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# *ENTEROMORPHA INTESTINALIS* AS VIABLE FEEDSTOCK FOR BIOETHANOL PRODUCTION

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## ABSTRACT

*Enteromorpha intestinalis* biomass were pretreated and subjected to fermentation. Acid pretreatment yielded 23.81±0.14 mg/g of reducing sugar. Acid pretreated biomass subjected to enzyme hydrolysis yielded 135 mg/g of reducing sugar. Separate hydrolysis and fermentation was carried out for acid hydrolysate using yeast strain isolated from Cashew fruit juice (CY) and Toddy juice (TY) Hydrolysate with CY strain yield 0.09g/g of ethanol and TY yielded 0.16g/g of ethanol achieving 17.26 and 31.25% theoretical efficiency respectively in SHF process. In SSF process, TY yielded higher ethanol yield of 0.31g/g achieving 61.54% theoretical efficiency and exhibiting thermotolerance ability.

**Keywords:** Bioethanol, *Enteromorpha intestinalis*, SHF, SSF, Thermotolerance

## 1. INTRODUCTION

Dwindling fossil fuel have posed threat to global economy. Coal-dependent nations like China and India are in urgent need of alternative fuels to secure its future energy and improve the environment, bioenergy is a promising solution for its Energy, Food and Environment trilemma (Qin et al., 2017).

Renewable & sustainable energy sources have come into existence due to serious environmental impact caused by non-renewable fossil fuels (Srivastava et al., 2017). Long standing energy problems in developing countries can be solved using renewable energy sources and technologies (Kumar et al., 2010). Biofuels derived from crop residues and bioenergy crops emerge as a great addition to renewable energy without compromising food production (Qin et al., 2017).

Bioethanol from sugar and starch are regarded as 1st generation biofuel. However, largescale production of this biomass damage the environment by the use of harmful pesticides, and valuable resources like arable land and enormous quantities of water. Bioethanol from lignocellulosic feedstock are regarded as 2nd generation biofuel. Lignocellulosic material constitutes world's largest bioethanol renewable resources belonging to second-generation feedstocks. Biofuel produced using lignocellulosic biomass originate from agricultural and forest residues (Limayem et al., 2012). However, obstacle in lignocellulosic biomass for conversion to biofuels are cost intensive pretreatment processes due to the presence of lignin molecule. Sustainability of first and second generation biofuels is questioned in connection with food versus fuel debate, carbon accounting and land use (Araujo et al., 2017)Therefore, algae are considered as 3rd generation feedstock for biofuel production. Advantages of algal biomass over first and second generation feedstocks are low land requirement for biomass production and high oil content with high productivity (Kumar and Sahoo, 2012; Behera et al., 2015). John et al., (2011), reviewed on potential of micro and macroalgal biomass as renewable source for bioethanol indicating that utilization of algal biomass for bioethanol production is undoubtedly a sustainable and eco-friendly approach for renewable biofuel production. Macroalgal biomass are rich in carbohydrate which are converted to bioethanol using microorganisms and lack lignin in algal biomass evades cost intensive delignification process. Higher growth rate and productivity is another advantage of algal biomass (John et al., 2011; Yanagisawa et al., 2011; Jung et al., 2013).

Macroalgal resources are distributed along the coast of India, rich resources are recorded from Tamil Nadu and Gujarat, whereas Mumbai, Goa, Karnataka and Kerala are fairly rich in them. Seaweeds have well established market for hydrocolloid (carrageenan, agar and alginate) production, since they are only natural source.

Red seaweeds are mostly utilized for extraction of carrageenan and agar, whereas alginate are extracted from brown seaweeds. The leftover residues rich in cellulose are utilized for biofuel production. Green seaweeds are mostly used for food purpose in Southeast Asian countries (Hebbale et al., 2017). Bioethanol has been obtained from all the three types of algae, however study indicates *Laminaria japonica*, *Eucheuma* spp., *Kappaphycus alvarezii*, *Undaria pinnatifida*, and *Gracilaria verrucosa* as the most promising feedstocks for biorefinery (Jung et al., 2013).

In India, bioethanol potential from red seaweed species *Kappaphycus alvarezii* (Khambaty et al.,2012), *Gracilaria verrucosa* (Kumar et al., 2013) and *Gracilaria corticata* (Baghel et al., 2016) and green seaweed species *Ulva fasciata* (Trivedi et al., 2013), *Ulva lactuca* (Trivedi et al., 2015) have been explored. Since red and brown seaweeds are already in use for extraction of hydrocolloid, this study focuses on bioethanol production from green seaweed *Enteromorpha intestinalis.*

Bioethanol production process for conversion of algal sugar to ethanol from macro algae involves three major processes such as pretreatment, saccharification and fermentation. Pretreatment involves acid hydrolysis of the biomass, which alters the structural integrity of the biomass and release sugars. Acid pretreatment increases the accessibility of enzyme for saccharification process, enzymes hydrolyze the cellulose present in algal cell walls to monosaccharides (Wei et al., 2013). Sugars released after acidic and enzymatic hydrolysis are subjected to fermentation through yeast organism to produce bioethanol (Trivedi et al., 2016).Conventional pretreatment processes are facing challenges in order to achieve higher sugar yield using environmentally friendly technique. Acid pretreatment leads to sugar degradation and results in inhibitor formation such as hydroxymethyl furfurals and levulinic acid which are detrimental for yeast microorganisms in fermentation process. Enzymes with higher cellulolytic activity are being isolated from various sources that can yield higher sugar from the biomass.

Predominantly utilized microorganism for fermentation is *Saccharomyces cerevisiae* that have ability to ferment hexose sugars to bioethanol. However, algal sugars constitute pentose sugar along with hexose sugar that are not fermented by *Saccharomyces cerevisiae* (Azhar et al., 2017). Therefore attempts have been done to identify and isolate yeast microorganism from various sources apart from investigating its efficacy in bioethanol production (Dhaliwal et al., 2011;Yuangsaard et al., 2013;Jutakanoke et al., 2014; Ruyters et al., 2015; Chamnpina et al., 2017).

This research explores the viability of, *Enteromorpha intestinalis* as suitable feedstock for bioethanol production. Reducing sugar from both acid and enzyme hydrolysis were subjected to fermentation using wild yeast strains.

## 2. MATERIALS AND METHODS

**2.1. Macroalgal sampling:** Seaweed samples were collected from Aghanashini estuary during low tide period and were cleaned thoroughly by rinsing in the seawater to remove epiphytes, which were dried in shade for 3-4 days. Thereafter the dried seaweeds were heated to 50-60ºC for 15-20 min, pulverized using mortar and pestle, and then sieved to get powder of < 0.1mm. These samples were stored in air tight covers for further analysis.

**2.2. Biochemical analysis:** Total carbohydrate analysis was performed by phenol-sulphuric acid method (Dubois et al., 1956) followed by the determination of cellulose composition using anthrone reagent method (Updegroff, 1969). Protein content was estimated by Lowry's method (Lowry *et al.,* 1951*).* Total lipid content of the sample was determined by Bligh & Dyer method (1959) gravimetrically using chloroform-methanol mixture. The experiment was performed in triplicates and the mean value was considered for further analyses (mean±SD).

**2.3. Pre-treatment process**

**2.3.1. Acid hydrolysis:** 100mg dried biomass was pretreated with 0.7N $H_2SO_4$ at 121ºC for 45 mins to extract sugars. The hydrolysate was made upto 100ml. After hydrolysis the hydrolysate was neutralized with 2N NaOH to obtain pH 6. The initial reducing sugar concentration was measured using DNS method.

**2.3.2. Enzyme hydrolysis:** Pretreated biomass was subjected to enzyme hydrolysis using enzyme (S9) extracted from marine bacteria. Enzyme hydrolysis was carried out at 55ºC for 36h and pH 6.8 (Potassium phosphate buffer). The sugar released was estimated every 6h using DNS method.

**2.4. Yeast Isolation and Fermentation**

**2.4.1. Yeast Isolation:** Yeast were isolated from Cashew fruit juice (CY) and Toddy juice (TY) and plated on YEPDA medium of composition 20g/L peptone, 10g/L yeast extract, 20g/L dextrose, 15g/L agar. Yeast suspension was maintained at 35ºC till $OD_{600}$ of 0.6 was achieved for further fermentation.

**2.4.2. Ethanol fermentation:** The hydrolysate obtained from acid pretreatment and enzyme pretreatment were subjected for fermentation using CY and TY. Separate hydrolysis and fermentation (SHF) was carried out where hydrolysate (obtained from acid pretreatment and enzyme hydrolysis) were inoculated with 6% v/v yeast seed culture (0.6 $OD_{600}$) and sealed with rubber flask to provide anaerobic condition, fermentation was carried out at 30ºC for 24h. Simultaneous Saccharification and Fermentation (SSF) was carried out using 2% (w/v) pretreated biomass and 6% (v/v) enzyme and yeast were added to the medium and fermented using CY and TY at 55ºC for 24h. The ethanol present in the fermented broth was analyzed using GC-FID.

## 3. RESULTS AND DISCUSSION

**3.1. Characterization of *E. intestinalis:*** *Enteromorpha (Ulva) intestinalis* is green algae belongs to *Ulvaceae* family. During favorable nutrient, salinity, light and temperature condition they grow profusely and occupy intertidal zones. *E.intestinalis* was collected from Aghanashini Estuary during the low tide period. *E.intestinalis* is composed of 40.1% total carbohydrate, 20.4% Protein, 2.8% Lipid. Elemental analysis such as carbon 33%, nitrogen 4.36% and hydrogen 6.44% were recorded. Cho et al., (2013) recorded 42.8% carbohydrate, 31.6% crude protein and 1.3% crude lipid.

**3.2. Pretreatment**

3.2.1. ***Dilute acid hydrolysis:*** Biomass treated using dilute acid yielded 23.81±0.14 mg/g of reducing sugar. Dilute acid pretreatment is most widely used process for extraction of reducing sugars from biomass. However drawback of this is degradation of sugars in to inhibitors such as hydroxymethyl furfural (HMF). Complementary to dilute acid pretreatment shortcomings are enzyme hydrolysis which do not release inhibitors (Jiang et al., 2016). Pretreatment of biomass is done to expose the cell constituents and cell wall materials for enzyme action (Ibrahim, 2012). Pretreatment enhances porosity of the biomass and reduces the crystallinity of the biopolymer cellulose.

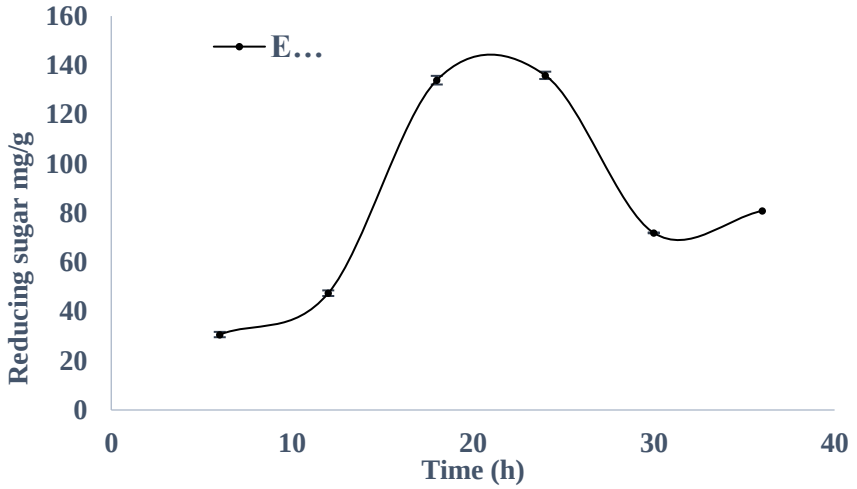


**Fig 1. .** Enzyme hydrolysis of pretreated *E.intestinalis* biomass

3.2.2. ***Enzyme hydrolysis:*** Enzyme hydrolysis was performed for acid pretreated *E.intestinalis* yielded 135 mg/g. Trivedi et al., (2015) isolated cellulase enzyme from *Cladosporium sphaerospermum* and subjected *Ulva lactuca*, green seaweed to enzyme hydrolysis and obtained 112 mg/g of reducing sugar. Kim et al., (2014) subjected hydrothermally pretreated *E. intestinalis* to enzyme hydrolysis using commercial enzymes Viscozyme L and Cellic CTec2 and obtained 20.1g/L of reducing sugar. Reducing sugar was seen to increase linearly with incubation period from 12 to 24h ranging from 47 mg/g to 133mg/g, and decreased beyond 24h to 74 mg/g. Similar trend was observed by Trivedi et al., (2015).

**3.2. Fermentation**

3.2.1. ***Separate hydrolysis and Fermentation (SHF):*** Fermentation was carried out by SHF method for 24h. Hydrolysate obtained from acid pretreatment were subjected to fermentation. Ethanol yield of 0.09g/g was obtained from 2.34 g reducing sugar and theoretical efficiency of 17.26% was achieved for hydrolysate with CY strain (table 1). Hydrolysate with TY strain yielded ethanol of 0.16g/g from 2.17g reducing sugar and theoretical yield of 31.25% efficiency was achieved. TY strain yielded higher efficiency than CY strain indicating its potential in producing ethanol from seaweed. Cho et al., (2013) achieved 30.5% theoretical yield from fermentation of *Enteromorpha intestinalis* using commercial yeast strain *Saccharomyces cerevisiae* KCTC 1126.

3.2.2. ***Simultaneous Saccharification and Fermentation (SSF):*** Higher ethanol yield was observed in SSF for EITY 0.31g/g whereas for EICY 0.14g/g of ethanol yield was recorded (table 1). SSF operated at higher temperature of 55ºC as enzyme gets activated at this

temperature. Lower yield in EICY is due to lower tolerance if temperature by CY strain. Higher temperature shortens the exponential phase of the yeast cell resulting in reduced ethanol production (Tesfaw and Assefa, 2014). TY strain exhibited tolerance to higher temperature and yielded higher ethanol.

### 3.3. Other value added products from *Enteromorpha*

Coastal areas, estuaries and semi-enclosed bays are prone to eutrophication which poses threat to underlying organisms. In order to detect eutrophication early, several indicators have been studied, macroalgae *Enteromorpha intestinalis* is one of the indicator for nutrient enrichment as it accumulates nutrients in its tissue (Fong et al., 1998). Other characteristics of *E.intestinalis* are adapted to variable environment, euryhaline in nature, eurythermal, tolerant of desiccation with low light saturation of photosynthesis. *Enteromorpha* sp. are exploited commercially for its varied chemical composition and quality. In China and Japan, *Enteromorpha* are cultivated for preparation of "aonori", which is included in variety of dishes, including raw salads, soups, cookies, meals and condiments (Ohono an Critchkey, 1993; Fong et al., 1998; Morales et al., 2005). Morales et al., (2005) estimated high protein digestibility for *E.intestinalis* indicating that the proteins are easily hydrolysed by the enzymes causing no risk for algae consumers. Fatty acid profile of *E.intestinalis* is highly relative to the other edible foods like soyabean and beans rich in PUFA, also long chain omega-3 fatty acid such as EPA (n-3) and DHA (n-3) content is greater when compared to other seaweeds and vegetables such as spinach and lettuce.

**Table 1: Estimation of ethanol**

| Process | Substrate | Initial sugar g/L | Final Sugar g/L | Fermented sugar g/L | Ethanol g/L | Ethanol yield g/g | % Theoretical yield |
|---|---|---|---|---|---|---|---|
| SHF | EI CY | 2.34 | 0.58 | 1.76 | 0.155 | 0.09 | 17.26 |
| | EI TY | 2.17 | 0.78 | 1.39 | 0.222 | 0.16 | 31.25 |
| SSF | EICY | 9.74 | 1.71 | 8.03 | 1.13 | 0.14 | 27.65 |
| | EITY | 9.91 | 2.81 | 7.10 | 2.23 | 0.31 | 61.54 |

## CONCLUSION

Macroalgae is an attractive biomass for bioethanol production as they are rich in carbohydrates which can be readily converted to bioethanol using appropriate yeast microorganisms. Wild strains have ability to convert the seaweed sugars to bioethanol. Highest ethanol yield of 0.31g/g was obtained for TY strain during SSF process indicating thermotolerance nature of TY strain.

*Enteromorpha intestinalis* are widely distributed along intertidal zones of estuaries and coastal ecosystem. Growth rate of *E. intestinalis* reaches up to 12.7% per day. *Enteromorpha* sp. were recorded in large amounts at the shores of Yellow Sea, which decayed faster and caused nuisance to the coastal seawater quality and ecological environment (Zhou et al., 2010). Similarly, in Aghanashini estuary, *E.intestinalis* is recorded in large quantities along the intertidal zone and gazni lands during monsoon and post monsoon. Availability of such large biomass quantity can be tapped for bioethanol production as well as for human consumption or extraction of value added products.

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