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Bioethanol from Marine Algae: A Solution to Global Warming Problem

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ABSTRACT: Ethanol fuel is an alternative to gasoline. Ethanol can be used as additive to gasoline, and also as a feed chemical in the transesterification process for biodiesel. Ethanol can be mass produced by fermentation of sugar or by hydration of ethylene from petroleum and other resource that may offer environmental and long term economic advantages over fossil fuels, like gasoline or diesel. A number of biofeed stock are currently being experimented for biofuel production, algae have emerged as one of the most promising sources for biofuel production. The marine ecosystem has vast resources of biomass with high to very high carbohydrate percentage. The marine biomass e.g. macro algae having very good potential for bioethanol production marine algae e.g. *Enteromorpha species* is used as the starting material for the bioethanol production in the present investigation. This algae is very rich in carbohydrate source ranging from 70-72%. Successful bioconversions of algal biomass to ethanol have been achieved by a series of different pretreatment, hydrolysis and fermentation. The processed biomasses was subjected to different pretreatments such as Nitric acid, steam flashing and dilute sulphuric acid (H_2SO_4), after which hydrolysis was undertaken chemically as well as enzymatically to accesses degradation of polysaccharides to their monomers or fermentable sugars. The percentage of fermentable sugar was obtained by such hydrolysis ranges from 0.75 mg/gm to 0.940 mg/gm in dry algae and 0.21 to 0.52 mg/gm in case of fresh algae as partial hydrolysis of different polysaccharides. The fermentation process used by industrial yeast (*Saccharomyses cerevisiae*) has the efficiency of utilization of glucose, fructose and sucrose etc. In addition to marine algae, in current years bioethanol is produced from a number of renewable biomasses. Bioethanol is a versatile transportable fuel and fuel additive that offers excellent performance and reduce air pollution compared to conventional fuel. Its production and use adds very little release of CO_2 to the atmosphere dramatically reducing the greenhouse gases responsible for global warming.

Keywords: Bioethanol, Marine algae, *Enteromorpha*, Alcohol, Fermentation.

INTRODUCTION

Population outburst together with increased motorization has led to an overwhelming increase in the demand for fuel. In the milieu of economical and environmental concern, algae capable of accumulating high starch/cellulose can serve as an excellent alternative to food crops for bioethanol production, a green fuel for sustainable future. "Algae and aquatic biomass has the potential to provide a new range of third generation biofuels, including jet fuels. Certain species of algae can produce ethanol during dark-anaerobic fermentation and thus serve as a direct source for ethanol production. Their high oil and biomass yields, widespread availability, absent (or very reduced) competition with agricultural land, high quality and versatility of the by-products, their efficient use as a mean to capture CO_2 and their suitability for wastewater treatments and other industrial plants make algae and aquatic biomass one of the most promising and attractive renewable sources for a fully sustainable and low-carbon economy portfolio." (Source: European Algae Biomass Association - EABA). The Sustainable Fuels from Marine Biomass project, Biomara, is a new UK and Irish joint project that aims to demonstrate the feasibility and viability of producing third generation biofuels from marine biomass. It will investigate the potential use of both macroalgae and microalgae as alternatives to terrestrial agri-fuel production. The first distinction that needs to be made is between macro algae (or seaweed) versus microalgae. Microalgae can provide several different types of renewable bio-fuels. These include methane produced by anaerobic digestion of the algal biomass (Spolaore *et al*., 2006) biodiesel derived from micro algal oil (Thomas, 2006; Rorssler *et al*., 1994 and Banerjee *et al*., 2002) and photo biologically produced bio-hydrogen (Gavrilescu and Chisti, 2005; Fedorov *et al*., 2005). The idea of using microalgae as a source of fuel is not new (Kapdan and Kargi, 2006) but it is now being taken seriously because of the escalating price of petroleum and, more significantly, the emerging concern about global warming that is

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associated with burning fossil fuels (Sawayama *et al*., 1995). Bio-ethanol is currently being produced by fermentation of sugars found in plants such as sugarcane and corn. Many social concerns have barred the adoption of their future use, so other feedstock is being considered as substitutes. Algae are one of that feedstock. Algae do not produce as much starch as corn, and do not have firm agricultural practices. However, there is reason to believe that algae will play a role in the future bio-ethanol market. In order to lower the cost of producing this fuel, other products from algae will have to be processed and sold. Bio-ethanol is one of those products. Using everything that algae have to offer is the best route towards more favorable economic models for these low-value high-volume products.

Seaweed/macro algae such as *Enteromorpha* species are abundantly available in our East Coastal marine ecology. More over it is reported to contain 70% carbohydrate (dry wt. basis).Therefore it appears that it is one of the suitable biomass which may be exploited for bioethanol production.

Accordingly the present investigation has been carried out by taking the *Enteromorpha* species as feed stock for bioethanol production.

In view of the above the present investigation followed different approaches which aims at meeting the following objectives.

1-Study of different pretreatment methods and their effects on production of fermentable carbohydrates.

2-Hydrolysis and conversion of pretreated biomass to fermentable sugars.

3-Alcoholic fermentation and estimation of bioethanol production.

MATERIALS AND METHODS

All the experiments leading to the production of bioethanol from marine algae were conducted in biochemistry laboratory of O.U.A.T, Bhubaneswar during the session 2009_2010. The algae materials which are used for the production of bioethanol were collected from outskirt of Kaliyugeswar, central sector of the Chilika Lake.

Plant Materials

Enteromorpha species of algae

1. Dry algae
2. Fresh algae

Estimation of Cellulose from Marine Algae (*Enteromorpha*)

For estimation of cellulose from algae, the algae were taken as dry or fresh. For drying algae cut in to pieces and then for dehydration of the biomass the materials were dried through hot air oven. The materials were powdered by mortar and pestle. Fresh algae were taken to remove the pigmentation. Methanol /cold acetone was added to it and was grinded by mortar and pestle to break the cell wall of algae. Then the grinded materials were centrifuged and the pellet was collected for estimation cellulose (Sadasivam and Manickam, 2008). A blank with distilled water and anthrone was taken. 100gm of cellulose was taken in a test tube for standard curve and the colo 2 measured at 630nm.

Estimation of Total Carbohydrate

The carbohydrate estimation was done by the method described in (Sadasivam and Manickam, 2008). A Standard graph was drawn by plotting concentration of the standard on x-axis v_s absorbance on y-axis. From the graph the carbohydrate present in the sample tube was calculated, by the following formula.

Amount of carbohydrate present in 100mg of the sample.

$$= \frac{\text{mg of glucose} \times 100}{\text{Volume of the test sample}}$$

Pretreatment of algal biomass

For pretreatment of algae 2 types of methods used.

*Physical pretreatment

*Chemical pretreatment

Physical pretreatment

In physical pretreatment, soaking the material overnight followed by steam explosion (raising to 120^0 C at 15 p.s.i. for 30 sec to 1 min) followed by reducing the pressure to normal as early as possible. The another method is without soaking steam flashing is done in a pressure cooker to dry matter in a test tube and treated with steam with high pressure.

Chemical pretreatment

(1) Nitric acid/Acetic acid reagent treatment

1 gm of sample was taken and 3ml. of acetic/nitric acid reagent added in a test tube, the tube was placed in a water bath for 100c for 30 min. After 30 min. the sample containing the tube was cooled and the contents were centrifuged for 15 min. at 10,000 r.p.m. After centrifugation the supernatant was discarded and the residue was washed with distilled water.

(2) Dilute Sulphuric acid treatment

3 ml of 1% H_2SO_4 was added with 1 gm of sample in a test tube. The tube containing the sample was placed in a boiling water bath 100^0C for 2 min. then the tube was cooled and the content was centrifuged for 15 min. The supernatant was discarded and the pellet was washed with distilled water.

(3) Cellulolysis (67% H_2SO_4)

After the pretreatment of the sample or algal biomass by physical and chemical treatment, the samples were treated with 10ml of 67% sulphuric acid.

(4) Cellulolysis (1% H_2SO_4)

After the pretreatment of the material, the hydrolysis was conducted with 10 ml of 1% H_2SO_4 incubated for 1hour in room temperature and the total carbohydrate and percentage of sugar was calculated.

Enzymatic hydrolysis

Isolation, purification &assay of cellulase

The extracellular cellulase was extracted from naturally rotting biomass, after isolating the microbial species and culturing in nutrient broth containing cellulose as inducer. The

crude enzyme extract was subjected to salting out followed by desalting. The resultant extract was taken as partially purified enzyme complex and assayed. For overall activity assay as described Ghose *et al.*,(1987). Accordingly 1 mg of total protein was assumed equivalent approximately 1 FPU activity. The enzyme content was estimated in terms of soluble protein by spectrophotometric method.

Preparation of reaction mixture

The reaction mixture was prepared by taking 1gm of sample broth fresh and dry suspended in 50mM phosphate buffer (pH 4.5) to which enzyme extract of 5 FPU per 100mg was added , and incubated at 30^0C for 4 days, up to which the hydrolysate was centrifuged and analyzed for reducing sugar content (DNS method) for subsequent fermentation process.

Estimation of fermentable sugar by DNS methods

The reaction mixture was prepared by taking 2 gm. of each sample in test tube which were pretreated chemically or physically. The chemically pretreated materials were with distilled water to remove acid. The fermentable sugar was estimated by the method (Sadasivam and Manickam, 2008). For standard curve (0-500µg) of glucose was taken in test tubes and a standard solution was prepared as proceeding as before by taking (0.5-1.5 ml) of concentrated solution and DNS reagent and Rochelle salt solution was added. Then O.D. was measured at 510 nm.

Alcoholic fermentation

Both hydrolysate and non hydrolysed material were taken for fermentation. To some pretreated material enzyme was added for hydrolysis. Alcoholic fermentation was done in two phases: Primary and Secondary fermentation.

2 gm of sample was pretreated material was washed thoroughly with distilled water. The samples were incubated with 1gm of commercial yeast at 37^0c in a thermostatic shaker in aerobic condition. After 3_4 days the conical flask containing all the materials were subjected to anaerobic incubation for 7 days. The primary alcohol of the content was estimated before and after fermentation. Secondary fermentation was done taking the fermentable of primary fermentation with sucrose for fermentation of existing yeast in the fermentation of another 7 days.

Experimental finding related to various experiments with the carbohydrate estimation, cellulose estimation and fermentable sugar estimation has been presented in Tables and Figures. Total polysaccharides such as cellulose and hemicellulose etc. are the basic starting material for conversion to bioethanol by using biotechnical tool. The process being involved such as pretreatment of biomass hydrolysis or cellulolysis of complex saccharide, estimation of hydrolysate for fermentable sugar are also presented in previous tables. Subsequent fermentation for production of ethanol in vitro has been summarized. Accordingly the various estimation and investigation are out line below for interpretation.

Moisture percentage calculated 92.23% found to be inaggreable with available literature. Therefore calculated where ever necessary are expressed on dry wt. basis.

The total carbohydrate content calculated from the dry sample and fresh sample on dry wt. basis comes to 71.08% and 64.15% respectively, which shows better suitability of biomass for direct conversion to bioethanol using proper biocatalyst even without pretreatment before hydrolysis of the polysaccharides available in the sample.

Available cellulose was estimated pretreatment of physical and chemical method, so as to render the cellulose accessible to hydrolysis. The Fig-1 shows the result analysis for cellulose content after eliminating the available carbohydrate. The result obtained as shown in the above table (55.0 mg/gm of dry matter and 715.0 mg/gm of dry matter). The result indicates that release of hydrolysable cellulose both in case of naturally dry and fresh sample comparatively higher in case of dilute acid pretreatment compared to Nitric acid pretreatment. More ever released cellulose content in case of naturally dry wt. sample in both the treatment is better than fresh sample.

Hydrolysable cellulose in case of dry algae is significantly higher than that of fresh sample.The pretreated sample were subjected to hydrolysis by both chemical and enzymatic means. The result of hydrolysis corresponds to estimation of fermentable sugar obtained by hydrolysis by standard protocol followed by other worker as per literature. The samples were hydrolyzed by 67% and 1% sulphuric acid and analyzed for fermentable simple sugar. The result has been shown in Table-4 and fig-3 and 4.

The result shows 880.0 mg/gm and 440 mg/gm. in case of hydrolysis by 67% sulphuric acid of the naturally dry and fresh biomass respectively, where as hydrolysis by 1%sulphuric acid yields 682.0 and 132.0 mg/gm. of sample.

RESULTS

Fig. 1. Total Carbohydrate Content of Algal Sample on Dry Wt. Basis

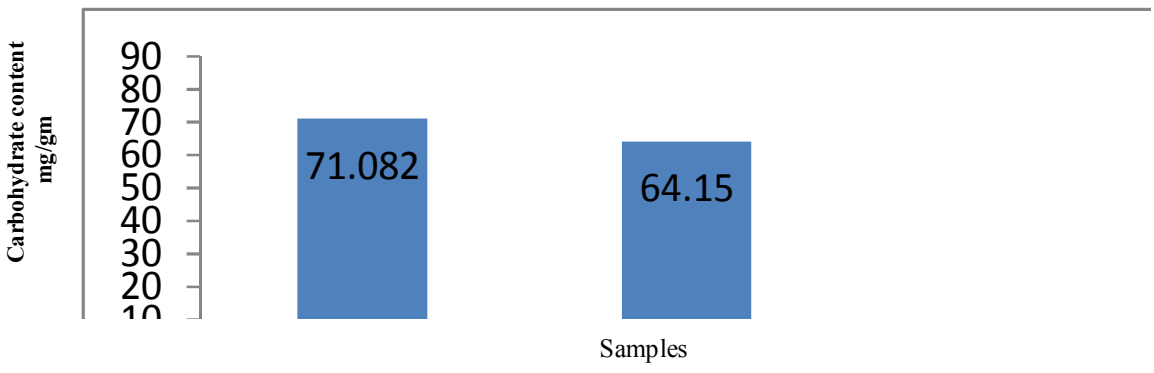

Fig. 2. Total Hydrolysable Cellulose Content of Algal Sample

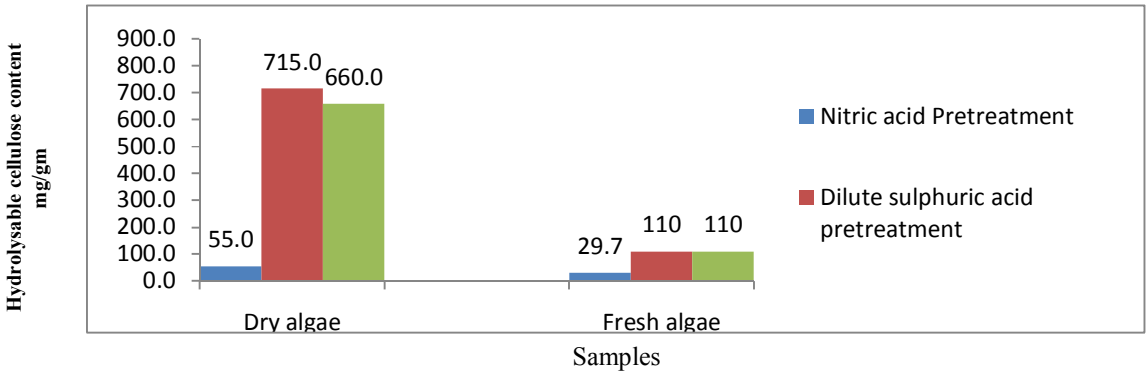

Fig. 3. Effect of Pretreatment on Chemical Hydrolysis (67% H_2SO_4)

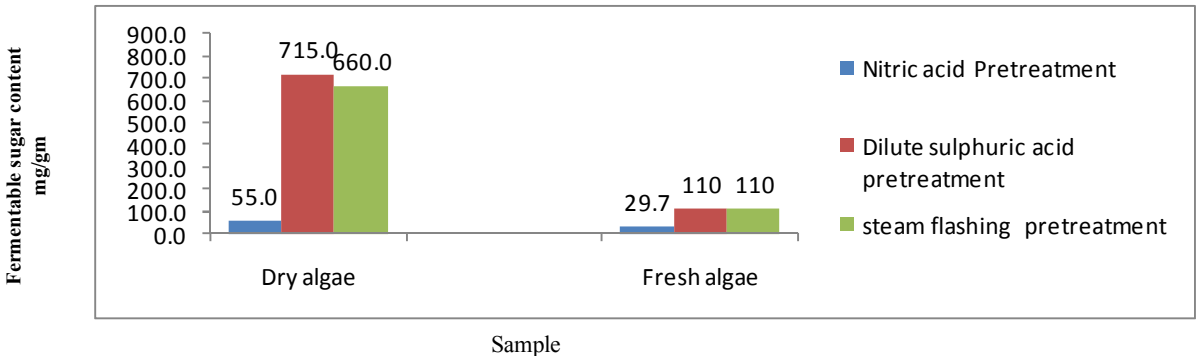

Fig. 4. Effect of Pretreatment on Chemical Hydrolysis (1% H_2SO_4)

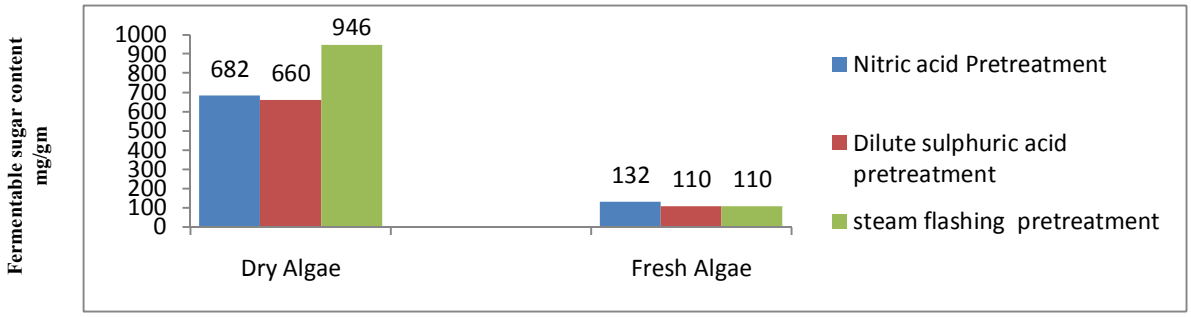

Fig. 5. Effect of Pretreatment on Enzymatic Hydrolysis

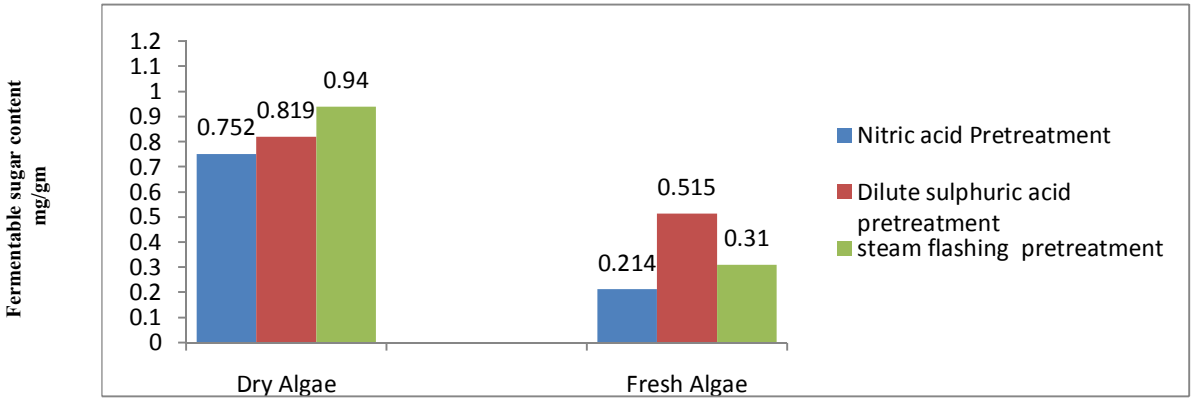

Table 1: P.A. Value of 1st Phase of Fermentation with and Without Enzymatic Hydrolysis

Samples	No. of pretreatments	Fermentation Without Enzymatic Hydrolysis			Fermentation With Enzymatic Hydrolysis		
		B.F P.A. value	A.F P.A. value	Diff.	B.F P.A. value	A.F P.A. value	Diff.
Dry Algae	Steam flashing	0.862	1.012	0.15	0.77	0.99	0.2256
	Dil. H_2SO_4	0.794	0.994	0.20	0.89	1.007	0.117
	Nitric acid	1.005	1.020	0.015	0.88	1.086	0.206
Fresh Algae	Steam flashing	0.990	1.0146	0.024	0.843	1.005	0.162

P.A.= Primary Alcohol, B.F.= Before Fermentation, A.F.= After Fermentation

Table -2: Summary Table Effect of Pretreatment on Cellulolysis

Pretreatments	Amount of fermentable sugar in mg./gm.			
	Dry Algae		Fresh Algae	
	Chemical	Enzymatic	Chemical	Enzymatic
Nitric acid	880.0	0.752	440.0	0.214
Dilute Sulphuric acid	770.0	0.819	242.0	0.310
Steam Flashing	**946.0**	**0.940**	**220.0**	**0.515**

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The yield of fermentable simple sugar in case of dry algae in both type of hydrolysis was more than that of fresh algae being 770 mg/gm. with $67\%H_2SO_4$ in case of dry algae and 242.0 mg./gm in case of fresh algae. Similarly 660.0 mg./gm. in case of dry algae with $1\%H_2SO_4$ and 110 mg./gm. in case of fresh algae.

Analysis for fermentable sugar yielded by chemical hydrolysis with both $67\%H_2SO_4$ and $1\%H_2SO_4$ after physical pretreatment of steam flashing was done and result is shown in steam flashing hydrolysis. The yield of fermentable sugar is found to be 836.0 mg/gm in case of dry algae and 220.0 mg/gm in case of fresh algae after hydrolysis with $67\%H_2SO_4$,and similarly after $1\%H_2SO_4$ hydrolysis 946.0mg/gm and 110.0mg/gm for dry algae and fresh algae respectively.

Extracellular cellulase/sample was isolated from the culture filtrate of the unidentified micro organism grown in presence of cellulose in the media. The protein content was estimated in terms of soluble protein by spectrophotometric methods and expressed in mg/ml, with BSA as standard .The activity of the enzyme in terms FPU/ml. The result showed as:

Average protein content = 28mg/ml
Average Activity =5.4 FPU/ml

The pretreated sample was subjected to enzymatic hydrolysis by using reaction mixture containing 0.5 gm substrate, enzyme (1F.P.U./gm) of material,50mM phosphate buffer(pH 4.5).The moisture was incubated for 4 days at 40^0c ,after which the hydrolysate were analyzed for fermentable sugar content by DNS method by taking glucose as standard. The results obtained are showed in Fig-5.

The fermentable sugar content was found to be 0.752 and0.214mg/gm of dry algae and fresh algae in case of Nitric acid pretreatment and 0.819 and 0.310 mg/gm of dry and fresh algae in case of dilute sulphuric acid pretreatment.The fermentable sugar content after Nitric acid pretreatment is comparatively higher than that of dilute sulphuric acid.

The fermentable sugar content after enzymatic hydrolysis of pretreated (steam flashing)biomass was estimated and found to be 0.940 mg/gm in case of dry algae and 0.515 mg/gm in case of fresh algae as shown in steam flashing pretreatment on enzymatic hydrolysis in Fig-5. The carbohydrate content was estimated before pretreatment and hydrolysis as shown in Table-1 was above 70%.Therefore direct fermentation was undertaken to observe the possibility and yield of ethanol after incubation with *Saccharomyces cerevisae* for a period of 7

days. The alcohol content of the ferment was evaluated by difference in P.A. value by hydrometric method and the result is summarized in Table-2.

The difference of P.A. representing mg of alcohol/ml of fermentation product was found to be highest in case of the fermentation of the hydrolysate with 1% sulphuric acid at 0.2 mg/ml in case of fresh algae where as that in case of fresh algae with steam flashing shows 0.024 mg/ml as the staring matter with 90% moisture. Therefore the yield of ethanol in both the cases appears to be apart. Alcoholic fermentation was carried out with hydrolysate of enzymatic hydrolysis and the conversion was evaluated as mentioned in Table-6.

Alcoholic fermentation was carried out with hydrolysate of enzymatic hydrolysis and the conversion was evaluated as mentioned above. The result was put in the following Table-6. The difference of P.A. value of enzymatic hydrolysate of dry algae after steam flashing pretreatment shows highest percentage of ethanol(difference of P.A. value 0.22), followed by that after Nitric acid pretreatment. Secondary fermentation samples with existing yeast cells was undertaken with sucrose as booster carbon source. The P.A. value as shown negligible result to be under taken in to account in the present investigation.

DISCUSSION

The total carbohydrate content in Fig-1 of the different sample of biomass as estimated on dry wt. basis (71.082 mg/gm.) is in confirmative with the finding of other workers (Haroon *et a*l., 2000) that shows suitability of the material to go for bioethanol conversion without undergoing combersion of methods of hydrolysis as it has been seen in case of alcoholic fermentation of unhydrolysed biomass as shown in Table-2.The cellulose content as estimated and recorded in Fig-4 and 5 for the pretreated material with Nitric acid, dilute sulphuric acid and steam flashing with the range of 55.0-715.0 mg/gm. in case of dry algae which shows the lowest in case of nitric acid pretreatment, which confirms that suitability of dilute sulphuric acid pretreatment compared to that of nitric acid pretreatment .It may be due to non accessibility of cellulose to hydrolysis after nitric acid pretreatment .More over degradation of materials and production of inhibitor may be accounted for the lower estimate of cellulose in case of nitric acid pretreated materials .As specific reports are not available from the literature in respect to *Enteromorpha* species ,the result can be compared with similar material and carbohydrate content such as water hycyanth, *(Eichhornia crassipes)* as reported by (Sauze *et al*., 1981).

As regards fermentable sugars obtained by hydrolysis of biomass both by chemical and enzymatic methods. There is no deviation on the finding of other workers such as (Phillipidis 1994; Wyman 2004).

Showing better result in case of enzymatic hydrolysis over chemical hydrolysis may be due to degradation of sum of fermentable sugar during acid hydrolysis .Although hydrolysis by dilute H_2SO_4 acid showed commentable result in terms of fermentable sugar in the hydrolysate (770.0 mg/gm) with still better yield after steam flashing pretreatment with 946.0 mg/gm.(Fig-4 and 5). The analysis of enzymatic hydrolysate of dry algae after steam flashing showed very low fermentable sugar that is 0.75 to 0.84 mg/gm.(Fig-5) in case of fresh algae is attributed to incomplete hydrolysis by exo cellulase used for hydrolysis for a very short incubation period, due to nature of cellulase complex acting on free chain of polysaccharides after action of endo-cellulase which is not available in the complex used Reported by(Montene *et al*., 1977)

The hydrolysate of different hydrolysis methods used after different pretreatments being subjected to alcoholic fermentation by yeast. Therefore the effect of pretreatment is indirectly related and can not be correlated quantitatively for the production of alcohol rather than it is the nature of hydrolysis which has direct relationship with alcoholic fermentation (Table-2). Percentage of reducing sugar being converted ethanol being standardize and reported to be 92 % (Wiman, 2005). The results obtained are no exception, to the finding of the other worker in the related field.

However due to abundance of biomass as a marine resource for exploitation in the bioconversion, detail study of hydrolysis protocol followed by fermentation needs prior to standardization and optimization of pH, temperature, reaction timing, enzyme substrate concentration and inhibitory factors, for better exploitation of the scope and prospect in future.

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