Preamble

Native cyanobacterial strains collected from local water bodies were cultured under optimal conditions with a view to maximize the biomass amount and carbohydrate content. Two strains *Lyngbya limnetica* and *Oscillatoria obscura* out of four were found to be suitable for yeilding maximum sugar after pretreatment and subsequent conversion to biobutanol. Using these strains growth studies were carried out to optimize the biomass and carbohydrate content under varying growth conditions. The biomass produced under optimal conditions was subjected to pretreatment and conversion to biobutanol through fermentation using *Clostridium* species. Fermentation conditions were also optimized and butanol recovery studies were performed using liquid-liquid extraction and perstraction techniques. Details of these experiments including equipment, materials used and procedure followed are described in detail in following sections.

3.1 Chemicals and Equipments

All chemicals used in the present study were of analytical grade purchased either from Sigma Aldrich (Germany), Himedia (Mumbai, India) or Merck (Germany). List of various equipment used for performing experiments and carrying out analysis are listed in Table 3.1.

3.2 Biomass Selection

3.2.1 Collection, cultivation and characterization of cyanobacterial biomass

Fresh cyanobacterial biomass was collected from four ponds located in different parts of Varanasi city (Ishwargangi, Durgakund, Indian Institute of Technology (BHU), and Institute of Agriculture Sciences, BHU) (Figure 3.1). Ponds selected for the collection of samples receive surface run of and are rich in nutrients that favor the growth of cyanobacterial biomass. The collected biomass was initially cultivated in the pond water itself for 5 days, washed repeatedly with distilled water to remove dirt and other impurities and then transferred to the growth media of a suitable composition as listed in Table 3.2. Culture flask was illuminated with an external visible light source mounted on the top of the growth chamber. The light intensity was measured using a lux meter and converted to the unit of $\mu molm^{-2}s^{-1}$. Initially the strains were cultivated under the light intensity of 49.0 $\mu molm^{-2}s^{-1}$ at 25±3°C incubation temperature with continuous shaking at 150 rpm. After 20 days of incubation, 2 mL of the media containing cells were transferred to a 100 mL Erlenmeyer flask containing 50 mL of BG-11 media and then cultivated again in the growth chamber to get healthy cells for further experiments.

Table 3.1: List of equipment used during experimentation

Equipments	Make	Purpose
Algae growth chamber	Designed inhouse	For cyanobacteria growth
Lux meter	Digital Lux Meter, HP-881A	To measure light intensity
Autoclave	INSTECH SYSTEMS, New Delhi	Sterilization
Hot air oven	HINDUSTAN SCIENTIFIC, New Delhi	Drying of biomass
Magnetic hot plate	IKA C-MAG HS 7, Germany	Pretreatment
Deep freezer	Voltas, India	Storage for culture
pH meter	ELICO LI 120, Hyderabad	Measuring media pH
Laminar air flow	SHIVAM Instruments, New Delhi	Creation of aseptic condition
Centrifuge	eltek TC 4100F, Mumbai, India	Sample preparation
Refrigerated centrifuge	Thermo Scientific, Sorvall ST16R, Germany	For concentrating thermolabile samples
BOD incubator shaker	Caltan, NSW-256, New Delhi	Sample incubation
UV-Vis Spectrophotometer	HITACHI U-2900, Tokyo, Japan	Bacterial growth, enzyme assay
Rotary flask shaker	SHI-168, Shivam instruments, Delhi	Sample mixing
Anaerobic sachet and indicator	BD GasPak^{TM}EZ 260001, USA	To maintain anaerobic condition
Gas chromatography	Nucon-5765, Hyderabad	Gaseous and liquid fuel analysis
High performance liquid chromatography	Dionex Ultimate 3000, Sunnyvale, California	Sugar and metabolites analysis
Membrane-solvent extraction unit	Designed inhouse	Recovery of butanol
Scanning Electron Microscopy	Zeiss EVO 18 Research SEM system, Germany	Study of biomass morphology
Fourier Transform Infrared Spectrometer	Thermo Fisher Nicolet 5700 FTIR, USA	For functional group analysis of biomass

Cyanobacterial biomass thus obtained was characterized in terms of solid density, bulk density, moisture content, ash content, volatile matter and fixed carbon content using the standard protocol proposed by NREL (Wychen and Laurens 2013a). According to this protocol the moisture and ash contents of the cyanobacterial biomass to be used as the sugar source, are the limiting conditions and should be less than 10% individually. On the basis of the results of characterization, cyanobacterial species *Lyngbya limnetica* and *Oscillatoria obscura* were selected for further studies.

Figure 3.1: Geographic location of different sample collection points

3.2.1.1 Scanning Electron Microscopic (SEM) Analysis

SEM analysis for both the selected strains was done to elucidate the morphology of the cells using SEM system. The freshly cultivated cells were collected by centrifugation at 5000 rpm, washed with phosphate-buffered saline (pH 7.0) and then incubated in 2.5% glutaraldehyde for 2 h. Incubated cells were washed twice with sodium phosphate buffer

(pH 7.2) and then serially treated with 20–100% alcohol to dehydrolyze the cell samples. The dehydrolyzed biomass was mounted on a stub and subjected to SEM analysis.

Table 3.2: BG-11 media composition

Chemicals	**Concentration (g/L)**	**Trace elements**	**Concentration (g/L)**
$NaNO_3 \cdot 4H_2O$	1.5	H_3BO_3	2.86
K_2HPO_4	0.04	$MnCl_2 \cdot 4H_2O$	1.81
$MgSO_4 \cdot 7H_2O$	0.075	$ZnSO_4 \cdot 7H_2O$	0.222
$CaCl_2 \cdot 2H_2O$	0.036	$NaMoO_4 \cdot 2H_2O$	0.39
Na_2CO_3	0.02	$CuSO_4 \cdot 5H_2O$	0.079
Ferric ammonium citrate	0.006	$Co(NO_3)_2 \cdot 6H_2O$	0.049
Citric acid	0.006		
EDTA (Na-salt)	0.001		
Trace elements	1 mL		

3.2.2 Batch growth of cyanobacterial biomass

To examine the growth pattern of selected cyanobacterial strains, batch growth studies were performed. Inoculum were prepared by centrifuging the cultivated cyanobacterial cells at 5000 rpm for 10 min. 0.2 g of inoculum was transferred to a 100 mL conical flask containing 50 mL of autoclaved media maintained at pH 8.3±0.2 under aseptic conditions. Flasks were incubated at 25±3°C for 20 days with continuous shaking at 150 rpm under 49.0 $\mu molm^{-2}s^{-1}$ light illumination using the 14:10 h light:dark cycle. Samples were collected after every 2-day intervals, centrifuged at 5000 rpm for 10 min and analyzed for drybiomass and carbohydrate content. The collected samples were kept at 70±2°C in a hot air

oven overnight to obtain the drybiomass for analyzing the carbohydrate content.

3.2.2.1 Carbohydrate analysis

The carbohydrate content of the dried biomass was analyzed using the phenol-sulfuric acid method (Efremenko et al. 2012; Ho et al. 2012). The two-step sulfuric acid hydrolysis was used to dissolve the cyanobacterial biomass and to prepare the hydrolysate containing total monomeric carbohydrate. As per the specified protocol, in the first step a known mass of cyanobacterial strains was treated with 72wt% of H_2SO_4 at 30°C for 1 h and in the second step 4wt% of H_2SO_4 was used to hydrolyze the biomass at 121°C for 1 h in an autoclave. The hydrolysate thus obtained, was cooled to room temperature and then centrifuged at 7000 rpm for 10 min to get a clear supernatant. A known volume of hydrolysate was maintained at pH 6.4±0.1 with the help of sodium-carbonate and used for the total carbohydrate analysis. 100 µL of the diluted hydrolysate was reacted with 100 µL of phenol (5% w/v) followed by further reaction with 500 µL of concentrated H_2SO_4 and was incubated first at the room temperature for 10 min and then placed in a water bath at 30±2°C for 20 min. The absorbance of this solution was measured at 490 nm using a UV-Vis spectrophotometer. A standard absorbance-concentration curve prepared for aqueous glucose solution was used to read the actual concentration. Further analysis of the extracted soluble carbohydrates was done by High Performance Liquid Chromatography (HPLC) equipped with Refractive Index Detector (RID) to evaluate the type of sugar monomers released from the treated biomass.

3.2.3 Manipulation of culture conditions

3.2.3.1 Effect of different operating parameters on growth of cyanobacterial biomass

To see the effect of shaking on the growth of *L. limnetica* and *O. obscura* a comparative analysis was done by keeping one set of the culture flasks under continuous shaking condition at 150 rpm and the other set under static condition. Effect of other parameters on the growth of cyanobacterial biomass was studied by varying the initial media pH (3–11), light intensity (14–81 $\mu molm^{-2}s^{-1}$ i.e. 1000–6000 lux), and incubation temperature (10–40°C). A set of 100 mL flasks containing 50 mL of autoclaved growth media was inoculated with an appropriate weight (0.2 g) of cyanobacterial biomass and cell growth was analyzed after 20 days of incubation in terms of drybiomass and carbohydrate contents for both the strains by collecting the samples after centrifugation at 5000 rpm for 10 min following the standard protocol as mentioned earlier (cf. Section 3.2.2.1).

3.2.3.2 Effect of nutrient limitation on cyanobacterial growth

To examine the effect of trace elements on the growth, one set of experiments was performed in its absence keeping other media constituents similar to that in BG-11 media. Flasks containing 50 mL of growth media were inoculated with 0.2 g of cyanobacterial inoculum and other parameters viz., incubation time, temperature, media pH, light intensity and shaking were kept constant at 20 days, 20±3°C, 9.0, 68 $\mu molm^{-2}s^{-1}$ and 150 rpm, respectively. The biomass from various flasks were collected after every 2-day intervals and analyzed for both drybiomass and carbohydrate content by following the standard protocol as described earlier (cf. Section 3.2.2).

Effects of nitrogen and phosphorus have also been examined in a similar fashion and under identical operating conditions. The source of nitrogen and phosphorus were $NaNO_3 \cdot 4H_2O$ and K_2HPO_4, respectively. During the manipulation of $NaNO_3 \cdot 4H_2O$ concentration (0–2.0 g/L) amount of K_2HPO_4 was kept constant as in P-2 media composition (i.e. 0.04 g/L) along with other trace elements. To see the effect of phosphorus on cyanobacterial growth and carbohydrate accumulation a separate set of experiments was conducted under PS condition keeping other components constant as prescribed in the BG-11 media. Other than the carbohydrate content change in lipid and protein concentrations has also been evaluated under NR and NS conditions by following the Bligh and Dyer method and Lowry's protocol, respectively (Bligh and Dyer 1959; Lowry et al. 1951).

3.2.4 Growth kinetics

Specific growth rate (μ) in terms of drybiomass per unit time defines the growth quite accurately (Xu and Boeing 2014). Specific growth rate was calculated from the slope of semi-logarithmic plot of biomass productivity versus time:

$$\mu = \frac{\ln\left(\frac{X_t}{X_0}\right)}{t - t_0} \qquad (3.1)$$

where μ = specific growth rate (d^{-1}), X_t = drybiomass concentration (g/L) at time t, X_0 = initial drybiomass concentration (g/L) at t = 0 and t = time (day).

3.2.5 Two-stage growth study

Two-stage growth study was carried out with the aim of increasing the total biomass and carbohydrate content in the selected strains. In the first stage, cyanobacterial strains were

cultured photoautotrophically in a 100 mL conical flask containing 50 mL of BG-11 media under the optimized conditions- light intensity- 68 $\mu molm^{-2}s^{-1}$, shaking- 150 rpm, incubation temperature- 20±3°C, pH- 9.0 and 14:10 h light:dark cycle with standard trace elements, $NaNO_3 \cdot 4H_2O$ and K_2HPO_4 (with concentrations as in BG-11 media) to get the maximum biomass growth on drybiomass basis. Initial batch growth studies for both the strains were performed for 20 days and no remarkable change in the drybiomass concentration was found after 16 days (cf. Section 3.2.2). Therefore, during Stage-I growth studies, flasks were incubated for 16 days in order to get the maximum drybiomass. Two set of flasks were placed for Stage-I growth analysis, the cyanobacterial biomass from one flask was recovered by centrifugation at 5000 rpm for 10 min and subjected to drybiomass and carbohydrate content analysis. Another set of culture flask was used as inoculum for Stage-II growth studies. From the results of batch growth studies under nitrogen-starvation (NS) and phosphorus-starvation (PS) conditions it is clear that effect of PS condition on carbohydrate accumulation is lower than the NS condition, so to get the large accumulation of carbohydrate within the biomass, the Stage-II cultivation was done under NS condition for 20 days keeping all other nutrient concentrations similar to BG-11 media and all operating parameters same as in Stage-I.

3.3 Pretreatment of biomass

3.3.1 Preparation and characterization of cyanobacterial biomass

The cyanobacterial biomass obtained from batch growth study was dried at 70±2°C in a hot air oven for 24 h, ground and sieved (150 μ) for use as raw material for pretreatment studies. SEM of the selected alga was done to examine the morphological changes and FTIR

spectroscopy to evaluate the vibrational frequency changes in the biomass before and after the pretreatment using acidic and alkaline reagents.

3.3.2 Batch pretreatment study

Initial pretreatment study was done with all the four collected biomass (cf. Section 3.2.1) to evaluate the efficiency of biomass in terms of sugar release under various pretreatment conditions. The impacts of acidic (H_2SO_4) and alkaline (NaOH) pretreatment reagents were tested at various temperature and time to release sugar from different biomass. Experiments were performed in 100 mL conical flasks containing 50 mL distilled water and 1.0 g of dry biomass without the use of any acid or alkali to get optimum treatment time and favourable temperature under ambient condition. The investigations were made in the temperature range of 40–100°C and for the treatment time range of 30–75 min. To minimize the energy involvement a lower temperature range was selected. The mixture was then centrifuged at 7000 rpm for 10 min and clear supernatant was adjusted to pH 6.8±0.2 and analyzed for the amount of total sugar released using the phenol-sulfuric acid method.

The efficacy of H_2SO_4 and NaOH (1 M) was tested for all the collected strains, other parameters viz., treatment temperature and time were kept constant at 100°C and 60 min, respectively. The hydrolysates thus obtained were centrifuged and their pH was adjusted to 6.8±0.2 with Na_2CO_3 and H_2SO_4 as per the requirement. Total sugar concentration was then analyzed spectrophotometrically at 490 nm following standard protocol mentioned earlier (cf. Section 3.2.2.1).

3.3.2.1 Optimization of pretreatment conditions through RSM

The RSM was applied to optimize the pretreatment conditions for biomass of *Lyngbya limnetica* selected from batch pretreatment study using Design Expert Software (8.0.7.1). It is a statistical technique that uses different experimental values from design to model and optimizes process wherein different independent variables (input parameter) affect the dependent response (Chatterjee et al. 2012; Myers and Montgomery 1995). Optimization of any process by this software is done in three steps i.e. performance of designed experiments, estimation of different coefficients, prediction of output variable and evaluation of the model adequacy (Myers and Montgomery 1995). From batch experiment it has been seen that H_2SO_4 was the best pretreatment agent so its concentration was selected as one of the input parameters. The operating parameters viz., treatment time (15–60 min), temperature (40–100°C) and H_2SO_4 concentration (0.5–2.5 M) were selected as the input variables and sugar release as the output response. Dilute acid concentration was used for pretreatment studies as concentrated acid is highly toxic, corrosive and harmful (Sun and Cheng 2002). Equation 3.2, relates the coded and actual values and the relation between response y and input variable x is expressed as shown in Equation 3.3.

$$x_i = (X_i - X_i^*)/\Delta X_i \quad (3.2)$$

where x_i = coded value of the ith input variable, X_i = uncoded value of the ith input variable, X_i^* = uncoded value of the ith input variable at center point and ΔX_i = step change value (Jung et al. 2011).

$$y = f(x_1, x_2, x_3, \dots\dots\dots\dots\dots, x_n) \quad (3.3)$$

where y is response of the system and $x_1, x_2, x_3, \ldots\ldots\ldots, x_n$ are input variables.

As Box-Behnken design (BBD) needs minimum number of experiments and the center points are used in this method to check the reproducibility. A second-order model was constructed with three-levels-three-factors to generate total 17 experimental runs (Betiku and Taiwo 2015; Kim and Han 2012). Following polynomial equation was used to compare the response with independent variables.

$$y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i<j} \beta_{ij} x_i x_j \quad (3.4)$$

where, β_0, β_i, β_{ii} and β_{ij} are constant, linear, quadratic and interaction coefficients, respectively.

The validity of statistical results thus obtained was examined using ANOVA. The maximum (+1) and minimum (-1) level of these parameters were 100 & 40°C, 60 & 15 min and 2.5 & 0.5 M for treatment temperature, time and acid concentration, respectively. The accuracy of the model equation was tested by the R^2 value whereas surface plot and regression equation gave the optimum values of different parameters. The cyanobacterial biomass of *Oscillatoria obscura* was also subjected to pretreatment conditions optimized by RSM to get the sugar for further fermentation study.

3.3.3 Other pretreatment strategies

3.3.3.1 Stepwise pretreatment

In this technique of pretreatment, cyanobacterial biomass of *L. limnetica* and *O. obscura* were subjected to H_2SO_4 treatment through a number of steps to get maximum possible sugar yield (Figure 3.2). In Step-I pretreatment 100 mL Erlenmeyer flask containing 50 mL

1.63 M H_2SO_4 and 1.0 g of dry cyanobacterial biomass was kept at 100°C for 60 min. The mixture was then subjected to steam treatment in an autoclave at 121°C for 45 min thereafter it was cooled to room temperature and centrifuged at 7000 rpm for 10 min to get a clear supernatant. The residue obtained after centrifugation was used for Step-II pretreatment using 1 M H_2SO_4 and NaOH separately at 100˚C for 60 min. After cooling the treated biomass was then centrifuged again at 7000 rpm for 10 min and the supernatants thus obtained from Step-I and Step-II were subjected to sugar estimation using phenol-sulfuric acid method after adjusting the solution pH to 6.8 ± 0.2**.**

3.3.3.2 Lipid extraction and simultaneous use of cyanobacterial residue for sugar release

In the present study an attempt has been made to evaluate the potential of cyanobacterial biomass of *L. limnetica* and *O. obscura* for lipid extraction and subsequent utilization of residual biomass for sugar analysis (Figure 3.3). Modified Bligh and Dyer method was used to extract lipid by changing the ratio of solvent chloroform:methanol (1:1–2:1) to get the maximum lipid yield. For 2 mL of sample volume approx 8.0 mL of chloroform:methanol was mixed in an appropriate proportion and lipid was extracted by cell rupturing using mortar-pestle. Further 4.0 mL of chloroform was added in the mixture to complete the extraction process. A known volume of distilled water (4.0 mL) was added in the mixture to separate the chloroform and methanol layers and mixed well. This mixture was then incubated at 40ºC for 30 min for clear separation of layers.

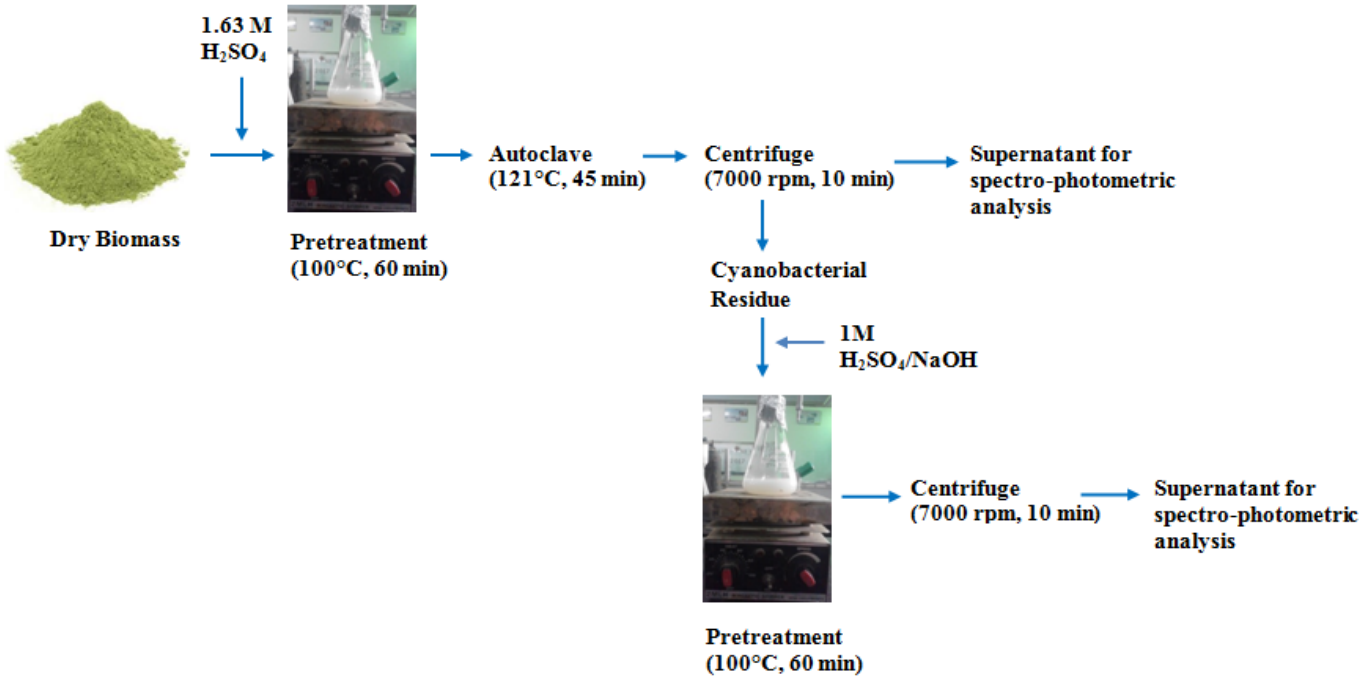

Figure 3.2: Two-step pretreatment of cyanobacterial biomass

After the incubation tubes containing sample were centrifuged at 5000 rpm for 10 min, bottom chloroform layer was separated in a pre-weighted container and placed in an oven for 60 min at 60ºC until a constant volume was achieved. Lipid extracted biomass was subsequently utilized for the acid pretreatment at optimized conditions (100°C for 60 min with 1.63 M H_2SO_4) to release sugars that were analyzed spectrophotometrically. Lipid content was calculated using the Eq. 3.5.

$$\text{Lipid Yield} = \frac{W_2 - W_1}{\text{sample weight}} \ (\text{g/g}) \qquad (3.5)$$

where, W_1 = initial weight of container (g), W_2 = final weight of container (g)

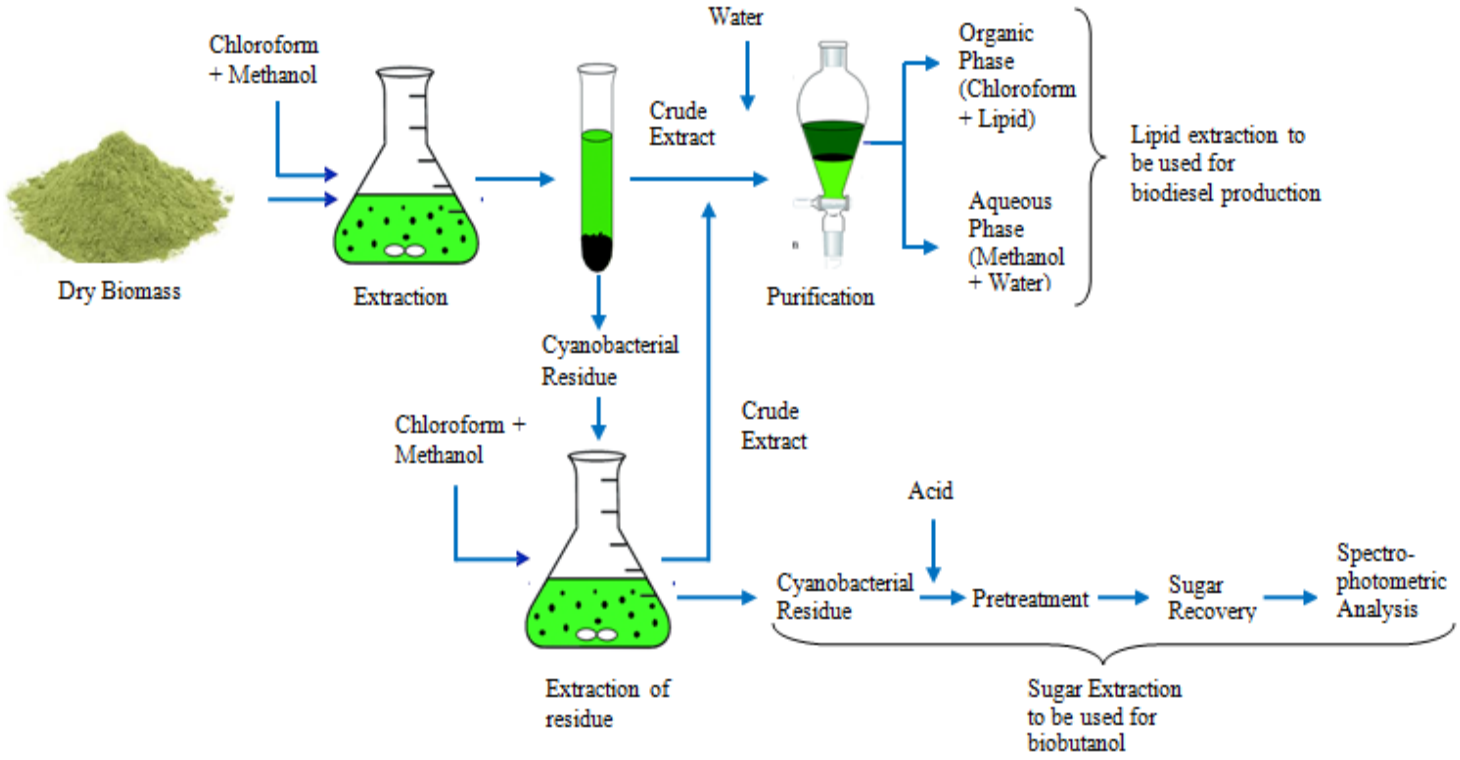

Figure 3.3: Simultaneous recovery of lipid and fermentable sugar from cyanobacterial biomass

3.4 Enzymatic hydrolysis

3.4.1 Preparation of inoculum for enzyme extraction

The fungal culture of *Aspergillus fumigatus* AA001 was obtained from Srivastava et al. (2015) and used for the production of enzyme in SSF. Fungal inoculum was prepared on PDA plates by using disc method of inoculation. Small discs of 0.5 cm diameter were cut and 2-3 such discs were placed on the fresh PDA plates under aseptic condition and then incubated at 37±2ºC for 2-3 days in an upside down position to obtain sufficient growth. Routine subculturing was done at an intervals of 25 days to maintain the metabolic activity of cells and fully grown cells were preserved on PDA plates for further use.

3.4.2 Solid state fermentation

Dried biomass of *L. limnetica* and *O. obscura* were used as substrate, in each case 5.0 g of the biomass was transferred in a 250 mL conical flask and moistened with 11.0 mL of Mandel and Weber media and mixed throughly. The media had (g/L): KH_2PO_4: 0.4; $MgSO_4 \cdot 7H_2O$: 0.3; $(NH_4)_2SO_4$: 1.4; $FeSO_4 \cdot 7H_2O$: 0.05; $MnSO_4 \cdot H_2O$: 0.016; $ZnSO_4 \cdot 7H_2O$: 0.014; $CoCl_2 \cdot 6H_2O$: 0.01; Peptone: 0.75, and was maintained at pH 4.7±0.2. The moistened cyanobacterial biomass was sterilized in an autoclave at 121ºC for 30 min at 15 psi pressure, cooled and subsequently inoculated with 1.0 mL of the spore suspension under aseptic condition and incubated at 42±2ºC for 4-5 days. At the end of the incubation time, enzyme produced was recovered by adding 50 mL of sodium citrate buffer (pH 4.6±0.2) and mixed at 150 rpm for 30 min on a rotary shaker.

Slurry thus obtained was filtered through glass wool and the filtrate was then centrifuged at 5000 rpm for 7 min at 4ºC to remove debris and the suparnatant was used for the enzyme activity (FPase (FP), β-glucosidase (βG), CMcase (EG), protein) tests and hydrolysis of cyanobacterial residue obtained after acidic pretreatment. Effect of Fe_3O_4/Alginate nanocomposite (obtained from Srivastava et al. 2015) on enzyme production was also evaluted by adding various concentrations (0.5-2.0 mM) of Fe_3O_4/Alg nanocomposite in the mixture (MSM+Biomass) before sterilization. Further treatment of the mixture was done in a manner similar to that used for mixture without nanocomposite and subjected to enzyme assay to see the effect of nanocomposite on various enzyme activities (FP, βG, EG).

3.4.3 Enzyme assays

The FP and EG activities were measured by following the protocol reported by Ghose (1987) while the βG activity was assayed following the procedure of Kubicek (1982). Concentration of reducing sugar was determined by following Miller's DNS method using glucose as standard (Miller 1959). One unit (IU) of filter paper activity is defined as the amount of enzyme required to release 1 μmol of glucose per minute from the filter paper (Whatman No. 1) under specified conditions. Similarly one unit of β-glucosidase activity is the amount of enzyme essential for liberating 1 μmol of *p*-nitrophenol per minute from paranitrophenyl-β-D glucopyranoside (pNPG).

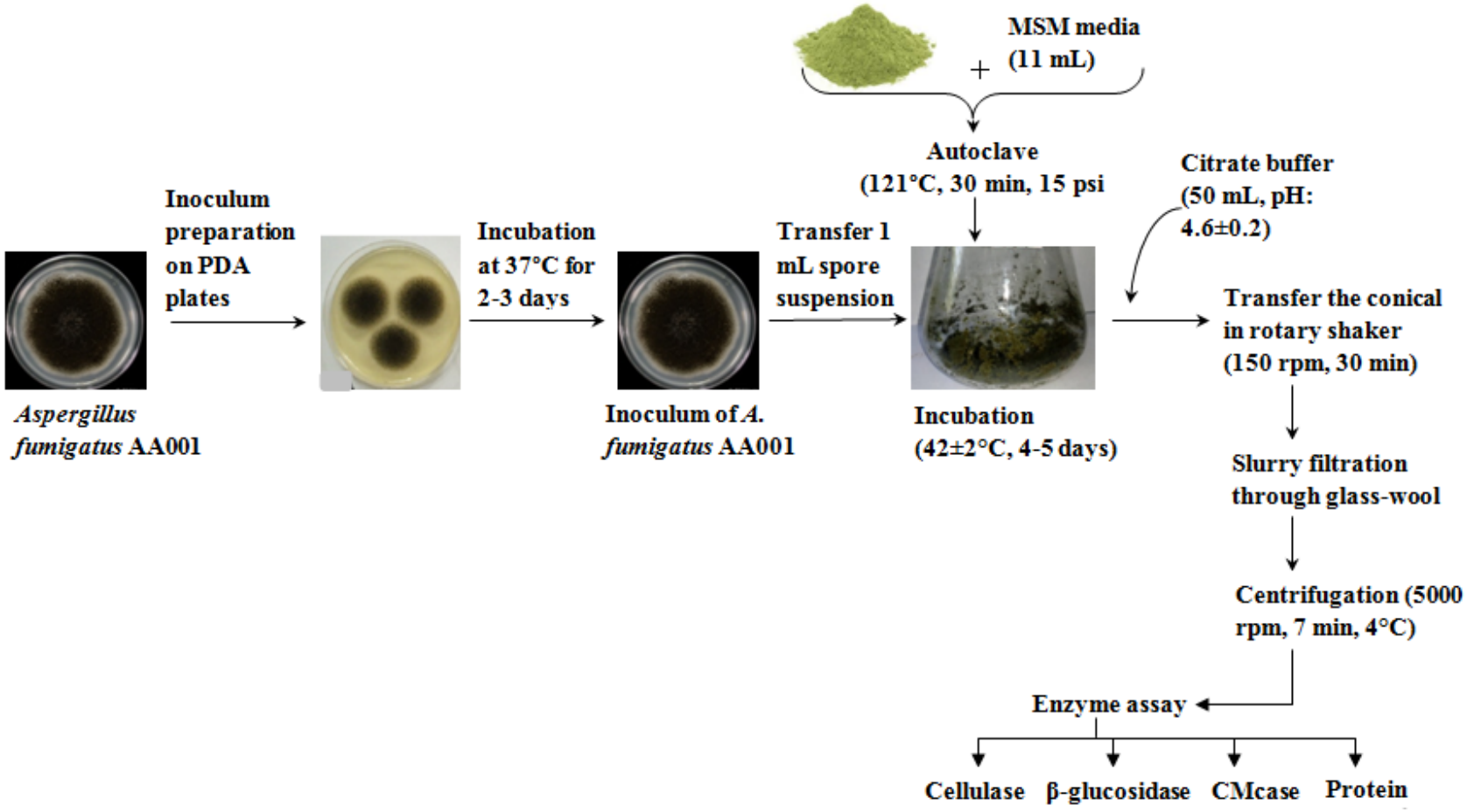

Figure 3.4: Stepwise incubation and extraction of enzyme

3.4.3.1 Preparation of standard curve for glucose

Preparation of DNS reagent: 10.0 g of 3,5-dinitrosalicylic acid (DNS) was mixed with 500 mL of 1% NaOH solution and dissolved properly. Then 2.0 g of phenol, 0.5 g of sodium sulphite and 200 g of Rochelle salt were added in the solution and mixed gently to dissolve all the components completely and the final reagent volume was made up to 1000 mL.

Glucose solution of different dilutions (0.1–1.0 mg/mL) were prepared from the stock glucose solution (10 mg/mL) in test tubes maintaining the total volume in each case at 1.5 mL. 3.0 mL of DNS reagent was added in each tube, these were then incubated in boiling water bath for 15 mins and there after were transferred to a cold water bath to stabilize the color. Solution of every tube including the blank was diluted with 20.0 mL distilled water and mixed properly. The absorbance of diluted solution was measured at 540 nm using a UV-Vis spectrophotometer. A linear standard curve was drawn between the absorbance and the glucose concentration.

3.4.3.2 Filter paper assay

Filter paper activity of enzyme was measured by using different tubes having solutions, the reagent blank (1.5 mL citrate buffer (0.05 M, 4.8±0.2 pH)), the substrate blank (filter paper (50 mg) & 1.5 mL buffer), the enzyme blank (1.0 mL buffer & 0.5 mL enzyme dilution) and the enzyme substrate (1.0 mL buffer, 0.5 mL enzyme dilution & filter paper). These test tubes were incubated at 50±3°C for 60 min and then DNS reagent (3.0 mL) was mixed to stop the enzyme action. All the tubes were placed in the boiling water bath for 15 min and then in the cold water bath to lower down the temperature and stabilize the color forming reaction. Reaction mixture of each tube was mixed properly after addition of 20 mL distilled

water and absorbance was measured at 540 nm. Filter paper activity of the enzyme was calculated by using the formula:

$$\text{Enzyme activity (U/mL)} = \frac{\text{ES} - \text{EB} - \text{SB}}{\text{Glucose (1 mg/ml) OD}} \times 0.185 \times \text{dilution} \quad (3.6)$$

3.4.3.3 Endoglucanase assay

Carboxymethyl cellulase activity (CMCase) of enzyme was calculated by preparing reaction test tubes in fashion similar to that mentioned earlier (cf. Section 3.4.3.2). Initially 0.5 mL of diluted enzyme solution was heated to 50°C for 5 min and then 0.5 mL of 2% carboxymethyl cellulose was mixed as substrate. All the reaction tubes were incubated at 50±3°C for 30 min and then 3.0 mL of DNS reagent was mixed in each tube and then the tubes were incubated for 15 min in boiling water. The tubes were then quickly transferred to a cold water bath to stabilize the color. Content of each tube was diluted by adding 20 mL of distilled water and mixed well by inverting the tube several times. The absorbance of the color was recorded at 540 nm and the activity was calculated using the Eq. 3.7.

$$\text{Enzyme activity(U/mL)} = \frac{\text{ES} - \text{EB} - \text{SB}}{\text{Glucose (1 mg/ml) OD}} \times 0.37 \times \text{dilution} \quad (3.7)$$

3.4.3.4 β-glucosidase assay

pNPG was used as substrate for measuring the βG activity of enzyme. All the reaction tubes were prepared in the same way as mentioned earlier (cf. Section 3.4.3.2) using acetate buffer (pH: 5.0) for dilution of enzyme. Diluted enzyme (0.5 mL) was maintained to 50°C for 5 min and then 0.5 mL of 5.0 mM pNPG was added to enzyme-substrate and substrate-blank reagent tubes and these were then incubated at 50±3°C for 30 min. Immediately 1.0 mL of glycine NaOH (pH: 10-11) was added in each tube and after proper mixing tubes were again

incubated at 34±3°C for 1-2 min for color development. Absorbance of the final solution was measured at 405 nm and activity was calculated using the formula:

$$\text{Enzyme activity (U/mL)} = \frac{\text{ES} - \text{EB} - \text{SB}}{2.25} \times \frac{(7.2 \times 2 \times \text{dilution})}{(1.0 \times 30)} \quad (3.8)$$

3.4.3.5 Extracellular protein estimation

Preparation of solution A: This solution is a mixture of 3 different reagents (i) Reagent-1: 2.0 g of Na_2CO_3 and 4.0 g of NaOH dissolved in 100 mL distiiled water; (ii) Reagent-2: 1.0 g of $CuSO_4 \cdot 5H_2O$ dissolved in 100 mL distilled water; (iii) Reagent-3: 2.0 g Na-K-tartarate dissolved in 100 mL distilled water. For the preparation of final Solution A, 1.0 mL of Reagent-2, 1.0 mL of Reagent-3, and 100 mL of Reagent-1 were mixed in the listed order.

Protein content of the enzyme was estimated by the Lowry method using bovine serum albumin (BSA) as standard (Lowry et al. 1951). Different dilutions of BSA (20-100 ppm) were prepared using distilled water to give a final volume of 1.0 mL from the stock BSA solution (1000 ppm) and pure distilled water was used as the blank. 4.5 mL of Solution A was added in each test tube and then incubated for 15 min in dark at room temperature. After incubation 0.5 mL of freshly prepared Folin reagent (2 N) was added in each tube and vortexed immediately to mix the contents properly. Again the samples were incubated at room temperature for 30 min in dark to develop the color. Absorbance of the colored samples was measured at 660 nm. Unknown samples were also treated following the same procedure and concentration was obtained using the standard protein curve.

3.4.4 Thermal stability test of enzyme in the presence of Fe_3O_4/Alginate nanocomposite

Thermal stability of the extracted enzyme was evaluated in the presence of Fe_3O_4/Alg

nanocomposite on the basis of filter paper activity because in a multi-component enzyme complex system it measures the overall activity (Rawat et al. 2014). 1.0 mM of nanocomposite was used for incubating the enzyme at temperatures of 45–75ºC for 3 h. Enzyme incubation without nanocomposite was used as control. Filter paper activity of both the control and the nanocomposite mixed samples were investigated following the protocol mentioned earlier (cf. Section 3.4.3.2). Effect of incubation time (0-6 h) was also investigated by performing similar experiments with 1.0 mM nanocomposite at optimized temperature and results were calculated in terms of relative activity of enzyme (w.r.t. original extracted enzyme).

3.4.5 Enzymatic hydrolysis of pretreated cyanobacterial biomass

The extracted enzyme was further utilized for the hydrolysis of the selected pretreated cyanobacterial residue of *L. limnetica* and *O. obscura*. Flasks containing cyanobacterial biomass at 10% (w/v) loading were used for saccharification study with 0.1 M citrate buffer (pH 5.8±0.2) and Fe_3O_4/Alg nanocomposite treated enzyme incubated at 55°C at 120 rpm in a BOD incubator shaker for 48 h. Hydrolysis of biomass with commercially available celluclast (C2730, Sigma-Aldrich) and *L. limnetica* extracted enzyme (with high βG activity) was also done to compare the efficacy of extracted enzyme. Enzymatic hydrolysis of untreated biomass was used as control in both the cases. Sampling was done at every 3 h interval by collecting the small fraction of liquid from the flask and heating for 10 min in boiling water to inactivate the enzyme. The solution thus obtained was centrifuged at 7000 rpm for 7 min and the supernatant was collected and analyzed for the presence of reducing sugars using HPLC (Srivastava et al. 2015).

3.5 Butanol fermentation study

3.5.1 Preparation of inoculum

Freeze-dried culture of *Clostridium beijerinckii* ATCC 35702 was procured from American Type Culture Collection (ATCC), Manassas, USA. Spores were activated at 37°C for 48 h in Modified ATCC 1441 medium (Table 3.3) according to the protocol suggested by the ATCC in an anaerobic chamber. Anaerobic condition was maintained by using anaerobic sachet with the catalyst and an indicator to confirm the anaerobic environment (Figure 3.5). Appropriate volume of modified ATCC 1441 media was purged with oxygen free nitrogen gas for 5 min and then autoclaved at 121°C and 15 psi pressure for 15 min. The inoculum was prepared by transferring 5% (v/v) of the actively growing cells to growth media under aseptic condition. Initially inoculated media was incubated at 70±2ºC for 7 min for activation of spores after that the flask with inoculated media was transferred to a BOD incubator-shaker maintained at 37°C and 120 rpm. Nearly 16 h grown culture was used as the inoculum to inoculate the fermentative media (P-2 media).

Figure 3.5: Anaerobic inoculum preparation and maintenance

Subculturing of the *Clostridium beijerinckii* ATCC 35702 cells was done in 20-day interval and 30% glycerol stock was used to preserve the cells at -20ºC in deep freezer to maintain the viability of the microorganism. SEM analysis of the stored culture (at 4°C) and freshly prepared inoculum was done following the protocol mentioned earlier (cf. Section 3.2.1.1).

Table 3.3: ATCC 1441 growth media

Chemicals	**Concentration (g/L)**
Glucose	5.0
Yeast extract	10.0
KH_2PO_4	0.75
K_2HPO_4	0.75
$MgSO_4$	0.02
$MnSO_4 \cdot H_2O$	0.01
$FeSO_4 \cdot 7H_2O$	0.01
NaCl	1.0
L-Cysteine·HCl	0.5
Asparagine·H_2O	2.0
$(NH_4)_2SO_4$	2.0
pH	6.6 ± 0.05

3.5.2 Effect of various process parameters

Batch fermentation studies were conducted with glucose as C-source to optimize various process parameters such as initial inoculum concentration (5–15%), media pH (3-11), glucose concentration (30–90 g/L), yeast extract concentration (0–7.5 g/L) and $FeSO_4$ concentration (0-0.02 g/L) that largely affect the butanol production. These parameters were varied in a prescribed manner using a set of 100 mL serum bottles containing 80 mL of P-2

media (Table 3.4). Prepared media was firstly purged with oxygen free nitrogen gas for 10 min and then autoclaved at 121°C and 15 psi for 15 min. Actively growing cells were transferred to the serum bottles aseptically and anaerobic condition was maintained by filling the oxygen free nitrogen gas in the head space volume. Serum bottles were placed in BOD incubator with shaker at 120 rpm and 37ºC and sampling of gaseous (for cumulative gas production) and liquid phases (for solvent production, sugar consumption, bacterial growth, intermediate metabolites) was done after every 24 h interval with the monitoring of change in media pH also done. Fermentation experiments were performed with *C. acetobutylicum* MTCC 11274 and *C. acetobutylicum* NCIM 2877 cultures using glucose as C-source.

3.5.3 Preparation of cyanobacterial hydrolysate for fermentation

The hydrolysates of harvested cyanobacterial biomass of *L. limnetica* and *O. obscura* were prepared separately at pre-optimized biomass loading of 4% drybiomass (w/w). Mixture was initially treated with 1.63 M H_2SO_4 at 100ºC for 60 min followed by autoclaving at 121ºC and 15 psi for 45 min then centrifugation at 5000 rpm for 10 min, at 4ºC to get a clear supernatant. Initial pH of hydrolysate was adjusted to 5.6±0.2 with 2 M NaOH and it was again centrifuged at 5000 rpm for 10 min to separate the precipitate if any and the supernatant was stored at -20ºC for fermentation study.

Prepared hydrolysate was further utilized for fermentation study by supplementing the standard P-2 media composition (except glucose) and optimized yeast extract and iron concentration (5.0 g/L & 20.0 mg/L respectively). The initial pH of the prepared media was

Table 3.4: Fermentation media (P-2 media) composition

Chemicals	**Concentration (g/L)**
Glucose	30.0
Yeast extract	5.0
Ammonium acetate	2.2
KH_2PO_4	0.75
K_2HPO_4	0.75
$MgSO_4$	0.2
$MnSO_4 \cdot 7H_2O$	0.01
$FeSO_4 \cdot 7H_2O$	0.01
NaCl	1.0
L-Cysteine·HCl	0.5
p-amino benzoic acid	0.001
Biotin	0.01 mg/L

adjusted to 6.1±0.2 because at optimized glucose media pH (i.e. 5.0) cyanobacterial hydrolysate fermentation resulted in a lower concentration of butanol. Equal concentration of glucose supplemented media was used as control. The hydrolysate and glucose media thus obtained were purged with oxygen free nitrogen gas for 10 min and then autoclaved at 121ºC and 15 psi for 15 min. After cooling the serum bottles 10% (v/v) bacterial inoculums was transferred to each bottle under sterile condition. The anaerobic conditions within the bottles were maintained as mentioned earlier (cf. Section 3.5.2) and then these were incubated in orbital-shaker incubator with shaking of 120 rpm and 37ºC. Sampling for gaseous and liquid phase analysis was done after every 24 h interval. Further similar fermentation studies with both the cyanobacterial hydrolysate were performed by supplementing the media with 10.0 g/L of initial glucose concentration.

3.5.4 Analysis of end products and metabolites

Samples of broth were collected after definite time intervals and analyzed for bacterial growth spectrophotometrically at 600 nm and then centrifuged at 5000 rpm for 7 min and supernatant obtained was used for the analysis of fermentation products. Bacterial culture used for the fermentation study was a non-ethanol producing strain. Acetone and butanol produced during the fermentation process were analyzed using a gas chromatograph equipped with flame ionization detector (FID). Carbowax-20M (80-100 mess) column was used for the separation of acetone, butanol and internal standard (8 g/L isobutanol). Injector and detector temperatures were maintained at 220 and 230°C, respectively and oven temperature was set to increase from 100 to 210°C at the rate of 20°C/min. Nitrogen was used as the carrier gas at a flow rate of 40 mL/min, hydrogen and oxygen were set at a flow rates of 30 and 300 mL/min, respectively for igniting the flame. Quantitative estimation of unknown concentrations of acetone and butanol were done from the standard plot prepared separately in terms of ratio of the area for a known concentration of acetone (or butanol) to the area of isobutanol (8.0 g/L) versus concentration. Gaseous phase analysis was done using a thermal conductivity detector (TCD) and Porapak-Q column (60-80 mesh, 2Mx1.8”×2mm SS). Initial oven temperature was maintained at 100ºC and analysis was performed in isothermal mode with auxiliary and detector temperature at 150ºC. Nitrogen was used as carrier gas with flow rate of 40 mL/min.

Sugars were analyzed using an ultra-high-performance liquid chromatograph (UHPLC) equipped with RID. Different intermediate fermentative metabolites were detected using C-18 column (Serial no 010380) and UV detector at 294 nm. Degassed HPLC grade distilled

water was used as the mobile phase at a flow rate of 1.0 mL/min. For sugar analysis, the column oven temperature was maintained at 80ºC and RID at 50ºC while for metabolites oven temperature was set at 35°C. For the analysis of samples through UHPLC, centrifuged samples were diluted and then filtered through 0.22 μm nylon membrane (Randisc Nylon SF, India) directly into HPLC vials. Detection of the peaks was done by comparing with the standard peak retention times and concentration was calculated from the area of peaks for known standards.

3.5.5 Continuous stirred tank reactor fermentation

Biobutanol fermentation study was performed in a 1000 mL bioreactor with a working volume of 700 mL at the optimized conditions in batch mode (Figure 3.6). Experiments were performed with both glucose and cyanobacterial hydrolysate as C-source and cumulative gas production was measured by the water-displacement method. Media for each case was prepared as mentioned earlier (cf. Section 3.5.2 and 3.5.3). 10% (v/v) fresh inoculum of *Clostridium beijerinckii* ATCC 35702 was transferred to the bioreactor aseptically and oxygen free nitrogen gas was sparged in the head-space volume to maintain the anaerobic environment. Inoculated bioreactor was placed on a magnetic stirrer and was maintained at the temperature of 37±2ºC with continuous mixing of 80 rpm. Sampling was done after every 24 h interval and the analysis of the biomass growth, solvent concentration, sugars and metabolites concentration by following the protocol mentioned earlier (cf. Section 3.5.4).

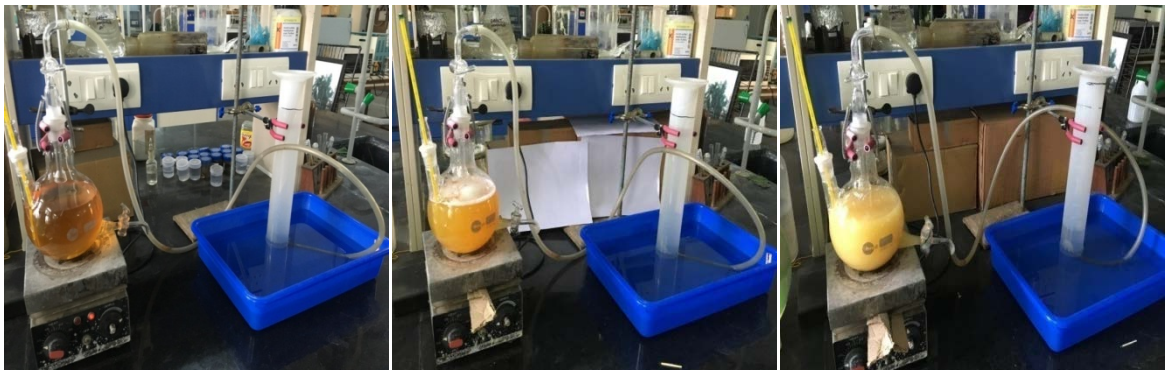

Figure 3.6: Continuous stirred tank reactor study for butanol fermentation

The correlation between various nutrients fed into the reactor and the products formed due to the consumption of nutrients and residual nutrients within the reactor after process completion was obtained by performing the mass balance in terms of C-balance using the proposed metabolic pathway. Mass balance within a closed system can be explained in a better way by understanding the Eq. 3.9.

Mass In = Mass Out + Mass Stored (3.9)

To evaluate various biokinetic parameters, a Monod type model proposed by Mercier was employed to the data obtained from the CSTR study. These models have been defined as kinetic model for product formation (Eq. 3.10), biomass growth (Eq. 3.11) and substrate consumption (Eq. 3.12) (Mercier et al. 1992). Models were fitted to the experimental data of butanol production, bacterial growth and sugar consumption using Matlab 9.2.0.538062 to estimate the biokinetic parameters involved in the butanol fermentation process (cf. Appendix).

$$P = \frac{P_{max}\ P_0 e^{P_t t}}{(P_{max} - P_0) + P_0 e^{P_t t}} \quad (3.10)$$

$$X = \frac{X_{max}\ X_0 e^{\mu t}}{(X_{max} - X_0) + X_0 e^{\mu t}} \quad (3.11)$$

$$S = S_0 - \frac{P - P_0}{Y_{P/S}} - \frac{X - X_0}{Y_{X/S}} \quad (3.12)$$

where, P = product concentration (g/L); P_0 = initial product concentration (g/L); P_{max} = maximum product concentration (g/L); P_t = kinetic constant; X = biomass absorbance (at 600 nm); X_{max} = maximum biomass concentration; X_0 = initial biomass concentration; μ = specific growth rate (h^{-1}); S = substrate concentration (g/L); S_0 = initial substrate concentration (g/L); $Y_{P/S}$ = product yield; $Y_{X/S}$ = biomass yield.

3.6 Recovery of butanol

Fermentation broth obtained after CSTR study was used for the recovery of butanol content produced during fermentation. In the present study liquid-liquid extraction and membrane-assisted solvent recovery techniques have been selected on the basis of their low energy consumption and higher solvent recovery efficiency (cf. Table 2.6).

3.6.1 Liquid-liquid extraction

On the basis of the distribution coefficient for butanol (Eq. 3.13), selectivity of butanol recovery (Eq. 3.14) and toxicity for *Clostridium* strain, three chemicals have been selected for the separation of butanol from fermentation broth. Selection parameters used are listed in Table 3.5.

$$\text{Distribution coefficient} = \frac{\text{mass fraction in organic phase}}{\text{mass fraction in aqoeous phase}} \quad (3.13)$$

$$\text{Selectivity} = \frac{\text{distribution coefficient of butanol}}{\text{distribution coefficient of water}} \quad (3.14)$$

Table 3.5: Solvent used in liquid-liquid extraction

Solvent	Toxicity	Selectivity	Distribution coefficient (kg/kg)
Hexane	Non-toxic	2700	0.5
Castor oil	Non-toxic	270	2.6
Oleyl alcohol	Non toxic	-	4

Source: Groot et al. (1990)

Fermentation broth containing acetone, butanol, organic acids and other media components was transferred to an extraction vessel and extractant was mixed in equal ratio (volume based, broth:extractant). The mixture was continuously stirred by using a magnetic stirrer at 120 rpm at 35ºC and sample was collected at different time interval for GC analysis by following the protocol mentioned earlier (cf. Section 3.5.4) to calculate the amount of butanol recovered. After the completion of extraction process, mixture was transferred to a separating funnel for the separation of organic and aqueous phases. Organic phase was collected from the top of the funnel and subjected to analysis of butanol to calculate the percentage recovery (Eq. 3.15) from fermentation broth.

$$\text{Percentage butanol recovered} = \frac{\text{Amount butanol recovered}}{\text{Initial butanol concentration}} \times 100 \qquad (3.15)$$

3.6.2 Perstraction/Membrane-assisted solvent extraction

Self-designed membrane-assisted solvent extraction device was used for the recovery of butanol from fermnetation broth (Figure 3.7). Various components of the separation unit are discussed below.

1) **Reaction and separation vessels:** Fermentation broth containing solvents was filled in the reaction vessel. Magnetic stirrer is used at the bottom of both the vessels to maintain

uniformity of liquid mixture. Separation vessel consists of a conical flask for storage of extractant and a tubular section for holding the membrane and to pass feed solution. Two lines are attached to this tubular section one to carry feed solution and other to take out the reflux. To maintain the liquid level thereby pressure on the upstream side of the membrane a by-pass is also provided after peristaltic pump.

2) **Temperature Bath:** The temperature bath consisted of two thermostats that are used to achieve the reaction temperature (maximum 100°C) and one submerged pump to make the uniform temperature throughout the system by circulating the water within the temperature bath.

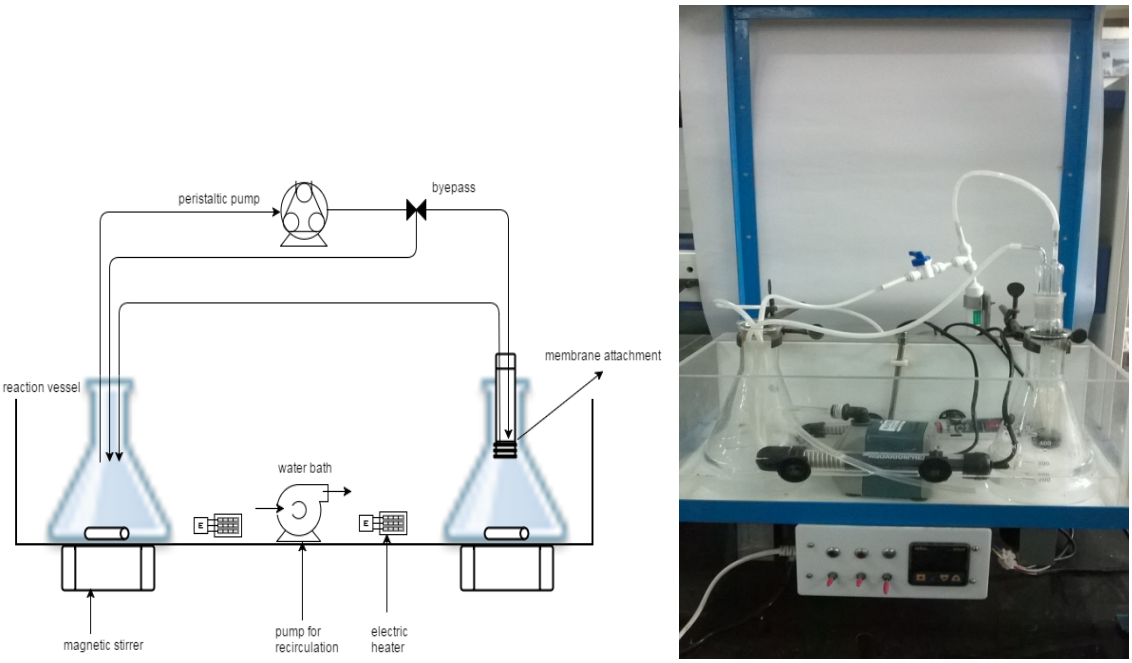

Figure 3.7: Membrane-assisted solvent extraction device

Various membranes (silicon, nitrile, natural rubber (latex), polypropylene) in combination with different extactants (hexane, oleyl alcohol) have been used to recover butanol. For an

efficient separation the membrane should possess a permselective property that can permit permeation of the desired solvent preferentially without allowing other media constituents. Selection of extractants has been done on the basis of their distribution coefficient and selectivity for butanol. From Table 3.5, it can be seen that hexane possess high selectivity for butanol while oleyl alcohol is characterized with high distribution coefficient.

Initial experiments were performed with an aqueous butanol solution (20 g/L) to get an idea of mass transfer through these membranes. Solution was pumped through the peristaltic pump and passed to the tubing attached to separation vessel. The inlet and reflux flow rates of the aqueous phase were found as 152 mL/min and 251 mL/min, respectively, and the by-pass can be controlled to the maximum rate of 153 mL/min to avoid overflow from the tubing. Experiments were performed until an equilibrium phase was reached, it was different for different membranes and extractant combinations. Initial sampling was done at every 2 h interval but after reaching the saturation stage the extractant sampling time was increased to 4 h. Samples were prepared and analyzed by using the protocol mentioned earlier (cf. Section 3.5.4). After optimization of the membrane and extractant combination, best combination was used for the recovery of butanol from glucose as well as cyanobacterial hydrolysate fermented media and percentage recovery was calculated using Eq. 3.11.