MATERIALS AND METHODS

3.1 Chemicals and reagents

The chemicals and reagents used in the lab experiments were mostly procured from companies such as S.D. Fine Chemicals (India); Merck (India); Himedia (Mumbai, India); Sisco Research Laboratories (Mumbai, India); Qualigens Fine Chemicals Ltd. (India) and Sigma-Aldrich (Bangalore-India).

3.2 Instruments and equipment used

In Table 3.1, the list of equipment and instruments mainly used for the experiments and analysis are mentioned.

Table 3.1 List of equipment used for experimentations

Sl. No.	Equipments/Instruments	Manufacturing Company
1.	Laminar Air Flow	Clean air
2.	Hot Air Oven	Ikon Instruments
3.	Centrifuge	Thermo fisher
4.	Shaking Incubator	Orbitek (Scigenics Biotech)
5.	pH meter	Esico
6.	UV/Vis Spectrophotometer	Shimadzu (UV-1800)
7	Thermocycler	Labnet Multi Gene II
8.	Air Lift Reactors	Indigenously designed
9.	Lyophilizer	Labconco
10.	Thermoblock	Genei
11.	Vortex Mixer	Moxcare Products Inc.
12.	Stirred Tank Bioreactor	Scigenics India Pvt. Ltd.
13.	Light Microscope	Olympus CKX53
14.	Scanning Electron Microscope	Evo 18 Research, Zeiss SEM
16.	Gas Chromatograph	Nucon (5765)
17.	Gel Documentation System	Bio-Rad

3.3 Isolation and identification of microalgae

The microalgae samples were collected from different water bodies in Varanasi i.e. Bank of River Ganga at Ravidas Ghat and Ramnagar, Durgakund pond, and BHU Pond. The microalgae were grown in Bolds Basal Media (BBM) agar plates and single colonies of different microalgae were transferred in separate Petri plates. The composition of BBM media is given in Table 3.2. Microalgae were characterized based on morphology. They were observed under a compound microscope (Magnus-MLX) at 1000X magnification and pictures were taken using a compound microscope (Olympus-CKX53) at 400X magnification. Initially, ten different microalgae were isolated and grown in conical flasks at 25°C and 120 rpm. Microalgae were chosen based on high carbohydrate content and two different microalgae were chosen for further study. The microalgae were also identified based on the 5.8S rRNA gene sequence.

3.3.1 Extraction of DNA

About 20mg of lyophilized microalgae biomass was homogenized in the mortar with 5 ml of STE buffer extraction (400mM sucrose, 50mM Tris pH7.8, 20 mM EDTA-Na_2, 0.2% bovine serum albumin, 0.2% beta-mercaptoethanol, and last two components were added just before the experiment). The homogenate was filtered through a 50 μl nylon mess and the extract was centrifuged at 1000rpm for 20 to 30 min. The pellet was collected while the supernatant was discarded. The CTAB buffer (2% CTAB, 2% polyvinylpyrrolidone, 1.4M NaCl, 20mM EDTA pH 8.0, and 100 mM Tris HCl pH 8.0) was added to the pellet and the sample was heated in a thermoblock at 65°C for 1 h. One volume of chloroform: isoamyl alcohol (24:1) was added to the pellet and mixed for 10 min, and further centrifuged for 30 min at 13200 rpm. The aqueous phase was collected into a clean microcentrifuge tube and the rest was

discarded. Two volume of ethanol was added with 0.1 volume of sodium acetate 3M pH 5.2 and mixed gently. The sample was put at -20°C and afterwards centrifuged for 30 min at 13200 rpm. The supernatant was discarded and the pellet was washed in 70 % ethanol and dried at room temperature. The pellet was dissolved in 50 µl of TE buffer and the quantity and size of isolated were checked by agarose gel electrophoresis (Varela-Álvarez et al., 2006).

3.3.2 Amplification of DNA

DNA sample about 15ng was used as a template for 18S rRNA gene PCR amplification performed with Ready-to-use PCR Kit (ThermoFisher Scientific, USA). The PCR Amplification was performed in a thermocycler (Labnet Multi Gene II) with the primers ITS1 forward, 5’-TCCGTAGGTGAACCTGCGG 3’and ITS2 reverse 5’-GCTGCGTTCTTCATCGATGC 3’(Grzebyk et al., 1998). The PCR reaction mixture consisted of a 25 µl solution containing 1 µl of DNA template, 1 µl of forward primer, 1 µl of reverse primer, 12.5 of 2X PCR *Taq* mix and 9.5 sterile water. The PCR amplification reaction was performed under the following cycling conditions: Preheating at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 1 min; Annealing at 63°C for 1 min; extension at 72°C for 1 min followed by another 10 min extension at 72°C. The final PCR product was separated on 1 % TAE agarose gel and the amplified DNA sequence was approximately 350 basepairs. The DNA sequence was further purified by DNA purification kit, QIA quick (Qiagen, Germeny). The purified DNA was sequenced using automated sequencer ABI 3130 genetic analyzer and sequencing analysis software V5.2 (ABI USA).

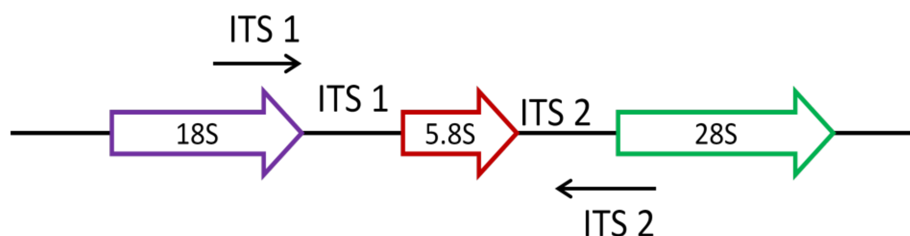

Figure 3.1: The arrangement of 18s, 5.8s and 28s rRNA genes

3.3.3 Blast and phylogenetic analysis

Resultant sequences were analysed with BLASTN against nucleotide collection for species identification. Maximum likelihood phylogenetic tree of microalgae 5.8S rRNA gene was constructed with MEGA 6.0 software. Numbers above branches indicate bootstrap values of maximum likelihood analysis from 1000 replicates.

3.4 Growth study of microalgae

Both isolated microalgae were grown initially in three different growth media i.e. BBM, BG11, and CHU M10 media with different inoculums volume (0.5%, 2% and 5%) at different temperatures (20°C, 25°C, 30°C, 35°C and 40°C), pH (6.0, 6.5, 7.0 7.5 and 8.0) and agitation rates (0, 90, 120, and 150) . The composition of BBM, BG11 and CHU M10 media are shown in table 3.2, 3.3, and 3.4 respectively. The effects of these parameters were observed as a change in the growth profiles and specific growth rates. The stock solutions for each of the ingredients were prepared in distilled water and autoclaved at 15 psi pressure for 15 min at a temperature of 121°C, and were stored until subsequent use.

Ten millilitres of microalgal culture was used to inoculate 200 mL of media. The algal culture was grown autotrophically 28°C in an environmental chamber with different shaking conditions, and illuminated with cool inflorescence lamps at an intensity of 60 µmol m^{-2} s^{-1}

in a 12:12 h light: dark period. Sampling was done periodically to measure the growth of the alga at a wavelength of 688 nm. The cell mass of the microalgae was determined by plotting a graph between absorbance and dry cell weight. The algal growth was also measured by dry cell weight analysis (Singh et al., 2018; Wang et al., 2010).

Table 3.2: Composition of Bold's Basal Medium (BBM)

Stocks	Chemicals	Per 400ml
1	$NaNO_3$	10.0 g
2	$MgSO_4.7H_2O$	3.0 g
3	NaCl	1.0 g
4	K_2HPO_4	3.0 g
5	KH_2PO_4	7.0 g
6	$CaCl_2.2H_2O$	1.0 g
7	**Trace elements (autoclave to dissolve)**	**Per liter**
	$ZnSO_4.7H_2O$	8.82 g
	$MnCl_2.4H_2O$	1.44 g
	MoO3	0.71 g
	$CuSO_4.5H_2O$	1.57 g
	$Co(NO_3).6H_2O$	0.49 g
8	H_3BO_3	11.42 g
9	EDTA	50.0 g
	KOH	31.0 g
10	$FeSO_4.7H_2O$	4.98 g
11	H_2SO_4 (Conc.)	1.0 ml

For BBM media preparation, stock solutions from 1-6 are taken 10.0 ml each and stock solutions 7 -11 are taken 1.0 ml each and distilled water is added to make up to 1 litre.

Table 3.3: Composition of BG11 (Blue – Green) medium

Stocks	Chemicals	Per 500ml
1	$NaNO_3$	75.0 g
2	K_2HPO_4	2.0 g
3.	$MgSO_4.7H_2O$	3.75 g
4	$CaCl_2.2H_2O$	1.8 g
5	Citric acid	0.30
6	Ammonium ferric citrate green	0.30 g
7	EDTA Na_2	0.05 g
8	Na_2CO_3	1.0 g
9	**Trace elements (autoclave to dissolve)**	**Per litre**
	$ZnSO_4.7H_2O$	0.22 g
	$MnCl_2.4H_2O$	1.84 g
	$NaMoO4.2H_2O$	0.39 g
	$CuSO_4.5H_2O$	0.08 g
	$Co(NO_3).6H_2O$	0.05 g
	H_3BO_3	2.86 g

For BG11 media preparation, stock solutions from 1-8 are taken 10.0 ml each and stock solution 9 is taken 1.0 ml and distilled water is added to make up to 1 litre.

Table 3.4 Composition of CHU M 10 media

Stocks	Chemicals	Per 100ml
1	$Ca(NO_3)_2.4H_2O$	2.0 mg
2	KH_2PO_4	0.062 mg
3.	$MgSO_4$. $7H_2O$	2.5 mg
4	Na_2CO_3	2.0 mg
5	$Na_2SiO_3.9H_2O$	2.5 mg
6	HCl	0.025 mL
7	$Na_2EDTA.2H_2O$	0.2 mg
8	$FeCl_3.6H_2O$	0.1 mg
9	H_3BO_3	0.248
10	$MnCl_2.4H_2O$	0.139 mg
11	$(NH_4)Mo_7O_{24}.4H_2O$	0.1 mg
12	Vitamin B_{12}	1 µg
13	Thiamine HCl	0.1 µg
14	Biotin	0.1 µg

The media was autoclaved at 15 psi for 15 minutes. Stocks 5 and 6 were autoclaved separately in 100ml deionized water to reduce precipitation and then rest of medium (autoclaved) was added aseptically when cooled and Made up to 1 litre with deionized water. The pH was adjusted with 1M NaOH or 1 M HCl.

3.4.1 Dry cell weight analysis

For the dry cell weight analysis, 5 ml of sample was withdrawn and centrifuged it at 5000 rpm for 10 min and the supernatant was decanted whereas the pellet was transferred in an

aluminium cup and further the aluminium cups were put into a hot air oven at 80°C for 24 h till the constant weight was obtained.

3.4.2 Characterization of microalgae

3.4.2.1 Total carbohydrate analysis

A10 mg of algal biomass was dissolved in 10 ml of distilled water. Then, 3 ml of 96% (v/v) sulphuric acid was added in 1 ml of algal sample. One ml of 5 % (w/v) phenol was added in the reaction mixture. The mixture was put at room temperature for 30 minutes to cool down. The total sugar was estimated in algal samples by taking the absorbance at 490 nm. Glucose was used to plot the standard curve. For individual sugar analysis, 100 mg of algal biomass was hydrolyzed by sulfuric acid in two stages. In the first stage, biomass was treated with 72 wt % sulfuric acid for 1 h at 30°C and in the second stage, by 4 wt % sulfuric acid for 1 h at 121°C in an autoclave). Ceramic filtering crucibles were used for separation of acid-insoluble residues. A soluble form of carbohydrates (xylose, arabinose, mannose, glucose and galactose) were analyzed by Waters HPLC with 2414 RI Detector (Dubois et al., 1956; Laurens et al., 2012).

3.4.2.2 Starch analysis

The starch content in microalgae was measured by starch (HK) assay kit (SA20-1KT) purchased from Sigma Aldrich. Sigma starch assay kit was used and 500 mg of biomass was mixed with 5 mL of 8 M HCl and 20 mL of DMSO which was incubated at 50°C (water bath) for 30 min. 4−5 pH was maintained using 5 M NaOH and made up to 100 mL. 1ml of the solubilized sample was treated with a Starch Assay Reagent with amyloglucosidase enzyme for 15 mins in a water bath to 60°C. Then, 1 ml of the mixture was incubated with Glucose Assay Reagent which contained ATP, glucose-6-phosphate dehydrogenase, NAD,

hexokinase,) for 15 mins. The absorbance of the resulting mixture was determined using a spectrophotometer at 340 nm.

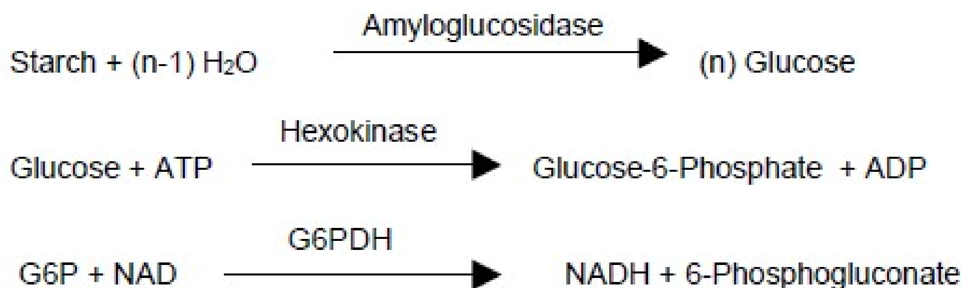

Figure 3.2: Reactions involved in the starch determination

3.4.2.3 Lipid analysis

200 mg algal biomass was measured in single thickness cotton cellulosic thimbles and enclosed using a filter made of glass fibre. A standard Soxhlet apparatus was shown in Figure 3.3 and used for reflux of 210 ml of chloroform and methanol in a ratio of 2:1 (v/v) over the thimble, between 12 to 20 h and a syphon rate of 6−8 times. The extracted sample was then transferred to a separating funnel. The aliquot was mixed 0.7−0.75% NaCl aqueous solution and a ratio of 3:8:4 NaCl (aq)/ chloroform/ methanol was added to eliminate the non-lipid content from the solvent as described by Folch *et al*.11 The biphasic mixture was left for settling for about 12 h. The lower lipid-containing organic phase was then transferred into a pre-weighed round-bottom flask from which the solvent was taken out with the help of a vacuum rotary evaporator at a temperature of 30− 35°C. The flasks were then kept at 40°C in a vacuum oven for the purpose of drying. The amount of lipid present was estimated gravimetric method (Folch et al., 1957; Bligh and Dyer, 1959).

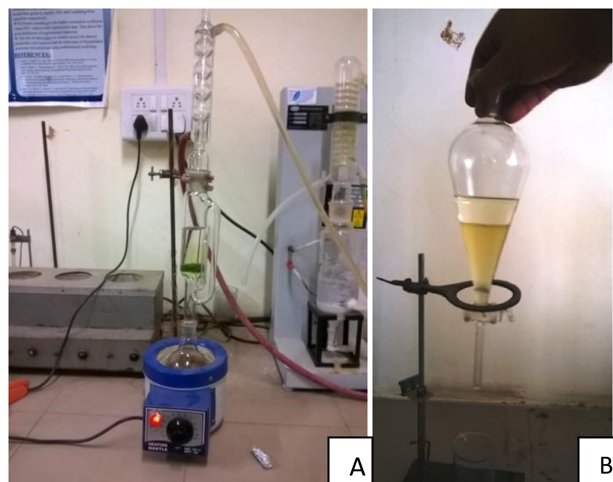

Figure 3.3: A setup of soxhlet apparatus (A), separating funnel containing chloroform with crude lipid mixture and methanol (B)

3.4.2.4 Protein analysis (Folin Lowry method)

1 mg of dried biomass was cleaned with phosphate buffered saline solution. It was then solubilized in 1 ml of alkaline copper tartrate solution (Reagent A). Then, the solubilized sample was further diluted in Reagent A making up the volume 500 μL and was further mixed with 4 mL Folin reagent (Reagent B), This mixture was kept for 15 mins and then analyzed using a spectrophotometer at 750 nm (maximum absorbance). Based on the BSA standard curve, the concentration of the sample was calculated (López et al., 2010).

3.4.2.5 Moisture content

100mg of dried algal biomass was taken in a ceramic crucible and put it into a hot air oven at 105°C for overnight. The sample was cooled into desiccator at room temperature. The sample was weighted and moisture content was determined by subtracting the present value from the former value(Van Wychen and Laurens, 2016).

3.4.2.6 Ash Value

After calculating the moisture content, the oven dried microalgae sample was taken on silica crucible and put into muffle furnace at 575°C until constant weight was obtained. The ash content was determined by subtraction the crucible weight(Van Wychen and Laurens, 2016).

3.4.2.7 CHNS analysis

Simultaneous determination of major components such as carbon, hydrogen, sulfur and nitrogen was carried out by combustion in CHNS analyzer (Euro EA3000 CHNS-O Analyzer). Gas was detected and quantified using thermal conductivity detector (TCD). Helium (He) was used as a carrier gas at a flow rate of 600 mL min^{-1}. Relative percent of oxygen was calculated by deducting the relative percent of C, H, N, and S from 100.

3.4.3 Measurement of Specific growth rate (μ)

The net specific growth rate of microalgae was calculated by giving equation.

$$\frac{dX}{dt} = \mu . x$$

Where X is the cell mass concentration, μ is the specific growth rate and t is time. This equation can be rewritten as

$$\mu = \frac{ln\, N_2 - ln\, N_1}{t_2 - t_1}$$

Where N_2 and N_1 are the biomass concentration at time t_1 and t_2 respectively. The specific growth rate was given in the exponential phase of the growth cycle.

3.4.4 Biomass productivity (P_X)

The biomass productivity is the amount of cell mass generated per unit time per unit volume and its units may be g/L/h or g/L/d. The biomass productivity is represented as by given equation.

$$P_X = \frac{X_2 - X_1}{t_2 - t_1}$$

Where X_2 and X_1 are the biomass concentration at time t_1 and t_2 respectively.

3.4.5 Carbohydrate productivity(P_C)

The carbohydrate productivity is the amount of carbohydrate generated per unit time per unit volume and its units may be g/L/h or g/L/d. The biomass productivity is represented as by given equation.

$$P_X = \frac{C_2 - C_1}{t_2 - t_1}$$

Where X_2 and X_1 are the carbohydrate concentration at time t_1 and t_2 respectively.

3.4.6 Starch productivity(P_S)

The carbohydrate productivity is the amount of carbohydrate generated per unit time per unit volume and its units may be g/L/h or g/L/d. The biomass productivity is represented as by given equation.

$$P_S = \frac{S_2 - S_1}{t_2 - t_1}$$

Where S_2 and S_1 are the starch concentration at time t_1 and t_2 respectively.

3.5 The growth of microalgae in different photobioreactors

Microalgae were grown in different photobioreactors to achieve high biomass yield. There were four photobioreactors used for the microalgal growth study. There are bubble column, stirred tank, internal loop airlift and external loop photobioreactors.

3.5.1 Bubble column photobioreactor

The bubble column reactor was fabricated using plexiglass (polymethyl methacrylate) and shown in Figure 3.4. The working volume of bubble column reactor was 1litre. The internal

diameter of the bubble column was 2.3 cm and the length was 80cm. The microalgae were grown at 30°C and initial pH of 7.0 in BG11 media. The bubble column was operated with a flow rate of 0.2vvm with air and 2% CO_2. The microalgae were grown in BG11 into bubble column reactor media with the light intensity of 10000 Lux.

Figure 3.4: The bubble column photobioreactor used for microalgal growth

3.5.2 Stirrer tank photobioreactor

The Stirred tank bioreactor (Scigenics India Pvt ltd.) was used in the study and shown in Figure 3.5. The working volume in stirred tank reactor was kept 3 liters and the all the parameters like temperature, pH and agitation were kept constant and at optimum values throughout the study. The microalgae, *C. sorokiniana* and *T. obliquus* were grown in stirred tank photobioreactor with 5% (150ml) of inoculums volume having a cell density of 520 mg/L for 12 days. The flow rate of gas used in the study was 0.2vvm. The light intensity was

maintained at 10000 lux using the cool incandescent light bulbs. The growth of microalgae was measured with air and 2% CO_2 by measuring the samples periodically.

Figure 3.5: Set up of stirred tank photobioreactor

3.5.3 Internal loop and external loop airlift photobioreactors

The internal loop and external loop airlift photobioreactors were fabricated using plexiglass (polymethyl methacrylate). The working volume of the internal loop and external loop ALPBR were 1 litre and 2 litres respectively. The configurations of the airlift reactors are mentioned in Table 3.5. The schematic diagram of internal loop ALPBR and external loop ALPBR are shown in Figure 3.6(A) and 3.6 (B) respectively. The microalgae were grown in BG11 media into photobioreactors with a flow rate of 0.2 vvm of air and 2% CO_2. The growths of microalgae were measured by taking the absorbance at 688nm and also by dry weight analysis method. After the growth, the microalgae biomass was harvested and used for the estimation of biomass, carbohydrate and starch productivities.

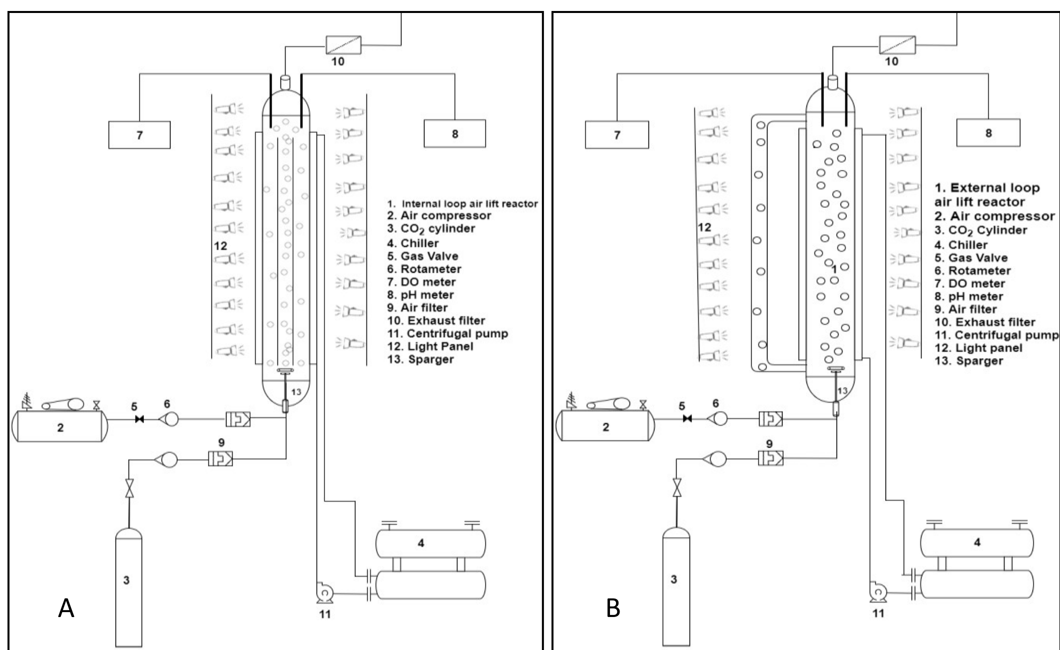

Figure 3.6: - Schematic diagram of internal loop photobioreactor (A) and external loop photobioreactor (B)

Table 3.5: The configurations of the internal and external airlift photobioreactors

Reactor Parameters	**Internal loop Airlift reactor**	**External loop Airlift reactor**
The working volume of the reactor	1 L	2 L
Column diameter	60mm	60mm
Column height	650mm	800mm
Draft tube diameter	40mm	40mm
Draft tube height	350mm	450mm
The height of draft tube above the reactor base	80mm	250mm
Single Sparger Nozzle	0.5mm	0.5mm

3.6 Carbohydrate enhancement strategies

3.6.1 Using antibiotics

3.6.1.1 Protein synthesis inhibition (cycloheximide treatment)

Cycloheximide was added into the harvested microalgae culture and a final concentration of 1 mg/L was maintained. The presence of cycloheximide (1 mg/L) prevented microalgal protein synthesis and leads to the accumulation of carbohydrate inside the cells. The microalgae were grown in optimum conditions upto stationary phase and then harvested. The harvested cells were again put in media containing cycloheximide and grown for five days in presence of cycloheximide at 150 rpm and 30°C. The samples were taken periodically and the cellular content of microalgae mainly carbohydrate, starch and lipid were measured.

3.6.1.2 Lipid synthesis inhibition (cerulenin treatment)

A 200µl of stock solution (0.5 mg/mL) of cerulenin was added to make the final concentration of cerulenin (2 mg/L) in 100 ml of microalgae culture. when the growth of microalgae reached to the stationary phase. Both the control and treated culture were grown in an incubator at 150 rpm and 30°C temperature for 5 days. The carbohydrate, starch, and lipid content in both treated and untreated cultures were measured. The effects of cerulenin in both microalgae were observed with respect to time in terms of carbohydrate, starch and lipid content.

3.6.2 The growth of microalgae under nutrient limitation

Nutrient limitation study was performed in the absence of specific nutrients (nitrogen, phosphorus and sulphur) in media. The microalgae were grown in stress conditions to enhance the carbohydrate content.

3.6.2.1 Under nitrogen limitation growth

The microalgae were grown in BG11 media to the early stationary phase at 30°C and 25°C for *C. sorokiniana* and *T. obliquus* respectively with 150 rpm and then cells were harvested by centrifugation. The cells were again re-suspended in BG11 nitrogen depleted media and grown for five days at the same culture conditions. The samples were taken out periodically to measure the carbohydrate, starch and lipid content. Each experiment was performed in triplicate.

3.6.2.2 Under phosphorus limitation growth

The microalgae were grown in BG11 media to the early stationary phase with the same condition as for nitrogen limitation and then cells were harvested by centrifugation. The cells were again re-suspended in BG11phosphorus depleted media and grown for five days at the same culture conditions. The samples were taken out periodically to measure the carbohydrate, starch and lipid content. Each experiment was performed in triplicate.

3.6.2.3 Under sulphur limitation growth

The microalgae were grown in BG11 media to the early stationary phase with the same condition as for nitrogen limitation and then cells were harvested by centrifugation. The cells were again re-suspended in BG11phosphorus depleted media and grown for five days at the same culture conditions. The samples were taken out periodically to measure the carbohydrate, starch and lipid content. Each experiment was performed in triplicate.

Initially, nutrient limitation study was performed in shake flask and further used in air lift reactor to achieve high biomass content.

3.7 Pretreatment of microalgae

The pretreatment of microalgae was performed with different methods and the pretreatment efficiency of these methods was measured by calculating the amount of carbohydrate released per gram of total carbohydrate present in the cells. The pretreatment efficiency and dissolved carbohydrate yield are calculated by the given equation.

$$\text{Extraction Efficiency} = \frac{(\text{g of dissolved carbohydrate})}{(\text{g of total carbohydrate})} \times 100$$

$$\text{Dissolved carbohydrate yield} = \frac{(\text{g of dissolved carbohydrate})}{(\text{g of algae})}$$

The cell breakage by different pretreatments was also confirmed by visualizing the cells under the microscope and scanning electron microscopy. An Olympus-CKX53 microscope was employed in the bright field configuration, using between 10X and 40 X objectives, 40X objective was used for all counting procedures. The microscope was equipped with a digital camera to take the microalgae photographs.

3.7.1 Acidic pretreatment

Since the pre-treatment with sulfuric or hydrochloric acid had very similar results, the former was selected since sulfate is a nutrient present in the yeast fermentation mediums. Sulfate also has a lower toxicity to yeasts when compared to chloride, and it is easily removed by neutralizing with calcium carbonate ($CaCO_3$). After neutralization, calcium sulfate ($CaSO_4$), a product of the chemical reaction, was removed using a centrifuge operated at 8000 rpm for 15min. A 10 ml of a sample having microalgae concentration of 1 mg/mL was used for acid pretreatment study. The optimum sulfuric acid concentration was determined by performing different experiments in the different concentration (0.25, 0.5, 1.0, 2.0, and 3.0 N) of sulphuric acid. Acid pre-treatment of microalgae was performed at 121°C for 30 min for

cellular disruption and sugar extraction, which was measured by phenol-sulphuric acid method (Laurens et al., 2015; Nguyen et al., 2009).

3.7.2 Alkali pretreatment

A 10 ml of the sample having a microalgae concentration of 1 mg/mL was used for alkali treatment. In microalgae sample, different concentrations (0.5, 1, 2, 3, 4 and 5.0%) of NaOH were added and autoclaved at 121°C for 30 min. The samples were cooled down and centrifuged at 5000 rpm for 5 min. The supernatant was taken and sugars concentration was estimated to measure the pretreatment efficiency by phenol-sulphuric acid method (Harun et al., 2011). The optimum acid concentration was used for further study.

3.7.3 Hydroxyl radical-aided thermal pretreatment

The hydroxyl radicals produced during the Fenton's reaction take part in the hydroxyl aided thermal pretreatment. In this pretreatment, different concentration of hydrogen peroxide was used and iron sulphate was used as a catalyst for Fenton's reaction. The different hydrogen concentrations were taken as 0.06, 0.12, 0.18, 0.24 and 0.30 g/L. The iron sulphate was taken 11.9 mM for every reaction. A 10 ml of microalgae having a concentration of 1 mg/mL was taken and added hydrogen peroxide and iron sulphate. The mixtures were autoclaved for thermal treatment at 121°C for 30 min. After the reaction, the samples were cooled down and centrifuged at 4000 rpm for 5min. The supernatant was further used for the dissolved carbohydrate analysis using phenol-sulphuric acid method (Gao et al., 2015).

3.7.4 Ultrasonication pretreatment

The ultrasonic device used for microalgae pretreatment was an ultrasonic processor UP200S (Hielscher – Ultrasound Technology, Germany) with a working frequency of 24 kHz and a

rated supplied power of 200W from the ultrasonic generator used. The instrument was fitted with a sonotrode (microtip S_2). The sonicator was operated at 100% amplitude and 0.5 cycles. The acoustic power generated by the instrument was reported as 600(W/m^2). A 10 ml of microalgae sample having cell mass concentration of 1mg/mL was pretreated at 100% amplitude and 0.5 cycles for 5, 10, 20, 30, 40, 50 and 60 min. The samples were centrifuged at 5000 rpm for 10 minutes. After cell breakage, the partially digested carbohydrate was released into the supernatant, which was measured by Phenol sulphuric acid method. In this method, a 100 µL of supernatant from was taken from each of centrifuged pretreated samples. Samples were made up to 1 ml. A 3 ml of 72% H_2SO_4 and 1 ml of 5% phenol were added in each sample. The samples were heated at 90°C for 5 minutes. The samples were cooled to room temperature and the absorbance was taken at 490nm (Masuko et al., 2005).

3.7.5 Ultrasonication followed by enzymatic pretreatment

In the first step, ultrasonication of microalgae biomass was performed as mentioned in the ultrasonication pretreatment section. After the ultrasonication, algal carbohydrate is hydrolyzed by enzymes. There were three enzymes; α-amylase (1300U/g), amyloglucosidase (from *Aspergillus niger*, 6U/mg) and cellulase (from *T. reesei* ATCC 2692, ≥ 1 unit/mg solid) were used for hydrolysis of carbohydrate, mainly starch and cellulose. After adding the enzymes in ultrasonicated microalga biomass, the reaction was performed at pH 5.5, temperature 30°C for 24h at 120 rpm. The reaction was stopped by putting the samples into a boiling water bath. The samples were cooled down and centrifuged at 4000 rpm for 5 min. The supernatant was taken to measure the released reducing sugar after the hydrolysis by DNS method (Shokrkar et al., 2017).

3.8 Fermentation

3.8.1 Yeast culture and culture conditions

The yeast, *Saccharomyces cerevisiae* NCIM 3494, was also procured from NCIM, National Chemical Laboratories Pune, India. Yeast culture was revived on MGYP media, which has the following composition as 3.0 g/L of malt extract, 10.0 g/L of glucose, 5.0 g/L of peptone, 3.0 g/L of yeast extract, and pH 6.0. The yeast culture was grown in MGYP media at 30°C and 150 rpm for 48 h. The fresh culture broth of 5% was used as inoculums for ethanol fermentation. Growth and monitoring of yeast cells were done by using both turbidity measurement at OD_{660} (UV/VIS Spectrophotometer, UV 3000+ LABINDIA) and standard dry cell weight method (Quirós et al., 2007).

3.8.2 Separate hydrolysis and fermentation (SHF)

Fermentation of algal carbohydrate was done in two-step process. In the first step, carbohydrates are hydrolyzed into simple sugars and in the second step; the reducing sugars were utilized by yeast *Saccharomyces cerevisiae* for production of ethanol**.** For the carbohydrate hydrolysis, two methods were adopted one acidic and another is enzymatic treatment. In the acidic pretreatment, both pretreatment and hydrolysis took place simultaneously.

3.8.2.1 Acid hydrolysis

In acid hydrolysis, the different concentrations (5, 10, 15, 20, 25 % w/v) of microalgae biomass were mixed separately with 1N H_2SO_4 at 180 rpm for 5 min. The resulting slurries were autoclaved at 121°C for 30 min. After hydrolysis, the samples were cooled down to room temperature. Centrifugation of samples was performed at 4000 rpm for 5 min to remove the cellular debris and the supernatant was taken to measure the total reducing sugar

present in the sample. The supernatant of pH was adjusted to 5.5 with solid $CaCO_3$ and the precipitate of $CaSO_4$ was removed by centrifugation. The supernatant was taken for fermentation.

3.8.2.2 Enzymatic hydrolysis

In enzymatic hydrolysis, the different concentrations (5, 10, 15, 20, 25 % w/v) of microalgae biomass were pretreated with ultrasonication and further hydrolyzed by alpha amylase, amyloglucosidase and cellulase enzymes. After adding the enzymes in ultrasonicated microalga biomass, the reaction was performed at pH 5.5, temperature 30°C for 24h at 120 rpm. The reaction was stopped by putting the samples into a boiling water bath. The samples were cooled down and centrifuged at 4000 rpm for 5 min. The supernatants were taken for the fermentation.

3.8.2.3 Fermentation of algal carbohydrate

After the acidic or enzymatic hydrolysis of algal biomass, the other nutrients necessary for ethanol production were added into the fermentation media. The fermentation media was prepared in 250 mL of Erlenmeyer flask by adding all the components as the composition given in Table 3.6. The hydrolyzed sugar was added into fermentation media as the main substrate for ethanol fermentation. The pH was adjusted 5.5 using 1 N NaOH and 1 N HCl. The media was autoclaved at 121°C for 15 minutes and cooled down to room temperature. After cooling, a 5% (v/v) of active cells of yeast *Saccharomyces cerevisiae* NCIM 3494 as inoculums. The ethanol fermentation was done at 30°C for 64 h under non-agitated and shaking (120 rpm) condition. In fermentation broth, glucose, ethanol and cell mass were analyzed at regular intervals. Glucose obtained from carbohydrate hydrolysis was measured

by DNS method (Miller, 1959). The estimation of ethanol during fermentation was done by using gas chromatography method (Stackler and Christensen, 1974). The growth kinetics of *S. cerevisiae* was calculated by measuring the weight of dry cell mass.

Table 3.6 Composition of fermentation media used in SHF of *C. sorokiniana*

Fermentation media	Acidic Hydrolysis	Enzymatic Hydrolysis
Glucose obtained from algal carbohydrate	24.62 g	25.14
KH_2PO_4	0.2 g	0.2 g
$(NH_4)_2SO_4$	1.0 g	1.0 g
$MgSO_4.7H_2O$	0.1 g	0.1 g
Yeast extract	0.2 g	0.2 g
Distilled water	200 mL	200 mL
pH	5.5	5.5

Table 3.7 Composition of fermentation media used in SHF of *T. obliquus*

Fermentation media	Acidic Hydrolysis	Enzymatic Hydrolysis
Glucose obtained from algal carbohydrate	27.6 g	27.66
KH_2PO_4	0.2 g	0.2 g
$(NH_4)_2SO_4$	1.0 g	1.0 g
$MgSO_4.7H_2O$	0.1 g	0.1 g
Yeast extract	0.2 g	0.2 g
Distilled water	200 mL	200 mL
pH	5.5	5.5

3.8.3 Simultaneous saccharification and fermentation (SSF)

Fermentation of algal carbohydrate was performed through Simultaneous Saccharification and Fermentation (SSF). In this process, firstly algal biomass was pretreated with ultrasonication so that all the carbohydrate content come out of the cells. After the pretreatment, the carbohydrate was hydrolyzed to glucose by enzymes (α-amylase (1300U/g), amyloglucosidase (from *Aspergillus niger*, 6U/mg) and cellulase (from *T. reesei* ATCC 2692, ≥ 1 unit/mg solid)) and after that *S. cerevisiae* strain was used for fermentation of reducing sugars to bioethanol. The other nutrients required for the growth of *S. cerevisiae* and production of ethanol were added into the sonicated algal sample to make a fermentation media. Then, this fermentation medium was autoclaved at 15 psi for 15 minutes to sterilize the media. The initial pH of the medium was adjusted to 5.5 using 0.1 N NaOH and 1 N HCl (Asada et al., 2012). The composition of fermentation media is given in Table 3.8.

After the sterilization, the fermentation media was cooled down to room temperature. A 100 mg of α-amylase, 50mg of amyloglucosidase and 50 mg of cellulose were added aseptically to that media. At the same time, 5% of yeast inoculum was added into fermentation media containing algal carbohydrate as the main carbon substrate. The culture broth was incubated at 30°C for 64 hours under non-agitated and shaking (120 rpm) conditions. Samples were collected at different time intervals, and analysis of total carbohydrate, glucose, ethanol and cell mass were performed accordingly. Glucose obtained from carbohydrate hydrolysis was measured by the DNS method (Hodge, 1962). The residual carbohydrate concentration during fermentation was measured using phenol-sulphuric acid method (Masuko et al., 2005). The estimation of ethanol as end product during fermentation was done by gas

chromatographic method (Stackler and Christensen, 1974). The yeast cell mass was measured by taking the absorbance at 600 nm and also by a dry weight method.

Table 3.8 Composition of fermentation media used in SSF

Fermentation media	Amount
Algal Biomass	50 g
KH_2PO_4	0.2 g
$(NH_4)_2SO_4$	1.0 g
$MgSO_4.7H_2O$	0.1 g
Yeast extract	0.2 g
Distilled water	200 mL
pH	5.5

3.8.4 Estimation of ethanol

The ethanol concentrations in the broth during the fermentation process were determined using gas chromatography (Nucon 5765) equipped with a flame ionization detector (FID) and Porapak-Q column (2 m length and 0.125 inch ID). In the gas chromatograph, the injector, detector, and oven temperatures were set to 190°C, 250°C, and 165°C, respectively. Nitrogen gas was utilized as a carrier at a flow rate of 30 mL/minute while Hydrogen as a fuel gas and Oxygen helped in burning. The standard calibration curve for ethanol concentration was plotted against time by injecting different concentrations of standard ethanol solution ranging from 0.1 to 10.0 % (v/v) ethanol in water. A fixed percentage of 5% (v/v) of n-propanol is added as internal standard in each ethanol samples. 1.0 µL of ethanol sample was injected into the gas chromatograph and the retention time was determined as 2.65 min (Stackler and Christensen, 1974).

3.9 Mathematical modelling for production of ethanol from microalgae

3.9.1 Mathematical modelling of SHF for production of ethanol from microalgae

The relationships during separate hydrolysis and fermentation of microalgae carbohydrate between the cell growth and substrate consumption, and cell growth and product synthesis have been given by Jiménez-Islas *et al.* using logistic, Pirt, and Luedeking-Piret equations [9]. The integration of the logistic equation is given in equation (1) which describes the relationship of cell biomass with time. The relationship between substrate consumption and cell growth is given by equation 2 which is obtained by dividing the Pirt equation by the logistic equation followed by integration. Equation 3 describes the relation between product synthesis and cell growth and obtained by dividing the Luedeking-Piret equations by the logistic equation followed by integration(Jiménez-Islas et al., 2014; Soto-Cruz et al., 2002).

$$X\,(t) = \frac{X_{max}}{1+\left(\frac{X_{max}}{X_0}-1\right)e^{-\mu\,t}} \quad (1)$$

$$G(X) = G_0 - \frac{1}{Y_{X/S}}\,(X - X_0) - \frac{m.X_{max}}{\mu}\ln\left(\frac{X_{max}-X_0}{X_{max}-X}\right) \quad (2)$$

$$E(X) = E + \alpha(X - X_0) + \frac{\beta.X_{max}}{\mu}\ln\left(\frac{X_{max}-X_0}{X_{max}-X}\right) \quad (3)$$

Where α and β are growth associated and non-growth associated factor respectively. The experimental data of fermentation processes were compared with model data of equations 1-3. The simulation program was designed in such a way that it can minimize normalized error (the sum of difference square of model data and experimental data) in solver function. The different kinetic parameters for microbial growth, sugar consumption, and ethanol production were evaluated by fitting the experimental data with equations 1-3. The experimental data with model data were analysed by using the regression curve fitting with statistical significance (p=0.05).

3.9.2 Mathematical modelling of SSF for production of ethanol from microalgae

The unstructured model, for ethanol fermentation through SSF, is mainly composed of four variables: Carbohydrate (*S*), glucose (*G*), bioethanol (*E*) and cell biomass (*X*).

3.9.2.1 Saccharification of algal carbohydrate to glucose

In the SSF process**,** microalgae carbohydrate was enzymatically hydrolyzed into glucose that was further utilized by yeast cells. The following equation gives the net glucose accumulation rate.

$$\frac{d[G]}{dt} = (Rate\ of\ glucose\ formation\ - Rate\ of\ glucose\ utilization)$$

$$\frac{d[G]}{dt} = r_{\mathrm{f}} - r_{\mathrm{u}} \quad (4)$$

Where, rf is the rate of glucose formation, and ru is the rate of glucose consumption/utilization by yeast during SSF process (Eklund and Zacchi, 1995).

Michaelis-Menten kinetics was used to describe the enzymatic saccharification of carbohydrate to glucose. Thus,

$$r_{\mathrm{s}} = V_{\mathrm{m}} \frac{S}{k_{\mathrm{m}} + S} \quad (5)$$

Where, r_{s} is the rate of saccharification of carbohydrate to glucose. The proposed model in the saccharification process is based on the model to describe carbohydrate saccharification (Kroumov et al., 2006).

The enzyme concentration was considered as constant for model simplification. The mass transfer limitations and variation in enzyme structure were not studied in the model. Ethanol was found as the primary product; other byproducts were not considered in this study because it is very complex to model each byproduct during fermentation.

3.9.2.2 Microbial growth

During SSF, glucose produced from saccharification of starch was utilized for ethanol production and growth of the cells. The specific growth rate of *S. cerevisiae* was given by Monod equation as-

$$\mu = \mu_{\mathrm{m}} \frac{G}{k_s + G} \tag{6}$$

Where, μ is the specific growth rate of yeast cells, G is the glucose concentration, $\mu_m$is mthe aximum specific growth rate, and k_s is the saturation constant. The substrate inhibition caused by the high concentration of glucose was not considered in the model equation because glucose did not accumulate up to an inhibitory level in the SSF process. The effect of ethanol on yeast cells has been neglected because lowthe concentration of ethanol was produced (Jang and Chou, 2013).

The growth rate of microorganisms is assumed to be directly proportional to its dry cell weight. The following equation gives the growth rate of cells.

$$\frac{dX}{dt} = \mu X \tag{7}$$

Where x is the dry cell weight. Some of the sugar produced during saccharification was consumed by yeast cells for its maintenance. The sugar consumed for maintenance is directly proportional to the dry cell weight. Mathematically, it is described by the given equation.

$$r_{\mathrm{m}} = m_{\mathrm{s}} X \tag{8}$$

Where, r_m is the consumption rate for cell maintenance, and m_s is the maintenance coefficient for yeast cells (Jiménez-Islas et al., 2014).

3.9.2.3 Fermentation of glucose to ethanol

Ethanol is produced from fermentation of glucose by *S. cerevisiae.* The production of ethanol is assumed to be directly proportional to the cell concentration and substrate, i.e. glucose. The following equation described the ethanol production rate.

$$r_{\mathrm{E}} = q_{\mathrm{m}}.\ G.X \tag{9}$$

Where, q_m is the maximum ethanol production rate (Jang and Chou, 2013).

3.9.2.4 A mathematical model of SSF

The design of SSF is described as based on the above-mentioned rate of reactions.

Carbohydrate depletion rate:-

$$-\frac{dS}{dt} = V_{\mathrm{m}} \frac{S}{k_{\mathrm{m}} + S} \tag{10}$$

Cell mass growth rate:-

$$\frac{dX}{dt} = \mu_{\mathrm{m}} \frac{G}{k_{\mathrm{s}} + G} X \tag{11}$$

Ethanol production rate:-

$$\frac{dE}{dt} = q_{\mathrm{m}} \frac{G}{k_{s1} + G}.X \tag{12}$$

Glucose accumulation rate: - In equation 1, r_{f} the rate of glucose formation is directly related to the carbohydrate depletion and can be calculated by multiplying 1.11 in r_{s}, the carbohydrate utilization. Hence,

$$r_{\mathrm{f}} = 1.11 * r_{\mathrm{s}}$$

$$r_{\mathrm{f}} = 1.11\, V_{\mathrm{m}} \frac{S}{k_{\mathrm{m}} + S} \tag{13}$$

(Glucose utilized during ethanol fermentation) = (Glucose utilized for Ethanol production + Glucose utilized for cell growth + Glucose used for cell maintenance)

$$r_u = \frac{1}{Y_{p/s}}\frac{dE}{dt} + \frac{1}{Y_{x/s}}\frac{dX}{dt} + m_s X \quad (14)$$

And r_u, the rate of glucose utilization is the summation of rates of glucose used for ethanol production, cell growth and cellular maintenance (Hari Krishna and Chowdary, 2000; Liu et al., 2014).

Therefore,

$$\frac{d[G]}{dt} = 1.11\, V_m \frac{S}{k_m + S} - \left[\frac{1}{Y_{p/s}}\frac{dE}{dt} + \frac{1}{Y_{x/s}}\frac{dX}{dt} + m_s X\right] \quad (15)$$

These model equations were used for the prediction of different variables of fermentation by minimizing the normalized error among kinetic data with model data.