Heterotrophic and mixotrophic cultivation of *Chlorella pyrenoidosa* and the enzymatic hydrolysis of its biomass for the synthesis of third generation bioethanol

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The present study has been carried out with a view of evaluating a green alga *Chlorella pyrenoidosa* as a potential candidate for the production of reducing sugars using an enzyme cocktail of multiple carbohydrates produced on site for the fermentation into bioethanol. The ability of *C. pyrenoidosa* to grow similarly fast on different carbon sources and light has been studied in Fog's medium in heterotrophic and mixotrophic cultures. The high cells densities of mixotrophic cultures demonstrated that the growth-stimulating effects of light and carbon utilization were better as compared to the effects of glucose in heterotrophic condition. Maximum biomass yield of 1.2 g/l was achieved with 1% Glucose and 0.2% KNO_3 after 7 days of incubation at 28^{o}C. The algal biomass was steam pretreated and hydrolyzed by a cocktail of multiple carbohydrases produced by solid state culture of a laboratory isolate belonging to *Aspergillus* sp. on wheat bran exhibiting the yields of 86, 35, 74, 1947, 61, 17000 and 1388 IU/g dry wheat bran for CMCase, FPase, β-glucosidase, xylanase, mannanase, α-amylase and glucoamylase respectively. The enzyme cocktail worked well in the hydrolysis of algal biomass at 50^{o}C and produced total reducing sugars amounting to 429 mg/g of dried biomass revealing carbohydrate conversion efficiency of 96% after 48 h of hydrolysis. The released sugars may be fermented using suitable yeast strains for the production of third generation bioethanol.

HETEROTROPHIC AND MIXOTROPHIC CULTIVATION OF *Chlorella pyrenoidosa* AND THE ENZYMATIC HYDROLYSIS OF ITS BIOMASS FOR THE SYNTHESIS OF THIRD GENERATION BIOETHANOL

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ABSTRACT

The present study has been carried out with a view of evaluating a green alga *Chlorella pyrenoidosa* as a potential feedstock for the production of reducing sugars, using a laboratory produced enzyme cocktail of multiple carbohydrases, for the fermentation into bioethanol. The ability of *C. pyrenoidosa* to grow similarly fast on different carbon sources and light has been studied in Fog's medium in heterotrophic and mixotrophic cultures. The high cells densities of mixotrophic cultures demonstrated that the growth-stimulating effects of light and carbon utilization were better as compared to the effects of glucose in heterotrophic condition. Maximum biomass yield of 1.2 g/l was achieved with 1% Glucose and 0.2% KNO_3 after 7 days of incubation at 28°C. The algal biomass was steam pretreated and hydrolyzed by a cocktail of multiple carbohydrases produced by solid state culture of a laboratory isolate belonging to *Aspergillus* sp. on wheat bran exhibiting the yields of 86, 35, 74, 1947, 61, 17000 and 1388 IU/g dry wheat bran for CMCase, FPase, β-glucosidase, xylanase, mannanase, α-amylase and gluco-amylase respectively. The enzyme cocktail worked well in the hydrolysis of algal biomass at 50°C and produced total reducing sugars amounting to 429 mg/g of dried biomass revealing carbohydrate conversion efficiency of 96% after 48 h of hydrolysis. The released sugars may be fermented using suitable yeast strains for the production of third generation bioethanol.

INTRODUCTION

Energy and environmental issues are among the major concerns faced by the global community today and the sustainable development of society is possible only if these two issues are properly addressed. Biofuels have been recognized as the potential alternatives to petroleum-derived transportation fuels. Of the various biofuels, ethanol has been most acceptable liquid fuel all over the world and is being used as a blend of gasoline in various proportions. It is generally produced from sugar cane, cereal grains or root tubers at the moment and these residues also act as staple foods in various parts of the world so there is a growing pressure to look for some alternative biomass residues which don't compete with human food. Lignocellulosic biomass, in the form of agricultural residues, is also being worked upon as potential candidates for making the way forward in biofuel research. However, the integration of lignin and its crystalline nature make it difficult to hydrolyze and in view of this, algal biomass appears to be a promising feedstock for the production of third generation of alcohol.

Chlorella pyrenoidosa is a widely available micro-algae with several commercial applications for food and nutritional purposes. It has also revealed great potential as future industrial bioenergy producer due to its robustness, high growth rate, and high carbohydrate and lipid contents, and can be cultured under autotrophic, heterotrophic and mixotrophic conditions. However, in presence of organic carbon sources, it produces higher biomass with better carbohydrate contents than its autotrophic cultures. Mixotrophic growth occurs when CO_2 and organic carbon are assimilated in cells simultaneously. The mixotrophy overcomes problems associated with the growth of phototrophic algae viz. light limitation at high cell densities and dark coloured (opaque) waste waters. Even though the biomass is significantly higher compared to autotrophic growth, the cost of glucose could contribute about 80% of the total cost of growth medium making mixotrophic algae cultivation economically unfeasible. Cheap carbon sources such as crude glycerol from biodiesel industry, sugars from industrial and agricultural waste, cellulosic materials and cane molasses offer great promise for the cultivation of mixotrophic algae. Certain species of algae have the ability to produce high levels of carbohydrates instead of lipids as reserve polymers. These species are ideal candidates for the production of bioethanol as carbohydrates from algae can be extracted to produce fermentable sugars. In addition, microalgae-based carbohydrates are mainly in the form of starch and cellulose (with the absence of lignin), are thus much easier to convert to monosaccharides when compared with lignocellulosic materials. Many researchers have reported that the genus of *Chlorella* possess has a high carbohydrate content, especially the species of *Chlorella vulgaris*, with carbohydrates being 37-55% of its dry weight. The carbohydrates in green algae mainly come from starch in chloroplasts and cellulose and hemicellulose on cell walls.

Exploitation of algal biomass for ethanol production is largely dependent upon the availability of a cocktail of carbohydrases due to heterogeneous composition of this feedstock. The cost of such an enzyme mixture is quite high and there is a need to develop a low cost method for large scale co-production of multiple carbohydrases and this is possible if we employ some simple technologies like solid state fermentation (SSF) involving low cost biomass residues for simultaneously inducing a cocktail of carbohydrate depolymerizing enzymes by a suitable microorganism. The aim of present work was to optimize cultivation conditions of *C. pyrenoidosa* and enzymatic hydrolysis of its biomass by cellulase, hemicellulase and amylase complex produced by solid state culture of *Aspergillus niger* NS-2.

MATERIALS AND METHODS

Microorganism and culture medium: *Chlorella pyrenoidosa* was obtained from National Collection of Industrial Microorganism, National Chemical Laboratory, Pune. This was maintained and cultivated in Fog's medium having the following composition (mg/L): $MgSO_4.7H_2O$ (200), K_2HPO_4 (200), $CaCl_2.H_2O$ (100), Fe-EDTA solution (5 ml) prepared by adding 0.00745g/l Na_2EDTA and 0.00557 g/l $FeSO_4.7H_2O$; H_3BO_3 (286), $MnCl_2.4H_2O$ (181), $ZnSO_4.7H_2O$ (22), $Na_2MoO_4.2H_2O$ (39), $CuSO_4.5H_2O$ (8), KNO_3 (200), pH 7.5.

Cultivation conditions:

Heterotrophic: Heterotrophic cultivation of *Chlorella pyrenoidosa,* was carried out in a 250 ml Erlenmeyer flask containing 150 ml medium, supplemented with 1% glucose at 28°C with continuous shaking (130 rpm) under dark conditions at 28°C.

Mixotrophic: Mixotrophic cultivation was carried out in a 250-ml Erlenmeyer flask containing 150 ml medium, supplemented with 1% glucose at 28°C with continuous shaking (130 rpm) under continuous illumination with fluorescent tube light.

Optimization of cultural conditions for cultivation under Mixotrophic conditions: The cultural conditions for mixotrophic cultivation were standardized by varying the carbon and nitrogen sources and the initial pH of Fog's medium. The effect of carbon sources was studied by replacing glucose with either of the other carbon sources including fructose, galactose, arabinose, xylose, carboxymethyl cellulose, inulin, starch and maltose at a level of 1%. The effect of nitrogen sources was studied by substituting KNO_3 with either of the other nitrogen sources including urea, ammonia, nitrate, nitrite, yeast extract and soybean meal at a level of 0.2%. The effect of pH was studied by varying the initial pH of the medium from 2.0 - 11.0. As glucose produced the maximum biomass, the effect of its concentration was also studied on biomass yield.

Measurement of cell count and dry weight: For cell count, 1ml of algal suspension was withdrawn and then direct microscopic count was determined using Neubauer hemocytometer and was represented as cell density per ml. Growth were also determined as dry cell weight after centrifuging the cultural broth at 5000 rpm for 15 min, washing twice with double distilled water and then drying the cell pellet at 70°C till constant weight.

Measurement of specific growth rate: The specific growth rate(μ) was calculated by the equation:

$$\mu\,(g/l) = \frac{1}{t}\ln\frac{Xm}{X0}$$

where *Xm* and *X*0 are the concentrations of biomass at the end and at the beginning of a batch run, respectively, and *t* is the duration of the run.

Determination of carbohydrate content of biomass: Total carbohydrate content of microalgal biomass was determined at the beginning of stationary growth phase by anthrone reagent method.

Production of multiple carbohydrases under solid state fermentation: The co-production of Cellulases, hemicellulases and amylases was carried out by the Solid state culture of *Aspergillus niger* NS-2, already available in our laboratory. Solid state fermentation of was carried out in 250ml Erlenmeyer flasks containing 5.0 g of wheat bran with distilled water to obtain final substrate to moisture ratio of 1: 1.5. Flasks were autoclaved at 121°C, cooled and inoculated with 5 discs (7mm) of 72h old culture of *A. niger* NS-2 grown on PDA followed by incubation at 30°C for 4 days. The cellulase enzymes were extracted by adding 100 ml of tap water to the flask keeping on a water bath shaker at 37°C (150 rpm) for 30-45 min. Thereafter, the contents of the flask were filtered through metallic sieve and the solid residue was pressed to release left over liquid. The volume of liquid obtained was measured and centrifuged at 10000 rpm for 10 min, the clear supernatant was used as a source of enzymes.

Enzyme assay: The enzyme supernatant was analyzed in terms of various components including endo-ß-1,4-glucanase (CMCase) , exo-ß-1,4-glucanase (FPase), ß-glucosidase, ß-1,4-xylanase, mannanase, α-amylase, glucoamylase using the standard procedures. The enzyme activities for various components except α-amylase have been expressed in terms of number of moles of end product formed from carboxymethyl cellulose, whatmann filter paper, salicin, xylan, locust bean gum and soluble starch respectively, in one minute at 50°C. α-amylase activity has been expressed as equivalent to the reduction of starch-iodine color by 10% in 10 min at 50°C.

Enzymatic hydrolysis of C. *pyrenoidosa* biomass: One gram of oven dried algal biomass was suspended in 5 ml of 0.1 M acetate buffer (pH 4.5) taken in 100 ml Erlenmeyer flask and steam pretreated at 121°C for 20 min. This was then cooled and supplemented with 3 ml of crude enzyme cocktail from solid state cultures of *A. niger* NS-2 with enzyme to substrate ratio 12 U CMCase, 5 U FPase, 11 U β-glucosidase, 290 U xylanase, 9 U mannanase, 2550 U α-amylase, 69 U glucoamylase /g substrate. and 10mg/ml of chloramphenicol. Total volume in the flask was made to 10 ml with 0.1 M acetate buffer, pH 4.5. The mixture was incubated at 50°C in a water bath shaker at 150 rpm for 48 h and the samples were withdrawn at regular intervals of 24 h, centrifuged at 10,000 rpm for 10 min and supernatant analyzed for total reducing sugars.

Carbohydrate conversion as percentage of the theoretical reducing sugars yield was calculated from the equation which involves the transfer of carbohydrates to sugar $(C_6H_{10}O_5)_n + {}_nH_2O \rightarrow (C_6H_{12}O_6)_n$ was computed by using the following formula:

Carbohydrate conversion (%) = [Reducing sugars]/ (1.11× f × [biomass]) × 100

where [Reducing sugars] is the total reducing sugar concentration (g), [Biomass] is dry algal biomass concentration used in enzymatic hydrolysis; f is the carbohydrate fraction (in terms of glucose) in dry biomass (g/g) and 1.11 is the factor that corresponds to the mass balance of the conversion of polysaccharides to sugars.

RESULTS AND DISCUSSION

The present study has yielded a process for laboratory cultivation of a microalgal strain of *Chlorella pyrenoidosa* under heterotrophic and mixotrophic conditions and bioconversion of carbohydrates of this biomass into fermentable sugars using this in-house produced enzyme system yielding total reducing sugars of 429mg/g of dry biomass.

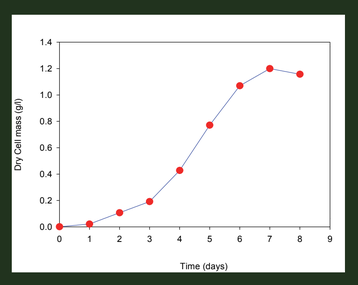

Fig. 1. Growth kinetics *of C. pyrenoidosa* in batch culture.

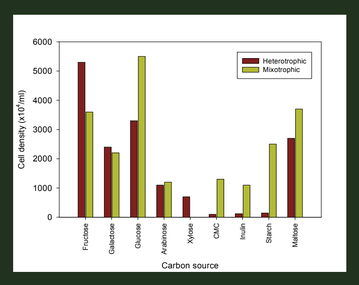

Fig. 2. Effect of carbon sources on growth of *C. pyrenoidosa.*

Table 1. Effect of various types of carbon and nitrogen sources on biomass concentration and specific growth rate.

Variables	Biomass yield (g/l)		Specific growth rate (μ)	
	Mixotrophic	Heterotrophic	Mixotrophic	Heterotrophic
Fructose (1%)	0.78	1.15	0.29	0.32
Galactose (1%)	0.48	0.52	0.26	0.27
Glucose (1%)	1.20	0.72	0.32	0.29
Arabinose (1%)	0.26	0.24	0.22	0.22
Xylose(1%)	0.19	0.15	0.21	0.19
CarboxyMethyl Cellulose (1%)	0.28	0.02	0.23	0.07
Inulin (1%)	0.24	0.02	0.22	008
Starch (1%)	0.54	0.03	0.27	0.09
Maltose (1%)	0.80	0.58	0.29	0.13
Urea (0.2%)	1.09	1.02	0.31	0.31
Ammonium persulphate (0.2%)	0.87	0.39	0.30	0.25
Nitrate (0.2%)	1.20	0.91	0.32	0.30
Nitrite (0.2%)	1.17	0.87	0.32	0.30
Yeast extract (0.2%)	1.04	0.69	0.31	0.28
Soybean meal (0.2%)	1.04	0.50	0.31	0.26

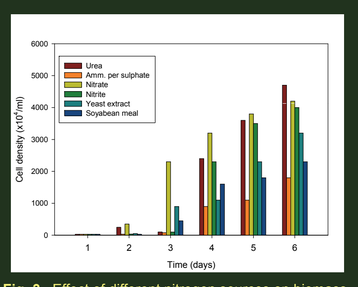

Fig. 3. Effect of different nitrogen sources on biomass production under heterotrophic cultivation.

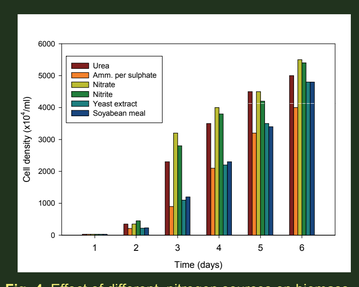

Fig. 4. Effect of different nitrogen sources on biomass production under mixotrophic cultivation.

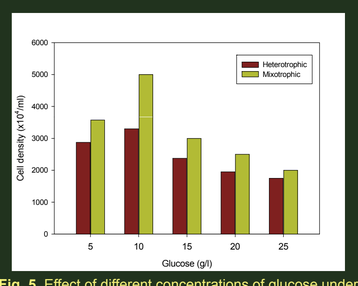

Fig. 5. Effect of different concentrations of glucose under heterotrophic and mixotrophic cultivation.

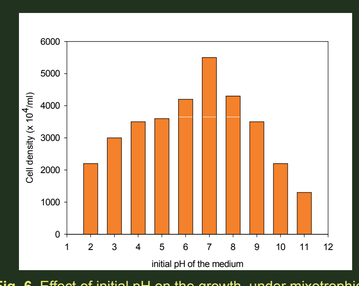

Fig. 6. Effect of initial pH on the growth under mixotrophic cultivation.

Table 2. Pattern of total reducing sugars formation during enzymatic hydrolysis of algal biomass

Time (h)	Total reducing sugars (mg/g) of biomass	Carbohydrate conversion efficiency * (%)
0	227.04	51.0
24	357.64	80.5
48	429.00	96.5

*on the basis of total carbohydrate content of 40.3 on dry weight basis