Preamble

This chapter presents and discusses the results obtained for each step of butanol production and recovery. The growth of cyanobacterial strains of *Lyngbya limnetica* and *Oscillatoria obscura* selected from local water bodies was optimized for enhanced carbohydrate content that was subjected to further pretreatment to maximize the sugar release. Discussion on parameters optimization for butanol fermentation with glucose media and further cyanobacterial sugar fermentation at optimized conditions to get large butanol yield has been made. A continuous stirred tank fermentation was used to obtain butanol production. The shake-flask and constant stirred tank fermentation results are compared with a view to obtain some useful information for scale-up purpose. Results of mass balance, kinetic modeling and butanol recovery are also discussed to get a clear idea of the overall production process and its efficiency.

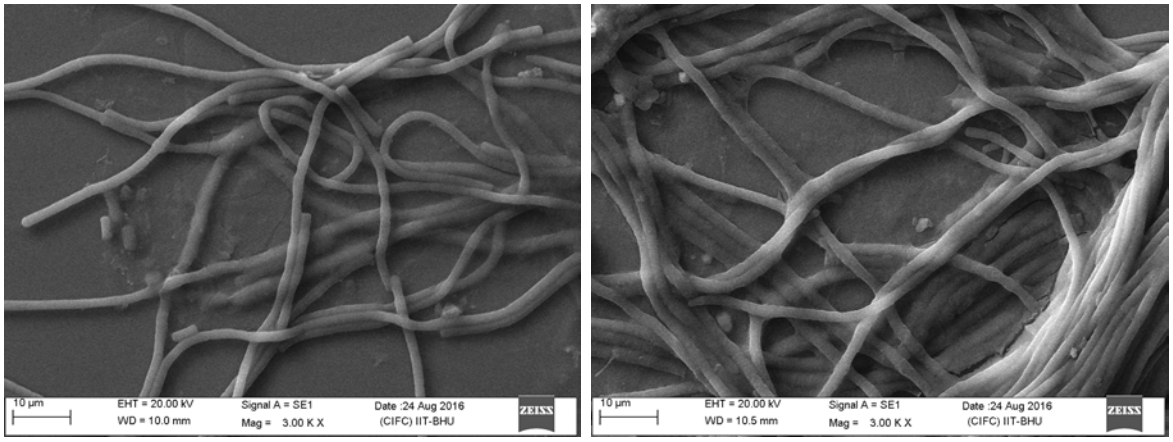
4.1 Biomass selection

4.1.1 Identification and characterization of collected biomass

Collected biomass was identified microscopically by the Botanical Survey of India, Ministry of Environment & Forests, Kolkata, India and was found to be cyanobacterial strains of *Lyngbya limnetica*, *Lyngbya subtilis*, *Oscillatoria obscura*, and consortium of *Chlorella vulgaris* and *Oscillatoria subbrevis*. Characterization of the dried cyanobacterial biomass was done in terms of bulk density, solid density, moisture content, ash content, fixed carbon content and volatile matter content (Table 4.1) to select the biomass suitable for further studies. Excess moisture content reduces the effectiveness of the pretreatment agent whereas high ash content may act as a deterrent to hydrolysis due to possible toxicity of accompanying metallic elements (Wychen and Laurens 2013b). According to the NREL protocol biomass to be used for pretreatment purpose to extract soluble carbohydrate content should possess ≤10% (dry basis) of ash and moisture contents (Wychen and Laurens 2013b). From Table 4.1, it is seen that the moisture content of all the four collected biomass is within the acceptable range (for *L. limnetica* and *O. obscura* it is well within this limit). The ash contents of *L. limnetica* and *O. obscura* are 11.7 and 13.61%, respectively, which are slightly higher than the suggested limit while that of *L. subtilis* and consortia of *C. vulgaris* and *O. subbrevis* are beyond the acceptable range. Therefore, on the basis of these results *L. limnetica* and *O. obscura* have been selected for further studies. The SEM analysis of the selected biomass was done to confirm the microscope identification result. A single type of filamentous structure (Figure 4.1a, b) also confirms the results obtained from the Botanical Survey of India, Kolkata.

Table 4.1: Characterization of dried cyanobacterial biomass

Parameter	***L. limnetica***	***L. subtilis***	***O. obscura***	**Consortia**
Bulk density (Kg/m^3)	392.8	438.2	373.8	509.2
Solid density (Kg/m^3)	1952	1709	2019	1686
Moisture content (%)	3.544	11.87	5.963	13.48
Ash content (%)	11.7	23.55	13.61	30.83
Volatile matter content (%)	32.93	28.22	29.19	22.89
Fixed carbon content (%)	51.83	36.36	51.24	32.80



(a) **(b)**

Figure 4.1: SEM images of cyanobacterial strains: **(a)** *L. limnetica*, **(b)** *O. obscura*

4.1.2 Batch growth of cyanobacterial biomass

Growth pattern of both the selected cyanobacterial strains was studied in terms of their drybiomass and carbohydrate content (Figure 4.2). It is seen that for both the strains the adaptation phase exists up to 4 days, it is then followed by a sharp biomass growth up to 14 days with stationary phase starting after 16^{th} day. During the exponential growth phase, drybiomass has increased from 0.226 to 1.232 g/L for *L. limnetica* and from 0.294 to 1.206 g/L for *O. obscura*. While the total carbohydrate content has decreased continuously from 0.316 to 0.201 g/g of drybiomass (g/g db) for *L. limnetica* and from 0.279 to 0.179 g/g db

for *O. obscura* during 20 days of incubation period. Mollers et al. (2014) has also reported reduction in the carbohydrate concentration (0.6 to 0.4 g/g db) and increase in drybiomass concentration (0.9 to 3.7 g/L) in *Synechococcus* sp. The possible reason for this could be the lower accumulation of carbohydrate and higher for other components such as lipids and proteins. Kim et al. (2016) reported a larger biomass growth under nitrogen-replete conditions with lower carbohydrate and higher lipid and protein concentrations.

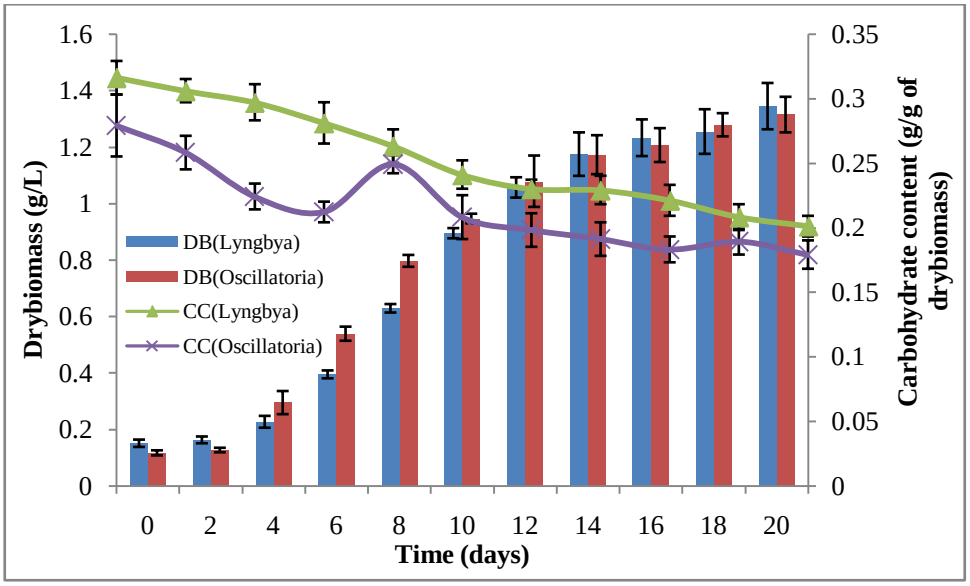


Figure 4.2: Growth pattern of cyanobacterial strains. In all cases inoculum size, incubation period, incubation temperature, media pH, light intensity, and volume of media are kept constant at 0.2 g, 20 days, 25±3°C, 9.0, 49.0 µmolm^{-2}s^{-1} and 50 mL, respectively

4.1.3 Effect of different operating parameters

4.1.3.1 Effect of shaking

Effect of shaking on the growth of the strains has been examined under continuous shaking and static conditions. Growth was estimated in terms of drybiomass and carbohydrate content after 20 days of incubation period. The maximum drybiomass was found to be 1.283 and 1.216 g/L with shaking and 0.649 and 0.563 g/L without shaking for *L. limnetica* and *O.*

obscura, respectively. The carbohydrate content under shaking condition has been found as 0.219 and 0.192 g/g db and as 0.179 and 0.151 g/g db under static condition for *L. limnetica* and *O. obscura*, respectively. Kirrolia et al. (2012) used green microalgae *Chlorococcum* sp. and observed a decrease in carbohydrate under static mode and attributed it to the non-homogenous mixing of nutrients and deposition of biomass at the bottom. The specific growth rate under static condition has been found to be 0.073 d^{-1} for *L. limnetica* and 0.079 d^{-1} for *O. obscura*, whereas under shaking condition it has been found as 0.107 d^{-1} and 0.117 d^{-1}, respectively (Table 4.2).

Table 4.2: Specific growth rate at different operating parameters

	Parameters	**Range**	**Strains**	
			L. limnetica	***O. obscura***
Specific growth rate (day^{-1})	**Shaking (rpm)**	**0**	0.073	0.079
		150	0.107	0.117
	Media pH	**3**	0.004	0.007
		5	0.027	0.029
		7	0.065	0.098
		9	0.104	0.118
		11	0.013	0.028
	Incubation temperature (°C)	**10**	-0.017	-0.031
		20	0.103	0.117
		30	0.094	0.109
		40	0.074	0.080
	Light intensity ($\mu molm^{-2}s^{-1}$)	**14**	0.055	0.047
		27	0.066	0.082
		41	0.075	0.095
		54	0.106	0.118
		68	0.109	0.122
		81	0.095	0.091

4.1.3.2 Effect of media pH

Effect of pH on the cyanobacterial growth was studied by varying the pH from 3 to 11. At pH 3, the growth of biomass in terms of drybiomass and total carbohydrate content has been found to be the lowest, as 0.162 g/L and 0.113 g/g db and 0.134 g/L and 0.116 g/g db for *L. limnetica* and *O. obscura*, respectively, during the 20 days incubation period (Figure 4.3). The maximum growth was observed at pH 9 with drybiomass and carbohydrate content of 1.196 g/L, 0.219 g/g db and 1.226 g/L, 0.192 g/g db, respectively. The limited availability of carbon from CO_2 at both higher and lower pH as well as the lower affinity of biomass for free CO_2 result in the reduced photosynthetic efficiency (Rotatore and Colman 1991). This could be the possible reason for slow growth of biomass at pH 3 and 11 that resulted in reduced drybiomass and carbohydrate contents.

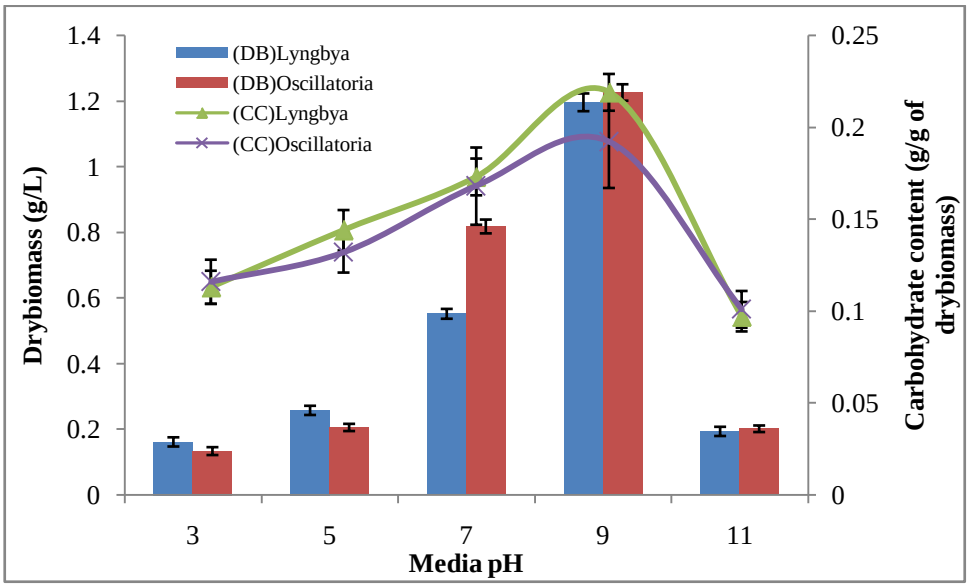


Figure 4.3: Effect of pH on growth of cyanobacterial strains. Inoculum size: 0.2 g, incubation period: 20 days, incubation temperature: 25±3°C, light intensity: 49 µmolm^{-2}s^{-1}, and media volume: 50 mL

A slightly alkaline media pH (i.e. pH 9) favors the growth and results in the highest specific growth rates of 0.104 and 0.118 d^{-1} for *L. limnetica* and *O. obscura*, respectively (Table 4.2). Several earlier studies with different cyanobacterial strains such as *Nannochloropsis salina* (Bartley et al. 2014), *Halomicronema hongdechloris* (Li et al. 2014b) and *Synechococcus* PCC 7002 (Silva et al. 2017) have also reported similar results.

4.1.3.3 Effect of incubation temperature

The effect of temperature on the growth of two cyanobacterial strains was studied in the temperature range of 10 to 40°C in terms of drybiomass and carbohydrate content. These results are shown in Figure 4.4. Optimum growth temperature for both the strains has been found to be 20°C and the maximum drybiomass and carbohydrate contents are obtained as 1.186 g/L and 0.255 g/g db for *L. limnetica* and 1.216 g/L and 0.201 g/g db for *O. obscura*. The specific growth rate for two strains increased dramatically on increasing the incubation temperature up to the optimum value after that it decreased. The highest values were found to be 0.103 and 0.117 d^{-1} for *L. limnetica* and *O. obscura*, respectively. Mackey et al. (2013) studied the effect of temperature (15-27°C) on the growth and photosynthetic yield of diverse strains of *Synechococcus* sp. and found 21-25°C as the optimal temperature range that favored the growth as well as carbohydrate accumulation. At the optimum temperature the carbon and nitrogen utilization capacity of cells increases significantly hence the growth rate (Converti et al. 2009). Lower temperature reduces the rate of CO_2 fixation resulting in reduced electron transport (Demeter et al. 1995) and higher temperature affects the cellular physiology and causes denaturation of essential metabolites (enzymes/proteins) resulting in lower CO_2 fixation (Kumar and Das 2014). Ho et al. (2012) showed that the rate of CO_2

fixation directly affected the carbohydrate content in *Scenedesmus obliquus*. This could also be the possible reason for the lower carbohydrate concentrations obtained at temperatures above and below the optimum for the two cyanobacterial strains studied in this work.

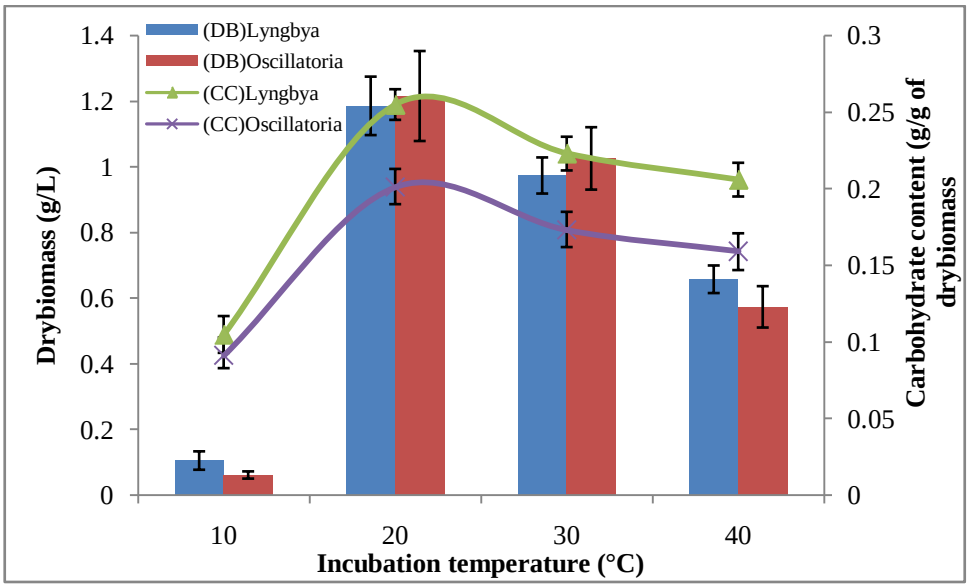


Figure 4.4: Effect of incubation temperature on growth of cyanobacterial strains. Inoculum size: 0.2 g, incubation period: 20 days, media pH: 9.0, light intensity: 49.0 µmolm^{-2}s^{-1}, and media volume: 50 mL

4.1.3.4 Effect of light intensity

Effect of light intensity (14–81 µmolm^{-2}s^{-1}) on the growth of isolated cyanobacterial strains and carbohydrate content has also been examined and the results are shown in Figure 4.5. From this figure, it is evident that with increase in the light intensity from 14 to 68 µmolm^{-2}s^{-1}, the drybiomass and carbohydrate content increased from 0.448 to 1.34 g/L and 0.138 to 0.226 g/g db, respectively for *L. limnetica* and from 0.298 to 1.32 g/L and 0.146 to 0.197 g/g db, respectively for *O. obscura*. At 81.0 µmolm^{-2}s^{-1}, a sharp decrease in biomass as well as carbohydrate content was obtained. *Synechococcus* sp. is the extensively utilized

cyanobacteria for bioalcohol production, and the growth optimization study of this strain has revealed that the light intensity in the range of 200-250 µmolm^{-2}s^{-1} is the saturation intensity required for large biomass growth and carbohydrate accumulation, and it varies from species to species and becomes independent at the end phase of the cell cycle (Chow et al. 2015; Dyble et al. 2006; Möllers et al. 2014; Vitova et al. 2015). Carneiro et al. (2009) reported that the growth of *Cylindrospermopsis raciborskii* was low below the saturation intensity (i.e., 100 µmolm^{-2}s^{-1}) and was unaffected above it. Based on the present results, 68 µmolm^{-2}s^{-1} can be considered as the saturation intensity for the selected strains. The lower carbohydrate content at 81.0 µmolm^{-2}s^{-1} may be attributed to the photo-degradation of metabolic components resulting in suppression of the growth of biomass as well as accumulation of different metabolites (Singh and Singh 2015).

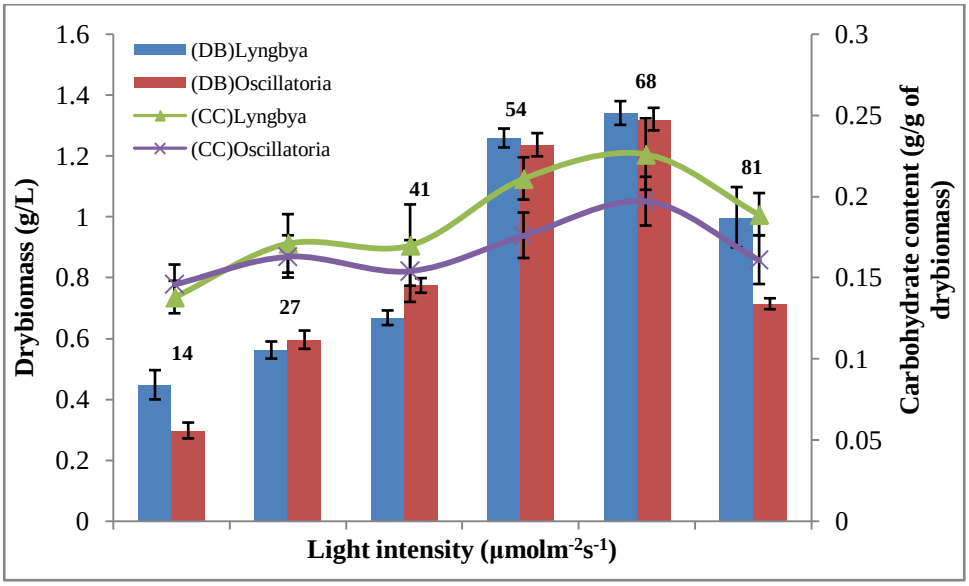


Figure 4.5: Effect of light intensity on growth of cyanobacterial strains. Inoculum size: 0.2 g, incubation period: 20 days, incubation temperature: 20±3°C, media pH: 9.0, and media volume: 50 mL (**Note:** Intensity values are shown as numbers above bars)

The specific growth rates of *L. limnetica* and *O. obscura* increased with increasing light intensity and were the highest (0.109 and 0.122 d^{-1}, respectively) at 68.0 $\mu molm^{-2}s^{-1}$ and then decreased (0.095 and 0.091 d^{-1}, respectively) at the higher intensity (81.0 $\mu molm^{-2}s^{-1}$). This is in confirmation with the results reported by Khajepour et al. (2015). The higher light intensity causes photo-inhibition by disrupting the chloroplast and inactivating the enzymes required for CO_2 fixation and the resultant slower growth rate (Khajepour et al. 2015).

4.1.4 Effect of nutrient limitation on cyanobacteria growth

4.1.4.1 Effect of trace elements concentration

The growth and carbohydrate accumulation in *L. limnetica* and *O. obscura* were evaluated separately in the presence and absence of trace elements. Drybiomass concentration (during the exponential growth phase) increased significantly from 0.162 to 1.334 g/L for *L. limnetica* and from 0.126 to 1.278 g/L for *O. obscura* in presence of trace elements, while in the absence it resulted in a maximum of 0.578 and 0.518 g/L, respectively (Figure 4.6). Large biomass growth and suppressed carbohydrate contents were obtained in the presence of trace elements in both the cyanobacterial strains. Dammak et al. (2017) studied the effect of trace elements on the growth of *Tetraselmis* sp. and proved that trace elements are essential for the activities of photosystems I and II and support the biomass growth and resultant lower carbohydrate concentration. These results thus confirm that the presence of trace elements for the growth of biomass in large amount is important.

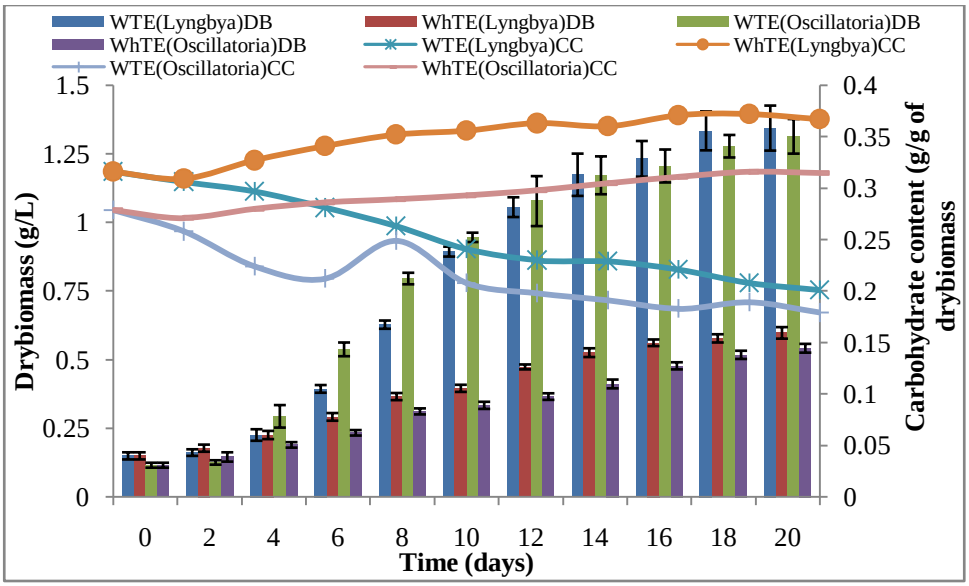


Figure 4.6: Effect of trace elements on growth of cyanobacterial strains. Inoculum size: 0.2 g, incubation period: 20 days, incubation temperature: 20±3°C, media pH: 9.0, light intensity: 68.0 µmolm^{-2}s^{-1}, and media volume: 50 mL

4.1.4.2 Effect of nitrogen concentration

Figure 4.7 shows the qualitative effect of nitrogen supplementation on the appearance and the apparent density of cyanobacterial biomass. Under N-starvation condition, slow growth (in terms of drybiomass) was observed and was found to be maximum as 1.344 and 1.314 g/L for *L. limnetica* and *O. obscura*, respectively, when inoculated in 1.5 g/L of $NaNO_3 \cdot 4H_2O$ containing media (Figure 4.8a, b). Further increase in $NaNO_3$ concentration to 2.0 g/L reduced the growth of biomass for both the strains. Depraetere et al. (2015) reported induced carbohydrate accumulation within the biomass of *Arthrospira* sp. PCC 8005 under N-deficient conditions and explained it on the basis of conversion of metabolites under stressed condition. A similar carbohydrate accumulation behavior was observed in the present study with a maximum concentration of 0.66 g/g db for *L. limnetica* and 0.621 g/g db for *O. obscura*. As the concentration of $NaNO_3$ increased, carbohydrate content

decreased sharply in both the cases and it is in confirmation with the results obtained by Möllers et al. (2014). This may be due to the lower carbohydrate yield with biomass and shift of the metabolism towards the synthesis of lipids and proteins (Kim et al. 2016).

Monitoring of pH during growth studies indicated that there was no change in the media pH during N-starvation condition while it changed from 9.0 to 10.0±0.3 in 20 days of incubation period when both the strains were cultured in N-rich media. This could be another reason for lower carbohydrate accumulation in both the strains, under alkaline pH. Lower carbohydrate accumulation in marine diatom *Skeletonema costatum* was also reported by Taraldsvik and Myklestad (2000).

(a) **(b)**

Figure 4.7: Effect of N-rich and N-starvation conditions on biomass density and appearance: **(a)** *L. limnetica*, **(b)** *O. obscura*

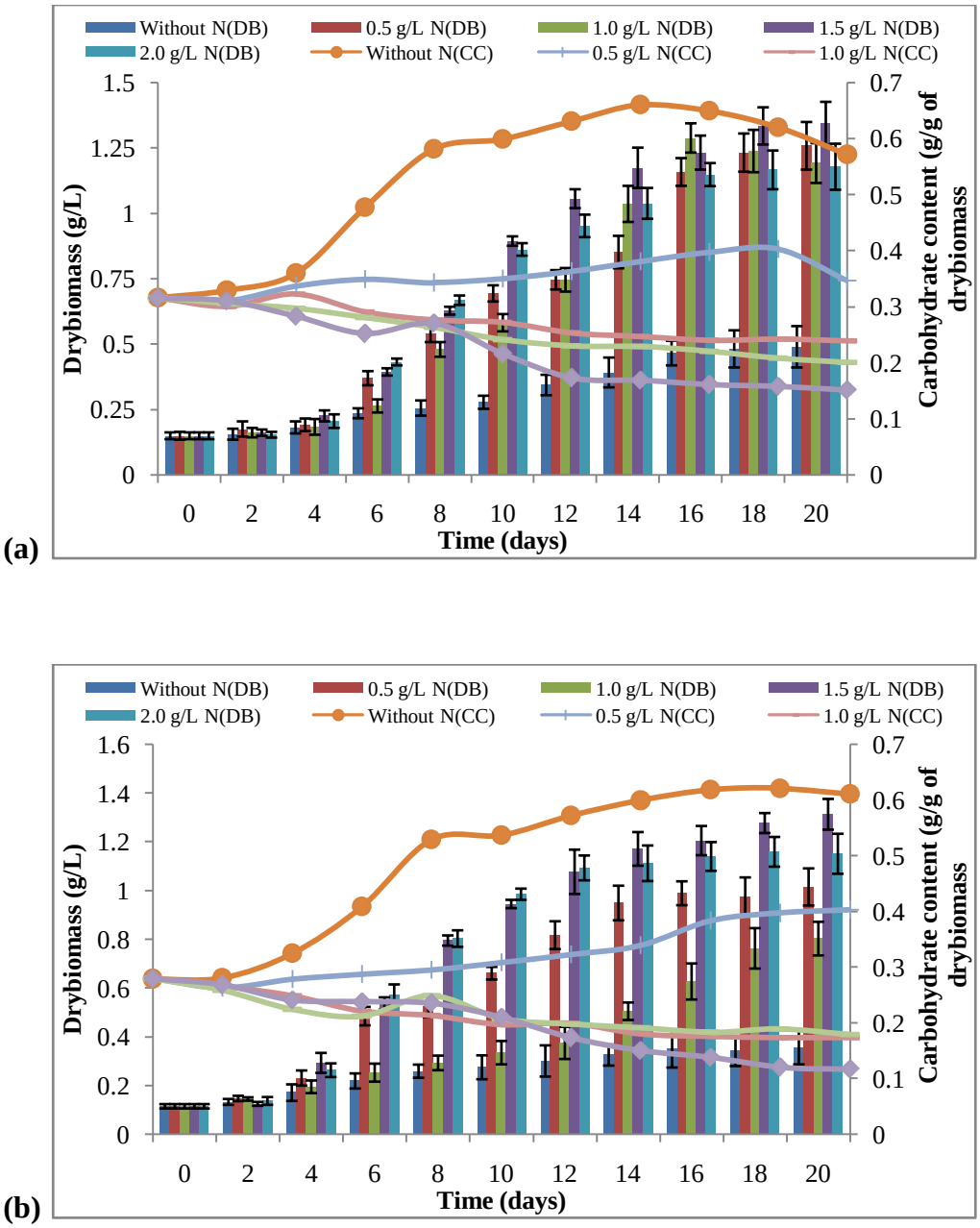


Figure 4.8: Effect of nitrogen concentration on the growth of cyanobacterial strains: **(a)** *L. limnetica*, **(b)** *O. obscura*; Inoculum size: 0.2 g, incubation period: 20 days, incubation temperature: 20±3°C, media pH: 9.0, light intensity: 68.0 µmolm^{-2}s^{-1}, and media volume: 50 mL

4.1.4.3 Effect of phosphorus concentration

Effect of P-starvation condition on growth was studied by examining the growth of two strains in presence (as per the standard media composition) and absence of phosphorus. Results of these experiments are shown in Figure 4.9. Under P-starvation condition, growth in terms of drybiomass have been found as 0.612 g/L for *L. limnetica* and 0.608 g/L for *O. obscura* after 20 days of incubation, but are better than those under the N-starvation condition. The carbohydrate accumulation on the other hand has been found to be lower, with maximum values being 0.445 and 0.404 g/g db for the two strains, respectively, under the studied condition. This confirms that effect of nitrogen on the growth and carbohydrate accumulation is more pronounced than for phosphorus. This may be due to large conversion of proteins and peptides to carbohydrates under N-starvation condition (Ho et al. 2012; Sun et al. 2014).

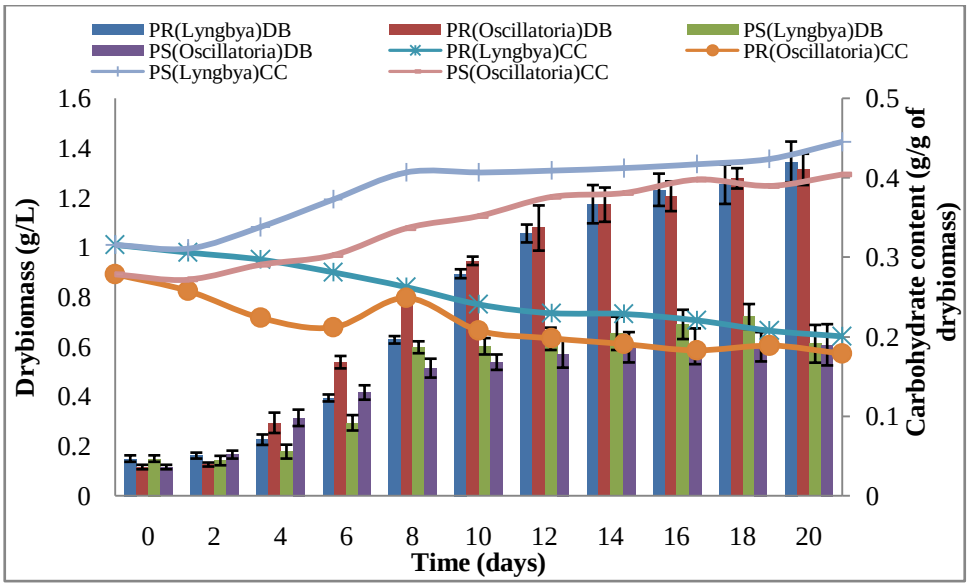


Figure 4.9: Effect of phosphorus concentration on cyanobacterial growth. Inoculum size: 0.2 g, incubation period: 20 days, incubation temperature: 20±3°C, media pH: 9.0, light intensity: 68.0 µmolm^{-2}s^{-1}, and media volume: 50 mL

Comparison of specific growth rates under various nutrient limiting conditions has revealed that the absence of nitrogen and trace elements affect the growth rates appreciably while phosphorus has only a moderate effect (Table 4.3). The highest specific growth rate observed for *L. limnetica* is 0.187 d^{-1} and for *O. obscura* it is 0.215 d^{-1} under N-rich conditions. Under N-starvation conditions, it decreased to 0.078 d^{-1} for *L. limnetica* and 0.074 d^{-1} for *O. obscura*. From these results, it is clear that *O. obscura* is more sensitive to nitrogen availability.

Table 4.3: Specific growth rate of cyanobacterial strains under nutrient limiting conditions

S. No.	Strains	Specific growth rate (day^{-1})			
		N-rich	N-starvation	P-starvation	TE-starvation
1.	***L. limnetica***	0.187	0.078	0.113	0.067
2.	***O. obscura***	0.215	0.074	0.107	0.073

Table 4.4: Compositional analysis of cyanobacterial biomass (as % dry cell weight)

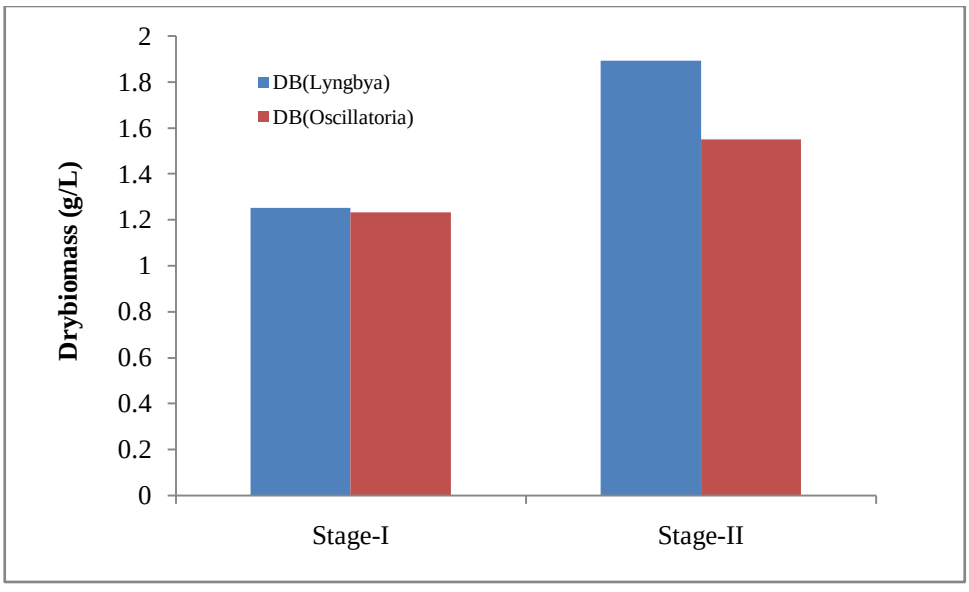
		Carbohydrate	Lipid	Protein	Ash
L. limnetica	**N-rich**	20.1	5.92	7.71	11.7
	N-starvation	66.0	7.18	2.48	7.219
O. obscura	**N-rich**	17.9	4.02	9.03	13.61
	N-starvation	62.1	6.64	1.93	9.451

Table 4.4 shows the change in various components of selected cyanobacterial strains under N-rich and N-starvation condition on 20th day of growth experiment.

4.1.5 Two-stage growth study

Growth studies during Stage-I and Stage-II were carried out by estimating the drybiomass and carbohydrate contents for both the strains (Figure 4.10a, b). Maximum biomass growths of 1.252 and 1.231 g/L were achieved for *L. limnetica* and *O. obscura*, respectively in 16 days of incubation period during Stage-I. After this, the recovered cells were used for Stage-II growth studies keeping other parameters same as in Stage-I. Due to the unavailability of the N-source, slower biomass growth has been observed during Stage-II while a remarkable increase in carbohydrate content has been found in 20 days of incubation period for both the strains. Maximum drybiomass and carbohydrate contents of 1.892 g/L and 0.691 g/g db for *L. limnetica* and 1.548 g/L and 0.652 g/g db for *O. obscura*, respectively were obtained. From Figure 4.10a and b, it is clear that carbohydrate accumulation rates in both of the strains under N-starvation conditions are much faster than the biomass growth as explained earlier (cf. Section 4.1.4) and is also in agreement with the results reported by Dragone et al. (2011).

(a)



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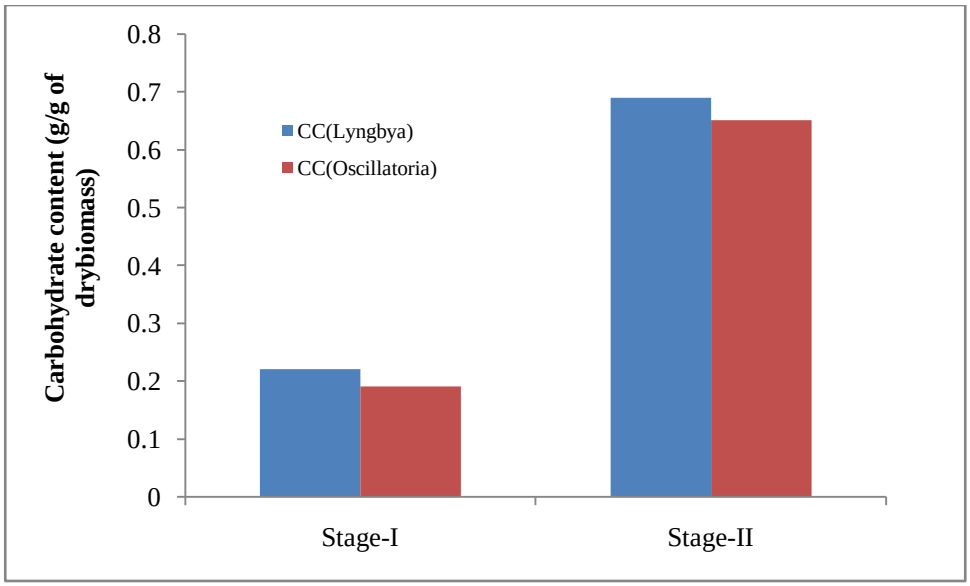


Figure 4.10: Two-stage growth study of *L. limnetica* and *O. obscura* biomass: **(a)** Drybiomass, **(b)** Carbohydrate content; Stage-I: Nitrogen-rich; Stage-II: Nitrogen-starved

The maximum carbohydrate productivity of 0.423 $gL^{-1}d^{-1}$ is obtained from *L. limnetica* which is comparable to that reported by other workers for different cyanobacterial strains (Table 4.5).

4.2 Biomass Pretreatment

4.2.1 Batch pretreatment

All the four collected biomass were tested for sugar release at different temperatures and time, and the results thus obtained are shown in Figure 4.11a and b. From Figure 4.11a, it is clear that with increase in temperature sugar release increases gradually in all the cases, and the maximum 0.059 g/g of sugar was obtained from *L. limnetica* at 100°C within 60 min of treatment time. Whereas, minimum 0.014 g/g db of sugar was found in the case of consortium of *C. vulgaris* and *O. subbrevis* at 40°C within 60 min that increased up to 0.036 g/g db at 100°C. Less carbohydrate accumulation during growth or reduced cell wall

destruction during the pretreatment could be the possible reason of lower sugar release from the biomass of *L. subtilis*, *O. obscura*, and consortium of *C. vulgaris* & *O. subbrevis*.

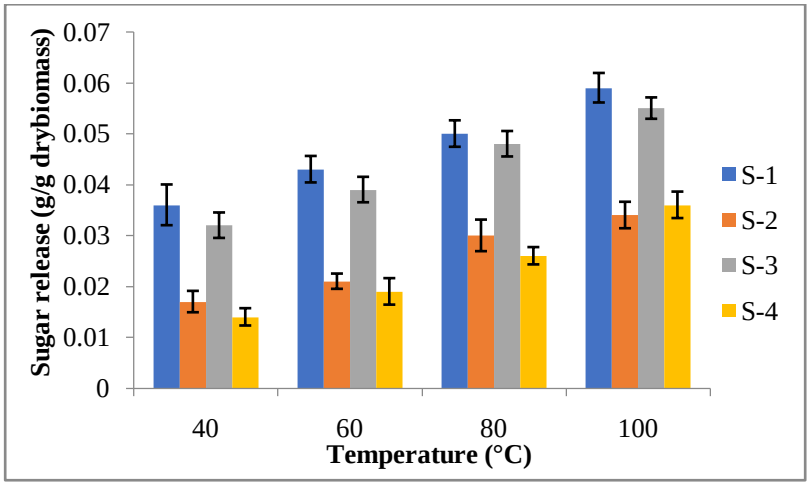
Table 4.5: Biomass and carbohydrate productivity of various cyanobacterial strains

Cyanobacterial strains	**Biomass productivity ($gL^{-1}d^{-1}$)**	**Carbohydrate productivity ($gL^{-1}d^{-1}$)**	**References**
Arthrospira platensis	0.277	0.212 (glycogen)	Hasunuma et al. (2013)
Synechococcus sp. PCC 7002	1.25	0.5 (glycogen)	Aikawa et al. (2014)
Synechocystis sp. PCC6803	0.05	0.112	Hasunuma et al. (2013)
Synechococcus elongatus PCC7942	0.953	0.144	Chow et al. (2015)
Synechococcus elongatus PCC7942 ieAB	1.239	0.564	Chow et al. (2015)
Aphanizomenon ovalisporum	0.1	NA	Mendez et al. (2015)
Anabaena plactonica	0.12	NA	Mendez et al. (2015)
Borzia trilocularis	0.1	NA	Mendez et al. (2015)
Lyngbya limnetica	0.088	0.423	Present study
Oscillatoria obscura	0.078	0.351	Present study

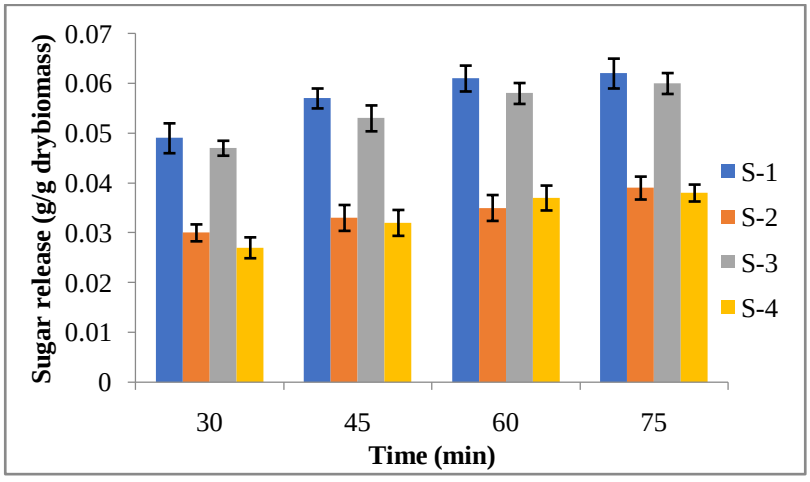
Source: Chow et al. (2015); NA: not available

Effect of treatment time on sugar release has been studied by keeping the temperature constant at 100°C and varying the time from 30 to 75 min (Figure 4.11b). The maximum sugar obtained was 0.062 g/g db from *L. limnetica* in 75 min at 100°C, whereas that in 60 min was 0.061. As the sugar release in 60 and 75 min of treatment time are nearly similar

for *L. limnetica*, the optimum time has been taken as 60 min. It can be attributed to nearly complete bond breakage of the complex polymeric carbohydrate structure during first hour of treatment itself. No significant increase in sugar release has been observed in the case of other studied biomass also by increasing treatment time from 60 to 75 min. Sugar releases of 0.035, 0.058, and 0.037 g/g db were obtained from *L. subtilis*, *O. obscura*, and consortium of *C. vulgaris* and *O. subbrevis*, respectively, after 60 min of pretreatment.



(a)



(b)

(c)

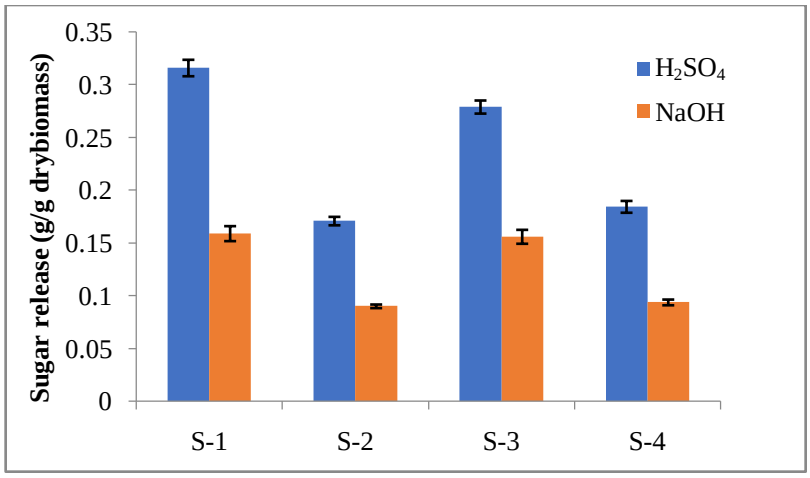


Figure 4.11: Effect of operating parameters on sugar release from different strains: **(a)** Pretreatment temperature, **(b)** Pretreatment time, **(c)** Pretreatment agents;
(S-1: *Lyngbya limnetica*; S-2: *Lyngbya subtilis*; S-3: *Oscillatoria obscura*; S-4: *Chlorella vulgaris* & *Oscillatoria subbrevis*)

The efficacies of H_2SO_4 and NaOH (each of strength 1.0 M) as pretreatment agents were evaluated with different strains as shown in Figure 4.11c. As microalgal/cyanobacterial biomass contains very little amount of lignin and only dilute acid pretreatment at an appropriate temperature is sufficient for the recovery of high soluble sugars (Davis et al. 2014). It can be seen from Figure 4.11c, that acid treatment is more efficient compared to alkali for all the biomass. Sugar release of 0.316 g/g db was found for *L. limnetica* when it was treated with H_2SO_4 at 100°C for 60 min, whereas only 0.159 g/g of sugar was obtained when treated with NaOH. Similar effect of acid treatment has also been observed in case of other strains. Castro et al. (2015) have also used acid hydrolysis along with increasing temperature for the treatment of wastewater microalgae. In this case, maximum sugar yield was 0.174 g/g of dry algae with 1.5 M H_2SO_4 at 80-90°C in 120 min.

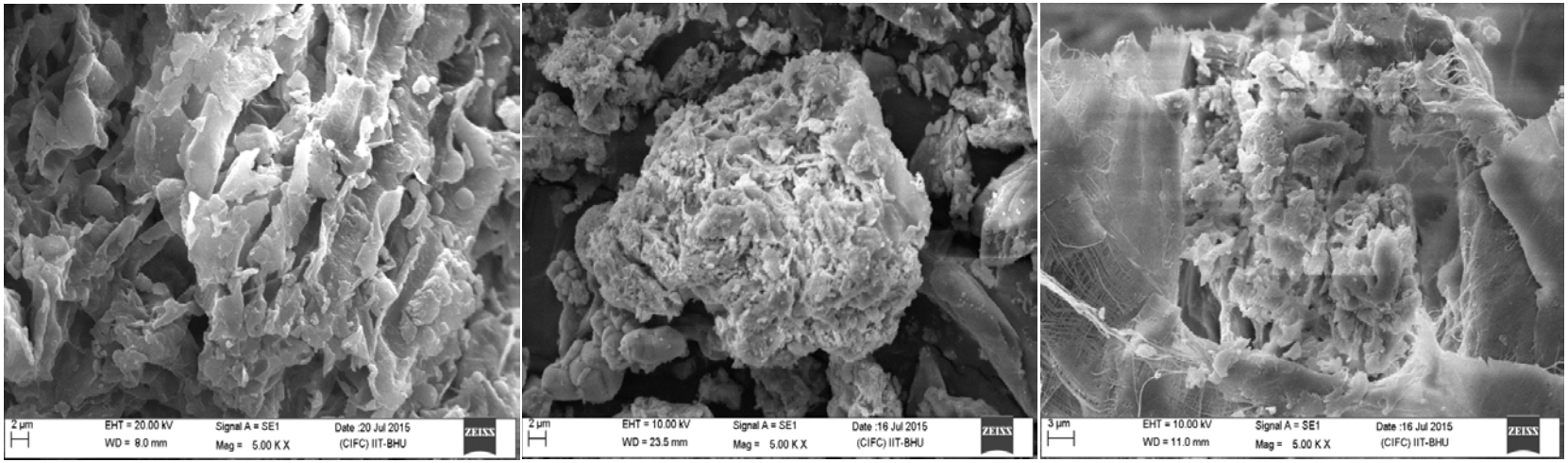
4.2.2 Biomass characterization

4.2.2.1 Scanning Electron Microscopy

The SEM image of dry untreated biomass of *L. limnetica* is shown in Figure 4.12a, whereas alkali (NaOH) and acid (H_2SO_4) treated images are shown in Figure 4.12b and 4.12c, respectively. It can be clearly inferred from these SEM images that acid pretreatment is more effective (cell thinning and large cell damage) compared to alkali treatment and this confirms its suitability for higher sugar yield.

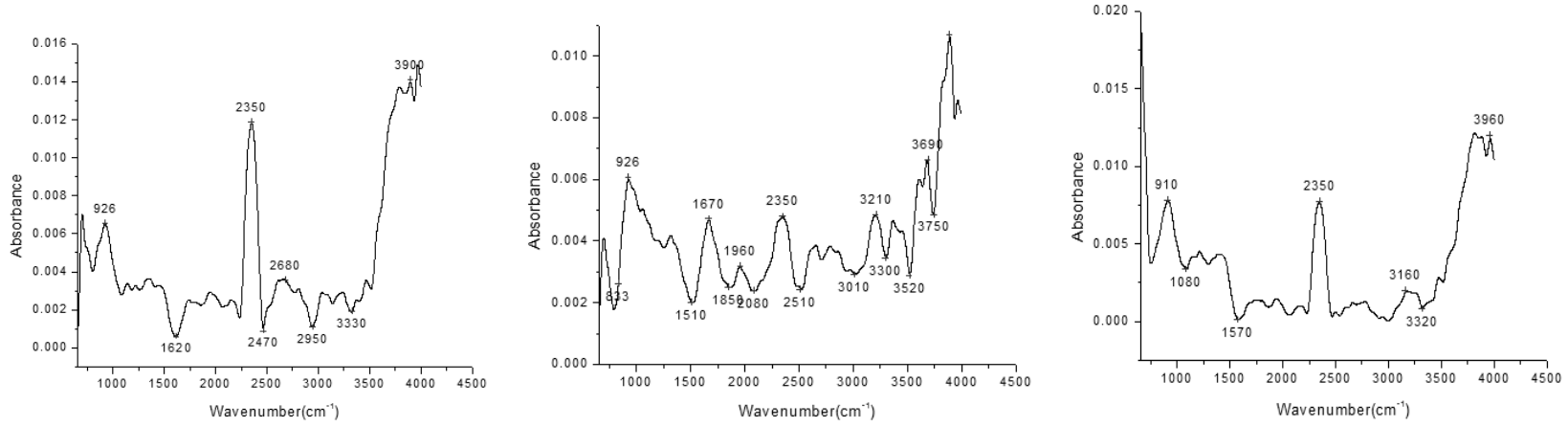
4.2.2.2 Fourier Transform Infrared spectroscopy

The FTIR spectroscopy was done to evaluate the change in chemical structure through shift in vibrational frequencies caused by various pretreatment agents. Figure 4.13a–c represents FTIR spectra of drybiomass of untreated, NaOH and H_2SO_4-treated *L. limnetica* biomass, respectively. As can be seen that all these spectra exhibit some common peaks at 910-926 and 2,350 cm^{-1} which correspond to =C-H bending and C≡C, respectively (Bandikari et al. 2014). Whereas peak at 3,300-3,330 cm^{-1} corresponds to O-H stretch (H-bonded) for every spectrum other than that for NaOH treated biomass for which it corresponds to C-H stretch. Certain new peaks can also be seen in the treated biomass at 1,510-1,570, 3,520-3,750, 833, and 1,080 cm^{-1} confirming the presence of C=C, O-H stretching, =C-H bending, and C-O stretching, respectively, which are not there in the raw biomass.



(a) **(b)** **(c)**

Figure 4.12: SEM images of *L. limnetica*: **(a)** Drybiomass, **(b)** NaOH treated biomass, **(c)** H_2SO_4 treated biomass



(a) **(b)** **(c)**

Figure 4.13: FTIR spectra of *L. limnetica*: **(a)** Drybiomass, **(b)** NaOH treated biomass, **(c)** H_2SO_4 treated biomass

4.2.3 Optimization of pretreatment conditions through RSM

As the sugar release from *L. limnetica* biomass was found to be maximum during the batch pretreatment study, hence only this cyanobacterial strain was selected for RSM analysis. The statistical design made by the software and the responses found at specified conditions are shown in Table 4.6. Quadratic process order was selected for the regression analysis of the experimental data using ANOVA to improve the validity of predictions. As the ratio of maximum (0.33 g/g db) and minimum sugar release (0.12 g/g db) was 2.75, which was less than 3, so no transformation was required. The responses of the BBD thus obtained were then fitted to a second-order polynomial equation, resulting into final regression equation in terms of coded factors. The expression thus obtained for sugar release is given in Eq. 4.1:

$$\text{Sugar release} = +0.30 + 0.07 * \text{A} + 0.032 * \text{B} + 0.016 * \text{C} - 0.013 * \text{AB} + 0.022 * \text{AC} - 0.006 * \text{BC} - 0.056 * \text{A}^2 - 0.012 * \text{B}^2 - 0.034 * \text{C}^2 \quad (4.1)$$

The statistical parameters such as R^2, adjusted R^2, and predicted R^2 were obtained as 0.997, 0.992, and 0.947, respectively, indicating suitability of the model to represent the relationship between the selected input variables and response. The significant regressions achieved through ANOVA analysis as shown in Table 4.7, established the validity of the model equation.

Table 4.6: Experimental design for pretreatment of cyanobacterial biomass

Run	Temperature (A, °C)	Time (B, min)	Acid concentration (C, molar)	Sugar release (R, g/g)	
				Observed	Predicted
1	70	15	0.5	0.20	0.20
2	100	37.5	2.5	0.32	0.32
3	40	37.5	0.5	0.16	0.15
4	70	60	2.5	0.30	0.30
5	40	15	1.5	0.12	0.12
6	70	37.5	1.5	0.30	0.30
7	70	37.5	1.5	0.30	0.30
8	40	37.5	2.5	0.14	0.14
9	70	37.5	1.5	0.30	0.30
10	100	60	1.5	0.33	0.33
11	70	37.5	1.5	0.30	0.30
12	100	15	1.5	0.29	0.29
13	40	60	1.5	0.21	0.21
14	70	15	2.5	0.25	0.25
15	70	60	0.5	0.28	0.28
16	70	37.5	1.5	0.30	0.30
17	100	37.5	0.5	0.25	0.25

As can be seen, the F-value and Prob > F values for the model are 232.6 and <0.0001, respectively. The Prob > F value less than 0.05 indicates the significance of the model. From Table 4.7 it is evident that A, B, C, A^2, B^2, C^2, AB and AC are significant model terms for sugar release from the biomass of *L. limnetica*. Whereas, the conjugate effect of treatment time (B) and acid concentration (C) has been found to be insignificant for sugar release. The conjugate effects of treatment temperature with time and acid concentration (i.e., AB and

AC) as shown in Figure 4.14 and 4.15, respectively, verify their significance for sugar release from cyanobacterial biomass.

Table 4.7: Results of the regression analysis of the design

Source	F-value	Prob>F
Model	232.6	<0.0001
A-Temperature	1138.9	<0.0001
B-Time	234.49	<0.0001
C-Concentration	57.98	0.0001
AB	18.67	0.0035
AC	56.99	0.0001
BC	4.49	0.0719
A^2	382.37	<0.0001
B^2	18.18	0.0037
C^2	137.61	<0.0001

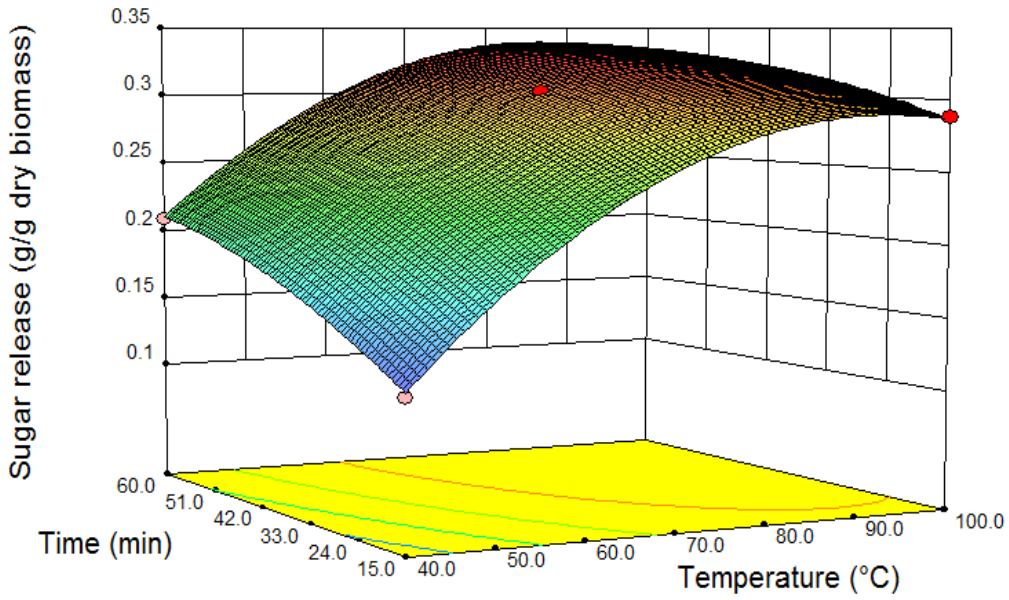


Figure 4.14: Combined effect of treatment temperature and time on sugar release at constant acid concentration of 1.5 M

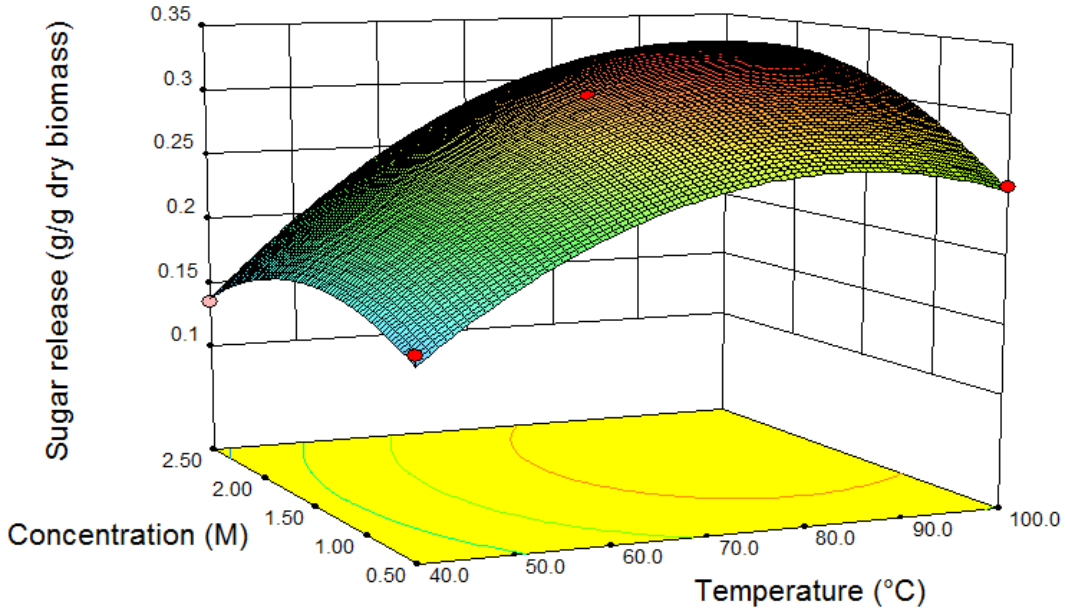


Figure 4.15: Combined effect of treatment temperature and acid concentration on sugar release at constant treatment time of 37.5 min

Combined effect of temperature and time on pretreatment is shown in Figure 4.14, at constant acid concentration (1.5 M). From this figure it is evident that the sugar release is directly proportional to both treatment time and temperature. With increase in the treatment temperature from 40 to 100°C, sugar yield increased from 0.122 to 0.288 g/g db in 15 min of incubation and from 0.211 to 0.333 g/g db in 60 min of treatment time. Similar effects were also observed in case of change in treatment time. The results shown in the figure validate the higher release of sugar at higher temperature and longer incubation period, as this facilitates the bond breakage within the complex carbohydrate structure (Jung et al. 2011).

The interactive effect of H_2SO_4 concentration and treatment temperature at constant treatment time of 37.5 min can be seen in Figure 4.15. Increased sugar release with increase in temperature is revalidated in this case also, whereas the effect of acid concentration on

sugar release is a bit complex. As can be seen from Figure 4.15 the sugar release has varied from 0.249 to 0.326 g/g db at 100°C for acid concentrations of 0.5-2.5 M, however at 40°C for similar acid concentrations, the release reduced from 0.152 to 0.14 g/g db. The decreased yield for 2.5 M acid pretreatment at 40°C can be attributed to the lower bond breakage at lower temperature, along with denaturation of available sugar at higher acid concentration. The maximum sugar release thus achieved was 0.326 g/g db at 100°C within 37.5 min incubation time. The reduction in sugar release even with increased concentration of acid at lower temperature (40°C) as observed in the present investigation can also be validated with similar results obtained at 60°C by Jung et al. (2011). In this study, H_2 yield increased when the acid concentration was reduced from 12 to 6% for the pretreatment of *Laminaria japonica* biomass. It is pertinent to mention here that H_2 yield is directly proportional to the sugar release.

The Design Expert Software has generated the optimum response as 0.33 g/g db of sugar release from *L. limnetica* at 100°C and 59.19 min of incubation using 1.63 M H_2SO_4 as the pretreatment agent. The confirmatory experiment performed at the same operating conditions resulted in 0.324 g/g of sugar yield which is very close to the estimated value.

Figure 4.16 shows the effect of treatment temperature and time on sugar release at optimized acid concentration (1.63 M) generated from the Design Expert Software. This figure reveals that sugar yield increases from 0.121 to 0.294 g/g and 0.209 to 0.333 g/g, respectively, for treatment temperatures of 40 and 100°C and constant time periods of 15 and 60 min in each case. Combined effect of acid concentration and treatment temperature on sugar release at constant treatment time (59.19 min) as optimized by the software can be seen in Figure 4.17.

With the increase in acid concentration from 0.5 to 2.5 M, sugar release increased from 0.261 to 0.332 g/g db at 100°C, whereas at lower temperature (40°C) sugar concentration decreased from 0.189 to 0.167 g/g db validating our experimental findings as well as those of Jung et al. (2011). Comparison of Figures 4.15 and 4.17 again confirms lower sugar release at the combination of higher acid concentration and lower temperature, probably due to the denaturation of sugar molecules. The optimized sugar release as shown in Figure 4.16 and 4.17 is very close to the experimental values as shown in Figure 4.14 and 4.15 within the selected input data range. The experiments conducted with the optimized conditions through RSM resulted in 0.33 g of sugar release per gram of dry cyanobacterial biomass which is a good yield as compared with the findings of other workers (Table 4.8).

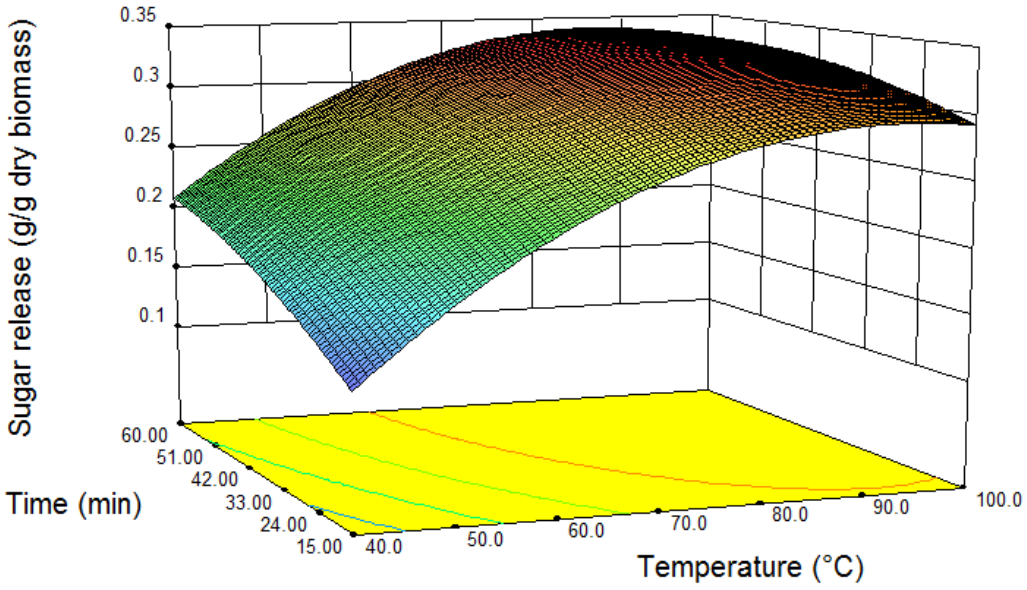


Figure 4.16: Combined effect of treatment temperature and time on sugar release optimized by the software at constant acid concentration of 1.63 M

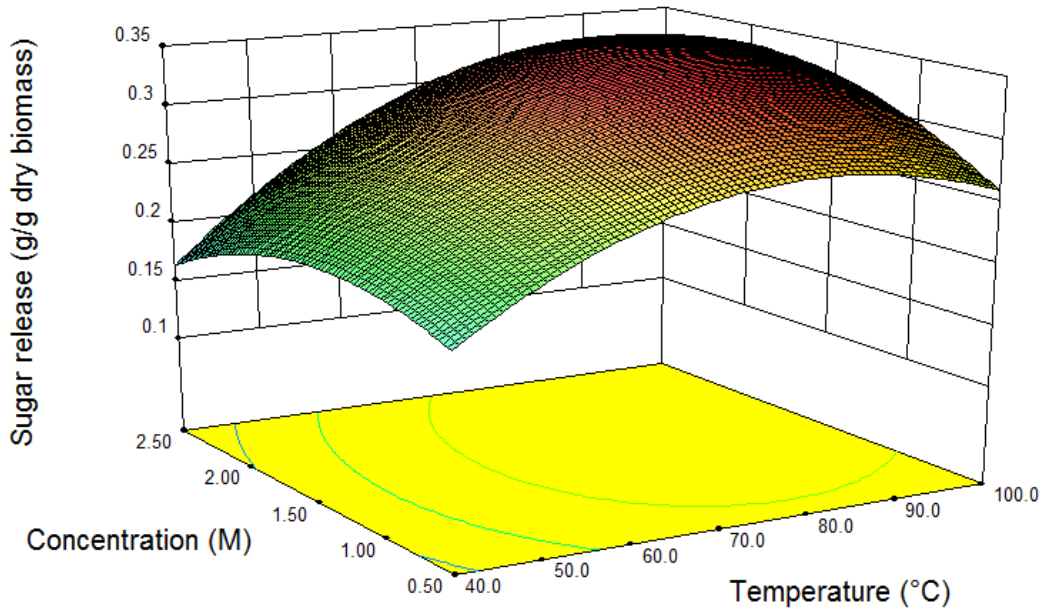


Figure 4.17: Combined effect of treatment temperature and acid concentration on sugar release optimized by the software at constant treatment time of 59.19 min

Table 4.8: Sugar release from different biomass by various pretreatment agents

Biomass	Pretreatment	Sugar yield (g/g db)	Reference
Mixed microalgae (*Scenedesmus*, *Chlorella*, *Micromonas*, *Ankistrosdemus*, *Chlamydomonas*)	H_2SO_4 (1.5 M)	0.182	(Castro et al. 2015)
Wastewater algae	H_2SO_4 (1 M) followed by NaOH (5 M)	0.35	(Ellis et al. 2012)
Arthrospira platensis	0.1 mM H_2SO_4	0.25	(Efremenko et al. 2012)
Chlorella sorokiniana	2% H_2SO_4	0.39	(Cheng et al. 2015)
Ulva lactuca	6% NaOH or 7.5% H_2SO_4 (w/w of dry matter)	0.2	(Van der Wal et al. 2013)
Lyngbya limnetica	1.63 M H_2SO_4	0.33	This study

4.2.4 Other pretreatment strategies

4.2.4.1 Stepwise pretreatment

The first step of pretreatment of *L. limnetica* and *O. obscura* at optimized conditions (1.63 M H_2SO_4 at 100°C for 60 min) resulted into 0.303 and 0.261 g/g sugar release on the drybiomass basis. Cyanobacterial residue obtained from the first step was separately subjected to H_2SO_4 and NaOH treatments (1 M at 100°C for 60 min) to get the residual sugar content from the biomass. Smaller fractions of sugar i.e. 0.013 & 0.009 and 0.027 & 0.018 g/g db were released from the cyanobacterial residue of *L. limnetica* and *O. obscura* treated with H_2SO_4 and NaOH, respectively. The lower sugar yield found during the second step could be either due to the denaturation of monomeric molecules or due to the effective removal of sugar monomers during the first step itself.

4.2.4.2 Lipid extraction and utilization of cyanobacterial residue for sugar release

Potential of cyanobacterial biomass of *L. limnetica* and *O. obscura* was evaluated for lipid extraction and subsequent utilization of the residual biomass for sugar release to be used for butanol fermentation. Simple grinding method was employed to rupture the cell wall of the biomass and maximum 0.123 and 0.139 g/g db lipid was extracted with a mixture of chloroform and methanol (2:1 v/v) and then the lipid extracted biomass was subsequently utilized for the acid pretreatment at optimized conditions to release sugar (Figure 4.18a and b). Nearly 0.162 and 0.167 g/g of sugar was found from the residue obtained from the first step while maximum 0.283 and 0.241 g/g of sugar release was found from 1:1.5 (chloroform:methanol) lipid extracted biomass residue. Smoothening of the residual biomass surface was observed when it was treated with high chloroform concentration to extract

lipid. This could be the possible reason of lower sugar yield from both the residual biomass.

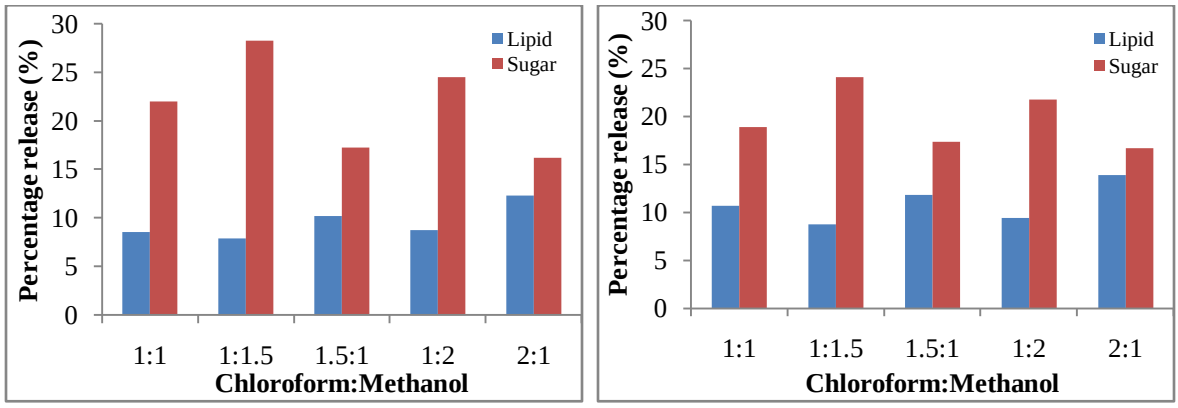


Figure 4.18: Sequential recovery of lipid and carbohydrate: **(a)** *L. limnetica*, **(b)** *O. obscura*

4.3 Enzymatic hydrolysis

4.3.1 Enzyme extraction

Enzyme extraction study was done by SSF using both the species and efficiency was compared with that of commercially available enzymes by performing enzyme assay (Table 4.9). FP and EG activities of the extracted enzymes have been found to be lower than those for the commercial enzymes for both the species while the βG activity was found to be much higher specially for *L. limnetica*. Thus it can be concluded that cyanobacterial biomass are excellent substrates for enzyme extraction, however activity is still lower for the effective hydrolysis of biomass. For further improvement in the enzyme activity effect of Fe_3O_4/Alg NC was evaluated by varying its concentration in a prescribed manner (Figure 4.19). Results of batch extraction experiments lead to the conclusion that the enzyme obtained from SSF of *L. limnetica* biomass possess higher βG activity hence for further enzymatic activity enhancement experiments only *L. limnetica* biomass was selected. From Figure 4.19 it is clear that the presence of appropriate concentration of NC in the production media affects

the enzymatic activity appreciably. The FP activity of the extracted enzyme increased with NC concentration and was found to be maximum 30.63 IU/gds with 1.0 mM i.e. almost double than the control (enzyme without NC). Similar increment in activity pattern was obtained for βG and EG activities with Fe_3O_4/Alg NC and nearly 41 and 37% increase in activities were obtained, respectively. All enzymatic activities reduced (FP: 19.83; βG: 75.48; EG: 82.95) with further increase in NC concentration but were higher than those for the control (FP: 15.37; βG: 71.68; EG: 78.29). Toxicity of NC to fungal cells could be a possible reason for reduced enzymatic activity at higher NC concentrations. These results are in agreement with the results obtained by Srivastava et al. (2015). Enzyme produced by the SSF of rice straw and wheat bran (5:1) showed a decline in the activity pattern with Fe_3O_4/Alg NC concentrations higher than the 0.5 mM, indicating the toxic effect of NC above 0.5 mM concentration.

Table 4.9: Enzymatic activities of commercial and extracted enzymes

Enzymes		**FPase (IU/mL)**	**β-glucosidase (IU/mL)**	**Endoglucanase (IU/mL)**	**Protein (g/L)**
Sigma		57.73	0.725	78.90	120.66
Varuna		20.12	0.896	3.499	8.651
SRL		-	1.747	-	-
Cyanobacteria without nanocomposite	***L. limnetica***	1.537	7.168	7.829	2.278
	O. obscura	0.938	4.146	5.617	1.406

4.3.1.1 Thermal stability of extracted enzyme in the presence of Fe_3O_4/Alg nanocomposite

The thermal stability of the extracted enzyme was tested in terms of relative activity by varying the incubation temperature (45-75°C) and exposure time (1–6 h). Figure 4.20a, shows a comparison of the enzyme stabilities with and without Fe_3O_4/Alg NC at different temperatures.

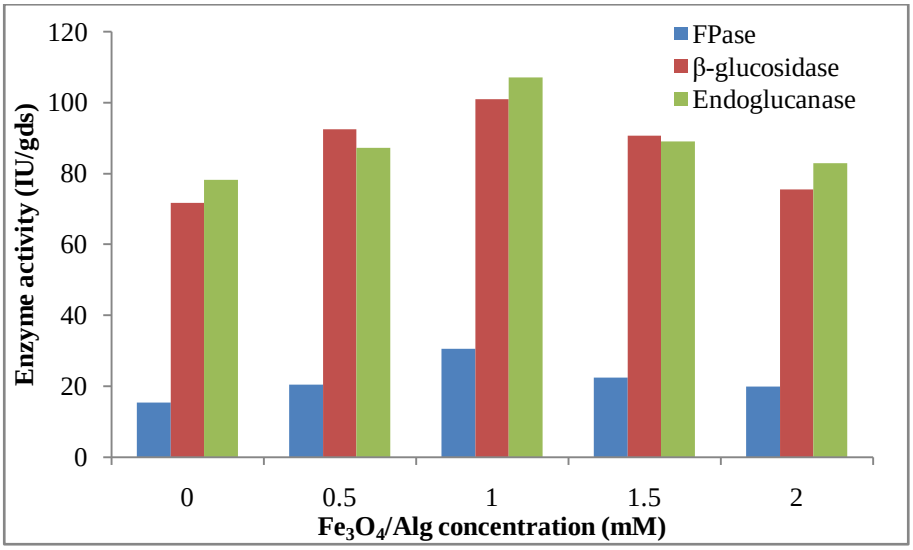
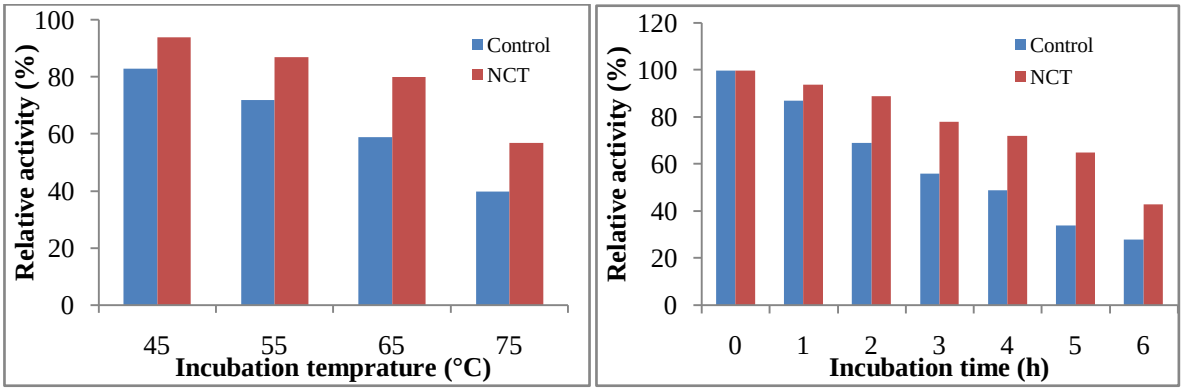


Figure 4.19: Effect of Fe_3O_4/Alg concentration on the activity of extracted enzyme

From this figure it is seen that at a temperature of 75°C stability increased effectively from 40 to 57% with 1.0 mM Fe_3O_4/Alg NC while at 65°C it increased from 59 to 80%. Nearly 9-fold higher enzyme activity for pectate lyase was reported by Mukhopadhyaya et al. (2012) in the presence of hydroxyapatite nanoparticles at a temperature of 90°C. Application of nanostructure for such purpose increases the interation between enzyme and substrate at high temperature and lowers down the activation energy of the nanostructure-enzyme system resulting in an improved enzyme stability (Dutta et al. 2014b).

Figure 4.20b shows the effect of incubation time on the thermal stability of 1.0 mM Fe_3O_4/Alg nanocomposite treated enzyme at 65°C along with that of control. Under studied conditions nearly 56% of the relative activity was maintained by the untreated enzyme whereas nanocomposite treatment improved this value to 78% in 3 h incubation. Sharp decline in the stability was found with both treated and untreated enzyme i.e. 43 and 28%, respectively in 6 h of incubation. These results clearly show that Fe_3O_4/Alg NC improves and maintains the quality and stability of enzyme in comparison to untreated enzyme showing its commercial relevance at high temperature. Enhanced thermal stability of crude cellulase has also been reported by Srivastava et al. (2016) with the application of ZnO nanoparticles. Nearly 64% of the relative activity was maintained at 65°C for 9 h while untreated cellulase lost nearly 50% of its activity within 4 h at the same temperature. Stability results obtained in the present study are in good agreement with the results of Srivastava et al. (2016).



(a) **(b)**

Figure 4.20: Thermal stability of enzyme in terms of relative activity at different: **(a)** Incubation temperature, **(b)** Incubation time

4.3.1.2 Enzymatic hydrolysis of pretreated cyanobacterial biomass

The enzymatic digestibility of the pretreated biomass of *L. limnetica* and *O. obscura* was done using extracted as well as commercial enzymes. Hydrolysis of both the pretreated biomass residues with *L. limnetica* extracted enzyme resulted in no sugar release while negligible concentration was found from untreated biomass. To confirm the results of extracted enzyme hydrolysis again hydrolysis of the pretreated biomass was performed with commercial cellulase C2730 and extrated enzyme (mixed due to high βG activity) using untreated biomass hydrolysis as control. Nearly 0.836 and 1.306 g/L of glucose release was found for untreated *L. limnetica* and *O. obscura* biomass, respectively while insignificant sugar concentration was obtained from the pretreated residues. These results showed the efficacy of the pretreatment step, as insignificant amount of sugar monomers were obtained after enzymatic hydrolysis. So for further fermentation experiments cyanobacterial hydrolysate was prepared using dilute acid pretreatment/hydrolysis method as mentioned earlier (cf. Section 3.5.3). Castro et al. (2015) also concluded that only acid pretreatment/hydrolysis of wastewater microalgal biomass is sufficient enough to get large soluble carbohydrate concentration.

4.4 Fermentation Study

4.4.1 Growth pattern of Clostridium strain

Figure 4.21 shows the growth curve of *C. beijerinckii* ATCC 35702 inocualted in the growth media and incubated at 37°C. This figure depicts that the initial lag phase exists up to 2 h, followed by log phase (exponential phase) for next 14-16 h (16-18 h of inoculation) and then cells enter the stationary phase of growth due to insufficient availability of nutrients.

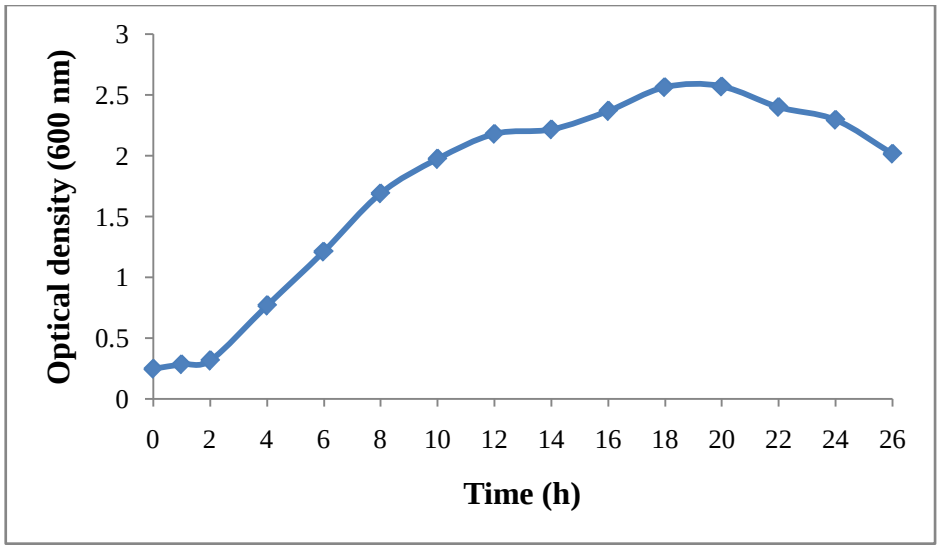
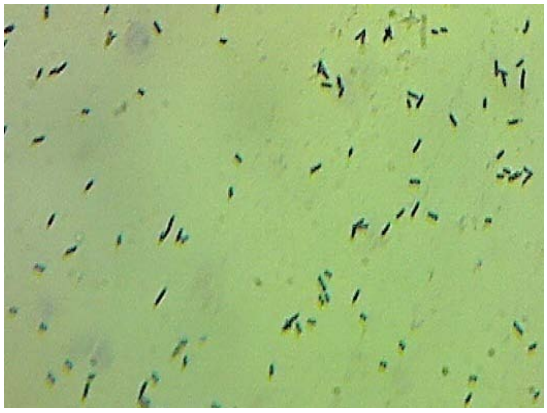
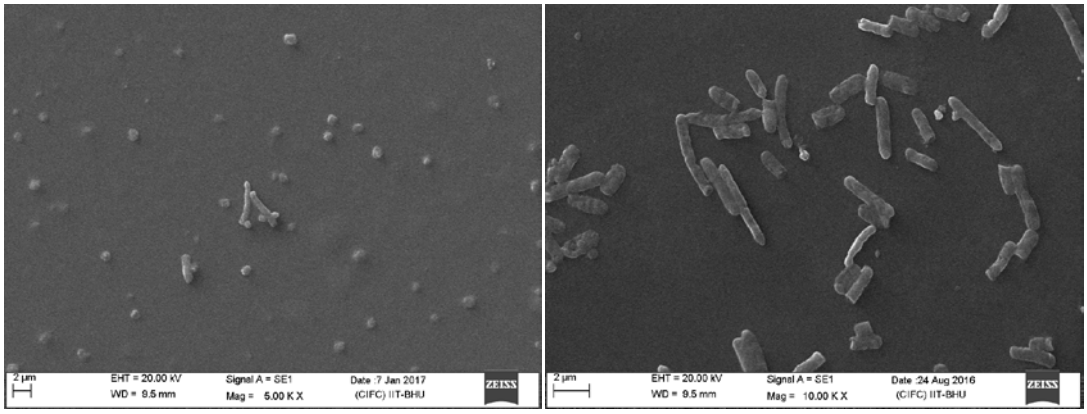


Figure 4.21: Growth curve of *C. beijerinckii* ATCC 35702



(a)



(b) **(c)**

Figure 4.22: Clostridial strain: **(a)** Gram staining, **(b)** SEM image of Clostridial spores, **(c)** SEM image of growing Clostrial cells

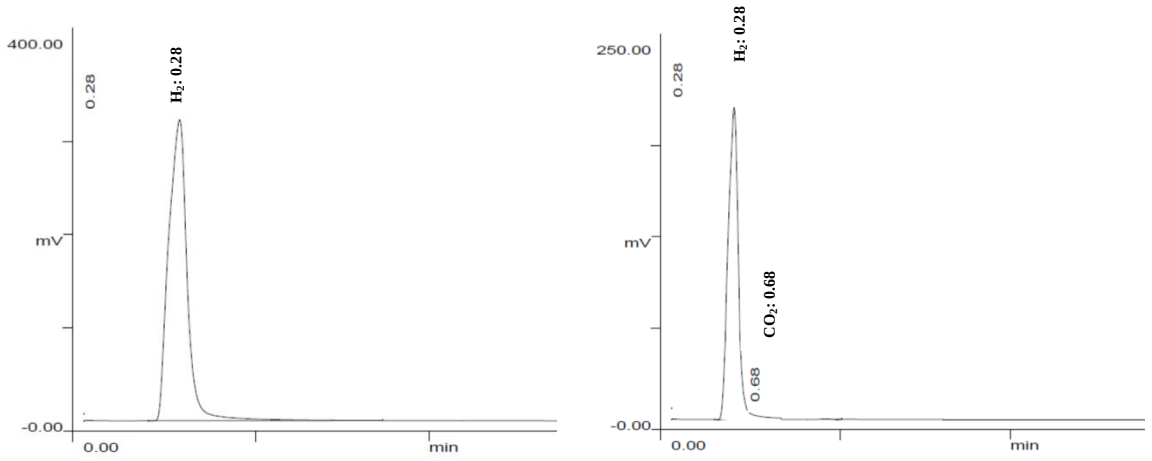
As it can be seen the maximum cell growth is taking place during 14-18 h so to get a maximum healthy count of cells nearly 16 h grown culture was selected as the inoculum for fermentation studies.

Gram staining of freshly prepared inoculum (16 h old culture) was done following the standard protocol proposed by H.C. Gram (Brock 1999), to study and confirm the type (positive or negative) and shape of the cells. Figure 4.22a shows the photomicrographs (60X) of purple stained, rod shaped cells of *Clostridium beijerinckii*. The SEM analysis of the cells grown under favorable and unfavorable conditions was done to get a clear idea about change in cell morphology due to change in the environmental conditions. Figures 4.22b and c, show the stage of endospore formation (unfavorable condition) and growth phase inoculum (favorable condition), respectively.

4.4.2 Butanol fermentation using glucose as the carbon source

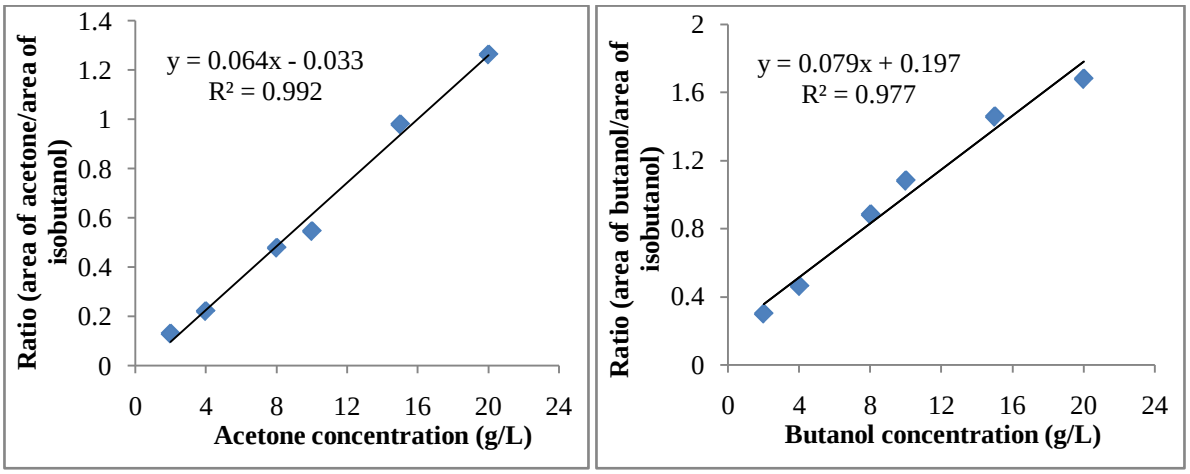
Initial batch fermentation studies were performed with glucose supplemented media using *C. beijerinckii* ATCC 35702. The procured clostridium is a non-ethanol producing strain (confirmed from GC chromatogram).

Figure 4.23: Serum bottles with crimped cap for anaerobic fermentation study



(a) **(b)**

Figure 4.24: Gas chromatograms of **(a)** Standard hydrogen gas sample **(b)** Gas mixture from batch reactor



(a) **(b)**

Figure 4.25: Standard plot for Gas Chromatoghraphic analysis: **(a)** Acetone, **(b)** Butanol

Figure 4.23 shows the set-up that was used for batch butanol production. Growth of bacteria with consumption of glucose and thereby butanol production is shown on one graph and acetone production along with cumulative gas production on another graph. From Figure 4.24b, it is clear that the gas produced during fermentation was a mixture of H_2 and CO_2.

For quantitative estimation of acetone and butanol produced during fermentation, standard curves were prepared separately as shown in Figure 4.25a and b.

4.4.2.1 Effect of initial inoculum concentration

Variation in initial inoculum concentration affects the pattern of glucose consumption thereby butanol production. From Figure 4.26 it is observed that with increase in the inoculum concentration from 5 to 10%, the glucose consumption increased from 56.48 to 86.34% in 7 days of incubation. Maximum butanol production has been found as 8.837 g/L with 2.785 g/L of acetone and 2025 mL/L of gas production from 30 g/L of initial glucose supplementation and media pH of 6.0±0.2. Further increase in the inoculum concentration (15%) resulted in lower glucose consumption (83.45%) with almost similar butanol production of 8.67 g/L. As the difference between the butanol production and yield with 10 and 15% inoculum concentrations has been found to be negligible therefore for further study 10% initial inoculum dose has been selected. Dada et al. (2012) reported 34 and 99% sugar consumption from rice bran hydrolysate during ABE production with initial inoculum concentrations of 0.5 and 1.5 g/L, respectively. Lower glucose consumption with low inoculum concentration could be because of low cell/substrate ratio (Dada et al. 2012), while lower glucose consumption at higher inoculum loading may be due to the end-product inhibition.

4.4.2.2 Effect of initial media pH

The effect of initial pH of fermentation media on AB production is shown in Figure 4.27, other parameters viz., initial glucose concentration, initial inoculum concentration,

incubation temperature are kept constant at 30.0 g/L, 10%, 37°C, respectively. The pH of media is an important parameter that affects the entire metabolic shift (acidogenesis to solventogenesis). No growth was observed at lower initial pH (3.0) while at high alkaline initial pH (11.0) sufficient growth was there with 71.356% glucose consumption. The probable reason for this could be the precipitate formation soon after maintaining the media pH that actually lowered the initial pH between 5.0 to 6.0, while for an initial pH of 9.0 the actual value was found as 7.6±0.2. During the fermentation in first 24 h, the media pH was found to be 4.7±0.3 in all cases (except for pH 3). This decrease in pH was due to the increased concentration of acetic and butyric acids. Media pH remained constant for the next few hours and again a slight increase (5.3±0.3) was observed at 72 h. Decrease in the media pH confirms that fermentation process is in the acidogenic phase, further increase in pH value is probably due to the phase change from acidogenic to solventogenic.

Figure 4.27, shows the effect of initial media pH on butanol production. No butanol titer was found at lower initial pH 3.0 while small concentrations of butanol, 0.166 and 0.168 g/L were found at alkaline pH of 9.0 and 11.0, respectively. As per the earlier discussion large biomass growth was observed at pH 11.0 (with original pH 5.5±0.5) but butanol concentration was very low. This may be due to the addition of large quantity of alkali that hampered the metabolic activity of microorganisms. Maximum butanol (10.45 g/L) and acetone production (3.061 g/L) was found with 91.28% glucose consumption and 2338 mL/L of gas production at initial media pH of 5.0 under studied conditions. According to the present observation the optimal pH for *C. beijerinckii* ATCC 35702 is 5.0 with highest butanol yield of 0.381 g/g of glucose. During the initial phase of production (72 h) butanol

productivity has been found to be higher (3.186 g/L/d) after that due to the slow production rate productivity decreased to 1.492 g/L/d. This result is quite similar to the observations of Tsai et al. (2014) who reported the high production rate at an initial pH of 4.5. Present results are also in agreement with those of Yang et al. (2013), who reported pH 5.3 as the optimal pH for butanol production at which complete glucose consumption was observed with resultant 11.9 g/L of butanol concentration.

4.4.2.3 Effect of yeast extract concentration

Yeast extract is the main N-source added in the fermentation media and ammonium acetate is the another N-supplement that was kept constant during the whole experimentation. Figure 4.28 depicts the impact of yeast extract concentration on butanol production and it is seen that in the absence of yeast extract butanol yield is low (0.169 g/g) but with increase in concentration up to 5.0 g/L the yield increased to 0.381 g/g glucose. Further increase in yeast extract concentration decreased the butanol production but the yield remained almost the same (0.387 g/g). Presence of yeast extract in the fermentation media supports the bacterial growth as well as butanol production (Rajagopalan 2016).

The lower butanol concentration (2.6 g/L) with less bacterial growth (1.522 OD) observed in the present work could be because of the absence of yeast extract in the fermentation media. Cheng et al. (2012) optimized the yeast extract concentration using RSM for the butanol production using mixed Clostridial bacteria and concluded that the maximum butanol concentration of 12.40 g/L could be obtained at 60 g/L initial glucose concentration and 5.133 g/L of yeast extract supplementation.

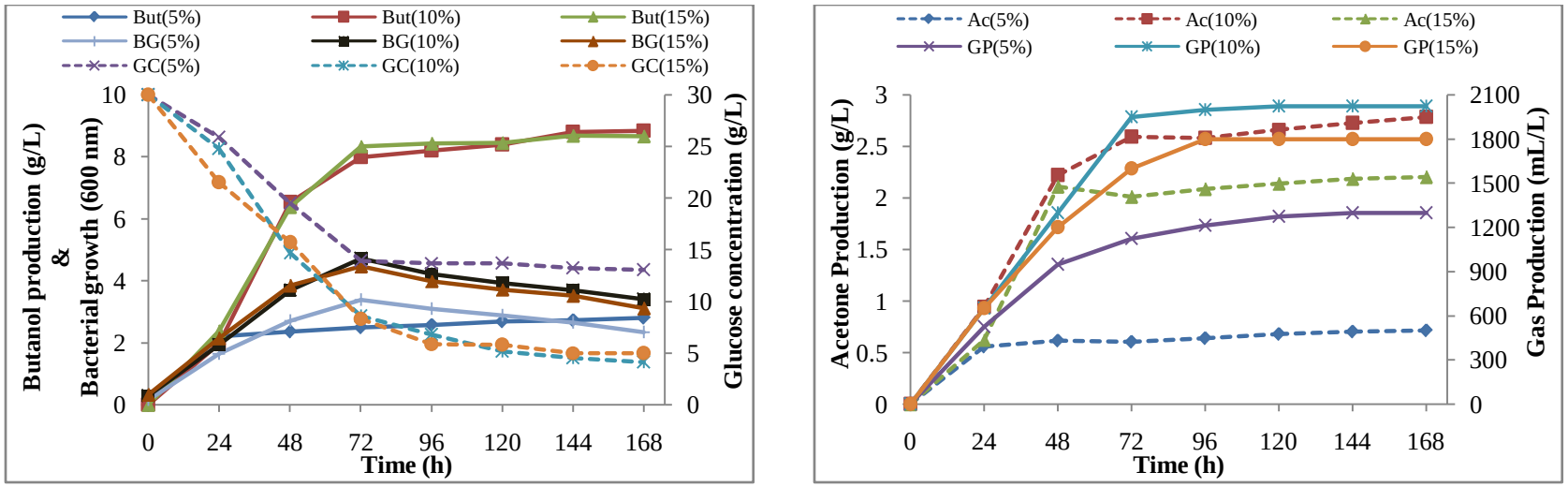


Figure 4.26: Effect of inoculum concentration on butanol production

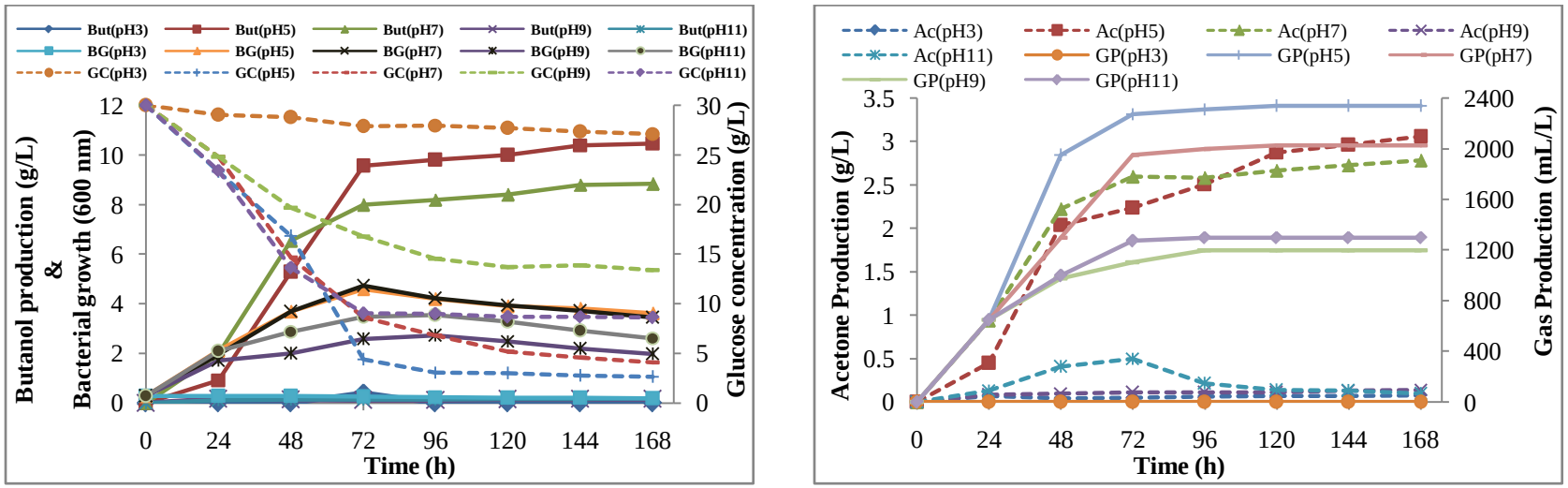


Figure 4.27: Effect of initial media pH on butanol production

The observations of the present study are in agreement with the results obtained by Cheng et al. (2012).

4.4.2.4 Effect of initial glucose concentration

Figure 4.29 shows the effect of initial concentration of glucose (C-source) on butanol production with 10% inoculum, at initial pH of 5.0±0.2, and with other media compositions kept constant as in P-2 media. It was observed that with increase in the glucose concentration from 30 to 90 g/L, the rate of glucose consumption decreased sharply. Percentage glucose consumption was found as 93% with 30 g/L of initial glucose supplementation, however with further increase in the glucose concentration decreased the efficiency of microorganisms and nearly 78.47, 60.32 and 12.32% glucose consumptions were found with 50, 70 and 90 g/L of initial glucose concentrations, respectively. Lower glucose consumption with increase in concentration could be due to the substrate inhibition (Huang et al. 2015). With increase in initial glucose concentration from 30 to 50 g/L, the butanol productivity (maximum at 72 h) increased from 3.186 to 3.381 g/L/d, after that a sharp decline was observed and was found as 0.276 g/L/d with 90 g/L of initial glucose supplementation. This result confirms the substrate-level inhibition during butanol production. Based on these observations, 50 g/L initial glucose concentration is suggested as the optimum concentration for *C. beijerinckii* ATCC 35702.

During butanol fermentation the C/N ratio plays a key role in the synthesis of solvents and other metabolites (Table 4.10). In the present study the effect of C/N ratio has been investigated by keeping the yeast extract concentration constant as 5.0 g/L. The C/N ratio of

10.0 has been found as the optimum with maximum butanol concentration of 13.41 g/L. Further increase in this ratio from 14.0 and 18.0 resulted in lower butanol productions of 10.95 and 1.483 g/L, respectively. From these results it can be concluded that a high C/N ratio is completely unfavorable for butanol production while at a lower ratio butanol concentration has been found to be in a range comparable with the optimal value with higher butyric acid and lower acetic acid concentrations. Al-Shorgani et al. (2016) studied the effect of C/N ratio in the range of 5-120 by keeping the glucose supplementation fixed at 50 g/L. The optimal ratio was found as 12.8 with a maximum butanol concentration of 9.21 g/L and a gradual decrease in the production was observed with further increase in the C/N ratio.

Table 4.10: Effect of C/N (Glucose/Yeast Extract) ratio on butanol production

C/N ratio	Growth (OD)	Solvent (g/L)		Acid production (g/L)	
		Acetone	Butanol	Acetic	Butyric
6	3.621	3.061	10.45	0.991	2.905
10	3.628	3.22	13.41	0.774	2.618
14	3.387	2.202	10.95	1.231	3.216
18	1.301	0.571	1.483	0.472	1.013

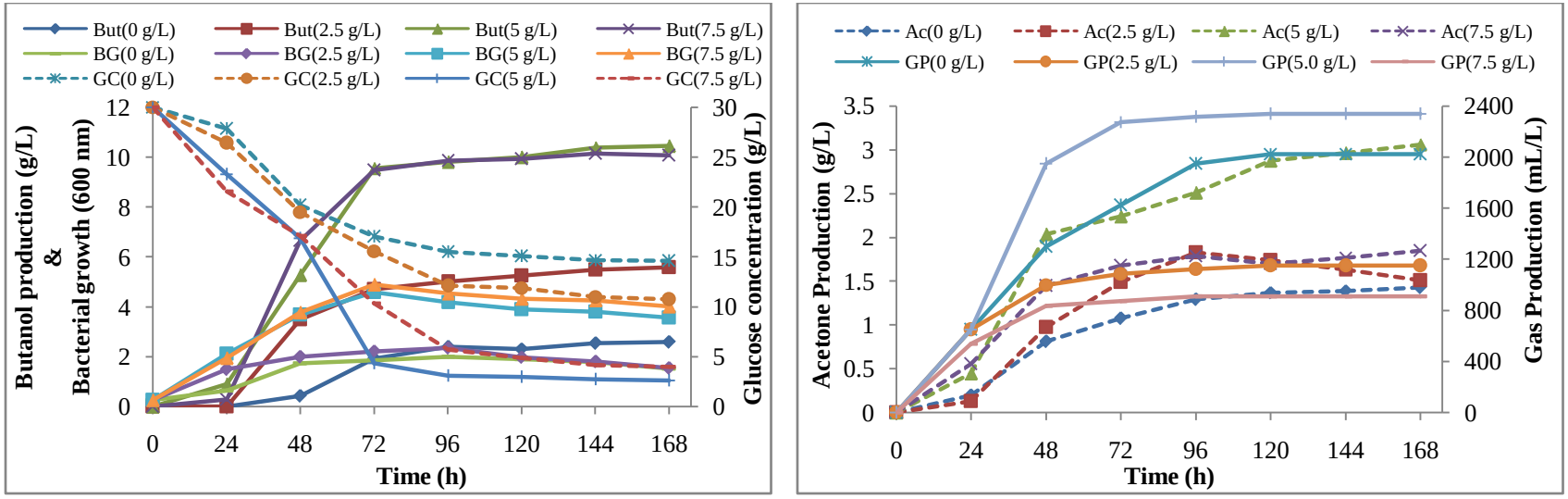


Figure 4.28: Effect of yeast extract concentration on butanol production

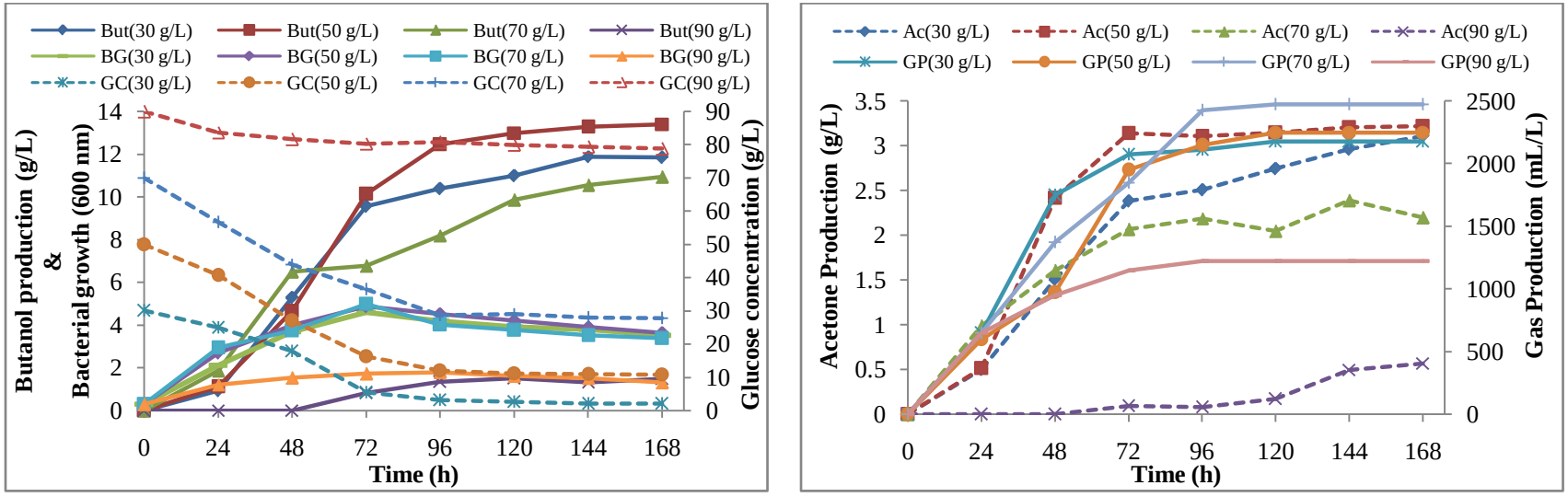


Figure 4.29: Effect of initial glucose concentration on butanol production

4.4.2.5 Effect of other parameters

The presence of some microelements and vitamins in the fermentation media supports the metabolic activity resulting in better solvent productivity (Soni et al. 1987). Table 4.11 shows the effect of PABA and $FeSO_4 \cdot 7H_2O$ on butanol production. Other parameters such as initial glucose and yeast extract concentrations, initial media pH and inoculum concentration were kept constant at 30 g/L, 5 g/L, 5.0 and 10%, respectively. Soni et al. (1987) concluded that with yeast extract as N-source the concentration of PABA did not affect the production of butanol appreciably.This has been confirmed by the present study also. Under PABA-starvation condition relatively slow microbial activity was found with a comparable solvent yield of 0.422 g/g of glucose. Further increase in PABA concentration by 5-times of that in the original media (concentration: 1.0 mg/L) did not affect the overall solvent yield while a marginal change in the butanol concentration from 10.45 to 11.09 g/L was found by varying the PABA concentration from 1.0 to 5.0 mg/L, respectively. Therefore for further study 1.0 mg/L PABA concentration has been selected as the optimum.

Small quantity of $FeSO_4 \cdot 7H_2O$ in the fermentation media also affects the butanol production. From Table 4.11 it is clear that with increase in $FeSO_4$ concentration from 5 to 20 mg/L, the total solvent yield increased upto 0.534 g/g with nearly 93% of glucose consumption. Further increase in salt concentration leads to decrease in the butanol concentration. This might be due to the suppressed enzymatic activity (Junelles et al. 1988). Concentration of acetic and butyric acids were found to be affected largely with increase in the concentration of $FeSO_4$. Cells growing at low iron concentration (5 mg/L) produced small quantity of acetic and butyric acids (0.461 and 1.272 g/L, respectively) compared to the optimized

concentration (20 mg/L) and were found as 1.174 and 3.002 g/L, respectively. The butanol production increased up to the optimum $FeSO_4 \cdot 7H_2O$ concentration (0.52 g/L) after that a sharp decline in the production was observed due to the reduced metabolic activity. Cheng et al. (2012) studied the effect of $FeSO_4 \cdot 7H_2O$ on butanol production over a wide concentration range (0.05 - 0.95 g/L) and obtained results similar to those obtained in the present study.

Table 4.11: Effect of micronutrients on butanol production

Parameter	Concentration (mg/L)	Butanol (g/L)	Acetone (g/L)	Total solvent yield (g/g)	Gas production (mL/L)
PABA	0	9.26	1.784	0.422	2296
	1	10.45	3.061	0.493	2338
	3	10.82	3.005	0.501	2435
	5	11.09	2.969	0.506	2316
$FeSO_4 \cdot 7H_2O$	5	7.693	1.872	0.348	1275
	10	10.45	3.061	0.493	2338
	20	11.86	3.107	0.534	2176
	30	9.678	2.451	0.49	2452

4.4.3 Butanol fermentation with other Clostridial strains

Production of butanol has also been attempted with *C. acetobutylicum* MTCC 11274 and *C. acetobutylicum* NCIM 2877 cultures. No butanol titer was observed with MTCC culture while nearly 1.734 g/L of butanol was produced by NCIM culture using 30.0 g/L of initial glucose concentration and optimized process parameters. Due to the lower butanol titer these strains were not used for further fermentation study.

4.4.4 Cyanobacterial hydrolysate as carbon source

4.4.4.1 Effect of biomass loading

Hydrolysates prepared from cyanobacterial biomass were found to contain various sugars such as glucose, xylose, galactose, arabinose in case of *L. limnetica* biomass and glucose, xylose, galactose in case of *O. obscura* biomass (Table 4.12). The total carbohydrate content in *L. limnetica* hydrolysate varied from 10.573 to 14.371 g/L with increase in biomass loading from 2 to 6%, respectively. In case of *L. limnetica* the increase in glucose concentration has been majorily found with increase in loading from 2 to 4% while further increase in biomass loading resulted only in a nominal increase in glucose concentration as well as total carbohydrate content. On the basis of these results obtained with *L. limnetica*, only 2 and 4% loadings were selected for *O. obscura* to check the type of monomeric sugars available in the biomass and the impact of loading on the concnetration of these sugars. Nearly 1.713 g/L increase in the total carbohydrate was found at 4% loading of *O. obscura*, therefore, for further fermentation study 4% biomass loading has been selected as the optimum value for both the biomass types. Cruz et al. (2013) reported large increase in xylose and lignin concentrations with increase in biomass loading of switchgrass from 3 to 50% while reduced glucose recovery at loadings more than 10%.

A detailed discussion on the two-stage grown biomass carbohydrate yield has been made earlier (cf. Section 4.1.5). Table 4.13 shows the carbohydrate profiles of *L. limnetica* and *O. obscura* biomass obtained under two-stage growth conditions. The maximum total carbohydrate under N-starvation condition was found as 20.31 g/L for *L. limnetica* and 18.27 g/L for *O. obscura* with 4% biomass loading. The observed increase in the

carbohydrate concentration is mainly responsible for increase in the glucose concentration. The increase in glucose concentration was found to be 5.29 g/L for *L. limnetica* and 5.706 g/L for *O. obscura* under NS conditions. Möllers et al. (2014) have also reported nearly 60% w/w of dry weight increment in N-depleted *Synechococcus* biomass. It must be remembered here that the sugar profile varies from species to species.

Table 4.12: Carbohydrate profiles at different biomass loadings

Carbohydrate (g/L)	***L. limnetica***			***O. obscura***	
	2%	**4%**	**6%**	**2%**	**4%**
Glucose	8.123	9.793	10.09	7.821	8.974
Xylose	1.759	2.647	2.945	1.382	1.701
Galactose	0.103	0.118	0.299	0.329	0.57
Arabinose	0.588	0.893	1.035	-	-
Total	10.57	13.45	14.37	9.532	11.25

Table 4.13: Carbohydrate profiles under two-stage growth conditions

Carbohydrate (g/L)	***L. limnetica***	***O. obscura***
Glucose	15.08	14.68
Xylose	2.826	2.374
Galactose	0.718	1.219
Arabinose	1.682	-
Total	20.31	18.27

4.4.4.2 Fermentation of cyanobacterial hydrolysate prepared from strains grown under normal condition

The cyanobacterial hydrolysate prepared at optimized pretreatment conditions with 4% biomass loading resulted in nearly 13.45 g/L and 11.25 g/L of total carbohydrate from *L. limnetica* and *O. obscura*, respectively. The biomass residue obtained after the pretreatment and further subjecting to complete digestion following the protocol mentioned earlier (cf. Section 3.2.2.1) resulted in nearly 1.941 and 1.881 g/L of total carbohydrate from *L. limnetica* and *O. obscura*, respectively. Lower carbohydrate yield after second step of pretreatment shows the efficiency of conditions used for the first step of pretreatment.

The *L. limnetica* and *O. obscura* hydrolysate-based media were used for butanol fermentation at optimized conditions: 10% inoculum concentration; initial hydrolysate pH 6.1±0.2; 5.0 g/L of yeast extract concentration; 1.0 mg/L of PABA and 20 mg/L of $FeSO_4 \cdot 7H_2O$. Van der Wal et al. (2013) have also concluded that butanol fermentation with *Ulva lactuca* hydrolysate using *C. beijerinckii* culture required a media pH of 6.0-6.4. As shown in Figure 4.30, fermentation using *L. limnetica* hydrolysate resulted in a total AB concentration of 4.3 g/L and 3.579 g/L using *O. obscura* hydrolysate at 120 h. This accounted for 3.79 and 3.162 g/L of butanol concentrations, with nearly 84 and 91% of total carbohydrate consumption, respectively.

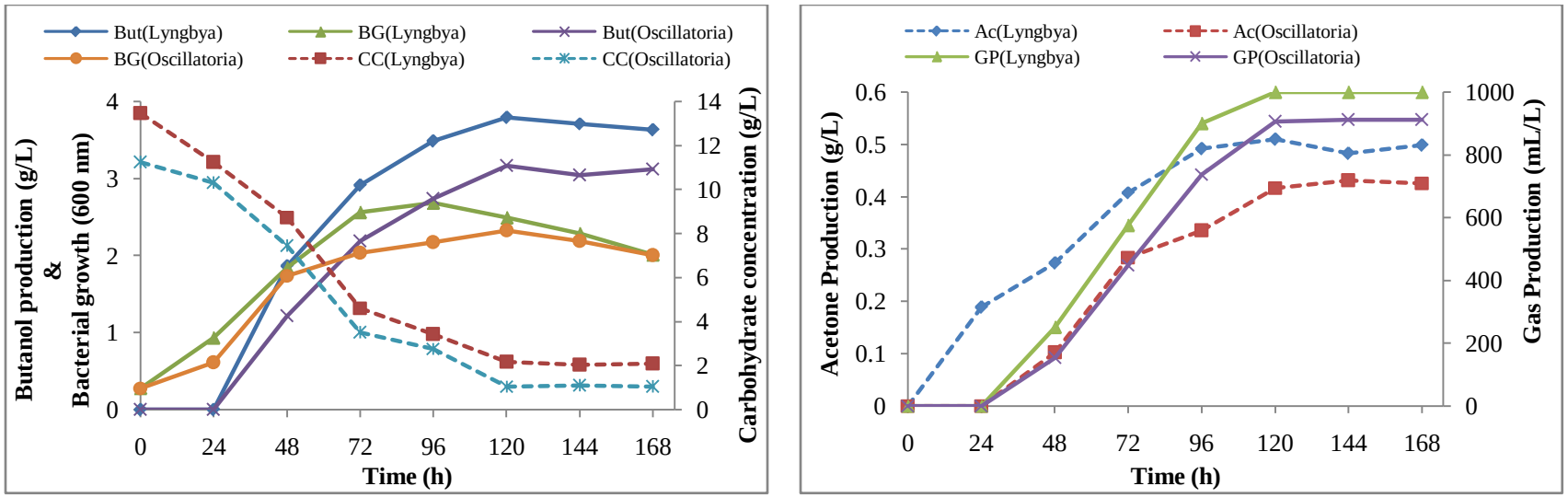


Figure 4.30: Cyanobacteria grown, pretreated & fermented at optimized conditions

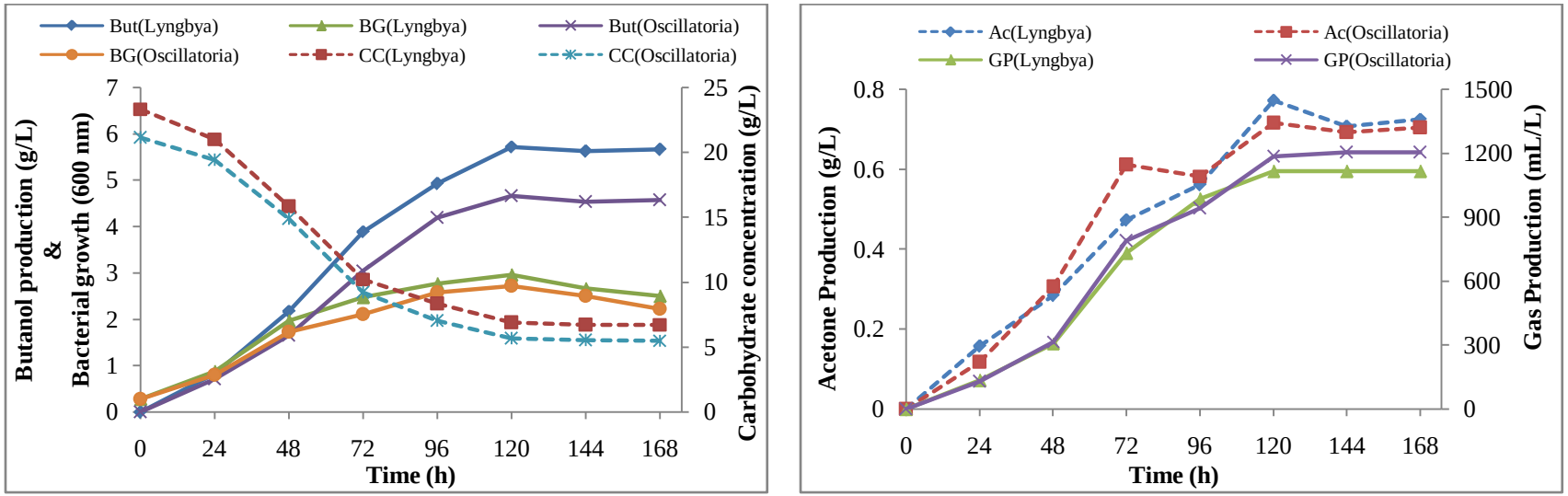


Figure 4.31: Fermentation of cyanobacterial hydrolysate supplemented with glucose

With 10 g/L of glucose supplementation, the total AB production has increased to 6.492 and 5.381 g/L from *L. limnetica* and *O. obscura* hydrolysate, respectively (Figure 4.31). This has increased the butnol productivity from 0.758 to 1.144 g/L/d from *L. limnetica* hydrolysate and 0.632 to 0.933 g/L/d from *O. obscura* hydrolysate. Addition of glucose in the hydrolysate supports the cell growth and thereby fermentation resulting in higher sugar conversion efficiency to solvents (Ellis et al. 2012). Here, the total AB yield increased from 0.381 to 0.392 g/g with *L. limnetica* while little change (0.352 to 0.345 g/g) was observed with *O. obscura*. It is seen that the glucose supplementation increases the butanol concentration but does not affect the solvent yield remarkably at lower cyanobacterial glucose concentration in the fermentation media.

4.4.4.3 Fermentation of cyanobacterial hydrolysate prepeared from strains grown under two-stage conditions

The hydrolysate was prepared from two-stage grown cyanobacteria with 4% biomass loading and tested for the available sugars through HPLC that were found as 20.31 and 18.27 g/L of total carbohydrate for *L. limnetica* and *O. obscura*, respectively. Due to the lower yield of carbohydrate content from the residual biomass further treatment was not done at this stage.

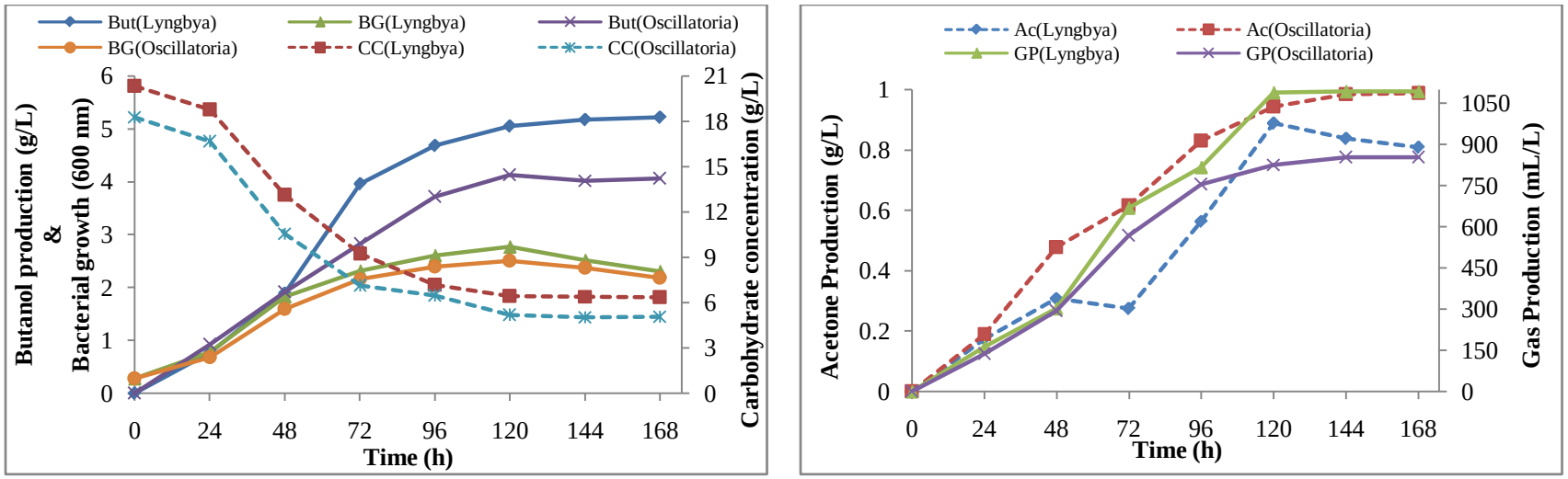


Figure 4.32: Two-stage grown cyanobacteria, pretreated & fermented at optimized conditions

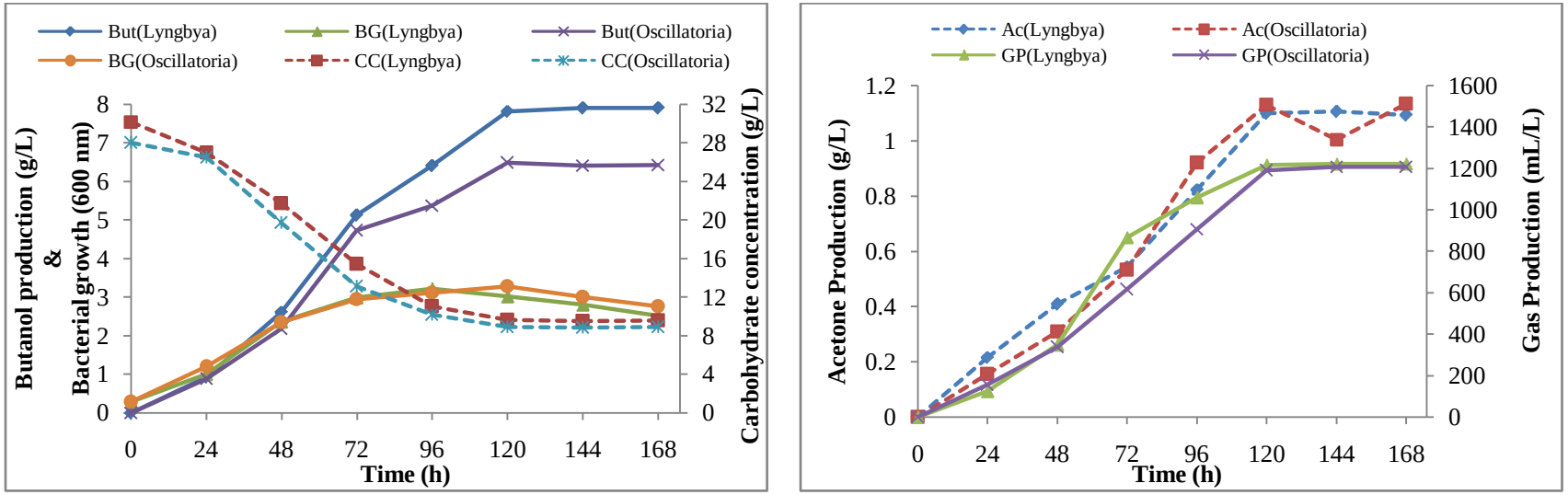


Figure 4.33: Fermentation of two-stage grown cyanobacterial hydrolysate supplemented with glucose

Figure 4.32 shows the butanol production from the hydrolysates prepared from biomass of *L. limnetica* and *O. obscura* grown under two-stage growth conditions. A sharp increase in butanol and acetone concentrations can be seen with both the strains up to 5 days with nearly 68-72% of carbohydrate consumption but after that no remarkable change is observed. With increase in the total carbohydrate concentration (compared to normally grown biomass) butanol productivity increased to 1.01 and 0.826 g/L/d for *L. limnetica* and *O. obscura*, respectively. This is the first ever attempt that has been made to utilize the two-stage grown biomass for butanol fermentation.

To see the effect of glucose supplementation on the hydrolysate fermentation 10 g/L of glucose was mixed and fermented (Figure 4.33). Production of butanol for *L. limnetica* hydrolysate increased from 5.219 to 7.912 g/L with a corresponding improved AB yield of 0.428 to 0.431 g/g carbohydrate and for *O. obscura* hydrolysate from 4.13 to 6.489 g/L with a corresponding increased AB yield of 0.387 to 0.394 g/g carbohydrate. It is confirmed that glucose supplementation improves the butanol production without affecting the overall solvent yield. This is due to the fact that the glucose supplementation in cyanobacterial hydrolysate does not improve the rate of initial cell proliferation (Ellis et al. 2012).

Figure 4.34 compares the productivity of butanol from both the cyanobacterial hydrolysates under four different production conditions. As discussed earlier (cf. Section 4.4.4.2 & 4.4.4.3) glucose supplementation improves the butanol production as well as productivity with a maximum of 1.565 g/L/d for the *L. limnetica* biomass (obtained from the two-stage

growth experiments) hydrolysate when supplemented with 10.0 g/L of glucose at the optimized conditions.

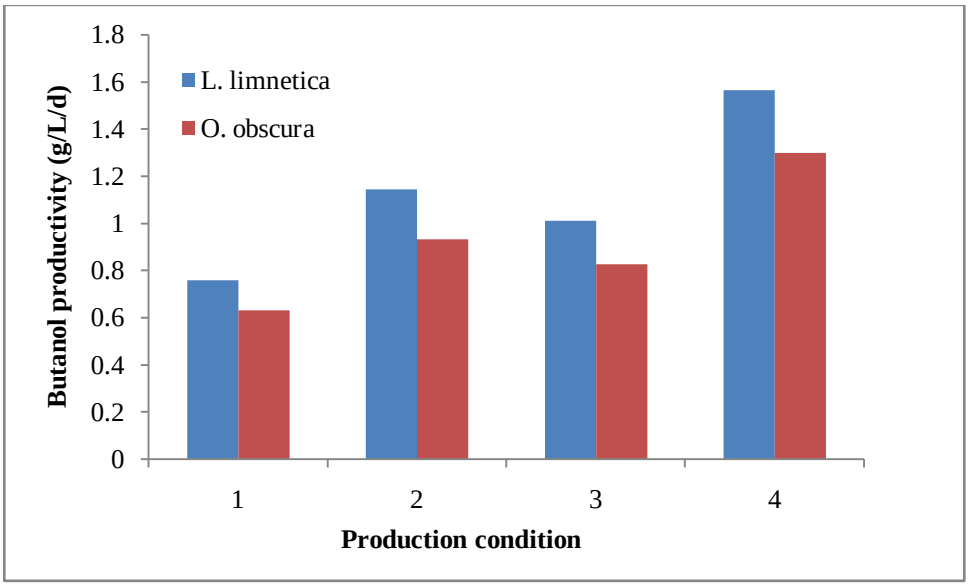


Figure 4.34: Comparison of butanol productivity from cyanobacterial hydrolysate fermented under various conditions*(1,2,3,4)

1: Cyanobacteria grown, pretreated & fermented at optimized conditions
2: Cyanobacterial hydrolysate supplemented with glucose
3: Two-stage grown cyanobacteria, pretreated & fermented at optimized conditions
4: Two-stage grown cyanobacterial hydrolysate supplemented with glucose

4.4.5 Continuous stirred tank reactor (CSTR)

The fermentation in CSTR was performed separately under optimized conditions using glucose, cyanobacterial hydrolysate (*L. limnetica*) and hydrolysate supplemented with glucose (10.0 g/L). Maximum butanol (16.193 g/L) was produced from 50 g/L of initial glucose concentration with butanol and total AB yields of 0.4 and 0.482 g/g sugar, respectively. Table 4.14, shows the initial sugar concentration and the concentration at the time of maximum butanol production together with the concentrations of major acids and solvents. Apart from the glucose fermented media, maximum butanol productivity of 2.218

g/L/d was obtained with *L. limnetica* hydrolysate supplemented with 10.0 g/L of glucose. From Table 4.14, it is clear that *C. beijerinckii* ATCC 35702 mainly utilized glucose (~86%), leaving approx 60% of the pentoses (xylose & arabinose). Similar sugar consumption pattern was also found with *L. limnetica* hydrolysate supplemented with glucose. Though large butanol production (8.873 g/L) was found with glucose supplemented hydrolysate, but the highest yield (0.421 g/g sugar) obtained with pure cyanobacterial hydrolysate, proves its industrial feasibility.

Figure 4.35 shows the comparison of butanol concentration produced in shake flasks and CSTR. In all cases CSTR butanol concentration has been found to be higher than that for the shake flask.

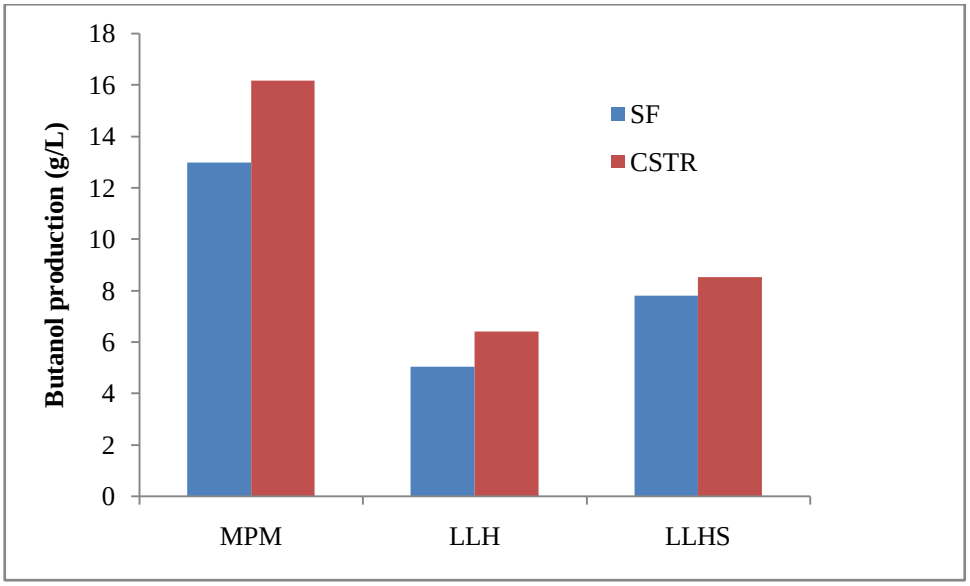


Figure 4.35: Comparison of shake-flask and CSTR butanol production (at 120 h)

Table 4.14: Fermentation in CSTR with glucose and cyanobacterial hydrolysate

	MPM	**LLH**	**LLHS**
Sugars at t = 0 h (g/L)			
Glucose	50.0	15.08	24.91
Xylose	-	2.826	2.71
Arabinose	-	1.682	1.788
Galactose	-	0.718	0.873
Total	50.0	20.31	30.28
Sugars at t = 120 h* (g/L)			
Glucose	9.553	2.112	4.75
Xylose	-	1.673	1.827
Arabinose	-	1.033	0.951
Galactose	-	0.209	0.316
Total	9.553	5.027	7.844
Products at t = 120 h*(g/L)			
Acetic acid	1.049	0.863	1.172
Butyric acid	3.273	2.495	2.785
Gas (mL/L)	2026	956	1009
Butanol	16.19	6.428	8.873
Acetone	3.309	1.213	1.196
Total solvent	19.50	7.641	10.07
Butanol yield (g/g sugar)	0.4	0.421	0.396

h*: For LLH & LLHS, t = 96 h

4.4.5.1 Mass-balance for CSTR

The biochemical changes taking place within the bioreactor can be explained in a better way through the substrate conversion pathway. Figure 4.36 shows the fermentation pathway that has been proposed on the basis of the results obtained during CSTR studies as well as the available published information.

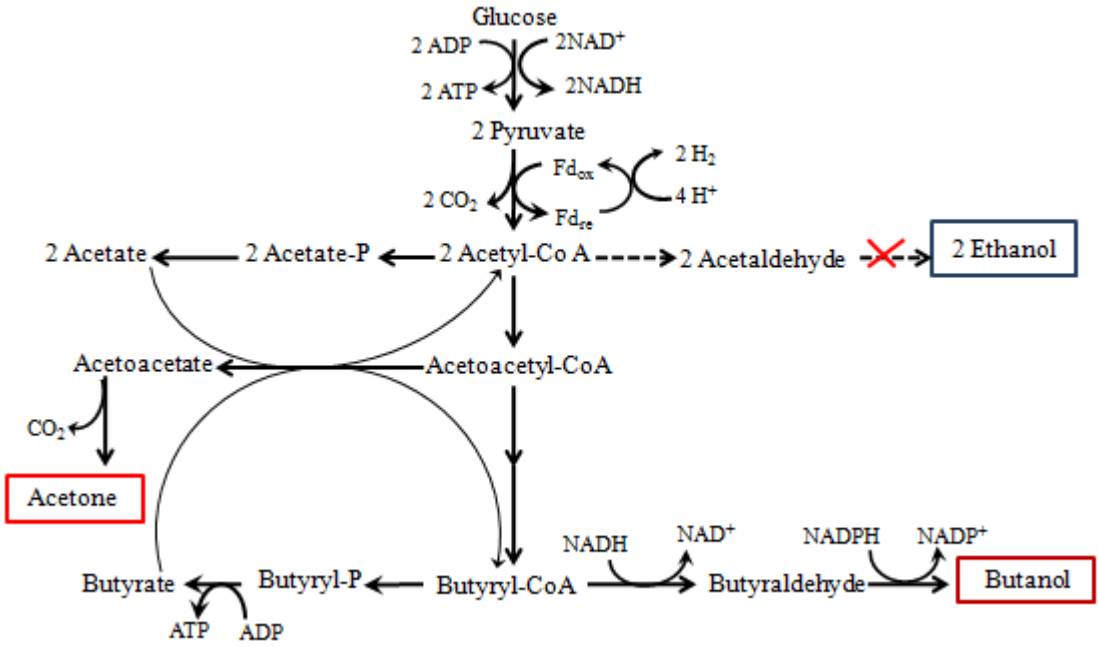


Figure 4.36: Proposed fermentation pathway for butanol production

It has been mentioned earlier that the ATCC procured culture does not possess ethanol production properties, probably because of the absence of acetaldehyde dehydrogenase and ethanol dehydrogenase producing genes. On the basis of the proposed fermentation pathway different biochemical reactions occuring within the system can be expressed in terms of Eq. 4.2-4.11. Major extracellular compounds produced during fermentation are acetone, butanol, acetate and butyrate along with some other intermediates and majorily hydrogen gas

$$\mathbf{C_6H_{12}O_6} + 2ADP + 2NAD^+ \longrightarrow 2C_3H_4O_3 + 2ATP + 2NADH \quad (4.2)$$
(Glucose)

$$2C_3H_4O_3 + 2CoA + 2Fd \longrightarrow 2C_{23}H_{38}N_7O_{17}P_3S + 2CO_2 + 2FdH_2 \quad (4.3)$$

$$C_{23}H_{38}N_7O_{17}P_3S + ADP + Pi \longrightarrow \mathbf{CH_3COOH} + ATP + CoA \quad (4.4)$$
(Acetate)

$$2C_{23}H_{38}N_7O_{17}P_3S + 2NADH \longrightarrow C_{25}H_{42}N_7O_{17}P_3S + CoA + 2NAD^+ \quad (4.5)$$

$$2C_{23}H_{38}N_7O_{17}P_3S + ADP + Pi \longrightarrow \mathbf{CH_3COCH_3} + CO_2 + 2CoA + ATP \quad (4.6)$$
(Acetone)

$$C_{25}H_{42}N_7O_{17}P_3S + ADP + Pi \longrightarrow \mathbf{C_4H_8O_2} + ATP + CoA \quad (4.7)$$
(Butyrate)

$$2C_{23}H_{38}N_7O_{17}P_3S + C_4H_8O_2 \longrightarrow \mathbf{CH_3COCH_3} + CO_2 + C_{25}H_{42}N_7O_{17}P_3S + CoA \quad (4.8)$$
(Acetone)

$$C_4H_8O_2 + ATP + CoA \longrightarrow C_{25}H_{42}N_7O_{17}P_3S + ADP + Pi \quad (4.9)$$

$$C_{25}H_{42}N_7O_{17}P_3S + 2NADH \longrightarrow \mathbf{C_4H_9OH} + CoA + 2NAD^+ \quad (4.10)$$

(Butanol)

$$Fd_{red} + 2H^+ \longrightarrow Fd_{ox} + H_2 \quad (4.11)$$
$$Fd_{red} + NAD^+ \longrightarrow Fd_{ox} + NADH + H^+$$

Mass balance calculations are necessary for verifying the composition at every step of the solvent production. In order to understand the balance between input materials and output products C-balance was done for glucose fermented and cyanobacterial fermented media. Individual component balance (in terms of C-balance) was selected only to avoid the complexity with other elemental and water balances. Figure 4.37 shows the total input and out moles of various carbon containing components at the maximum butanol production stage (120 h). In the fermentation media the main C-source is glucose, however, some other components (biotin, ammonium acetate, and cysteine hydrochloride) added to it act as indirect C-source hence are also considered during the mass balance. Total of 1.732 C-moles

were fed into the media and fermentation resulted in an output of 1.228 C-moles with highest mole of butanol (0.218) in addition to 0.057, 0.017, and 0.037 moles for acetone, acetate and butyrate, respectively. Only 0.053 mole of glucose was found in the fermntation media at the end of the experiments therefore, it was concluded from the mass balance equation (Eq. 3.9) that the remaining 0.186 C-moles were utilized in the bacterial growth and other metabolic activities within the system.

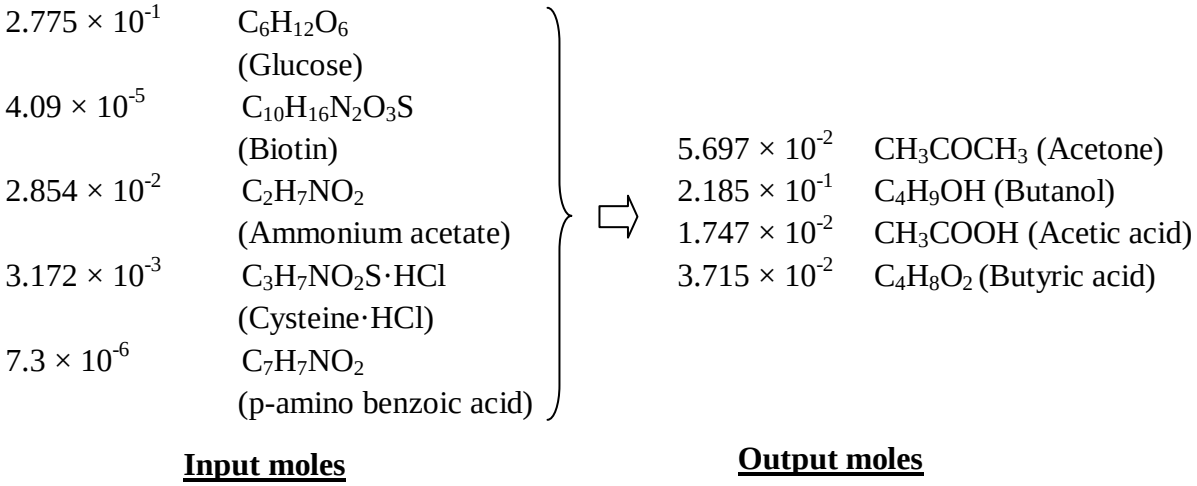


Figure 4.37: C-balance at optimized conditions using glucose as the main C-source

Figure 4.38, shows the C-balance of cyanobacterial hydrolysate fermentation. Total input C-moles was calculated as 0.743 including 0.084 moles glucose, 0.004 mole galactose, 0.011 mole xylose and 0.019 mole arabinose. Fermentation resulted in nearly 0.552 C-moles consumption for the synthesis of 0.087, 0.021, 0.014 and 0.028 moles of butanol, acetone, acetate and butyrate, respectively. Here the complete C-balance during fermentation proved the efficiency of the process with maximum sugar consumption being used in butanol synthesis.

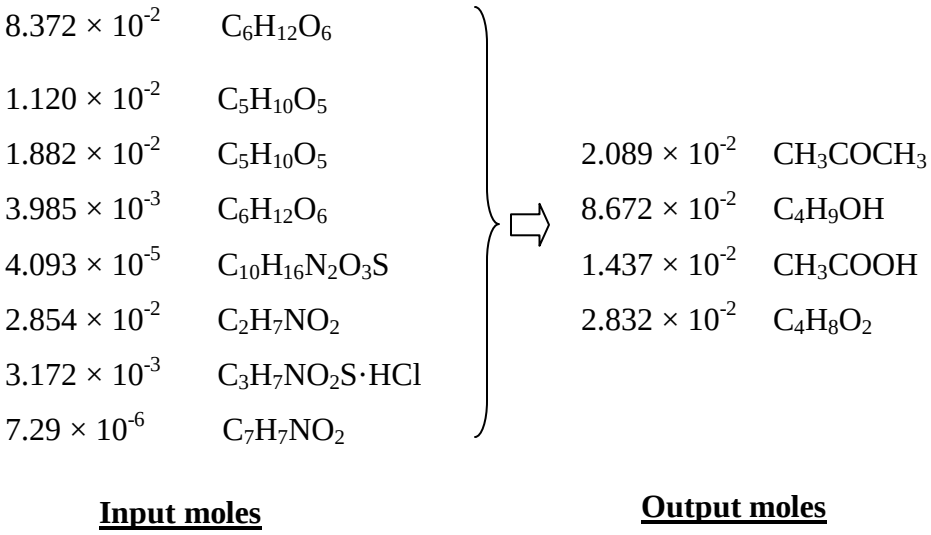


Figure 4.38: C-balance at optimized conditions using *L. limnetica* hydrolysate as the main C-source

4.4.5.2 Kinetic modeling of butanol production, bacterial growth & sugar consumption

The Mercier model, a Monod kinetics based model, was fitted to the CSTR data of butanol production, Clostridium growth, and sugar consumption using Matlab 9.2.0.538062 to estimate the biokinetic parameters involved in the fermentation process. Mercier et al. (1992) proposed this unstructured logistic model to describe the kinetics of cell growth, substrate utilization and product synthesis, that was further used by Rodrigues et al. (2006) and Daverey and Pakshirajan (2010) to explain the kinetics of biosurfactant and sophorolipids production, respectively. Table 4.15, shows various biokinetic parameters calculated by fitting the experimental values to the model. Figure 4.39, shows the patterns of predicted and the experimental values. A high value of regression coefficients (R^2) ($\geq$0.93) in all conditions, shows that the experimental values are well described by this model. The values of kinetic constant (P_t), presented in Table 4.15 have been found to be slightly higher than those reported by Ranjan et al. (2013b) i.e. 0.029. This might be attributed to the

difference in the nature of biomass sources used. Close aggrement between model predictions and experimental values confirm the feasibility of optimized operating parameters that control the butanol production. The P_{max} value predicted from the model for glucose (15.93 g/L), *L. limnetica* (6.394 g/L) and *L. limnetica* supplemented with glucose (8.784 g/L) fermented media are in good agreement with those obtained experimentally i.e. 16.19 g/L, 6.428 g/L and 8.873 g/L, respectively. The values of specific growth rate (μ) predicted are in good agreement with the values obtained by Ranjan et al. (2013b). The high values of $Y_{P/S}$ and $Y_{X/S}$ obtained for *L. limnetica* hydrolysate fermentation confirm its suitability for industrial exploitation.

Table 4.15: Various biokinetic parameters estimated using Mercier model and experimental results obtained during CSTR studies

Parameters	Kinetic parameters	Sugar source		
		Glucose	*L. limnetica*	*L. limnetica*+Glucose
Biobutanol production $P = \frac{P_{max} P_0 e^{P_t t}}{(P_{max} - P_0) + P_0 e^{P_t t}}$	P_0	2.742	0.878	0.966
	P_{max}	15.93	6.394	8.784
	P_t	0.034	0.042	0.04
	R^2	0.987	0.992	0.986
Biomass growth $X = \frac{X_{max} X_0 e^{\mu t}}{(X_{max} - X_0) + X_0 e^{\mu t}}$	X_0	1.689	0.777	1.034
	X_{max}	6.017	3.365	3.843
	μ	0.03	0.026	0.028
	R^2	0.942	0.932	0.959
Sugar consumption $S = S_0 - \frac{P - P_0}{Y_{P/S}} - \frac{X - X_0}{Y_{X/S}}$	S_0	49.32	20.18	30.30
	$Y_{P/S}$	0.394	0.473	0.546
	$Y_{X/S}$	5.639	1.607	0.497
	R^2	0.998	0.996	0.998

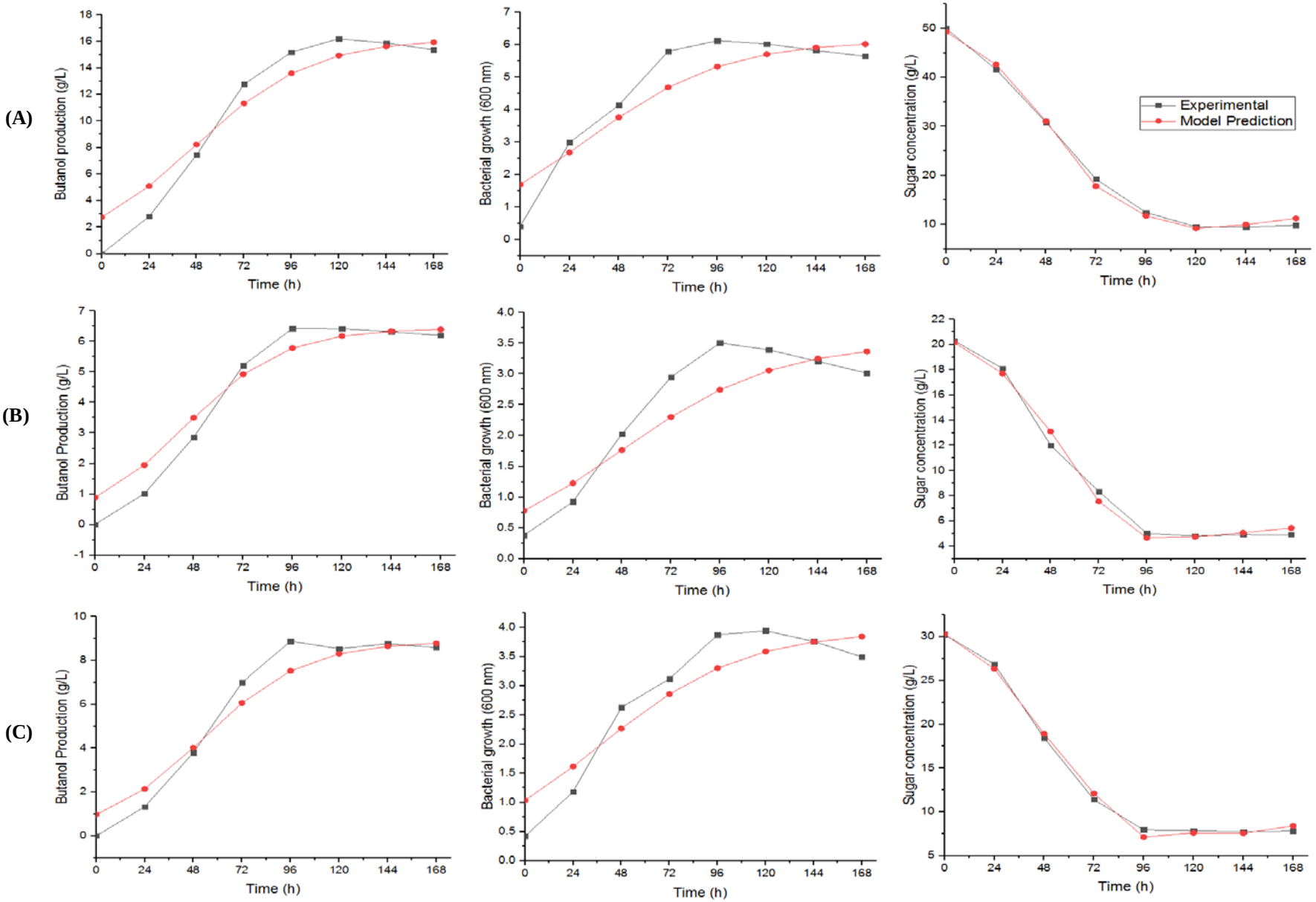

(A)
(B)
(C)
Butanol production (g/L)
Bacterial growth (600 nm)
Sugar concentration (g/L)
Butanol Production (g/L)
Time (h)
Experimental
Model Prediction

Figure 4.39: Comparison of experimental data with model prediction: for Column **(I)** Butanol production, Column **(II)** Bacterial growth, Column (III) Sugar consumption; Row (A) Butanol fermentation with Glucose as C-source, Row **(B)** *L. limnetica* hydrolysate as C-source, and Row **(C)** *L. limnetica* hydrolysate supplemented with glucose as C-source.

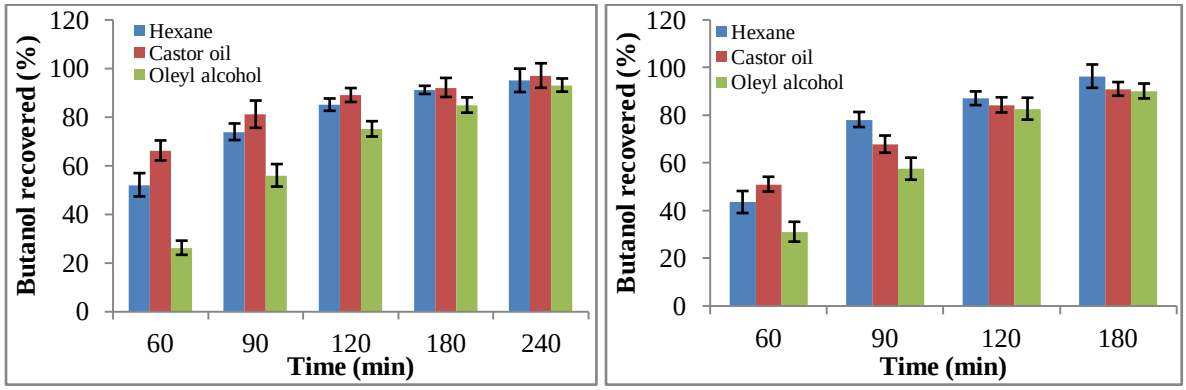
4.5 Recovery of Butanol

4.5.1 Liquid-liquid extraction

Three extractants hexane, castor oil, and oleyl alcohol were selected for butanol recovery from batch fermentation broth on the basis of their distribution coefficients, non-toxic nature and selectivity for butanol from an aqueous solution. Continuous stirring of the mixture was done to increase the mass transfer rate and ensure better solvent dispersion. Figure 4.40a, shows the percentage butanol recovered from the glucose fermented media. More than 50% butanol was recovered using hexane and castor oil within 60 min, however, at the end of the extraction process i.e. 4 h, nearly 97% recovery was obtained with castor oil and 95% with hexane. Initial recovery rate with oleyl alcohol was found to be less but at the end nearly 93% butanol recovery was achieved. From here it is concluded that recovery of butanol in castor oil is better in comparison to other two studied extractants but the major drawback associated with this is the foam formation (Figure 4.41a) during mixing that restricts its application.

Similar butanol extraction pattern was also observed for the cyanobacterial hydrolysate fermented media (Figure 4.40b). Initial butanol extraction was found to be better with castor oil and nearly 51% recovery was achieved within 60 min. Butanol recovery rate of hexane increased after 60 min and at the end of the extraction process i.e. 3 h, hexane was found as

the best extractant with maximum 96% of butanol recovery. Nearly 90 and 91% butanol recovery were achieved with oleyl alcohol and castor oil, respectively. Oleyl alcohol has been reported as efficient extractant for butanol recovery by Qureshi and Maddox (1995). However, the present results show that probably due to emulsion formation its efficiency is slightly lower compared to those of other two extractants.



(a) **(b)**

Figure 4.40: Butanol extraction: **(a)** Glucose fermented broth, **(b)** *L. limnetica* hydrolysate

(a) **(b)** **(c)**

Figure 4.41: Butanol extraction using various extractants: **(a)** Castor oil, **(b)** Hexane, **(c)** Oleyl alcohol

Liquid-liquid extraction is an efficient butanol recovery technique as has been shown through experiments, however some problems are encountered during and after the completion of extraction. These are-

(1) Emulsion formation with castor oil and oleyl alcohol as shown in Figure 4.41a and c.

(2) Distillation of extractants resulted in an incomplete distillation with castor oil due to burning of the oil and foam content at higher temperatures while hexane and oleyl alcohol were separated easily but were with contamination of butanol restricting their use for further butanol recovery.

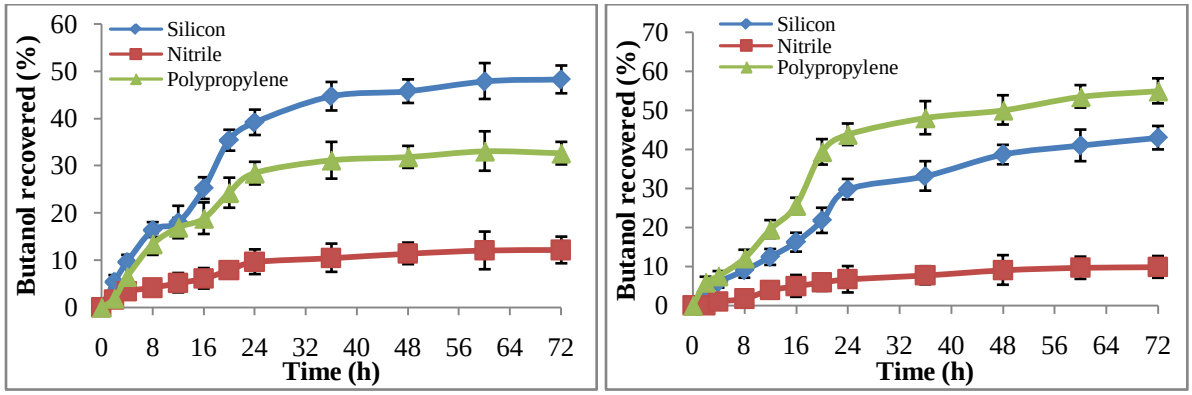
(3) Fermentation broth obtained after liquid-liquid extraction process was further tested for bacterial growth. No growth was found in any of the containers that confirmed the removal of essential nutrients from the fermentation broth, as was observed by Groot et al. (1990).

4.5.2 Membrane-assisted solvent extraction

Initially the experiments were performed with various combinations of extractant and solvent using aqueous solution of butanol to find out the best membrane-extractant combination in terms of butanol recovery. Figure 4.42a, shows the butanol recovery using hexane as the extractant with three different membranes. Among the three membranes used maximum butanol (48%) was recovered with silicone membrane in 72 h. Butanol recovery slowed down after 36 h to approx 45% recovery. It can be said that an equlibrium has reached, and the membrane is no more selective for particular concentration of butanol. Further to increase the butanol recovery some modification in the membrane system is required to increase the driving force for butanol towards the extractant side. As can be see

from Figure 4.42a with nitrile membrane equilibrium has been achieved within 24 h and only 10% recovery has been obtained at this stage. No butanol recovery was observed with latex and hexane, membrane-solvent combination, as this was also concluded by Groot et al. (1990) who found that latex is not permeable to butanol.

With the use of oleyl alcohol as extractant in combination with the polypropylene membrane butanol recovery increased from 31 to 48% within 36 h (Figure 4.42b). With hexane-polypropylene combination equilibrium was achieved in 36 h while for oleyl alcohol-polypropylene this equilibrium time was 60 h with nearly 54% butanol recovery. So on the basis of the results obtained, oleyl alcohol with polyprolene membrane has been selected for further recovery of butanol from the fermentation broth.



(a) **(b)**

Figure 4.42: Membrane-asisted solvent extraction from aqueous butanol solution: **(a)** Hexane, **(b)** Oleyl alcohol

Butanol recovery from glucose as well as cyanobacterial hydrolysate fermented broth was carried out with oleyl alcohol in combination with polypropylene membrane to get maximum recovery (Figure 4.43). Initial butanol concentration in glucose and

cyanobacterial fermented media was 16.19 and 8.873 g/L, respectively. At equilibrium (48 h) 50 and 53% butanol was recovered from glucose and cyanobacterial hydrolysate fermented media, respectively. After this point butanol selectivity through polypropylene membrane decreased resulting in slow rate of separation. By comparing the results obtained for butanol recovery using oleyl alcohol-polypropylene combination, it has been found that approximately 11.0, 8.696 and 4.966 g/L butanol was recovered from the aqueous butanol solution, glucose and cyanobacterial hydrolysate fermented media, respectively.

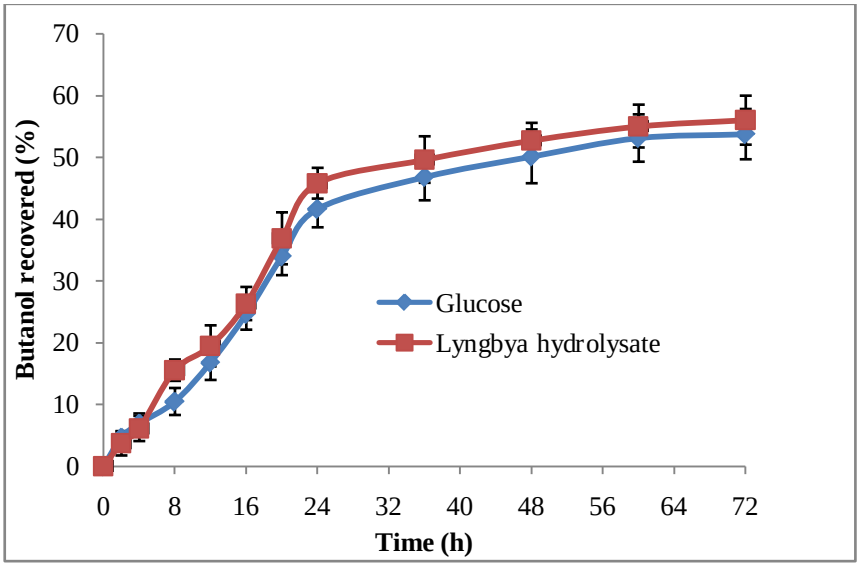


Figure 4.43: Membrane-assisted solvent extraction with glucose and cyanobacterial hydrolysate media using oleyl alcohol and polypropylene membrane

These results show that recovery of a solvent depends not only on the selection of a suitable and efficient extractant and membrane combination but also on the characteristics of the liquid mixture that control the equilibrium point for a particular solvent. During butanol recovery from its aqueous solution a mild swelling of the membrane was observed, that might be due to the prolonged exposure to the solvent, however, with fermented media

swelling was observed along with the membrane smoothness. Presence of various nutrient components and cells in the fermentation broth could be the probable reason for membrane smoothness and resultant lower recovery rate with the same extractant–membrane combination.