Bioethanol Production from Carbohydrate-Enriched Biomass of *Chlorella minutissima* by extraction of carbohydrates through hydrolysis and fermentation technology using *Saccharomyces cerevisiae* 182

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***ABSTRACT*: In the present study the potential of bioethanol production using carbohydrate-enriched biomass of the *Chlorella minutissima* was studied. The saccharification of the carbohydrate-enriched biomass, tow acids (H_2SO_4 and HNO_3) was investigated. Each acid were used at three concentrations, 2.5 N, 1 N and 0.5 N and for each acid concentration the saccharification was conducted under three temperatures (40 °C, 60 °C and 100 °C). Higher acid concentrations gave in general higher reducing sugars (RS) yields (%, gRS/gTotal sugars) with higher rates, while the increase in temperature lead to higher rates at lower acid concentration. The hydrolysates then were used as substrate for ethanolic fermentation by *Saccharomyces cerevisiae* 182 strain. The bioethanol yield (%, gEtOH/gBiomass) was significantly affected by the acid concentration used for the saccharification of the carbohydrates. The highest bioethanol yields of 16.32% ± 0.98% (gEtOH/gBiomass) and 16.27% ± 0.95% (gEtOH/gBiomass) were obtained in hydrolysates produced with HNO_3 0.5 N and H_2SO_4 1 N, respectively.**

***Keywords*: *Chlorella minutissima*; bioethanol; fermentation; microalgae; pretreatment; acid hydrolysis.**

1. INTRODUCTION

Microalgae are photosynthetic single cell organisms, composed of many different types of sugars like mannitol, glucose, starch, etc. Some algae are known to have cellulose material in their cell composition. Many algae are known to store oil droplets as their storage food materials for these reason algae are the potential candidates for biofuel like Bioethanol. Fast growth of the world population and rapid development of emerging economies have led to a sharp increase in global energy consumption [1; 2]. The *Chlorella minutissima*, a microalga is also known to accumulate high content of starch. The accumulated starch can be used for production of ethanol [3].

The cultivation of microalgae in medium with reduced nitrogen and phosphorus, important elements for the metabolism of microalgae, allows lipids and carbohydrates to be preferentially synthesized [4]. The increase in carbohydrate concentration of microalgal biomass favors its use as alternative raw material for bioethanol production [5].

Thus, the third generation bioethanol is focused on the use of algae as raw material. Algae can be considered as a suitable feedstock for bioethanol production due to the ease of cultivation and abundance [6]. Algae biomass would make available as advantageous substrate for production of ethanol due to its ubiquitous nature. The fermentation of carbohydrate present in algae biomass, to ethanol is achieved by *Saccharomyces cerevisiae*. Algae are considered to be most important source for the production of clean and renewable energy. The production of bioethanol from algae involves some previous steps before hydrolysis and fermentation which involve a drying process with the aim to preserve the crude extract and prevent the algae from gelling [7] and a size reduction to increase surface area.

Under stress conditions, such as nutrient starvation or high light intensity, some microalgal species accumulate carbohydrates in their biomass, which can increase to a significantly high level (a content of up to 65%) [8]. *Scenedesmus obliquus* is a very versatile microalga as raw material for biofuels production [9].

The *Saccharomyces cerevisiae* produces ethanol as its major fermentation product. The robust yeast can operate in a wide pH range, and can tolerate high levels of ethanol and other inhibitory compounds when compared to other fermentative microbes [10; 11; 12; 13]. Algae metabolic engineering forms the basis for 4th generation biofuel production. It uses recombinant DNA and other biological and bioengineering techniques for directed modification of cellular metabolism and properties through the introduction, deletion, and/or modification of algal metabolic networks to create or enhance biofuel production [14].

Chlorella minutissima, a microalga is also known to accumulate high content of starch. The accumulated starch can be used for production of ethanol. The microorganisms commonly used for ethanol production in fermentations are *Saccharomyces cerevisiae* (yeast) and *Zymomonas mobilis* (bacterium) with limited substrate range. There are needs for development of ethanol producing

improved strains using genetic manipulation so that high efficiency and high ethanol tolerant strains can be produced. The process of anaerobic digestion performance using carbohydrate-enriched biomass of *Chlorella minutissima*. The carbohydrate enrichment is achieved after the cultivation of *Chlorella minutissima* under phosphorus limitation conditions [2; 3].

2. MATERIALS AND METHODS

2.1 Sample Collection: Green Microalgae (*Chlorella minutissima*) were purchased from centre for conservation and utilization of blue green algae (CCUBGA) Division of Microbiology, ICAR, and Indian Agricultural Research Institute New Delhi- 110012. The yeast (*Saccharomyces cerevisiae* 182 strain) purchased from (IMTECH), *Chandigarh*.
The samples were subculture using Modified Zarrouk's medium Composition [15] by making media distilled water. Further subculture it by replacing the media pH 9.5.

2.2 ACID HYDROLYSIS

The dilute acid hydrolysis process. *Chlorella minutissima* was harvested by filtration after settling and bio-flocculation for 30 min. The filtrate biomass was re-suspended in deionized water in concentration of about 12–13 g/L. Aliquots (25 mL) of concentrate *chlorella minutissima* were treating with acids. The acids use was H_2SO_4, HNO_3. For each acid, two concentrations were examine, namely 2.5 N, 1 N, 0.5 N. The treat biomass was close on polypropylene tubes with screw caps and place in a water-bath. For each acids concentration three temperatures were use, namely 40 °C, 60 °C, 100 ^{0}C [15]. 50 ml of identify algal culture was taken in a centrifuge tube. 1:3 ratio of distilled water is added to the sample and centrifuge at 5000 rpm for 10 mins. Algae species were hydrolyzed in dilute 1ml of (0.70% H_2SO_4 /HNO_3) and were hate at 105°C for 6 hrs. Then the samples were neutralizing by adding $CaCO_3$ (2M). Samples were again centrifuge at 5000 rpm for 10 mins. Samples were then evaporating in water bath. Filtration process carried out to filter the extract [16].

2.3 YEAST USED FOR FERMENTATION

The inoculation media for yeast cultivation strain was prepared as 3g/l of yeast extract, 10g/l of peptone, and 20g/l of dextrose in a 1L Erlenmeyer flasks with distilled water [17]. The solution was divided into 250 mL Erlenmeyer flasks everywhere each flask contains 100 mL of solution with cotton plugs autoclaved at 121°C for 20 min. After sterilization, inoculation *Saccharomyces cerevisiae* 182 strain of were transferred into each flask. The preserved flasks were then placed into a rotary shaker at temperature 30°C for 68 h at 150 rpm.

Yeast fermentation was carried out in 250 mL Erlenmeyer flasks containing pretreated microalgae and supplemented with nutrients the hydrolysed biomass of *Chlorella minutissima* was subsequently adjusted to pH 4.5 (± 0.1) with NaOH. To the hydrolysates the following nutrients were added NH_4SO_4 (2 g/L), K_2HPO_4 (1 g/L), KH_2PO_4 (1 g/L), $ZnSO_4$ (0.2 g/L), $MgSO_4$ (0.2 g/L) and yeast extract (2 g/L) and the transfer of yeast inoculums. Due to evaluate the potential of the hydrolysates for bioethanol production, the samples were inoculated with *Saccharomyces cerevisiae*. The flasks were closed with cotton plugs through had been inserted for the removal of CO_2 produced during the experimental period (48 h). The samples were withdrawn after 24 h and for 7 days where, the ethanol content and residual sugars were analyzed. Conversion of the ethanol was produced ethanol and the initial sugar content in the fermentation medium [18].

2.4 REFRACTOMETRIC ANALYSIS OF ETHANOL CONCENTRATION

Abbe's Refractometer is used to measure substances dissolved in water and certain oils. It is based on the principle of light refraction through liquids. Preparations of 10, 20, 30, 40, 50, 60, 70, 80 and 90% ethanol were made by mixing appropriate volumes of absolute ethanol and distilled water. The equal volume of the different ethanol concentrations and the refractive index of each of the ethanol solutions determined. Clean the surface of prism first with alcohol and then with avetone using cotton and allow it to dry. Using a dropper put 2-3 drops of given liquid b/w prisms and press them together. Allow the light to fall on mirror. Adjust the mirror to reflect maximum light into the prism box. Rotate the prism box by moving lever until the boundary b/w shaded and bright parts appear in the field of view. If a band of colors appear in the light shade boundary make it sharp by rotating the compensator. Adjust the lever so that light shade boundary passes exactly through the centre of cross wire. Read the refractive index directly on the scale. Take 3 set of readings and find the average of all the readings [19].

2.5 ETHANOL DETERMINATION BY DICHROMATE TEST

Dichromate solution (1.0ml) and 2.9 ml of the concentrated perchloric acid were added to 100 µL of the sample in a 10 mL Erlenmeyer flask. The solution was homogenized, left to stand for 20 min at 25 ^{0}C and then diluted to the mark with water. The absorbance (A1) was measured at 267 nm against a 3.0M perchlorate aqueous solution. This procedure was applied to a second 100µl volume of the alcohol-free sample as blank (in this case, the solvent was evaporated under vacuum, at room temperature, before adding the reagents). Ao was the new absorbance value measured at 267 nm. Alcohol content of the sample was calculated as [20]:
Volumetric alcohol content (%) = 3(Ao-A1) 11.51D/0.78934v.
Where:
D= dilution factor of the sample
V= volume (ml) of the diluted sample withdrawn for the analysis.

(Ao-A1) = amount of chromium (VI) reduced to chromium (III) in the oxidation reaction of ethanol to acetic acid.

3. RESULT AND DISCUSSION

Chlorella minutissima, a microalga was also known to accumulate high content of starch. The accumulated starch can be used for production of ethanol. Microalgae carbohydrate content usually increases at nitrogen starvation, for the reason that all the carbon structure produced during metabolic process capacity in directed towards carbohydrate and lipid and that increased the carbohydrate content. The microorganisms commonly used for ethanol production in fermentations are *Saccharomyces cerevisiae.*

3.1 Growth of *C. minutissima* Microalgae

Growth of *Chlorella minutissima* was determined by measuring OD of the algae with a spectrophotometer at 670 nm. OD (optical density) was proportional to the density of the algal population in the suspension. An increased growth of *Chlorella minutissima* was achieved by optimizing different parameters, such as temperature, light intensity, type of culture media, and additional nutrients. Light and CO_2 supply was ensured throughout the culture. The effect of a parameter on algal growth was determined by the increase in biomass and sugar content of *Chlorella minutissima* culture (**Figure 1).** The highest OD of *Chlorella minutissima* was observed in the culture grown under culture medium. Duration and intensity of light have a direct effect on photosynthesis and growth of microalgae.

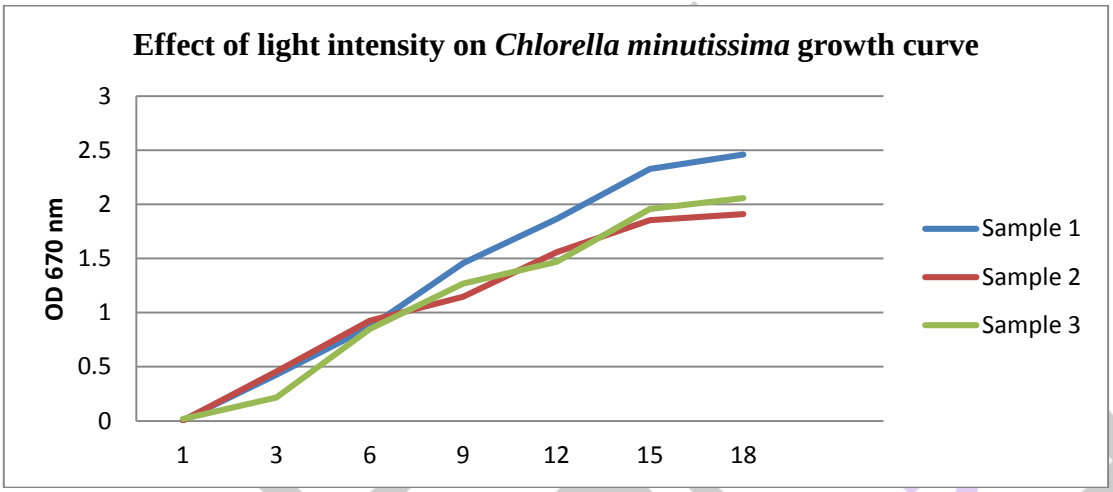

Figure 1. Effect of light intensity on *Chlorella minutissima* growth curve

Growth of *C. minutissima* was monitored by optical density measurement. Although there were studies in which microalgae have less doubling time than 7 days, it is a remarkable growth because the aeration may overcome stress in photobioreactor environment and microalgae may need more time to multiply. According to [21;22] also reported that doubling time of microalgae can be up to 6–7 days in 100 ml of flask. *Chlorella minutissima* cultured in the modified Zarrouk's medium showed an increased OD and carbohydrate concentration in comparison to that cultured in the Zarrouk's medium and BBM medium.

3.2 ACID PRETREATMENT

Acid pretreatment promotes the breakdown of hemicellulose fiber and releasing other types of sugars such as glucose, xylose and arabinose. Followed by acid pretreatment, DNS test was performed **(Figure: 2).** The polysaccharides present in *Chlorella minutissima* algae were indicated by increased colour in DNS test. After applying optimal pretreatment methods, the reducible sugars were prepared for fermentation.

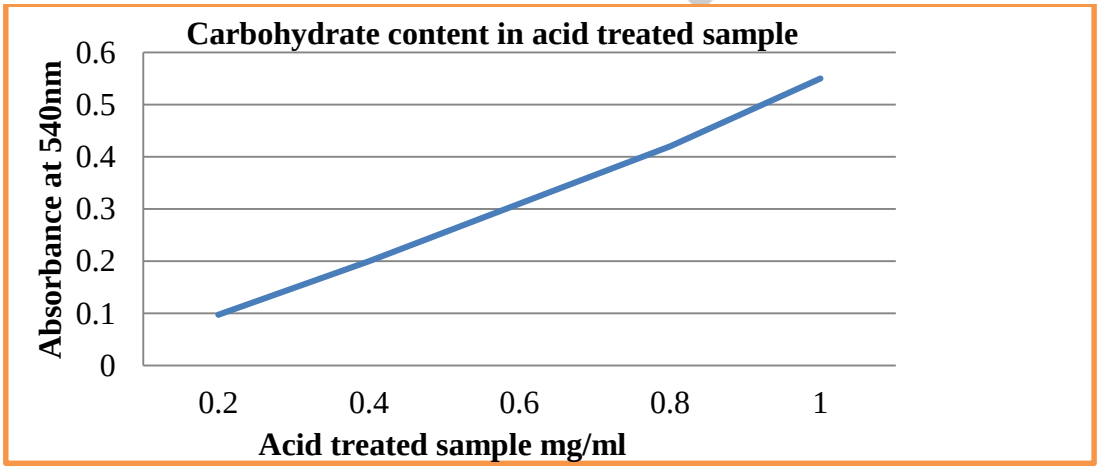

Figure: 2 Carbohydrate content in acid treated sample curve in DNS method

[23] Demonstrated that variations in the concentrations of reactants can cause changes in the amount of released sugars improving or improvement the result of reaction and extraction.

Few studies had revealed that the performance of acid hydrolysis depends strongly to the cell wall rigidity of species [9]. For instance, [24] concluded that the treatment of *Chlorella vulgaris* biomass with hydrochloric acid obtained the highest sugar conversion yield (92.5%); while [25] reported that 69.5 % of sugar yield is obtained by the treatment of *Golenkinia sp.* biomass with 2.0% (w/w) of sulphuric acid at 150 °C for 15 minutes.

Higher concentration of sulphuric acid at 3%, recorded lowest yields of glucose (0.240 g per g) and bioethanol (0.119 g g-1per g) of spent biomass achieved maximum glucose conversion of 0.276 g g-1in 1% H_2SO_4 pretreated unrinsed wheat straw and maximum bioethanol yield of 0.092 g/g from rinsed wheat straw. [26] reported maximum bioethanol yield of 7.2 gL-1 from 15 gL-1of microalgae which is treated using 1% H_2SO_4 for 25 min at 100^0C.

3.3 REFRACTOMETRIC ANALYSIS

The refractive index of the ethanol concentration can be read as a standard curve of the refractive indices of ethanol solutions of different concentrations. The Refractometric method of determining ethanol concentration was both relatively rapid and easy to use and could determine the ethanol concentration to increasing level of ethanol volume. The following result shows the refractive indices of the sample obtained by fermentation diluted to different concentrations **(Figure: 3).**

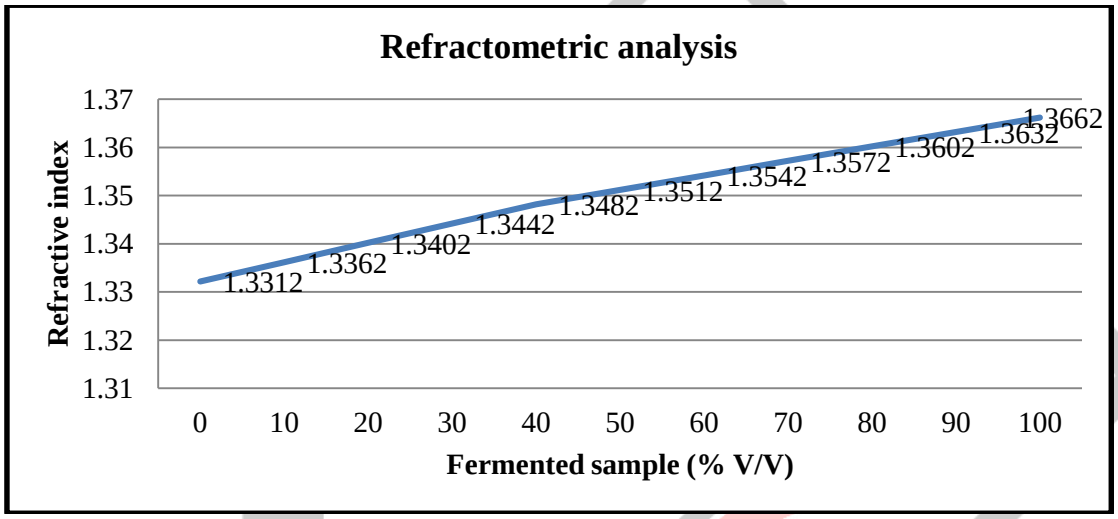

Figure: 3 Refractometric analyses

As described above, the measurement of the refraction index is in the case of beer at least a three-dimensional problem, largely influenced by the variables alcohol concentration and extract concentration. Schild *et al.*, already proposed these influences of alcohol and extract on the refraction index [27]. A common way to take this influence of alcohol into account is the additional measurement of a second parameter (e.g., the specific weight).

3.4 ETHANOL DETERMINATION BY DICHROMATE TEST

To determine the alcohol content, the fermented sample was subjected to potassium dichromate analysis followed by UV spectroscopy. The final volumetric alcohol content obtained was 85%.

The present study potassium dichromate inhibits cell concentration of *P. subcapitata*. The present findings coincide with findings of [28] who reported that Zinc Oxide Nanoparticles inhibit the cell concentration of *Chlorella vulgaris*. Similarly, [29] reported that endosulfan reduced the cell count of treated of diatom Ditylum brightwellii, Prorocentrum minimum and alga *Tetraselmis suecica*. At 4 mg/l concentration inhibit more than 90% growth rate on 1st, 2nd and 3rd day of treatments. The inhibition of growth rate was increased while concentration of potassium dichromate increases. The present finding supported with earlier findings [30; 31].

The previous study, *Chlorella minutissima* was cultivated for bioethanol production, and applications of acid and alkaline pretreatments were conducted before the fermentation, and effects of solution concentration, pretreatment time and pretreatment temperature on bioethanol yield were investigated. Following acid pretreatment was performed at H_2SO_4, HNO_3. *Chlorella minutissima* was cultured in short time with enhanced growth rate and 48% increased quantities of carbohydrates.

The present study refractometric method of determining alcohol concentration was both relatively rapid and easy to use and can determine ethanol concentration to increasing level of ethanol volume. The following result show purity of ethanol increase with increasing concentration. The higher yield of bioethanol was ethanol dichromate test analysis obtained from the 85% higher volumetric alcohol content respectively.

4. CONCLUTIONS

Acid pretreatment hydrolysis was performed with H_2SO_4 and HNO_3. The present study was performed with two acids at three different concentrations (2.5 N, 1 N, and 0.5 N) and for each acid concentration, the hydrolysis was conducted under three different temperatures (40 °C, 60 °C, 80 °C). Highest sugar concentrations were observed at 80, 60 and 40 minutes. *Chlorella minutissima was* cultured in short time with enhanced growth rate and 48% increased quantities of carbohydrates. Increasing acid concentration and process temperature hydrolysis rates are improved. Nevertheless, the acid concentration and the resulting salt concentration of the hydrolysates affect the bioethanol production. At 0.5 N and 1N acid concentrations the hydrolysates could be fermented by *S. cerevisiae* yeast with a bioethanol yield of about 16.26% g EtOH per g of dry *Chlorella minutissima* biomass.

Acid hydrolyzed sample was further processed to obtain fermentable sugars as well as other value added products. *Chlorella minutissima* was cultured in short time with enhanced growth rate and increased quantities of carbohydrates. The Refractometric method of determining alcohol concentration was both relatively rapid and easy to use and can determine ethanol concentration to increasing level of ethanol volume. The result shows an increase in refractive indices with increasing concentration of the fermented sample. A higher yield of alcohol was obtained by dichromate test analysis. 85% of volumetric alcohol content was reported.

Due to the raw materials of lignocellulosic bioethanol are cost effective, renewable and abundant the production of the second-generation bioethanol is costly because of the high recalcitrance of lignocellulosic raw materials. The alternative renewable source of biomass for production of bioethanol, which is grouped under third-generation bioethanol. To a greater extent, the major drawbacks of first-generation and second generation bioethanol are overcome by the algae bioethanol.

5. FUTURE PROSPECTS

- Future research in this area is wide open to various direction and with promising aspects such as,
- To increase the carbohydrate content of microalgae
- Suitable selection of yeast and bacterial strain for improved fermentation
- Selection of potential algal strains
- Isolation and identification of microalgae strains
- Optimization of their growth in various environments
- Most difficult thing in ethanol production is to select a proper strain of microbe, so that they can ferment the algal biomass more effectively.

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