SUMMARY AND CONCLUSIONS

For third-generation bioethanol production, seven microalgae were isolated from Ravidas Ghat, Durgakund pond and BHU pond, Varanasi. Two of the seven microalgae were selected based on high starch content. On the basis of rRNA gene profile and phylogenetic analysis, the two microalgae were identified as *Chlorella sorokiniana* (KY348331) and *Tetradesmus obliquus* IIT-BHU BC01 (MH045839). Gene sequences of the isolated strains were submitted at National Center for Biotechnology Information (NCBI), USA. The PCR amplified DNA sequences of two microalgae were found to contain 330 bp and 252 bp for *C. sorokiniana and T. obliquus* IIT-BHU BC01 respectively. The BLAST analysis for both microalgae was performed. The phylogenetic tree of both microalgae was also constructed.

After characterization of the two microalgae, various cell growth studies were performed so as to obtain maximum biomass for bioethanol production. Various parameters affecting the growth of microalgae such as growth media, inoculum size, temperature, and pH were studied. A growth study was also carried out in different types of photobioreactors to evaluate the effect of their design on microalgae cultivation.

Microalgae, *C. sorokiniana and T. obliquus* exhibited higher productivity in BG11 media. Thus, BG11 was chosen as growth media for further studies. The optimum temperatures for the growth of *C. sorokiniana and T. obliquus* were found as 30^{o}C and 25^{o}C respectively. Inoculum size of 5% (v/v) (average cell density about 10^8 cells/ ml) provided the higher productivities in both the cultures. The optimum pH for *C. sorokiniana* and *T. obliquus* was found as pH 7.5. In STR, the specific growth rates of microalgae were directly correlated to the agitation rate. Among the different photobioreactors used, the external airlift photobioreactor provided the higher biomass, carbohydrate and starch productivities with 2% CO_2 (v/v) supply. The external loop airlift photobioreactor has the high surface area for illumination and thus more growth was achieved.

The carbohydrate content of microalgae was increased from 34.0 % to 51.7% and 39.4% to 56.3 % in *C. sorokiniana* and *T. obliquus* respectively within two days in the presence of cycloheximide. However, the carbohydrate content decreased when the microalgae growth were continued for further days. Similarly, the starch content was increased to its maximum from 22.5% to 35.2% and 26.2% to 37.4% in *C. sorokiniana* and *T. obliquus* respectively within two days after cycloheximide treatment. In the presence of cerulenin, the carbohydrate content of microalgae was increased from 34.0% to 45.3% and 39.4% to 48.5 % in *C. sorokiniana* and *T. obliquus* respectively. The starch content increased from 22.5% (w/w) to 29.3% (w/w) and 26.3% (w/w) to 32.3% (w/w) in *C. sorokiniana* and *T. obliquus* respectively. Under the nitrogen, phosphorus and sulphur limitation conditions, the carbohydrate and starch content were increased in both the microalgae. However, nitrogen limitation condition was found as a better strategy in both microalgae for carbohydrate and starch enhancement than phosphorus and sulphur limitation strategies.

After growth study, different pretreatment strategies were evaluated to identify the optimum method for maximum conversion of intracellular carbohydrate to fermentable sugar. The sugar concentration was increased as the concentration of H_2SO_4 increased from 0.25N to 1N. However, above the 1N acid concentration, sugar concentration was decreased as some of the sugar was degraded at high acid concentration. In alkali pretreatment method, the sugar concentration in the suspension after the pretreatment was increased as the concentration of NaOH was increased from 1N to 4 N**.** 4N NaOH provided the higher sugar release. Further increase in NaOH concentration doesn't have any significant change in sugar release.

In hydroxyl radical-aided thermal pretreatment, as the concentration of H_2O_2 increased from 0.06 to 0.18mg/L the sugar released from the microalgae was also increased. However, a further increase in H_2O_2 concentration doesn't increase the sugar release. In the ultrasonication method, the sugar released from the cell was increased as the duration for sonication was increased up to 60 min.

Other strategies included ultrasonication and ultrasonication followed by enzymatic hydrolysis. It was observed that through ultrasonication followed by enzymatic hydrolysis 49.9 and 55.5% of sugar was released from *C. sorokiniana* and *T. obliquus* respectively. Thus, it was the best method for pretreatment of both microalgae.

It was concluded from the study that the highest sugar release was obtained with ultrasonication followed by enzymatic pretreatment among the other pretreatment methods. The ultrasonication followed by enzymatic pretreatment provided 91.3% and 97.4% (g of dissolved carbohydrate per g of total carbohydrate) from *C. sorokiniana* and *T. obliquus* respectively. Therefore the enzymatic pretreatment (coupled with ultrasonication) is the best method for microalgae pretreatment as most of the sugar was released. The microalgae cell breakage study was further confirmed by SEM study.

As suggested by literature bioethanol production can be carried out by two methods. One is Separate Hydrolysis and Fermentation (SHF) and the other is Simultaneous Saccharification and fermentation (SSF). Thus, studies were conducted to evaluate the effect of both methods on the bioethanol production by the two isolated strains of microalgae. Both the procedures were carried out under agitated and non-agitated condition. For carrying out hydrolysis in SHF, acidic and enzymatic (coupled with ultrasonication) methods were evaluated. The bioethanol production was evaluated for each case.

C. sorokiniana produced 29.5 g/L and 40.0 g/L of bioethanol by acidic and enzymatic hydrolysis respectively under non- agitated SHF condition. It produced 40.7 and 53.1 g/L of bioethanol by acidic and enzymatic hydrolysis respectively under agitated SHF condition. When SSF was carried out, the same microalgae yielded 38.0 and 58.1 g/L of bioethanol under non- agitated and agitated conditions respectively.

T. obliquus, on the other hand, produced 33 and 44.3 g/L of bioethanol by acidic and enzymatic hydrolysis respectively under non- agitated SHF condition. It exhibited the production of 45.9 and 59.6 g/L of bioethanol by acidic and enzymatic hydrolysis respectively under agitated SHF condition. The concentrations of bioethanol obtained using *T. obliquus* by SSF were 41.8 and 64.2 g/L under non- agitated and agitated conditions respectively.

Thus, the results show that *T. obliquus* gave maximum production of bioethanol under agitated SSF condition probably due to better substrate utilization and conversion efficiency. Further, the process modelling for bioethanol production by *T. obliquus* was carried out under different fermentation conditions. For this, unstructured models were suggested for both SSF and SHF under agitated and non-agitated conditions using enzymatic hydrolysis. The model could efficiently predict the experimental results in all cases, which was evident by high coefficients of determination (R^2 values). Kinetic parameters for variation of total carbohydrate, glucose, cell mass and ethanol were evaluated. Results show that the values of $Y_{x/s}$, $Y_{p/s}$ and m_s were obtained as 0.041 gg^{-1}, 0.477 gg^{-1} and 0.089 $gg^{-1}h^{-1}$ respectively under agitated SSF condition when bioethanol production was carried out using *T. obliquus*.

Thus, present study proposed the use of *T. obliquus IIT-BHU BC01* for bioethanol production by shaking at 150 rpm under SSF condition using ultrasonication followed by enzymatic hydrolysis by a combination of α- amylase, amyloglucosidase and cellulase.

Future scope of this work includes cultivation of *T. obliquus* on various kinds of wastewater to couple the wastewater treatment and bioethanol production processes as well as optimization of the scale-up conditions for the suggested process.