

***Dunaliellatertiolecta* Microalgae as biological feedstock, for bioethanol production**

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Abstract

The microalgae named *Dunaliellatertiolecta*, holds a high quantity of sugars such as starch and cellulose due to which it has gained more attention in the biofuel industry. Microalgal extract carbohydrate can be utilized as a source for the output of ethyl alcohol or ethanol. The purpose of the present work is to study, how the sugar extract of marine algae can possibly be used by investigating the usage of *Nakaseomycesdelphensis* for ethanol production. With the help of fermentation process the produced ethanol is distilled and measured qualitatively by laboratory model distillation unit and GC chromatography respectively. The measurements will be in comparison with the standard analytical grade ethanol. This data will give us the potential of marine algae in the output of bioethanol.

Keywords

Ethanol; Fermentation; Carbohydrate Extraction; Yeast; Marine algae, Glucose.

Introduction

The biotech world would be developed by utilizing second generation biomass, the major lignocellulosic-agro-residues in terms of volumes generated such as rice straw, wheat stalk, sugarcane tops & switchgrass, bagasse so the war between Food Vs Fuel will be solving [1]. Microalgae known as third generation feedstock, produce high biomass yields without requiring no additional lands, minimal water use, and mitigate atmospheric CO₂ [2] and hold the potential to be cultivated in a containment offshore [3]. Microalgae can convert approximately 6 percent of the total incident radiation, into new biomass [4] By using a technique microalgal sugar are relieved and simply fermented to ethanol using fermentative microorganisms [5]. In the recent past a lot of attention was given to production of bio-ethanol from microalgal feedstock can be taken out to produce fermentable sugars that was estimated approximately 5000–15,000 gal of ethanol/acre/year (46,760–140,290 L/ha) [6]. Productions of bio-ethanol is the aim of present investigation. *Nakaseomycesdelphensis* belong to the *Saccharomyces* higher level then it might be possible that bio-ethanol productivity much higher than others. *Dunaliellatertiolecta* microalgae use as

a feedstock for bio-ethanol production with *Nakaseomycesdelphensis*. The screening of microalgae biomass is an eco-friendly and sustainable approach for quantitative and qualitative biofuel production.

Materials and Methods

Preparation of the Microalgal Culture Medium

A microalgae SAG 13.86 cultivated in the “artificial seawater medium”, as proposed by SAG (Sammlung von Algenkulturen Göttingen, 2008). This was microalgae's standard medium. It was cultivated in large scale approx 180 L capacity for algal biomass production. Microalgal Cultures were illuminated using fluorescent light, 12 h light/12 h dark cycle and 18°C temperature with continuous aeration.

Measuring Algae Concentrations through Spectrophotometer

Applying a UV/VIS spectrophotometer distinguishes their specific growth rate and measuring optical density at wavelength 680 nm [7].

Measurement of Dry Biomass

For dry biomass measurement take 10 mL every second day of algal samples were filtered on to predried and weighed GF/C fibre filters, oven dried overnight at 60°C, and reweighed using an analytical scale [7].

Harvest Algal Biomass

Dewatering of microalgal cultures by flocculation method. In this method algae was harvested from 1L culture by the addition of 0.4% ferric chloride in the algal culture, then dry it in the oven for 2 days at 60°C. Dry algal biomass was used further carbohydrate estimation [8].

Medium Preparation for Yeast

Nakaseomycesdelphensis MTCC 114 obtained as a lyophilized culture from IMTECH, CHD was revived on malt yeast broth and was further cultured over malt yeast agar medium for its use in the further process [9]. Yeast growth was monitored by measuring the optical density (OD) at 600nm (OD₆₀₀) after every 2-3h. A calibration curve was built based on the hour and OD₆₀₀ measured from the yeast culture [10].

Hydrolysis Process

In process of acidic hydrolysis, Take various amounts of sugar concentration (0.1–10 g) and hydrochloric acid solution 0.3M, 0.5M, 1M concentration were mixed at 60°C up to 15–60 min. After hydrolysis, neutralised it with Basic treatment which was done by saturated sodium carbonate than centrifuged at 4°C and 5000 g for 20 min, and the supernatant containing the released sugars were collected as acidic hydrolysate, then calculate sugar content in this [11].

Reducing Sugar Concentration Analysis (Algal carbohydrate estimation)

The concentration of the total sugars in a sample was determined by Anthrone–sulphuric acid method. In first step carbohydrates are hydrolysed into simple sugars with help of dilute hydrochloric acid. Resultant compound with Anthrone seems in green color with an absorption maximum at 620 nm [12]. Consider the concentration of the standard on the X-axis versus absorbance on the Y-axis plot a standard graph and calculated the quantity of carbohydrate present in the sample tube.

Yeast Fermentation for Ethanol Production Using Microalgal Biomass as a Substrate

After acid hydrolysis batch culture was run away in a 250mL Erlenmeyer flask containing 100mL of malt, yeast broth made with either 1 g/100mL of glucose or approximately 1g/100mL of pretreated algal hydrolyzate sugar, and approx. (1% v/v) yeast inoculum added under the following conditions: pH 6.2, temperature 25°C and agitation at 150 rpm in a rotary shaker [8]. The temperature, pH and sugar concentration were increased in a format or experimental design (Minitab) in order to examine its effect on the specific growth rate of yeast or the effect of variable parameters like pH, temperature and total reducing sugars was examined on yeast growth rate.

Statistical Analysis

The statistical software MINITAB was used for design of experiments, regression and graphical analyses of the information obtained, and statistical analysis of the model to evaluate the analysis of variation (ANOVA).

Ethanol Concentration Analysis

(i) UV Spectrophotometer (Spectrophotometric determination of ethanol)

After distillation process 1 mL of chromic acid solution which was prepared with 0.5g of potassium dichromate dissolved in 5mL of distilled water and 45 mL of sulphuric acid was added to 1 mL of the sample and kept in a water bath at 80°C for 10 min. It was then cooled and the absorbance was measured at 600 nm. A standard curve was prepared by using

different concentrations (1–10% v/v) of ethanol as a standard for measuring ethanol concentration [13].

Analysis of ethanol with the help of Gas Chromatography

Gas Chromatography was connected to an integrator and computer system to determine retention time of ethanol. Standard solutions of ethanol (EtOH) were prepared in distilled water. It was contained 1% v/v of *n*-butanol as an internal standard, extracted and injected. In further process broth sample was transferred to an eppendorf tube and that tubes were centrifuged (8,000 g for 2 min at R.T.) to sediment yeast cells. Supernatant were formed, than transfer this supernatant into a new tube and then 5 µl of *n*-butanol (as internal standard) were added and the tube was vortexed. In the next step, 1 ml of ethyl acetate was added, and vortexing at maximum speed for 5 min. At the last step, for separation of facilitate phase the tubes were centrifuged at 5,000 g for 2 min at R.T., and finally separate the organic phase (upper) for GC analysis. Oven temperature was initially set at 50°C and then raised at the rate of 7°C/min to 100°C, thus making a total run time of 7 min [14]. In GC had some other conditions like FID: 250°C; H₂, 25 PSI, equivalent to 25 ml/minute; air, 2 PSI, equivalent to 100 ml/min; gain sight of 'medium'. N₂ was used as carrier gas with set pressure control which is 24 PSI constant; equivalent to 27 ml/min.

Results and Discussion

Growth Rate of Microalgae Strain

Fig. 1. Graph shows the growth of marine microalgae *Dunaliellatertiolecta*

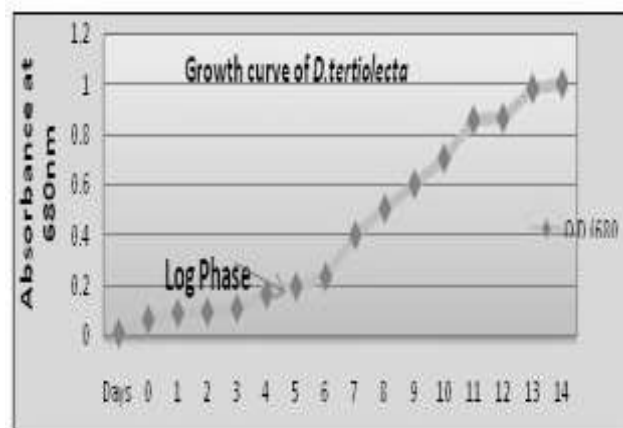


Fig.1 shows the *Dunaliellatertiolecta* strain had a low growth rate in the first 5 days, due to accommodate to the new environmental conditions provided. The maximum cell density (approximately 1.3×10^7 cells/mL) was attained after 14 days of culture and the specific growth rate was 0.364 per day.

The genus *Dunaliella* had potential as a rich, renewable biomass for use in the fermentative

production of butanol and ethanol [15]. Previous studies detailed about the growth of organism as was observed with the utilization of fluorescent light by the organism, and the maximum cell density (approximately 1.2×10^7 cells/mL) was observed [16]. In present study the cell density approximately 1.3×10^7 cells/mL was achieved. Fluorescent light and optimum media conditions supported the culture to increase algal biomass to produce ethanol. The response of selected species of microalgae such as *Dunaliellatertiolecta* were recorded and dry weight of algal species per unit volume is 1.06 g was observed after harvesting process, means large scale culture volumes for experiment setup 1 is 180 L and dry weight of algal is 190.8 g.

Acid Hydrolysis of *Dunaliellatertiolecta*

Table 1. Different amount of reducing sugar present on various time and acid conc.

Time(min.) Acid	15 min	30 min	45min	60min
Conc.				
0.3M	0.008g	0.009g	0.01g	0.02g
0.5M	0.009g	0.02g	0.03g	0.01g
1M	0.01g	0.01g	0.009g	0.009g

Table 1 shows Acid hydrolysis of *Dunaliellatertiolecta* had reducing sugar concentration of algal hydrolysates that increased correspondingly with the duration considered at concentration of 0.3M, but it decreased after 60 min at concentration of 0.5M and 1M. It was clearly recorded that on completion of 45 minutes, reducing sugar was 0.01g at 0.3M acid concentration and then 0.03g at 0.5M acid concentration. Further, it became suddenly high because of high acid concentrations and then deduction in the reducing sugar was observed at 1M. Too much amount of acid could not extract reducing sugar successfully. In previous studies 0.5M diluted acid-mediated saccharification of the residual biomass was produce 14.8 mg/mL reducing sugar [17]. Hence specific time and molar concentration produced higher amount of reducing sugar. Various studies have suggested that the temperature was considered as effective in reducing sugar extraction from the organism selected. Monosaccharide's could not be extracted from *C.reinhardtii* (previous research) under increased temperature conditions [18]. High acid concentration and longer hydrolysis duration were the two situations that might cause monosaccharide's decomposition during the process of hydrolysis. In this study, the highest reducing sugar obtained after hydrolysis with 0.5 M HCl acid

for 45 min, resulted in 0.03g reducing sugar present in 0.1g algal powder.

Reducing Sugar Concentration Analysis

Algal carbohydrate estimation was performed with the utilization Anthrone Sulphuric acid method. Firstly carbohydrate was hydrolysed into monosaccharide's by using HCl after which it was analysed how much sugar was present in algal supernatant. Pretreatment improved the conversion of carbohydrates, irrespective of the study discussed, *Dunaliellatertiolecta* microalgae contained 21-87% glucose [19]. From Eq.(1) it was identified that the concentration of sugar in sample was 0.15mg/mL.

$$C_{uk} = (A_{uk}/A_{st.}) \times C_{St.} \quad \dots\dots\dots(1)$$

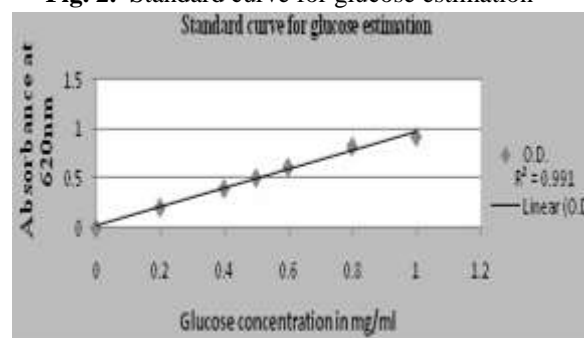
C_{uk} = Concentration of unknown sample,

$C_{St.}$ = Concentration of Standard

A_{uk} = Absorbance of unknown sample

$A_{St.}$ = Absorbance of standard

Fig. 2. Standard curve for glucose estimation



Amount of carbohydrate present in 0.1g of algal sample was 0.03g. In previous studies, maximum amount of reducing sugars approximately 14.8 mg/mL was observed, and total carbohydrates of the residual biomass was approximately 51.9% (w/w) [17]. In the present work, the percent of total carbohydrate of the residual biomass was about 30% (w/w). 0.32 g glucose present in 100 g algal biomass as shown in Fig.2. Hence it was observed that 3.12 g algal dry biomass contained 1g glucose. Therefore, large scale of dry biomass 190.8g algae obtained 60.8g glucose. The biomass carbohydrate content depends on the microalgal species and on the cultivation and environmental conditions. Several microalgae species, such as *Porphyridium cruentum* (40–57 %), *Spirogyra sp.* (33–64 %), etc., have high innate carbohydrate content. The control of some of the cultivation or environmental conditions seems to be an affordable approach for the microalgal biomass composition manipulation, in order for the biomass carbohydrate accumulation to be increased or maximized.

Growth Rate of Microorganism Strain

Nakaseomyces delphensis 114 was obtained from MTCC, IMTECH, Chandigarh. Starting inoculate

culture was used for growth kinetic. *Nakaseomyisdelpensis* was observed with cream colony colour and oval shape. The environmental factors that influence the growth of a microorganism such as substrate, temperature, and pH [20]. Yeast growth was monitored by measuring the optical density (OD) at 600 nm after every 2-3 h. Under optimization, in control conditions 12-32 h was observed as the exponential phase of yeast. And the maximum cell density (approximately 6.9×10^7 cells/mL) were achieved in turn increment in the specific growth rate was 0.201 per hour. In previous studies, specific growth rate of *Nakaseomycesdelpensis* was almost same (0.202 per hour) at appropriate growth conditions since there was no difference in specific growth rate but variation in environmental condition surely effected the microorganism growth [21]. *Saccharomyces cerevisia* with *Arthrospiraplatensis* was produced, high amount of ethanol yield that is 16.27 was obtained. And again *Saccharomyces cerevisia* with *Scenedesmusobliquus* was produced 2.24 ethanol yield [22]. Bio-ethanol yield was fluctuating, it was entirely dependent on fermentation conditions, So experimental work was planned based on fluctuations of some key process conditions such as

different temperature, pH and reducing sugar concentration in order to investigate their influence along the production procedure.

Yeast Fermentation for Ethanol Production Using Microalgae Biomass As A Substrate

3.5.1. Statistical Analysis

Experimental Design was used to detect out the optimal concentrations of three selected variables described and to study interactions amongst them. Each variable pH(4.2,5.2,6.2), temperature(25°C, 30°C, 35°C), reducing sugar(1g, 2g, 3g) was analyzed at 3 different levels. A full-factorial experimental design combining a total of 3 replicates in totality of 81 experimental setups, was employed. The experimental runs were carried out according to a 3^3 means (27) general full factorial design for the three identified independent variables. 0.201 per hour Specific growth rate of control at pH 6.2, 25°C temp. and 1 g reducing sugar. And the experimental (Sample 14) high response values (Specific growth rate) is 0.205 at pH 5.2, 30°C temp. and 2g reducing sugar. The efficiency of the full-factorial experimental design was checked by the analysis of variance (ANOVA) presented in Table 2.

Table 2. Analysis of Variance for specific growth rate

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	2	0.0051854	0.0051854	0.0025927	1555.61	0.000
T	2	0.0016191	0.0016191	0.0008096	485.74	0.000
RS	2	0.0002987	0.0002987	0.0001493	89.61	0.000
pH*T	4	0.0002220	0.0002220	0.0000555	33.30	0.000
pH*RS	4	0.0000304	0.0000304	0.0000076	4.56	0.003
T*RS	4	0.0000066	0.0000066	0.0000017	1.00	0.418
pH*T*RS	8	0.0000165	0.0000165	0.0000021	1.24	0.297
Error	54	0.0000900	0.0000900	0.0000017		
Total	80	0.0074687				

pH, T= Temperature, RS=Reducing Sugar

The *F*-value was higher than the critical values, so it can be indicated that the general linear model is highly statistically significant at 95% confidence level, and the predicted model presents a relatively high determination coefficient ($R^2 = 0.9879$), explaining 98.79% of the variability in the response. The value of R^2 was 0.9879, indicating that about 1.21% variation was not explained by the model. An R^2 value higher than 0.80 indicates that the model fits the experimental data reasonably well or the data

fit well with the model and move over a convincingly good estimate of the response of the system in the experimental range decided. The *P*-values were used to distinguish the effects of each factor on the specific growth rate. A *P*-value below 0.05 indicates that the model terms are significant. The *p*-value is used as a substitute for refusal points to provide the smallest level of significance ($\alpha = 0.05$) at which the null hypothesis would be rejected. This indicated that smaller the *p*-value, the stronger

the verification is in favour of the alternative hypothesis.

$S = 0.00129099$; $R^2 = 98.79\%$; $R^2(\text{adj}) = 98.21\%$
 SS= sum of squares; DF= degrees of freedom; MS= mean square;

Considering the current work, 2g reducing sugar with 5.2pH and 30°C temperature there were best interactions with a more significant value of specific growth rate. During the early studies it was observed that 30gL⁻¹ microalgae biomass yielded 0.1946 gL⁻¹ of ethanol while 10gL⁻¹ resulted in 0.1197 gL⁻¹ of ethanol [8]. A study by Cromie *et al.* reported that higher microalgae biomass concentration possibly reduced the ethanol production levels during fermentation process. This was due to the reason that generation of higher glucose concentration tends to accumulate inhibitors and produce high ethanol concentration in the fermentation broth, hence generating more toxic chemicals in the fermentation environment [23] provided to the organism. Other previous studies about the temperature, the optimum growth was observed at temperatures below 30°C and the maximum temperature above 37°C in case of *Saccharomyces bayanus* [24]. Higher temperatures affected the growth and metabolism of yeast cell thus resulted in a significant reduction in ethanol concentration [25]. It had also been reported that higher temperatures can easily kill the yeast cells due to the rupturing of the membrane. Hence the present work gave a view that at lower temperature ethanol production efficiency increases along with the active metabolism of the organism. In previous reports maximum productivity and growth were obtained at pH 5.2 for the domestic strain *Y. lipolytic* [26]. Full factorial design were employed for the optimization of fermentation medium conditions for the production of bio-ethanol by *Nakaseomyces sp.*

Optimization of Fermentation Process

Effects of Experimental Variables on Specific growth rate of the organism Fermentation process was held out by yeast, three parameters were used to evaluate the effects of pH (4.2 to 6.2), temperature (25°C to 35°C) and reducing sugar concentration (1g/100mL to 3g/100mL) on specific growth rate of *Nakaseomycesdelphensis* for higher ethanol production.

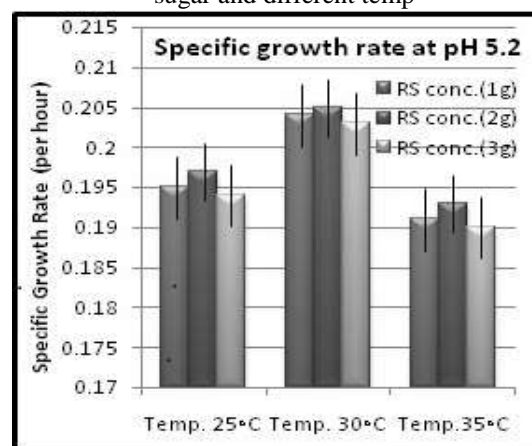
In this study, the effect of the specific growth rate on the physiology and fermentative capacity of an ethanol producing strain *Nakaseomycesdelphensis* was studied. At specific growth rates (μ_{max}) was below to the optimum or control growth rate was attained, glucose metabolism was fully respiratory and was effective on low fermentative capacity. When the specific growth rate was above the control specific growth

rate, respiro-fermentative metabolism set in, with higher ethanol production rates. This fermentative capacity increased with increasing growth rates.

Effect of pH

The specific growth rate reached a maximum at a pH of 5.2. But it was not very sensitive to pH 6.2, because 6.2 pH was the optimum pH of *Nakaseomycesdelphensis* as cited in the literature. Further, decreased sharply at pH 4.2 since at low pH yeast multiplication was inhibited, in turn proper growth was not observed at 4.2 pH. The optimum pH for *Nakaseomycesdelphensis* fermentation was pH 5.2. At 6.2 pH, higher specific growth rate (0.202 per hour) was obtained at 30°C temperature and reducing sugar 2g.

Fig. 3. Specific growth rate at pH 5.2 with reducing sugar and different temp



When the pH decrease like 6.2 to 5.2, specific growth rate increased (0.205 per hour) but at same condition, temperature 30°C and reducing sugar 2g as given in Fig.3. And again when the pH decreased 5.2 to 4.2, specific growth rate is also decreased (0.19 per hour) but the condition is temperature 30°C and reducing sugar 2g. On 35°C specific growth rate is too low because of temperature effect. In the present study it was investigated that at optimum condition for higher growth of microorganism from microalgae at 5.2 pH. In earlier studies, *Ziziphus mauritiana* fruit pulp was used along with *Saccharomyces cerevisiae* [27]. The rate of ethanol production observed was maximum at pH 6 and on lowest pH carbon dioxide production presumably low because the low pH encourages the production of acid instead of alcohol [28]. Thus, pH plays an important role in the growth and adaptation of microorganism.

Effect of sugar concentration

Specific growth rate obtained was maximum at 2g/100mL reducing sugar concentration. Specific growth rate was found to decrease slightly as the reducing sugar concentration was increased from

1 to 3g/100mL and dropped significantly at 3g/100mL.

When the reducing sugar concentration increased like 1 to 3g/100mL, specific growth rate was decreased on temperature 30°C and pH 5.2. High substrate concentrations were inhibitory to fermentation due to osmotic stress. Earlier the studies explored the suitability of microalgae (*Chlorococum sp.*) as a substrate for bio-ethanol production via yeast (*Saccharomyces bayanus*) under different fermentation conditions. Results had shown maximum ethanol concentration of 3.83 g L⁻¹ obtained from 10 g L⁻¹ [8]. Previously it was reported that batch fermentation system was used to produce ethanol using an osmotolerant *S. cerevisiae* (US3) immobilized in calcium alginate. Fermentation was carried out with initial concentration of 150, 200, 250 g glucose per litre at 30°C. The maximum amount of ethanol produced by immobilization VS3 cells using 150, 200 and 250 g/L glucose was 72.5, 93 and 83 g ethanol per litre at 30°C after 48h [29]. The present data indicated that the specific growth rate was lower in concentration above 2g/100mL. Substrate inhibition probably reduced the specific growth rate. When substrate concentration increased, osmotic pressure became pronounced that immensely effected fermentation efficiency. The optimum initial reducing sugar concentration for fermentation was 2g/100mL that gave the maximum specific growth rate for the production of higher ethanol yield.

Effect of temperature on fermentation

To optimize the fermentation temperature, fermentation was carried out at 25, 30 and 35°C. The maximum specific growth rate was achieved at 30°C and slightly dropped at 35°C. At higher temperature more than 30°C, during fermentation the cell viability and productivity of the organism decreased. Maintenance of high cell viability was the major characteristic of fermentation so as to retrieve high ethanol yield. The optimum temperature for *Nakaseomyces delphensis* was 25°C but as per the results obtained 30°C must be considered as the maximum fermentable temperature for the organism.

At 25°C optimum temperature, specific growth rate was increased (0.2 per hour) but the combining conditions were pH 6.2 and reducing sugar 2g. Cells were more comfortable at this temperature and pH because it was the optimum condition of *Nakaseomyces delphensis* strain. When the temperature increased from 25 to 30°C, specific growth rate also increased (0.205 per hour) but the conditions were pH 5.2 and reducing sugar 2g at the time of consideration for 30°C. Cells were more viable on 5.2 acidic pH and 30°C temperature. When the temperature increased from 35°C, specific growth rate was decreased at pH 5.2 and reducing sugar 2g/100mL. On higher temperature and more

acidic condition the cell growth was inhibited, then that higher specific growth rate was kept at pH 6.2 than 5.2 pH. In previous studies *S. cerevisiae* (Zymaflore VL1) and *S. Cerevisiae* (Uvaferm CM) had shown higher growth rate and maximum biomass concentration at 25°C [30]. As expected, the growth of species varied according to the temperature, i.e. a higher growth rate was observed with higher temperature.

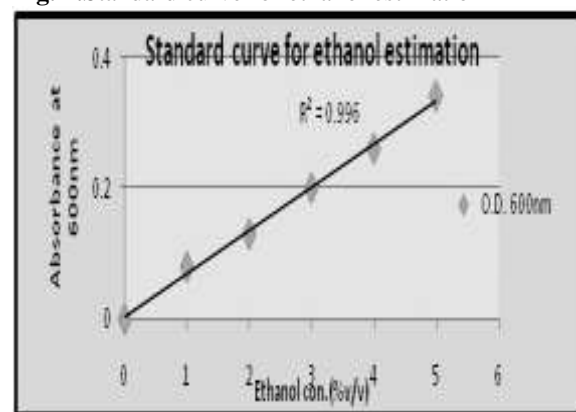
Thus, microbial growth rate and conversion of carbon source to biomass increased with increasing temperature, while the ethanol yield (Y^{P/s}) decreased utilizing substrate at specific conditions. Also, higher temperature decreased cell viability and productivity. In this study higher ethanol production was obtained at 30°C with effective specific growth rate without facing any external problem.

Ethanol production

Ethanol concentration determination by spectrophotometer

After optimizing the various parameters like pH, temperature, sugar concentration, etc. The optimum condition of previous experiments was considered, i.e. 2% sugar concentration, pH 5.2 and temperature 30°C for 100 ml medium to further carry out the experiment. After carrying out the fermentation, the samples were analysed using spectrophotometer and compared with the standard run of absolute ethanol (95%).

Fig. 4. Standard curve for ethanol estimation



Applying Eq.(1) ethanol percentage was estimated experimental ethanol production from marine algae using yeast fermentation was 0.8% v/v of ethanol from 2 grams of biomass, and 1% v/v ethanol from 2g of sugar that was treated as control as shown in the Fig.4.

Fig.5 (a) shows, consumption of control and microalgal Sugar for ethanol production by fermentation process. Control means that fermentation with original incubation medium. Maximum ethanol concentration of control was 0.789 g/mL and an ethanol yield was 0.39g/g and ethanol production from marine algae was 0

.6312 g/mL and an ethanol yield were 0.31 g/g as shown in Fig.5 (b).

Sugar consumption held at the time of exponential phase in fermentation was considered. Control sugar and microalgal sugar were consumed and simultaneously ethanol was produced. After 10 h of bio-ethanol fermentation, the concentration of the reducing sugar decreased with time, and the concentration of bio-ethanol further increased gradually, clearly indicating that the biomass of *D. tertiolecta* 13.86 were used as fermentation sugars.

Fig. 5. (a) Sugar consumption for ethanol production by fermentation process.

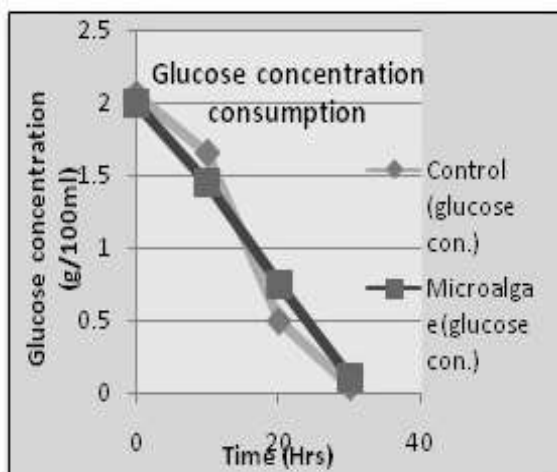
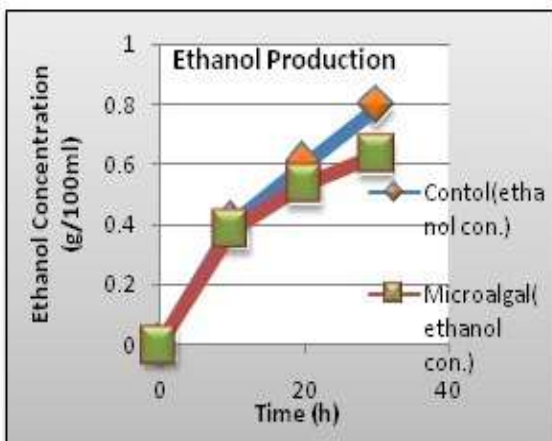


Fig.5.(b) Ethanol production graph with control and microalgal ethanol concentration



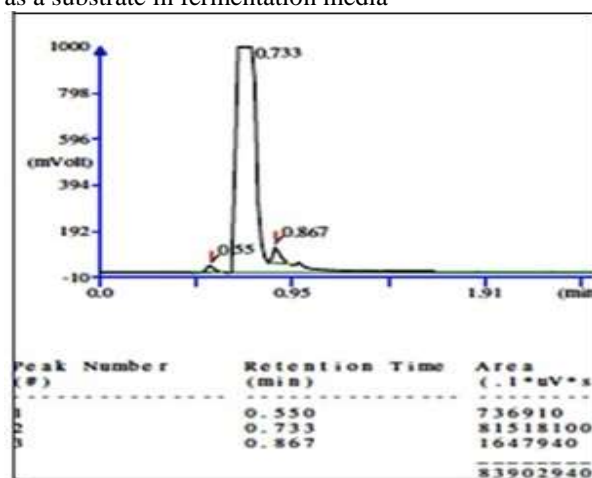
In the fermentation process, sugar was consumed and simultaneously ethanol was produced. From the control sugar, ethanol production is high in comparison to microalgal ethanol. Because of inhibitory effect that was found to be high in microalgal fermentation and yeast adaptation was slow in microalgal sugar environment. Furfural and 5-hydroxy-methyl-furfural (HMF) were known as strong inhibitor compounds present in the hydrolysate. Also algal reducing sugar environment not suited for proper microorganism growth. High

concentration of furfural, HMF resulted in less fermentability of the hydrolyzate. The fermentation yield of bio-ethanol based on the residual biomass was considered along with glucose that came out to be 0.31 g ethanol/g residual biomass and 0.394 g ethanol/g glucose, respectively.

Analysis of ethanol by Gas Chromatography

Gas Chromatography was connected to an integrator and computer system to determine retention time of ethanol and internal standard peak. GC provides a rapid and very effective method of finding out a certain unknown by using this retention parameter in Fig.6

Fig. 6. Chromatogram for microalgal reducing sugar as a substrate in fermentation media



Retention time of control and sample was almost same as std. ethanol retention time as shown in Table 3. This was indicated that ethanol was present in control and sample. A qualitative analysis of ethanol was done with the help of GC.

Table 3. Qualitative analysis of ethanol with the help of GC

S.No.	Sample Detail	Retention Time (minutes)
1.	Std. Ethanol	0.650
2.	Control (Glucose)	0.717
3.	Sample (microalgal reducing sugar)	0.733

This study proved bio-ethanol was produced using pretreated algal sugar as carbon source and *Nakaseomyces delphensis*114 yeast culture. In this case the ethanol yield is 0.31g/g by using acid pretreated microalgae *Dunaliellatertiolecta* biomass and *Nakaseomycesdelphensis*yeast combination. And also unidentified monosaccharide were not used for ethanol fermentation by *Nakaseomyces delphensis*114. This amount of ethanol was higher

than previous studies for example microalgae *Dunaliellatertiolecta*biomass and *S. cerevisiae* produce 0.14 g ethanol/g residual biomass bio-ethanol yield with help of enzymatic hydrolysis [31].

From microalgae, bio-ethanol production on a large scale can be possible by using carbohydrate-rich microalgae as the feedstock. For example, Ethanol production by the SHF process with dilute acid hydrolysis (1% H₂SO₄) of the microalgal *C. vulgaris* FSPE biomass and *Pseudomonas sp.* CL3 was more effective, producing 11.66 g/L of ethanol within 12 h with a yield of 87.59% of the theoretical one [32]. The main focus was the valuable cell components of microalgae need to be utilized to enhance the economic feasibility of microalgalbiorefinery. The residual biomass can be utilized for the production of bio-ethanol because it holds a great amount of polysaccharide.

Conclusion

Microalgae possesses many good properties to be considered as feedstock for ethanol output. *N. delphensis* yeast produced 0.31g/g ethanol by using *D. tertiolecta* microalgal biomass. Simple acid pretreatment converted the biomass to the polysaccharide and then to monosaccharide sugars. In Acid hydrolysis, percentage of acid and time both are effected on to the reaction; prolonged hydrolysis time might cause monosaccharide's decomposition at high acid concentration. 0.5 M HCl and 45 minutes had shown the best result for acid hydrolysis. Extraction of carbohydrate or pretreatment of algae was carried out by acid treatment. After acid hydrolysis 30.0 mg/mL of reducing sugar was retrieved.

The factorial design was used to find the factors that were significant, the optimum conditions obtained were: 5.2 pH, 2g sugar and 30°C that was the best optimum conditions with the predicted response of 0.205 per hour specific growth rate of ethanol production. The outcome revealed that Bio-ethanol was produced by *Nakaseomycesdelphensis*, and the ethanol yield was 0.31 g ethanol/g residual biomass. The experiment design MINITAB suggests new possibilities and techniques to improve the production of bio-energy by microorganisms. But research and development of microalgae biofuel is still in its infancy. There are also too many technical problems. For example difficulty to find one kind of perfect microalgae with high carbohydrate content that is easy to harvest. There are few of commercial microalgae production companies. There for, Market acceptance is still unclear.

Marine microalgae might be more suitable as a renewable energy source for Bio-ethanol production from carbohydrate. And also indicate that the residual biomass of microalgae after biodiesel production could be used for Bio-ethanol fermentation, which may enhance the environmental

cultivation possibilities without any competition with food crops. This study can help the industrialists/scientific community to choose the best ethanol producing strain to produce environment friendly bio-ethanol.

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