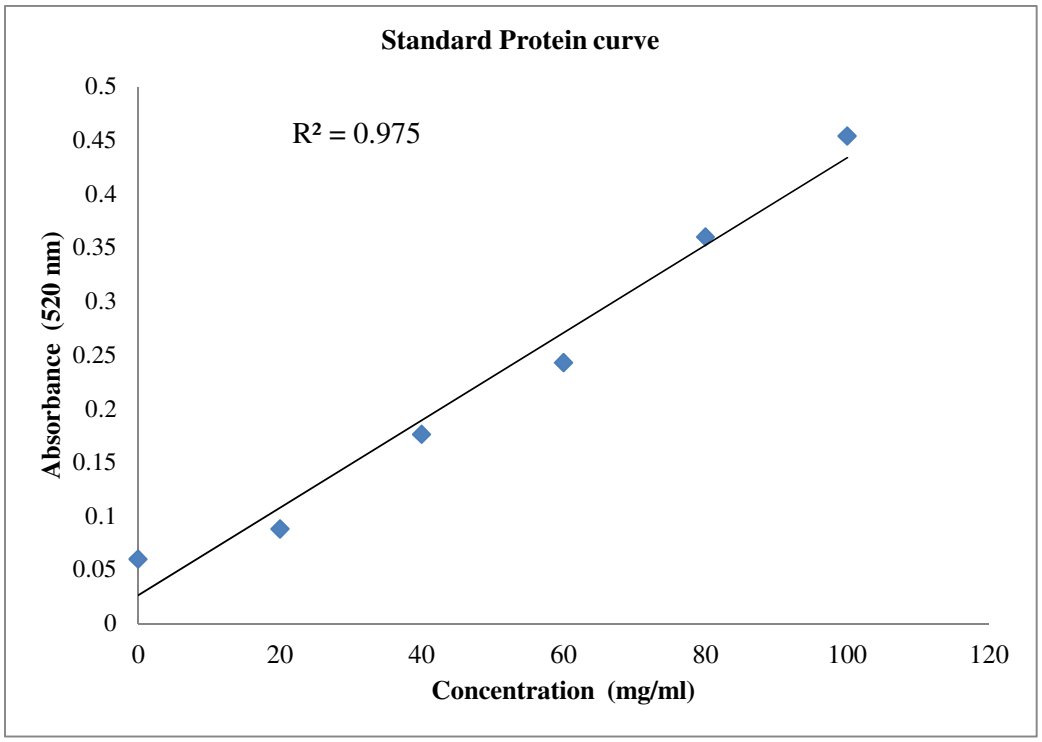


Fig 3.4 Standard curve for protein estimation

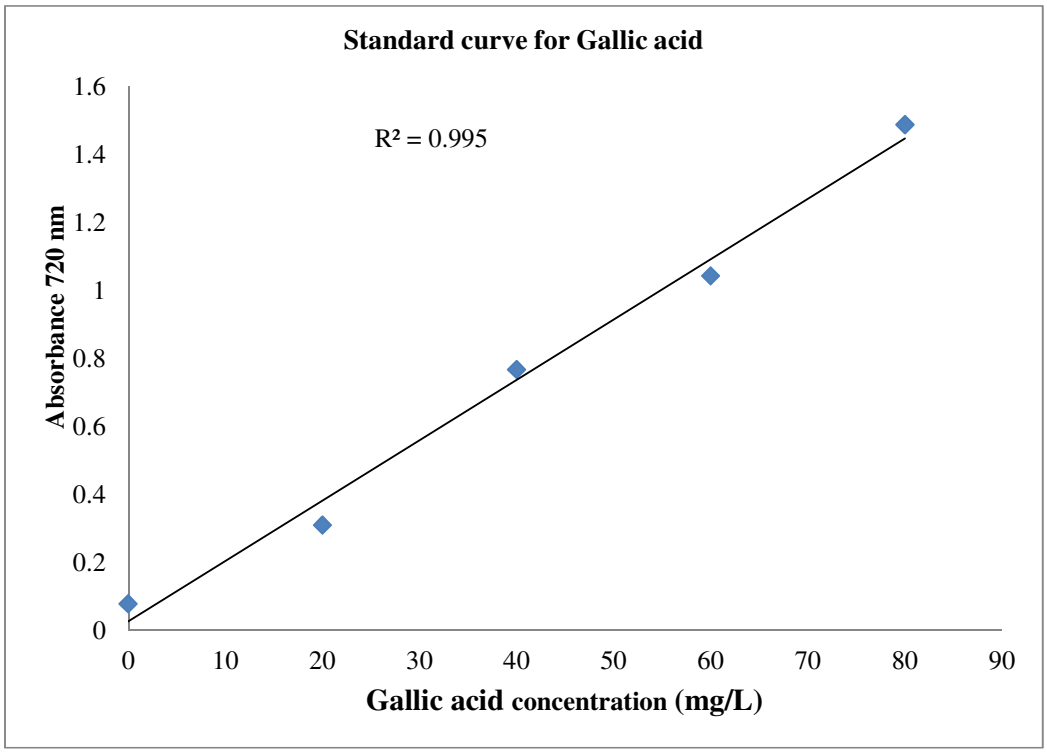


Fig 3.5 Standard curve for Gallic acid estimation

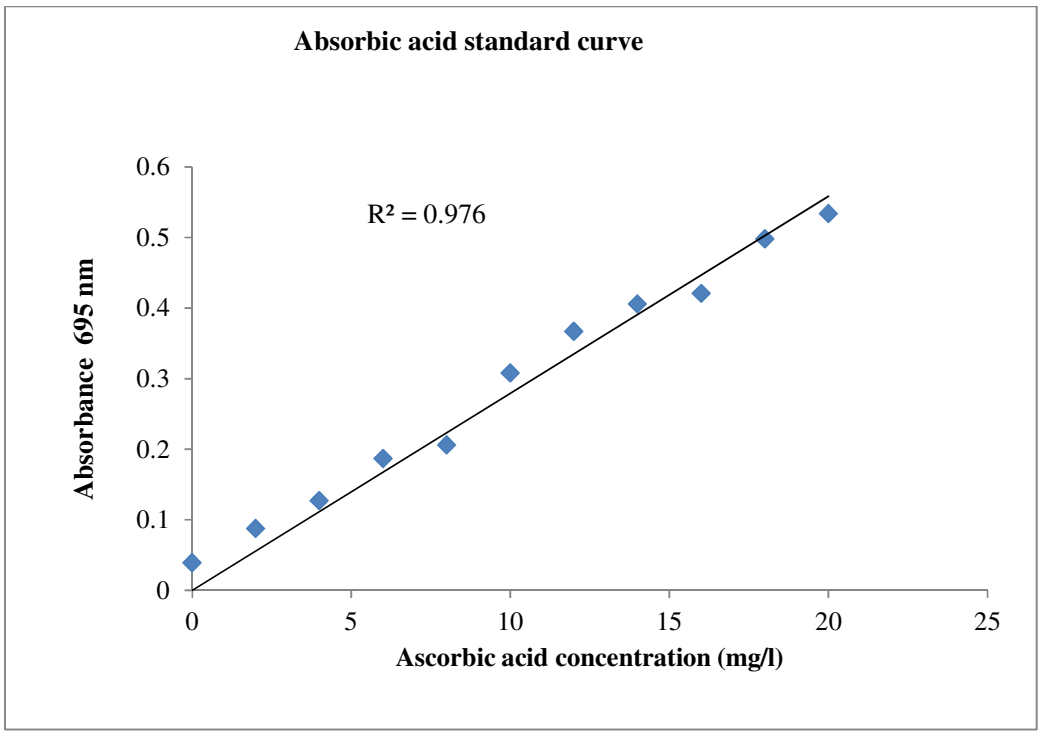


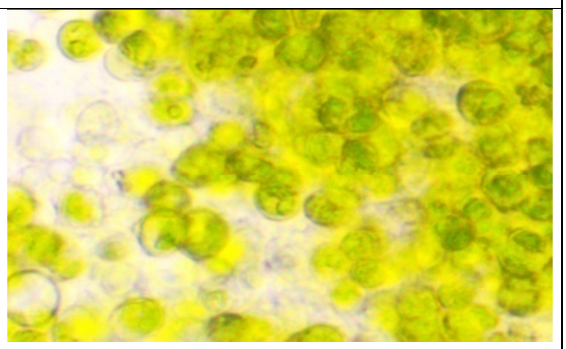
Fig 3.6 Standard curve for ascorbic acid

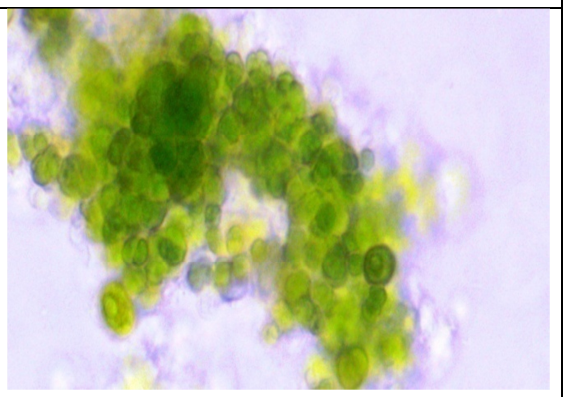
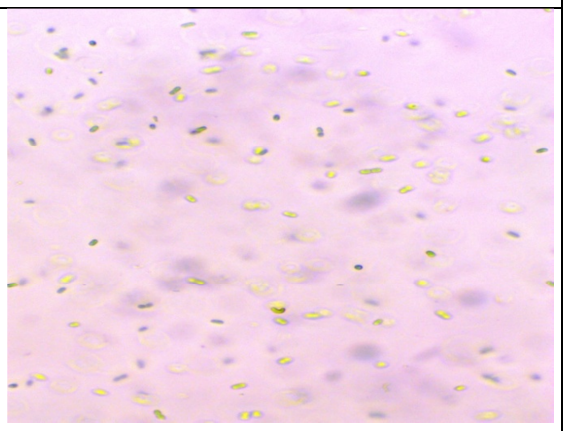
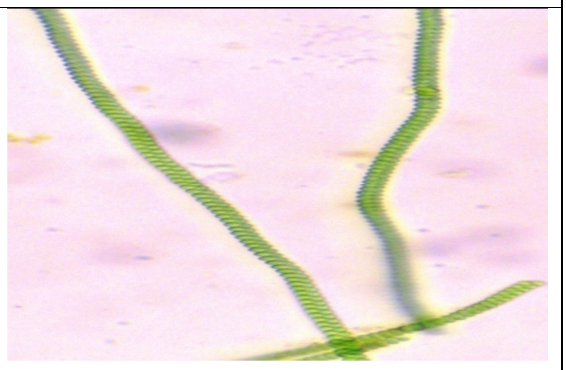
CHAPTER IV

RESULTS AND DISCUSSION

A total of six strains, five stress tolerant microalgal strains viz. BGLR4, BGLR7, BGLR10, BGLR18, BGLRS (Dar 2017) already available at Biogas laboratory, Department of Renewable Energy Engineering, College of Agricultural Engineering and Technology, PAU, Ludhiana and one standard strain *Spirulina* sp. NCIM 5143 procured from National Collection of Industrial Microorganisms (NCIM) laboratory, Pune, India were used in the present study as per Table 4.1. These strains were observed under microscope (40×) using the Olympus 528293 microscope (Magnus Icon Freedom Model) with the Debro 5.1 Megapixel digital camera and the Toup view software program. Strains BGLR4, BGLR10, BGLRS and BGLR18 showed spherical to oval cells while BGLR7 and *Spirulina* sp. NCIM 5143 showed filamentous and large cells.

Table 4.1 Microalgal strains used in the present study

Microalgal strains	**Cell shape and size**	**Other morphological characteristics**	***Cell morphology**
BGLR4	Spherical and medium cells	Unicellular cells, occur singly or in aggregates, pyrenoid is present in each cell	
BGLR7	Filamentous and large cells	Uniform trichomes, heterocysts generally intercalary which are larger than vegetative cells	

BGLR10	Spherical and small cells	Cells have visible transparent sheath	
BGLR18	Oval and small cells	Unicellular cells which occur singly or in aggregates	
BGLRS	Spherical or slightly oblonged and small cells	Coenobia of two or four cells inside the parental mother cells	
Spirulina sp. NCIM 5143	Filamentous and large cells	Long unicellular filamentous cells that grow in the form of a tight coiled right or left handed helix	

* (40×) using the Olympus 528293 microscope (Magnus Icon Freedom Model) with the Debro 5.1 Megapixel digital camera and the Top view software program.

4.1.1 Growth profile of stress tolerant microalgae strains on various media and dairy wastewater

Six microalgal strains viz. BGLR4, BGLR7, BGLR10, BGLR18, BGLRS and one standard strain *Spirulina* sp. NCIM 5143 were evaluated for growth profile on four different media viz. Blue green -11 (BG-11), Bold's basal medium (BBM), Algal culture medium (ACM), Zarrouk's medium (ZM) as well as on different concentrations of unsterilized dairy wastewater (20%, 40%. 60%, 80% and 100%) (Plate VII a, b and c). Growth was evaluated mainly in terms of optical density (O.D. at 750 nm) at every 3rd day, chlorophyll content (mg/l) at every 5th day and dry biomass weight (g/l) produced after 30 days. In case of commercial media, different strains showed difference in growth profile due to diverse nutrient environment. However, in case of dairy wastewater, all the microalgae strains survived at different concentrations of dairy wastewater but differing in their growth rate. For each strain, absorbance first increase with increase in concentration from 20 to 60% then decreased or became nearly constant from 80 to 100% except *Spirulina* sp. NCIM 5143 in which growth increased with increase in concentration from 20 to 100%. Measurement of optical density is a common and indirect means of microalgal biomass determination (Duygu *et al* 2018). Results from table 4.2 showed that strain BGLR4 showed highest absorbance (0.06) on BG-11 medium followed by ACM and BBM (0.05) and ZM (0.04). However, highest absorbance (1.47) in dairy wastewater was found at 60% concentration followed by 40% (1.43). Similarly, BGLR7 showed highest absorbance (0.25) on BG-11 and lowest (0.13) on ZM. Also, highest absorbance (2.32) in dairy wastewater was shown at 60% concentration (Table 4.3). Strain BGLR10 showed highest absorbance (0.36) on BG-11 medium and on 60% dairy wastewater (1.36) (Table 4.4). Highest absorbance in BBM (0.88) was shown by strain BGLR18 followed by BG-11 (0.50), ACM (0.25) and ZM (0.19) and in dairy wastewater, 60% is the most preferred concentration showing absorbance of 2.59 followed by 40%, 20%, 80% and 100% which showed absorbance values of 2.55, 2.27, 2.14 and 1.78 respectively (Table 4.5). Highest value of absorbance for strain BGLRS for commercial media and dairy wastewater was recorded in ACM (0.31) medium and on 60% concentration of dairy wastewater (2.53) (Table 4.6). For standard strain *Spirulina* sp. NCIM 5143, highest absorbance (0.21) was recorded on ZM followed by BBM, BG-11, ACM which showed absorbance of 0.20, 0.19, 0.18 respectively and for dairy wastewater 100% concentration showed highest absorbance (1.77) (Table 4.7). Like other micro-organisms growth cycle of microalgae is divided into four stages : lag phase, exponential phase, stationary phase and lysis or death phase (Feng *et al* 2011). The curves represent the absorbance values taken for 30 days in different media (Fig 4.1 a-f) showed the lag phase of very short duration in all the strains in various media. All the strains have a long exponential phase and continued to remain in this phase till the end of experimental growth period i.e. 30 days. No

a)

b)

c)

Plate VII a) and b) Microalgae grown on different concentrations of dairy wastewater under artificial LED light system, temperature =28±2□C at lab scale

stationary or death phase was detected in case of commercial media. This may be due to the reason that long time is required for decay phase to commence (Kodihalli *et al* 2018) but in present study cultivation time was only 30 days. For dairy wastewater, all the microalgae strains grown on dairy wastewater showed a small or no prominent lag phase. However, exponential phase was lasted upto 21st day in all microalgae strains except *Spirulina* sp. NCIM 5143 where it continued for 30 days. In strains BGLR4, BGLR7, BGLR10, BGLR18, BGLRS exponential growth phase was followed by stationary phase whereby nearly constant absorbance value was observed after 21st day. In all the strains death or decline phase was not evident till the end of the experimental growth period i.e. 30 days.

Similarly, chlorophyll profile of all the six strains showed the same trend as in case of absorbance. For BGLR4, maximum value of chlorophyll (1.77 mg L^{-1}) was found on BG-11 medium and on 60% dairy wastewater (2.06 mg L^{-1}) (Table 4.8) (Fig 4.2 a). Similarly, BGLR7 showed maximum value of chlorophyll (1.48 mg L^{-1}) on BG-11 followed by ACM (1.30 mg L^{-1}), BBM (1.18 mg L^{-1}) and ZM (1.03 mg L^{-1}). For dairy wastewater, maximum chlorophyll (2.63 mg L^{-1}) value was found in 60% concentration followed by 40% (2.48 mg L^{-1}), 20% (2.26 mg L^{-1}), 80% (1.47 mg L^{-1}) and 100% (1.31 mg L^{-1}) (Table 4.9) (Fig 4.2 b). Also, strain BGLR10 showed maximum chlorophyll (1.83 mg L^{-1}) on BG-11 and on 60% concentration of dairy wastewater (2.56 mg L^{-1}) (Table 4.10) (Fig 4.2 c). Out of the four media studied, BGLR18 showed maximum chlorophyll content of 2.78 mg L^{-1} in BBM and 4.69 mg L^{-1} in 60% dairy wastewater (Table 4.11) (Fig 4.2 d). Maximum chlorophyll (2.19 mg L^{-1}) for BGLRS was in ACM medium and in 60% dairy wastewater (3.58 mg L^{-1}) (Table 4.12) (Fig 4.2 e). However, standard strain *Spirulina* sp. NCIM 5143 recorded highest chlorophyll content (2.00 mg L^{-1}) on ZM and on 100% concentration of dairy wastewater (4.59 mg L^{-1}) (Table 4.13) (Fig 4.2 f).

Strains BGLRS (0.63 g L^{-1}), BGLR18 (0.44 g L^{-1}), *Spirulina* sp. NCIM 5143 (0.28 g L^{-1}) showed maximum biomass on ACM, BBM and ZM respectively while BGLR7, BGLR4 and BGLR10 produced maximum biomass 0.15 , 0.12 and 0.04 g L^{-1} respectively on BG-11. Strains BGLR4, BGLR7, BGLR10 , BGLR18, BGLRS showed maximum biomass values of 0.14, 0.09, 0.09, 0.21, 0.19 g L^{-1} on the combination of 60% dairy wastewater and 40% respective media while for *Spirulina* sp. NCIM 5143, highest biomass (0.15 g L^{-1}) was recorded on 100% dairy wastewater concentration (Table 4.14-4.19).

This showed that most favorable medium for strains BGLR4, BGLR7, BGLR10 was BG-11 and for BGLR18, BGLRS and *Spirulina* sp. NCIM 5143 was BBM, ACM and ZM. Our studies are in corroboration with those of Marimuthu and Jayaraman (2018) who showed that optimal growth medium for growth of *Chlorosarcinopsis eremi* is Bold's basal medium (BBM). Pandey *et al* (2010) reported that most favorable media for *Spirulina maxima* is Zarrouk's media. Sharma *et al* (2016) also found that BG-11 is the most efficient and

economical medium for microalgae. Similarly, Lobakova *et al* (2016) also reported that maximum biomass accumulation by three strains of microalgae *Desmodesmus* (namely 1Pm66B, 2C166E and 3Dp86E-1) was on BG-11. This may be due the fact that BG-11 is nutrient sufficient medium for growth of microalgae as it contains sodium nitrate, $MgSO_4$ etc (Lobakova *et al* 2016) and also it contains trace metals like Mn, Zn, Cu, Co etc essential for biological growth. Nitrogen is of utmost importance for growth of microalge as it is required for the synthesis of protein, nucleic acids and chlorophyll molecules (Lourenço *et al* 2004). Mg act as a cofactor of some key enzymes required for the operation of metabolic pathway (Esakkimuthu *et al* 2016). Zn is required as a cofactor in many enzymes like carbonic anhydrase, alkaline phosphatase, RNA polymerase, reverse transcriptase, and other enzymes (Raven *et al* 1999, Sunda 2012). Cu is essential for the action of transcriptional regulators, oxido-reductases and in chaperones or storage (Festa and Thiele 2011). In photosynthetic organisms, oxygen-evolving complex of Photosystem II requires Mn for its functioning and it is also required for maintenance of structure of chloroplast (Rodriguez and Ho 2018). In case of dairy wastewater combination of 60% dairy waste water and 40% respective medium was most favorable concentration for all the strains except *Spirulina* sp. NCIM 5143 which preferred 100% concentration of dairy wastewater. The increase in microalgal growth from 20-60% dairy wastewater might be due to availability of sufficient nutrients for microalgal growth because at lower concentrations nutrient levels are not enough to support microalgae growth. Further reduction in growth with increase in concentrations (i.e. 80% and 100% in the case of present study) may be due to increase in organic load unfavorable for microalgal growth (Lu *et al* 2016). The rationale behind is that higher concentrations of organic matter lead to unfavourable C/N ratio for microalgal growth and activation of self-protection mechanism occurs which further affect the growth (Stehfest *et al* 2005). Our results are in corroboration with those of Kothari *et al* (2012) who reported that algal strain *Chlorella pyrenoidosa* showed highest growth at 75% concentration of dairy wastewater.

Other parameters i.e. protein, carbohydrates and lipids were also estimated for different concentrations of dairy wastewater for different microalgal strains (Table 4.14-4.19). Highest protein content was shown by *Spirulina* sp. NCIM 5143 (119.17 mg L^{-1}) on ZM followed by BGLRS (86.42 mg L^{-1}) on ACM, BGLR18 (67.95 mg L^{-1}) and BGLR10 (31.87 mg L^{-1}) on BBM and BGLR4 (9.54 mg L^{-1}) and BGLR7 (4.42 mg L^{-1}) on BG-11 (Table 4.14-4.19) while in dairy wastewater highest protein content was recorded in *Spirulina* sp. NCIM 5143 (162.33 mg L^{-1}) and lowest (0.24 mg L^{-1}) in BGLR10 at 100% dairy wastewater. Highest carbohydrate content was obtained in BGLRS (234.79 mg L^{-1}) in ACM whereas lowest (7.32 mg L^{-1}) in ZM by strain BGLR7. Maximum carbohydrate was produced by BGLRS (137.67 mg L^{-1}) at 60% concentration and lowest by BGLR7 (12.34 mg L^{-1}) at 20% concentration (Table 4.14-4.19). Similarly, highest lipid was produced by BGLRS (134.46

mg L^{-1}) in ACM followed by 120.90 in BG-11, 98.42 mg L^{-1} in BBM and 77.68 mg L^{-1} in ZM. Lowest lipid value was recorded for strain BGLR7 (5.72 mg L^{-1}) in BBM. For dairy wastewater, lipid concentration ranged from 23.90 mg L^{-1} to 266.40 mg L^{-1} with maximum value observed for BGLRS (266.40 mg L^{-1}) followed by BGLR18 (240.10 mg L^{-1}) at 60% concentration and lowest by BGLR7 (23.90 mg L^{-1}) at 100% concentration (Table 4.14-4.19). Bajwa *et al* (2017) also observed similar trend in different parameters viz. biomass yield, chlorophyll content, total carbohydrate, protein and lipid production for four microalgae strains (*Chlorococcum aquaticum,, Scenedesmus obliquus, Nannochloropsis oculata, Chlorella pyrenoidosa*) grown on five different inorganic medium including BG-11, BBM, Modified HS CHU#10, Modified Hoagland Medium, Half strength CH#10 medium. Different strains of microalgae evaluated in present study show different rates of growth in different media. This is because different species of microalgae have different physiological requirements in their natural habitats and culture conditions (Falkowski 1984).

The growth kinetics of six microalgae strains were also studied by Logistic model. Logistic growth equation fit the growth kinetics as evident by the high coefficient of determination (R^2) and low sum of squared deviations (SSD) values (Table 4.20). Biological or kinetic parameters like A (asymptote value); μ (growth rate); λ (lag time) as well as statistical parameters were calculated. Models are used to describe the behavior of microorganisms under different physical or chemical conditions such as temperature, pH, and water activity. According to Zwietering *et al* (1990) bacterial specific growth rate starts at a zero value and in certain time period reaches the maximal value (μ_m) resulting in lag time (λ) and finally reaches a stage where growth reaches zero so that an asymptote (A) is reached.

The asymptote A determines the biomass production potential which ranged from 0.65 g L^{-1} to 1.87 g L^{-1}. The highest value was obtained for BGLR18 (1.87) followed by BGLRS (1.57), BGLR10 (1.49), BGLR4 (1.05), BGLR7 (0.90) and *Spirulina* sp. NCIM 5143 (0.65). The growth rate per day (μ (day^{-1})) ranged from 0.01 to 0.07 day^{-1}. Highest growth per day was shown by BGLRS (0.07 day^{-1}) followed by BGLR18 (0.06 day^{-1}). Lag time (λ) varied between 0.29 to 9.74 days (Table 4.20).

In case of dairy wastewater highest value of asymptote A was obtained for BGLR18 (2.81) followed by BGLRS (2.67), BGLR7 (2.53) at 60% concentration of dairy wastewater and *Spirulina* sp. NCIM 5143 (2.02) at 100% concentration of dairy wastewater, BGLR10 (1.65), BGLR4 (1.63) at 60% concentration. The growth rate per day (μ (day^{-1})) ranged from 0.16 to 0.64 day^{-1}. Highest growth per day is shown by BGLR18 (0.64 day^{-1}) followed by BGLR7 (0.61 day^{-1}), *Spirulina* sp. NCIM 5143 (0.25 day^{-1}), BGLRS (0.21 day^{-1}), BGLR4 (0.20 day^{-1}) and BGLR10 (0.16 day^{-1}). Lag time (λ) was 0.00 for all the strains in dairy wastewater (Table 4.20).

Table 4.2 Growth profile of BGLR4 on various media and dairy wastewater (DWW)

Growth media	Optical density (750 nm) Incubation period (Days)											Average
	0	3	6	9	12	15	18	21	24	27	30	
*BG-11	0.00	0.00	0.02	0.04	0.05	0.05	0.07	0.07	0.10	0.10	0.11	0.06
ACM	0.00	0.00	0.02	0.04	0.06	0.05	0.05	0.08	0.09	0.10	0.11	0.05
BBM	0.00	0.01	0.03	0.05	0.05	0.06	0.06	0.08	0.08	0.09	0.09	0.05
ZM	0.00	0.01	0.02	0.03	0.04	0.04	0.05	0.05	0.05	0.05	0.06	0.04
DWW+BG-11 (20% +80%)	0.57	0.79	1.00	1.16	1.29	1.38	1.56	1.65	1.47	1.43	1.43	1.31
DWW+BG-11 (40% +60%)	0.76	0.88	1.00	1.19	1.30	1.40	1.68	1.77	1.69	1.68	1.68	1.43
DWW+BG-11 (60% +40%)	0.90	0.99	1.11	1.29	1.35	1.46	1.69	1.80	1.68	1.70	1.69	1.47
DWW+BG-11 (80% +20%)	0.47	0.58	0.99	1.11	1.28	1.37	1.46	1.57	1.49	1.49	1.49	1.28
DWW+BG-11 (100%)	0.35	0.58	0.90	1.10	1.13	1.19	1.25	1.38	1.38	1.38	1.37	1.16
C.D. (0.05) Growth media=0.103 Incubation period (Days)=0.113 Growth media × Incubation period (Days) = 0.340												

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period : 30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11=Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water, Values are means of triplicates.

Table 4.3 Growth profile of BGLR7 on various media and dairy wastewater (DWW)

Growth media	Optical density (750 nm) Incubation period (Days)											Average
	0	3	6	9	12	15	18	21	24	27	30	
*BG-11	0.03	0.08	0.16	0.20	0.22	0.24	0.28	0.29	0.31	0.35	0.35	0.25
ACM	0.03	0.05	0.10	0.13	0.15	0.16	0.17	0.18	0.18	0.21	0.23	0.14
BBM	0.01	0.03	0.08	0.11	0.13	0.16	0.17	0.19	0.20	0.22	0.23	0.14
ZM	0.02	0.05	0.09	0.11	0.12	0.13	0.15	0.17	0.19	0.19	0.21	0.13
DWW+BG-11 (20% +80%)	0.97	1.58	1.80	1.90	1.99	2.15	2.23	2.48	2.40	2.39	2.37	2.02
DWW+BG-11 (40% +60%)	1.01	1.79	1.88	2.00	2.23	2.46	2.58	2.70	2.58	2.57	2.57	2.22
DWW+BG-11 (60% +40%)	1.10	1.89	1.99	2.23	2.44	2.60	2.71	2.78	2.58	2.58	2.58	2.32
DWW+BG-11 (80% +20%)	0.85	1.43	1.58	1.79	1.88	2.00	2.20	2.30	2.18	2.17	2.17	1.87
DWW+BG-11 (100%)	0.79	1.03	1.23	1.50	1.59	1.89	2.13	2.15	2.09	2.09	2.09	1.69
C.D. (0.05) Growth media=0.112 Incubation period (Days)=0.123 Growth media × Incubation period (Days) = 0.370												

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period : 30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11=Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water, Values are means of triplicates.

Table 4.4 Growth profile of BGLR10 on various media and dairy wastewater (DWW)

Growth media	Optical density (750 nm) Incubation period (Days)											Average
	0	3	6	9	12	15	18	21	24	27	30	
*BG-11	0.07	0.13	0.16	0.22	0.32	0.35	0.38	0.42	0.53	0.61	0.74	0.36
ACM	0.07	0.13	0.13	0.21	0.30	0.31	0.33	0.35	0.43	0.52	0.61	0.31
BBM	0.08	0.13	0.18	0.20	0.22	0.25	0.30	0.30	0.32	0.34	0.46	0.25
ZM	0.09	0.11	0.13	0.15	0.16	0.18	0.19	0.20	0.25	0.30	0.31	0.19
DWW+BG-11 (20% +80%)	0.35	0.61	0.72	0.82	0.95	0.98	1.01	1.11	1.10	1.10	1.09	0.89
DWW+BG-11 (40% +60%)	0.57	0.79	0.90	0.97	1.17	1.22	1.44	1.68	1.58	1.58	1.57	1.22
DWW+BG-11 (60% +40%)	0.61	0.90	0.99	1.24	1.34	1.46	1.57	1.62	1.79	1.76	1.71	1.36
DWW+BG-11 (80% +20%)	0.11	0.60	0.69	0.79	0.82	0.89	0.94	1.00	1.00	0.98	0.98	0.89
DWW+BG-11 (100%)	0.10	0.52	0.61	0.69	0.75	0.80	0.85	0.96	0.95	0.95	0.95	0.74
C.D. (0.05) Growth media=0.062 Incubation period (Days)=0.069 Growth media × Incubation period (Days) = 0.207												

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period : 30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11=Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water, Values are means of triplicates.

Table 4.5 Growth profile of BGLR18 on various media and dairy wastewater (DWW)

Growth media	Optical density (750 nm) Incubation period (Days)											Average
	0	3	6	9	12	15	18	21	24	27	30	
BG-11	0.02	0.06	0.18	0.23	0.44	0.53	0.64	0.84	0.94	1.00	0.59	0.50
ACM	0.02	0.03	0.03	0.08	0.13	0.19	0.22	0.37	0.60	0.75	0.33	0.25
*BBM	0.12	0.44	0.44	0.65	0.83	0.90	1.00	1.24	1.44	1.64	1	0.88
ZM	0.01	0.16	0.16	0.19	0.19	0.20	0.23	0.22	0.23	0.26	0.19	0.19
DWW+BBM (20% +80%)	1.02	2.00	2.00	2.15	2.29	2.49	2.56	2.73	2.68	2.66	2.42	2.27
DWW+BBM (40% +60%)	1.12	2.35	2.35	2.57	2.68	2.77	2.90	2.90	2.90	2.87	2.66	2.55
DWW+BBM (60% +40%)	1.23	2.37	2.37	2.60	2.79	2.79	2.90	2.96	2.89	2.88	2.70	2.59
DWW+BBM (80% +20%)	1.01	1.93	1.93	1.96	2.27	2.46	2.50	2.58	2.35	2.31	2.25	2.14
DWW+BBM (100%)	0.90	1.32	1.32	1.51	1.61	1.90	2.24	2.35	2.30	2.24	1.88	1.78
C.D. (0.05) Growth media=0.196 Incubation period (Days)=0.178 Growth media × Incubation period (Days) = N.S.												

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period : 30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11=Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water, Values are means of triplicates.

Table 4.6 Growth profile of BGLRS on various media and dairy wastewater (DWW)

Growth media	Optical density (750 nm) Incubation period (Days)											Average
	0	3	6	9	12	15	18	21	24	27	30	
BG-11	0.02	0.06	0.14	0.17	0.18	0.26	0.30	0.32	0.38	0.42	0.45	0.25
*ACM	0.07	0.13	0.16	0.22	0.32	0.35	0.38	0.42	0.38	0.42	0.53	0.31
BBM	0.02	0.05	0.12	0.17	0.19	0.21	0.22	0.28	0.30	0.31	0.39	0.21
ZM	0.01	0.05	0.10	0.15	0.18	0.20	0.21	0.27	0.29	0.30	0.30	0.19
DWW+ACM (20% +80%)	1.00	1.78	2.00	2.00	2.00	2.45	2.62	2.96	2.68	2.59	2.66	2.25
DWW+ACM (40% +60%)	1.13	1.45	1.78	1.00	2.79	2.79	2.90	2.96	2.89	2.90	2.88	2.32
DWW+ACM (60% +40%)	1.12	1.92	2.35	2.57	2.68	2.77	2.90	2.90	2.90	2.85	2.87	2.53
DWW+ACM (80% +20%)	1.01	1.81	1.93	1.96	2.27	2.46	2.50	2.58	2.35	2.33	2.31	2.14
DWW+ACM (100%)	0.87	1.00	1.25	1.46	1.50	1.88	2.23	2.30	2.30	2.24	2.24	1.75
C.D. (0.05) Growth media=0.139 Incubation period (Days)=0.126 Growth media× Incubation period (Days) =0.417												

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period : 30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11=Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water, Values are means of triplicates.

Table 4.7 Growth profile of *Spirulina* sp. NCIM 5143 on various media and dairy wastewater (DWW)

Growth media	Optical density (750 nm)											Average
	Incubation period (Days)											
	0	3	6	9	12	15	18	21	24	27	30	
BG-11	0.01	0.04	0.10	0.14	0.18	0.20	0.20	0.28	0.30	0.32	0.36	0.19
ACM	0.03	0.07	0.13	0.16	0.17	0.17	0.18	0.21	0.25	0.30	0.31	0.18
BBM	0.01	0.03	0.08	0.15	0.19	0.22	0.23	0.24	0.32	0.32	0.36	0.20
*ZM	0.02	0.06	0.07	0.16	0.18	0.20	0.22	0.25	0.35	0.40	0.43	0.21
DWW+ZM (20% +80%)	0.68	0.88	1.12	1.23	1.36	1.47	1.58	1.66	1.70	1.81	1.88	1.40
DWW+ZM (40% +60%)	0.90	1.19	1.25	1.26	1.47	1.52	1.62	1.72	1.80	1.90	1.93	1.51
DWW+ZM (60% +40%)	0.99	1.21	1.30	1.31	1.49	1.68	1.79	1.90	1.91	1.97	2.20	1.61
DWW+ZM (80% +20%)	1.07	1.28	1.36	1.45	1.57	1.77	1.88	1.99	1.99	2.06	2.11	1.68
DWW+ZM (100%)	1.13	1.30	1.41	1.51	1.62	1.82	2.10	2.11	2.12	2.14	2.23	1.77
C.D. (0.05) Growth media=0.069 Incubation period (Days)=0.076 Growth media × Incubation period (Days) = 0.229												

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period : 30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11=Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water, Values are means of triplicates.

Table 4.8 Chlorophyll profile of BGLR4 biomass grown on various media and dairy wastewater (DWW)

Growth media	Chlorophyll (mg/L)						Average
	Incubation period (Days)						
	5	10	15	20	25	30	
*BG-11	0.70	0.93	1.83	2.13	2.56	2.49	1.77
ACM	0.46	0.90	1.49	1.88	2.26	2.12	1.52
BBM	0.41	0.89	1.19	1.74	1.99	1.87	1.35
ZM	0.10	0.19	1.03	1.67	1.80	1.75	1.26
DWW+BG-11 (20% +80%)	1.01	1.33	1.61	2.01	2.38	2.38	1.79
DWW+BG-11 (40% +60%)	1.47	1.66	1.86	2.17	2.30	2.33	1.96
DWW+BG-11 (60% +40%)	1.52	1.55	2.03	2.29	2.49	2.49	2.06
DWW+BG-11 (80% +20%)	0.94	0.96	1.07	2.02	2.27	2.27	1.59
DWW+BG-11 (100%)	0.88	0.93	1.25	1.85	2.12	2.12	1.53
C.D. (0.05) Growth media=0.104 Incubation period (Days)=0.085 Growth media × Incubation period (Days) = 0.255							

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11= Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water,Values are means of triplicates.

Table 4.9 Chlorophyll profile of BGLR7 biomass grown on various media and dairy wastewater (DWW)

Growth media	Chlorophyll (mg/L)						Average
	Incubation period (Days)						
	5	10	15	20	25	30	
*BG-11	1.08	1.14	1.24	1.52	2.07	1.83	1.48
ACM	0.90	1.13	1.19	1.29	1.76	1.52	1.30
BBM	0.50	1.01	1.11	1.28	1.67	1.50	1.18
ZM	0.45	0.88	1.08	1.08	1.44	1.29	1.03
DWW+ACM (20% +80%)	1.83	2.00	2.04	2.50	2.61	2.61	2.26
DWW+ACM (40% +60%)	1.93	2.04	2.06	2.88	2.98	2.98	2.48
DWW+ACM (60% +40%)	2.30	2.09	2.11	2.98	3.15	3.15	2.63
DWW+ACM (80% +20%)	1.07	1.27	1.47	1.67	1.67	1.67	1.47
DWW+ACM (100%)	0.96	1.12	1.22	1.56	1.51	1.51	1.31
C.D. (0.05) Growth media=0.167 Incubation period (Days)=0.136 Growth media × Incubation period (Days) =0.409							

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11= Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water,Values are means of triplicates.

Table 4.10 Chlorophyll profile of BGLR10 biomass grown on various media and dairy wastewater (DWW)

Growth media	Chlorophyll (mg/L)						Average
	Incubation period (Days)						
	5	10	15	20	25	30	
*BG-11	1.23	1.22	1.65	2.08	2.51	2.33	1.83
ACM	0.95	1.02	1.05	1.26	1.94	1.69	1.32
BBM	0.85	0.97	1.03	1.22	1.69	1.45	1.20
ZM	0.84	0.97	1.02	1.08	1.58	1.09	1.10
DWW+BG-11 (20% +80%)	0.33	0.90	1.05	1.30	1.11	1.11	0.97
DWW+BG-11 (40% +60%)	1.15	1.90	2.24	2.52	2.73	2.73	2.21
DWW+BG-11 (60% +40%)	2.09	2.28	2.57	2.61	2.89	2.89	2.56
DWW+BG-11 (80% +20%)	0.85	0.62	0.73	0.88	0.95	1.00	0.84
DWW+BG-11 (100%)	0.32	0.42	0.55	0.76	1.41	1.43	0.82
C.D. (0.05) Growth media=0.108 Incubation period (Days)=0.088 Growth media ×Incubation period (Days) =0.264							

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11= Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water,Values are means of triplicates.

Table 4.11 Chlorophyll profile of BGLR18 biomass grown on various media and dairy wastewater (DWW)

Growth media	Chlorophyll (mg/L)						Average
	Incubation period (Days)						
	5	10	15	20	25	30	
BG-11	1.58	1.62	1.76	2.06	2.50	2.32	1.97
ACM	0.88	1.58	1.62	2.02	2.41	1.84	1.73
*BBM	1.66	1.84	1.90	3.59	3.97	3.74	2.78
ZM	0.46	0.93	1.46	1.87	2.37	2.12	1.54
DWW+BBM (20% +80%)	1.51	2.51	3.10	3.38	3.69	3.75	2.99
DWW+BBM (40% +60%)	2.02	4.04	4.49	4.76	5.12	5.12	4.26
DWW+BBM (60% +40%)	2.04	4.45	4.45	4.80	6.19	6.19	4.69
DWW+BBM (80% +20%)	1.17	2.40	3.03	3.28	3.38	3.28	2.76
DWW+BBM (100%)	1.08	2.40	2.92	2.93	3.05	3.04	2.57
C.D. (0.05) Growth media=0.155 Incubation period (Days)=0.127 Growth media× Incubation period (Days) =0.380							

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11= Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water,Values are means of triplicates.

Table 4.12 Chlorophyll profile of BGLRS biomass grown on various media and dairy wastewater (DWW)

Growth media	Chlorophyll (mg/L)						Average
	Incubation period (Days)						
	5	10	15	20	25	30	
BG-11	0.86	0.97	1.69	1.88	2.44	2.14	1.66
*ACM	1.44	1.94	2.13	2.15	3.12	2.38	2.19
BBM	0.86	0.82	1.43	1.71	2.24	2.15	1.53
ZM	0.32	0.80	1.05	1.39	1.98	1.68	1.20
DWW+BG-11 (20% +80%)	1.11	2.50	3.20	3.69	3.84	3.84	3.03
DWW+BG-11 (40% +60%)	1.35	3.10	3.37	3.92	4.28	4.28	3.38
DWW+BG-11 (60% +40%)	1.64	3.35	3.60	4.12	4.39	4.39	3.58
DWW+BG-11 (80% +20%)	0.84	1.83	2.22	2.75	2.84	2.85	2.22
DWW+BG-11 (100%)	0.82	1.14	1.46	1.59	1.69	1.74	1.41
C.D. (0.05) Growth media=0.271 Incubation period (Days)=0.222 Growth media × Incubation period (Days) =0.665							

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11= Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water,Values are means of triplicates.

Table 4.13 Chlorophyll profile of *Spirulina* sp. NCIM 5143 biomass grown on various media and dairy wastewater (DWW)

Growth media	Chlorophyll (mg/L)						Average
	Incubation period (Days)						
	5	10	15	20	25	30	
BG-11	0.86	0.97	1.69	1.88	2.14	2.44	1.66
ACM	0.32	0.80	1.05	1.39	1.68	1.99	1.21
BBM	0.86	0.82	1.43	1.71	2.15	2.24	1.54
*ZM	1.43	1.94	2.13	2.11	2.15	2.23	2.00
DWW+ZM (20% +80%)	2.82	2.98	3.48	3.81	3.92	4.21	3.54
DWW+ZM (40% +60%)	2.97	3.18	3.31	4.65	4.86	5.17	4.02
DWW+ZM (60% +40%)	3.01	3.48	3.75	4.65	4.77	4.98	4.11
DWW+ZM (80% +20%)	3.50	3.99	4.05	4.82	5.13	5.57	4.51
DWW+ZM (100%)	3.60	4.03	4.10	4.93	5.23	5.63	4.59
C.D. (0.05) Growth media=0.119 Incubation period (Days)=0.097 Growth media × Incubation period (Days) =0.291							

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11= Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water,Values are means of triplicates.

Table 4.14 Biomass and biochemical constituents of BGLR4 biomass grown on various media and dairy wastewater (DWW)

Growth Media	Weight of wet biomass (g/L)	Weight of dry biomass (g/L)	Protein (mg/L)	Carbohydrate (mg/L)	Lipid (mg/L)
*BG-11	5.23[a]	0.12[a]	9.54[a]	70.67[c]	24.46[h]
ACM	2.02[c]	0.05[b]	1.29[d]	68.41[e]	70.90[f]
BBM	1.87[c]	0.06[b]	2.25[c]	13.75[i]	14.87[i]
ZM	3.31[b]	0.07[b]	2.22[c]	48.57[h]	38.42[g]
DWW+BG-11 (20% +80%)	2.12[c]	0.05[b]	1.32[d]	68.92[d]	136.30[c]
DWW+BG-11 (40% +60%)	3.26[b]	0.11[a]	1.33[d]	86.25[b]	212.60[b]
DWW+BG-11 (60% +40%)	4.55[a]	0.14[a]	3.00[b]	98.38[a]	230.60[a]
DWW+BG-11 (80% +20%)	2.00[c]	0.05[b]	1.21[e]	55.52[f]	131.89[d]
DWW+BG-11 (100%)	1.42[c]	0.04[b]	1.12[f]	52.87[g]	108.40[e]

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11= Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water,Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

Table 4.15 Biomass and biochemical constituents of BGLR7 biomass grown on various media and dairy wastewater (DWW)

Growth Media	Weight of wet biomass (g/L)	Weight of dry biomass (g/L)	Protein (mg/L)	Carbohydrate (mg/L)	Lipid (mg/L)
*BG-11	2.57 ^{c}	0.15 ^{a}	4.42^{a}	50.75 ^{a}	69.16^{d}
ACM	1.83^{e}	0.13 ^{a}	2.73^{d}	41.82^{c}	20.72^{h}
BBM	2.24 ^{d}	0.12$^{a\ b}$	2.95^{c}	18.76^{g}	5.72^{i}
ZM	1.55 ^{f}	0.06 ^{cd}	1.45^{h}	7.32^{i}	48.98^{e}
DWW+BG-11 (20% +80%)	3.10 ^{b}	0.05 ^{d}	1.21^{i}	12.34^{h}	81.93^{c}
DWW+BG-11 (40% +60%)	3.11^{b}	0.05 ^{d}	1.66^{f}	29.34^{e}	84.21^{b}
DWW+BG-11 (60% +40%)	3.24^{a}	0.09 ^{bc}	3.21^{b}	47.36^{b}	93.89^{a}
DWW+BG-11 (80% +20%)	2.23 ^{d}	0.01^{e}	2.55^{e}	31.14^{d}	34.52^{f}
DWW+BG-11 (100%)	1.86 ^{e}	0.01 ^{e}	1.49^{g}	26.16^{f}	23.90^{g}

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11= Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water,Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

Table 4.16 Biomass and biochemical constituents of BGLR10 biomass grown on various media and dairy wastewater (DWW)

Growth Media	Weight of wet biomass (g/L)	Weight of dry biomass (g/L)	Protein (mg/L)	Carbohydrate (mg/L)	Lipid (mg/L)
*BG-11	2.82^{b}	0.04^{bc}	12.46^{c}	34.09^{f}	112.69^{e}
ACM	1.93^{e}	0.04^{bc}	21.36^{b}	45.07^{e}	112.60^{e}
BBM	1.73^{f}	0.02^{c}	31.87^{a}	54.79^{d}	26.98^{g}
ZM	1.42^{g}	0.02^{c}	21.39^{b}	65.14^{c}	23.99^{g}
DWW+BG-11 (20% +80%)	2.27^{c}	0.07^{ab}	0.86^{g}	66.39^{c}	49.00^{f}
DWW+BG-11 (40% +60%)	2.74^{b}	0.09^{a}	1.54^{f}	33.86^{f}	143.86^{b}
DWW+BG-11 (60% +40%)	5.25^{a}	0.09^{a}	2.73^{d}	70.62^{b}	185.43^{a}
DWW+BG-11 (80% +20%)	2.14^{d}	0.06^{abc}	1.77^{e}	66.00^{c}	123.52^{c}
DWW+BG-11 (100%)	1.01^{h}	0.05^{abc}	0.24^{h}	119.40^{a}	118.10^{d}

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11= Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water,Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

Table 4.17 Biomass and biochemical constituents of BGLR18 biomass grown on various media and dairy wastewater (DWW)

Growth Media	Weight of wet biomass (g/L)	Weight of dry biomass (g/L)	Protein (mg/L)	Carbohydrate (mg/L)	Lipid (mg/L)
BG-11	1.24^{e}	0.12^{bc}	29.20^{h}	60.24^{f}	19.16^{h}
ACM	1.36^{de}	0.09^{bc}	38.49^{g}	84.22^{d}	23.00^{g}
*BBM	1.51^{cd}	0.44^{a}	67.95^{f}	105.55^{b}	23.71^{f}
ZM	0.90^{f}	0.08^{c}	25.04^{i}	133.73^{a}	17.64^{i}
DWW+BBM (20% +80%)	1.79^{b}	0.09^{bc}	89.23^{c}	42.98^{i}	64.59^{e}
DWW+BBM (40% +60%)	1.82^{b}	0.11^{bc}	90.49^{b}	75.77^{e}	127.10^{d}
DWW+BBM (60% +40%)	3.02^{a}	0.21^{b}	112.90^{a}	85.44^{c}	240.10^{a}
DWW+BBM (80% +20%)	1.70^{bc}	0.06^{c}	78.57^{d}	59.12^{g}	202.50^{b}
DWW+BBM (100%)	1.56^{cd}	0.06^{c}	68.86^{e}	45.98^{h}	178.40^{c}

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11= Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water,Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

Table 4.18 Biomass and biochemical constituents of BGLRS biomass grown on various media and dairy wastewater (DWW)

Growth Media	Weight of wet biomass (g/L)	Weight of dry biomass (g/L)	Protein (mg/L)	Carbohydrate (mg/L)	Lipid (mg/L)
BG-11	2.18^{e}	0.61^{a}	75.55^{e}	109.26^{f}	120.90^{g}
*ACM	2.59^{d}	0.63^{a}	86.42^{b}	234.79^{a}	134.46^{f}
BBM	2.02^{e}	0.49^{b}	81.82^{c}	174.22^{c}	98.42^{h}
ZM	1.58^{f}	0.08^{de}	78.23^{d}	181.33^{b}	77.68^{i}
DWW+ACM (20% +80%)	2.87^{c}	0.04^{de}	40.87^{g}	66.39^{g}	168.01^{e}
DWW+ACM (40% +60%)	3.28^{b}	0.11^{d}	61.38^{f}	119.50^{e}	178.80^{d}
DWW+ACM (60% +40%)	5.23^{a}	0.19^{c}	92.42^{a}	137.67^{d}	266.40^{a}
DWW+ACM (80% +20%)	2.78^{c}	0.04^{de}	37.83^{h}	66.00^{g}	213.01^{b}
DWW+ACM (100%)	2.75^{cd}	0.03^{e}	33.14^{i}	61.00^{h}	195.02^{c}

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11= Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water,Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

Table 4.19 Biomass and biochemical constituents of *Spirulina* sp. NCIM 5143 biomass grown on various media and dairy wastewater (DWW)

Growth Media	Weight of wet biomass (g/L)	Weight of dry biomass (g/L)	Protein (mg/L)	Carbohydrate (mg/L)	Lipid (mg/L)
BG-11	1.23^{h}	0.11^{bcd}	90.63^{h}	34.89^{e}	29.87^{f}
ACM	3.77^{b}	0.12^{bc}	87.42^{i}	45.54^{c}	17.20^{h}
BBM	2.40^{d}	0.10^{cde}	98.30^{g}	74.90^{a}	11.52^{i}
*ZM	4.06^{a}	0.28^{a}	119.17^{d}	22.33^{h}	19.69^{g}
DWW+ACM (20% +80%)	1.82^{g}	0.02^{g}	98.68^{f}	16.70^{i}	48.24^{e}
DWW+ACM (40% +60%)	1.92^{f}	0.04^{fg}	115.99^{e}	33.24^{g}	50.73^{d}
DWW+ACM (60% +40%)	2.69^{c}	0.06^{efg}	145.42^{c}	34.58^{f}	63.16^{c}
DWW+ACM (80% +20%)	2.70^{c}	0.07^{def}	153.99^{b}	36.33^{d}	82.70^{b}
DWW+ACM (100%)	2.80^{c}	0.15^{b}	162.33^{a}	49.72^{b}	99.80^{a}

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11= Blue green-11 medium, BBM= Bold's basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water,Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

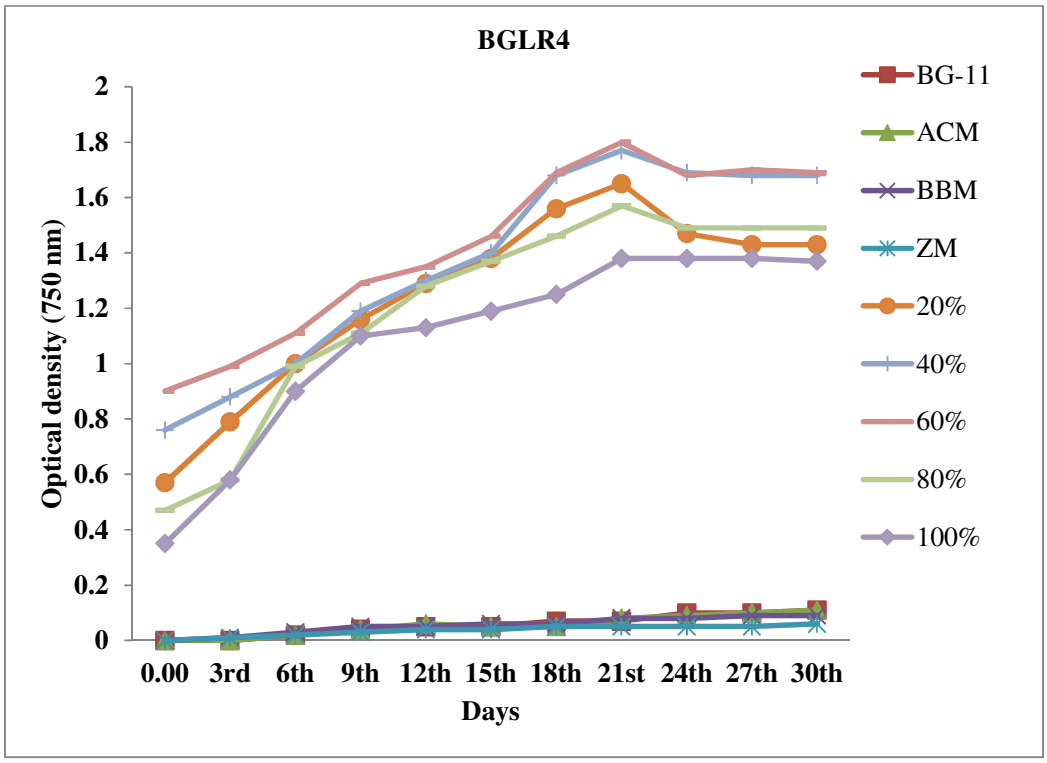


Fig. 4.1a. Growth curve of BGLR4 on various media and dairy wastewater

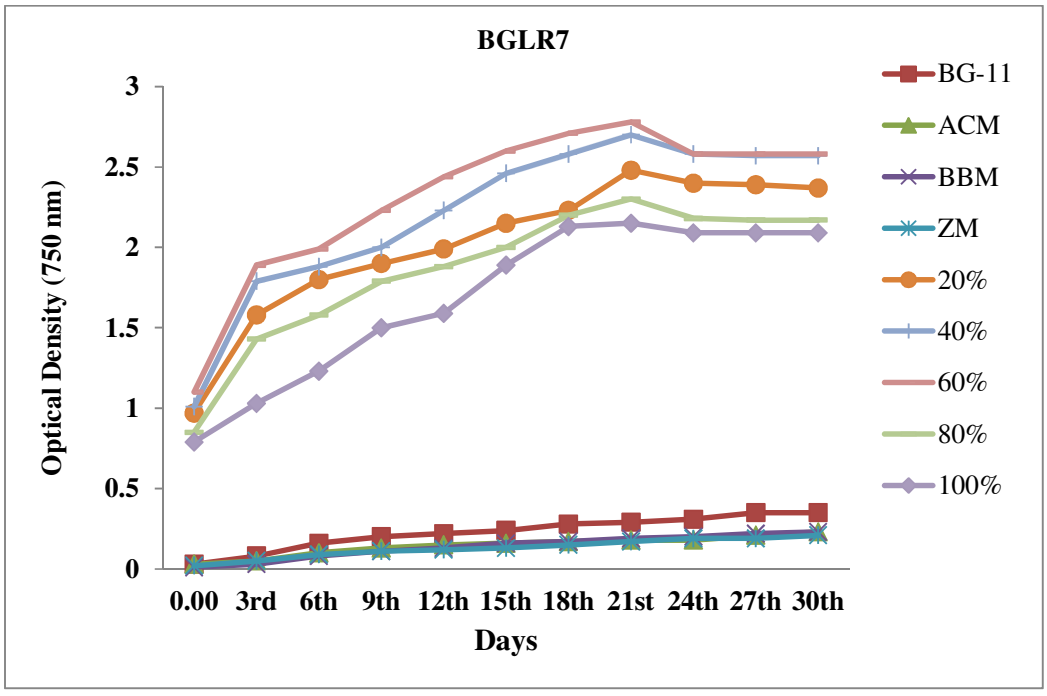


Fig. 4.1 b. Growth curve of BGLR7 on various media and dairy wastewater

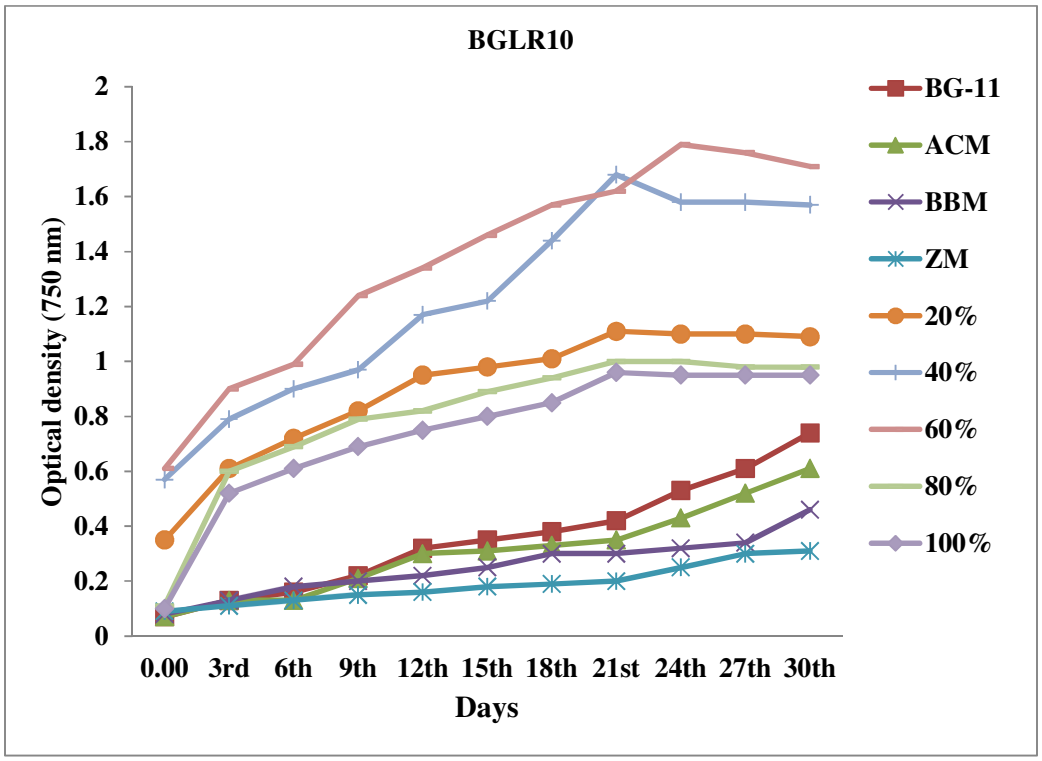


Fig. 4.1 c. Growth curve of BGLR10 on various media and dairy wastewater

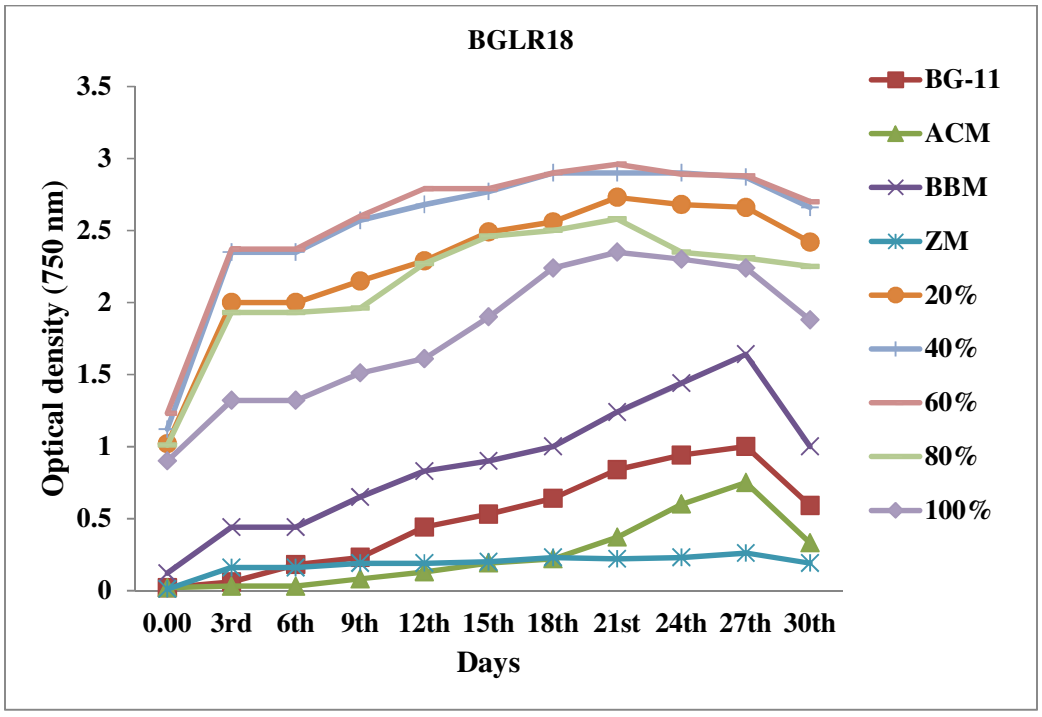


Fig. 4.1 d. Growth curve of BGLR18 on various media and dairy wastewater

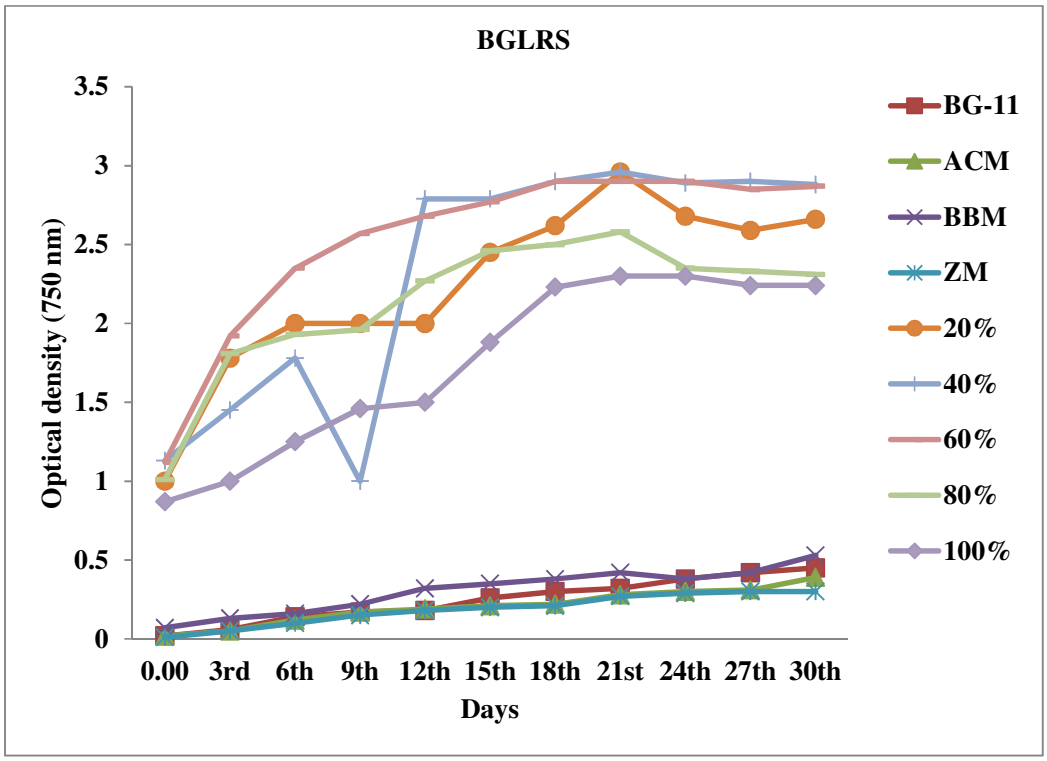


Fig. 4.1 e Growth curve of BGLRS on various media and dairy wastewater

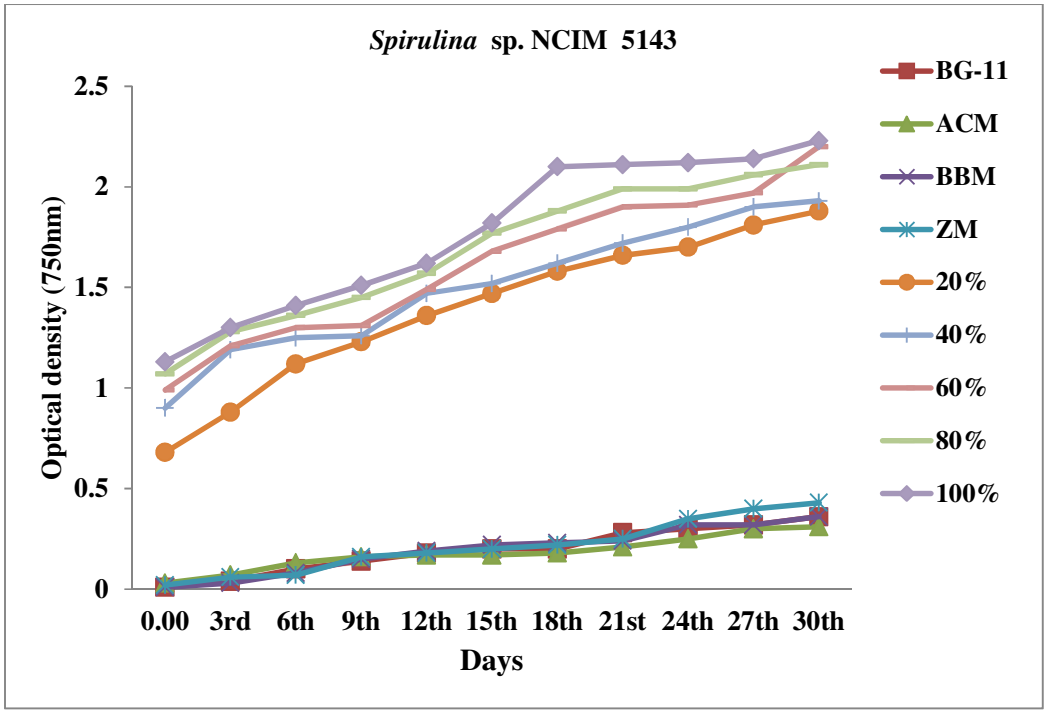


Fig. 4.1 f Growth curve of *Spirulina* sp. NCIM 5143 on various media and dairy wastewater

Table 4.20 Modified Logistic model for microalagal strains (A is asymptote value; μ is growth rate; λ is lag time; $R^2$is coefficient of determination; SSD is sum of squared deviations)

Microalgal strains	Biological parameters			Statistical parameters	
	A (g L^{-1})	μ (day^{-1})	λ (days)	R^2	SSD
BGLR4 (BG-11)	1.05	0.03	6.05	0.96	0.01
BGLR7 (BG-11)	0.90	0.01	0.72	0.99	0.01
BGLR10 (BG-11)	1.49	0.04	9.74	0.95	0.00
BGLR18 (BBM)	1.87	0.06	0.29	0.99	0.05
BGLRS (ACM)	1.57	0.07	7.14	0.97	0.01
Spirulina sp. NCIM 5143 (ZM)	0.65	0.02	4.26	0.98	0.01
BGLR4 (60% DWW +20% BG-11)	1.63	0.20	0.00	0.95	0.82
BGLR7 (60% DWW +20% BG-11)	2.53	0.61	0.00	0.95	1.08
BGLR10 (60% DWW +20% BG-11)	1.65	0.16	0.00	0.99	0.44
BGLR18 (60% DWW +20% BBM)	2.81	0.64	0.00	0.94	1.03
BGLRS (60% DWW +20% ACM)	2.67	0.21	0.00	0.96	1.22
Spirulina sp. NCIM 5143 (100% DWW)	2.02	0.25	0.00	0.97	1.46

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, Values are calculated for the media in which these cultures showed highest biomass production in screening experiment, BG-11=Blue green-11 medium, BBM= Bold basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water.

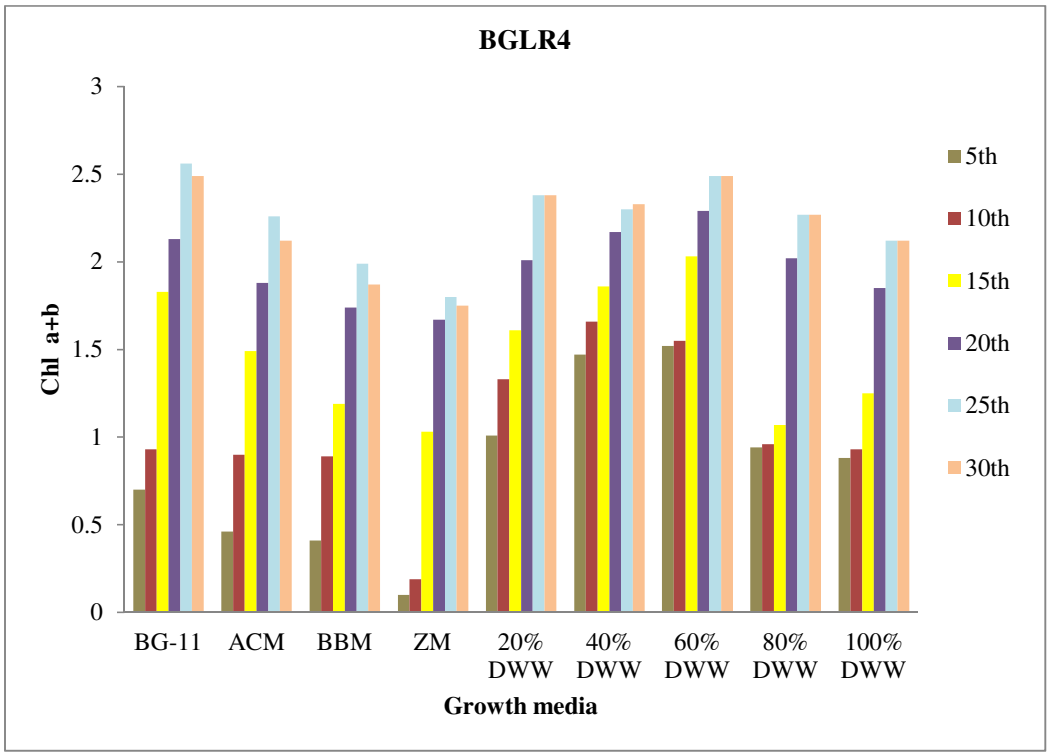


Fig. 4.2 a Chlorophyll Profile of BGLR4 on dairy wastewater and different media

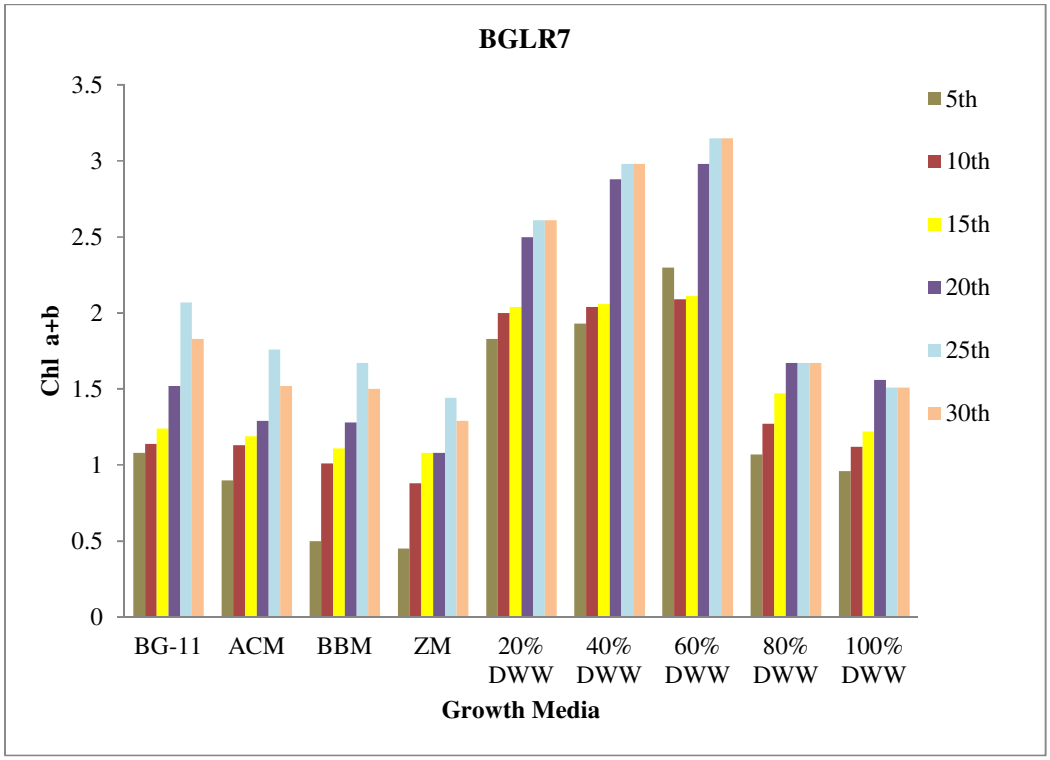


Fig. 4.2 b Chlorophyll Profile of BGLR7 on dairy wastewater and different media

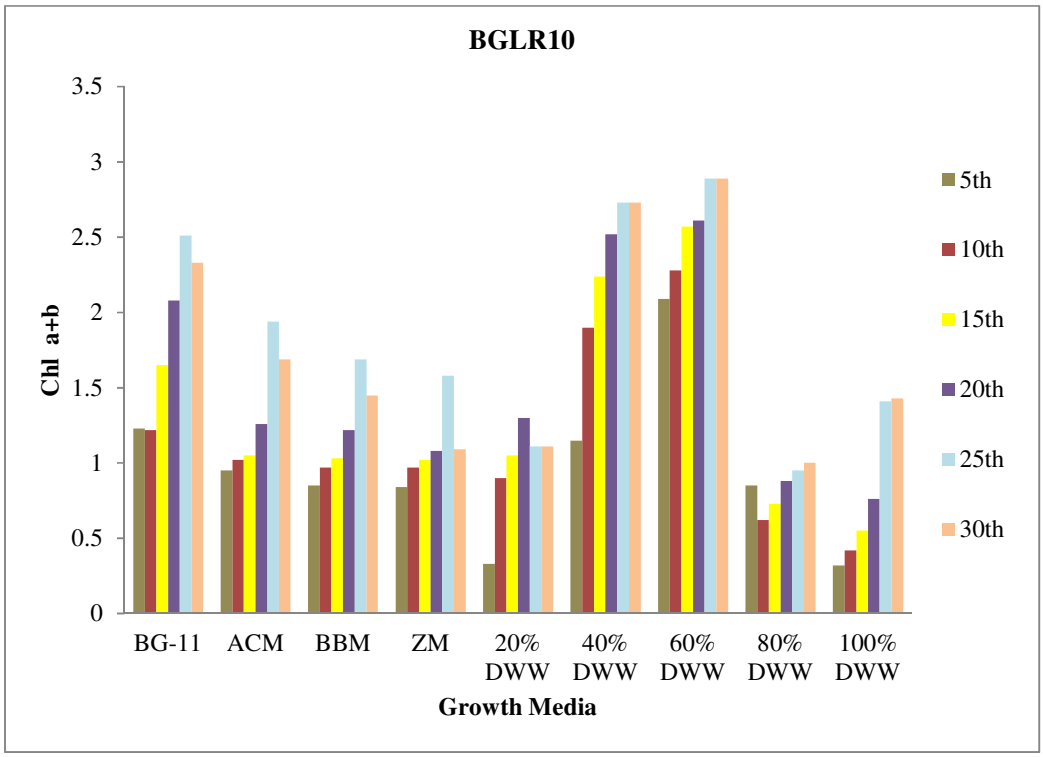


Fig. 4.2 c Chlorophyll Profile of BGLR10 on dairy wastewater and different media

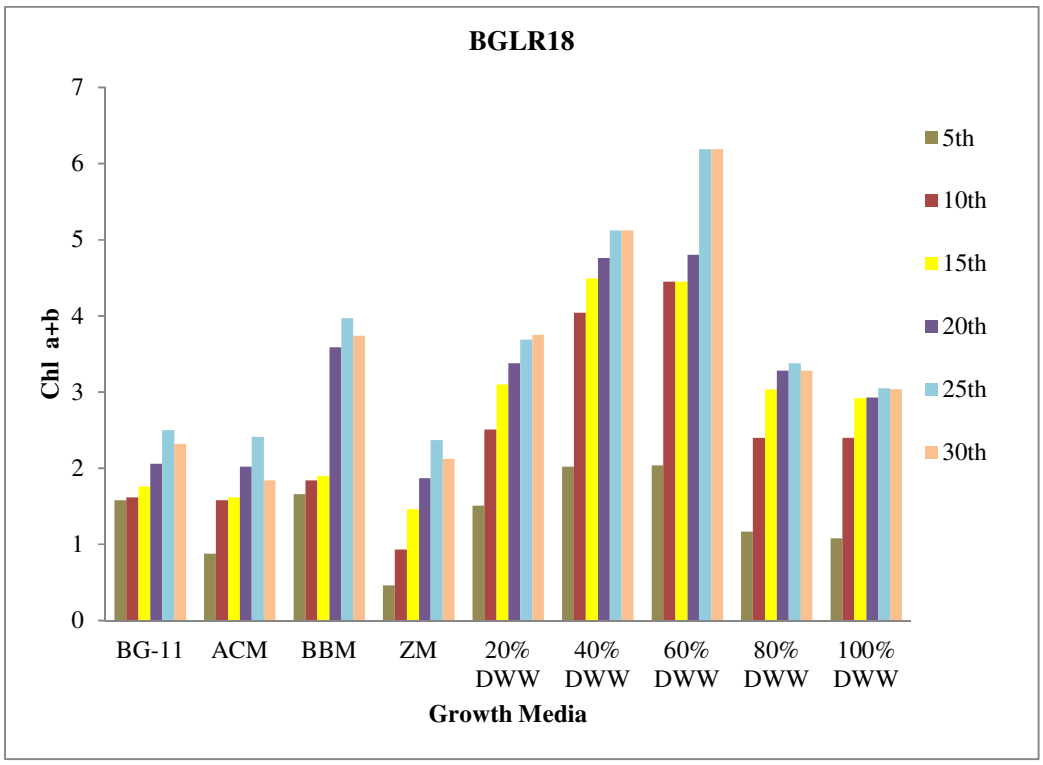


Fig. 4.2 d Chlorophyll Profile of BGLR18 on dairy wastewater and different media

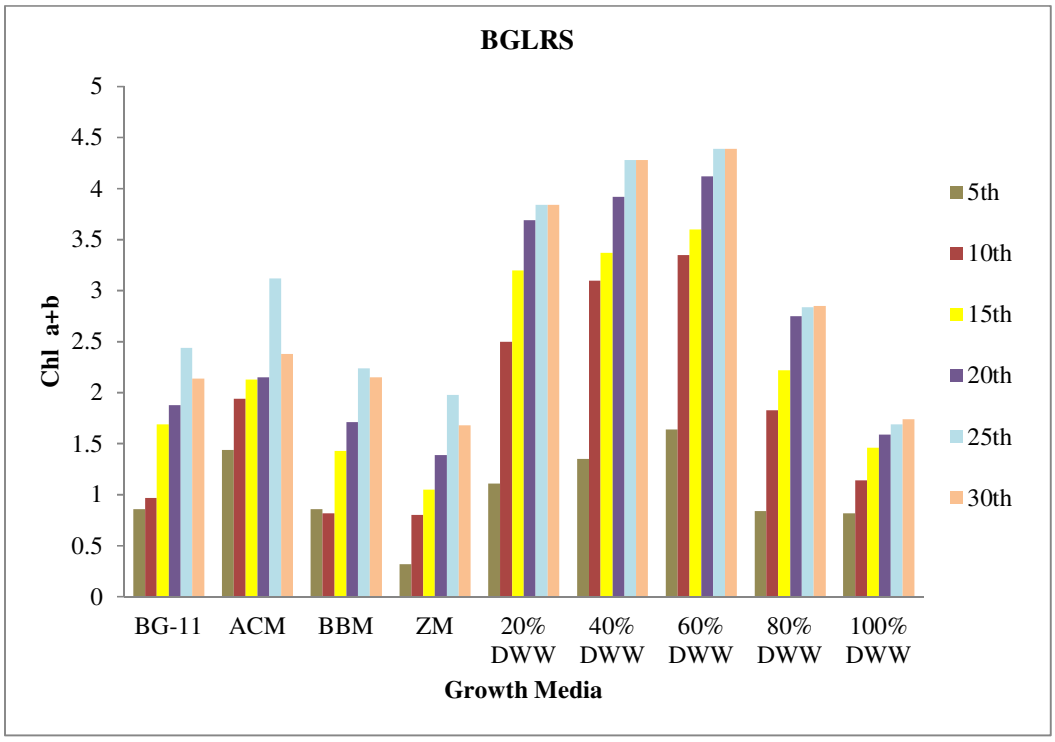


Fig. 4.2 e Chlorophyll Profile of BGLRS on dairy wastewater and different media

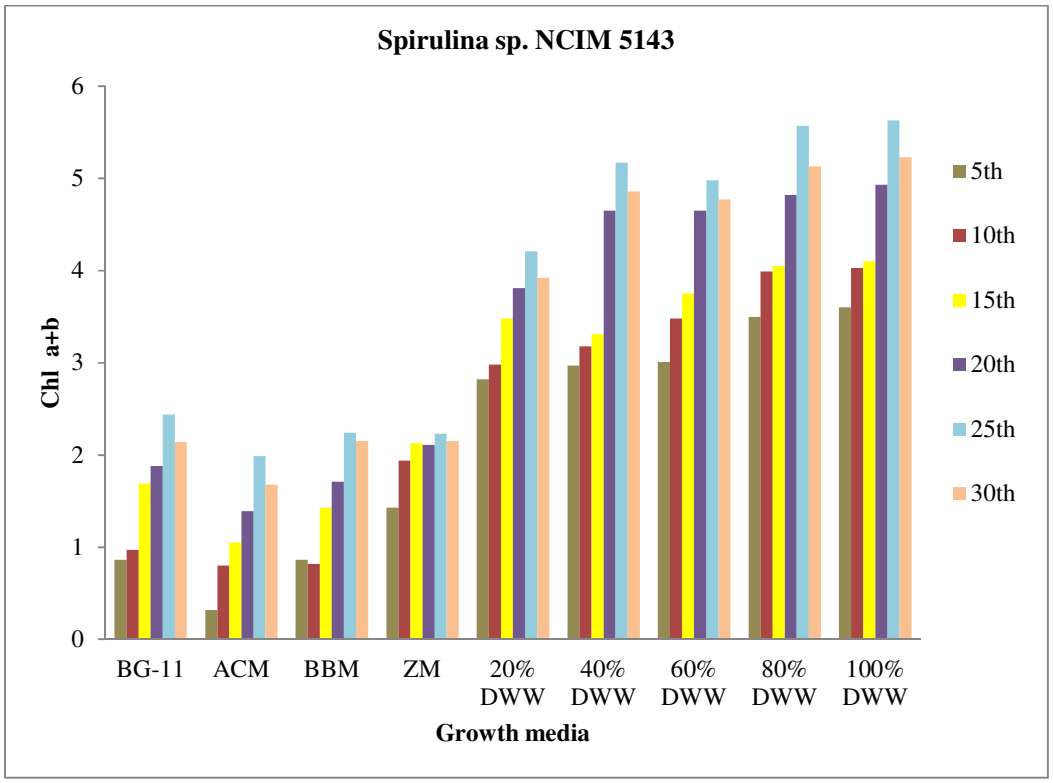


Fig. 4.2 f Chlorophyll Profile of *Spirulina* sp. NCIM 5143 on dairy wastewater and different media

4.1.2 Physico-chemical analysis of dairy wastewater before and after treatment with stress tolerant microalgae strains

Dairy wastewater collected from nearby dairy was analyzed for various physico-chemical parameters viz. temperature, pH, colour, odour, dissolved oxygen (DO), biological Oxygen Demand (BOD), chemical Oxygen Demand (COD), total Solids (TS), total Dissolved Solids (TDS), total Suspended solids (TSS), total Phosphorus (TP), total kjeldahl nitrogen (TKN), crude protein content and chloride concentration (Cl^-) by standard methods (Table 4.21). Dairy wastewater collected in the morning immediately after milk processings had the temperature of 42°C. The dairy wastewater was off white in colour and acidic in pH (6.5) and had pungent smell. This may be contributed by the organic matter decomposition or presence of various aromatic and volatile organic compounds (Singh *et al* 1998) or due to the activity of micro-organisms present (Nagarajan and Shasikumar 2002). The acidic nature of pH was attributed to the decomposition of lactose present in milk into lactic acid under aerobic conditions (Joseph 1995). No dissolved oxygen was present in the dairy wastewater. The BOD (11,000 mg/l) and COD (11,500) content found in dairy wastewater were on higher scale and closely matched. Fats, nutrients, lactose, detergents, sanitizing agents, casein and inorganic salts contribute to high BOD and COD values of dairy wastewater (Porwal *et al* 2015). Because of the presence of easily biodegradable organic compounds in the food industry wastewaters, their COD and BOD values are often closely matched (Finnish Food and Drink Industries' Federation 2005). Also, higher COD may be contributed by the higher organic matter in wastewater as COD is a measure of non-biodegradable organic matter in biologically treated industrial effluents (Malaviya and Rathore 2001). Dairy industry wastewater has a typical white color and a high nutrient level as well as organic matter content (Kushwaha *et al* 2010). The concentration of total solids (24,190.00 mg/l), total dissolved solids (TDS) (23,600.00 mg/l) and total suspended solids (TSS) (1,000.00 mg/l) were also in the higher range. Compounds like carbonates, bicarbonates, chlorides, sulfate, phosphate, nitrate, Ca, Mg, Na, K, Mn and organic matter constitutes the total solids content of wastewater (Sahana and Shirnalli 2018). Higher concentration of biodegradable organic matter in dairy waste water may be responsible for the higher concentration of total suspended solids (Kotteswari *et al* 2012). The presence of high concentration of ions like Na^+ and Cl^- is due to the use of large amount of alkaline cleaners in dairy plant (Demirel *et al* 2005). The values of total phosphorus (TP), total kjeldahl nitrogen (TKN), crude protein content and chlorides were 10.88 mg/l, 195.55 mg/l, 1319.96 mg/l and 850 mg/l respectively. Thus, the nutrient composition of dairy wastewater showed that it was an ideal medium for the growth of microalgae.

Six microalgae strains viz. BGLR4, BGLR7, BGLR10, BGLR18, BGLRS and *Spirulina* sp. NCIM 5143 grown on dairy waste were also evaluated for bioremediation

potential to affect various physico-chemical parameters of dairy wastewater like COD, BOD, TS, TDS, TSS, Cl^-, TKN, TP after a period of 30 days (Table 4.21). All strains showed maximum percent reduction in various physico-chemical parameters at 60% while *Spirulina* sp. NCIM 5143 at 100%. Highest BOD percent reduction was given by BGLR18 and BGLRS (63.64%) followed by BGLR7 (45.45%), BGLR4 (40.91%), BGLR10 (36.36%) and *Spirulina sp.* NCIM 5143 (27.27%). Removal of dissolved organic compounds and its derivatives might be the reason behind reduction of BOD levels (Kotteswari *et al* 2012). Maximum percent reduction in COD was found in BGLR18 and BGLRS (56.52%) followed by BGLR7 and *Spirulina* sp. NCIM 5143 (47.82%), BGLR4 and BGLR10 (43.48%). The reduction of COD content by different microalgal strains at different concentrations might be due to inherent potential of microalgae to utilize the organic compounds as an energy source besides carbon dioxide as COD is indirect measure of organic compounds present in wastewater (Hu *et al* 2012). More or less similar trend was found in case of other remaining parameters viz. TS, TDS, TSS, TKN, crude protein content, TP and Cl^-. Maximum percent reduction in TS (99.99%) was shown by strains BGLR7, BGLR18, BGLRS and *Spirulina* sp. NCIM 5143 followed by BGLR4 (98.99%) and BGLR10 (98.97%). While highest percent reduction values in TDS (99.99%) was given by BGLR18, BGLRS, BGLR7 and *Spirulina* sp. NCIM 5143 and lowest was found in BGLR10 (98.90%). Similarly, BGLR18 and BGLRS gave maximum percent reduction (99.99%) in TSS and BGLR10 gave minimum value of 98.51%. The maximum percent reduction of total solids, total suspended solids and total dissolved solids of dairy wastewater was observed after treatment with microalgae. This may be due to the conversion of the already present total suspended solids into dissolved materials which is further required for uptake by algae and assimilation (Rao *et al* 2011). Strain BGLR18 reduced the TP content to maximal value of 54.84% followed by BGLRS (53.93%), *Spirulina* sp. NCIM 5143 (50.77%), BGLR7 (49.82%), BGLR4 (45.25%), BGLR10 (37.48%). Nitrogen and Phosphorus are two most important macronutrients required for microalgal growth. Deficiency of Phosphorus affects chlorophyll synthesis and growth and metabolism of cells because it is required for the operation of Calvin cycle and many phosphorylation syntheses (Liang *et al* 2013). Percent reduction in TKN and crude protein content were highest in BGLR18 with values of 68.66% and 68.65% respectively. Reduction in total phosphorus and total kjeldahl nitrogen may be due to the utilization of phosphorus by algae for growth and development, to synthesize its biomass and and intracellular polysphosphate compounds, phospholipids, adenosine triphosphates (ATP) and nucleic acids which are assimilated as inorganic orthophosphate, preferably as $H_2PO_4^-$ or HPO_4^{2-} (Ding *et al* 2015, Becker 1994). As compared to nitrate, phosphorus removal mechanism is complex as it is usually assimilated in the form of orthophosphate. It is used for growth, nucleic acid synthesis and for value-added compounds like astaxanthin and polyunsaturated fatty acids etc.

Table 4.21 Physico-chemical parameters of dairy wastewater before and after microalgal growth

Growth media	Biological Oxygen Demand (mg/L)	Chemical Oxygen Demand (mg/L)	Total Solids (mg/L)	Total Dissolved Solids (mg/L)	Total Suspended Solids (mg/L)	Total Phosphorus mg/L)	Total Kjeldahl Nitrogen (mg/L)	Crude Protein content (mg/L)	Chloride concentration (mg/L)
Dairy wastewater	11,000.00	11,500.00	24,190.00	23,600.00	1,000.00	10.88	195.55	1319.96	850.00
BGLR4	6,000.00 (40.91%)^{c}	5,000.00 (43.48%)^{c}	243.52 (98.99%)^{b}	237.54 (98.99%)^{b}	11.22 (98.88%)^{b}	5.96 (45.25%)^{e}	110.00 (43.75%)^{d}	0.07 (43.74%)^{e}	85.00 (90.00%)^{b}
BGLR7	6,000.00 (45.45%)^{b}	5,000.00 (47.82%)^{b}	0.01 (99.99%)^{a}	0.02 (99.99%)^{a}	0.22 (99.98%)^{a}	5.46 (49.82%)^{d}	78.80 (59.70%)^{b}	0.05 (59.70%)^{c}	85.00 (90.00%)^{b}
BGLR10	8,000.00 (36.36%)^{d}	5,000.00 (43.48%)^{c}	250.04 (98.97%)^{b}	259.82 (98.90%)^{c}	14.85 (98.51%)^{c}	6.80 (37.48%)^{f}	115.00 (41.19)^{e}	0.08 (50.70%)^{c}	95.00 (88.82%)^{c}
BGLR18	4,000.00 (63.64%)^{a}	5,000.00 (56.52%)^{a}	0.14 (99.99%)^{a}	0.12 (99.99%)^{a}	0.02 (99.99%)^{a}	5.01 (54.84%)^{a}	96.25 (59.70%)^{b}	0.06 (59.77%)^{b}	55.00 (93.53%)^{a}
BGLRS	4,000.00 (63.64%)^{a}	5,000.00 (56.52%)^{a}	2.22 (99.99%)^{a}	0.04 (99.99%)^{a}	0.02 (99.99%)^{a}	4.91 (53.93%)^{b}	61.29 (68.66%)^{a}	0.04 (68.65%)^{a}	55.00 (93.53%)^{a}
Spirulina sp. NCIM 5143	8,000.00 (27.27%)^{e}	6,000.00 (47.82%)^{b}	0.06 (99.99%)^{a}	2.06 (99.99%)^{a}	0.20 (99.98%)^{a}	6.70 (50.77%)^{c}	96.25 (50.78%)^{c}	0.06 (50.77%)^{d}	55.00 (93.53%)^{a}

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11=Blue green-11 medium, BBM= Bold basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water, Values in parentheses showed percent reduction, Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

Table 4.22 Percent reduction in various physico-chemical parameters of dairy waste water after growth of BGLR4

Growth media	Biological Oxygen Demand (mg/L)	Chemical Oxygen Demand (mg/L)	Total Solids (mg/L)	Total Dissolved Solids (mg/L)	Total Suspended Solids (mg/L)	Total Phosphorus mg/L)	Total Kjeldahl Nitrogen (mg/L)	Crude Protein content (mg/L)	Chloride concentration (mg/L)
DWW+ BG-11 (20% +80%)	9,000.00 (36.36)^{b}	5,000.00 (43.48)^{a}	246.30 (98.98)^{a}	251.98 (98.93)^{bc}	16.95 (98.30)^{d}	7.70 (29.25)^{c}	113.83 (41.79)^{b}	0.07 (41.78)^{b}	75.00 (91.18)^{a}
DWW+ BG-11 (40% +60%)	8,000.00 (27.27)^{c}	5,000.00 (43.48)^{a}	245.38 (98.98)^{a}	244.00 (98.97)^{ab}	14.17 (98.58)^{b}	6.90 (36.57)^{b}	115.00 (41.19)^{c}	0.08 (41.19)^{c}	85.00 (90.00)^{b}
DWW+ BG-11 (60% +40%)	6,000.00 (40.91)^{a}	5,000.00 (43.48)^{a}	243.52 (98.99)^{a}	237.54 (98.99)^{a}	11.22 (98.88)^{a}	5.96 (45.25)^{a}	110.00 (43.75)^{a}	0.07 (43.74)^{a}	85.00 (90.00)^{b}
DWW+ BG-11 (80% +20%)	8,000.00 (27.27)^{c}	9,000.00 (21.74)^{b}	266.32 (98.90)^{b}	264.22 (98.88)^{c}	14.92 (98.51)^{c}	9.19 (15.54)^{d}	131.34 (32.84)^{d}	0.09 (32.83)^{d}	95.00 (88.82)^{c}
DWW+ BG-11 (100%)	10,000.00 (9.09)^{d}	10,000.00 (13.04)^{c}	284.00 (98.82)^{c}	278.88 (98.82)^{d}	15.10 (98.50)^{c}	9.44 (13.25)^{e}	131.34 (32.84)^{d}	0.09 (32.83)^{d}	95.00 (88.82)^{c}

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11=Blue green-11 medium, BBM= Bold basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water, Values in parentheses showed percent reduction, Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

Table 4.23 Percent reduction in various physico-chemical parameters of dairy waste water after growth of BGLR7

Growth media	Biological Oxygen Demand (mg/L)	Chemical Oxygen Demand (mg/L)	Total Solids (mg/L)	Total Dissolved Solids (mg/L)	Total Suspended Solids (mg/L)	Total Phosphorus mg/L)	Total Kjeldahl Nitrogen (mg/L)	Crude Protein content (mg/L)	Chloride concentration (mg/L)
DWW+ BG-11 (20% +80%)	8,000.00 (27.27)^{c}	5,000.00 (43.48)^{c}	0.42 (99.99)^{a}	0.24 (99.99)^{a}	0.22 (99.98)^{a}	7.30 (32.90)^{b}	127.00 (36.05)^{c}	0.08 (35.05)^{a}	105.00 (87.65)^{a}
DWW+ BG-11 (40% +60%)	7,000.00 (36.36)^{b}	6,000.00 (52.17)^{a}	0.06 (99.99)^{a}	0.20 (99.99)^{a}	0.25 (99.97)^{a}	5.46 (49.82)^{a}	119.00 (39.146)^{b}	0.08 (39.14)^{a}	85.00 (90.00)^{a}
DWW+ BG-11 (60% +40%)	6,000.00 (45.45)^{a}	5,000.00 (47.82)^{b}	0.01 (99.99)^{a}	0.02 (99.99)^{a}	0.22 (99.98)^{a}	5.46 (49.82)^{a}	78.80 (59.703)^{a}	0.05 (59.77)^{a}	85.00 (90.00)^{a}
DWW+ BG-11 (80% +20%)	10,000.00 (9.09)^{d}	9,000.00 (21.74)^{d}	4.44 (99.98)^{a}	4.12 (99.98)^{a}	0.40 (99.91)^{b}	9.19 (15.54)^{c}	145.00 (25.850)^{d}	0.09 (25.85)^{a}	95.00 (88.82)^{a}
DWW+ BG-11 (100%)	10,000.00 (9.09)^{d}	9,000.00 (21.74)^{d}	15.16 (99.94)^{b}	14.76 (99.94)^{b}	0.50 (99.95)^{ab}	9.45 (13.25)^{d}	155.00 (20.736)^{e}	0.10 (20.73)^{a}	95.00 (88.82)^{a}

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11=Blue green-11 medium, BBM= Bold basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water, Values in parentheses showed percent reduction, Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

Table 4.24 Percent reduction in various physico-chemical parameters of dairy waste water after growth of BGLR10

Growth media	Biological Oxygen Demand (mg/L)	Chemical Oxygen Demand (mg/L)	Total Solids (mg/L)	Total Dissolved Solids (mg/L)	Total Suspended Solids (mg/L)	Total Phosphorus mg/L)	Total Kjeldahl Nitrogen (mg/L)	Crude Protein content (mg/L)	Chloride concentration (mg/L)
DWW+ BG-11 (20% +80%)	10,000.00 (27.27)^{b}	7,000.00 (39.13)^{c}	252.88 (98.95)^{a}	268.82 (98.96)^{a}	17.95 (33.82)^{c}	7.20 (33.82)^{c}	113.83 (41.78)^{b}	0.08 (41.78)^{b}	75.00 (91.18)^{a}
DWW+ BG-11 (40% +60%)	10,000.00 (27.27)^{b}	4,000.00 (47.83)^{a}	266.56 (98.89)^{b}	267.48 (98.87)^{b}	15.25 (98.47)^{a}	6.95 (36.10)^{b}	96.25 (50.77)^{a}	0.06 (41.19)^{a}	95.00 (88.82)^{b}
DWW+ BG-11 (60% +40%)	8,000.00 (36.36)^{a}	5,000.00 (43.48)^{b}	250.04 (98.97)^{a}	259.82 (98.90)^{b}	14.85 (98.51)^{a}	6.80 (37.48)^{a}	115.00 (41.19)^{c}	0.08 (50.77)^{c}	95.00 (88.82)^{b}
DWW+ BG-11 (80% +20%)	6,000.00 (9.09)^{c}	9,000.00 (21.74)^{d}	281.76 (98.84)^{b}	270.68 (98.85)^{b}	20.15 (97.98)^{b}	8.19 (24.68)^{d}	131.34 (32.83)^{d}	0.09 (32.83)^{d}	105.00 (87.65)^{c}
DWW+ BG-11 (100%)	6,000.00 (9.09)^{c}	11,000.00 (13.04)^{e}	281.74 (98.84)^{b}	286.90 (98.78)^{c}	20.72 (97.93)^{b}	8.69 (20.11)^{e}	148.86 (23.87)^{e}	0.10 (23.87)^{e}	115.00 (86.47)^{d}

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11=Blue green-11 medium, BBM= Bold basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water, Values in parentheses showed percent reduction, Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

Table 4.25 Percent reduction in various physico-chemical parameters of dairy waste water after growth of BGLR18

Growth media	Biological Oxygen Demand (mg/L)	Chemical Oxygen Demand (mg/L)	Total Solids (mg/L)	Total Dissolved Solids (mg/L)	Total Suspended Solids (mg/L)	Total Phosphorus mg/L)	Total Kjeldahl Nitrogen (mg/L)	Crude Protein content (mg/L)	Chloride concentration (mg/L)
DWW+BBM (20% +80%)	6,000.00 (45.45)^{c}	6,000.00 (47.82)^{b}	0.42 (99.99)^{a}	0.32 (99.99) ^{a}	0.125 (99.99) ^{a}	6.55 (39.76)^{c}	113.83 (41.79)^{c}	0.08 (41.78)^{c}	75.00 (91.17)^{d}
DWW+BBM (40% +60%)	4,000.00 (60.64)^{b}	5,000.00 (56.52)^{a}	0.16 (99.99) ^{a}	0.10 (99.99) ^{a}	0.075 (99.99) ^{a}	5.01 (53.94)^{b}	96.25 (50.78)^{b}	0.06 (50.77)^{b}	65.00 (92.35)^{b}
DWW+BBM (60% +40%)	4,000.00 (63.64)^{a}	5,000.00 (56.52)^{a}	0.14 (99.99) ^{a}	0.12 (99.99) ^{a}	0.025 (99.99) ^{a}	5.01 (54.84) ^{a}	96.25 (59.70) ^{a}	0.06 (59.70) ^{a}	55.00 (93.53) ^{a}
DWW+BBM (80% +20%)	8,000.00 (27.27)^{d}	8,000.00 (30.43)^{c}	5.00 (99.98) ^{a}	4.90 (99.98) ^{a}	0.125 (99.99) ^{a}	7.70 (29.25)^{d}	120.00 (38.63)^{d}	0.08 (38.63)^{d}	70.00 (91.76)^{c}
DWW+BBM (100%)	8,000.00 (27.27)^{d}	9,000.00 (21.74)^{d}	9.04 (99.96) ^{a}	8.82 (99.96) ^{a}	0.275 (99.97) ^{a}	8.69 (20.11)^{e}	128.00 (34.54)^{e}	0.08 (34.54)^{e}	80.00 (90.59)^{c}

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11=Blue green-11 medium, BBM= Bold basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water, Values in parentheses showed percent reduction, Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

Table 4.26 Percent reduction in various physico-chemical parameters of dairy waste water after growth of BGLRS

Growth media	Biological Oxygen Demand (mg/L)	Chemical Oxygen Demand (mg/L)	Total Solids (mg/L)	Total Dissolved Solids (mg/L)	Total Suspended Solids (mg/L)	Total Phosphorus mg/L)	Total Kjeldahl Nitrogen (mg/L)	Crude Protein content (mg/L)	Chloride concentration (mg/L)
DWW+ACM (20% +80%)	5,000.00 (54.54)^{b}	5,000.00 (56.52)^{a}	18.12 (99.99)^{a}	0.30 (99.99)^{a}	0.37 (99.96)^{a}	5.91 (45.71)^{c}	78.80 (59.70)^{b}	0.05 (59.70)^{b}	65.00 (92.35)^{ab}
DWW+ACM (40% +60%)	4,000.00 (63.64)^{a}	5,000.00 (56.52)^{a}	2.50 (99.99)^{a}	0.12 (99.99)^{a}	0.27 (99.97)^{a}	5.56 (48.91)^{b}	78.80 (59.70)^{b}	0.05 (59.70)^{b}	55.00 (93.53)^{a}
DWW+ACM (60% +40%)	4,000.00 (63.64)^{a}	5,000.00 (56.52)^{a}	2.22 (99.99)^{a}	0.04 (99.99)^{a}	0.02 (99.99)^{a}	4.91 (53.93)^{a}	61.29 (68.66)^{a}	0.04 (68.65)^{a}	55.00 (93.53)^{a}
DWW+ACM (80% +20%)	7,000.00 (36.34)^{c}	8,000.00 (30.43)^{b}	1.56 (99.99)^{a}	0.26 (99.99)^{a}	0.50 (99.95)^{a}	8.19 (24.68)^{d}	85.00 (56.53)^{c}	0.05 (56.53)^{c}	75.00 (91.18)^{ab}
DWW+ACM (100%)	8,000.00 (27.27)^{d}	8,000.00 (30.43)^{b}	0.92 (99.93)^{b}	17.58 (99.92)^{b}	0.65 (99.94)^{a}	8.69 (20.11)^{e}	89.00 (54.49)^{d}	0.06 (54.49)^{d}	85.00 (90.00)^{b}

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark) , Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11=Blue green-11 medium, BBM= Bold basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water, Values in parentheses showed percent reduction, Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

Table 4.27 Percent reduction in various physico-chemical parameters of dairy waste water after growth of *Spirulina* sp. NCIM 5143

Growth media	Biological Oxygen Demand (mg/L)	Chemical Oxygen Demand (mg/L)	Total Solids (mg/L)	Total Dissolved Solids (mg/L)	Total Suspended Solids (mg/L)	Total Phosphorus mg/L)	Total Kjeldahl Nitrogen (mg/L)	Crude Protein content (mg/L)	Chloride concentration (mg/L)
DWW+ZM (20% +80%)	10,000.00 (9.09)^{c}	10,000.00 (13.04)^{c}	0.14 (99.92)^{b}	17.94 (99.92)^{b}	0.35 (99.96)^{a}	6.40 (32.82)^{d}	131.34 (32.83)^{d}	0.08 (32.83)^{d}	75.00 (91.18)^{c}
DWW+ZM (40% +60%)	10,000.00 (9.09)^{c}	10,000.00 (13.04)^{c}	0.10 (99.99)^{a}	2.34 (99.99)^{a}	0.20 (99.98)^{a}	6.60 (41.77)^{c}	113.83 (41.79)^{c}	0.07 (41.78)^{c}	65.00 (92.35)^{b}
DWW+ZM (60% +40%)	8,000.00 (27.27)^{b}	6,000.00 (47.82)^{b}	0.06 (99.99)^{a}	2.06 (99.99)^{a}	0.20 (99.98)^{a}	6.70 (50.77)^{b}	96.25 (50.78)^{b}	0.06 (50.77)^{b}	55.00 (93.53)^{a}
DWW+ZM (80% +20%)	6,000.00 (45.45)^{a}	6,000.00 (47.82)^{b}	0.66 (99.99)^{a}	0.04 (99.99)^{a}	0.22 (99.98)^{a}	7.40 (59.69)^{a}	78.80 (59.70)^{a}	0.05 (59.70)^{a}	55.00 (93.53)^{a}
DWW+ZM (100%)	6,000.00 (45.45)^{a}	5,000.00 (56.52)^{a}	18.10 (99.99)^{a}	0.78 (99.99)^{a}	0.17 (99.98)^{a}	7.90 (59.69)^{a}	78.80 (59.70)^{a}	0.05 (59.70)^{a}	65.00 (92.35)^{b}

Cultural conditions : Temperature (28±2 °C), light intensity (4000 Lux) and photoperiod (16:8 light:dark), Incubation period :30 days, *indicates media in which microalgal strains showed highest biomass production, BG-11=Blue green-11 medium, BBM= Bold basal medium, ZM= Zarrouk's medium, ACM= Algal culture medium, DWW= Dairy waste water, Values in parentheses showed percent reduction, Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

(Chen and Chen 2006). Similarly, chloride content was reduced maximally in BGLR18, BGLRS and *Spirulina* sp. NCIM 5143 (93.53%) followed by BGLR7 and BGLR4 (90.00%), BGLR10 (88.82%). Overall, strains BGLR18 and BGLRS showed maximum percent reduction in various physico-chemical parameters (Table 4.22 to 4.27).

4.2 Optimization of cultural conditions of selected microalgal strains by Response Surface Methodology (RSM)

4.2.1 Optimization of cultural conditions BGLR18

Response surface methodology is an easy and efficient method for screening of important factors rapidly among large number of factors and optimizing culture conditions (Zhang *et al* 2012, Qin *et al* 2013). In the present study, the interactive effect of five significant factors (independent variables) viz. light intensity, pH, temperature, incubation period, inoculum concentration on five responses (dependent variables) viz. biomass, chlorophyll, carbohydrates, lipids, proteins production by strain BGLR18 were determined statistically using response surface methodology (RSM) based on central composite design (Table 4.28). Statgraphics Centurian XVI.I. was adopted for the optimization experiment. A matrix consisting of 24 experimental runs generated by the software was applied for maximizing the values of five response variables. The experimental design matrix, the observed and predicted desirability and observed values for responses are given in Table 4.29. The results obtained were analyzed by ANOVA (analysis of variance) which is required for testing the model significance are shown in Table 4.30-4.34. A considerable variation was observed for various response variable evaluated. Based on the predicted responses form the fitted model, the most desirable run corresponds to run 23.

Significance of the coefficients and interaction strength of variables was evaluated by the P-value and F value. If $P < 0.05$ (Low P value) then it indicates higher significance of the corresponding coefficients and vice-versa (Nazir *et al* 2018). As a general statistical rule, larger t, F and smaller P values indicate that the corresponding coefficient terms are significant (Li *et al* 2008). The number of effects shown in table 4.30-4.34 having P value less than 0.05 in biomass, chlorophyll, carbohydrate, lipids and proteins are 11, 18, 20, 20 and 16 respectively which showed that they are significantly different from zero at the 95.0% confidence level. In our study, five responses under study could be described well by CCD model with relatively high coefficient of determination as indicated by the ANOVA. 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, AD, AE, BC, BD and quadratic terms A^2 and E^2 were significant model terms. For chlorophyll content AB, AC, AE, BC, BD, BE, CD, DE interactive terms and A^2, B^2, C^2, D^2 and E^2 quadratic terms were found to be significant model terms. The interactive (AB, AC, AD, AE, BC, BD, BE, CD,

CE, DE) and quadratic terms (A^2, B^2, C^2, D^2 and E^2) acted as significant effects for carbohydrate. Similarly, for lipids the interactive terms AB, AC, AD, AE, BC, BD, BE, CD, CE, DE and quadratic (A^2, B^2, C^2, D^2, E^2) were significant model terms whereas for protein the significant model terms were interactive AB, AC, AD, AE, BC, BD, BE, CD, CE, DE and quadratic E^2. R^2 value is an estimate of amount of variability in the observed response can be explained by the experimental parameters and their interaction (Reddy *et al* 2008). The R^2 of the model for biomass, chlorophyll, carbohydrates, lipids and proteins were 92.77%, 99.92%, 99.98%, 99.99%, 98.17% respectively (Table 4.35). This can be interpreted as, that 92.77%, 99.92%, 99.98%, 99.99%, 98.17% of variability in the responses (biomass, chlorophyll, carbohydrates, lipids and proteins) respectively could be explained by the model and that only 7.23, 0.08, 0.02, 0.01, 1.83% of the total variance for the 1st five responses could not be explained by the model. The predicted and observed values of various responses in our study are very much close as can be seen in Fig. 4.3 (a-e). The values for all the five response variables almost fall on the line of fit, which indicates the significance and accuracy of the model. The adjusted R arranges the R values for the sample size and for the number of variables in the model (Kanmani *et al* 2012). The value of the adjusted R^2 (adjusted coefficient of determination) was 86.15%, 99.62%, 99.84%, 99.95% and 97.26% for biomass, chlorophyll, carbohydrate, lipid and protein respectively (Table 4.35).

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

Biomass = 1.29565 - 0.610702*A + 0.0736193*B + 0.000125052*C - 0.00439531*D + 0.0978015*E + 0.0390207*A^2 - 0.00401563*A*B + 0.00160937*A*D - 0.00629861*A*E - 0.00000359063*B*C - 0.000363125*B*D - 0.00510084*E^2

Chlorophyll = -72.8996 + 12.1148*A + 2.14682*B + 0.0100645*C - 2.37115*D - 1.45716*E - 0.68719*A^2 + 0.0105268*A*B + 0.0000369997*A*C + 0.064196*A*E - 0.0369959*B^2 - 0.0000227787*B*C + 0.00187499*B*D - 0.00339808*B*E - 7.85822E-7*C^2 - 0.00000735194*C*D + 0.0384885*D^2 + 0.00347225*D*E + 0.0803761*E^2

Carbohydrate = -2786.73 - 514.214*A + 125.028*B + 2.01452*C - 50.7535*D - 190.614*E + 16.0797*A^2 + 13.0095*A*B - 0.0248313*A*C - 5.55375*A*D + 17.6044*A*E - 3.5067*B^2 - 0.00979063*B*C - 0.278125*B*D + 1.38772*B*E - 0.000123793*C^2 + 0.00233362*C*D - 0.00850694*C*E + 1.57671*D^2 - 2.0625*D*E + 11.1882*E^2

Lipid = -212.058 + 175.122*A - 54.7675*B - 0.201715*C + 64.7322*D + 36.3778*E - 12.5186*A^2 + 0.87545*A*B + 0.0014985*A*C + 0.694575*A*D -

$0.687806*A*E + 0.604087*B^2 + 0.0005533*B*C + 0.170485*B*D - 0.166161*B*E + 0.0000127047*C^2 + 0.000522675*C*D + 0.000256972*C*E - 1.30887*D^2 - 0.344606*D*E - 1.40349*E^2$

Protein = $-148.252 + 9.49051*A + 2.67834*B + 0.00440997*C + 3.19957*D + 0.754104*E - 0.278538*A*B + 0.00162818*A*C - 0.318926*A*D - 0.712183*A*E - 0.000286905*B*C + 0.0518064*B*D + 0.122767*B*E - 0.000323768*C*D - 0.000717109*C*E + 0.143162*D*E + 0.326097*E^2$

Hence, according to the results obtained from the model for different responses investigated in the study and also from the above mentioned regression equations, pH of 10.02, temperature of 20.00°C, the light intensity of 7599.41 Lux for a growth period of 21.75 day and inoculum concentration of 1.00% were found to be the the optimal and most desired conditions (Table 4.36).

Model validation

Further experiments were carried out to check the validity of the model under the estimated optimum conditions generated by the software. So, a validation experiment was conducted by cultivating BGLR18 under optimal conditions (pH of 10.02, 20.00 °C of temperature, the light intensity of 7599.41 Lux, 21.75 days at inoculum concentration of 1%) suggested by the model in the BBM medium. The experimental value for different responses (biomass, chlorophyll, carbohydrate, lipid and protein) was found to be 0.20 gL^{-1}, 7.79 mgL^{-1}, 925mgL^{-1}, 109mgL^{-1} and 1.37mgL^{-1} respectively. The values of responses obtained are close to that obtained from CCD of RSM and model predicted values at optimum.

4.2.1.1 3D Response surface plots for various interactions of cultural factors for biomass production

i) pH versus temperature

The interaction between pH and temperature was found to be significant as it showed the p-value of 0.00. It is obvious from the fig. 4.4 a that high pH (9.5 to 10) and low temperature (20-24°C) has favourable effect on biomass productivity by BGLR18 biomass. Biomass productivity decrease on lower pH values (7.5) and at high temperature values (40°C) (Fig. 4.4 a).

ii) pH versus growth period

The interaction between pH and growth period was significant. High pH and longer incubation period has positive effect on biomass production (Fig.4.4 b)

iii) pH versus inoculum concentration

Lower p-value of 0.00 indicates the significant interaction between pH and inoculum concentration keeping all other independent variable constant at optimum levels. As shown in fig. 4.4 c it is clear that high pH (9.5 to 10) and lower inoculum concentration (1 to 2%) tends

to have a positive effect on response (dependent) variable i.e. biomass.

iv) Temperature versus light intensity

The interaction between temperature and light intensity showed a significant interaction as shown in table 4.30 (p-value =0.00). Fig 4.4 d revealed that low temperature (20 to 24◦C) and high light intensity (8000 Lux) is most favourable and produce more biomass if all other independent variables are kept fixed at optimum levels.

v) Temperature versus growth period

Interaction between temperature and growth period was significant (table 4.30) (fig. 4.4 e). Lower temperature (20 to 24°C) and lower growth period produce a favourable effect on biomass productivity. A slight decrease in biomass productivity was observed when growth period was extended to 40 days.

Table 4.28 Different factors (independent variables) used in the study

Name of the independent factor	Units	Low	High	Levels
pH	-	7.50	11.50	-1, 0, +1
Light	Lux	4000.00	8000.00	-1, 0, +1
Temperature	◦C	20.00	40.00	-1, 0, +1
Inoculum concentration	%	1.00	10.00	-1, 0, +1
Growth period	Days	20.00	40.00	-1, 0, +1

Where -1= lowest level; 0= middle level and +1= highest level

Table 4.29 : Central Composite Design (CCD) and response values for different responses

Run	pH	Temperature (°C)	Light (Lux)	Growth period (Days)	Inoculum concentration (%)	Biomass (g/L)	Chlorophyll (mg/L)	Carbohydrate (mg/L)	Lipid (mg/L)	Protein (mg/L)	Observed desirability	Predicted desirability
1.00	11.50	40.00	8000.00	20.00	1.00	0.45	1.00	35.00	5.23	7.89	0.14	0.14
2.00	7.50	40.00	4000.00	40.00	10.00	0.30	1.19	0.00	3.33	95.87	0.16	0.16
3.00	11.50	20.00	8000.00	40.00	1.00	0.67	1.30	217.98	49.88	3.50	0.25	0.26
4.00	11.50	20.00	4000.00	20.00	1.00	0.34	1.27	57.98	62.56	0.33	0.20	0.20
5.00	7.50	20.00	8000.00	40.00	10.00	0.23	2.79	1100.00	81.00	3.12	0.32	0.32
6.00	11.50	40.00	8000.00	40.00	10.00	0.12	1.62	103.00	54.00	0.89	0.17	0.17
7.00	7.50	40.00	8000.00	40.00	1.00	0.23	0.80	109.00	56.67	0.40	0.17	0.17
8.00	7.50	40.00	8000.00	20.00	10.00	0.32	0.17	53.00	54.00	0.61	0.12	0.13
9.00	7.50	30.00	6000.00	30.00	5.50	0.34	3.40	980.00	130.00	1.65	0.38	0.38
10.00	11.50	40.00	4000.00	40.00	1.00	0.45	0.70	0.00	57.43	0.00	0.18	0.18
11.00	9.50	40.00	6000.00	30.00	5.50	0.23	1.68	267.00	193.88	7.09	0.30	0.30
12.00	11.50	20.00	8000.00	20.00	10.00	0.34	4.59	879.00	64.99	2.21	0.35	0.35
13.00	9.50	20.00	6000.00	30.00	5.50	0.14	3.01	709.00	245.55	0.00	0.32	0.32
14.00	9.50	30.00	8000.00	30.00	5.50	0.35	3.12	401.00	209.00	0.00	0.36	0.34
15.00	11.50	40.00	4000.00	20.00	10.00	0.12	1.90	1515.00	7.00	0.00	0.16	0.16
16.00	9.50	30.00	6000.00	30.00	5.50	0.24	6.01	815.00	160.00	0.00	0.39	0.39
17.00	9.50	30.00	6000.00	40.00	5.50	0.23	9.59	853.00	19.88	3.21	0.29	0.30
18.00	11.50	20.00	4000.00	40.00	10.00	0.21	2.20	107.00	21.98	2.23	0.18	0.17
19.00	9.50	30.00	4000.00	30.00	5.50	0.25	2.68	286.00	211.25	5.57	0.34	0.35
20.00	7.50	20.00	8000.00	20.00	1.00	0.23	4.38	1360.00	101.32	1.79	0.37	0.37
21.00	7.50	20.00	4000.00	40.00	1.00	0.06	2.34	700.00	98.00	1.79	0.21	0.20
22.00	7.50	40.00	4000.00	20.00	1.00	0.56	2.19	1.90	36.43	0.00	0.22	0.22
23.00	9.50	30.00	6000.00	30.00	1.00	0.21	7.50	940.00	128.00	1.65	0.40	0.40
24.00	7.50	20.00	4000.00	20.00	10.00	0.03	2.34	775.90	226.00	0.54	0.15	0.00

Table 4.30 Analysis of variance (ANOVA) and regression coefficients for the quadratic model for various independent variables for BGLR18 biomass

Source	Sum of squares	Df	Mean - square	F-ratio	P-value	Regression coefficients
A:pH	0.04	1.00	0.04	12.10	0.00	-0.61
B: Temperature	0.02	1.00	0.02	5.35	0.04	0.07
C:Light intensity	0.02	1.00	0.02	6.99	0.02	0.00
E: Inoculum concentration	0.11	1.00	0.11	35.41	0.00	0.10
AA	0.04	1.00	0.04	13.40	0.00	0.04
AB	0.10	1.00	0.10	33.35	0.00	0.00
AD	0.02	1.00	0.02	5.36	0.04	0.00
AE	0.05	1.00	0.05	16.62	0.00	-0.01
BC	0.08	1.00	0.08	26.67	0.00	0.00
BD	0.02	1.00	0.02	6.82	0.02	0.00
EE	0.02	1.00	0.02	5.87	0.03	-0.01
Total error	0.04	12.00	0.00			
Total (corr.)	0.51	23.00				
Constant						1.30

Table 4.31 Analysis of variance (ANOVA) and regression coefficients for the quadratic model for various independent variables for BGLR18 chlorophyll

Source	Sum of squares	Df	Mean - square	F-ratio	P-value	Regression coefficients
A:pH	0.17	1.00	0.17	8.86	0.03	12.11
B: Temperature	9.34	1.00	9.34	496.76	0.00	2.15
C:Light intensity	0.49	1.00	0.49	25.87	0.00	0.01
D: Growth period	1.50	1.00	1.50	79.93	0.00	-2.37
E: Inoculum concentration	0.50	1.00	0.50	26.37	0.00	-1.46
AA	9.48	1.00	9.48	504.21	0.00	-0.69
AB	0.71	1.00	0.71	37.71	0.00	0.01
AC	0.35	1.00	0.35	18.64	0.01	0.00
AE	5.34	1.00	5.34	284.02	0.00	0.06
BB	27.37	1.00	27.37	1455.67	0.00	-0.04
BC	3.32	1.00	3.32	176.59	0.00	0.00
BD	0.56	1.00	0.56	29.91	0.00	0.00
BE	0.37	1.00	0.37	19.89	0.01	0.00
CC	19.76	1.00	19.76	1050.81	0.00	0.00
CD	0.35	1.00	0.35	18.40	0.01	0.00
DD	18.59	1.00	18.59	988.55	0.00	0.04
DE	0.39	1.00	0.39	20.77	0.01	0.00
EE	3.32	1.00	3.32	176.78	0.00	0.08
Total error	0.09	5.00	0.02			
Total (corr.)	115.03	23.00				
Constant						-72.90

Table 4.32 Analysis of variance (ANOVA) and regression coefficients for the quadratic model for various independent variables for BGLR18 carbohydrate

Source	Sum of squares	Df	Mean - square	F-ratio	P-value	Regression coefficients
A:pH	88795.20	1.00	88795.20	249.95	0.00	-514.21
B: Temperature	811946.00	1.00	811946.00	2285.55	0.00	125.03
C: Light intensity	36829.00	1.00	36829.00	103.67	0.00	2.01
D: Growth period	341977.00	1.00	341977.00	962.63	0.00	-50.75
E: Inoculum concentration	262386.00	1.00	262386.00	738.59	0.00	-190.61
AA	5191.41	1.00	5191.41	14.61	0.03	16.08
AB	1083180.00	1.00	1083180.00	3049.05	0.00	13.01
AC	157847.00	1.00	157847.00	444.33	0.00	-0.02
AD	197402.00	1.00	197402.00	555.67	0.00	-5.55
AE	401652.00	1.00	401652.00	1130.61	0.00	17.60
BB	245939.00	1.00	245939.00	692.29	0.00	-3.51
BC	613481.00	1.00	613481.00	1726.89	0.00	-0.01
BD	12376.60	1.00	12376.60	34.84	0.01	-0.28
BE	62395.00	1.00	62395.00	175.64	0.00	1.39
CC	490387.00	1.00	490387.00	1380.39	0.00	0.00
CD	34853.20	1.00	34853.20	98.11	0.00	0.00
CE	93789.10	1.00	93789.10	264.01	0.00	-0.01
DD	31197.10	1.00	31197.10	87.82	0.00	1.58
DE	137827.00	1.00	137827.00	387.97	0.00	-2.06
EE	64414.10	1.00	64414.10	181.32	0.00	11.19
Total error	1065.76	3.00	355.25			
Total (corr.)	5092040.00	23.00				
Constant						-2.786.73

Table 4.33 Analysis of variance (ANOVA) and regression coefficients for the quadratic model for various independent variables for BGLR18 lipid

Source	Sum of squares	Df	Mean – square	F-ratio	P-value	Regression coefficients
A:pH	6973.83	1.00	6973.83	2579.84	0.00	175.12
B: Temperature	12977.40	1.00	12977.40	4800.73	0.00	-54.77
C: Light intensity	127.44	1.00	127.44	47.14	0.01	-0.20
D: Growth period	1149.34	1.00	1149.34	425.18	0.00	64.73
E: Inoculum concentration	126.61	1.00	126.61	46.84	0.01	36.38
AA	3146.60	1.00	3146.60	1164.02	0.00	-12.52
AB	4905.04	1.00	4905.04	1814.53	0.00	0.88
AC	574.85	1.00	574.85	212.65	0.00	0.00
AD	3087.58	1.00	3087.58	1142.19	0.00	0.69
AE	613.11	1.00	613.11	226.81	0.00	-0.69
BB	7298.41	1.00	7298.41	2699.91	0.00	0.60
BC	1959.30	1.00	1959.30	724.81	0.00	0.00
BD	4650.42	1.00	4650.42	1720.34	0.00	0.17
BE	894.55	1.00	894.55	330.92	0.00	-0.17
CC	5165.07	1.00	5165.07	1910.72	0.00	0.00
CD	1748.41	1.00	1748.41	646.79	0.00	0.00
CE	85.58	1.00	85.58	31.66	0.01	0.00
DD	21498.20	1.00	21498.20	7952.86	0.00	-1.31
DE	3847.60	1.00	3847.60	1423.35	0.00	-0.34
EE	1013.62	1.00	1013.62	374.97	0.00	-1.40
Total error	8.11	3.00	2.70			
Total (corr.)	131740.00	23.00				
Constant						-212.06

Table 4.34 Analysis of variance (ANOVA) and regression coefficients for the quadratic model for various independent variables for BGLR18 protein

Source	Sum of squares	Df	Mean - square	F-ratio	P-value	Regression coefficients
A:pH	449.26	1.00	449.26	43.99	0.00	9.49
B: Temperature	525.28	1.00	525.28	51.43	0.00	2.68
C: Light intensity	410.10	1.00	410.10	40.15	0.00	0.00
D: Growth period	545.25	1.00	545.25	53.39	0.00	3.20
E: Inoculum concentration	536.58	1.00	536.58	52.54	0.00	0.75
AB	496.54	1.00	496.54	48.62	0.00	-0.28
AC	678.65	1.00	678.65	66.45	0.00	0.00
AD	650.97	1.00	650.97	63.74	0.00	-0.32
AE	657.34	1.00	657.34	64.36	0.00	-0.71
BC	526.81	1.00	526.81	51.58	0.00	0.00
BD	429.43	1.00	429.43	42.05	0.00	0.05
BE	488.33	1.00	488.33	47.81	0.00	0.12
CD	670.88	1.00	670.88	65.69	0.00	0.00
CE	666.46	1.00	666.46	65.25	0.00	0.00
DE	664.05	1.00	664.05	65.02	0.00	0.14
EE	213.41	1.00	213.41	20.90	0.00	0.33
Total error	71.49	7.00	71.49			
Total (corr.)	8569.65	23.00	10.21			
Constant						-148.25

Table 4.35 Analysis of the experimental results of Central Composite Design

Model	Biomass	Chlorophyll	Carbohydrate	Lipid	Protein
Transformation	None	None	None	None	None
Model d.f.	11.00	18.00	20.00	20.00	16.00
P-value	0.00	0.00	0.00	0.00	0.00
Error d.f.	12.00	5.00	3.00	3.00	7.00
Stnd. error	0.06	0.14	18.85	1.64	3.20
R-squared	92.77	99.92	99.98	99.99	99.17
Adj. R-squared	86.15	99.62	99.84	99.95	97.26

Table 4.36 Factor setting at optimum

Factors	
pH	10.02
Temperature (◦C)	20.00
Light intensity (LUX)	7599.41
Incubation period (Days)	21.75
Inoculum concentration (%)	1.00

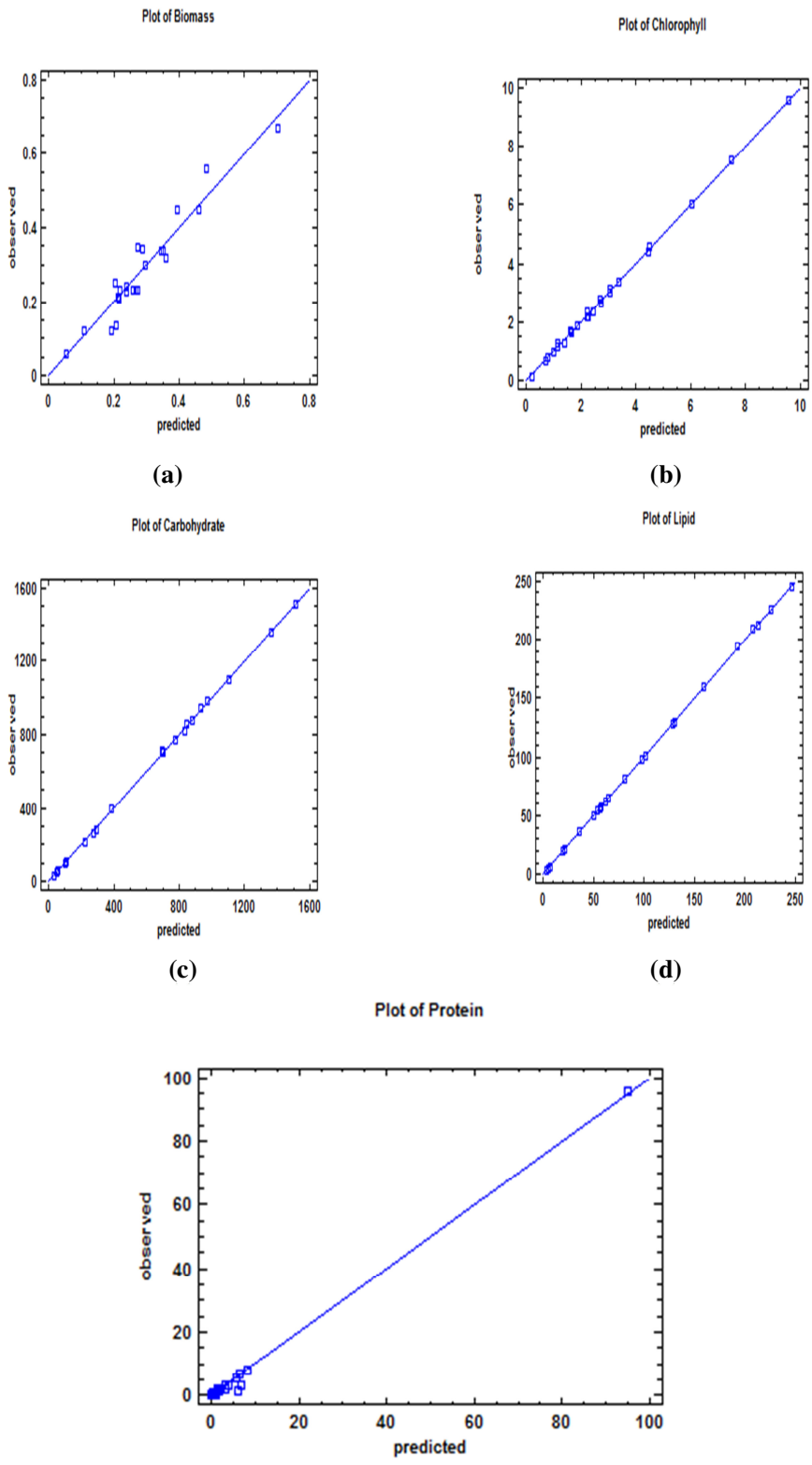
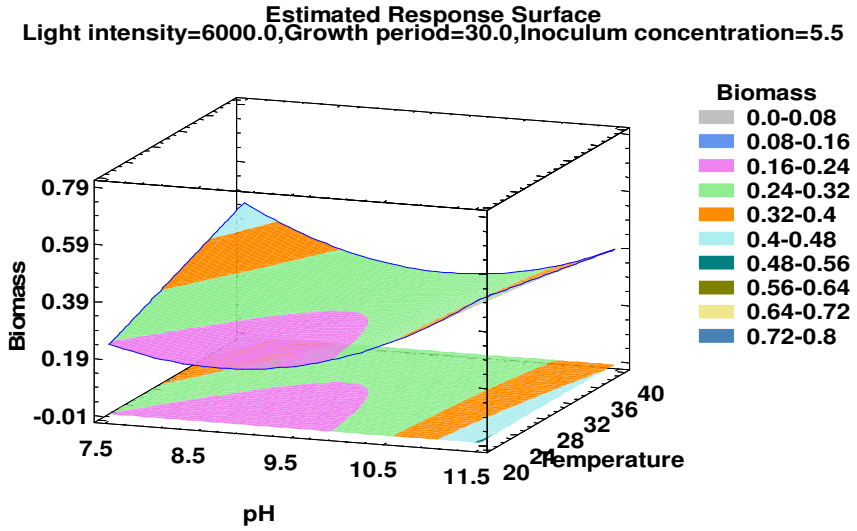
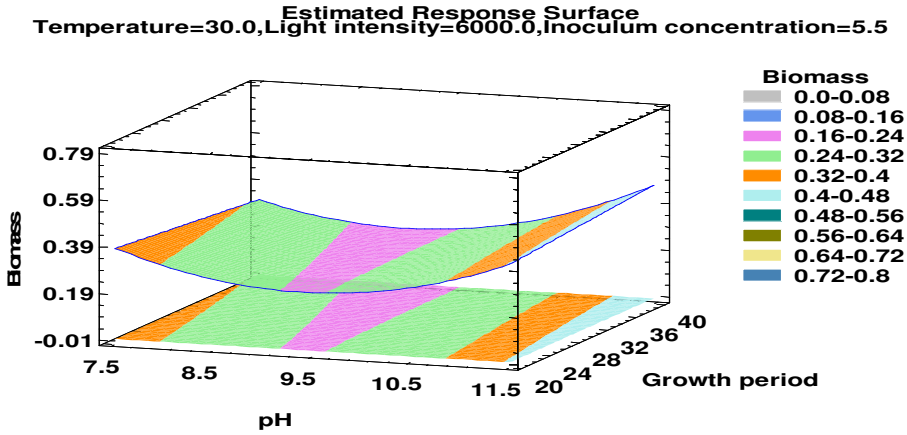


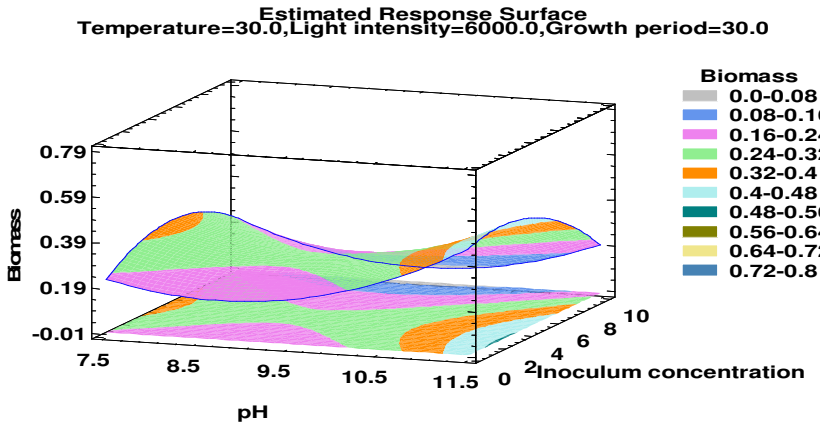
Fig 4.3 Predicted versus observed results of various response variables a). Biomass b). Chlorophyll c). Carbohydrate d). Lipid e). Protein


Estimated Response Surface
Light intensity=6000.0,Growth period=30.0,Inoculum concentration=5.5
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0.79
0.59
0.39
0.19
-0.01
7.5
8.5
9.5
10.5
11.5
pH
20
24
28
32
36
40
Temperature

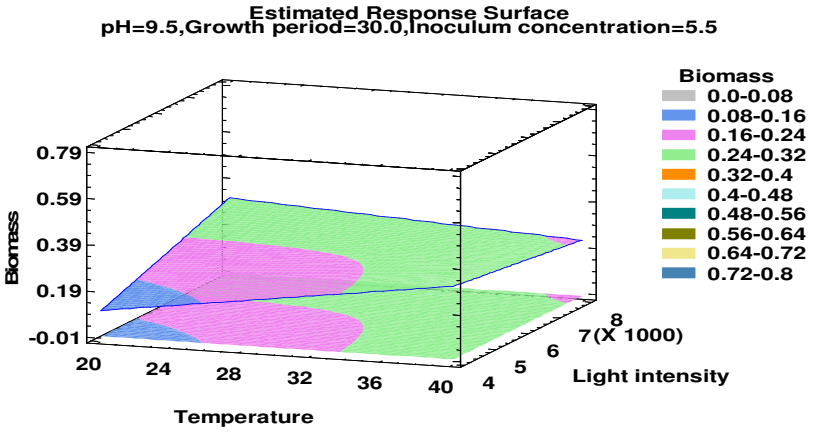
(a)


Estimated Response Surface
Temperature=30.0,Light intensity=6000.0,Inoculum concentration=5.5
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0.79
0.59
0.39
0.19
-0.01
7.5
8.5
9.5
10.5
11.5
pH
20
24
28
32
36
40
Growth period

(b)


Estimated Response Surface
Temperature=30.0,Light intensity=6000.0,Growth period=30.0
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0.79
0.59
0.39
0.19
-0.01
7.5
8.5
9.5
10.5
11.5
pH
0
2
4
6
8
10
Inoculum concentration

(c)



(d)

(e)

Fig. 4.4 3D Response surface plots for various interactions of independent variables for BGLR18 biomass (a-e)

4.2.2 Optimization of cultural conditions of BGLRS

Microalgal strain BGLRS was studied for optimization by RSM using CCD. A matrix consisting of 24 experimental runs generated by the software was applied for maximizing the values of five response variables. The experimental design matrix, the observed and predicted desirability and observed values for responses are given in Table 4.37. The results obtained were analyzed by ANOVA (analysis of variance) which is required for testing the model significance are shown in Table 4.38-4.42. A considerable variation was observed for various response variable evaluated. Based on the predicted responses form the fitted model, the most desirable run corresponds to run 18.

The number of effects shown in table 4.38-4.42 having P value less than 0.05 in biomass, chlorophyll, carbohydrate, lipids and proteins are 20, 17, 15, 18 and 15 respectively which showed that they are significantly different from zero at the 95.0% confidence level. In our study, five responses under study could be described well by CCD model with relatively high coefficient of determination as indicated by the ANOVA. The effect of five variables namely pH, temperature, light intensity, growth period, incubation period were significant as their p-value were less than 0.05 (Table 4.38-4.42). 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, AE, BC, BD, BE, CD, CE, DE and quadratic terms A^2, B^2, C^2, D^2 and E^2 were significant model terms. For chlorophyll content AB, AE, BC, BD, BE, CD, DE interactive terms and A^2, B^2, C^2, D^2 and E^2 quadratic terms were found to be significant model terms. The interactive (AC, AD, AE, BE, CE, DE) and quadratic terms (B^2, C^2, D^2 and E^2) acted as significant effects for carbohydrate. Similarly, for lipids the

interactive terms AB, AC, AD, AE, BC, BD, BE, CD, DE and quadratic (B^2, C^2, D^2, E^2) were significant model terms whereas for protein the significant model terms were interactive AB, AC, AD, AE, BC, BD, BE, CD, CE, DE. The R^2 of the model for biomass, chlorophyll, carbohydrates, lipids and proteins were 99.88%, 99.81%, 99.14%, 99.08%, 96.40% respectively (Table 4.43). This can be interpreted as, that of 99.88%, 99.81%, 99.14%, 99.08%, 96.40% variability in the responses (biomass, chlorophyll, carbohydrates, lipids and proteins) respectively could be explained by the model and that only 0.12, 0.19, 0.86, 0.92, 3.6% of the total variance for the 1st five responses could not be explained by the model. The predicted and observed values of various responses in our study are very much close as can be seen in Fig. 4.5 (a-e). The value of the Adjusted R^2 (adjusted coefficient of determination) was 99.06% for biomass, 99.28%, 97.53%, 95.79% and 89.65% for chlorophyll, carbohydrate, lipid and protein respectively (Table 4.43).The final regression equations for the biomass production, chlorophyll, carbohydrate, lipid and protein, which have been fitted to the data are given below:

Biomass = $1.29565 - 0.610702*A + 0.0736193*B + 0.000125052*C - 0.00439531*D + 0.0978015*E + 0.0390207*A^2 - 0.00401563*A*B + 0.00160937*A*D - 0.00629861*A*E - 0.00000359063*B*C - 0.000363125*B*D - 0.00510084*E^2$

Chlorophyll = $-72.8996 + 12.1148*A + 2.14682*B + 0.0100645*C - 2.37115*D - 1.45716*E - 0.68719*A^2 + 0.0105268*A*B + 0.0000369997*A*C + 0.064196*A*E - 0.0369959*B^2 - 0.0000227787*B*C + 0.00187499*B*D - 0.00339808*B*E - 7.85822E-7*C^2 - 0.00000735194*C*D + 0.0384885*D^2 + 0.00347225*D*E + 0.0803761*E^2$

Carbohydrate = $-2786.73 - 514.214*A + 125.028*B + 2.01452*C - 50.7535*D - 190.614*E + 16.0797*A^2 + 13.0095*A*B - 0.0248313*A*C - 5.55375*A*D + 17.6044*A*E - 3.5067*B^2 - 0.00979063*B*C - 0.278125*B*D + 1.38772*B*E - 0.000123793*C^2 + 0.00233362*C*D - 0.00850694*C*E + 1.57671*D^2 - 2.0625*D*E + 11.1882*E^2$

Lipid = $-212.058 + 175.122*A - 54.7675*B - 0.201715*C + 64.7322*D + 36.3778*E - 12.5186*A^2 + 0.87545*A*B + 0.0014985*A*C + 0.694575*A*D - 0.687806*A*E + 0.604087*B^2 + 0.0005533*B*C + 0.170485*B*D - 0.166161*B*E + 0.0000127047*C^2 + 0.000522675*C*D + 0.000256972*C*E - 1.30887*D^2 - 0.344606*D*E - 1.40349*E^2$

Protein = $-148.252 + 9.49051*A + 2.67834*B + 0.00440997*C + 3.19957*D + 0.754104*E - 0.278538*A*B + 0.00162818*A*C - 0.318926*A*D - 0.712183*A*E - 0.000286905*B*C + 0.0518064*B*D + 0.122767*B*E - 0.000323768*C*D - 0.000717109*C*E + 0.143162*D*E + 0.326097*E^2$

Hence, according to the results obtained from the model for different responses investigated in the study and also form the above mentioned regression equations, pH of 11.17, temperature of 26.03 °C, the light intensity of 4000.02 Lux for a growth period of 39.37 days and inoculum concentration of 10.00% were found to be the the optimal and most desired conditions (Table 4.44).

Model validation

Further experiments were carried out to check the validity of the model under the estimated optimum conditions generated by the software. So, a validation experiment was conducted by cultivating BGLRS under optimal conditions (pH of 11.17, 26.03 °C of temperature, the light intensity of 4000.02 Lux, 39.37 days at inoculum concentration of 10.00%) suggested by the model in the ACM medium. The experimental value for different responses (biomass, chlorophyll, carbohydrate, lipid and protein) was found to be 0.712gL^{-1}, 3.450 mgL^{-1}, 1450 mgL^{-1}, 312.0 mgL^{-1} and 8.012 mgL^{-1} respectively. The values of responses obtained are close to that obtained from CCD of RSM and model predicted values at optimum.

4.2.2.1 3D Response surface plots for various interactions of cultural factors for biomass production

i) pH versus temperature

The interaction between pH and temperature showed a significant p-value of 0.00 (table 4.38) (Fig. 4.6 a). High pH (10.5 to 11) and lower temperature range (26-28°C) was conducive for higher biomass productivity of BGLRS biomass keeping all other factors constant at optimum. Also a decrease biomass content was evidenced at lower pH and higher temperature.

ii) pH versus light intensity

In case of interaction between pH and light intensity, higher pH (10.5 to 11) and lower light intensity (4000 Lux) was proved to beneficial for higher biomass productivity of BGLRS strain (fig 4.6 b).

iii) pH versus growth period

As shown in Fig. 4.8 c) the interaction between the two is favourable for BGLRS biomass. It showed the lower p-value of 0.00. Higher pH (10.5 to 11) and longer growth period (40 days) has beneficial effect on higher biomass productivity. It is evident from the fig 4.6c that biomass production also increases with increase in both pH and growth period.

iv) pH versus inoculum concentration

Similarly, in case of interaction between pH and inoculum concentration higher pH (10.5 to 11) range and higher inoculum concentration (10%) provide conducive conditions for maximum biomass productivity (fig. 4.6 d). It was observed that with increase in pH and inoculum concentration biomass productivity increased sharply.

v) Temperature versus light intensity

A favourable and significant interaction between temperature and light intensity was observed (Fig 4.6 e). Lower temperature range (24 to 26 °C) and high light intensity (8000 lux) showed positive effect on biomass.

vi) Temperature versus growth period

As obvious from fig 4.6 f) that lower temperature showed favourable effect on biomass productivity. Also, biomass production increased when growth period was extended to 40 days.

vii) Temperature versus inoculum concentration

The p-value (0.00) of interaction between temperature and inoculum concentration (table 4.40) indicated a significant interaction. Lower temperature (20-24°C) range and higher inoculum concentration (10%) increased the biomass values (fig.4.6 g).

viii) Light intensity versus growth period

In another interaction study between light intensity and growth period lower light intensity (4000 Lux) and longer growth period was advantageous for biomass (fig. 4.6 h).

Table 4.37 Central Composite Design (CCD) and response values for different responses

Run	pH	Temperature (◦C)	Light (Lux)	Growth period (Days)	Inoculum concentration (%)	Biomass (g/L)	Chlorophyll (mg/L)	Carbohydrate (g/L)	Lipid (g/L)	Protein (g/L)	Observed desirability	Predicted desirability
1.00	11.50	40.00	8000.00	20.00	1.00	0.50	0.98	25.00	15.00	7.78	0.22	0.21
2.00	7.50	40.00	4000.00	40.00	10.00	0.28	1.22	0.00	4.00	95.00	0.26	0.24
3.00	11.50	20.00	8000.00	40.00	1.00	0.60	1.30	170.00	48.00	3.45	0.27	0.26
4.00	11.50	20.00	4000.00	20.00	1.00	0.30	1.22	98.00	57.00	0.30	0.22	0.23
5.00	7.50	20.00	8000.00	40.00	10.00	0.24	2.49	1157.00	82.00	3.01	0.34	0.33
6.00	11.50	40.00	8000.00	40.00	10.00	0.13	1.60	101.00	57.00	0.88	0.19	0.20
7.00	7.50	40.00	8000.00	40.00	1.00	0.22	0.87	56.00	83.00	0.43	0.20	0.20
8.00	7.50	40.00	8000.00	20.00	10.00	0.30	0.26	45.00	62.00	0.60	0.17	0.17
9.00	7.50	30.00	6000.00	30.00	5.50	0.32	3.39	1135.00	132.00	1.62	0.40	0.44
10.00	11.50	40.00	4000.00	40.00	1.00	0.41	0.69	0.00	58.03	0.00	0.20	0.19
11.00	9.50	40.00	6000.00	30.00	5.50	0.26	1.69	325.00	192.88	6.60	0.31	0.31
12.00	11.50	20.00	8000.00	20.00	10.00	0.19	4.56	914.00	65.00	2.10	0.33	0.33
13.00	9.50	20.00	6000.00	30.00	5.50	0.18	3.02	817.00	278.00	0.00	0.36	0.37
14.00	9.50	30.00	8000.00	30.00	5.50	0.29	3.00	1616.00	209.00	0.00	0.42	0.42
15.00	11.50	40.00	4000.00	20.00	10.00	0.24	1.89	0.00	6.88	0.00	0.18	0.18
16.00	9.50	30.00	6000.00	30.00	5.50	0.26	6.00	1082.00	158.00	0.00	0.42	0.45
17.00	9.50	30.00	6000.00	40.00	5.50	0.22	9.40	140.00	18.96	3.28	0.29	0.31
18.00	11.50	20.00	4000.00	40.00	10.00	0.70	3.35	1479.00	298.00	7.99	0.59	0.58
19.00	9.50	30.00	4000.00	30.00	5.50	0.31	2.53	1415.00	231.00	5.68	0.44	0.47
20.00	7.50	20.00	8000.00	20.00	1.00	0.23	4.38	702.00	97.08	1.87	0.35	0.33
21.00	7.50	20.00	4000.00	40.00	1.00	0.05	2.40	7.09	97.00	1.57	0.16	0.15
22.00	7.50	40.00	4000.00	20.00	1.00	0.55	2.21	34.01	38.09	0.00	0.25	0.25
23.00	9.50	30.00	6000.00	30.00	1.00	0.22	7.67	1184.00	128.77	1.62	0.43	0.42
24.00	7.50	20.00	4000.00	20.00	10.00	0.02	2.20	800.03	25.00	0.60	0.10	0.00

ix) Light intensity versus inoculum concentration

Fig. 4.6 i showed interaction between light intensity and inoculums concentration. In this interaction study, higher biomass productivity was favoured by lower light intensity and and higher inoculums concentration.

x) Growth period versus inoculum concentration

Higher growth period (40 days) and higher inoculum concentration (10%) favoured maximum biomass in case of the interaction between growth period and inoculums concentration (Fig 4.6 j) when all other factors are kept fixed at optimum levels. Biomass increase was observed with increased growth period and increased inoculum concentration

Table 4.38 Analysis of variance (ANOVA) and regression coefficients for the quadratic model for various independent variables for BGLRS biomass

Source	Sum of squares	Df	Mean - square	F-ratio	P-value	Regression coefficients
A:pH	0.09	1.00	0.09	357.15	0.00	-0.60
B: Temperature	0.01	1.00	0.01	33.92	0.01	0.10
C:Light intensity	0.00	1.00	0.00	5.99	0.09	0.00
D: Growth period	0.01	1.00	0.01	23.78	0.02	0.25
E: Inoculum concentration	0.04	1.00	0.04	152.49	0.00	0.07
AA	0.03	1.00	0.03	117.39	0.00	0.04
AB	0.11	1.00	0.11	448.08	0.00	0.00
AC	0.01	1.00	0.01	24.32	0.02	0.00
AD	0.05	1.00	0.05	221.71	0.00	0.00
AE	0.01	1.00	0.01	29.63	0.01	0.00
BB	0.00	1.00	0.00	5.89	0.09	0.00
BC	0.02	1.00	0.02	64.56	0.00	0.00
BD	0.12	1.00	0.12	499.95	0.00	0.00
BE	0.03	1.00	0.03	126.30	0.00	0.00
CC	0.01	1.00	0.01	25.93	0.01	0.00
CD	0.01	1.00	0.01	32.93	0.01	0.00
CE	0.02	1.00	0.02	96.04	0.00	0.00
DD	0.00	1.00	0.00	8.94	0.06	0.00
DE	0.05	1.00	0.05	206.71	0.00	0.00
EE	0.01	1.00	0.01	29.57	0.01	0.00
Total error	0.00	3.00	0.00			
Total (corr.)	0.60	23.00				
Constant						0.93

Table 4.39 Analysis of variance (ANOVA) and regression coefficients for the quadratic model for various independent variables for BGLRS Chlorophyll

Source	Sum of squares	Df	Mean - square	F-ratio	P-value	Regression coefficients
A: pH	0.11	1.00	0.11	0.32	0.59	11.98
B: Temperature	10.15	1.00	10.15	285.99	0.00	2.17
C:Light intensity	0.17	1.00	0.17	4.67	0.07	0.01
D: Growth period	0.90	1.00	0.90	25.27	0.00	-2.18
E: Inoculum concentration	0.78	1.00	0.78	21.92	0.00	-1.70
AA	8.78	1.00	8.78	247.37	0.00	-0.66
AB	0.17	1.00	0.17	4.78	0.07	0.01
AE	7.42	1.00	7.42	208.96	0.00	0.08
BB	26.69	1.00	26.69	751.69	0.00	-0.37
BC	2.16	1.00	2.16	60.76	0.00	0.00
BD	0.22	1.00	0.22	6.16	0.05	0.00
BE	0.60	1.00	0.60	16.78	0.01	0.00
CC	21.02	1.00	21.02	592.06	0.00	0.00
CD	1.03	1.00	1.03	28.98	0.00	0.00
DD	16.51	1.00	16.51	465.17	0.00	0.04
DE	0.67	1.00	0.67	18.97	0.00	0.00
EE	4.44	1.00	4.44	125.12	0.00	0.09
Total error	0.21	6.00	0.04			
Total (corr.)	113.56	23.00				
Constant						-76.81

Table 4.40 Analysis of variance (ANOVA) and regression coefficients for the quadratic model for various independent variables for BGLRS Carbohydrate

Source	Sum of squares	Df	Mean - square	F-ratio	P-value	Regression coefficients
A: pH	179.39	1.00	179.39	0.02	0.88	-1.14
B: Temperature	1715690.00	1.00	1715690.00	214.85	0.00	305.26
C: Light intensity	50442.30	1.00	50442.30	6.32	0.04	-1.00
D: Growth period	7420.33	1.00	7420.33	0.93	0.36	537.70
E: Inoculum concentration	733811.00	1.00	733811.00	91.89	0.00	-79.34
AC	137988.00	1.00	137988.00	17.28	0.00	-0.02
AD	72085.00	1.00	72085.00	9.03	0.02	3.36
AE	62259.00	1.00	62259.00	7.80	0.02	6.93
BB	557859.00	1.00	557859.00	69.86	0.00	-5.18
BE	698039.00	1.00	698039.00	87.41	0.00	-4.64
CC	378416.00	1.00	378416.00	47.39	0.00	0.00
CE	47953.30	1.00	47953.30	6.01	0.04	-0.01
DD	1317800.00	1.00	1317800.00	165.02	0.00	-9.66
DE	160779.00	1.00	160779.00	20.13	0.00	2.23
EE	137955.00	1.00	137955.00	17.28	0.00	15.44
Total error	63883.90	8.00	7985.49			
Total (corr.)	7444040.00	23.00				
Constant						-8102.51

Table 4.41 Analysis of variance (ANOVA) and regression coefficients for the quadratic model for various independent variables for BGLRS Lipid

Source	Sum of squares	Df	Mean - square	F-ratio	P-value	Regression coefficients
A:pH	1272.17	1.00	1272.17	4.18	0.10	21.17
B: Temperature	15617.30	1.00	15617.30	51.27	0.00	-37.02
C: Light intensity	517.56	1.00	517.56	1.70	0.25	-0.10
D: Growth period	8635.84	1.00	8635.84	28.35	0.00	108.02
E: Inoculum concentration	598.08	1.00	598.08	1.96	0.22	-2.68
AB	2945.78	1.00	2945.78	9.67	0.03	-0.68
AC	9746.63	1.00	9746.63	31.99	0.00	-0.01
AD	4668.99	1.00	4668.99	15.33	0.01	0.85
AE	9556.04	1.00	9556.04	31.37	0.00	2.72
BB	9509.67	1.00	9509.67	31.22	0.00	0.68
BC	5436.11	1.00	5436.11	17.84	0.01	0.00
BD	2521.55	1.00	2521.55	8.28	0.03	-0.13
BE	3456.26	1.00	3456.26	11.35	0.02	-0.33
CC	5704.70	1.00	5704.70	18.73	0.01	0.00
CD	5592.80	1.00	5592.80	18.36	0.01	0.00
DD	44056.50	1.00	44056.50	144.62	0.00	-1.77
DE	2582.16	1.00	2582.16	8.48	0.03	0.28
EE	1997.53	1.00	1997.53	6.56	0.05	-1.86
Total error	1523.18	5.00	304.64			
Total (corr.)	166387.00	23.00				
Constant						-699.57

Table 4.42 Analysis of variance (ANOVA) and regression coefficients for the quadratic model for various independent variables for BGLRS Protein

Source	Sum of squares	Df	Mean - square	F-ratio	P-value	Regression coefficients
A: pH	342.27	1.00	342.27	9.04	0.02	10.02
B: Temperature	454.07	1.00	454.07	12.00	0.01	2.83
C: Light intensity	460.22	1.00	460.22	12.16	0.01	0.01
D: Growth period	546.79	1.00	546.79	14.45	0.01	3.15
E: Inoculum concentration	575.00	1.00	575.00	15.19	0.00	4.24
AB	554.20	1.00	554.20	14.65	0.01	-0.29
AC	590.20	1.00	590.20	15.60	0.00	0.00
AD	561.71	1.00	561.71	14.84	0.00	-0.30
AE	574.71	1.00	574.71	15.19	0.00	-0.67
BC	454.65	1.00	454.65	12.01	0.01	0.00
BD	368.49	1.00	368.49	9.74	0.01	0.05
BE	417.86	1.00	417.86	11.04	0.01	0.11
CD	732.22	1.00	732.22	19.35	0.00	0.00
CE	737.88	1.00	737.88	19.50	0.00	0.00
DE	729.95	1.00	729.95	19.29	0.00	0.15
Total error	302.74	8.00	37.84			
Total (corr.)	8405.76	23.00				
Constant						-160.49

Table 4.43 Analysis of the experimental results of Central Composite Design

Model	Biomass	Chlorophyll	Carbohydrate	Lipid	Protein
Transformation	None	None	None	None	None
Model d.f.	20.00	17.00	15.00	18.00	15.00
P-value	0.00	0.00	0.00	0.00	0.00
Error d.f.	3.00	6.00	8.00	5.00	8.00
Stnd. error	0.02	0.19	89.36	17.45	6.15
R-squared	99.88	99.81	99.14	99.08	96.40
Adj. R-squared	99.06	99.28	97.53	95.79	89.65

Table 4.44 Factor setting at optimum

Factors	
pH	11.17
Temperature (◦C)	26.03
Light intensity (LUX)	4000.02
Incubation period (Days)	39.37
Inoculum concentration (%)	10.00

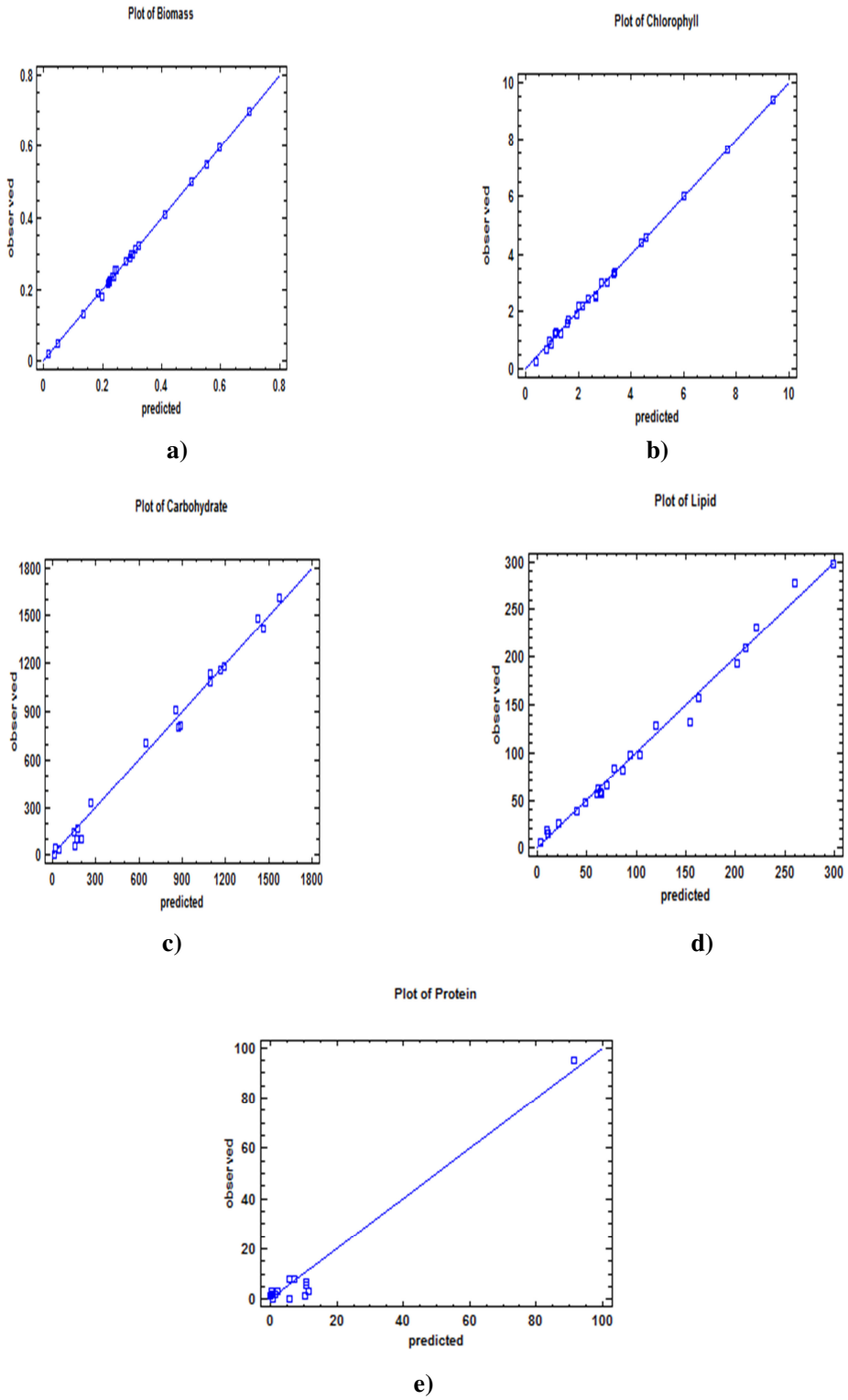
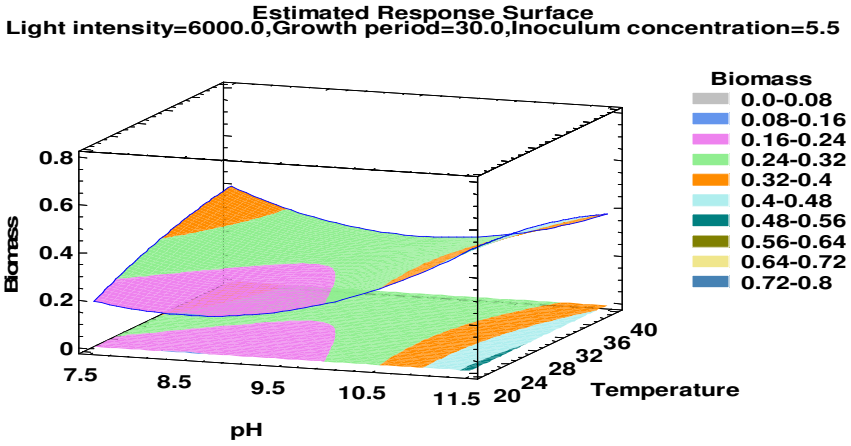
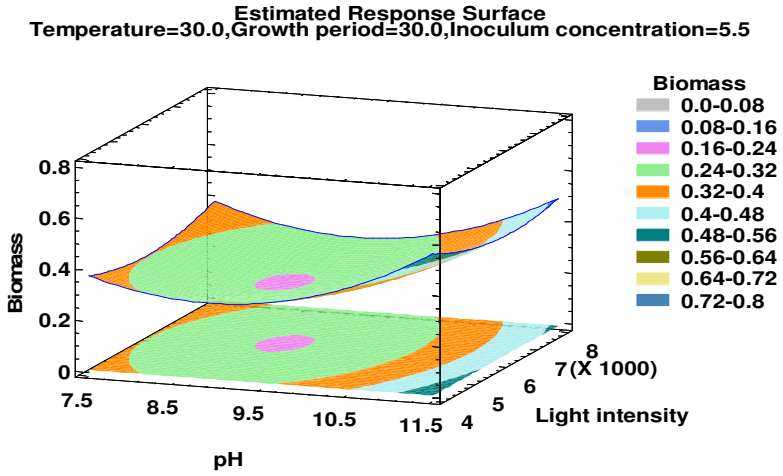


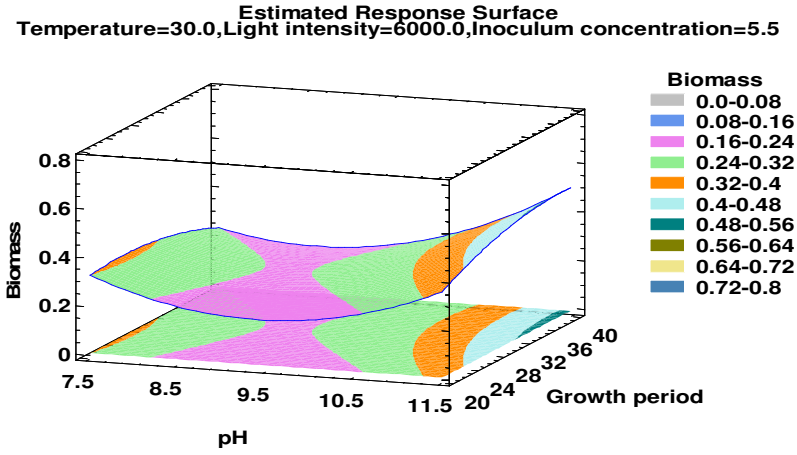
Fig 4.5 Predicted versus observed results of various response variables a) Biomass b) Chlorophyll c) Carbohydrate d) Lipid e) Protein


Estimated Response Surface
Light intensity=6000.0,Growth period=30.0,Inoculum concentration=5.5
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0
0.2
0.4
0.6
0.8
7.5
8.5
9.5
10.5
11.5
pH
20
24
28
32
36
40
Temperature

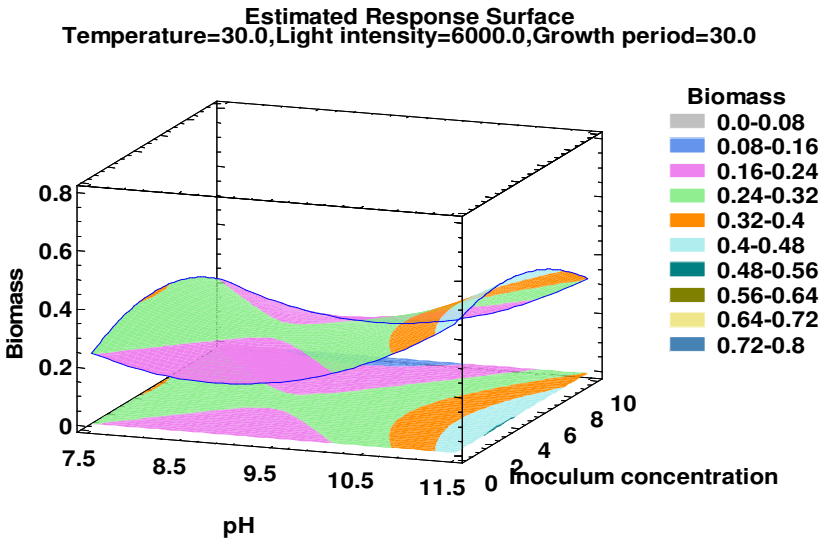
(a)


Estimated Response Surface
Temperature=30.0,Growth period=30.0,Inoculum concentration=5.5
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0
0.2
0.4
0.6
0.8
7.5
8.5
9.5
10.5
11.5
pH
4
5
6
7(X 1000)
8
Light intensity

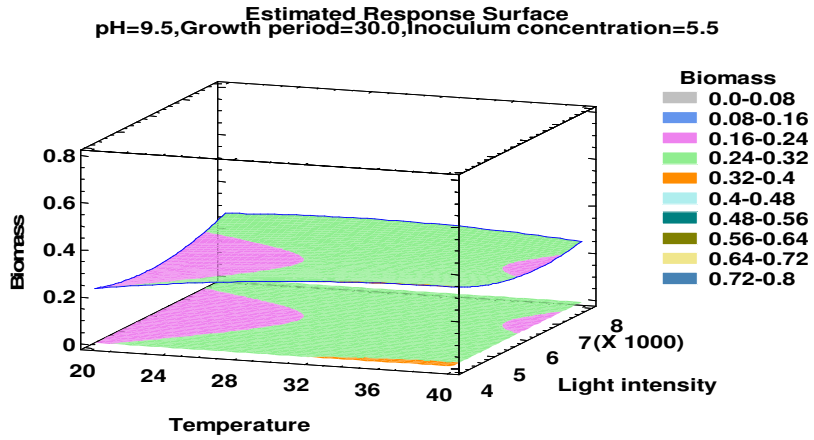
(b)


Estimated Response Surface
Temperature=30.0,Light intensity=6000.0,Inoculum concentration=5.5
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0
0.2
0.4
0.6
0.8
7.5
8.5
9.5
10.5
11.5
pH
20
24
28
32
36
40
Growth period

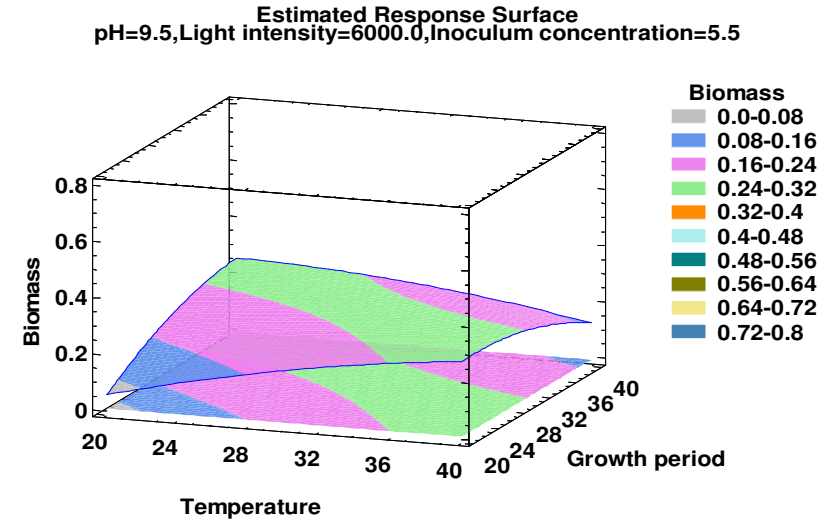
(c)


Estimated Response Surface
Temperature=30.0,Light intensity=6000.0,Growth period=30.0
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0.8
0.6
0.4
0.2
0
7.5
8.5
9.5
10.5
11.5
pH
0
2
4
6
8
10
Inoculum concentration

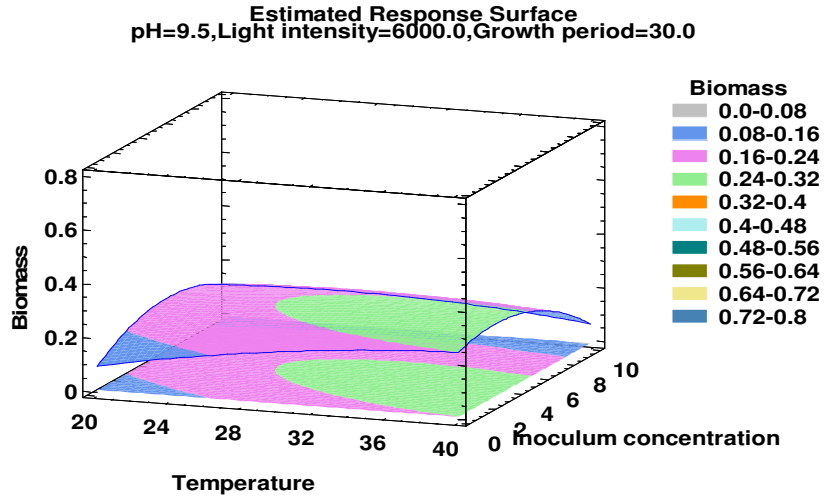
(d)


Estimated Response Surface
pH=9.5,Growth period=30.0,Inoculum concentration=5.5
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0.8
0.6
0.4
0.2
0
20
24
28
32
36
40
Temperature
4
5
6
7(X 1000)
8
Light intensity

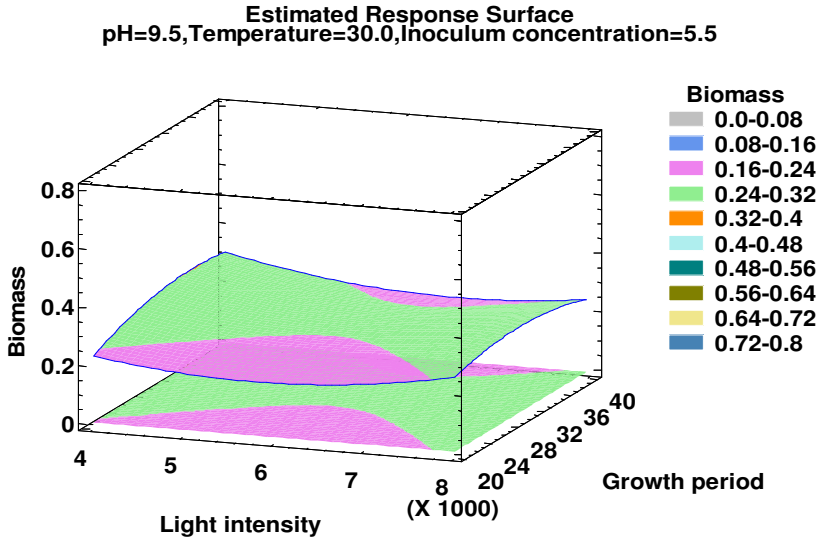
(e)


Estimated Response Surface
pH=9.5,Light intensity=6000.0,Inoculum concentration=5.5
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0.8
0.6
0.4
0.2
0
20
24
28
32
36
40
Temperature
20
24
28
32
36
40
Growth period

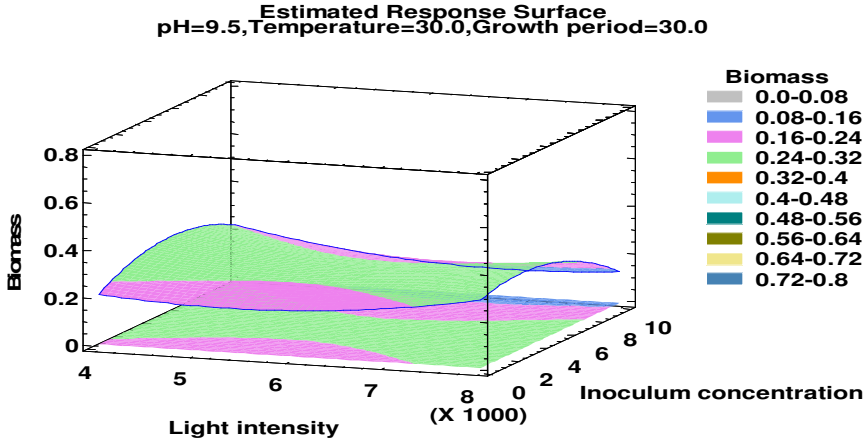
(f)


Estimated Response Surface
pH=9.5,Light intensity=6000.0,Growth period=30.0
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0.8
0.6
0.4
0.2
0
20
24
28
32
36
40
Temperature
0
2
4
6
8
10
Inoculum concentration

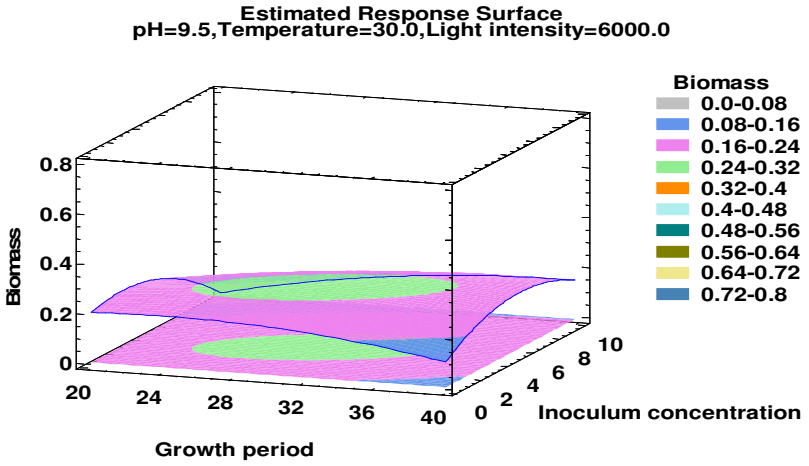
(g)


Estimated Response Surface
pH=9.5,Temperature=30.0,Inoculum concentration=5.5
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0.8
0.6
0.4
0.2
0
4
5
6
7
8
(X 1000)
Light intensity
20
24
28
32
36
40
Growth period

(h)


Estimated Response Surface
pH=9.5,Temperature=30.0,Growth period=30.0
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0.8
0.6
0.4
0.2
0
4
5
6
7
8
(X 1000)
Light intensity
0
2
4
6
8
10
Inoculum concentration

(i)



(j)

Fig. 4.6 3D Response surface plots for plots for various interactions of independent variables for BGLRS biomass (a-j)

4.2.3 Optimization of cultural conditions of *Spirulina* sp. NCIM 5143

Cultural conditions of *Spirulina* sp. NCIM 5143 was also optimized by RSM. The experimental design matrix, the observed and predicted desirability and observed values for responses are given in Table 4.45. The results obtained were analyzed by ANOVA (analysis of variance) which is required for testing the model significance are shown in Table 4.46-4.50. A considerable variation was observed for various response variable evaluated. Based on the predicted responses form the fitted model, the most desirable run corresponds to run 23.

The number of effects shown in Table 4.46-4.50 having P value less than 0.05 in biomass, chlorophyll, carbohydrate, lipids and proteins are 18, 20, 20, 20 and 16 respectively which showed that they are significantly different from zero at the 95.0% confidence level. In our study, five responses under study could be described well by CCD model with relatively high coefficient of determination as indicated by the ANOVA. The linear terms were significant model terms in all the cases. For biomass interactive AB, AC, AD, AE, BC, BD, BE,CD, DE and quadratic terms A^2 , B^2, C^2, E^2 were significant model terms. For chlorophyll content AB, AE, BB, BC, BD, BE, CD, DD, DE, EE interactive terms and A^2, B^2, C^2, D^2 and E^2 quadratic terms were found to be significant model terms. The interactive (AB, AC, AD, AE, BC,BD, BE, CD, CE, DE) and quadratic terms (A^2, B^2, C^2 and D^2) acted as significant effects for carbohydrate. Similarly, for lipids the interactive terms AB, AC, AD, AE, BC, BD, BE, CD, CE, DE and quadratic (A^2 , B^2, C^2, D^2, E^2) were significant model terms whereas for protein the significant model terms were interactive AB, AC, AD, AE, BC, BD, BE, CD, CE, DE and quadratic E^2. The R^2 of the model for biomass, chlorophyll, carbohydrates, lipids and

proteins were 99.94%, 100.00 %, 100.00%, 100.00 %, 99.26% respectively (Table 4.51). This can be interpreted as, that of 99.94%, 100.00 %, 100.00%, 100.00 %, 99.26% variability in the responses (biomass, chlorophyll, carbohydrates, lipids and proteins) respectively could be explained by the model and that only 0.06, 0.00, 0.00, 0.00, 0.74 % of the total variance for the ist five responses could not be explained by the model. The predicted and observed values of various responses in our study are very much close as can be seen in Fig. 4.7 (a-e). The value of the adjusted R^2 (adjusted coefficient of determination) was 99.75% for biomass, 99.99%, 99.98%, 99.99% and 97.57 % for chlorophyll, carbohydrate, lipid and protein respectively (Table 4.51). The final regression equations for the biomass production, chlorophyll, carbohydrate, lipid and protein, which have been fitted to the data are given below:

Biomass = $1.2728 - 0.578885*A + 0.0978292*B - 0.0000585763*C - 0.000145736*D + 0.0948296*E + 0.0359062*A^2 - 0.00409375*A*B + 0.00000753125*A*C + 0.00106875*A*D - 0.00673611*A*E - 0.000413791*B^2 - 0.0000036125*B*C - 0.0002625*B*D - 0.000233333*B*E + 1.11552E-8*C^2 - 7.375E-7*C*D + 0.000394444*D*E - 0.00460494*E^2$

Chlorophyll = $-74.0181 + 12.2841*A + 2.17975*B + 0.0100382*C - 2.36232*D - 1.51166*E - 0.691392*A^2 + 0.00860156*A*B + 0.0000398516*A*C - 0.00282594*A*D + 0.0688771*A*E - 0.0371198*B^2 - 0.0000235516*B*C + 0.00183394*B*D - 0.00376153*B*E - 7.84119E-7*C^2 - 0.00000837406*C*D + 0.00000426181*C*E + 0.0389401*D^2 + 0.00295625*D*E + 0.0809201*E^2$

Carbohydrate = $-6869.64 - 39.5955*A + 255.827*B - 0.829617*C + 480.076*D - 17.0063*E - 4.41188*A^2 + 4.32497*A*B - 0.00103453*A*C - 0.981969*A*D - 2.43063*A*E - 4.89311*B^2 - 0.00549997*B*C + 0.700019*B*D - 2.59415*B*E + 0.0000827974*C^2 + 0.00173197*C*D + 0.00334729*C*E - 8.49889*D^2 + 0.247264*D*E + 10.6709*E^2$

Lipid = $-188.292 + 171.617*A - 55.3851*B - 0.203684*C + 65.4068*D + 35.5096*E - 12.4841*A^2 + 0.924984*A*B + 0.00158852*A*C + 0.717266*A*D - 0.669271*A*E + 0.606407*B^2 + 0.000552766*B*C + 0.172191*B*D - 0.167437*B*E + 0.0000127752*C^2 + 0.000524484*C*D + 0.00031066*C*E - 1.32518*D^2 - 0.340007*D*E - 1.3701*E^2$

Protein = $-153.066 + 9.78539*A + 2.78594*B + 0.00465803*C + 3.25086*D + 0.854501*E - 0.283853*A*B + 0.00165073*A*C - 0.325603*A*D - 0.734215*A*E - 0.000299353*B*C + 0.0521544*B*D + 0.123546*B*E - 0.000323353*C*D - 0.000727549*C*E + 0.146879*D*E + 0.332985*E^2$

Hence, according to the results obtained from the model for different responses investigated in the study and also from the above mentioned regression equations, pH of 11.671, temperature of 26.0391 °C, the light intensity of 4000.02 Lux for a growth period of 39.372 day and inoculums concentration of 10.00% were found to be the the optimal and most desired conditions (Table 4.52).

Model validation

Further experiments were carried out to check the validity of the model under the estimated optimum conditions generated by the software. So, a validation experiment was conducted by cultivating BGLR18 under optimal conditions (pH of 11.671, 26.0391 °C of temperature, the light intensity of 4000.02 Lux, 39.372 days at inoculum concentration of 10.00%) suggested by the model in the ACM medium. The experimental value for different responses (biomass, chlorophyll, carbohydrate, lipid and protein) was found to be 0.712 gL^{-1}, 3.450 mgL^{-1}, 1450 mgL^{-1}, 312.0 mgL^{-1} and 8.012 mgL^{-1} respectively. The values of responses obtained are close to that obtained from CCD of RSM and model predicted values at optimum.

4.2.3.1 3D Response surface plots for various interactions of cultural factors for biomass production

i) pH versus temperature

The significant interaction between pH and temperature (p-value=0.00, table 4.46) (Fig 4.8 a) showed that higher a pH and lower temperature favours high biomass production in *Spirulina* sp. NCIM 5143 strain.

ii) pH versus light intensity

With the interaction of high pH and high light intensity produced a positive effect on biomass was observed (Fig.4.8 b). Maximum biomass productivity was observed at light intensity of 8000 Lux.

iii) pH versus growth period

In case of growth period, biomass productivity increased with increase in number of days from 24 to 40 days (4.8 c)

iv) pH versus inoculum concentration

A significant interaction between pH and inoculum concentration was evidenced by table 4.48. For maximum biomass production higher pH and low inoculums concentration was conducive (fig. 4.8 d).

v) Temperature versus light intensity

As shown by 3D response surface plot of interaction temperature and light intensity (Fig. 4.8 e), maximum biomass (0.72 to 0.8) was produced at temperature of 24°C and light intensity of 8000 Lux. Therefore, interaction between low temperature and high light intensity

was favourable for response variable (biomass) at fixed value of all other factors at optimum level.

vi) Temperature versus growth period

Lower temperature and lower growth period interaction produced beneficial conditions for biomass (fig. 4.8 f)

vii) Temperature and inoculum concentration

The interaction between temperature and inoculums concentration was significant which was indicated by their lower p-value of 0.00 (table 4.46) (fig 4.8 g). Lower temperature and lower inoculum concentration produced positive effect. However,it was also observed that biomass concentration increased abruptly at higher inoculum concentration (8 to10%).

viii) Light intensity versus growth period

Higher light intensity increased biomass values from 0.24 to 0.32g/L (fig.4.8 h). Also, shorter growth period was favourable. However, further increasing the growth period does not have any significant effect on biomass which tends to be remained constant even after increasing the growth period.

ix) Growth period versus inoculum concentration

In an another interaction study between growth period and inoculum concentration, p-value indicated 0.00 significant interaction (Table 4.46). Highest biomass at lower inoculum concentration and shorter growth period. At shorter growth period of 24 days biomass value obtained was 0.24 to 0.32 g/L as shown by 3D response surface plot in Fig 4.8 i.

Table 4.45 : Central Composite Design (CCD) and response values for different responses

Run	pH	Temperature (°C)	Light (Lux)	Growth period (Days)	Inoculum concentration (%)	Biomass (g/L)	Chlorophyll (mg/L)	Carbohydrate (g/L)	Lipid (g/L)	Protein (g/L)	Observed desirability	Predicted desirability
1.00	11.50	40.00	8000.00	20.00	1.00	0.44	0.93	0.00	5.87	8.09	0.13	0.13
2.00	7.50	40.00	4000.00	40.00	10.00	0.39	1.18	0.00	2.70	97.65	0.14	0.14
3.00	11.50	20.00	8000.00	40.00	1.00	0.64	1.30	150.00	48.00	4.50	0.23	0.25
4.00	11.50	20.00	4000.00	20.00	1.00	0.31	1.20	90.12	60.30	0.46	0.21	0.21
5.00	7.50	20.00	8000.00	40.00	10.00	0.21	2.67	1100.00	82.01	4.60	0.32	0.32
6.00	11.50	40.00	8000.00	40.00	10.00	0.12	1.50	67.00	58.00	1.20	0.17	0.18
7.00	7.50	40.00	8000.00	40.00	1.00	0.22	0.88	21.00	55.00	0.59	0.17	0.17
8.00	7.50	40.00	8000.00	20.00	10.00	0.31	0.18	13.99	54.32	0.65	0.14	0.14
9.00	7.50	30.00	6000.00	30.00	5.50	0.33	3.37	1121.00	130.98	2.34	0.40	0.42
10.00	11.50	40.00	4000.00	40.00	1.00	0.41	0.69	0.00	58.60	0.00	0.19	0.19
11.00	9.50	40.00	6000.00	30.00	5.50	0.23	1.59	329.00	193.88	7.89	0.31	0.31
12.00	11.50	20.00	8000.00	20.00	10.00	0.34	4.71	881.00	64.86	2.80	0.36	0.37
13.00	9.50	20.00	6000.00	30.00	5.50	0.14	3.01	800.00	247.20	0.00	0.34	0.32
14.00	9.50	30.00	8000.00	30.00	5.50	0.31	3.01	1502.00	209.00	0.00	0.43	0.40
15.00	11.50	40.00	4000.00	20.00	10.00	0.12	1.87	2.10	7.22	0.00	0.11	0.11
16.00	9.50	30.00	6000.00	30.00	5.50	0.23	5.98	1059.00	159.09	0.00	0.41	0.42
17.00	9.50	30.00	6000.00	40.00	5.50	0.22	9.59	139.01	19.00	3.56	0.24	0.25
18.00	11.50	20.00	4000.00	40.00	10.00	0.20	2.23	85.00	20.55	2.53	0.17	0.17
19.00	9.50	30.00	4000.00	30.00	5.50	0.24	2.74	1268.00	213.00	5.89	0.42	0.42
20.00	7.50	20.00	8000.00	20.00	1.00	0.23	4.41	680.00	101.90	2.11	0.34	0.34
21.00	7.50	20.00	4000.00	40.00	1.00	0.05	2.41	22.00	98.01	1.67	0.16	0.16
22.00	7.50	40.00	4000.00	20.00	1.00	0.49	2.35	26.00	37.00	0.00	0.23	0.22
23.00	9.50	30.00	6000.00	30.00	1.00	0.20	7.50	1148.00	129.00	1.98	0.42	0.42
24.00	7.50	20.00	4000.00	20.00	10.00	0.02	2.30	780.00	227.12	0.68	0.14	0.00

Table 4.46 Analysis of variance (ANOVA) and regression coefficients for the quadratic model for various independent variables for *Spirulina* sp. NCIM 5143 Biomass

Source	Sum of squares	Df	Mean - square	F-ratio	P-value	Regression coefficients
A: pH	0.03	1.00	0.03	545.22	0.00	-0.58
B: Temperature	0.02	1.00	0.02	380.59	0.00	0.10
C: Light intensity	0.02	1.00	0.02	378.02	0.00	0.00
D: Growth period	0.00		0.00	0.50	0.51	0.00
E: Inoculum concentration	0.07	1.00	0.07	1443.24	0.00	0.09
AA	0.03	1.00	0.03	573.13	0.00	0.04
AB	0.11	1.00	0.11	2110.82	0.00	0.00
AC	0.01		0.01	285.76	0.00	0.00
AD	0.01	1.00	0.01	143.87	0.00	0.00
AE	0.06	1.00	0.06	1157.31	0.00	-0.01
BB	0.00		0.00	70.14	0.00	0.00
BC	0.08	1.00	0.08	1643.70	0.00	0.00
BD	0.01	1.00	0.01	216.97	0.00	0.00
BE	0.00	1.00	0.00	34.72	0.00	0.00
CC	0.00	1.00	0.00	81.57	0.00	0.00
CD	0.00	1.00	0.00	68.51	0.00	0.00
DE	0.01	1.00	0.01	99.21	0.00	0.00
EE	0.01	1.00	0.01	241.59	0.00	0.00
Total error	0.00	5.00	0.00			
Total (corr.)	0.46	23.00				
Constant						1.27

Table 4.47 Analysis of variance (ANOVA) and regression coefficients for the quadratic model for various independent variables for *Spirulina* sp. NCIM 5143 Chlorophyll

Source	Sum of squares	Df	Mean - square	F-ratio	P-value	Regression coefficients
A: pH	0.24	1.00	0.24	393.32	0.00	12.28
B: Temperature	9.51	1.00	9.51	15598.87	0.00	2.18
C: Light intensity	0.38	1.00	0.38	629.31	0.00	0.01
D: Growth period	1.62	1.00	1.62	2649.49	0.00	-2.36
E: Inoculum concentration	0.38	1.00	0.38	623.57	0.00	-1.51
AA	9.60	1.00	9.60	15736.11	0.00	-0.69
AB	0.47	1.00	0.47	776.34	0.00	0.01
AC	0.41	1.00	0.41	666.58	0.00	0.00
AD	0.05	1.00	0.05	83.80	0.00	0.00
AE	6.15	1.00	6.15	10080.29	0.00	0.07
BB	27.56	1.00	27.56	45181.37	0.00	-0.04
BC	3.55	1.00	3.55	5820.20	0.00	0.00
BD	0.54	1.00	0.54	882.28	0.00	0.00
BE	0.46	1.00	0.46	751.61	0.00	0.00
CC	19.68	1.00	19.68	32257.68	0.00	0.00
CD	0.45	1.00	0.45	735.82	0.00	0.00
CE	0.02	1.00	0.02	38.59	0.01	0.00
DD	19.03	1.00	19.03	31197.64	0.00	0.04
DE	0.28	1.00	0.28	464.24	0.00	0.00
DE	3.37	1.00	3.37	5524.47	0.00	0.08
Total error	0.00	3.00	0.00			
Total (corr.)	115.89	23.00				
Constant						-74.02

Table 4.48 Analysis of variance (ANOVA) and regression coefficients for the quadratic model for various independent variables for *Spirulina* sp. NCIM 5143 Carbohydrate

Source	Sum of squares	Df	Mean - square	F-ratio	P-value	Regression coefficients
A:pH	117089.00	1.00	117089.00	2932.17	0.00	-39.60
B: Temperature	947160.00	1.00	947160.00	23719.01	0.00	255.83
C: Light intensity	254843.00	1.00	254843.00	6381.84	0.00	-0.83
D: Growth period	66429.30	1.00	66429.30	1663.54	0.00	480.08
E: Inoculum concentration	236227.00	1.00	236227.00	5915.64	0.00	-17.01
AA	390.82	1.00	390.82	9.79	0.05	-4.41
AB	119714.00	1.00	119714.00	2997.91	0.00	4.32
AC	273.99	1.00	273.99	6.86	0.08	0.00
AD	6171.28	1.00	6171.28	154.54	0.00	-0.98
AE	7656.69	1.00	7656.69	191.74	0.00	-2.43
BB	478850.00	1.00	478850.00	11991.46	0.00	-4.89
BC	193598.00	1.00	193598.00	4848.12	0.00	-0.01
BD	78404.20	1.00	78404.20	1963.42	0.00	0.70
BE	218040.00	1.00	218040.00	5460.21	0.00	-2.59
CC	219373.00	1.00	219373.00	5493.59	0.00	0.00
CD	19198.20	1.00	19198.20	480.77	0.00	0.00
CE	14520.90	1.00	14520.90	363.63	0.00	0.00
DD	906429.00	1.00	906429.00	22699.01	0.00	-8.50
DE	1980.92	1.00	1980.92	49.61	0.01	0.25
DE	58595.50	1.00	58595.50	1467.36	0.00	10.67
Total error	119.80	3.00	39.93			
Total (corr.)	6122950.00	23.00				
Constant						-6869.64

Table 4.49 Analysis of variance (ANOVA) and regression coefficients for the quadratic model for various independent variables for *Spirulina* sp. NCIM 5143 Lipid

Source	Sum of squares	Df	Mean - square	F-ratio	P-value	Regression coefficients
A: pH	7028.84	1.00	7028.84	15831.46	0.00	171.62
B: Temperature	12659.30	1.00	12659.30	28513.31	0.00	-55.39
C: Light intensity	115.24	1.00	115.24	259.56	0.00	-0.20
D: Growth period	1151.84	1.00	1151.84	2594.34	0.00	65.41
E: Inoculum concentration	168.97	1.00	168.97	380.58	0.00	35.51
AA	3129.28	1.00	3129.28	7048.25	0.00	-12.48
AB	5475.82	1.00	5475.82	12333.49	0.00	0.92
AC	645.99	1.00	645.99	1454.99	0.00	0.00
AD	3292.61	1.00	3292.61	7416.13	0.00	0.72
AE	580.51	1.00	580.51	1307.51	0.00	-0.67
BB	7354.59	1.00	7354.59	16565.15	0.00	0.61
BC	1955.52	1.00	1955.52	4404.53	0.00	0.00
BD	4743.94	1.00	4743.94	10685.04	0.00	0.17
BE	908.34	1.00	908.34	2045.92	0.00	-0.17
CC	5222.56	1.00	5222.56	11763.07	0.00	0.00
CD	1760.54	1.00	1760.54	3965.36	0.00	0.00
CE	125.08	1.00	125.08	281.72	0.00	0.00
DD	22037.20	1.00	22037.20	49635.73	0.00	-1.33
DE	3745.59	1.00	3745.59	8436.41	0.00	-0.34
DE	965.97	1.00	965.97	2175.71	0.00	-1.37
Total error	1.33	3.00	0.44			
Total (corr.)	133223.00	23.00				
Constant						-188.29

Table 4.50 Analysis of variance (ANOVA) and regression coefficients for the quadratic model for various independent variables for *Spirulina* sp. NCIM 5143

Protein

Source	Sum of squares	Df	Mean - square	F-ratio	P-value	Regression coefficients
A: pH	467.10	1.00	467.10	49.96	0.00	9.79
B: Temperature	519.74	1.00	519.74	55.60	0.00	2.79
C: Light intensity	395.15	1.00	395.15	42.27	0.00	0.00
D: Growth period	586.71	1.00	586.71	62.76	0.00	3.25
E: Inoculum concentration	570.66	1.00	570.66	61.04	0.00	0.85
AB	515.67	1.00	515.67	55.16	0.00	-0.28
AC	697.58	1.00	697.58	74.62	0.00	0.00
AD	678.51	1.00	678.51	72.58	0.00	-0.33
AE	698.64	1.00	698.64	74.73	0.00	-0.73
BC	573.52	1.00	573.52	61.35	0.00	0.00
BD	435.21	1.00	435.21	46.55	0.00	0.05
BE	494.54	1.00	494.54	52.90	0.00	0.12
CD	669.17	1.00	669.17	71.58	0.00	0.00
CE	686.01	1.00	686.01	73.38	0.00	0.00
DE	698.98	1.00	698.98	74.77	0.00	0.15
EE	222.52	1.00	222.52	23.80	0.00	0.33
Total error	65.44	7.00	23.80			
Total (corr.)	8858.38	23.00				
Constant						-153.07

Table 4.51 Analysis of the experimental results of Central Composite Design

Model	Biomass	Chlorophyll	Carbohydrate	Lipid	Protein
Transformation	None	None	None	None	None
Model d.f.	18.00	20.00	20.00	20.00	16.00
P-value	0.00	0.00	0.00	0.00	0.00
Error d.f.	5.00	3.00	3.00	3.00	7.00
Stnd. error	0.01	0.02	6.32	0.67	3.06
R-squared	99.94	100.00	100.00	100.00	99.26
Adj. R-squared	99.75	99.99	99.98	99.99	97.57

Table 4.52 Factor setting at optimum

Factors	
pH	10.35
Temperature (◦C)	24.38
Light intensity (LUX)	8000.00
Incubation period (Days)	25.13
Inoculum concentration (%)	1.00

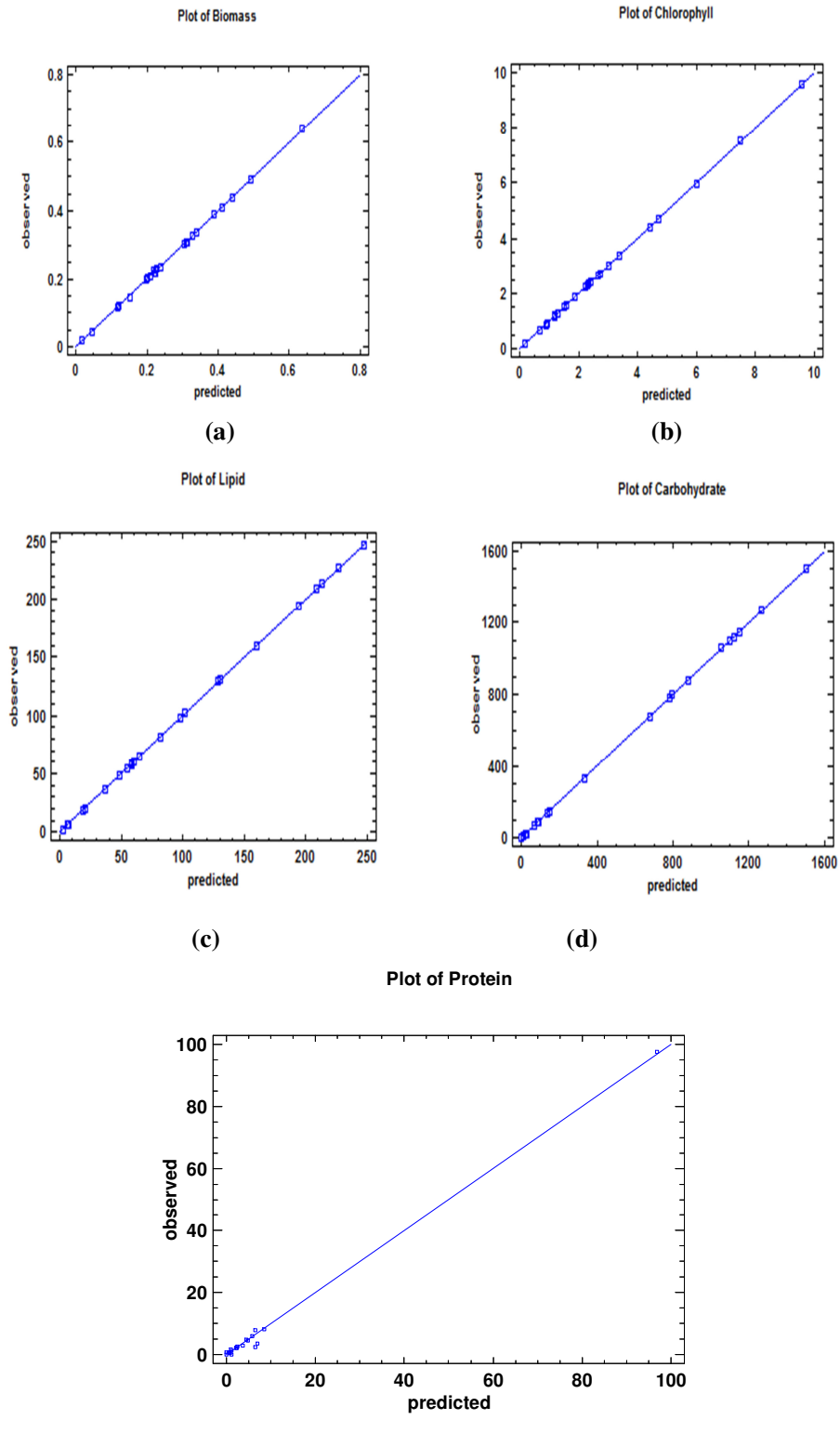
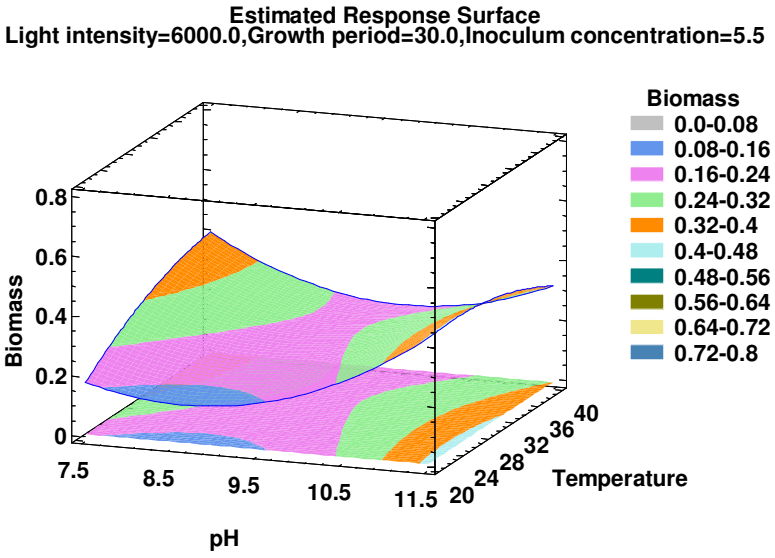
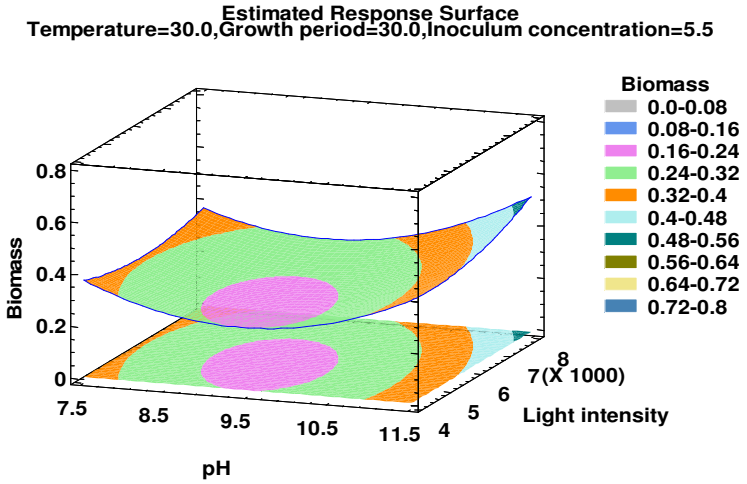


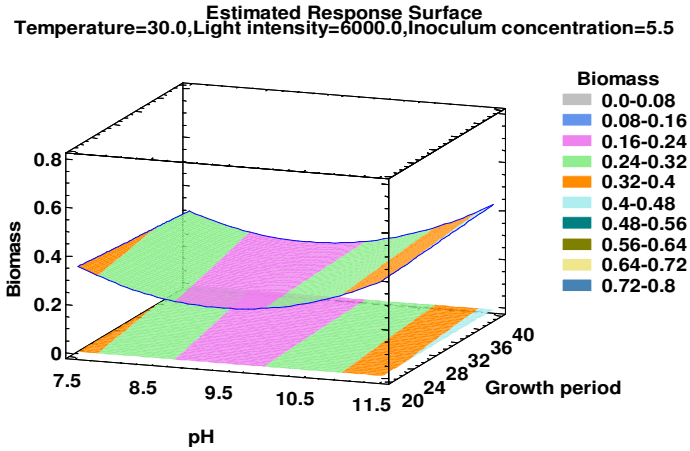
Fig. 4.7 Predicted versus observed results of various response variables a) Biomass b) Chlorophyll c) Carbohydrate d) Lipid e) Protein


Estimated Response Surface
Light intensity=6000.0,Growth period=30.0,Inoculum concentration=5.5
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0.8
0.6
0.4
0.2
0
7.5
8.5
9.5
10.5
11.5
pH
20
24
28
32
36
40
Temperature

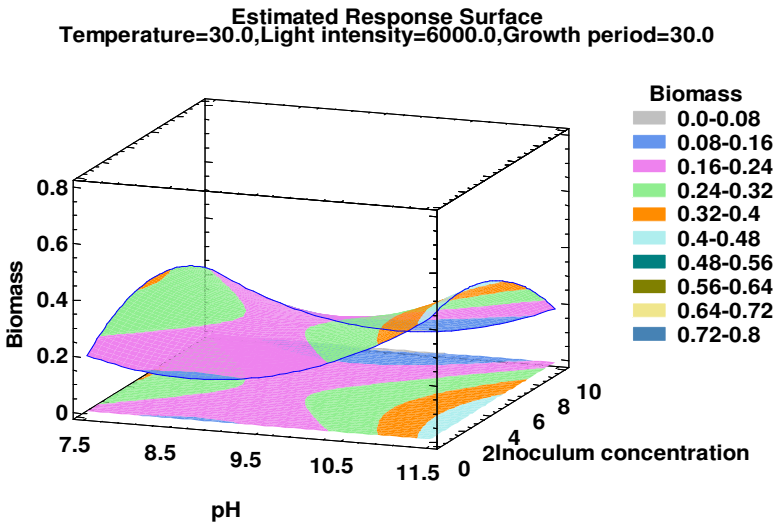
(a)


Estimated Response Surface
Temperature=30.0,Growth period=30.0,Inoculum concentration=5.5
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0.8
0.6
0.4
0.2
0
7.5
8.5
9.5
10.5
11.5
pH
4
5
6
7(X 1000)
8
Light intensity

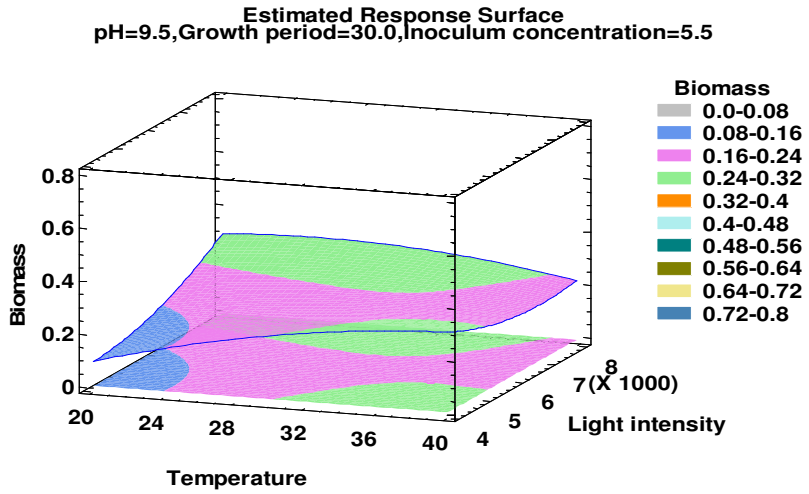
(b)


Estimated Response Surface
Temperature=30.0,Light intensity=6000.0,Inoculum concentration=5.5
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0.8
0.6
0.4
0.2
0
7.5
8.5
9.5
10.5
11.5
pH
20
24
28
32
36
40
Growth period

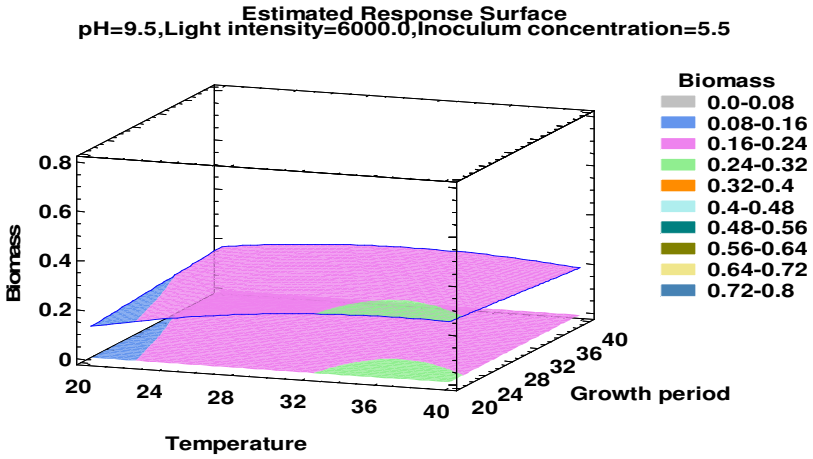
(c)


Estimated Response Surface
Temperature=30.0,Light intensity=6000.0,Growth period=30.0
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0.8
0.6
0.4
0.2
0
7.5
8.5
9.5
10.5
11.5
pH
0
2
4
6
8
10
Inoculum concentration

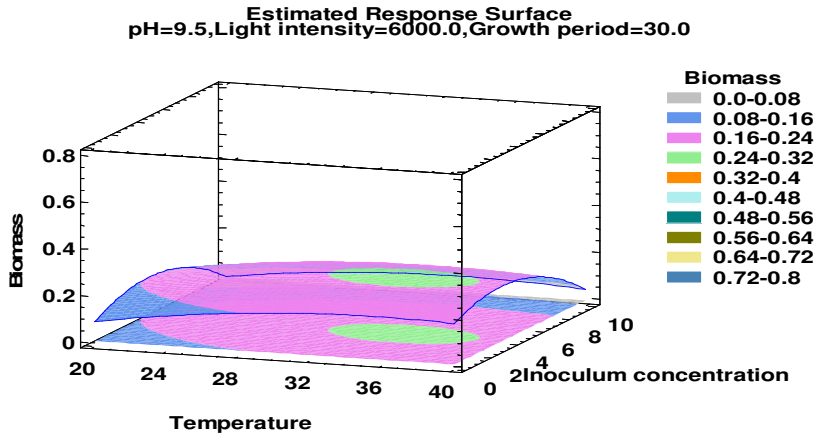
(d)


Estimated Response Surface
pH=9.5,Growth period=30.0,Inoculum concentration=5.5
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0.8
0.6
0.4
0.2
0
20
24
28
32
36
40
Temperature
4
5
6
7(X 1000)
8
Light intensity

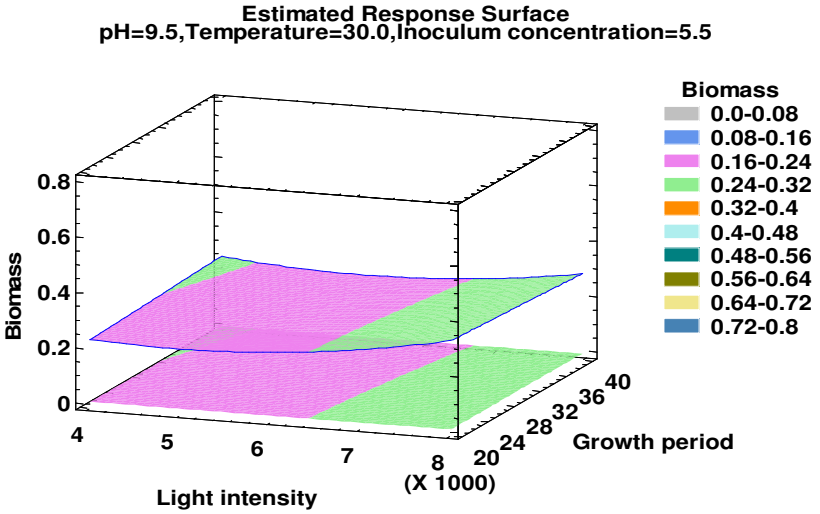
(e)


Estimated Response Surface
pH=9.5,Light intensity=6000.0,Inoculum concentration=5.5
Biomass
0.0-0.08
0.08-0.16
0.16-0.24
0.24-0.32
0.32-0.4
0.4-0.48
0.48-0.56
0.56-0.64
0.64-0.72
0.72-0.8
Biomass
0.8
0.6
0.4
0.2
0
20
24
28
32
36
40
Temperature
20
24
28
32
36
40
Growth period

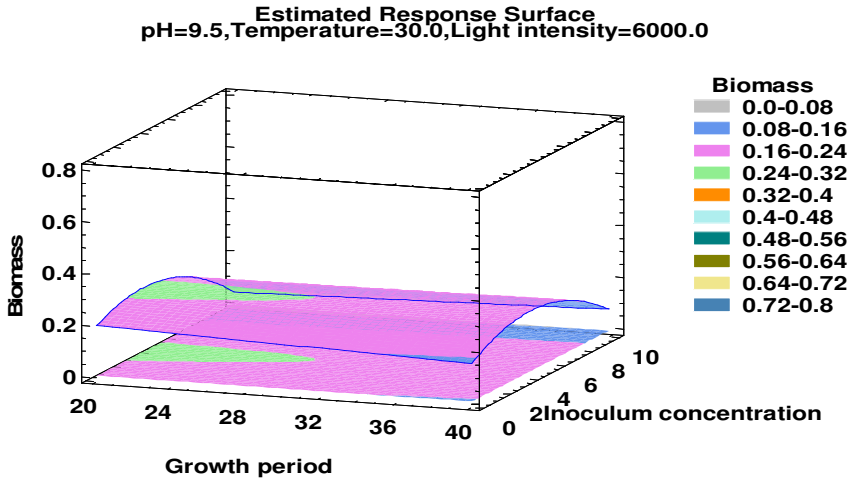
(f)



(g)



(h)



(i)

Fig. 4.8 **3D Response surface plots for various interactions of independent variables for *Spirulina* sp. NCIM 5143 biomass (a-i)**

4.3.1 Molecular identification of selected microalgae strains

Three most efficient microalgae strains viz. BGLR18, BGLRS and *Spirulina* sp. NCIM 5143 were selected for further evaluation on the basis of production of maximum biomass, protein, carbohydrate and lipids and also highest percent reduction of various physico-chemical parameters of dairy wastewater.

Out of these, two strains were identified at molecular level. The strain BGLR18 was found to be *Chlorosarcinopsis eremi* with close resemblance to *Chlorosarcinopsis eremi*, internal transcribed spacer I, partial sequence 5.8 S ribosomal RNA gene with 96% match. The strain BGLRS was found to be *Scenedesmus* sp. *MKB* with close resemblance to *Scenedesmus* sp. *MKB*, 18S RNA gene partial sequence, internal transcriber spacer I, 5.8 S ribosomal RNA gene and internal transcriber spacer 2 complete sequence with 99% match. The agarose gel analysis of the genomic DNA extracted from strain BGLR18 and BGLRS is shown below in fig. 4.9 and phylogenetic tree analysis of BGLR18 and BGLRS in fig. 4.10 a and b respectively.

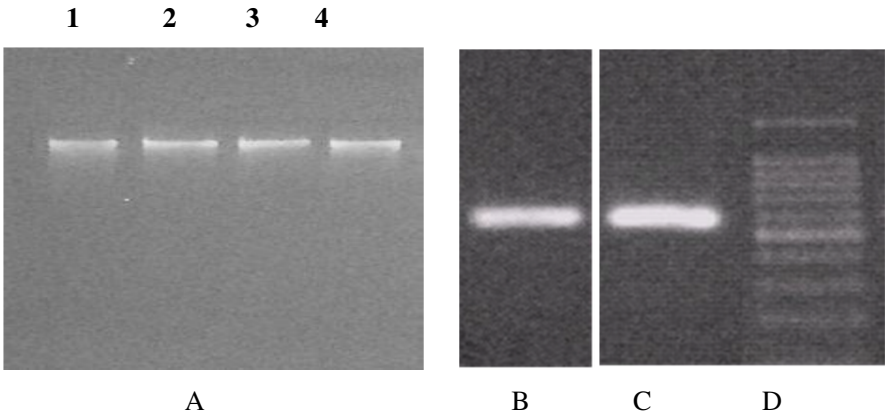


Fig. 4.9 A) Genomic DNA from samples loaded in 1% agarose gel.

B) PCR amplification of ITS fragment from BGLRS

C) PCR amplification of ITS fragment from BGLR18.

D) Ladder

The 571 bp nucleotide sequence of PCR amplified ITS region of BGLR18 is given below:

TCTGGCGTTCTTTGATATGCTTAAGTTCACCGGGTCGTCTAGCTTAACTGACGCCA
GTTAGAAGTTAATAAACTCGACGTAATTTAAAAACGCCCCTCTTCTGATACACAT
GCTGATCAAAAGAAAAAACTCCAACCGTTCCGAGAAACAGTTTCCATTTTTGCGT
TCATATTATCGATACACAACTAGCAGTCAATCAAACCTGCCAGGAAATCCTGGCG
CAATGTCTGCATTCAAAGACGTAGTGTTCAAAAAGATTTGCAATGCACACTCCCT
TCATCATTCGCTATGTTTTTCATCGTAATAGGAGCCTAGGCATCCATTGTACAGCT
TTGTTCATTGTCTTGAGGAAGATAAGAGGACTTCTCCTTTGTTTGACCATCAAGAG
ACCTTCGCCCATATCTCACACACCCAATCGACCGGAAGGGGTAAACGCCCACTGG
CTCTCCCCAGTTCCAAAGACACCGACCAGATGCGAGAGGGACAGGCTGAGCACA

GATGAGTAGCCCCGTTTGGGTTTTTTTTTGGAGGATTATTTTTTTTGAGGAGGGTT
TTGGGATTATCTGGCGT

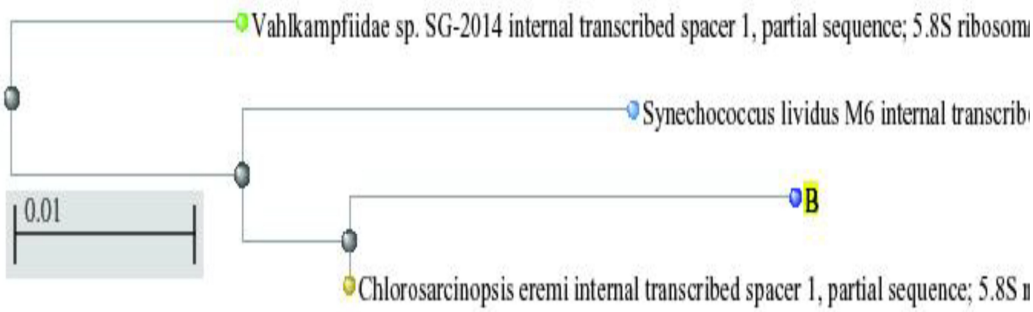


Fig 4.10 a). Phylogenetic tree of BGLR18 The tree was constructed by Weighbor (a weighted version of Neighbor Joining that gives significantly less weight to the longer distances in the distance matrix) with alpahabet size 4 and length size 1000 and 100 rounds of bootstrap resampling (Bruno *et al* 2000).

The 615 bp nucleotide sequence of PCR amplified ITS region of BGLRS is given below:

CACAATGTGAACCTCAACGTTCCGTGCCCTGGCTTGCCAGTGGGGCGACATGG
TCAACACCAGGTCGTACTCACAGCTGGGTGGGCATTGTTGCCTACTCAGTGGC
GCCTTGGCATGATCATACACCCATGCTAACCAATGATAAAACCAAACTTTGAA
GTTTGATTGATATTCATTGGCAATCTTAACCAAAGACAACTCTCAACAACGGA
TATCTTGGCTCTCGCAACGATGAAGAACGCAGCGAAATGCGATACGTAGTGTG
AATTGCAGAATTCCGTGAACCATCGAATCTTTGAACGCATATTGCGCTCGAGC
CTTCGGGCAAGAGCATGTCTGCCTCAGCGTCGGTTTAATCCCTCACCCCTCCCT
ATTATGGGTGCGTTGATCATGTGATCAGCCATTGGGGTGGATCTGGCTTCCCCA
ATCTCACTTGTTGCGATTGGGTTGGCTGAAGCACAGAGGCTTAAGCAAGGACC
CGATATGGGCTTCAACTGGATAGGTAGCAACGGCGTATGCCGACTACACGAAG
TTGTTGCTTGTGGACTTTGTTAGGAGCCGAGCAGGAACATGCCTTGTGCATGCA
TAAACTTTCGACCTGAGCTCAGGCAAGTA

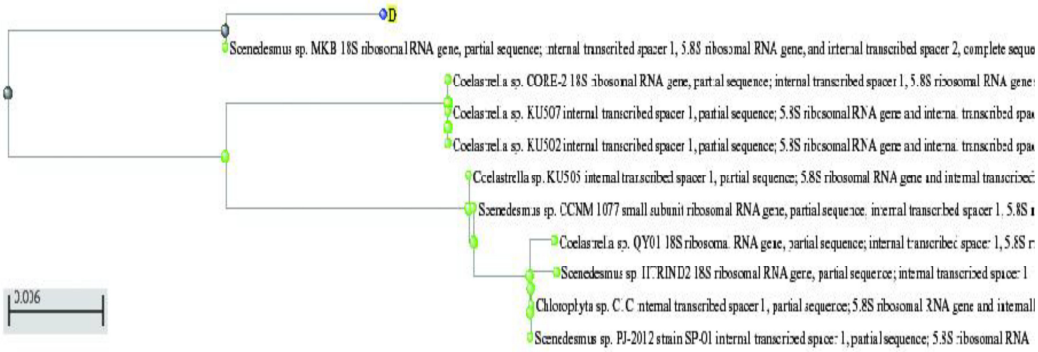


Fig. 4.10 b). Phylogenetic tree of BGLRS The tree was constructed by Weighbor (a weighted version of Neighbor Joining that gives significantly less weight to the longer distances in the distance matrix) with alphabet size 4 and length size 1000 and 100 rounds of bootstrap resampling (Bruno *et al* 2000).

4.3.2 Biochemical characterization of three selected microalgal strains

The three selected microalgal strains viz. BGLRS (*Scenedesmus sp. MKB*), BGLR18 (*Chlorosarcinopsis eremi*) and *Spirulina* sp. NCIM 5143 were tested for the total kjeldahl nitrogen (TKN), crude protein content, total phenolic content (TPC), antioxidant activity and the for the presence of various phytochemicals viz. quinones, tannins, saponins, terpenoids and steroids (Table 4.53). Highest TKN was shown by *Spirulina* sp. NCIM 5143 (7.14±0.49) followed by BGLR18 (*Chlorosarcinopsis eremi*) (3.64±0.49) and BGLRS (*Scenedesmus sp. MKB*) (2.73±0.30) and same trend was found in values of crude protein content where highest protein was present in *Spirulina* sp. NCIM 5143 (48.23±3.34) followed by BGLR18 (*Chlorosarcinopsis eremi*) (24.59±3.34) and BGLRS (*Scenedesmus sp. MKB*) (18.44±2.00). Results showed that highest phenolic content (8.88 ±1.93) was present in methanolic extracts of *Spirulina* sp. NCIM 5143 while lowest (0.49±0.04) in BGLR18 (*Chlorosarcinopsis eremi*). Maximum antioxidant activity (3.07±0.03) was shown by methanolic extract of *Spirulina* sp. NCIM 5143 followed by BGLRS (*Scenedesmus sp. MKB*) (2.07±0.09) and BGLR18 (*Chlorosarcinopsis eremi*) (1.86±0.14). DPPH radical scavenging activity test was done at 3 different concentrations of methanolic extracts of microalgal biomass viz. 250, 500 and 1000 µg/ml and percent inhibition values was calculated. All the three strains showed maximum% inhibition at highest concentration (1000 µg/ml) and minimum at lowest concentration (250 µg/ml). At 1000 µg/ml concentration of methanolic extract, BGLR18 (*Chlorosarcinopsis eremi*) and BGLRS (*Scenedesmus* sp. *MKB*) and *Spirulina* sp. NCIM 5143 showed maximum % inhibition of 57.27± 0.40, 67.79± 0.42 and 75.07±0.09 µg/ml respectively which is lower than control (ascorbic acid) (109.88±0.11 µg/ml) (Table 4.54) (Fig.4.11). In all the three strains, it was observed that with increase in concentration from 250 to 1000 µg/ml, absorbance decreases and hence% inhibition value increases. The scavenging activity of microalgal species towards DPPH radicals suggest that the extract had antioxidant activity through electron and hydrogen donor' mechanisms (El-Baky *et al* 2009). All the three selected strains were also evaluated for the presence of various phytochemicals viz. quinones, tannins, saponins, terpenoids and steroids. It was found that all the phytochemicals were present in *Spirulina* sp. NCIM 5143. BGLRS (*Scenedesmus sp. MKB*) showed the presence of tannins, saponins and steroids whereas in BGLR18 (*Chlorosarcinopsis eremi*) only saponins and terpenoids were detected. Our results for phytochemical screening of *Spirulina* sp. NCIM 5143 are in line with Mane and Chakraborty (2018) where all the phytochemicals has been found in methanolic extracts of *Spirulina platensis* (alkaloids, terpenoids, steroids, saponins, phenols and flavonoids tannins, coumarins, quinines). For BGLRS (*Scenedesmus sp. MKB*) and BGLR18 (*Chlorosarcinopsis eremi*) our results corroborate with Elayarani (2016) and Dhanalakshmi and Angayarkanni (2013) respectively. In *Scenedesmus arcutus*, phytocompounds viz. phytosterols, tannins, alkaloids, and saponins (Elayarani 2016) and in

Table 4.53 Biochemical characterization of three selected microalgal strains

Biochemical characteristics	**BGLR18 (*Chlorosarcinopsis eremi*)**	**BGLRS (*Scenedesmus sp. MKB*)**	***Spirulina* sp. NCIM 5143**
Total Kjeldahl Nitrogen (TKN) (%)	3.64±0.49[b]	2.73±0.30[b]	7.14±0.49 [a]
Crude protein content (%)	24.59±3.34 [b]	18.44±2.00 [b]	48.23±3.34 [a]
*Total Phenols (mg GAE g^{-1})	0.49±0.04 [b]	0.51±0.35 [b]	8.88±1.93[a]
*Antioxidant activity (mg AAE g^{-1})	1.86±0.14 [b]	2.07±0.09 [b]	3.07±0.03 [a]
*Quinones	-	-	+
*Tannins	-	+	+
*Saponins	+	+	+
*Terpenoids	+	-	+
*Steroids	-	+	+

Cultural conditions : Temperature (25±2 °C), light intensity (4000 LUX) and photoperiod (16:8 light:dark), *Methanolic extracts of microalgal biomass was used for evaluation, - = not present, += present, GAE= Gallic acid equivalent, AAE= Ascorbic acid equivalent, Values superscripted by different alphabets in column differ significantly (P≤0.05) from each other (Tukey's test).

Table 4.54 DPPH radical scavenging activity of microalgal strains

Microalgal strains	Concentration (µg/ml)		
	250	**500**	**1000**
BGLR18 (*Chlorosarcinopsis eremi*)	4.03±0.04^{d}	26.69±0.50^{d}	57.27±0.40^{d}
BGLRS (*Scenedesmus sp. MKB*)	11.91±0.14^{c}	39.35±0.07^{c}	67.79±0.42^{c}
Spirulina sp. NCIM 5143	20.95±0.11^{b}	41.20±0.30^{b}	75.07±0.09^{b}
Ascorbic acid (Standard)	80.00±0.12^{a}	90.00±0.45^{a}	109.88±0.11^{a}

Values superscripted by different alphabets in column differ significantly (P≤0.05) from each other (Tukey's test).

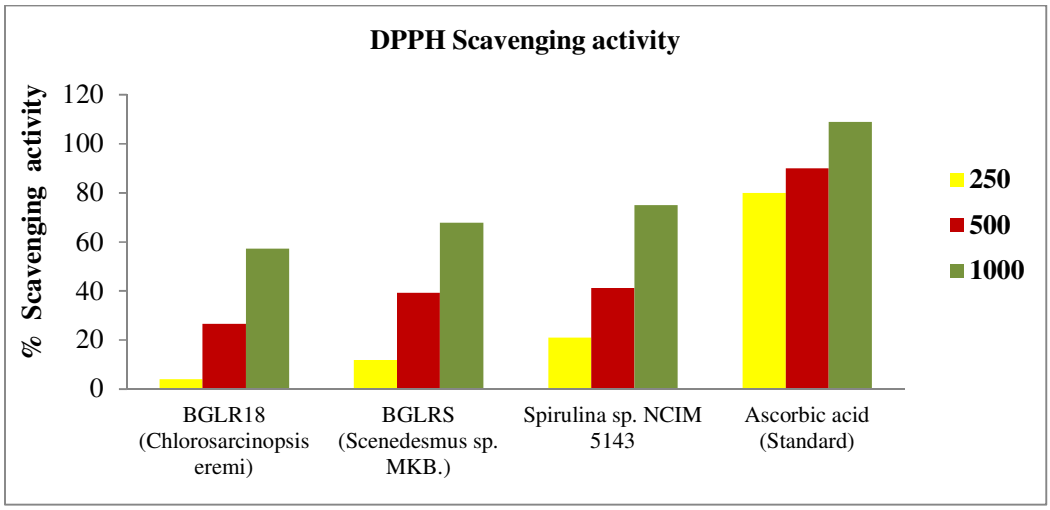


Fig. 4.11 DPPH radical scavenging activity of BGLR18, BGLRS, *Spirulina* sp. NCIM 5143 in comparison with ascorbic acid

Chlorosarcinopsis spp. alkaloids, cardiac glycosides, saponins and terpenoids (Dhanalakshmi and Angayarkanni 2013) were reported. Phytochemicals like phenols, flavanoids and tannins are powerful antioxidants which are anti-inflammatory, antimicrobial and anticancareous in action and glycosides are used in the treatment of congestive heart failure and cardiac arrhythmia (Balch and Balch 2000). Alkaloids are the important group of phytochemical compounds helpful in relieving pain, anxiety and depression (Jisika *et al* 1992).

4.3.3 Inductively coupled plasma-atomic emission spectroscopy of microalgal biomass

These three microalgae strains viz. BGLR18 (*Chlorosarcinopsis eremi*), BGLRS (*Scenedesmus* sp. *MKB.*) *Spirulina* sp. NCIM 5143 were also processed by the wet digestion method given by Hseu (2004) with little modifications for the determination of their elemental composition (Arsenic, boron, calcium, cadmium, chromium, copper, iron, magnesium, manganese, nickel, phosphorus, lead, sulphur, zinc) (Table 4.55). The concentrations of various elements were in mg/kg but converted to percent (%). A wide

variation is found in three microalgae strains in terms of concentration of fourteen nutrients as analyzed by ICP-AES. However, in each of the three strains concentration of heavy metals like Cd, Cr, As and Pb were negligible which assures its safety for consumption as mineral supplements. Many important elements like Calcium (Ca), Iron (Fe), Magnesium (Mg) were found to be highest in stress tolerant microalgae strain BGLRS (*Scenedesmus sp. MKB*) and BGLR18 (*Chlorosarcinopsis eremi*) as compared to standard strain *Spirulina* sp. NCIM 5143. BGLRS (*Scenedesmus sp. MKB*), BGLR18 (*Chlorosarcinopsis eremi*) and *Spirulina* sp. NCIM 5143 contain Ca as 3.40%, 0.88% and 0.21% respectively. This showed that BGLRS (*Scenedesmus sp. MKB*) contained 16.19 times and BGLR18 (*Chlorosarcinopsis eremi*) contained 4.19 times as much as Ca content of *Sprirulina* sp. NCIM 5413. Ca has function in various processes in human body including vascular contraction, vasodilation, muscle functions, nerve transmission, intracellular signaling and hormone secretion (Beto 2015). Iron (Fe) element was 0.42%, 0.09% and 0.07% in BGLRS (*Scenedesmus sp. MKB*), BGLR18 (*Chlorosarcinopsis eremi*) and *Spirulina* sp. NCIM 5143 respectively which revealed BGLRS (*Scenedesmus sp. MKB*) contained 1.28 times and BGLR18 (*Chlorosarcinopsis eremi*) contained 6 times as much as Fe than *Spirulina* sp. NCIM 5413. Iron is important for oxygen transport, role in cellular processes like DNA, RNA and protein synthesis, electron transport, cellular respiration, cell proliferation and differentiation and regulation of gene expression

Table 4.55 Elemental composition of microalgal strains by ICAP-AES

Element (%)	**Symbol**	***BGLR18 (*Chlorosarcinopsis eremi*)**	***BGLRS (*Scenedesmus sp. MKB*)**	***Spirulina* sp. NCIM 5143**
Arsenic	As	0.00	0.00	0.00
Boron	B	0.05	0.01	0.01
Calcium	Ca	0.88	3.40	0.21
Cadmium	Cd	0.00	0.00	0.00
Chromium	Cr	0.00	0.00	0.00
Copper	Cu	0.00	0.00	0.00
Iron	Fe	0.09	0.42	0.07
Magnesium	Mg	0.44	0.98	0.29
Manganese	Mn	0.18	0.01	0.01
Nickel	Ni	0.00	0.01	0.00
Phosphorus	P	0.38	2.82	0.86
Lead	Pb	0.00	0.00	0.00
Sulphur	S	2.73	3.01	2.92
Zinc	Zn	0.01	0.10	0.01

* Diacid (HNO_3:$HClO_4$ (3:1)) digested biomass

(Lieu *et al* 2001). Similarly, Mg was 3.38 times and 1.52 times as much as in BGLRS (*Scenedesmus sp. MKB*) (0.98%) and BGLR18 (*Chlorosarcinopsis eremi*) (0.44%) respectively than *Sprirulina* sp. NCIM 5413 (0.29%). Mg play an important role as a cofactor for a different metabolic reactions involving more than 300 enzymes within the human body (Viering *et al* 2017). Optimal concentrations of trace elements are also crucial for efficient anaerobic digestion as micro-organisms require these trace elements for their growth (Zhang *et al* 2011). Iron (Fe), cobalt (Co) and nickel (Ni) are considered most essential elements for the anaerobic digestion process (Moestedt *et al* 2015) and their importance has been discussed by many researchers in their study (Florencio *et al* 1994, Karlsson *et al* 2012, Kida *et al* 2001, Bayr *et al* 2012, Jarvis *et al* 1997, Gustavsson *et al* 2011). These results showed that both these stress tolerant microalgal strains have the potential to act as substrate for anaerobic digestion as well as mineral supplements.

4.3.4 GC-MS analysis of Microalgae strains

GC-MS analysis of methanolic extracts of three microalgae compounds revealed the presence of many bioactive compounds. It was found that in BGLR18 (*Chlorosarcinopsis eremi*), a total of eleven compounds (Table 4.56) (Fig. 4.12) were found with Phytol (21.56%), Heptadecane (19.37%); Hexadecanoic acid, methyl ester (18.46%), Trans-13-Octadecenoic acid, methyl ester (13.41%), 9,12-Octadecadienoic acid, methyl ester (10.33%) and Phthalic acid, 6-ethyl-3-octyl butyl ester (9.05%) as major compounds whereas eighteen compounds were found in strain BGLRS (*Scenedesmus sp. MKB*) (Table 4.57) (Fig. 4.13) which was highest among the three strains studied with phytol (22.93%), hexadecanoic acid, methyl ester (19.98%), Trans-13-Octadecenoic acid, methyl ester (19.53%) and 9,12-Octadecadienoic acid, methyl ester (20.09%) as major compounds. Microalgae strain *Spirulina* sp. NCIM 5143 showed the presence of twelve compounds (Table 4.58) (Fig. 4.14) and major compounds were as follows : phytol (26.15%), hexadecanoic acid, methyl ester (18.82%), Trans-13-Octadecenoic acid, methyl ester (22.31%) and 9,12-Octadecadienoic acid, methyl ester (20.75%). However, compounds detected in all the three strains were Phytol; Hexadecanoic acid, methyl ester; 9,12- Octadecadienoic acid, methyl ester and Trans-13-Octadecenoic acid, methyl ester. Beside these, compounds common between BGLR18 (*Chlorosarcinopsis eremi*) and BGLRS (*Scenedesmus sp. MKB*) were Hexadecanoic acid methyl ester; 1-Monolinoleoylglycerol trimethylsilyl ether; 9,12-Octadecadienoic acid, methyl ester; Trans-13-Octadecenoic acid, methyl ester; Phytol and Heptadecanoic acid, 16-methyl, methyl ester and between BGLR18 (*Chlorosarcinopsis eremi*) and *Spirulina* sp. NCIM 5143 were Hexadecanoic acid, methyl ester; 9,12-Octadecadienoic acid, methyl ester; Trans-13-Octadecenoic acid, methyl ester and Phytol. Also, the compounds common between BGLRS (*Scenedesmus sp. MKB*) *and Spirulina* sp. NCIM 5143 were Hexadecanoic acid, methyl ester; 9,12- Octadecadienoic acid, methyl ester and Trans-13-Octadecenoic acid,

methyl ester; Phytol; Cyclohexasiloxane, dodecamethyl and Cycloheptasiloxane, tetradecamethyl.

As it is revealed from above, that among other compounds phytol is present as major compound in BGLR18 (*Chlorosarcinopsis eremi*), BGLRS (*Scenedesmus sp. MKB*) and *Spirulina* sp. NCIM 5143 (at the retention time (RT) of 38.84, 38.86 and 38.87 respectively. Phytol is the component of various pharmaceutical used for the purpose of prophylaxis, prevention and treatment of hypercholesterolemia, for maintaining normal levels of cholesterol in serum, obesity, insulin resistance, diabetes, atherosclerosis and related cardiovascular diseases (Olofsson 2011). Many plants used as chinese medicines traditionally contains phytol which has pharmacological activity, low toxicity and costs (Yu *et al* 2009).

Heptadecane as found in methanolic extract of strain BGLR18 (*Chlorosarcinopsis eremi*) (19.37%) at RT of 30.62 was previously reported to be found in algae and many plant species and has antioxidant, anticancer and antimicrobial activity (Lee *et al* 2007, Mishra and Shree 2007).

The fatty acid compounds 9,12-Octadecadienoic acid, methyl ester found in BGLRS (*Scenedesmus* strain *MKB.*) (20.09%), BGLR18 (*Chlorosarcinopsis eremi*) (10.33%) and *Spirulina* sp. NCIM 5413 (20.75%) at the RT 38.52, 38.48 and 38.52 respectively has been reported to has anti-cancareous effect (Yu *et al* 2005). Trans-13-Octadecenoic acid, methyl ester in BGLRS (*Scenedesmus* strain *MKB.*) (19.53%), BGLR18 (*Chlorosarcinopsis eremi*) (13.41%) and *Spirulina* sp. NCIM 5413 (22.31%) at the RT 38.65, 38.62 and 38.66 respectively has anti-inflammatory, antiandrogenic, cancer preventive, dermatitigenic, irritant, antileukotriene-D4, hypocholesterolemic, 5-alpha reductase inhibitor, anemiagenic, insectifuge, flavor (Krishnamoorthy and Subramaniam 2014). Similarly, at the RT of 35.31, 35.29 and 35.32 in BGLRS (*Scenedesmus* strain *MKB.*) (19.98%), BGLR18 (*Chlorosarcinopsis eremi*) (18.46%) and *Spirulina* sp. NCIM 5413 (18.82%), Hexadecanoic acid, methyl ester was detected which was reported to has antibacterial and antifungal properties (Chandrasekaran *et al* 2011), anti-inflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, nematicide, insectifuge, antihistaminic, antieczemic, antiacne, alpha reductase inhibitor, antiandrogenic, antiarthritic, anticoronary (Krishnamoorthy and Subramaniam 2014).

Organosiloxane compounds like Cyclohexasiloxane dodecamethyl, Cycloheptasiloxane tetradecamethyl and Cyclooctasiloxane, hexadecamethyl were also detected in methanolic extracts BGLRS (*Scenedesmus* strain *MKB.*) and *Spirulina* sp. NCIM 5413 (Table 4.57 and 4.58). Cyclohexasiloxane dodecamethyl has antimicrobial, antifouling imunomodulatory and antitumor activities and Cycloheptasiloxane tetradecamethyl has skin-Conditioning Agent, fragrance, antimicrobial properties (Chaudhary and Tripathy 2015).

Table 4.56. Composition of different volatile compounds in BGLR18 (*Chlorosarcinopsis eremi*) analyzed through GC-MS

S.No.	Compound name	Molecular Formula	Area (%)	Retention time (RT)
1.	Heptadecane	$C_{17}H_{36}$	19.37	30.62
2.	Octatriacontyl pentafluoropropionate	$C_{41}H_{77}F_5O_2$	1.91	32.64
3.	2-Pentadecanone,6,10,14-trimethyl	$C_{18}H_{36}O$	1.75	33.61
4.	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	18.46	35.29
5.	Phthalic acid, 6-ethyl-3-octyl butyl ester	$C_{22}H_{34}O_4$	9.05	35.78
6.	1-Monolinoleoylglycerol trimethylsilyl ether	$C_{27}H_{54}O_4Si_2$	0.18	36.70
7.	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	10.33	38.48
8.	Trans-13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	13.41	38.62
9.	n-Propyl 11-octadecenoate	$C_{21}H_{40}O_2$	1.61	38.73
10.	Phytol	$C_{20}H_{40}O$	21.56	38.84
11.	Heptadecanoic acid, 16-methyl, methyl ester	$C_{19}H_{38}O_2$	2.37	39.13

Table 4.57 Composition of different volatile compounds in BGLRS (*Scenesesmus* sp. *MKB*.) analyzed through GC-MS

S.No.	Compound name	Molecular Formula	Area (%)	Retention time (RT)
1.	Silanediol, dimethyl	$C_2H_8O_2Si$	0.89	4.05
2.	Cyclotetrasiloxane, octamethyl	$C_8H_{24}O_4Si_4$	0.13	9.99
3.	Cyclotetrasiloxane, octamethyl	$C_8H_{24}O_4Si_4$	0.06	11.02
4.	Cyclopentasiloxane, decamethyl	$C_{10}H_{30}O_5Si_5$	0.52	16.06
5.	Cyclohexasiloxane, dodecamethyl	$C_{12}H_{36}O_6Si_6$	5.46	20.98
6.	Cycloheptasiloxane, tetradecamethyl	$C_{14}H_{42}O_7Si_7$	1.57	25.40
7.	Methyl tetradecanoate	$C_{15}H_{30}O_2$	0.33	31.11
8.	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	2.49	32.48
9.	Heptacosanoic acid, methyl ester	$C_{28}H_{56}O_2$	0.79	32.66
10.	2-Pentadecanone, 6,10,14-trimethyl	$C_{18}H_{36}O$	0.54	33.64
11.	i-Propyl 9-tetradecenoate	$C_{17}H_{32}O_2$	0.25	34.87
12.	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	19.98	35.31
13.	Phthalic acid, butyl 4-octyl Ester	$C_{20}H_{30}O_4$	0.24	35.81
14.	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	20.09	38.52
15.	Trans-13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	19.53	38.65
16.	1-Monolinoleoylglycerol trimethylsilyl ether	$C_{27}H_{54}O_4Si_2$	1.11	38.77
17.	Phytol	$C_{20}H_{40}O$	22.93	38.86
18.	Heptadecanoic acid, 16-methyl, methyl ester	$C_{19}H_{38}O_2$	3.10	39.16

Table 4.58 Composition of different volatile compounds in *Spirulina* sp. NCIM 5143 analyzed through GC-MS

S.No.	Compound name	Molecular Formula	Area (%)	Retention time (RT)
1.	Cyclohexasiloxane, dodecamethyl	$C_{12}H_{36}O_6Si_6$	4.87	20.99
2.	Cycloheptasiloxane, tetradecamethyl	$C_{14}H_{42}O_7Si_7$	1.33	25.41
3.	Cyclooctasiloxane, hexadecamethyl	$C_{16}H_{48}O_8Si_8$	0.03	29.34
4.	Z-3-Octadecen-1-ol acetate	$C_{20}H_{38}O_2$	0.13	33.64
5.	Docosanoic acid, docosyl ester	$C_{44}H_{88}O_2$	0.07	34.87
6.	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	18.82	35.32
7.	Dibutyl phthalate	$C_{16}H_{22}O_4$	0.24	35.82
8.	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	20.75	38.52
9.	Trans-13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	22.31	38.66
10.	6-Octadecenoic acid, methyl ester, (Z)	$C_{19}H_{36}O_2$	1.85	38.78
11.	Phytol	$C_{20}H_{40}O$	26.15	38.87
12.	Methyl stearate	$C_{19}H_{38}O_2$	3.46	39.17

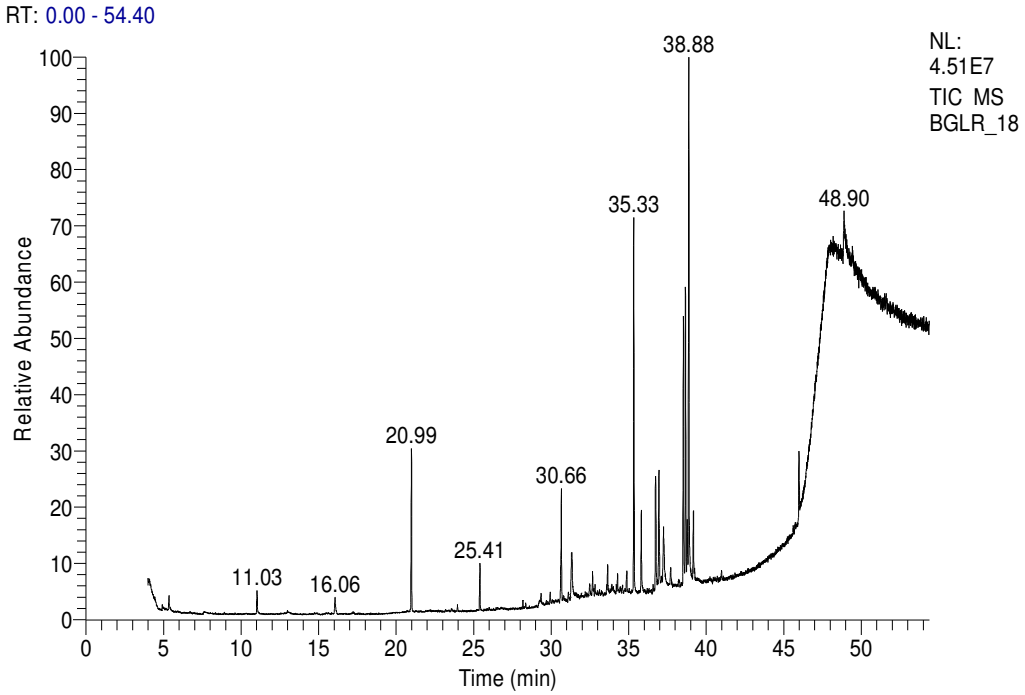


Fig. 4.12 Peaks showing various compounds revealed in GC-MS analysis of BGLR18 (*Chlorosarcinopsis eremi*)

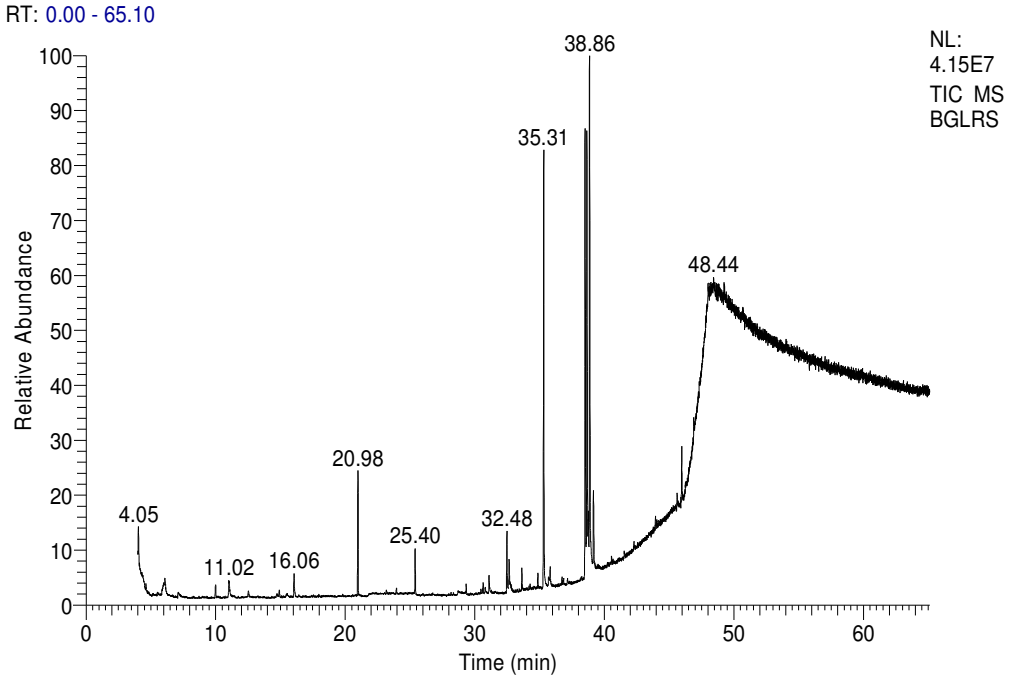


Fig. 4.13 Peaks showing various compounds revealed in GC-MS analysis of BGLRS (*Scenesesmus* sp. *MKB*.)

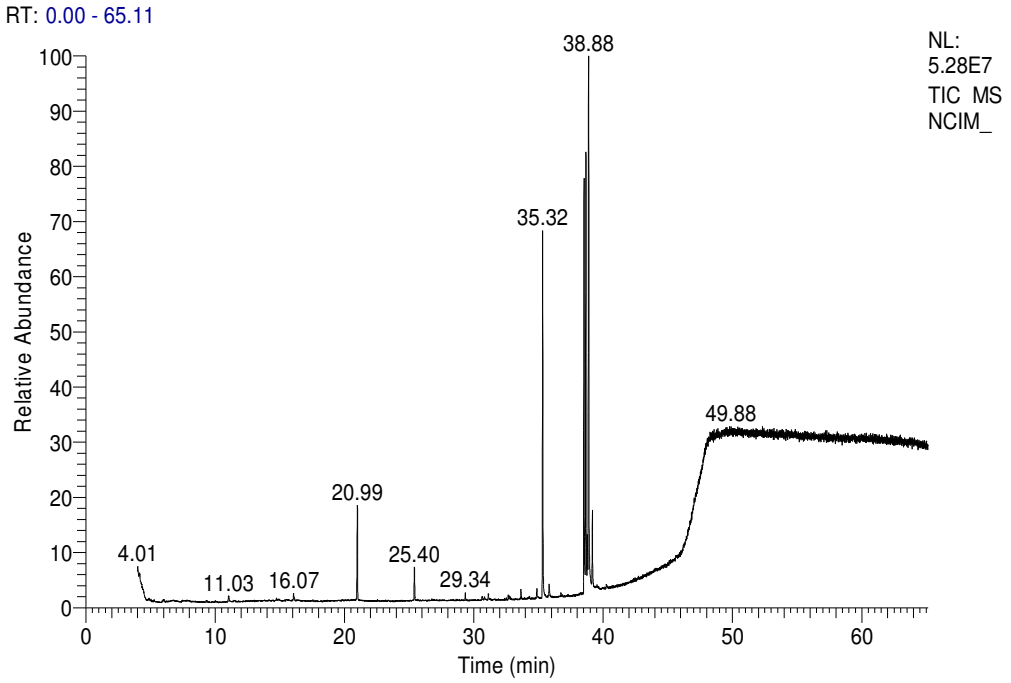


Fig. 4.14 Peaks showing various compounds revealed in GC-MS analysis of *Spirulina* sp. NCIM 5143

4.4 Proximate and chemical composition of three selected microalgae strains

Three selected microalgae strains were also analyzed for their proximate and chemical composition (Table 4.59). Results obtained showed that highest total solids was found in BGLRS (*Scenedesmus sp. MKB*) (2.70) followed by *Spirulina* sp. NCIM 5143 (2.04) and BGLR18 (*Chlorosarcinopsis eremi*) (1.50). Total solid is one of the very important parameters for biogas production and in our study microalgae strains showed low range of total solids. In his study, Baserja (1984) reported that anaerobic digestion process was slow and unstable at TS of 7% and overloading of digester occurred at 10% level. Highest volatile solids were present in BGLRS (*Scenedesmus sp. MKB*) (67.45) followed by BGLR18 (*Chlorosarcinopsis eremi*) (59.41) and *Spirulina* sp. NCIM 5143 (58.10). High volatile matter in microalgae suggests that it is ideal candidate for co-digestion for biogas production. In anaerobic digestion process, volatile fatty acids is an intermediate which represent the metabolic activity of fermentative bacteria and thus an important factor for determining anerobic biodigestbility (Wang *et al* 2019). BGLRS (*Scenedesmus sp. MKB*), *Spirulina* sp. NCIM 5143, BGLR18 (*Chlorosarcinopsis eremi*) have the ash content of 32.55, 41.90 and 40.59 respectively. Ash content has no direct energetic value but it constitutes the plant mineral portion which are essential for operating metabolism normally (Siegal-Willott *et al* 2010). Expected biogas yield and efficiency of anaerobic digestion process can be estimated by concentrations of total solids (TS) and volatile solid (VS). Total solids (TS) expressed as g/kg is a measure of dry matters of substrate. Volatile solids is the organic fraction of total

solids (TS) (Orhorhoro *et al* 2017, Ray *et al* 2013, Budiyono and Syaichurrozi 2014). Highest cellulose content was shown by BGLR18 (*Chlorosarcinopsis eremi*) (3.20) followed by BGLRS (*Scenedesmus sp. MKB*) (2.40) and *Spirulina* sp. NCIM 5143 (1.50). Highest hemicellulose content was found in BGLR18 (*Chlorosarcinopsis eremi*) (4.00) followed by BGLRS (*Scenedesmus sp. MKB*) (3.40) and *Spirulina* sp. NCIM 5143 (2.30). It has been reported that in higher plants, principal carbohydrate is cellulose but in algae this is not true (Rabemanolontsoa and Saka 2013). In algae such as brown and red algae, alginate as well as sulfated polysaccharides such as agar and carageenan are reported to be major part of the hemicellulosic saccharides (Gorham and Lewey 1984, McCandles 1981). Lignin content ranged from 0.20 to 0.70. It was believed previously that algae does not contain lignin but re-consideration of lignin content in plants is going on (Lewis and Yamamoto 1990, Novo-Uzal *et al* 2012). In primitive green algae, first, "lignin-like compounds have been detected (Delwiche *et al* 1989, Gunnison and Alexander 1975). Martone *et al* (2009) has reported that in red alga *Calliarthron* secondary walls and true lignin have been perceived and also this alga contains guaiacyl propane, *p*-hydroxyphenylpropane as well as syringyl propane units. Moreover, many studies have reported the lignin content of 15 g/kg in the green alga sea lettuce (Sitompul *et al* 2012) and between 141 and 175 g/kg in brown algae (Malihan *et al* 2012). Silica content was found highest in BGLRS (*Scenedesmus sp. MKB*) (2.40), *Spirulina* sp. NCIM 5143 (2.20) and BGLR18 (*Chlorosarcinopsis eremi*) (1.70).

Table 4.59 Proximate and chemical composition of microalgal strains

Proximate composition (%)	**BGLR18 (*Chlorosarcinopsis eremi*)**	**BGLRS (*Scenedesmus* sp. *MKB.*)**	***Spirulina sp.* NCIM 5143**
Total solids (TS)	1.50^{c}	2.70^{c}	2.04^{c}
Volatile solids (VS)	59.41^{a}	67.45^{a}	58.10^{a}
Ash	40.59^{b}	32.55^{b}	41.90^{b}
Chemical composition (%)			
NDF (Neutral Detergent Fibre)	7.90^{a}	7.60^{b}	5.10^{c}
ADF (Acid Detergent Fibre)	3.90^{b}	4.20^{a}	2.20^{c}
Cellulose	3.20^{a}	2.40^{b}	1.50^{c}
Hemicellulose	4.00^{a}	3.40^{b}	2.30^{c}
Lignin	0.70^{a}	0.30^{b}	0.20^{c}
Silica	1.70^{b}	2.40^{a}	2.20^{ab}

Cultural conditions : Temperature (25±2 °C), Values superscripted by different alphabets in column differ significantly (P≤0.05) from each other (Tukey's test).

4.5 Assessment of biogas potential of selected microalgae strains at lab scale

Three selected microalgal strains viz. BGLRS (*Scenedesmus sp. MKB*), BGLR18 (*Chlorosarcinopsis eremi*) and *Spirulina* sp. NCIM 5143 were evaluated for their biogas production potential at lab scale under batch mode in six different sets. These strains were studied alone and in co-digestion with paddy straw alongwith cowdung as substrate and biodigested slurry as inoculum. The biogas production was noted daily for a period of five months.

4.5.1.1 Biogas production potential of BGLRS (*Scenedesmus sp. MKB*)

Biogas production potential of BGLRS (*Scenedesmus sp. MKB*) was studied in terms of cumulative biogas production in terms of L/Kg of total solids added and consumed as well as L/Kg of volatile solids added and consumed (Table 4.60). The experiment was conducted in the month of October 2018 and continued till February 2019. It was found that highest biogas production (28.80 litres) was in digester C in terms of total solids added and (800.00 L/kg) and total solids consumed (870.00 L/kg). Again in terms of volatile solids added (872.73 L/kg) and consumed (997.94 L/kg) highest biogas production was in digester C which was higher than control (digester A) where total biogas production was 0.80 Litres and 29.63 L/kg, 43.93 L/kg , 38.10 L/kg and 57.58 L/kg in terms of total solids added, total solids consumed, volatile solids added and volatile solids consumed respectively.

In case of co-digestion experiment with paddy straw highest biogas production (60.18 litres) was in digester E in terms of total solids added (80.25 L/kg), total solids consumed (88.18 L/kg) and in terms of volatile solids added (88.51 L/kg) and consumed (133.24 L/kg) which was higher than control (digester B) where total biogas production was 48.56 litres, 63.90 L/kg in terms of total solids added, 73.16 L/kg total solids consumed, 74.71 L/kg volatile solids added and 107.75 L/kg volatile solids consumed (Table 4.60).

Ambarsari *et al* (2018) reported that co-digestion of *Chlorella* sp. "CD01" substrate with cow rumen fluid and manure as inoculum produced 314.5 mL biogas with yield of 43.23 mL/g TS and 1758 mL biogas with yield of 98.96 mL/g TS respectively.

4.5.1.2 Study of kinetics of biogas production BGLRS (*Scenedesmus sp. MKB*) biomass

The kinetics of biogas production by BGLRS (*Scenedesmus sp. MKB*) biomass and its co-digestion with paddy straw was studied by modified Gompterz equation. Results showed that digester C showed highest biogas production potential (P) 30.85 mLg^{-1} VS at a maximum biogas production rate (R_m) of 0.58 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 1.30 days followed by digester D with biogas production potential (P), biogas production rate (R_m) and a lag phase (λ) of 30.00 mLg^{-1} VS, 0.45 $mLg^{-1}d^{-1}$ and 0.00 days respectively at the end of 5 month period (150 days). Both sets showed higher values than control (digester C) in which biogas production potential (P) was 1.23 mLg^{-1} VS at a maximum biogas production rate (R_m) of 0.02 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 15.13 days (Table 4.61) (Fig. 4.15).

Similarly, digester C showed maximum percent reduction in volatile solids (7.80%) followed by D (4.81%) compared to control digester A (1.91%) (Table 4.61) (Fig. 4.15). In digester A negligible biogas was formed after the end of 150 days. Digester A contains only BGLRS (*Scenedesmus sp. MKB*) biomass only. The rigidity and recalcitrant nature of cell wall structure of BGLRS (*Scenedesmus sp. MKB*) biomass can be the main reason for negligible biogas production. In algal biorefinery and biofuel processes, the break down of cell walls to disintegrate membrane structure is a crucial problem (Kroger *et al* 2018). To make inner microalgal organic matter available to anerobic micro-organisms hydrolysis of cell wall is necessary (Passos and Ferrer 2014). Cell wall of *Scenedesmus* consists of glucose, mannose and galactose and is rigid (Takeda 1996). It has three layers : inner cellulosic layer, middle algaenan layer and outer pectic layer (Munoz and Gonzalez-Fernandez 2017). Algaenan is a recalcitrant, insoluble and non-hydrolysable substance present in many microalgae of chlorophyte division (green algae) (Allard *et al* 2002, Kroger *et al* 2018). Highly resistant aliphatic polymers are present in algaenan layer which make it difficult to break down (Scholz *et al* 2014, Kroger *et al* 2018).

As seen from above results, biogas production from microalgae BGLRS (*Scenedesmus sp. MKB*) biomass is low. The possible reason for this could below C/N ratio of algal biomass which can cause ammonium accumulation and inhibit process (Rincon *et al* 2018). However, the co-digestion of microalgae with other feedstocks having high C/N ratio like lignocellulosic biomass can improve the biogas production rate by adjusting the C/N ratio between the two (Li *et al* 2017, Thorin *et al* 2017). Therefore, co-digestion experiment was carried out in order to assess the co-digestion potential of paddy straw with microalgae under the same conditions. In co-digestion experiment, it was found that digester E had the maximum biogas production potential (P) of 68.58 mLg^{-1} VS at a maximum biogas production rate (R_m) of 1.34 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 0.66 days (Table 4.61) (Fig. 4.15) at the end of 5 months period. This was followed by digester F biogas production potential (P) of 22.00 mLg^{-1} VS at a maximum biogas production rate (R_m) of 0.21 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 1.31 days. Digester E showed higher biogas production than control (digester B) which showed biogas production potential (P) of 54.00 mLg^{-1} at a maximum biogas production rate (R_m) of 1.00 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 8.00 days. Also, percent reduction in volatile solids was highest in digester E (27.01%) which was greater than control digester B (20.61%). The modified Gompertz equation satisfactorily described biogas production with a goodness of fit (R^2) of 0.987, 0.992, 0.991, 0.997, 0.984 and 0.997 for digesters A, B, C, D, E and F respectively (Table 4.61) (Fig. 4.15).

4.5.1.3 Proximate and chemical composition of feedstock of BGLRS (*Scenedesmus sp. MKB*) before and after anerobic digestion process

Proximate and chemical composition of feedstock fed into different sets of digesters

A-F was determined before and after anaerobic digestion process. It was evident from the results represented in Table 4.62 that after anerobic digestion process of 150 days there was a notable decrease in total solids and volatile solids of feedstock as compared to before anaerobic digestion process of BGLRS (*Scenedesmus sp. MKB*) biomass. The decrease in volatile solids was due to its consumption during anaerobic process. In biomethanation process, after the initial hydrolysis step, fermentative bacteria use the small molecules to volatile fatty acids and alcohols which are further transformed by acetogens to acetate, carbon dioxide, and hydrogen (Tsapekos *et al* 2017) and finally acetate was converted by methanogens to methane (Shima *et al* 2002). Ash content after completion of digestion process was found to be increased. Ash content represent the plant mineral portion which are essential for operating metabolism normally (Siegal-Willott *et al* 2010). The increase in ash content was due to conversion of microalgal biomass to biogas as BGLRS (*Scenedesmus sp. MKB*) biomass has high mineral content revealed by ICP-AES analysis (as shown in Table 4.55). Other parameters like cellulose, hemicelluloses, lignin and silica content also showed a consequential difference after anaerobic digestion process compared to before anerobic digestion. There was decrease in cellulose, hemicelluloses and lignin content after digestion. The decrease is due to its hydrolysis in anaerobic digestion process. In hydrolysis phase which is the first step of biomethanation process facultative and obligate anaerobic bacteria release hydrolase enzymes which break down cellulose, carbohydrate, proteins and fats into monomers. However, there is slow degradation of lignocellulosic material and lignin (Boontian 2014). After anerobic digestion process of 150 days, maximum percent decrease in total solids (TS) and volatile solids (VS) was observed in digester E which is responsible for highest biogas production in it among all the digesters.

4.5.1.4 Effect of temperature (seasonal variation) on biogas production of BGLRS (*Scenedesmus sp. MKB*) biomass

A great variation was observed in biogas yield per month as the temperature varied greatly from October 2018 to February 2019 as shown in Table 4.63 (Fig.4.16). Biogas production decrease with decrease in temperature corresponds to weather and vice-versa. Maximum biogas production was observed in digester B (7,920.00 mL) followed by digester E (4,215.00 mL), digester F (4,032.00 mL), digester C (2,990.00 mL) and digester D (2,625.00 mL), digester A (100.00 mL) in the month of October where the average temperature was between 29 to 32°C. Lowest biogas production (5.00 mL) was found in month of January (average temperature was between 15 to 17 °C) in digester A, followed by digester F (17.5 mL), B (50.00 mL), D (382.00 mL), C (492.00 mL) and E (1,311.00 mL). Temperature plays a very important role in the process of anaerobic digestion. For anaerobic digestion process, temperature is mainly divided into three phases : Psychrophilic temperature <20° C, mesophilic temperature between 20°C to 40°C and thermophilic temperature between

40°C to 60°C and mesophilic is the mainly operational temperature in process (Singh and Pal 2016). The possible reason for the decrease in biogas production at low temperature is that methanogenic bacteria which ultimately produce methane (biogas) in anaerobic digestion process are very temperature sensitive organisms. As to utilization of volatile acids by methanogenic bacteria, temperature decrease leads to decrease of maximum specific growth of bacteria and ultimately biogas generation rate decrease (Dhadse *et al* 2012). Temperature significantly affects the growth and activity of methanogens (Griffin *et al* 1998). Pandey and Soupir (2012) in their study found that biogas production at 52.5°C and 37°C were 49 and 17 times higher than at 25°C.

Table 4.60 Biogas production potential of BGLRS (*Scenedesmus* sp. *MKB*.) biomass in combination with paddy straw

Digester	Composition of feedstock	Biogas (in Litres)	Cumulative biogas (L/Kg TS added)	Cumulative biogas (L/Kg VS added)	Cumulative biogas (L/Kg TS consumed)	Cumulative biogas (L/Kg VS consumed)
A	*Harvested Microalgal biomass	0.80^{f}	29.63^{e}	43.93^{e}	38.10^{e}	57.58^{e}
B	***Paddy straw	48.56^{b}	63.90^{d}	73.16^{d}	74.71^{d}	107.75^{d}
C	*Harvested microalgal biomass+¥cow dung + #biodigested slurry	28.80^{c}	800.00^{a}	870.00^{a}	872.73^{a}	997.94^{a}
D	**Microalgae biomass filtrate+¥ cow dung + # biodigested slurry	24.62^{d}	769.50^{b}	855.00^{b}	820.80^{b}	989.15^{b}
E	*Harvested Microalgal biomass + ¥cow dung + # biodigested slurry+*paddy straw	60.18^{a}	80.25^{c}	88.18^{c}	88.51^{c}	133.24^{c}
F	**Microalgal biomass filtrate +¥cow dung + # biodigested slurry+paddy straw	18.43^{e}	24.91^{f}	27.67^{f}	26.33^{f}	32.91^{f}

*Harvested microalgal biomass=1500g, **Microalgal biomass filtrate= 1500 mL, #Biodigested slurry =150 mL, ¥Cow dung=300g, ***Paddy straw=250g, Temperature range =15-32°C, Values superscripted by different alphabets in column differ significantly (P≤0.05) from each other (Tukey's test).

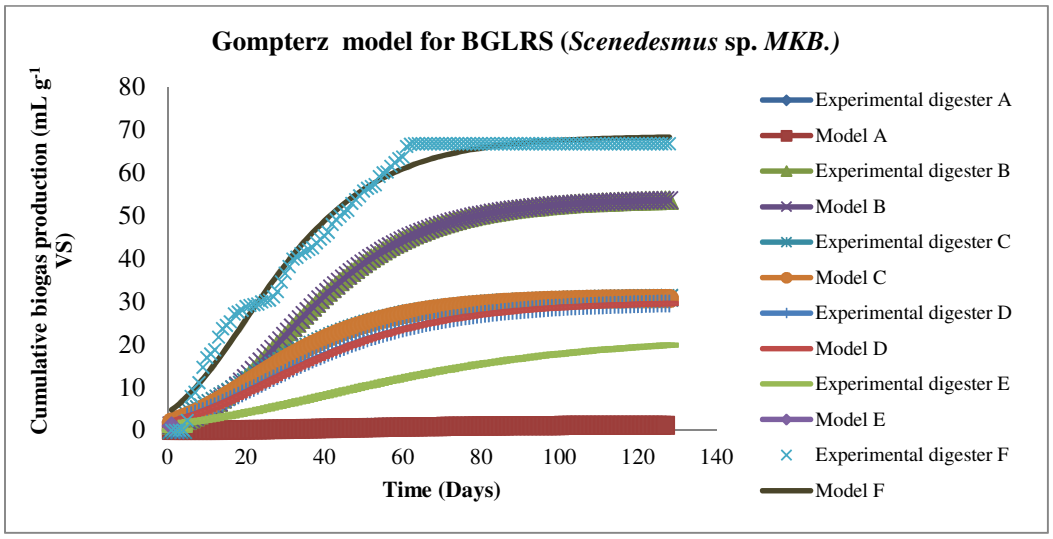


Fig 4.15 Variation and fitting of the cumulative biogas data of Biogas potential of BGLRS (*Scenedemus* sp. *MKB*.) with the Gompertz model for the different digesters (A-F) with time

Table 4.61 Estimated kinetic constants using modified Gompterz equation characteristics of the digesters A-F of BGLRS (*Scenedemus* sp. *MKB*.) biomass and its combination with paddy straw

Digester	Composition of feedstock	P (mLg^{-1} VS)	R_m ($mLg^{-1}d^{-1}$)	^ (d)	R^2	VSR (%)
A	*Harvested Microalgal biomass	1.23	0.02	15.13	0.987	1.91^{f}
B	***Paddy straw	54.00	1.00	8.00	0.992	20.61^{b}
C	*Harvested microalgal biomass+¥cow dung + #biodigested slurry	30.85	0.58	1.30	0.991	7.80^{d}
D	**Microalgae biomass filtrate+¥ cow dung + # biodigested slurry	30.00	0.45	0.00	0.997	4.81^{e}
E	*Harvested Microalgal biomass + ¥cow dung + # biodigested slurry+*paddy straw	68.58	1.34	0.66	0.984	27.01^{a}
F	**Microalgal biomass filtrate +¥cow dung + # biodigested slurry+paddy straw	22.00	0.21	1.31	0.997	11.11^{c}

Harvested microalgal biomass=1500g, **Microalgae filtrate= 1500 mL, #Biodigested slurry =150 mL, ¥Cow dung=300g, ***Paddy straw=250g, Temperature =15-32°C; P: ultimate biogas yield; R_m:maximum rate of biogas production; λ: lag phase; R^2: Coefficient of determination; VSR: volatile solid reduction, Values superscripted by different alphabets in column differ significantly (P≤0.05) from each other (Tukey's test).

Table 4.62 Proximate and chemical composition of feedstock of BGLRS (*Scenedemus* sp. *MKB*.) before and after digestion process

	Proximate composition (%)						Chemical composition (%)							
	Total solids (TS)		Volatile solids (VS)		Ash		Cellulose		Hemicellulose		Lignin		Silica	
Digester No.	Before digestion	After digestion	Before digestion	After digestion	Before digestion	After digestion	Before digestion	After digestion	Before digestion	After digestion	Before digestion	After digestion	Before digestion	After digestion
A	2.70^{b}	2.10^{c}	67.45^{c}	66.16^{e}	32.55^{a}	33.84^{a}	2.40^{f}	1.80^{f}	3.40^{e}	2.80^{f}	0.70^{c}	0.70^{c}	2.40^{d}	2.60^{d}
B	76.00^{a}	65.00^{b}	87.34^{b}	69.34^{d}	12.66^{b}	30.66^{c}	33.60^{c}	30.40^{a}	25.20^{c}	22.00^{b}	3.40^{a}	3.20^{a}	12.50^{a}	12.60^{a}
C	3.60^{b}	3.30^{c}	91.87^{a}	87.45^{a}	8.13^{c}	12.55^{f}	17.40^{e}	13.60^{d}	3.40^{e}	3.30^{e}	0.20^{d}	0.20^{c}	0.60^{e}	0.80^{f}
D	3.20^{b}	3.00^{c}	90.00^{ab}	82.98^{b}	10.00^{c}	17.02^{e}	20.80^{d}	11.20^{e}	14.20^{d}	12.60^{d}	0.40^{cd}	0.40^{c}	0.80^{e}	2.00^{e}
E	74.00^{a}	70.00^{a}	90.00^{ab}	80.00^{c}	10.00^{c}	20.00^{d}	37.60^{a}	29.60^{b}	26.60^{b}	20.60^{c}	1.60^{b}	1.50^{b}	5.40^{c}	5.90^{b}
F	75.00^{a}	68.00^{ab}	91.00^{ab}	66.42^{e}	9.00^{c}	33.58^{a}	37.00^{b}	27.40^{c}	33.60^{a}	30.60^{a}	1.80^{b}	1.80^{b}	6.60^{b}	4.40^{c}

Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

Table 4.63 Biogas production of BGLRS (*Scenedemus* sp. *MKB*.) in different months of the year due to temperature variation

Month	Temperature (°C)	Digesters Biogas produced (mL/250 g FS)					
		A	B	C	D	E	F
October	29-32	100.00^{f}	7,920.00^{a}	2,990.00^{a}	2,625.00^{a}	4,215.00^{b}	4,032.50^{c}
November	28-30	65.00^{f}	2,510.00^{c}	1,931.00^{b}	1,827.00^{b}	3,630.00^{b}	370.00^{e}
December	22-23	10.00^{e}	891.00^{c}	1,197.00^{c}	752.00^{c}	2,910.00^{a}	5.00^{f}
January	15-17	5.00^{e}	50.00^{d}	492.00^{e}	382.00^{e}	1,311.00^{a}	17.50^{e}
February	19-20	20.00^{f}	770.00^{c}	590.00^{d}	570.00^{d}	2,980.00^{a}	182.50^{e}
Total Biogas (ml/250 g FS)		200.00	12,141.00	7,200.00	6,156.00	15,046.00	4,607.50

Values superscripted by different alphabets in column differ significantly (P≤0.05) from each other (Tukey's test).

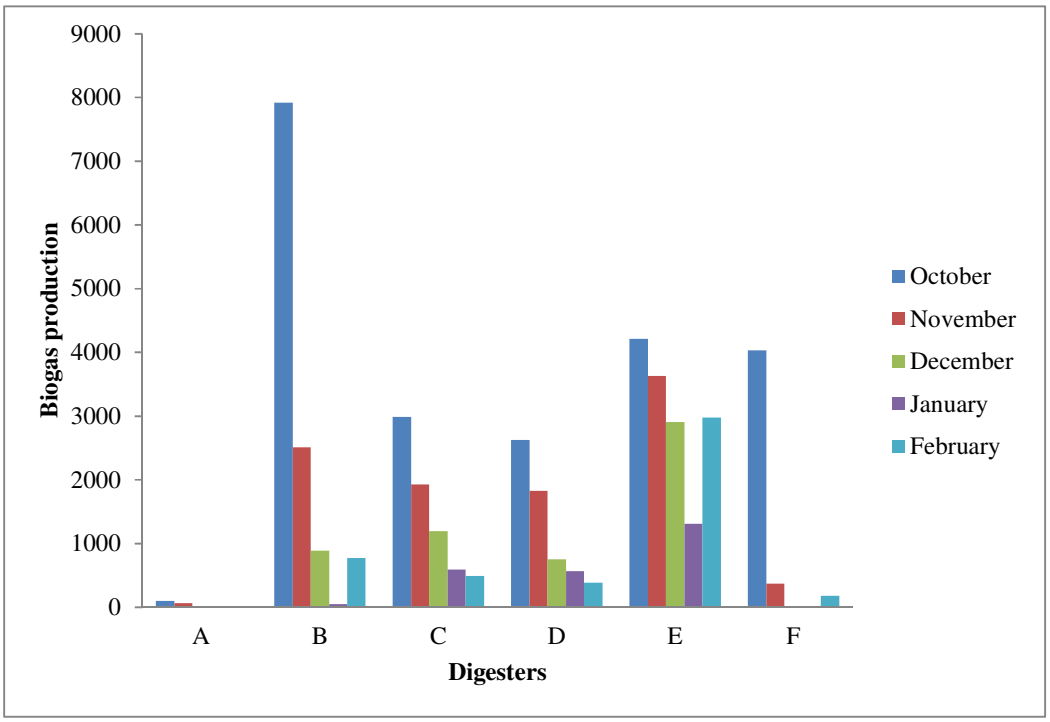


Fig 4.16 Variation in biogas production with temperature of BGLRS (*Scenedemus* sp. *MKB*.) biomass

4.5.2.1 Biogas production potential of BGLR18 (*Chlorosarcinopsis eremi*) biomass

The experiment was conducted in the month of January 2019 and continued till May 2019. It was found that highest biogas production (16.00 Litres) was in digester C in terms of

total solids added (516.13 L/kg) and total solids consumed (600.23 L/kg). Again in terms of volatile solids added (551.72 L/kg) and consumed (688.61 L/kg) highest biogas production was in digester C which was higher than control (digester A) where biogas production is 1 litre, 66.67 L/kg in terms of total solids added, 112.21 L/kg total solids consumed, 83.33 L/kg volatile solids added and 141.29 L/kg volatile consumed (Table 4.64)

Table 4.64 Biogas production potential of BGLR18 (*Chlorosarcinopsis eremi*) biomass in combination with paddy straw

Digester	Composition of feedstock	Biogas (in Litres)	Cumulative biogas (L/Kg TS added)	Cumulative biogas (L/Kg VS added)	Cumulative biogas (L/Kg TS consumed)	Cumulative biogas (L/Kg VS consumed)
A	*Harvested Microalgal biomass	1.00^{e}	66.67^{f}	112.21^{e}	83.33^{f}	141.29^{e}
B	***Paddy straw	58.64^{c}	78.06^{e}	111.15^{e}	80.35^{e}	123.13^{f}
C	*Harvested microalgal biomass+$^{¥}$cow dung + $^{\#}$biodigested slurry	16.00^{d}	516.13^{a}	600.23^{b}	551.72^{a}	688.61^{a}
D	**Microalgae biomass filtrate+$^{¥}$ cow dung + $^{\#}$ biodigested slurry	14.00^{d}	503.60^{b}	615.75^{a}	522.39^{b}	659.11^{b}
E	*Harvested Microalgal biomass + $^{¥}$cow dung + $^{\#}$ biodigested slurry+*paddy straw	168.46^{a}	213.51^{c}	294.25^{c}	231.80^{c}	374.60^{c}
F	**Microalgal biomass filtrate +$^{¥}$cow dung + $^{\#}$ biodigested slurry+paddy straw	154.14^{b}	197.03^{d}	281.00^{d}	209.30^{d}	336.91^{d}

*Harvested microalgal biomass=1500g, **Microalgal biomass filtrate= 1500 mL, $^{\#}$Biodigested slurry =150 mL, $^{¥}$Cow dung=300g, ***Paddy straw=250g, Temperature range =15-32°C, Values superscripted by different alphabets in column differ significantly (P≤0.05) from each other (Tukey's test)

In case of co-digestion experiment with paddy straw highest biogas production (168.46 Litres) was in Digester E in terms of total solids added and (213.51 L/kg) total solids consumed (294.25 L/kg) and in terms of volatile solids added (231.80 L/kg) and consumed (374.60 L/kg). Both the digesters showed higher biogas production than control digester B where total biogas production was 58.64 litres, 78.06 L/kg in terms of total solids added, 111.15 L/kg total solids consumed, 80.35 L/kg volatile solids added and 123.13 L/kg volatile consumed (Table 4.64).

4.5.2.2 Study of kinetics of biogas production BGLR18 (*Chlorosarcinopsis eremi*) biomass

As biogas production increased abruptly in the months of April and May. The kinetics of biogas production by BGLR18 (*Chlorosarcinopsis eremi*) biomass and its co-digestion with paddy straw was studied by modified Gompterz equation in two parts i.e. January 2019 to March 2019 and April 2019 to May 2019. In biogas production experiment form January 2019 to March 2019, it was observed that digester C showed highest biogas production potential (P) 8.93 mLg^{-1} VS at a maximum biogas production rate (R_m) of 0.12 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 5.38 days followed by digester D with biogas production potential (P), biogas production rate (R_m) and a lag phase (λ) of 8.26 mLg^{-1} VS, 0.29 $mLg^{-1}d^{-1}$ and 4.65 days respectively at the end of 5 month period (150 days). Both sets showed higher values than control (digester A) in which has biogas production potential (P) 0.54 mLg^{-1} VS at a maximum biogas production rate (R_m) of 0.04 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 14.19 days (Table 4.65) (Fig. 4.17).

Digester A contains only BGLR18 (*Chlorosarcinopsis eremi*) biomass only. The rigidity and recalcitrant nature of cell wall structure of BGLRS biomass can be the main reason for negligible biogas production. In algal biorefinery and biofuel processes, the break down of cell walls to disintegrate membrane structure is a crucial problem (Kroger *et al* 2018). To make inner microalgal organic matter available to anerobic micro-organisms hydrolysis of cell wall is necessary (Passos and Ferrer 2014). Cell wall of *Scenedesmus* consists of glucose, mannose and galactose and is rigid (Takeda 1996). It has three layers : inner cellulosic layer, middle algaenan layer and outer pectic layer (Munoz and Gonzalez-Fernandez 2017). Algaenan is a recalcitrant, insoluble and non-hydrolysable substance present in many microalgae of chlorophyte division (green algae) (Allard *et al* 2002, Kroger *et al* 2018). Highly resistant aliphatic polymers are present in algaenan layer which make it difficult to break down (Scholz *et al* 2014, Kroger *et al* 2018).

As seen from above results, biogas production from microalgae BGLR18 (*Chlorosarcinopsis eremi*) biomass is low. The possible reason for this could be low C/N ratio of algal biomass which can cause ammonium accumulation and inhibit process (Rincon *et al* 2018). However, the co-digestion microalgae with other feedstocks having high C/N ratio like

lignocellulosic biomass can improve the biogas production rate by adjusting the C/N ratio between the two (Li *et al* 2017, Thorin *et al* 2017). Therefore, co-digestion experiment was carried out in order to assess the co-digestion potential of paddy straw with microalgae under the same conditions as the above experiment. In co-digestion experiment, it was found that digester F had the maximum biogas production potential (P) of 19.91 mLg^{-1} VS at a maximum biogas production rate (R_m) of 0.74 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 9.98 days (Table 4.61) at the end of 5 months period. This was followed by digester E biogas production potential (P) of 16.23 mLg^{-1} VS at a maximum biogas production rate (R_m) of 0.51 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 0.00 days. Both of these were higher than control (digester B) which showed biogas production potential (P) of 9.74 mLg-^{1} at a maximum biogas production rate (R_m) of 0.27 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 8.97 days. The modified Gompertz equation satisfactorily described biogas production with a goodness of fit (R^2) of 0.923, 0.987, 0.992, 0.980, 0.985 and 0.976 for digesters A, B, C, D, E and F respectively (Table 4.65) (Fig. 4.17).

Table 4.65 Estimated kinetic constants using modified Gompterz equation characteristics of the digesters A-F of BGLR18 (*Chlorosarcinopsis eremi*) biomass and its combination with paddy straw (January to March 2019)

Digester	Composition of feedstock	P ($mLg^{-1}VS$)	R_m ($mLg^{-1}d^{-1}$)	^ (d)	R^2	VSR (%)
A	*Harvested Microalgal biomass	0.54	0.04	14.19	0.923	0.72^{f}
B	***Paddy straw	9.74	0.27	8.97	0.987	7.09^{c}
C	*Harvested microalgal biomass+¥cow dung + #biodigested slurry	8.93	0.12	5.38	0.992	6.82^{d}
D	**Microalgae biomass filtrate+¥ cow dung + # biodigested slurry	8.26	0.29	4.65	0.980	3.09^{e}
E	*Harvested Microalgal biomass + ¥cow dung + # biodigested slurry+*paddy straw	16.23	0.51	0.00	0.985	14.72^{a}
F	**Microalgal biomass filtrate +¥cow dung + # biodigested slurry+paddy straw	19.91	0.74	9.98	0.976	11.40^{b}

Harvested microalgal biomass=1500g, **Microalgae filtrate= 1500 mL, #Biodigested slurry =150 mL, ¥Cow dung=300g, ***Paddy straw=250g, Temperature =15-32°C; P: ultimate biogas yield; R_m:maximum rate of biogas production; λ: lag phase; R^2: Coefficient of determination; VSR: volatile solid reduction, Values superscripted by different alphabets in column differ significantly (P≤0.05) from each other (Tukey's test).

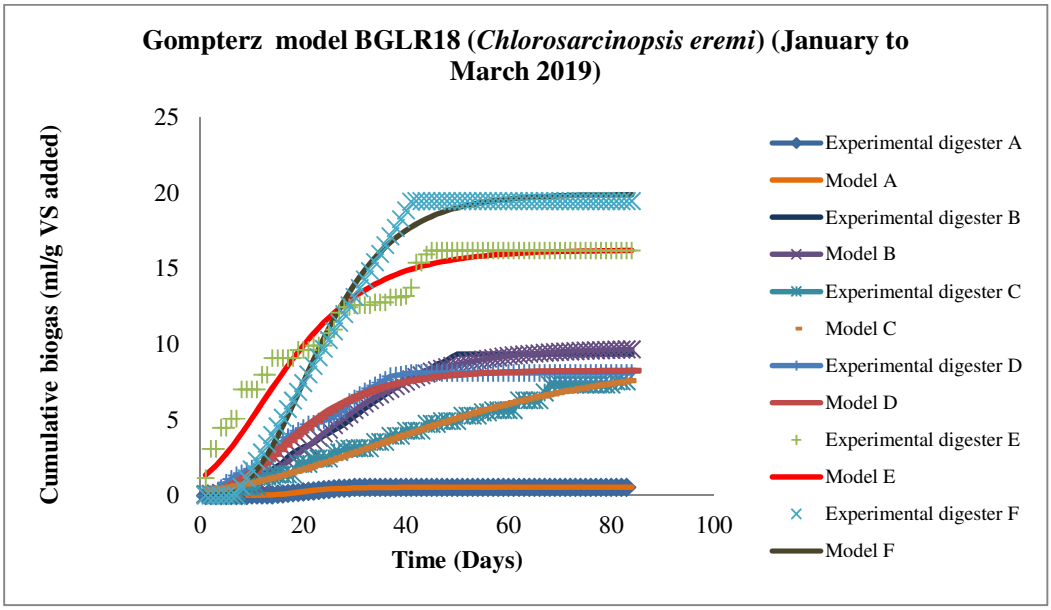


Fig. 4.17 Variation and fitting of the cumulative biogas data of Biogas potential of BGLR18 (*Chlorosarcinopsis eremi*) with the Gompertz model for the different digesters (A-F) with time (January to March 2019)

In biogas production experiment by BGLR18 (*Chlorosarcinopsis eremi*) biomass from April 2019 to May 2019, it was observed that digester C showed highest biogas production potential (P) 12.23 mLg^{-1} VS at a maximum biogas production rate (R_m) of 0.33 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 4.15 days followed by digester D with biogas production (P), biogas production rate (R_m) and a lag phase (λ) of 9.30 mLg^{-1} VS, 0.43 $mLg^{-1}d^{-1}$ and 9.09 days respectively at the end of 5 month period (150 days). Both sets showed higher values than control (digester A) in which has biogas production potential (P) 1.28 mLg^{-1} VS at a maximum biogas production rate (R_m) of 0.03 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 7.23 days. Also, percent reduction in volatile solids was highest in digester C (6.82%) which was greater than control digester A (0.72%) (Table 4.66) (Fig. 4.18).

In co-digestion experiment, it was found that digester E had the maximum biogas production potential (P) of 214.30 mLg^{-1} VS at a maximum biogas production rate (R_m) of 8.76 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 12.10 days (Table 4.66) at the end of 5 months period. This was followed by digester F with biogas production potential (P) of 198.85 mLg^{-1} VS at a maximum biogas production rate (R_m) of 8.93 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 10.08 days. Both of these are higher than control (digester B) which showed biogas production potential (P) of 77.31 mLg-^{1} at a maximum biogas production rate (R_m) of 3.14 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 13.60 days. Maximum percent reduction in volatile solids was in digester E (14.72%) which was greater than control digester B (7.09%). The modified Gompertz equation satisfactorily described biogas production with a goodness of fit (R^2) of 0.989,

0.959, 0.993, 0.976, 0.965 and 0.972 for digesters A, B, C, D, E and F respectively (Table 4.66) (Fig. 4.18).

4.5.2.3 Proximate and chemical composition of feedstock of BGLR18 (*Chlorosarcinopsis eremi*) before and after anerobic digestion process

Proximate and chemical composition of feedstock fed into different sets of digesters A-F was determined before and after anaerobic digestion process. It was evident from the results represented in Table 4.67 that after anerobic digestion process of 150 days there was a notable decrease in total solids and volatile solids of feedstock compared to before anaerobic digestion process of BGLR18 (*Chlorosarcinopsis eremi*) biomass. The decrease in volatile solids is due to its consumption during anaerobic process. In biomethanation process, after the initial hydrolysis step, fermentative bacteria use the small molecules to volatile fatty acids and alcohols which are further transformed by acetogens to acetate, carbon dioxide, and hydrogen (Tsapekos *et al* 2017) and finally acetate was converted by methanogens to methane (Shima *et al* 2002). Ash content after completion of digestion process was found to be increased. Ash content represent the plant mineral portion which are essential for operating metabolism normally (Siegal-Willott et al 2010). The increase in ash content was due to conversion of microalgal biomass to biogas as BGLR18 (*Chlorosarcinopsis eremi*) biomass has high mineral content revealed by ICP-AES analysis (as shown in Table 4.55). Other parameters like cellulose, hemicelluloses, lignin and silica content also showed a consequential difference after anaerobic digestion process compared to before anerobic digestion. There was decrease in cellulose, hemicelluloses and lignin content after digestion. The decrease is due to its hydrolysis in anaerobic digestion process. In hydrolysis phase which is the first step of biomethanation process facultative and obligate anaerobic bacteria release hydrolase enzymes which break down cellulose, carbohydrate, proteins and fats into monomers. However, there is slow degradation of Lignocellulosic material and lignin (Boontian 2014). After anerobic digestion process of 150 days, maximum percent decrease in total solids (TS) and volatile solids (VS) was observed in diegester E which is responsible for highest biogas production in it among all the digesters.

4.5.2.4 Effect of temperature (seasonal variation) on biogas production of BGLR18 (*Chlorosarcinopsis eremi*) biomass

A great variation was observed in biogas yield per month as the temperature varies greatly from January 2019 to May 2019 Table 4.68 (Fig.4.19). Biogas production decrease with decrease in temperature corresponds to weather and vice-versa. Maximum biogas production was observed in digester E (34,287.00 mL) followed by digester F (31,345.00 mL), digester B (11,820.00 mL), digester C (1,460.00 mL), digester D (1,184.00 mL) and digester A (110.00 mL) in the month of May where the average temperature was between 38 to 40°C. Lowest biogas production (10.00 ml) was found in month of January (average

temperature was between 15 to 17 ˚C) in digester A, followed by digester B (135.00 mL), F (255.00 mL), E (287.50 mL), D (454.00 mL) and C (500.00 mL).

Table 4.66 Estimated kinetic constants using modified Gompterz equation characteristics of the digesters A-F of BGLR18 (*Chlorosarcinopsis eremi*) biomass and its combination with paddy straw (April to May 2019)

Digester	Composition of feedstock	P ($mLg^{-1}VS$)	R_m ($mLg^{-1}d^{-1}$)	^ (d)	R^2	VSR (%)
A	*Harvested Microalgal biomass	1.28	0.03	7.23	0.989	0.72^{f}
B	***Paddy straw	77.31	3.14	13.60	0.959	7.09^{d}
C	*Harvested microalgal biomass+¥cow dung + #biodigested slurry	12.23	0.33	4.15	0.993	6.82^{c}
D	**Microalgae biomass filtrate+¥ cow dung + # biodigested slurry	9.30	0.43	9.09	0.976	3.09^{e}
E	*Harvested Microalgal biomass + ¥cow dung + # biodigested slurry+*paddy straw	214.30	8.76	12.10	0.965	14.72^{a}
F	**Microalgal biomass filtrate +¥cow dung + # biodigested slurry+paddy straw	198.85	8.93	10.08	0.972	11.40^{b}

Harvested microalgal biomass=1500g, **Microalgae filtrate= 1500 mL, #Biodigested slurry =150 mL, ¥Cow dung=300g, ***Paddy straw=250g, Temperature =15-32˚C; P: ultimate biogas yield; R_m:maximum rate of biogas production; λ: lag phase; R^2: Coefficient of determination; VSR: volatile solid reduction, Values superscripted by different alphabets in column differ significantly (P≤0.05) from each other (Tukey's test).

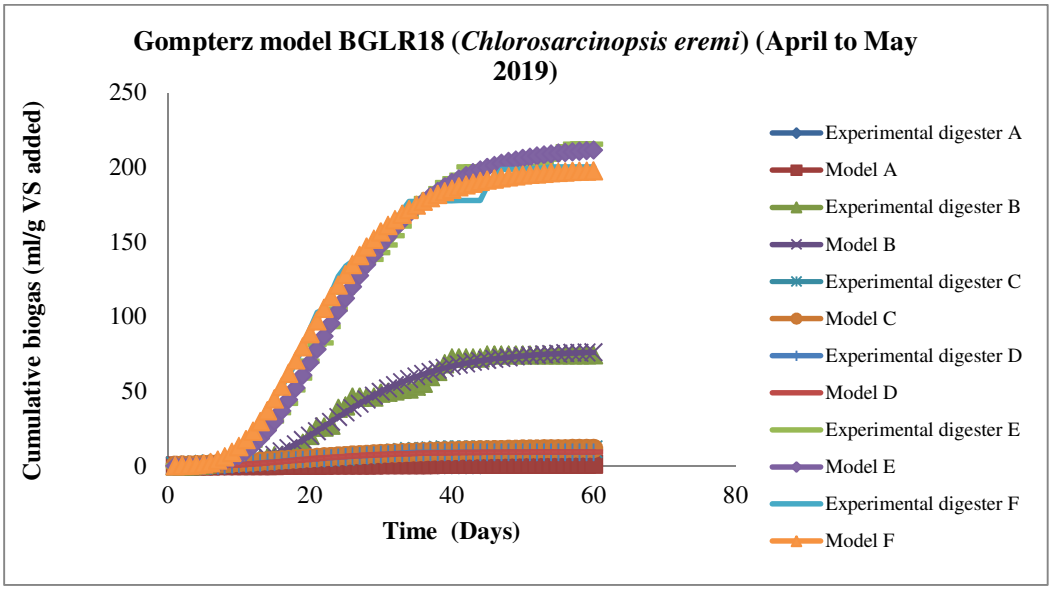


Fig. 4.18 Variation and fitting of the cumulative biogas data of Biogas potential of BGLR18 (*Chlorosarcinopsis eremi*) with the Gompertz model for the different digesters (A-F) with time (April to May 2019)

Table 4.67 Proximate and chemical composition of feedstock of BGLR18 (*Chlorosarcinopsis eremi*) before and after digestion process

	Proximate composition (%)						Chemical composition (%)							
	Total solids (TS)		Volatile solids (VS)		Ash		Cellulose		Hemicellulose		Lignin		Silica	
Digester No.	Before digestion	After digestion	Before digestion	After digestion	Before digestion	After digestion	Before digestion	After digestion	Before digestion	After digestion	Before digestion	After digestion	Before digestion	After digestion
A	1.50^{e}	1.20^{e}	59.41^{f}	58.98^{f}	40.59^{a}	41.02^{a}	3.20^{f}	3.00^{c}	4.00^{d}	3.80^{d}	0.70^{c}	0.60^{d}	1.70^{d}	1.80^{cd}
B	75.12^{c}	72.98^{b}	70.23^{d}	65.26^{c}	29.77^{c}	34.75^{d}	37.20^{a}	24.00^{a}	23.40^{a}	22.20^{a}	2.20^{a}	2.20^{a}	10.20^{a}	12.20^{a}
C	3.10^{d}	2.90^{d}	85.99^{a}	80.12^{a}	14.01^{f}	19.88^{f}	19.80^{d}	15.40^{b}	14.40^{c}	10.40^{c}	0.80^{c}	0.80^{d}	0.60^{e}	1.00^{d}
D	2.78^{d}	2.68^{d}	81.79^{b}	79.26^{b}	18.21^{e}	20.74^{e}	18.40^{e}	16.60^{b}	13.40^{c}	11.00^{c}	1.00^{c}	1.00^{cd}	0.80^{e}	1.40^{d}
E	78.23^{b}	73.25^{c}	70.12^{e}	62.12^{d}	29.88^{b}	37.88^{c}	28.20^{c}	25.40^{a}	20.80^{b}	18.00^{b}	1.60^{b}	1.40^{bc}	3.60^{c}	4.00^{bc}
F	78.90^{a}	72.68^{b}	72.56^{c}	61.88^{e}	27.44^{d}	38.12^{b}	31.60^{b}	27.00^{a}	20.20^{b}	13.20^{c}	1.80^{ab}	1.60^{b}	4.40^{b}	5.00^{b}

Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

Table 4.68 Biogas production of BGLR18 (*Chlorosarcinopsis eremi*) in different months of the year due to temperature variation

Month	Temperature (°C)	Digesters Biogas produced (mL/250 g FS)					
		A	B	C	D	E	F
January	15-17	10.00^{e}	135.00^{e}	500.00^{e}	454.00^{d}	287.50^{e}	255.00^{e}
February	19-20	20.00^{d}	705.00^{d}	540.00^{d}	600.00^{c}	300.00^{d}	1,555.00^{b}
March	26-27	50.00^{c}	800.00^{c}	580.00^{c}	600.00^{c}	2,350.00^{c}	1,600.00^{d}
April	28-30	60.00^{b}	1,200.00^{b}	920.00^{b}	662.00^{b}	4,890.00^{b}	3,780.00^{c}
May	38-40	110.00^{a}	11,820.00^{a}	1,460.00^{a}	1,184.00^{a}	34,287.00^{a}	31,345.00^{a}
Total Biogas (ml/250 g FS)		250.00	14,660.00	4,000.00	3,500.00	42,115.00	38,535.00

Values are means of triplicates, Values superscripted by different alphabets in column differ significantly (P≤0.05) from each other (Tukey's test).

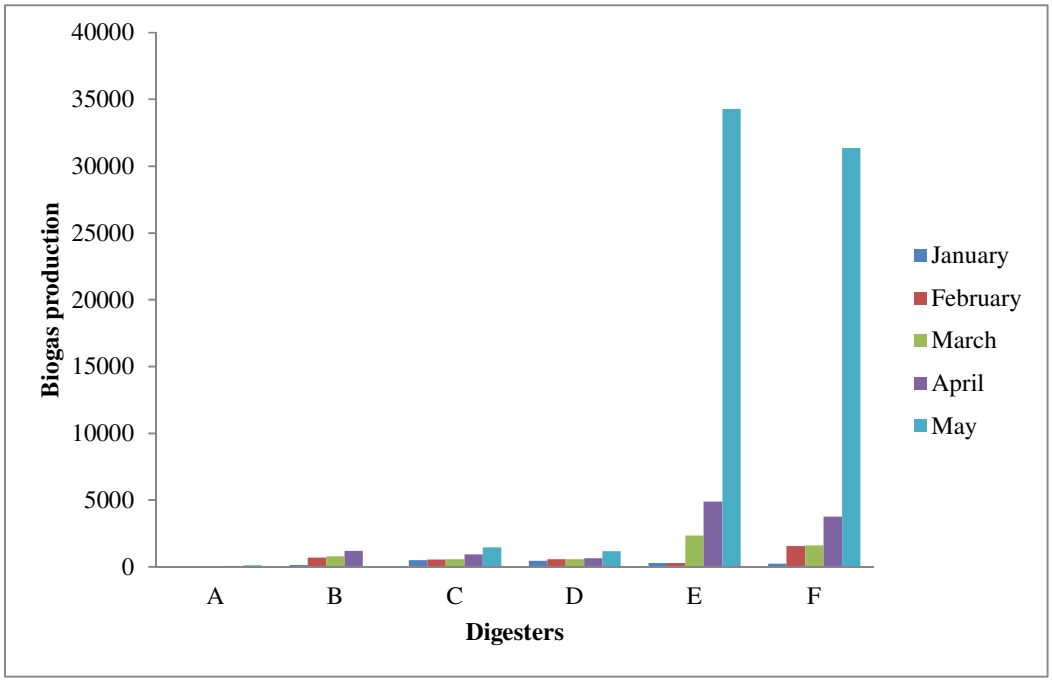


Fig 4.19 Variation in biogas production with temperature of BGLR18 (*Chlorosarcinopsis eremi*) biomass

4.4.5.3.1 Biogas production potential of *Spirulina* sp. NCIM 5143 biomass

Biogas production potential of *Spirulina* sp. NCIM 5143 was studied in terms of L/Kg of total solids added and consumed as well as L/Kg of volatile solids added and consumed (Table 4.69). The experiment was conducetd in the month of March 2019 and continued till July 2019. It was found that highest biogas production (20.82 Litres) was in digester C in terms of total solids added (427.52 L/kg), total solids consumed (502.96 L/kg), volatile solids added (473.18 L/kg) and consumed (614.52 L/kg) as compared to control (digester A) where biogas production was 0.36 Litres, 17.65 L/kg in terms of total solids added, 30.37 L/kg total solids consumed, 17.91 L/kg volatile solids added and 31.87 L/kg volatile consumed (Table 4.69).

In case of co-digestion experiment with paddy straw highest biogas production (158.97 Litres) was in digester E in terms of total solids added and (196.26 L/kg) total solids consumed (269.55 L/kg) and in terms of volatile solids added (233.78 L/kg) and consumed (401.61 L/kg) followed by digester F where total biogas production was 134.77 Litres and cumulative biogas production L/kg total solids added, total solids consumed, volatile solids added and volatile solids consumed was 168.46 L/kg, 234.14 L/kg, 179.69 L/kg and 282.00 L/kg respectively which were higher than control (digester B) where total biogas production was 31.68 Litres and 42.24 L/kg in terms of total solids added, 53.29 L/kg total solids consumed, 45.26 L/kg volatile solids added, 59.61 L/kg volatile solids consumed (Table 4.69).

Varol and Ugurlu (2016) reported that *Spirulina platensis* showed biogas production between 210 and 260 mL/g VS with volatile solid content of 89-93% and in continuous two-phase anerobic digestion system 525 ml biogas/g VS day with 60% reduction in volatile solids. They further reported that in co-digestion of *S. platensis* with waste sewage sludge biogas production of 640 ml biogas /g VS day with VS reduction of 62.5% was achieved.

4.4.5.3.2 Study of kinetics of biogas production *Spirulina* sp. NCIM 5143 biomass

As biogas production increased abruptly in the months of June and July. The kinetics of biogas production by BGLR18 (*Chlorosarcinopsis eremi*) biomass and its co-digestion with paddy straw was studied by modified Gompterz equation in two parts viz. March 2019 to May 2019 and June 2019 to July 2019. In biogas production experiment from March 2019 to May 2019 digester C showed highest biogas production (P) 7.74 mLg^{-1} VS at a maximum biogas production rate (R_m) of 0.21 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 2.98 days followed by digester D with biogas production (P), biogas production rate (R_m) and a lag phase (λ) of 4.65 mLg^{-1} VS, 0.13 $mLg^{-1}d^{-1}$ and 4.75 days respectively at the end of 5 month period (150 days). Both the Digester C and Digester D showed higher values than control (digester A) in which has biogas production potential (P) 0.24 mLg^{-1} VS at a maximum biogas production rate (R_m) of 0.05 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 16.69 days (Table 4.70) (Fig. 4.20).

In digester A negligible biogas was formed after the end of 150 days. Digester A contains only *Spirulina* sp. NCIM 5143 biomass only. As seen from above results, biogas production from microalgae *Spirulina* sp. NCIM 5143 biomass is low. The possible reason for this could be low C/N ratio of algal biomass which can cause ammonium accumulation and inhibit process (Rincon *et al* 2018). However, the co-digestion microalgae with other feedstocks having high C/N ratio like lignocellulosic biomass can improve the biogas production rate by adjusting the C/N ratio between the two (Li *et al* 2017, Thorin *et al* 2017). Therefore, co-digestion experiment was carried out in order to assess the co-digestion potential of paddy straw with microalgae under the same conditions as the above experiment. In co-digestion experiment, it was found that digester E produced the maximum biogas production potential (P) of 70.59 mLg^{-1} VS at a maximum biogas production rate (R_m) of 1.86 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 7.84 days at the end of 5 months period. This was followed by digester F biogas production potential (P) of 65.03 mLg^{-1} VS at a maximum biogas production rate (R_m) of 1.10 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 4.52 days. Both of these were higher than control (digester B) which showed biogas production potential (P) of 8.99 mLg-^{1} at a maximum biogas production rate (R_m) of 0.26 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 4.55 days. The modified Gompertz equation satisfactorily described biogas production with a goodness of fit (R^2) of 0.852, 0.989, 0.995, 0.993, 0.985 and 0.992 for digesters A, B, C, D, E and F respectively (Table 4.70) (Fig. 4.20).

From June 2019 to July 2019, digester C showed highest biogas production potential (P) 16.96 mLg^{-1} VS at a maximum biogas production rate (R_m) of 0.52 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 1.39 days followed by digester D with biogas production potential (P), biogas production rate (R_m) and a lag phase (λ) of 12.37 mLg^{-1} VS, 0.26 $mLg^{-1}d^{-1}$ and 0.00 days respectively at the end of 5 month period (150 days) (Table 4.62) (Fig. 4.13). Both digester C and D showed higher values than control (digester A) in which has biogas production (P) 0.29 mLg^{-1} VS at a maximum biogas production rate (R_m) of 0.02 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 4.77 days. After 150 days, maximum percent reduction in volatile solid content was in digester C (9.41%) followed by D (3.31%) wheras in control (digester B) was 3.27 % (Table 4.71) (Fig. 4.21).

In co-digestion experiment, it was found that digester E had the maximum biogas production potential (P) of 148.45 mLg^{-1} VS at a maximum biogas production rate (R_m) of 4.48 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 2.66 days (Table 4.71) at the end of 5 months period. This was followed by digester F which showed biogas production potential (P) of 108.59 mLg^{-1} VS at a maximum biogas production rate (R_m) of 2.35 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 2.32 days. Both of these were higher than control (digester B) which showed biogas production potential (P) of 31.32 mLg-^{1} at a maximum biogas production rate (R_m) of 0.85 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 0.00 days. Maximum percent reduction in volatile solid

content was in digester E (20.05%) followed by F (11.44%) as compared to control (digester B) (4.23%). The modified Gompertz equation satisfactorily described biogas production with a goodness of fit (R^2) of 0.894, 0.997, 0.992, 0.971, 0.979 and 0.960 for digesters A, B, C, D, E and F respectively (Table 4.71) (Fig. 4.21).

4.5.3.3 Proximate and chemical composition of feedstock of *Spirulina* sp. NCIM 5143 before and after anerobic digestion process

Results presented in Table 4.72 showed that after anerobic digestion process of 150 days there was a notable decrease in total solids and volatile solids of feedstock compared to before anaerobic digestion process of *Spirulina* sp. NCIM 5143 biomass. The decrease in volatile solids is due to its consumption during anaerobic process. In biomethanation process, after the initial hydrolysis step, fermentative bacteria use the small molecules to volatile fatty acids and alcohols which are further transformed by acetogens to acetate, carbon dioxide, and hydrogen (Tsapekos *et al* 2017) and finally acetate was converted by methanogens to methane (Shima *et al* 2002). Ash content after completion of digestion process was found to be increased. Ash content represent the plant mineral portion which are essential for operating metabolism normally (Siegal-Willott *et al* 2010). The increase in ash content was due to conversion of microalgal biomass to biogas as *Spirulina* sp. NCIM 5143 biomass has high mineral content revealed by ICP-AES analysis (as shown in Table 4.55). Other parameters like cellulose, hemicelluloses, lignin and silica content also showed a consequential difference after anaerobic digestion process compared to before anerobic digestion. There was decrease in cellulose, hemicelluloses and lignin content after digestion. The decrease is due to its hydrolysis in anaerobic digestion process. In hydrolysis phase which is the first step of biomethanation process facultative and obligate anaerobic bacteria release hydrolase enzymes which break down cellulose, carbohydrate, proteins and fats into monomers. However, there is slow degradation of Lignocellulosic material and lignin (Boontian 2014). After anerobic digestion process of 150 days, maximum percent decrease in Total solids (TS) and volatile solids (VS) was observed in diegester E which is responsible for highest biogas production in it among all the digesters.

4.5.3.4 Effect of temperature (seasonal variation) on biogas production of *Spirulina* sp. NCIM 5143 biomass

Biogas production was low initially from March to May while increased abruptly from June to July as shown in Table 4.73 (Fig.4.22). Biogas production decrease with decrease in temperature corresponds to weather and vice-versa. Maximum biogas production was observed in digester E (15,565.00 mL) followed by digester F (14,525.00 mL), digester B (3,800.00 mL), digester C (1,875.00 mL) and digester D (1,600.00 mL), digester A (30.00 mL) in the month of July where the average temperature was between 42 to 45 $^{\circ}$C. Lowest biogas production (10.00 mL) was found in month of March (average temperature was

between 26 to 27 °C) in digester C, followed by digester B (120.00 mL), C (230.00 mL), D (300.00 mL), E (652.00 mL) and F (1,412.00 mL).

Table 4.69 Biogas production potential of *Spirulina* sp. NCIM 5143 biomass in combination with paddy straw

Digester	Composition of feedstock	Biogas (in Litres)	Cumulative biogas (L/Kg TS added)	Cumulative biogas (L/Kg VS added)	Cumulative biogas (L/Kg TS consumed)	Cumulative biogas (L/Kg VS consumed)
A	*Harvested Microalgal biomass	0.36	17.65^{f}	30.37^{f}	17.91	31.87^{f}
B	***Paddy straw	31.68	42.24^{e}	53.29^{e}	45.26	59.61^{e}
C	*Harvested microalgal biomass+$^{¥}$cow dung + $^{\#}$cow dung slurry	20.82	427.52^{a}	502.96^{a}	473.18^{a}	614.52^{a}
D	**Microalgae biomass filtrate+$^{¥}$ cow dung + $^{\#}$cow dung slurry	14.20	315.56^{b}	375.66^{b}	338.10^{c}	416.27^{b}
E	*Harvested Microalgal biomass + $^{¥}$cow dung + $^{\#}$cow dung slurry *paddy straw	158.97	196.26^{c}	269.55^{c}	233.78	401.61^{c}
F	**Microalgal biomass filtrate +$^{¥}$cow dung + $^{\#}$cow dung slurry+paddy straw	134.77	168.46^{d}	234.14^{d}	179.69^{e}	282.00^{d}

Harvested microalgal biomass=1500g, **Microalgae filtrate= 1500 mL, $^{\#}$Biodigested slurry =150 mL, $^{¥}$Cow dung=300g, ***Paddy straw=250g, Temperature =15-32°C; P: ultimate biogas yield; R_m:maximum rate of biogas production; λ: lag phase; R^2: Coefficient of determination; VSR: volatile solid reduction, Values superscripted by different alphabets in column differ significantly (P≤0.05) from each other (Tukey's test).

Table 4.70 Estimated kinetic constants using modified Gompterz equation characteristics of the digesters A-F of *Spirulina* sp. NCIM 5143 biomass and its combination with paddy straw (March to May 2019)

Digester	Composition of feedstock	P ($mLg^{-1}VS$)	R_m ($mLg^{-1}d^{-1}$)	^ (d)	R^2	VSR (%)
A	*Harvested Microalgal biomass	0.24	0.05	16.69	0.852	3.27^{e}
B	***Paddy straw	8.99	0.26	4.55	0.989	4.23^{d}
C	*Harvested microalgal biomass+¥cow dung + #biodigested slurry	7.74	0.21	2.98	0.995	9.41^{c}
D	**Microalgae biomass filtrate+¥ cow dung + # biodigested slurry	4.65	0.13	4.75	0.993	3.31^{e}
E	*Harvested Microalgal biomass + ¥cow dung + # biodigested slurry+*paddy straw	70.59	1.86	7.84	0.985	20.05^{a}
F	**Microalgal biomass filtrate +¥cow dung + # biodigested slurry+paddy straw	65.03	1.10	4.52	0.992	11.44^{b}

* Harvested microalgal biomass=1500g, **Microalgae filtrate= 1500 mL, #Biodigested slurry =150 mL, ¥Cow dung=300g, ***Paddy straw=250g, Temperature =15-32°C; P: ultimate biogas yield; R_m:maximum rate of biogas production; λ: lag phase; R^2: Coefficient of determination; VSR: volatile solid reduction, Values superscripted by different alphabets in column differ significantly (P≤0.05) from each other (Tukey's test).

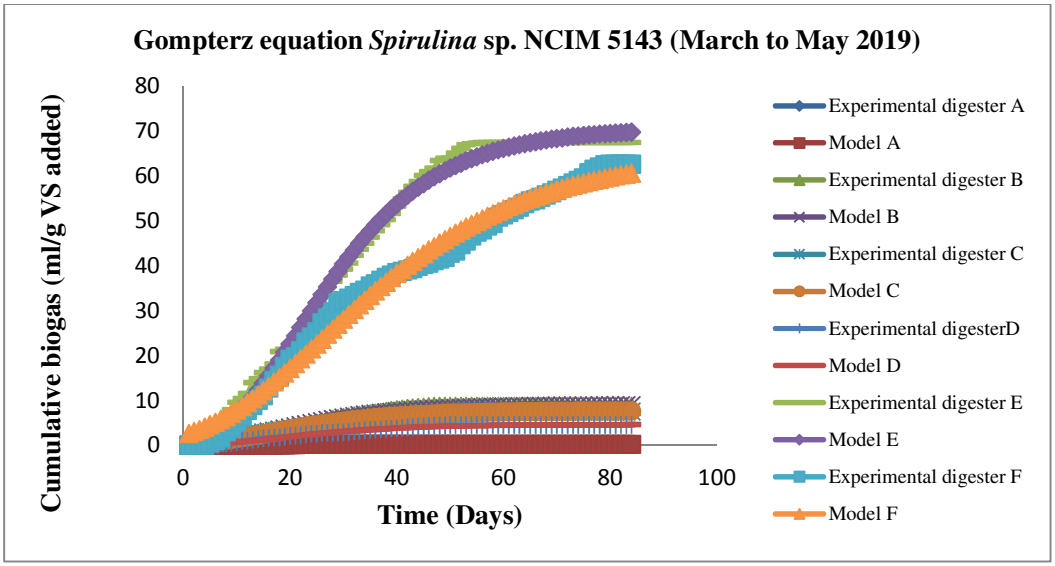


Fig. 4.20 Variation and fitting of the cumulative biogas data of Biogas potential of *Spirulina* sp. NCIM 5143 with the Gompertz model for the different digesters (A-F) with time (March to May 2019)

Table 4.71 Estimated kinetic constants using modified Gompterz equation characteristics of the digesters A-F of *Spirulina* sp. NCIM 5143 biomass and its combination with paddy straw (June to July 2019)

Digester	Composition of feedstock	P ($mLg^{-1}VS$)	R_m ($mLg^{-1}d^{-1}$)	^ (d)	R^2	VSR (%)
A	*Harvested Microalgal biomass	0.29	0.02	4.77	0.894	3.27^{e}
B	***Paddy straw	31.32	0.85	0.00	0.997	4.23^{d}
C	*Harvested microalgal biomass+¥cow dung + #biodigested slurry	16.96	0.52	1.39	0.992	9.41^{c}
D	**Microalgae biomass filtrate+¥ cow dung + # biodigested slurry	12.37	0.26	0.00	0.971	3.31^{e}
E	*Harvested Microalgal biomass + ¥cow dung + # biodigested slurry+*paddy straw	148.45	4.48	2.66	0.979	20.05^{a}
F	**Microalgal biomass filtrate +¥cow dung + # biodigested slurry+paddy straw	108.59	2.35	2.32	0.960	11.44^{b}

*Harvested microalgal biomass=1500g, **Microalgae filtrate= 1500 mL, #Biodigested slurry =150 mL, ¥Cow dung=300g, ***Paddy straw=250g, Temperature =15-32°C; P: ultimate biogas yield; R_m:maximum rate of biogas production; λ: lag phase; R^2: Coefficient of determination; VSR: volatile solid reduction, Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

Table 4.72 Proximate and chemical composition of feedstock of *Spirulina* sp. NCIM 5143 before and after digestion process

	Proximate composition (%)						Chemical composition (%)							
	Total solids (TS)		Volatile solids (VS)		Ash		Cellulose		Hemicellulose		Lignin		Silica	
Digester No.	Before digestion	After digestion	Before digestion	After digestion	Before digestion	After digestion	Before digestion	After digestion	re	After digestion	Before digestion	After digestion	Before digestion	After digestion
A	2.04^{c}	2.01^{b}	58.10^{d}	56.20^{d}	41.90^{a}	43.80^{a}	1.50^{f}	0.80^{f}	2.30^{e}	2.00^{d}	0.20^{b}	0.20^{b}	2.20^{d}	4.40^{de}
B	75.00^{b}	70.00^{a}	79.27^{b}	75.92^{b}	20.73^{c}	24.08^{c}	12.00^{d}	10.20^{d}	23.40^{a}	21.00^{a}	13.40^{a}	13.20^{a}	13.40^{a}	13.80^{ab}
C	4.87^{c}	4.40^{b}	85.00^{a}	77.00^{b}	15.00^{d}	23.00^{c}	27.40^{c}	22.40^{b}	11.80^{d}	7.20^{c}	0.40^{b}	0.20^{b}	2.60^{d}	4.00^{e}
D	4.50^{c}	4.20^{b}	84.00^{a}	81.22^{a}	16.00^{d}	18.78^{d}	9.60^{e}	9.20^{e}	15.40^{c}	13.45^{b}	0.40^{b}	0.20^{b}	6.00^{c}	8.00^{cd}
E	80.00^{a}	68.00^{a}	71.95^{c}	63.72^{c}	28.05^{b}	36.28^{b}	30.60^{b}	18.40^{c}	20.80^{b}	8.80^{c}	0.20^{b}	0.20^{b}	13.60^{a}	16.40^{a}
F	81.00^{a}	70.00^{a}	72.81^{c}	58.21^{d}	27.19^{b}	41.79^{a}	35.60^{a}	31.80^{a}	13.80^{b}	8.40^{c}	0.20^{b}	0.20^{b}	7.40^{c}	10.00^{bc}

Values superscripted by different alphabets in column differ significantly ($P \leq 0.05$) from each other (Tukey's test).

Table 4.73 Biogas production of *Spirulina* sp. NCIM 5143 in different months of the year due to temperature variation

Month	Temperature (°C)	Digesters Biogas produced (mL/250 g FS)					
		A	B	C	D	E	F
March	26-27	10.00^{f}	120.00^{f}	230.00^{e}	300.00^{c}	652.50^{b}	1,412.50^{a}
April	28-30	10.00^{e}	400.00^{c}	400.00^{c}	350.00^{d}	4,925.00^{a}	3,400.00^{b}
May	38-40	10.00^{f}	1,200.00^{c}	975.00^{d}	300.00^{e}	6,700.00^{a}	6,455.00^{b}
June	42-45	30.00^{f}	2,400.00^{c}	1,725.00^{d}	1,000.00^{e}	11,900.00^{a}	7,900.00^{b}
July	42-45	30.00^{f}	3,800.00^{c}	1,875.00^{d}	1,600.00^{e}	15,565.00^{a}	14,525.00^{b}
Total Biogas (ml/250 g FS)		90.00	7,920.00	5,205.00	3,550.00	39,742.50	33,692.50

Values are means of triplicates

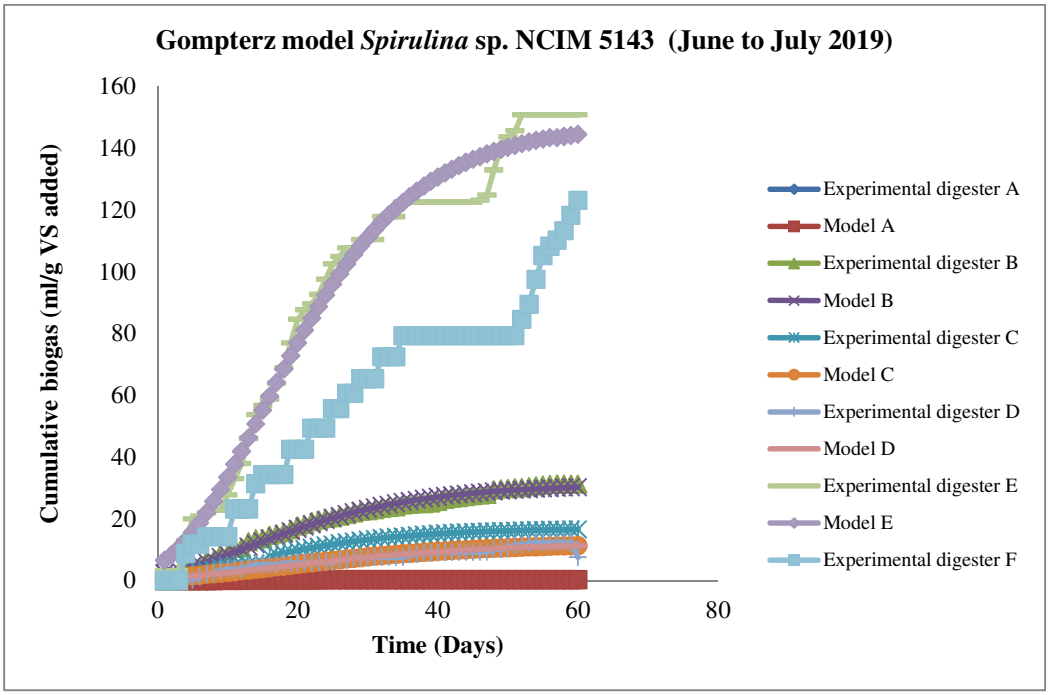


Fig. 4.21 Variation and fitting of the cumulative biogas data of Biogas potential of *Spirulina* sp. NCIM 5143 with the Gompertz model for the different digesters (A-F) with time (June to July 2019)

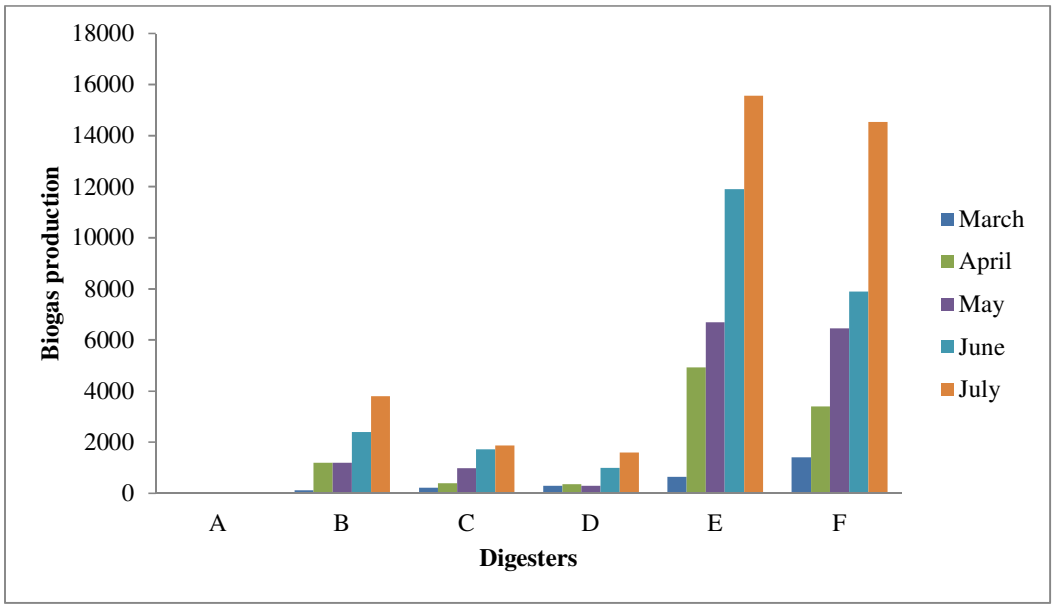


Fig. 4.22 Variation of biogas production with temperature of *Spirulina* sp. NCIM 5143 biomass

4.6 Assessment of biogas potential of BGLRS (*Scenedesmus sp. MKB*) strain at field level

BGLRS (*Scenedesmus sp. MKB*) was found to be a robust microalgal strain which could tolerate wide range of environmental conditions. So, co-digestion study of BGLRS (*Scenedesmus sp. MKB*) biomass with paddy straw was done for the assessment of its biogas production potential at field level. The study was conducted in the month of October 2018 and continued till February 2019. For evaluation of biogas potential of BGLRS (*Scenedesmus sp. MKB*) biomass, it was cultivated in 200 L open algal pond for the period of 6 months. After this, biomass was harvested by filtration through the muslin cloth. The biomass obtained was about 6.50 Kg. The feedstock added to the digester comprised of 50 kg paddy straw (soaked overnight), 10% biodigested slurry (5 kg) (act as inoculum) and 40% cow dung (20 kg) and 6.5 Kg microalgal biomass. The volume of biogas produced was measured by the gas meter (m^3/h) attached at the side of the digester and noted daily. It was found that total biogas production was 2,407 Litres and 31.67 L/kg in terms of total solids added and 33.95 L/kg total solids consumed. Again in terms of volatile solids added biogas production was 25.87 L/kg and 28.20 L/kg in terms of VS consumed (Table 4.74) The kinetics of biogas production field digester was also assessed by modified Gompterz equation. It was found that maximum biogas production potential (P) of 46,132.25 mLg^{-1} VS at a maximum biogas production rate (R_m) of 452.21 $mLg^{-1}d^{-1}$ with a lag phase (λ) of 0.00 days was obtained after 150 days of experimental period (Table 4.75) (Fig. 4.23). Also, percentage reduction in volatile solids after 150 days of digestion was 8.30%. The modified Gompertz equation satisfactorily described biogas production with a goodness of fit (R^2) of 0.991 for field digester. Proximate and chemical analysis of feedstock was done before and after anaerobic digestion process.

Total solids, volatile solids and ash content were 76.00, 93.06% and 6.94% respectively. Chemical composition revealed that it contains 40.00% cellulose, 28.90% hemicellulose, 2.56% lignin and 4.30% silica. However, after anaerobic digestion total solids (70.90%) and volatile solids (85.34) were reduced significantly. Ash content increased to 14.66% after digestion. Other parameters like cellulose (33.40), hemicelluloses (22.45%), lignin (2.45%) also decreased while silica content increased (5.10%) (Table 4.76). Study of effect of temperature on biogas production per month was also done. It was found that maximum biogas (585.00 Litres) was produced in the month of November where average temperature exists between 28 to 30°C which is considered ideal temperature range for mesophilic digestion. Lowest biogas production was produced in the month of January (323.00 Litres) where temperature ranges from 15 to 17° C not suitable for biogas production (Table 4.77) (Fig. 4.24).

Table 4.74 Biogas production in BGLRS (*Scenedesmus sp. MKB*) at field level in combination with paddy straw

Composition of feedstock	Biogas (Litres)	Cumulative biogas (L/Kg TS added)	Cumulative biogas (L/Kg VS added)	Cumulative biogas (L/Kg TS consumed)	Cumulative biogas (L/Kg VS consumed)
6.5 kg Harvested microalgal biomass+ 20 kg cow dung + 5 kg cow dung slurry+50 kg paddy straw	2,407	31.67	33.95	25.87	28.20

Table 4.75 Value of different parameters estimated from Gompertz model and volatile solid reduction (VSR) obtained for BGLRS (*Scenedesmus sp. MKB*) biomass

Composition of feedstock	P ($mLg^{-1}VS$)	R_m ($mLg^{-1}d^{-1}$)	Λ (d)	R^2	VSR (%)
6.5 kg Harvested microalgal biomass+ 20 kg cow dung + 5 kg cow dung slurry+50 kg paddy straw	46,132.25	452.21	0.00	0.991	8.30

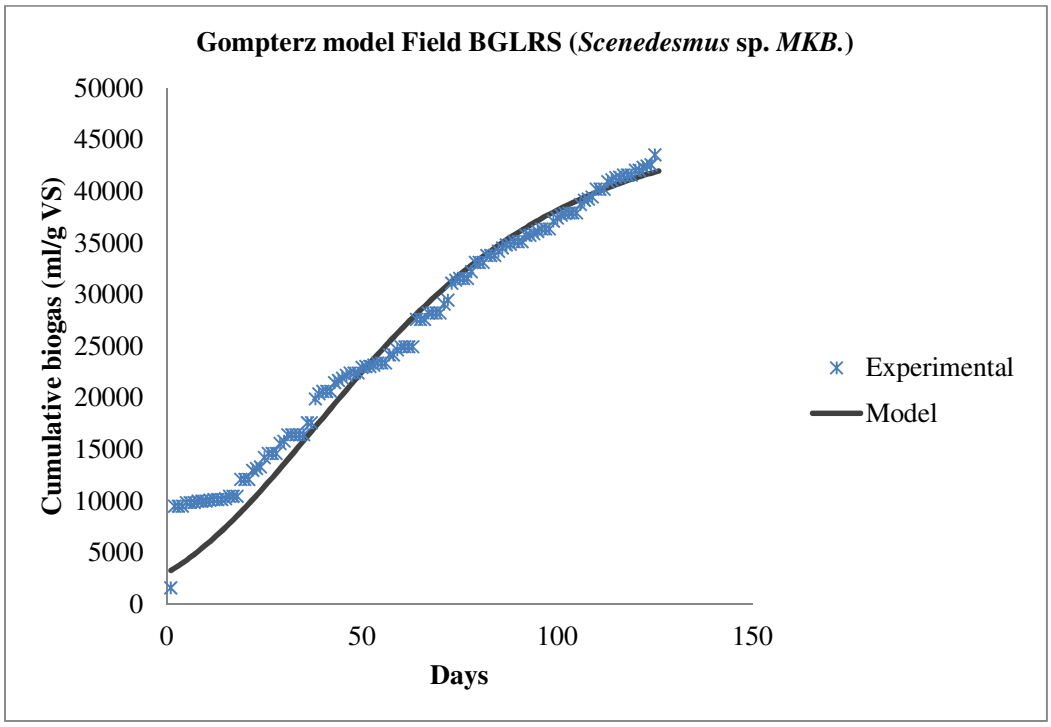


Fig. 4.23 Variation and fitting of the cumulative biogas data of Biogas potential of BGLRS (*Scenedesmus sp. MKB*) with the Gompertz model with time at field level

Table 4.76 Proximate and chemical composition of feedstock of BGLRS (*Scenedesmus sp. MKB*) before and after digestion process

Proximate composition (%)				**Chemical composition (%)**			
Before Digestion							
Digester No.	Total Solids (TS)	Volatile Solids (VS)	Ash	Cellulose	Hemicellulose	Lignin	Silica
	76.00	93.06	6.94	40.00	28.90	2.56	4.30
After Digestion							
	70.90	85.34	14.66	33.40	22.45	2.45	5.10

Table 4.77 Biogas production in BGLRS (*Scenedesmus sp. MKB*) during different months of the year due to temperature variation

Month	Temperature (°C)	Biogas production (in Litres)
October	29-32	551.00[b]
November	28-30	585.00[a]
December	22-23	465.00[d]
January	15-17	323.00[e]
February	19-20	483.00[c]
Total Biogas production (in Litres)		2,407.00

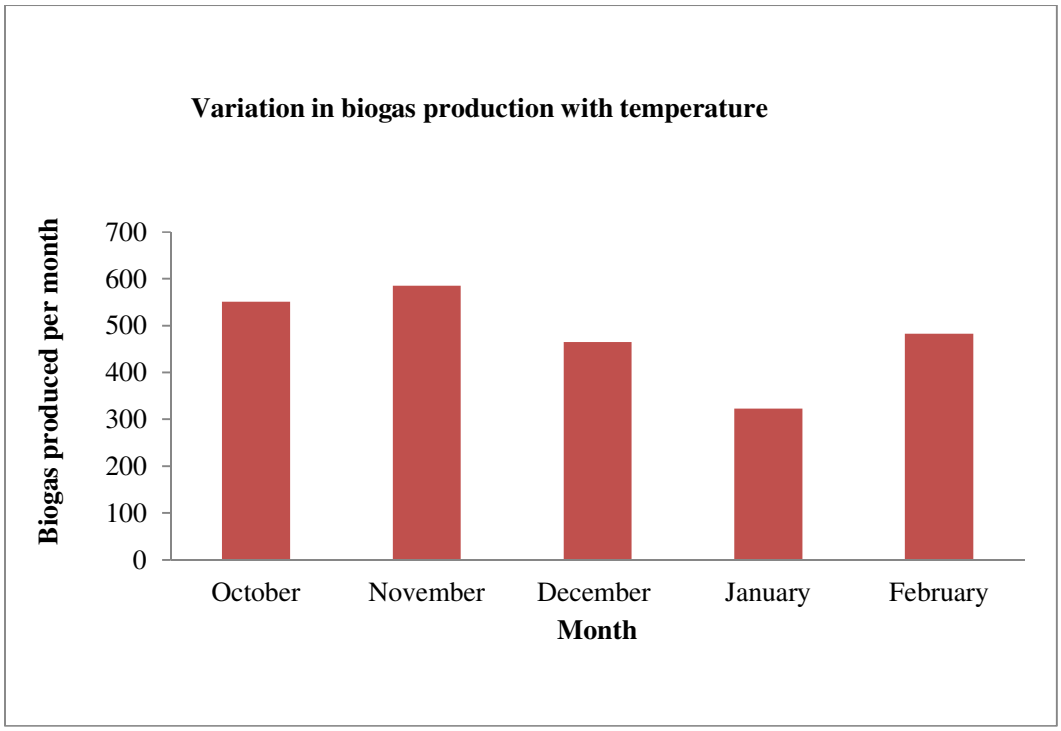


Fig. 4.24 Variation in biogas production with temperature at field level

CHAPTER V

SUMMARY

Microalgal biomass as co-feed stock in anaerobic digestion process to produce biogas has been receiving much attention now a days. Microalgal biomass can also be grown on various types of wastewater as they have the ability to use nutrients present in them which further results in treatment of wastewaters. Moreover, harvested microalgal biomass from wastewater treatment can be used as a co-feedstock for anaerobic digestion to produce biogas from various agricultural waste products like paddy straw, wheat straw etc.

Keeping these points in mind, present work was proposed with the following objectives:

- To screen and optimize the growth of stress tolerant microalgae strains for biomass productivity
- To characterize microalgae biomass for biogas production

A total of six strains, five stress tolerant microalgae strains viz. BGLR4, BGLR7, BGLR10, BGLR18, BGLRS and one standard strain *Spirulina* sp. NCIM 5143 were used in the present study. These strains were evaluated for biomass production on four different commercial media viz. Blue green-11 (BG-11), Bold's Basal Medium (BBM), Algal Culture Medium (ACM), Zarrouk's medium (ZM)) and on different concentrations (20, 40, 60, 80 and 100%) of unsterilized dairy wastewater. Growth was evaluated mainly in terms of optical density (O.D.) at 750 nm at every 3rd day, chlorophyll content (mg/l) at every 5th day and dry biomass weight (g/l) produced after 30 days. Microalgal strains BGLR4, BGLR7, BGLR10 showed highest absorbance, chlorophyll content and biomass weight (dry) in BG-11 while BGLR18, BGLRS and *Spirulina* sp. NCIM 5143 showed highest absorbance, chlorophyll content and dry biomass weight on BBM, ACM and ZM respectively. For dairy wastewater, highest absorbance, chlorophyll content and dry biomass weight for strains BGLR4, BGLR7, BGLR10, BGLR18 and BGLRS was with the combination of 60% dairy wastewater and 40% respective media expect *Spirulina* sp. NCIM 5143 which preferred 100% concentration of dairy wastewater. Highest protein content was shown by *Spirulina* sp. NCIM 5143 (119.17 mg L^{-1}) both on ZM and at 100% dairy wastewater concentration (162.33 mg L^{-1}). Highest carbohydrate content was recorded by BGLRS (234.79 mg L^{-1}) in ACM and combination of 60% dairy wastewater and 40% ACM medium (137.67 mg L^{-1}). Similary, highest lipid was produced by BGLRS (134.46 mg L^{-1}) in ACM and combination of 60% dairy wastewater and 40% ACM medium (266.40 mg L^{-1}).

The growth kinetics of six microalgae strains were also studied by Logistic

model. The asymptote A which determines the biomass production potential of the strain was highest for BGLR18 (1.87) followed by BGLRS (1.57), BGLR10 (1.49), BGLR4 (1.05), BGLR7 (0.90) and *Spirulina* sp. NCIM 5143 (0.65). Highest growth per day was shown by BGLRS (0.07 day^{-1}) followed by BGLR18 (0.06 day^{-1}). Lag time (λ) varied between 0.29 to 9.74 days.

In case of dairy wastewater highest value of asymptote A was obtained for BGLR18 (2.81) followed by BGLRS (2.67), BGLR7 (2.53) at combination of 60% dairy wastewater and 40% respective media *Spirulina* sp. NCIM 5143 (2.02) at 100% concentration of dairy wastewater, BGLR10 (1.65), BGLR4 (1.63) combination of 60% dairy wastewater and 40% respective media. The growth rate per day (μ)(day^{-1}) ranged from 0.16 to 0.25 day^{-1}.

Dairy wastewater used in the study for screening experiment was analyzed for various physico-chemical parameters. Bioremediation potential of six microalgae strains grown on dairy waste was evaluated via percent reduction in various physico-chemical parameters of dairy wastewater like COD, BOD, TS, TDS, TSS, Cl^-, TKN, TP after a period of 30 days. All strains showed maximum percent reduction in various physico-chemical parameters at combination of 60% dairy wastewater and 40% respective media while *Spirulina* sp. NCIM 5143 at 100%. Overall, it was found that two stress tolerant microalgal strains BGLR18 and BGLRS gave maximum percent reduction in various physico-chemical parameters of dairy wastewater.

BGLR18 and BGLRS were selected for further evaluation and compared with *Spirulina* sp. NCIM 5143. The interactive effect of five significant factors (independent variables) light intensity, pH, temperature, incubation period, inoculum concentration on five responses (dependent variables) viz. biomass, chlorophyll, carbohydrates, lipids, proteins production by three microalgal strains were determined statistically using response surface methodology (RSM) based on central composite design. by Statgraphics Centurian XVI.I. software. Optimum conditions of temperature, pH, growth period, light intensity and inoculum concentration for BGLR18 were 20.00°C, 10.02, 21.75 days, 7599.41 Lux, and 1.00% respectively; for BGLRS 26.03 °C, 11.17, 39.37 days, 4000.02 Lux and 10.00% respectively and for *Spirulina* sp. NCIM 5143 were 24.38 °C, 10.35, 25.13 days, 8000.00 Lux and 1.00% respectively.

Outsourcing for molecular identification of strains BGLR18 and BGRLS was done from Chromous Biotech Pvt. Ltd., Banglore, India. BGLR18 showed 96% resemblance with *Chlorosarcinopsis eremi* and BGLRS showed 99% resemblance with *Scenedesmus* sp. MKB.

The three selected strains BGLRS (*Scenedesmus sp. MKB*), BGLR18 (*Chlorosarcinopsis eremi*) and *Spirulina* sp.NCIM 5143 were also tested for various other

biochemical parameters. Highest TKN was shown by *Spirulina* sp. NCIM 5143 (7.14±0.21) followed by BGLR18 (*Chlorosarcinopsis eremi*) (3.64±0.49) and BGLRS (*Scenedesmus sp. MKB*) (2.73±0.28) and same trend was found in values of protein content where highest protein was present in *Spirulina* sp. NCIM 5143 (48.23±3.34) followed by BGLR18 (*Chlorosarcinopsis eremi*) (24.59±3.34) and BGLRS (*Scenedesmus sp. MKB*) (18.44±2.00). Highest phenolic content (9.68 ±3.08) was present in methanolic extracts of *Spirulina* sp. NCIM 5143 while lowest (0.49±0.04) in BGLR18. Maximum antioxidant activity (3.07±0.03) was shown by methanolic extract of *Spirulina* sp. NCIM 5143 followed by BGLRS (*Scenedesmus sp. MKB*) (2.07±0.09) and BGLR18 (*Chlorosarcinopsis eremi*) (1.86±0.14). It was found that maximum scavenging activity (% inhibition) (75.07±0.09) was shown by *Spirulina* sp. NCIM 5143 at highest concentration of methanolic extract i.e. 1000 µg/ml while lowest% scavenging activity (4.03±0.04%) was found in BGLR18 (*Chlorosarcinopsis eremi*) at lowest concentration i.e. 250 µg/ml. It was found that all the phytochemicals viz. quinones, tannins, saponins, terpenoids and steroids were present in *Spirulina* sp. NCIM 5143. BGLRS (*Scenedesmus sp. MKB*) showed the presence of tannins, saponins and steroids whereas in BGLR18 (*Chlorosarcinopsis eremi*) only saponins and terpenoids were detected..

Gas Chromatography-Mass Spectroscopy (GC-MS) analysis of three microalgal strains revealed the presence of many bioactive compounds. It was found that in BGLR18 (*Chlorosarcinopsis eremi*), a total of eleven compounds were found whereas eighteen compounds were found in strain BGLRS (*Scenedesmus sp. MKB*) which were highest among the three strains studied standard microalgal strain *Spirulina* sp. NCIM 5143 showed the presence of twelve compounds

These three microalgae strains were also tested for their elemental composition (Arsenic, boron, calcium, cadmium, chromium, copper, iron, Magnesium, Manganese, Nickel, Phosphorus, Nickel, lead, sulphur, zinc) from Department of Soil Science, College of Agriculture, PAU, Ludhiana and a wide variation was found in three microalgae strains in terms of nutrient concentration. However, in all the three strains heavy metals were negligible.

Proximate and chemical analysis showed that total solids ranged from 1.50 to 2.70. *Spirulina* sp. NCIM 5143 , BGLR18 (*Chlorosarcinopsis eremi*) and BGLRS (*Scenedesmus sp. MKB*) had the ash content of 41.90%, 40.59% and 32.55% respectively. Highest volatile solids were present in BGLRS (*Scenedesmus sp. MKB*) (67.45%) followed by BGLR18 (*Chlorosarcinopsis eremi*) (59.41) and *Spirulina* sp. NCIM 5143 (58.10). Highest cellulose content was shown by BGLR18 (*Chlorosarcinopsis eremi*) (3.20) followed by BGLRS (*Scenedesmus sp. MKB*) (2.40) and *Spirulina* sp. NCIM 5143 (1.50). Highest hemicellulose content was found in BGLR18 (*Chlorosarcinopsis eremi*)

(4.00) followed by BGLRS (*Scenedesmus sp. MKB*) (3.40) and *Spirulina* sp. NCIM 5143 (2.30). Lignin content ranged from 0.20 to 0.70. Silica content was found highest in BGLRS (*Scenedesmus sp. MKB*) (2.40), *Spirulina* sp. NCIM 5143 (2.20) and BGLR18 (*Chlorosarcinopsis eremi*) (1.70).

These strains were evaluated for their biogas production potential under batch mode at lab scale in six different sets. These were studied alone and and in co-digestion with paddy straw alongwith cow dung as inducer and biodigested slurry as inoculum. The biogas production was noted daily for a period of five months.

For strain BGLRS (*Scenedesmus sp. MKB*), experiment was conducted in October 2018 and continued till February 2019. Digester C showed highest amount of biogas (28.80 Litres) in terms of total solids added (800.00 l/kg) and total solids consumed (870.00 l/kg), volatile solids added (872.73 l/kg) and consumed (997.94 l/kg) and in co-digestion experiment with paddy straw highest biogas production (60.18 litres) was in digester E in terms of total solids added (80.25 l/kg), total solids consumed (88.18 l/kg) and in terms of volatile solids added (88.51 l/kg) and consumed (133.24 l/kg). The kinetics of biogas production by BGLRS (*Scenedesmus sp. MKB*) biomass studied by modified Gompterz equation revealed that (P) which is maximum biogas production potential is 30.85 mLg^{-1} VS at a biogas production rate (denoted by R_m) is 0.58 $mLg^{-1}d^{-1}$ and lag phase (λ) is 1.30 days for digester C and in case of co-digestion, digester E produced the maximum values of (P) (68.58 mLg^{-1} VS) and R_m (1.34 $mLg^{-1}d^{-1}$) with a lag phase (λ) of 0.66 days. Maximum biogas production was observed in the month of October where the average temperature was between 29 to 32°C and lowest biogas production was found in month of January (average temperature was between 15 to 17°C. Maximum percent reduction in volatile solids was in digester C (7.80%) and digester E (27.01%).

For evaluation of biogas production potential of BGLR18 (*Chlorosarcinopsis eremi*), experiment was conducted in the month of January 2019 and continued till May 2019. Highest biogas production (16.00 Litres) was in digester C in terms of total solids added (516.13 l/kg) and total solids consumed (600.23 l/kg), volatile solids added (551.72 l/kg) and consumed (688.61 l/kg) and in Digester E (168.46 litres) in terms of total solids added and (213.51 l/kg) total solids consumed (294.25 l/kg) and in terms of volatile solids added (231.80 l/kg) and consumed (374.60 l/kg) in case co-digestion studies. As biogas production increased abruptly in the months of April and May. The kinetics of biogas production by BGLR18 (*Chlorosarcinopsis eremi*) was studied by modified Gompterz equation in two parts viz. January 2019 to March 2019 and April 2019 to May 2019. From January 2019 to March 2019, it was observed that maximum values of P was 8.93

mLg^{-1} VS, R_m was 0.12 $mLg^{-1}d^{-1}$ and λ was 5.38 days was for digester C and in case of co-digestion experiment, P was 19.91 mLg^{-1} VS, R_m was 0.74 $mLg^{-1}d^{-1}$ and λ was 9.98 days for digester F.

From April 2019 to May 2019, again digester C and digester E (in co-digestion study) showed highest values for P, R_m and λ as 12.23 mLg^{-1} VS, 0.33 $mLg^{-1}d^{-1}$ and 4.15 days respectively and 214.30 mLg^{-1} VS, 8.76 $mLg^{-1}d^{-1}$ and 12.10 days respectively. Maximum percent reduction in volatile solids was in digester C (6.82%) and digester E (14.72%). Maximum biogas production was observed in the month of May where the average temperature was between 38 to 40°C and lowest biogas production was found in month of January (average temperature was between 15 to 17°C.

Biogas production potential of *Spirulina* sp. NCIM 5143 was studied from March 2019 to July 2019. Like, BGLRS (Scenedesmus sp. *MKB.*) and BGLR18 (*Chlorosarcinopsis eremi*), for *Spirulina* sp. NCIM 5143 biomass highest biogas was produced in digester C (20.82 litres), total solids added (427.52 l/kg), total solids consumed (502.96 l/kg), volatile solids added (473.18 l/kg) and consumed (614.52 l/kg) and in co-digestion studies with paddy straw maximum biogas production values were in digester E in terms of total solids added and (196.26 l/kg) total solids consumed (269.55 l/kg) and in terms of volatile solids added (233.78 l/kg) and consumed (401.61 l/kg). Similar to BGLRS (*Scenedesmus* sp. *MKB.*) and BGLR18 (*Chlorosarcinopsis eremi*), kinetic studies of *Spirulina* sp. NCIM 5143 was divided in two parts viz. March 2019 to May 2019 and June 2019 to July 2019. From March 2019 to May 2019 and from June 2019 to July 2019 all the biological parameters obtained from modified Gompterz equation and percent reduction in volatile solids were found to be maximum for digester C and digester E (for co-digestion studies). Biogas production was highest in the month of July where the average temperature was between 42 to 45 °C and lowest in the month of March where average temperature was between 26 to 27 °C.

Proximate and chemical composition of feedstock fed into different sets of digesters A-F was determined before and after anaerobic digestion process for three strains. Results showed that after anerobic digestion process of 150 days there was a notable decrease in total solids and volatile solids of feedstock compared to before anaerobic digestion process while ash content was increased. Cellulose, hemicelluloses and lignin content decreased after digestion while silica content increased.

Microalgae strain BGLRS (*Scenedesmus sp. MKB*) was mass cultivated in a 200 Litre open raceway pond for six months from April 2018 to September 2018. After that, biomass was harvested and used as co-feedstock with paddy straw for anerobic digestion at field scale for a period of 5 months from October 2018 to February 2019. Paddy straw

was soaked in water overnight. The feedstock added to the digester comprised of 50 kg paddy straw, 10% biodigested slurry (5 kg) (act as inoculum) and 40% cow dung (20 kg) and 6.5 Kg microalgal biomass. The volume of biogas produced was measured by the gas meter (m^3/h) automatically attached at the side of the digester. It was found that total biogas production was 2,407 litres and 31.67 l/kg in terms of total solids added and 33.95 l/kg total solids consumed, 25.87 l/kg volatile solids added and 28.20 l/kg VS consumed. The kinetics of biogas production in field digester was also assessed by modified Gompterz equation which showed that values of P and R_m were 46,132.25 mLg^{-1} VS and 452.21 $mLg^{-1}d^{-1}$ respectively with λ of 0.00 days and percentage reduction in volatile solids after 150 days of digestion was 8.30%. Proximate and chemical analysis of feedstock before and after anaerobic digestion process revealed that total solids, volatile solids, celluloses, hemicelluloses, lignin decreased by 6.71%, 8.29%, 16.5%, 22.31%, 4.29 % respectively while ash and silica content increased by 52.66 % and 15.68% respectively.

Conclusions

1. BGLR18 and BGLRS produced maximum biomass and showed highest percent reduction in BOD (63.64%) and COD (56.52%) content.
2. Optimum conditions for BGLR18 were : pH : 10.02, temperature : 20.00°C, the light intensity : 7599.41 Lux, growth period : 21.75 days and inoculum concentration : 1.00%. For BGLRS optimum conditions were : pH of 11.67, temperature of 26.03°C, the light intensity of 4000.02 Lux for a growth period of 39.37 days and inoculum concentration of 10.00% . For *Spirulina* sp. NCIM 5143 pH of 10.35, temperature of 24.38 °C, the light intensity of 8000.00 Lux for a growth period of 25.13 days and inoculum concentration of 1.00% were found to be the optimal and most desired conditions**.**
3. Identification at molecular level showed that strain BGLR18 and BGLRS showed closed resemblance to *Chlorosarcinopsis eremi*, and *Scenedesmus sp. MKB* respectively.
4. *Spirulina* sp. NCIM 5143 showed highest values of total nitrogen, total protein,total phenols, antioxidant activity, DPPH radical scavenging activity as compared to BGLR18 (*Chlorosarcinopsis eremi*) and BGLRS (*Scenedesmus sp. MKB*).
5. ICP-AES analysis showed that heavy metals were negligible in all the three strains while essential elements like Ca, Fe, P and Mg were present in higher concentrations in stress tolerant microalgal strains BGLR18 (*Chlorosarcinopsis eremi*) and BGLRS (*Scenedesmus sp. MKB*) than *Spirulina* sp. NCIM 5143.
6. GC-MS analysis of three microalgal strains showed the presence of many bioactive compounds

7. BGLRS (*Scenedesmus* sp. MKB) showed maximum biogas production.
8. Co-digestion of paddy straw with BGLR18 (*Chlorosarcinopsis eremi*) showed 65.19% increase in biogas production.

Future prospectives

1. Other agricultural wastes/wastewaters can be used as a low cost media for cultivation of other stress tolerant microalgae strains.
2. Optimization studies for various cultural factors of BGLR18 and BGLRS can be done on dairy wastewater and other wastes as well.
3. Biogas production potential of these strains can be increased with certain pretreatment methods.

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ANNEXURE I

Preparation of solutions for proximate and chemical analysis

Neutral Detergent Fibre (NDF) solution

Ingredients	**Quantity (gL^{-1})**
Sodium Lauryl Sulphate	30.00
Disodium ethylene diamine tetra-acetate (EDTA)	18.60
Sodium borate decahydrate (Borax)	6.80
Disodium hydrogen phosphate (Na_2HPO_4)	4.56

Method of preparation :

Initially, 30.00 g of Disodium ethylene diamine tetra-acetate (EDTA) and 6.80g of Sodium borate decahydrate (Borax) were dissolved in hot distilled water. After that 30.00g of Sodium Lauryl Sulphate was added. 4.56 g of Disodium hydrogen phosphate (Na_2HPO_4) was initially dissolved separately by heating slowly in distilled water and then added to the above prepared mixture. Finally, total volume was made 1 litre with distilled water and pH was in the range of 6.9-7.1.

Acid Detergent Fibre (NDF) solution

Ingredients	**Quantity**
Concentrated sulphuric acid	28.00 ml
Cetyl trimethyl ammonium bromide (CTAB)	20 gL^{-1}

Method of preparation:

Cetyl trimethyl ammonium bromide (CTAB) (20g) was dissolved in distilled water and concentrated sulphuric acid (28 ml) was added to it slowly along the walls of the container. Then the mixture was cooled and volume was made one litre with distilled water.

Method of prepration of 72% H_2SO_4 (Sulphuric acid)

72% H_2SO_4 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%

39.1/98×100=39.9 i.e. 40 ml

Hence, 72% H_2SO_4 was prepared by dissolving 40 ml of H_2SO_4 in 28 ml of distilled water.

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