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Phycoremediate Terrestrial Microalgae Serves Source for Bioethanol

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A B S T R A C T

Screened the all collected microalga samples from in and around the Western Ghats of Karnataka state for the bioethanol and biomass production. 62 terrestrial microalgae were collected from the fresh water bodies and only 5 samples used for bioethanol production on the basis of biochemical characteristics, were used for multiplication and fermentation study. Multiplication of algae was done by open ponds production system. Bioethanol production study was attempted by anaerobic fermentation using defatted algal residues. The hydrolyzed residue was used as substrate for fermentation using 5% of *Saccharomyces cerevisiae* inoculum. In fermentation setup biochemical components *viz.*, percent alcohol, total soluble sugars, total reducing sugars, proteins, pH, organic acids, microbial load and brix were estimated and used for standardization of fermentation time. The maximum bioethanol (61.76±1.12%) was observed at 21st days after fermentation in *Rhizoclonium hieroglyphicum* and minimum (57.51±0.99) in the *Spirogyra sp.* Based on study conducted, it can be suggested that terrestrial microalgae can be used for bioethanol production by growing in polluted or waste water.

Introduction

Microorganisms contain a wide variety of carbohydrates, both monomers and polymers. Different classes of algae produce specific types of polysaccharides, green algae produce starch as an energy store, consisting of both amylose and amylopectin. Microalgae can able to adapt new environments through the de novo synthesis and recycling of fatty acids to maintain the membrane characteristics (Williams and Laurens, 2010). Biomass energy sources have three generations development steps; first generation biofuel feedstocks which were derived from the food crops, such as sugarcane, corn, soyabean and

wheat. Paddy straws and switch grass were non-food raw materials become the second generation biofuel feedstocks. The third generation biofuel feedstock is algae. Algae will probably play an increasing role in the sustainable energy use field in the near future (Patil *et al.,* 2008). The sugars produced in the algae can be converted to lipids, proteins and carbohydrates, which are as the materials can be converted to the biofuel (Field, 1998). The algal carbohydrates are also of great importance for bioethanol production and some algae species possess more carbohydrates contents than lipids (Khan *et al.,* 2016). By using a technique microalgal sugar are relieved and simply fermented to ethanol using fermentative microorganisms (Singh *et al.,* 1995). Terrestrial microalgae collected from Western Ghats were grown very quickly to reduce the pH of waste water from alkaline to neutral or acidic and also reduces the TDS content of water so it can be used for bioremediation purpose of alkaline water (Manoj *et al.,* 2018). In the present study the potential of bioethanol production using carbohydrate-enriched biomass of terrestrial microalgae was done. The efficiency of hydrolyzed microalgal biomass (substrate) for bioethanol production using wine yeast was examined and discussed. Productions of bioethanol are the aim of present investigation.

Materials and Methods

Collection of samples

The experiments were carried out in the laboratory of Agriculture Biotechnology (Agriculture College Hassan), University of Agricultural Sciences, Bengaluru, Karnataka and also in the laboratory of Biochemistry and Biochemical Engineering, JIBB, SHUATS, Allahabad, U.P. India. Algal samples were collected in sterile plastic bags from different water bodies in and around the western ghats of Hassan and Kodagu district of Karnataka state. In these experiments collected 62 samples of terrestrial microalgae from rivers and ponds of fresh water bodies in and around western ghats and five were screened for bioethanol production. *Oedogonium sp.*, *Rhizoclonium sp.* and *Spirogyra* collected from the pond of Gadanahalli, Dandiganahalli and Hoovnalli villages, respectively (Hassan district), *Klebsormidium sp.* and *Rhizoclonium hieroglyphicum* collected from the rivers of Ikola and Kaggodu villages (Kodagu district). Collected algae were used for mass multiplication and were subjected to anaerobic fermentation after acid hydrolysis.

Multiplication of microalgae culture

Microalgae cultivation was done in plastic trays and open pond system with 50% shade. Collected algae were kept for multiplication under direct sunlight in plastic tray using 20% source water and 80% normal tap water and kept it under direct sunlight. Evaporation was controlled by fixing 50% shade net and maintaining the water level by adding extra normal water for every 5 days interval (Figure 1).

Open pond production method for multiplication of terrestrial microalgae

Small ponds were opened in the dimension of 4×5×2 feet and covered with low density plastic sheets to prevent the leaching of stored water for the culture of microalgae. R.O. residual dispense water whose pH 8.4 and urban waste water was used. In these two types of water six identified microalgae samples were grown. To reduce the errors in open field 3 replication was done. Provided 50% shade with green shade net to prevent the evaporation of water from ponds. In the small ponds for two treated water 10grams of wetted terrestrial collected microalgae was added and once in two days agitation was done manually

for equal distribution of microalgae in the pond (Figure 2).

Harvesting and drying of microalgae

A conventional filtration process is most appropriate for harvesting of relatively large size microalgae. In this method algae grown in small open ponds and plastic trays were filtered by conventional filtration method using sieves whose size 0.2mm. This method easily removes the excess water from the microalgae. It was an easy method for harvesting the large quantity microalgae (Mohn, 1980). Harvested microalgae were dry directly exposed to sun light for removing the excess water content and extracted lipid from these algae. Defatted algal biomass was used for bioethanol production.

Preparation of algal hydrolyses for substrate preparation

Substration preparation was done by hydrolyzed the dried defattted algal biomass. Hydrolysis of terrestrial microalgae was done by ground the dried microalgae to fine powder and crushed algae were autoclaved at 121^{o}C for 15min at 15Psi. Cooled the sample and the autoclaved crushed algae were homogenized treated with HCl (2.5N) for 3hrs, which helps to conversion of polysaccharides into monosaccharides. After hydrolysis, it neutralized with pinch of Na_2CO_3 (Chaudhary *et al.,* 2017; Harun *et al.,* 2014).

Bioethanol production by fermentation of defatted terrestrial microalgae

The yeast (*Saccharomyces cerevisiae*) is known for the production of Ethanol. Preserved cultured *Saccharomyces cerevisiae* was collected from the laboratory of biochemistry department of Agricultural biotechnology block, Agriculture College Hassan sub campus of UAS, Bangalore. In this experiment defatted algal residue hydrolyzed with 2.5N HCl was used as substrate for fermentation process. Yeast (*Saccharomyces cerevisiae*) was used for breaking the sugar contents in the algal residue to produce ethanol. Here five defatted 10g of hydrolyzed algae from various places were inoculated with 5% *S. cerevisiae* and 0.5% glucose were used as a starter for this experiment. 90ml of sterilized water was added to 250 ml conical flasks. Samples were taken for initial analysis that was considered as 0th day sample. Conical flasks were airtight by rubber cork fixed with 2 silicon pipes among two one medium sized silicon pipes was immersed to a test tube with water to ensure anaerobic condition. Another small pipe connected to stopper and syringe for analyze the sample in the interval of 3 days up to 21days with 8 parameter. The stoppers fixed checks the air entry to the anaerobic condition. This experiment was conducted in completely randomized design with three replications R_1, R_2 and R_3 to minimize errors (Figure 3).

Biochemical and physical characterization of terrestrial microalgae

Total soluble sugar (TSS) concentration analysis

The quantitative determination of TSS was done by Anthrone reagent method. In this method 100mg of hydrolysed samples were diluted to 100ml with distilled water than centrifuged at 10000 rpm for 10 minutes at 4^{o}C, and the supernatant containing total soluble sugars used for estimation.

Total soluble sugars estimated by taking 0.2ml of supernatant and made up the volume to 1 ml with distilled water. Added 4ml of Anthrone reagent and kept in boiling water bath for 8 minutes. Green to dark green colour was observed at 630nm (Hedge and Hofreiter, 1962).

$$\text{Total soluble sugar content (\%mg)} = \frac{\text{Sugar value from graph (mg)}}{\text{Aliquot sample used (0.2ml)}} \times \frac{\text{Total volume of extract (ml)}}{\text{weight of sample (mg)}} \times 100$$

Total reducing sugar (TRS) concentration analysis

Reducing sugar was estimated by3, 5-Dinitrosalicylic acid (DNS) method. In this method sugar was extracted from 100mg of dried sample with 10ml of hot 80% ethanol and centrifuged at 10000rpm for 10minutes.

The supernatant was kept in boiling water bath for evaporating ethanol and dissolved the sugar content by adding 10ml distilled water. These dissolved reducing sugars were analyzed by taking 0.2ml of aliquot and dilute it to 1ml with distilled water. Then added 3ml of DNS and kept in boiling water bath until colour develops.

After the colour has developed added 1ml of 40% Rochelle salt solution and mixed it in vertex mixture. Cooled the tubes and measured the absorbance at 510nm using spectrophotometer. Drew the standard graph and calculated the amount of total reducing sugar in the sample (Sadasivam and Manickam, 1992).

Estimation of protein

Total soluble protein was extracted by ground 0.5gm of the algal sample with 5ml of phosphate buffer (0.1M, pH 7.4); added pinch of polyvinylpyrrolidone, 2µl β mercapto ethanol and centrifuged at 6000rpm for 10min.Added equal amount of chilled acetone to supernatant and kept at -20 ^{0}C for 30min. Then centrifuged at 12000 rpm for 15min and discarded the supernatant and washed the pellet with 75% ethanol until white pellet appears. At last dissolved the pellet in 1ml of phosphate buffer (0.1M, pH 7.4) and used it for protein estimation (Lowry *et al.*, 1951)

Measuring pH, ^{o}Brix and microbial load of the terrestrial microalgae during fermentation period

pH was measured by using digital pH meter (ELICO LI615). Total soluble solid of natural sugars solution were measured by index of refraction. ^{o}Brix measured by taking a drop of sample & place it on refractometer surface and focused towards the light and observed the readings (Neto *et al.*, 2006). Microbial load was measured by taking 1ml of sample from the anaerobic setup and made up the volume to 2ml by adding distilled water. Shaken well and after 5minutes measured the absorbance at 600nm using spectrophotometer.

Estimation of titrable acidity of the fermentation setup

Taken 10ml sample in 100ml volumetric flask and the volume made up to 100ml. After that 10 ml aliquot was taken in a 100 ml conical flask and titrated against NaOH (0.1 N) solution using 2 drops of indicator. Total titrable acidity was expressed in percent of citric acid (gm/100ml of sample). The appearance of light pink colour denoted the end point (Normas Analíticas Do Instituto Adolfo Lutz, 1985). Titrable acidity of fermentation setup was calculated by using the formulae given below(Beshir *et al.*, 2015).

$$\text{Titrable acidity} = \frac{\text{volume of titrate} \times \text{Normality of titrate} \times \text{Eq. Weight of organic acid} \times 100}{\text{Volume of sample (g)} \times 1000}$$

Ethanol estimation

Ethanol estimation was done using potassium dichromate, ethanol is completely oxidized by potassium dichromate in presence of sulphuric acid. Acetic acid is formed at the end of this reaction (Williams and Reese, 1950). In this method 1ml of anaerobic maintained algae sample from each treatment was transferred to separate 250ml round bottom distillation flasks connected to the condenser and diluted

with 30ml of distilled water. The sample was distilled at 74-75^{0}C. The distillate was collected in 25ml of $K_2Cr_2O_7$ (0.23N) reagents kept at receiving end. The containing alcohol was collected till total volume of 45ml was obtained. Similarly standard (20-100mg ethanol) was carried out and distillate the sample. Standards were heated at 60^{0}Cfor 20 minutes by using water bath and cooled it. The volume made up to 50ml with distilled water and absorbance was measured at 600nm using spectrophotometer (Caputi *et al.,* 1968).

$2Cr_2O_7^{2-} + 3C_2H_5OH + 16H+ \rightarrow 4Cr^{3+} + 3CH_3COOH + 11H_2O$

Results and Discussion

Biochemical characterization of terrestrial microalgae

Terrestrial microalgae collected from different places such as *Rhizoclonium sp.* (Dandiganahalli), *Spirogyra sp.* (Hoovnalli), *Klebsormidium sp.* (Ikola), *Rhizoclonium hieroglyphicum* (Kaggodu) and *Oedogonium sp.* (Gadanahalli) had contain total soluble sugar 38.18 ± 0.95, 37.10 ± 0.11, 34.68 ± 0.04, 42.16 ± 0.04 and 35.36 ± 0.10%; total reducing sugar 24.40 ± 0.40, 24.10 ± 0.36, 23.80 ± 0.92, 23.90 ± 0.17 and 24.40 ± 0.27% respectively. The results obtained in the present study were statistically significant. Maximum amount of TSS and TRS showed in the *Rhizoclonium hieroglyphicum* and *Rhizoclonium sp.* respectively. Minimum observed in the *Klebsormidium sp.* These total soluble sugars are hydrolyzed by 2.5N HCl, which was helped in saccharification and these hydrolyzed sugar were used as the sugar source for yeast (*Saccharomyces cerevisiae*). These reducing sugars are monosaccharaides and disaccharides which were easily breakdown to produce ethanol by yeast (Canilha *et al.,* 2012). Total protein was estimated by Lowry method. The protein content of current studied terrestrial microalgae had 22.24 ± 0.03, 6.64 ± 0.03, 5.12 ± 0.16, 19.51 ± 0.04 and 5.73 ± 0.02 mg/g of protein, respectively. Maximum protein content was observed in *Rhizoclonium sp.* and minimum observed in the *Klebsormidium sp.* According to Peter *et al.,* (2010) higher protein content increases the lipid content in the microalgae and they observed 27-48% of protein in various species of microalgae (Table 1).

Physico-Biochemical characters changes during bioethanol production by fermentation of terrestrial microalgae

In the current study bioethanol were extracted by fermenting the algal biomass and standardized the ethanol production on the basis of biochemical changes *viz.* TSS, TRS, protein, pH, organic acids, microbial load, ^{o}brix and alcohol content of anaerobic fermentation setup of terrestrial microalgae in duration of 21 days showed below. The results of total soluble sugars were estimated from fermentation setup of terrestrial microalgae for every 3 days of interval from 0th day to 21st days. In present study the total soluble sugar decreased, the decreased percentage difference between 0th day and 9th day of *Rhizoclonium sp., Spirogyra sp., Klebsormidium sp., Rhizoclonium hieroglyphicum* and *Oedogonium sp.* had 59.06, *51.03,* 55.84, 44.75 and 46.45%, respectively. The maximum decreases of sugar were found in *Rhizoclonium sp.* (Dandiganahalli), where 35.54 ± 0.4% of total soluble sugar on 0th day decreased to 14.55 ± 0.75% on 9th day. This decreased percentage difference of 0th day and 9th day was 59.06%. It was mainly because of *Saccharomyces cerevisiae* which breakdown the sugar content during the fermentation period to ethanol. But after 12th to 18th days, TSS percentage was increased because of releasing sugar content from algal biomass inside fermentation set up and it is known as

secondary fermentation (Robinson, 2006). *Saccharomyces cerevisiae* utilized these sugar content so on 21st days total soluble sugar content were decreased in all present studied sample and decreased percentage difference between 0th day and 21stday was 61.93, *62.43*, 60.11, 58.03 and 59.29%, respectively. On 21st day also *Rhizoclonium sp.* has decreased total soluble sugar to 13.53 ± 1.25%, giving 61.93 % difference in this period. The values obtained in present study except 12th and 21st day all are statistically significant. These high carbohydrate content efficiently saccharified to monosugars by treated with sulfuric acid and these monosaccharaides serve as good substrates for the fermentative production (Sung-Soo Jang, 2012). These total soluble sugars are feedstocks for ethanol production by fermentation after hydrolysis (Chaudhary *et al.*, 2017) (Figure 4).

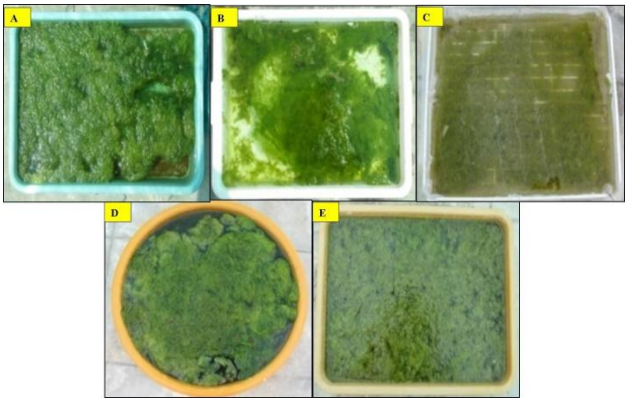
Total reducing sugar of samples was decreased and the decreased percentage difference between 0th day and 21st day of current studied samples were 30.70, 30.71, 32.27, 33.35 and 38.52%, respectively. The maximum reducing sugar decreased percentage difference were observed in *Oedogonium sp.* 38.52%, it as reducing sugar content on 0th day 24.40 ± 0.10% it was decreased gradually and on 21st day of fermentation reducing sugar concentration 15.00 ± 1.36% were observed.

The sugar content in all species of present study decreased gradually from 0th day to 21st days due the utilization of sugar by the microbes (*Saccharomyces cerevisiae*) (Yang and Wiegand, 1949), which breakdown the sugar content during the fermentation period and produced the ethanol. The results of TRS in present study are in accordance with the similar study done by Lalitesh *et al.*, (2017), who also observed decreased sugar content during fermentation on different species of microalgae (Figure 4).

The total proteins of samples are gradually increased and the increased percentage difference between 0th day and 21st day of present studied samples were 1.01, 33.79, 36.71, 33.06 and 3.14%, respectively. *Klebsormidium sp.* had maximum increased from 0th day to 21st day was 2.07 ± 0.53 to 2.83 ± 0.80 mg/g the increased percentage difference was 46.37%. During fermentation protein content were increased due to decreased sugar content. In present study protein content of *Klebsormidium sp.*were gradually increased from 3rd day to 21st days during anaerobic fermentation period are 1.55 ± 0.08, 1.62 ± 0.06, 1.72 ± 0.11, 1.95 ± 0.31 and 2.22 ± 0.50 mg/g, respectively. Protein, nitrogen and ash in the hydrolysate might be favorable ingredients supporting fermentation (Sung-Soo Jang, 2012). The value observed in present study was statistically significant. The result of present study is in accordance with the similar study done by Peter *et al.*, (2010). According to whom higher protein content increases production of other byproducts like enzymes, nucleic acids and organic acids which helps production of ethanol (Figure 4).

In this study pH were decreased, the decreased percentage difference between 0th day and 9th day of *Rhizoclonium sp.*, *Spirogyra sp.*, *Klebsormidium sp.*, *Rhizoclonium hieroglyphicum* and *Oedogonium sp.* had 16.13, 16.53, 15.43, 9.17 and 18.72%, respectively. The maximum decrease of sugar were found in *Oedogonium sp.*7.80 ± 0.20 of pH on 0th day and it decreased to 6.30 ± 0.05 pH on 9th day, the decreased percentage difference of 0th day and 9th day was 18.72%. It was mainly because of yeast (*Saccharomyces cerevisiae*) which breakdown the sugar content during the fermentation period which increases the microbial load and produced the ethanol. But after 12th to 18th day's pH percentages were increased, it because of increasing TSS inside anaerobic fermentation set up.

Fig.1 Multiplication of terrestrial microalgae from different location on plastic tray

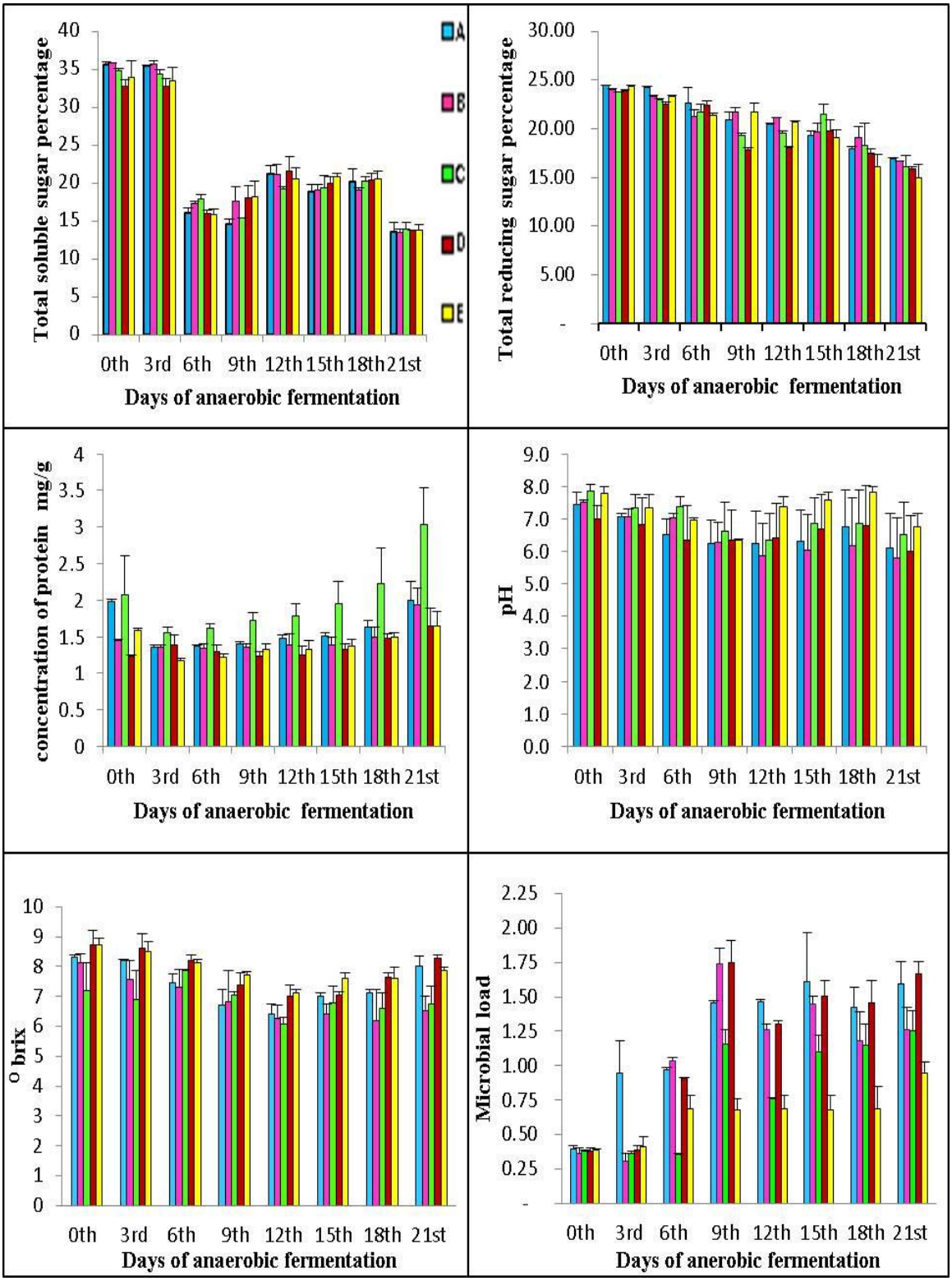


(A). *Rhizoclonium sp.* (Dandiganahalli), (B). *Spirogyra sp.* (Hoovnalli), (C). *Klebsormidium sp.* (Ikola), (D). *Rhizoclonium hieroglyphicum* (Kaggodu) and (E). *Oedogonium sp.* (Gadanahalli).

Fig.2 Multiplication of terrestrial microalgae by open pond system

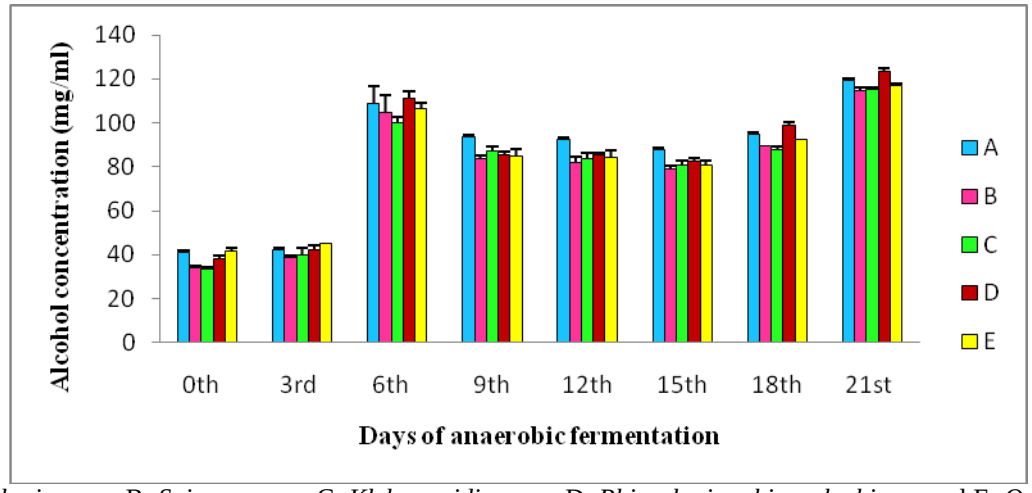
Fig.3 Anaerobic fermentation setup for five terrestrial microalgae collected from different places

Fig.4 Physio-biochemical analysis during fermentation period



A. *Rhizoclonium sp.*, B. *Spirogyra sp.*, C. *Klebsormidium sp.*, D. *Rhizoclonium hieroglyphicum* and E. *Oedogonium sp.*

Fig.5 Alcohol estimated from the fermentation setup of terrestrial microalgae



A. *Rhizoclonium sp.*, B. *Spirogyra sp.*, C. *Klebsormidium sp.*, D. *Rhizoclonium hieroglyphicum* and E. *Oedogonium sp.*

Table.1 Biochemical characterization of terrestrial microalgae

Sample	Total soluble sugars (%)	Total reducing sugars (%)	Protein (mg/g)
A	38.18 ± 0.95	24.40 ± 0.40	22.24 ± 0.03
B	37.10 ± 0.11	24.10 ± 0.36	06.64 ± 0.03
C	34.68 ± 0.04	23.80 ± 0.92	05.12 ± 0.16
D	42.16 ± 0.04	23.90 ± 0.17	19.51 ± 0.04
E	35.36 ± 0.10	24.40 ± 0.27	05.73 ± 0.02

A. *Rhizoclonium sp.*, B. *Spirogyra sp.*, C. *Klebsormidium sp.*, D. *Rhizoclonium hieroglyphicum* and E. *Oedogonium sp.*

The pH of samples on 21st day decreases because of yeast (*Saccharomyces cerevisiae*) utilized this sugar content so total soluble sugar content was decreased in all present studied samples (Yang and Wiegand, 1949) and increased the microbial growth. Decreased percentage difference between 0th day and 21st day was 18.0, 22.7, 17.1, 14.0 and 23.1%, respectively. The rate of ethanol production maximum at pH 6.0 was observed. pH during fermentation period act as an important role in the growth and adaptation of microorganism (Chaudhary *et al.*, 2017), in their study microorganism growth increased below pH 7 and maximum ethanol produced at pH 6 (Figure 4).

In present study the microbial load was increased. The 0th day optical densities of present studied five terrestrial microalgae are 0.40 ± 0.02, 0.36 ± 0.04, 0.38 ± 0.01, 0.38 ± 0.02 and 0.39 ± 0.01, respectively. On 9th day optical density was increased to 1.46 ± 0.01, 1.74 ± 0.11, 1.16 ± 0.10, 1.75 ± 0.16 and 0.68 ± 0.08, respectively. The maximum increases of microbial load were found in *Spirogyra sp.* Microbes were multiplies very quickly at acidic pH of 5.5 to 6.8. The environmental factors influence the growth of a microorganism such as substrate, temperature and pH (Dorn *et al.*, 2003). In present study the pH of 6.2 to 6.9 was observed on 9th day of fermentation so more microbial growth

were observed on that period. But after 12th to 18th days microbial load was decreased, it because of increasing TSS inside anaerobic fermentation set up. Microbial load was increased on 21st day, microbe's optical growth were 1.59 ± 0.6, 1.27 ± 0.16, 1.26 ± 0.14, 1.66 ± 0.09 and 0.95 ± 0.07, respectively. The rate of ethanol production maximum at pH 6.0 was observed. In present study the sugar concentration of samples during fermentation period was 12-36%. Concentration of carbohydrate significantly influences citric acid production. High sugar concentration induces increased glucose uptake and consequently enhanced citric acid production and on 21st day maximum ethanol production was observed. The organic acids are formed by utilizing carbon sources, nitrogen and other micronutrients released from microalgal residues during fermentation period (Vandenberghe *et al.,* 1999). Due the formation of organic acid pH of the fermented product was acidic (pH <7) it helps to control the contamination. At low pH other microorganism growth was very less. The result of present study is in accordance with the similar study done by (Whiting, 1976) (Figure 4).

Degree Brix were the indication of the potential alcohol in a wine when fermentation is completed(Vincent, 2011). In this experiment 0th day samples show higher brix value because of 0.5% sugar used as starter during fermentation along with substrate and inoculum of yeast. The initial day brix values of the present studied samples were 8.30 ± 0.10, 8.13 ± 0.31, 7.20 ± 0.92, 8.73 ± 0.46 and 8.73 ± 0.23, respectively. The brix value on 21st day are 8.00 ± 0.35, 6.53 ± 0.46, 6.77 ± 0.57, 8.27 ± 0.12 and 7.87 ± 0.12, respectively. Maximum of 8.27 degree brix was founded in *Rhizoclonium hieroglyphicum*, hence alcohol content also obtained maximum on same species (Figure 4).

The alcohol contents were high on 6th and 21st day of anaerobic fermentation. The alcohol quantity observed on 6th day of *Rhizoclonium sp., Spirogyra sp., Klebsormidium sp., Rhizoclonium hieroglyphicum* and *Oedogonium sp.* are 109.15, 104.57, 100.32, 111.11 and 106.37mg/ml, respectively. The maximum alcohol quantity was observed on 21st day of present studied samples are 119.28, 115.03, 115.35, 123.52 and 117.15mg/ml, respectively. The percentages of alcohol content on 21st day are 59.64 ± 0.79, 57.51 ± 0.99, 57.67 ± 0.51, 61.76 ± 1.12 and 58.57 ± 0.49%, respectively. The maximum alcohol percentage was founded on *Rhizoclonium hieroglyphicum* and *Rhizoclonium sp.* is 61.76 ± 1.12 and 59.64 ± 0.79%. It was mainly because of yeast (*Saccharomyces cerevisiae*) which breakdown the sugar content during the fermentation period and produced the ethanol(Yang and Wiegand, 1949). During the period of fermentation of 6th and 21st day carbohydrates content in fermentation setup decreases and microbial load increases, it is also a one reason for increased percentage of alcohol on 6th and 21st day. But after 12th to 18th day alcohol percentage were decreases, because of decreasing microbial load due to increase in pH inside fermentation set up. Yeast (*Saccharomyces cerevisiae*) utilizes the sugar content present in the algal biomass residues during fermentation period so on 21st days total soluble sugar content were decreased in all present studied samples which were advantage to produce more quantity of alcohol. Sugar consumption held at the time of exponential phase in fermentation was observed. Microalgal sugars were consumed and simultaneously ethanol was produced. At the period of bio-ethanol fermentation, the concentration of the reducing sugar decreased with respect to days, and the concentration of bio-ethanol further increased gradually, clearly indicating that present studied samples were used as fermentation sugars (Figure 5).

On the basis of above investigation it can be concluded that the bioethanol from terrestrial microalgae is technically feasible in this experiment. It can easily culture in open pond method for biomass production. By-products of algal oil extracts such as defatted algal residues can use as a substrate for bioethanol production. And can able to produce 62% of ethanol from it. *Rhizoclonium hieroglyphicum* grown in waste water showed good response and it was found that it reduces the pH of alkaline water and TDS content of waste water by algae. Based on study conducted, it can be suggested that terrestrial microalgae *Rhizoclonium hieroglyphicum* can be used for bioethanol production by growing in polluted or waste water.

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