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**TOTAL BIOMASS UTILIZATION OF *Spirogyra singularis* FOR RENEWABLE  
BIOFUEL PRODUCTION**

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**ABSTRACT**

In the coming era, there is an endless energy requirement accomplishing industrial development and population growth. The cost of energy has shot up to a new peak due to the excessive use of fossil fuels. Microalgae, with a rapid growth potential, having thousand times more oil magnitude than oil crops assures the most pragmatic renewable source of biomass for biofuel production. In the current study, various species of algae from fresh water bodies in and around Pune, India were isolated and identified. A commonly available filamentous alga, *Spirogyra singularis*, being dominant among the algal flora and rich in fat, carbohydrate and protein contents was selected for biochemical and physiological characterization. Algal oil was extracted using organic solvents to be transesterified into biodiesel. The de-oiled biomass was subjected to pretreatments followed by enzymatic hydrolysis and subsequently fermented by *Saccharomyces cerevisiae* to produce bioethanol. The isolated species yielded 20 % crude oil while the rate of conversion of polysaccharides to alcohol was 36.9 %. Enzymatic saccharification efficiency of algal biomass was enhanced by coupling with pretreatments. Acidic pretreatments yielded improved hydrolytic activity as compared to alkaline pretreatments. A cocktail of enzymes like amylase, glucoamylase, cellulase and pectinase were more effective than individual enzymes for hydrolysis of carbohydrates. The leftover biomass, rich in protein content is proposed to be used as an animal feed in the future studies. In India, research on microalgal based fuel production needs to be amplified to commercialize the process with economic feasibility.

**Keywords: Microalgae, Renewable, Biofuel, Bioethanol**

## INTRODUCTION

Economies around the world with their expanding population are facing land, food and fuel shortages besides running in a race to encounter monstrous pollution. Thus, world is sighting towards microalgae which is emerging as a green remedy to all such challenges [1]. Microalgae feedstock is one of the most feasible, sustainable, renewable alternatives for large scale biodiesel production for complete replacement of petro-diesel. [2]. Microalgae with higher multiplication rates have the potential to yield tremendously high oil magnitudes in comparison with traditional oil crops cultivation [3, 4]. In context of biofuel production, microalgae do not compete for land and food as much as in case of food crops [5]. Phycoremediation [6, 7, 8] enlightens role of microalgae in curbing water pollution and carbon dioxide consumption, thus proves its contribution in reducing carbon footprints, earning carbon credits, leading to reduction in Green House Gases effect and Global Warming [4]. Looking towards the increasing demand and depleting levels of fossil fuels, the US Energy Information Administration has forecasted that global oil consumption will rise by about 60% by 2020 (Institute for Analysis of Global Security). In India, a 20 % blending of biodiesel and bioethanol by

2017 is been proposed, but at present, the supply of biofuel outruns its demands.

*Spirogyra*, a common freshwater filamentous green algae belonging to the family *Zygnemataceae*, produces oxygen in the presence of sunlight and appears as a green accumulated mass floating on the surface of water.

The current study explores the potential of *Spirogyra singularis* for extraction of oil to be used for production of biodiesel and further utilizing the defatted algal biomass residue, rich in carbohydrates for bioethanol production [9, 10] with subsequent pretreatments, enzymatic hydrolysis and fermentation by *Saccharomyces cerevisiae*. The left over residual biomass rich in proteins is further being tried as a constituent of animal or poultry feed [11].

## MATERIALS AND METHODS

### Sample Collection

Microalgal samples were collected manually in sterile transparent polyethylene bags/bottles with the help of sieving nets from various localities of Pawana River, near Pimpri- Chinchawad township of Pune, Maharashtra in early spring.

### Isolation, Identification and Cultivation of Microalgae

From the collected microalgae samples, *Spirogyra* species were isolated by serial

dilution in flasks using enriched medium and then transferred to synthetic media. The samples were made free of contamination by providing washings with saline, tween 80 and distilled water. Biotin was added to eradicate algal grazers. The genus and species were identified under light microscope based on morphological studies and mode of reproduction with reference to the published algal monographs [12, 13]. Cultures were maintained in Bold Basal Medium [14] and Bristol Medium (H.C. Bold's modification of Bristol's recipe) at ambient temperature. An enrichment medium was made by adding soil extracts to distilled water. The artificial /synthetic medium was made by addition of media chemicals. Growth rate was determined based on cell density which was measured as PCV and dry cell weight [15].

Spirogyra is composed of unbranched filaments made up of cylindrical cells placed end to end, ranging from 10 to 100 micrometers in diameter. One or more ribbon shaped spirally arranged chloroplasts are present in the cytoplasm. Each chloroplast bears pyrenoids. A single nucleus is stranded in the centre of the vacuole by cytoplasmic strands. Asexual reproduction in algae takes place by fragmentation and sexual reproduction by conjugation. The microalgae species was identified as *Spirogyra singularis* based on

its morphological characteristics (Figure 1) [12].

#### Characterization of Algal Biomass

The solid contents were determined by drying the algal sample in a hot air oven at 60 °C for minimum 8hrs till constant weight was obtained [16]. Carbohydrate contents were determined by Phenol Sulphuric Acid Method, [17] soluble protein contents were estimated by Folin-Lowry's method, [18] hexoses were determined by DNSA method, [19] ash contents were determined in a muffle furnace [16] and the pigment identification was done with the help of TLC [20].

#### Oil Extraction

Fat/ Oil contents were extracted with a mixture of organic solvents such as hexane and diethyl ether (1: 1), chloroform and methanol (1: 1), ethanol and diethyl ether (1: 1) by refluxing in a soxhlet apparatus for 3hrs [15, 21, 22]. The extracted oil was made free of solvents by evaporation. The percent oil recovery was calculated by the formula as:

$$\% \text{ Oil Content} = \frac{\text{Mass of Oil Obtained}}{\text{Mass of Dried Algal Biomass}} \times 100$$

The carbohydrate contents of defatted residual algal biomass after oil extraction were subjected to pretreatment and enzymatic hydrolysis for further conversion to bioethanol.

### **Pretreatment, Enzymatic Hydrolysis and Fermentation of Defatted Algal Biomass**

The pretreatments enhance the hydrolysis of complex polysaccharides in algal biomass by decreasing the particle size, loosening the intact cell wall and thus, making the substrate more accessible for enzymatic attack. Pretreatments were carried out by soaking finely grinded microalgal samples in Alkali (1N NaOH) / Acid (1N HCl) and heating at 90 °C in hot air oven for 3 hrs. The samples were allowed to cool and incubated with bacterial alpha-amylase enzyme for starch liquefaction. To all the liquefied samples, a cocktail of crude commercial enzymes containing glucoamylase, cellulase, and pectinase were added for enzymatic hydrolysis. Also, each of these enzymes was added individually. After hydrolysis, determination of hexoses was done by DNSA method at different intervals of incubation. The fermentation of saccharified hydrolysate was carried by *S.cerevisiae var ellipsoideus* for 96 hrs at 25 °C to convert free hexoses into alcohol. The strain of *S. cerevisiae* was procured from culture collection of the institute [9]. A 5 % inoculum of yeast in YPD medium grown for 24hrs at 25 °C was transferred to the fermentation medium containing algal biomass hydrolysate with 0.5 % corn steep liquor and 0.5 % ammonium sulphate. Alcohol contents were determined by

potassium dichromate method [23]. The commercial enzymes were procured from Maps Enzyme Ltd. Ahmadabad. Heat stable Bacterial Alpha amylase, Palkozyme HT Plus (Standard Activity 40000 MAU/ML); Glucoamylase, Palkodex (Standard Activity 45000 MGU/ML); Cellulase from Trichoderma rezei, Palkosoft Super 720 (Standard Activity 26000 MCU/ML); Pectinase, Palkoscour APCL (5000 MPU/Gram).

## **RESULTS AND DISCUSSION**

### **Media Optimization**

The collected microalgal flora consisted of, *Spirogyra* sp., *Chlorella* sp., *Euglena* sp., algal grazers and rotifers. Amongst it, *Spirogyra* sp. was dominating which consisted of *S. singularis*, *S. cirumlineata* and *S.colligata*. *S. singularis* was considered in the present studies due to its abundance. The suitable season for the growth of *Spirogyra* was spring. In other unfavorable seasons, the filament gets converted to resistant spores. The BBM medium was found to be most suitable for its growth followed by D 11 medium (Graham 1984) [14]. Bristol Medium and BG 11 medium (Allen and Stanier 1968, Rippka et al. 1979) [14] did not prove to be effective for its growth. Feeding the culture with carbon dioxide initially accelerated the growth rate of the culture.

### Biochemical Composition

The 3 strains/isolates of *S.singularis* viz. st.1, st.2 and st.3 were isolated from 3 different water bodies around Pune. *Spirogyra* st.1 was collected from Tunnel, *Spirogyra* st.2 was collected from near Pawana riverside, at chinchwad area and *Spirogyra* st.3 was collected again from near Pawana river side, at ravet village, Pune and was characterized for their solids, moisture, fat, carbohydrates, protein and mineral contents (**Table 1**).

*Spirogyra* st.3 contained significant amount of carbohydrates along with the fat content as compared to *Spirogyra* st.2 and *Spirogyra* st.3 and thus was selected for this study. The fat and carbohydrate contents of this species were found to be more than that reported in earlier studies [15].

The beta carotene was the predominant pigment present in the algae as determined by TLC technique. It was confirmed by the Rf value which was calculated to be 0.89 [24].

### Solvent System

Three solvents systems were used for extraction of oil from algal cell viz. Methanol & Chloroform (1:1), Ethanol & Diethyl Ether (1:1) and Hexane & Diethyl ether (1:1). Methanol & Chloroform showed maximum oil extraction yields with three folds increase compared to that of Hexane & Diethyl ether solvents (**Figure 2**). The

algal biomass was subjected to ultrasonication prior to solvent extraction increased the efficiency of algal oil extraction. Similarly, magnetic stirring also enhanced the oil extraction but was found to be less effective as compared to ultrasonication.

### Saccharification of Algal Biomass

The different concentrations of algal biomass; 5, 10 and 15% were used for the saccharification process. It was observed that 10% concentration was found to be optimum, having maximum saccharification at 96 hrs of incubation using a cocktail of enzymes as compared to the individual enzymes [25]. The 15 % concentration of sample resulted into lower saccharification due to the increase in the viscosity, thus decreasing the accessibility of enzymes on a larger scale and making the sample difficult to handle [26, 27] (**Figure 4**).

### Pretreatment, Enzymatic Hydrolysis and Fermentation

#### Pretreatments

The pretreated samples with acid/alkali have shown higher rate of saccharification in comparison to untreated samples. Amongst the pretreatments, acid treatments have shown better saccharification results (**Table 2**).

Pretreatments coupled with enzymatic hydrolysis have shown a higher saccharification rate up to 64 % as

compared to only pretreatment or enzymatic hydrolysis alone. On pretreatment with acid, a certain amount of sugar was produced due to the acidic hydrolysis of carbohydrates in samples. Higher saccharification rate is the result of greater availability and exposure of substrate to enzymes (Table 2). Mixture of enzymes raised the saccharification rate to many folds producing significant levels of reducing sugars as compared to action of individual enzymes (Table 3).

**Fermentation:** The maximum bioconversion rate of carbohydrates into alcohol was found to be 39% in *Spirogyra* st.3 with acidic pretreatments [28]. Harun et.al explored the importance of alkaline pretreatment for bioethanol production but in the present study the acidic pre treatments were found to be more effective [25, 26].

De-fatted samples proved to contain high contents of carbohydrates and simultaneously produced maximum alcohol as compared to full fatted samples (Figure 3). The trial of left over biomass, rich in protein content (14.7 % approx.) is in progress to be used as an animal/poultry feed so as to utilize the whole algal biomass, to make the process economically feasible.

### Data Analysis

The data for comparison of solvent systems, pretreatments and saccharification along with the fermentation of hexoses to alcohol was analyzed for T Tests with the help of statistical software. Standard error, standard deviation and variance were calculated. Probability,  $P < 0.05$ , in all the data sets with respect to with  $\alpha$ , proved that the results were significant.

**Table 1: Biochemical Composition\* of the *Spirogyra singularis* Collected From 3 Different Sites, Denoted Here as *Spirogyra* st. 1, 2 and 3**

Isolates	Solids	Fat	Carbohydrate	Free sugar	Protein	Mineral
<i>Spirogyra</i> st.1	13.75	11.3	64	0.6	12.00	5.2
<i>Spirogyra</i> st.3	28.45	7.7	62	0.18	9.00	6.2
<i>Spirogyra</i> st.3	11.81	20.0	65.72	1.26	12.00	2.2

NOTE: \* Values in %

**Table 2: Reducing Sugar\*\* After Pretreatment with Alkali and Acid, Enzymatic hydrolysis only and Enzymatic Hydrolysis Combined With Pretreatment**

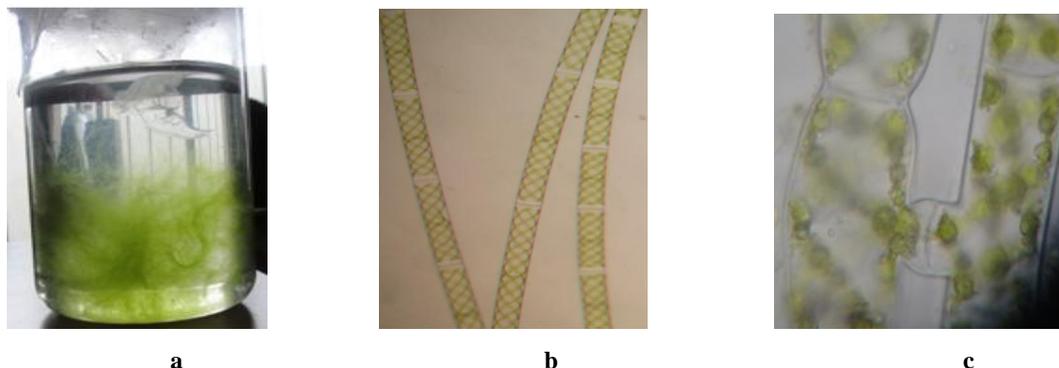
Isolates	Pretreatment with Alkali/ Acid		Enzymatic Hydrolysis (Without Pre-Treatment)	Enzymatic Hydrolysis With Pre-Treatments Alkali /Acid	
<i>Spirogyra</i> st.1	16	17.8	27.7	47.5	52.6
<i>Spirogyra</i> st.2	14.3	16.3	26	32.1	36.7
<i>Spirogyra</i> st.3	18	21.1	32	53.1	64.0

NOTE: \*\* Values in %

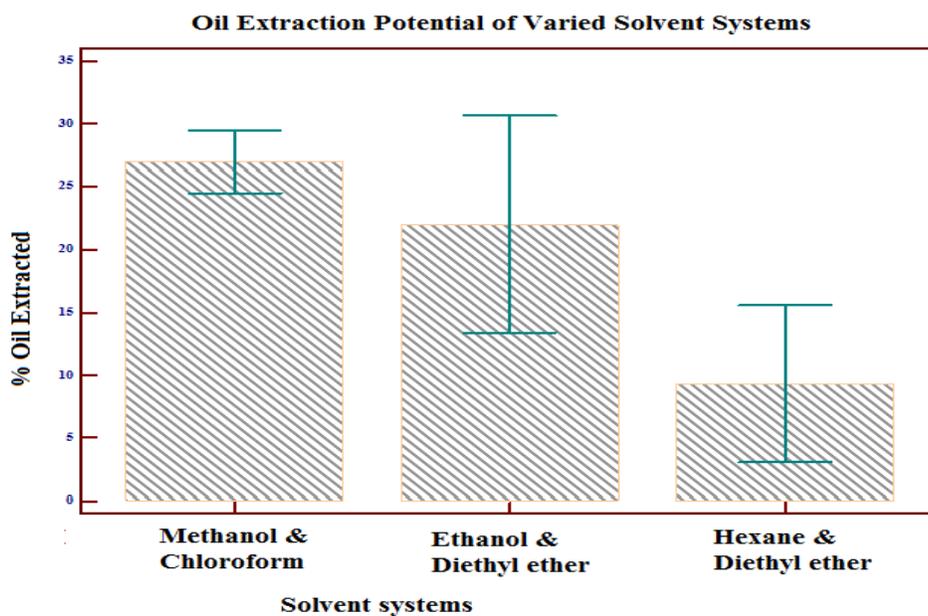
**Table 3: Reducing Sugar<sup>†</sup> Obtained After Alkali/Acid Pre-Treatment's with Individual Enzymes and Their Mixture**

Isolates	Alpha amylases & Glucoamylases Alkali / Acid		Cellulases Alkali / Acid		Pectinases Alkali / Acid		Mixture of enzymes Alkali / Acid	
	<i>Spirogyra</i> st.1	26.6	28.42	14.2	15.3	5.2	5.9	47.5
<i>Spirogyra</i> st.2	21.1	24.4	8.8	9.4	2.7	3.9	32.1	36.7
<i>Spirogyra</i> st.3	30.5	33.2	18.6	20.2	7.9	9.5	53.1	64.0

NOTE: <sup>†</sup> Values in %



**Figure 1: Depicts Growth, Morphology and Reproduction by Conjugation in Spirogyra sp. a) Growth of Culture in BBM Medium; b) Morphology of Filaments (40 x Magnification); c) Sexual Reproduction by Conjugation (100 x Magnification)**



**Figure 2: Oil Extraction Potential of Different Solvents (1:1)**

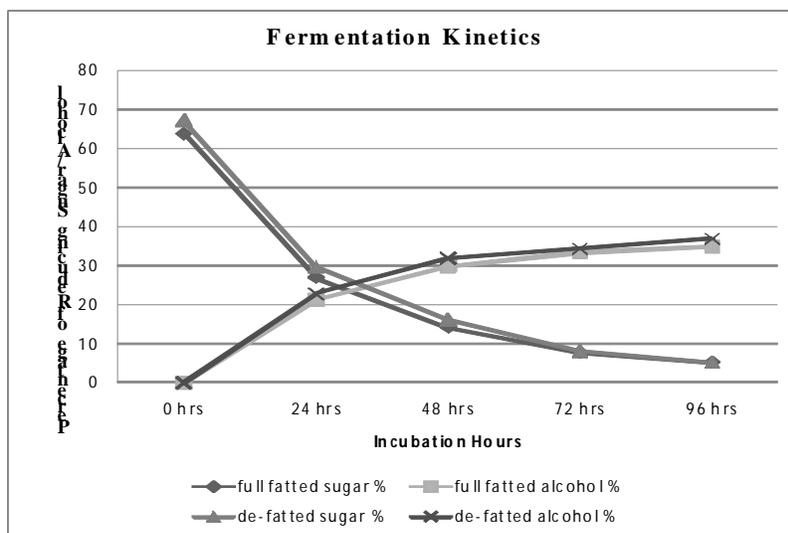


Figure 3: Fermentation Kinetics From Full Fatted and Defatted Algal Biomass

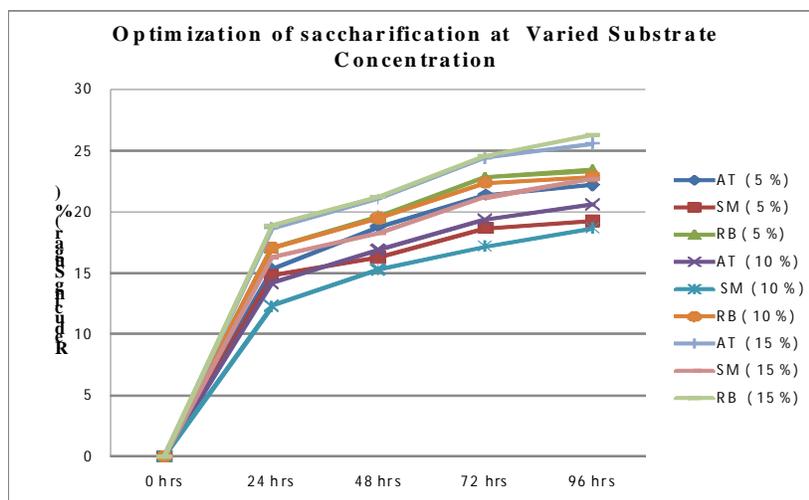


Figure 4: Optimization of Saccharification at 5, 10 & 15 % of Substrate Concentration at 0, 24, 48, 72 and 96 hours of Hydrolysis

**CONCLUSION**

*Spirogyra* sp. is easily available and grown at varied latitudes in India under their regional climatic conditions. This study deals with the maximum utilization of *Spirogyra* sp. biomass for two biofuel productions, biodiesel and bioethanol and

further using the residual left over for animal /poultry feed. The study proves that after the proper optimization of various parameters involved in the process, algal sample can be saccharified, fermented and optimum yields of alcohol can be achieved. The same process can be adopted for pilot

plants in India. The resultant crude oil can be converted into biodiesel and used as a biofuel in blending with diesel and the crude bioethanol produced can be further distilled and utilized as biofuel by blending with Petrol.

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