"Production of bioethanol from Green Seaweed, *Ulva* sp."

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, **"Production of Bioethanol from Green Seaweed, *Ulva* sp."** is based on the results of investigations carried out by me in the School of Biological Sciences and Biotechnology, Goa University under the supervision of Dr. Meghanath Prabhu and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation. I hereby authorize the Goa University authorities to upload this dissertation on the dissertation repository or anywhere else as the UGC regulations demand and make it available to any one as needed.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Production of Bioethanol from Green Seaweed, *Ulva* sp."** is a bonafide work carried out by **Ms Varisha Fatima** under my supervision in partial fulfilment of the requirements for the award of the degree of **Master of Science** in the Discipline Marine Biotechnology at the School of Biological Sciences & Biotechnology, Goa University.

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PREFACE

This thesis has been prepared as a part of my dissertation work to fulfil my master's degree. It is based on biofuel and its production from renewable sources. The vast detail on bioethanol is included in this report such as source of bioethanol, how to process it, what are the measures for production, what I get in the end, how it concluded. In this thesis, we're exploring how we can make bioethanol from green seaweed. It's like going on an adventure to find new ways to create energy that won't harm the environment. We're looking for different options to power our world while keeping nature safe. This study was carried to make environment sustainable and to aware people for contributing to making other renewable energy sources to protect the environment. Within these pages, we uncover the potential of green seaweed biomass, rich in carbohydrates and other valuable compounds, as a feedstock for bioethanol production. Through rigorous scientific inquiry and technological innovation, researchers have illuminated pathways toward harnessing the power of seaweed for renewable energy.

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ABBREVIATIONS

℃ - degree Celsius

µg – microgram

µL- microlitre

mg – milligram

g – gram

% - percentile

RT – room temperature

YEPD – Yeast Extract Potato Dextrose Agar

PSU – Practical Salinity Unit

BSA- Bovine Serum Albumin

B1 – Biomass before starch extraction

B2 – Biomass after starch extraction

GODPOD – Glucose oxidase peroxidase

DNSA – 3,5- Dinitrosalicylic Acid

ABSTRACT

Green seaweed *Ulva* sp. biomass (B1) and its residual biomass after starch extraction (B2) was used as a feedstock in the production of bioethanol, a renewable energy source. The biomass B1 and B2 underwent acidic pretreatment with 1 % v/v H_2SO_4, followed by enzymatic saccharification using a cocktail of enzymes (Viscozyme L) to convert complex polysaccharides into fermentable sugars. B2 biomass was composed of 59% of carbohydrate, 20% of protein, 10.08% of ash and 2.6% of starch, whereas B1 had 48% carbohydrate, 8% proteins, 3.7% starch and 18% of ash. After fermentation of both the biomass using *Saccharomyces cerevisiae* (Turbo yeast), at room temperature for 72 hours, the ethanol yield was found to be highest at 36 hours giving 273.67 ±0.29 mg/L of hydrolysate (B1) and 215.41 ±0.22 mg/L of hydrolysate (B2). Despite of the lower yield of ethanol in B2 biomass by 21% compared to B1, result suggest that valuable intermediate product, such as starch, can be extracted from seaweed biomass and the residual biomass can be used as a feedstock for bioethanol production.

Chapter – 1

INTRODUCTION

1.1 Background

Many countries depend on fossil fuel consumption for their economic development such as in industries, transportation sector and various other areas. As the global demand for energy increases rapidly, it increases concerns about dwindling fossil fuel reserves, climate change and environmental sustainability (Wei et al., 2013). This heightened demand has resulted in rising fuel costs and increased emissions of greenhouse gases (GHGs), thereby posing significant challenges to the stability of the planet's climate (Ramachandra et al., 2017). 75% of global greenhouse emission is due to fossil fuels (Osman, 2021). This rapid depletion and deterioration of environment due to continuous use of fossil fuels has led to development of renewable energy source which helps in neutralization of carbon (Chen, 2022). This renewable energy source is biofuel such as bioethanol, biodiesel, and biogas. Among these, bioethanol is the best alternative to fossil fuels, as it is carbon neutral. Bioethanol stands out as a promising alternative which mitigate the effect of greenhouse gases and reduces the use of non-renewable energy sources (Vo Hoang Nhat et al., 2018). The demand of bioethanol will increase to 3.4 million barrel per day by 2024 (Kim et al., 2017). Using bioethanol on a large scale has some problems. One big issue is that it competes with food crops for land and water (Bušić et al., 2018). This means there might not be enough land and water for growing both food crops and for making bioethanol. Also, there are conflicts about whether using traditional crops such as corn and sugarcane for bioethanol is a good idea in the long run. This is because it could affect things such as food security and how land is used (Somma et al., 2010).

Bioethanol can be derived from various feedstocks biomass, which have organic molecule that can be converted into various fuels and chemical or biochemical processes (John et al., 2011). The production of biofuels from grains and oil crops is constrained by limited

cultivable land on Earth. Additionally, replacing food crops with energy crops can drive up food prices, disproportionately affecting impoverished communities. Given the current population growth, there's a crucial problem, whether to allocate food crops for bioethanol production or to fulfil the nutritional needs of a growing population. Utilizing food materials for ethanol production can amplify issues of food scarcity (Singh et al., 2022). Furthermore, extensive cultivation of energy crops raises concerns about pollution from fertilizers and pesticides, soil erosion, decreased crop diversity, loss of ecosystem services related to biocontrol.(Subhadra & Edwards, 2010).

In this context, macroalgal biomass emerge as a potential source for biofuels with high photosynthetic activity(Herrmann et al., 2015). Macroalgae are plentiful globally, particularly in nations with extensive coastlines like Japan, The Philippines, Malaysia, Singapore, Thailand, numerous European countries, The United States, and Australia (Khambhaty et al., 2012). India has nearly 7 lakh tonne of standing stock of seaweed (Sudhakar et al., 2020). Many of these are drift seaweed which is still unexplored (Klnc et al., 2013). In 2008, macroalgae production reached 15.5 million tonnes in fresh weight, with approximately 93% of it possessing significant commercial worth (Kraan, 2013). Seaweeds are richest source of fermentable carbohydrates including cellulose and hemicellulose, pigments and phycocolloids (Adams et al., 2011). Green seaweed offers several advantages over traditional feedstocks, including its abundance, rapid growth rates, minimal land and freshwater requirements, and negligible competition with food crops (Demirbas, 2008). Moreover, green seaweed cultivation does not rely on arable land, making it suitable for marginal or non-arable coastal areas, thus avoiding potential conflicts with food production (Dismukes et al., 2008). The consumption of seaweeds is on the rise because of their natural composition. However, they have a high moisture content, typically ranging from 80% to 90%. On a dry weight basis, seaweeds contain approximately 50% carbohydrates, 1% to 3%

lipids, and 7% to 38% minerals rendering it as potential biomass for fuel production. Their protein content varies widely, ranging from 10% to 47%, and they contain significant amounts of essential amino acids (El-Said & El-Sikaily, 2013). Various species of *Ulva* are used in biorefinery processes, serving different purposes. Ben Yahmed et al. (2016), explored bioethanol and biogas production. Trivedi et al. (2016), extract mineral-rich liquid extract (MRLE), lipids, ulvan, and cellulose from *Ulva fasciata*. Magnusson et al. (2016), demonstrated the salt extraction potential of *Ulva ohnoi* and *U. tepida*, with residual biomass utilized for protein, fertilizer, animal feed, and fuel. Gajaria et al. (2017) and Mhatre et al. (2019), studied *Ulva lactuca* for MRLE, lipids, ulvan, protein, cellulose, and methane. Pezoa-Conte et al. (2015) reported on *U. rigida*, providing carbohydrates and salt, along with concentrated protein. Characterization of residual biomass was done after oil extraction, and significant difference in composition was found in biomass before and after extraction (Pardilhó et al., 2021).

The main goal of this work was to produce a renewable energy source using green seaweed which will work as an alternative to fossil fuel. It will reduce fossil fuel emission, release of greenhouse gases, reduce the competition with land-based feedstocks. Seaweeds are potential source also because of its abundance, high carbohydrate content and its property to sequester carbon which will ultimately mitigate the effect of fossil fuel.

1.2 Aims and Objectives

The aim of this study was to produce and compare bioethanol from green seaweed (*Ulva* sp.) using biomass before and after extraction of starch.

Objectives –

1. Collection and in vitro cultivation of green seaweed.
2. Extraction of starch and characterization of biomass before(B1) and after(B2) the extraction process.
3. Comparative assessment of bioethanol production from seaweed biomass before starch extraction(B1) and after starch extraction (B2).

1.3 Hypothesis

Using green seaweed for making bioethanol is an efficient approach. We believe that if these carbohydrates get converted into sugars that can be fermented by certain microorganisms, will result in significant ethanol yields. Additionally, we anticipate that optimizing process parameters such as pretreatment methods, enzyme activity, and fermentation conditions will enhance bioethanol production efficiency from green seaweed biomass. Ultimately, the hypothesis of this study is to compare the bioethanol production from *Ulva* sp. biomass before extraction and after starch extraction.

1.4 Scope of the study

Seaweeds provides a sustainable and plentiful source of raw material with valuable chemical properties. Producing bioethanol from green seaweeds has a lot of potential. Using seaweed for bioethanol can help reduce environmental problems linked to fossil fuels. By improving

how we grow and process seaweed, we can make the most of it and create more opportunities for businesses. We can also use bioethanol as an alternative to fossil fuel. It will minimize the usage of terrestrial crops, making it available at large scale.

Chapter - 2

LITERATURE REVIEW

Data showed that 110 billion litres of ethanol was produced globally in 2020. Bioethanol production is constantly increasing annually, and there is prediction that by 2024, the worldwide bioethanol production and its consumption will increase nearly to 134.5 billion litres (Kumar et al., 2010). According to Statista Research Department the global bioethanol production in 2023 climbed to 29.5 billion gallons which is 1.4 billion times increase from last year, in which India share is 2%. Despite huge production, it cannot prove as effective and efficient transportation fuel. Bioethanol production faces many challenges such as feedstock availability vary from season to season and depends on geographical locations (Balat, 2008).

Bioethanol is a flammable liquid, volatile and colourless with density of 789kg/m^3 at 294K and molecular weight of 46.07g. It has both hydroxyl group and the shortness of carbon chain making it less polar and more viscous than other organic compounds. It is miscible with many organic solvents like acetic acid, toluene, glycerol, ethylene glycol, ether, acetone as well as with water (Baeyens, 2014). Bioethanol is produced from edible feedstock such as sweet sorghum, cassava, sugar beet, corn, and sugarcane (Offei et al., 2018). In context of research, bioethanol production is classified into three categories: first generation, second generation and third generation.

First-generation bioethanol relies on food feedstocks such as corn, sugarcane, and vegetable oils, raising concerns about environmental and socio-economic impacts(Buijs et al., 2013) Resource depletion, water shortages, and pollution from overfertilization are significant worries, given the conflict with food production (Jambo et al., 2016). Despite these challenges, biofuels offer lower greenhouse gas emissions than fossil fuels (Sarris & Papanikolaou, 2016). Second-generation bioethanol, derived from non-food sources like

lignocellulosic biomass, offers a sustainable alternative (Poland et al., 2018). However, third-generation bioethanol from macroalgae shows promise due to its high protein, lipid, and carbohydrate content, without the need for agricultural land or pesticides (Gengiah et al., 2023; Offei et al., 2018). Seaweed maintains carbon neutrality and has higher photosynthetic efficiency compared to terrestrial biomass (Tan et al., 2020).

All the seaweeds vary in their carbohydrate content which serve as supporting structural tissue for their cell wall. The total carbohydrate content of the red seaweeds varies from 27-66% (*E. cottonii and G. amansii*). These carbohydrates are primarily polysaccharides, including cellulose, agar, and carrageenan (which are found in agarophytes and carrageenan) (Offei et al., 2018). The hydrophilic galactans that make up agar are l-galactopyranose units with alternating α-1,3 and β-1,4 linkages. On the other hand, α-linked galactopyranose units in the d-configuration are present in carrageenans. Agar is produced commercially from a variety of *Gelidium* and *Gracilaria* species. The simplest extraction process entails heating the seaweed in water for a few hours. Agar is obtained by filtering out seaweed residue after dissolution. With supplementary uses in microbiology and pharmaceuticals, the food industry is its principal application. Repetitive oligosaccharide units of 3-linked β-d-galactopyranose and 5-linked α-d-galactopyranose make up carrageenan, which is present in carrageenophytes and is composed of linear sulphated galactans (S. Y. Lee et al., 2014). Certain species, such as *Kappaphycus, Chondrus,* and *Eucheuma*, are used for the commercial extraction of carrageenan. There are several varieties of carrageenan, such as *iota* (which forms elastic gels with calcium salts), *kappa* (which forms stiff gels with potassium salts), and *lambda* (which forms a viscous solution without gels). These varieties are distinguished by their distinct structures and gelling qualities. Selective hybrid carrageenan extraction is made possible by enzymatic extraction, which enables the targeted production of gelation properties. Hydrolysis has shown commercial cellulase enzymes to

be effective. Carrageenan is used in enzyme immobilisation procedures and in the food industry, especially in dairy products (Rhein-Knudsen et al., 2015).

Brown seaweed has total carbohydrate content in range of 40-60% (*S. fulvellum and L. digitata*). Laminarin, mannitol, cellulose, alginate, and fucoidan are among the diverse polysaccharides that make up this mixture. Brown seaweed's main storage polysaccharide, laminarin, has a β-1,3 glucan chain and occasionally contains β-1,3 linkages, particularly in kelps like *Laminaria* sp (Ravanal et al., 2019). In brown seaweed, mannitol, which is produced by mannose reduction, has an osmoregulatory role. Laminarin and mannitol concentrations fluctuate seasonally, peaking in June and July and declining in winter, in line with variations in the weather. While mannitol must be oxidised to fructose by mannitol dehydrogenase, laminarin is hydrolysed enzymatically by laminarinase and cellulases to liberate glucose monomers (Borines et al., 2011). Up to 50% of the carbohydrates found in brown seaweeds are made up of alginate, also known as alginic acid. It is essential for maximising the recovery of bioethanol during yeast fermentation and is made up of recurrent chains of mannuronic and guluronic acids joined by 1,4-glucosidic bonds (J. Y. Lee et al., 2013). The main components of fucoidans in brown seaweeds are sulphated ester groups and l-fucose, which includes sulphated fucogalacturonans in species like *Laminaria* and *Sargassum*. Fucoidan has been isolated from a variety of species, including *Undaria, Laminaria,* and *Sargassum*. It has been thoroughly studied for its biological and pharmacological qualities, including antioxidant, anticoagulant, antiviral, and therapeutic effects. *Laminaria hyperborea's* dry matter composition was found to contain 0–30% laminarin, 4-55% mannitol, and 17–34% alginate, according to a biochemical analysis.

Green seaweed contains 45-59% of carbohydrate (Chen et al., 2015). It mainly includes polysaccharides like starch, ulvan and cellulose. Ulvan is a sulphated polysaccharide consist of oligosaccharide units of L-rhamnose-3-sulfate, D-xylose-2-sulfate, and units of uronic

acid. Ulvan are basically found in *Ulva* sp. (Lee et al., 2014). Although it is soluble in water and has a wide range of applications in the food, pharmaceutical, and chemical industries, other thickening agents such as agar and alginate that are made from red and brown seaweeds, respectively, pose a serious threat to this substance (Trivedi et al., 2016). Although they have different configurations, the glucose units that make up cellulose and starch in green seaweed and plants are monomeric in form. The difference is due to the anomeric carbon (C1) structure, which is α- in starch and β- in cellulose (Lahaye & Robic, 2007). The stable crystalline structure of cellulose is created by its regular linear chain with 1,4-β-glycosidic connections arranged in parallel linear arrays. Because of the hydrogen and Van der Waals connections that maintain this stable chain, cellulose is strong and incredibly resistant to physical and enzymatic degradation. A loosely linked, open helical structure is exhibited by starch, which facilitates its solubilization through enzyme action, chemical reactions, or physical degradation (Thygesen et al., 2005).

Starch in green seaweed is stored in their chloroplast or found surrounding pyrenoids. *Ulva rigida* has seasonal variations in its starch content, which can account for up to 32% of its dry weight (Prabhu et al., 2019). Moreover, blue light exposure and nutrient stress can greatly raise the starch concentration in seaweeds. Although this carbohydrate has been used to produce bioenergy (Korzen et al., 2015), the method used now entails hydrolyzing the entire biomass to produce monosaccharides that can be fermented. Remember, though, that native starch granules are required for several of the previously mentioned biorefinery applications. Remarkably, no techniques for isolating these granules for biorefinery applications have been reported (Milledge et al., 2014). Bioethanol is produced from carbohydrate content of seaweed, it constitutes 40-70% dry matter, and this gets converted into fermentable sugars (Offei et al., 2018).

Starch and sugar-rich food crops are the source of first-generation bioethanol, a liquid biofuel designed for use in automobiles (Ho et al., 2014). *Saccharomyces cerevisiae* cannot efficiently break down complex carbohydrates, so hydrolysis is required for both starchy and lignocellulosic materials (Balat et al., 2008).

Bioethanol production is mainly done through three stages: pretreatment, hydrolysis, and fermentation. Hydrolysis and fermentation can be separate or simultaneous due to their effect on ethanol production (Borines et al., 2013). Cultivation of macroalgae seaweed was also done in various ways. 4.5% total starch content of the dry weight of *U. ohnoi* was obtained after grown for two weeks in artificial seawater supplemented with nutrients (6.4 g/m^3 of N2 and 0.97 g/m^3 of P) (Prabhu et al., 2019).

Ulva sp. was cultured in 600 l and 40 l tanks filled with filtered seawater and constant aeration, nutrients (0.1mM NH_4Cl and 0.1mM NaH_2PO_4) were given on a weekly basis and observed for specific growth rate during different season (Qarri & Israel, 2020). Studies on the long-term cultivation of *U. lactuca* in 600L tanks have shown that seasonal variations generally have an impact on the growth potential of cultivated seaweeds (Gengiah et al., 2023). According to another study, increased summertime temperature and irradiance levels had a positive effect on growth of *G. conforta* in a 40L tank (Alongi et al., 2014).

Various pretreatments methods are involved to extract carbohydrate, lipid etc. There was very little reduction of sugar when boiling water was used as a pretreatment. It was also observed that a considerable number of oligosaccharides can be effectively released using liquid hot water pretreatment (Bixler & Porse, 2011). The difference in sugar content between different types of biomasses highlights the complex composition of biomass by separating its structural and carbohydrate components. Plant biomass is an essential resource for the synthesis of fuels and is primarily composed of lignin, cellulose, and hemicellulose.

The crucial step in the production of biofuels is the conversion of biomass into sugars. The passage emphasises the need for an ideal strategy that maximises sugar yield while minimising energy input, emphasising the significance of a carefully chosen pretreatment process in line with the characteristics of the biomass (Ramachandra & Hebbale, 2020). First generation biomass requires no pretreatment, in case of second generation, it requires pretreatment due to presence of recalcitrant. Four different pretreatment techniques were applied to the biomass of *U. lactuca*: ionic liquid, alkaline, liquid hot water, and ethanol organo-solvent treatments. With values of 80.8 g/100 g DW and 62.9 g/100 g DW, respectively, the organo-solvent and liquid hot water treatments showed the highest glucan recovery among them (El Sayed & Ibrahim, 2016). Sugar was extracted from lignocellulosic biomass by exposing it to 200°C and 121°C steam, Sulphite treatment to prevent recalcitrance (Ramachandra & Hebbale, 2020). One particularly important handling and pre-treatment method is size reduction or milling. This procedure increases the biomass's surface area, which helps the catalytic action during the fermentation and hydrolysis phases (Tan et al., 2020).

After pretreatment, the biomass needs to be hydrolysed, where the complex sugars such as agar, carrageenan, ulvan, alginate, mannitol, cellulose and laminarin breaks into simple sugars such as arabinose, xylose, fucose, galactose, glucose, and mannose for fermentation to ethanol (Offei et al., 2018). Hydrolysis of seaweed depends on various treatments for bioethanol production. The most important treatments are dilute alkaline hydrolysis, dilute acid hydrolysis and enzymatic hydrolysis. Strong acids are used like H_2SO_4 and HCl, 2% sulfuric acid at 121 for 30 min followed by enzymatic hydrolysis gives good ethanol yield (Yanagisawa et al., 2013). *G. amansii* was treated with 0.05-0.2 N $Ca(OH)_2$ at 121°C for 15 min, it formed gel and alkaline treatment was not pursued further (N.-J. Kim et al., 2011). No advantage of alkaline hydrolysis was seen over acid hydrolysis. Alpha-amylase and

glucoamylase enzymes were used for hydrolysis gives 0.136g of bioethanol/g Dry weight (Brockmann et al., 2015). *Ulva pertusa* biomass was hydrolysed by a crude enzyme (isolated from mid gut gland of scallops) containing cellulase and amylase with simultaneous fermentation by *Saccharomyces cerevisiae* gives 7.2g/l of ethanol at 35℃ for 72h (Yanagisawa et al., 2013). The enzymatic hydrolysis of glucans was started after 30 minutes of acid hydrolysis using 2% sulfuric acid at 121°C to raise the ethanol concentration. A final ethanol concentration of 27.5 g/L was obtained through this process (Yanagisawa et al., 2011). *Ulva* sp. was hydrolysed using three commercially available enzymes: α-amylase obtained from *Bacillus amyloliquafaciens*, cellulase derived from *Aspergillus niger*, and amyloglucosidase for 77 h at different temperature of 70℃, 45℃, 60℃ in 250 ml flask (Qarri & Israel, 2020). Water hydrolysis of *Ulva sp.* in a batch reactor under suitable conditions: temperature 180℃, 220℃, and 260℃ at time 10 min, 20 min, and 40 min, seawater salinity 38.2gr/l gives a major release of monosaccharides, polyhydroxyalkanoates and hydrochar (Steinbruch et al., 2020).

Fermentation is a stage where an organism converts the reducing sugar to ethanol. There are various techniques like Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Co-fermentation (SSCF), Simultaneous Saccharification and Fermentation (SSF). Sequential hydrolysis and fermentation (SHF) are the process of carrying out the hydrolysis and fermentation steps one after the other, whereas simultaneous saccharification and fermentation (SSF) is the process of carrying out these steps simultaneously (Offei et al., 2018). When *Saccharina japonica, Undaria pinnatifida*, and *Porphyra* were exposed to SSF in a study using the *Pichia angophorae* KCTC strain, 7.7 g/L of ethanol were produced. Even though the SHF process is quicker, yeast microorganisms are greatly impacted by the inhibitors from the acid pretreatment. Because the released sugars are easily metabolised by yeast microorganisms, SSF is typically

preferred over SHF because it results in a faster rate of ethanol production and lower capital costs. The temperature differential between the ideal range for cellulase (50 °C) and fermenting microbes (35 °C) makes SSF unfavourable. Both SHF and SSF procedures were used in the cases of *Ulva (Enteromorpha) intestinalis or Enteromorpha intestinalis*, producing ethanol yields of 8.6 g/L and 7.6 g/L with fermentation efficiencies of 30.5% and 29.6%, respectively (Jang et al., 2012). The suboptimal temperature of 30 °C compared to the ideal temperature of 55 °C for enzyme activity and the conversion of ethanol by yeast to acetic acid were blamed for the lower ethanol yield in SSF (Ramachandra & Hebbale, 2020). The sulphated polysaccharide Ulvan mainly consist of repeated units of dissacharide like xylose, glucorin acid and iduronic acid (Rocha et al., 2022). Researchers used a xylose-fermenting yeast (strain 39), a genetically modified xylose-utilizing variant of *Saccharomyces cerevisiae*, and an ethanologenic recombinant of *Escherichia coli* (*E. coli*) to aid in the conversion of xylose into ethanol. Both the ethanologenic *E. coli* and the xylose-consuming *S. cerevisiae* showed that they could make ethanol from xylose, with ethanol yields exceeding 0.4 g-ethanol/g-xylose (Tan et al., 2020).

SSF is superior to SHF reported by (H. M. Kim et al., 2015) for bioethanol production, when observed in *Gelidium amansii,* 76.9% ethanol yield was obtained after 24h. A single strain or a combination of strains are being applied to make use of sugars. Fermentation tests were carried out using three yeast strains, *Pichia angophorae, Pachysolen tannophilus,* and *Kluyveromyces marxianus*, along with one bacterium, *Zymobacter palmae* T109, in the case of Laminaran and mannitol derived from *L. hyperborea*. According to the findings, only *P. angophorae* was able to ferment mannitol and laminaran efficiently, especially at higher oxygen transfer rates, which produced 0.43 g of ethanol per g of substrate. *Zymobacter palmae* was found to be able to use mannitol and produce 0.37 g ethanol/g mannitol, but only in the presence of lower oxygen rates in the fermentation media. Furthermore, the

fermentation of mannitol by E. coli KO11 demonstrated efficacy, yielding 0.41 g (Sarris & Papanikolaou, 2016).

This study focused on seaweed cultivation, hydrolysis of biomass (B1) with starch and biomass(B2) without starch, and fermentation to produce ethanol. Various research has been done on ethanol production from algal biomass and residual biomass. It's a comparative study to know how starch affect the efficiency and yield of bioethanol. Residual biomass of *Ulva lactuca* after extraction of biodiesel were further processed for bioethanol production. It was found out that the maximum bioethanol conversion was observed to be 2.8 ±0.12 mg/ml, and the highest yield of fermentable sugars observed was 13.48mg/ml (Gengiah et al., 2023).

Chapter - 3

METHODOLOGY

3.1. Collection of seaweed samples

Seaweed samples were collected from three different locations in Goa, India. The first collection was done on 28th October 2023 from Vagator beach, Goa-403509, India (latitude: 15.59993°, longitude: 73.734145°). The second sample was collected in month of November from Baga beach Goa-403509, India (latitude:15.561747°, longitude: 73.746295°). The third sampling site was Anjuna beach, Goa-403509, India (latitude:15.577206°, longitude: 73.739685°) on December 19th ,2023 (Fig. 1). All these samples were collected during low tide from intertidal zone.

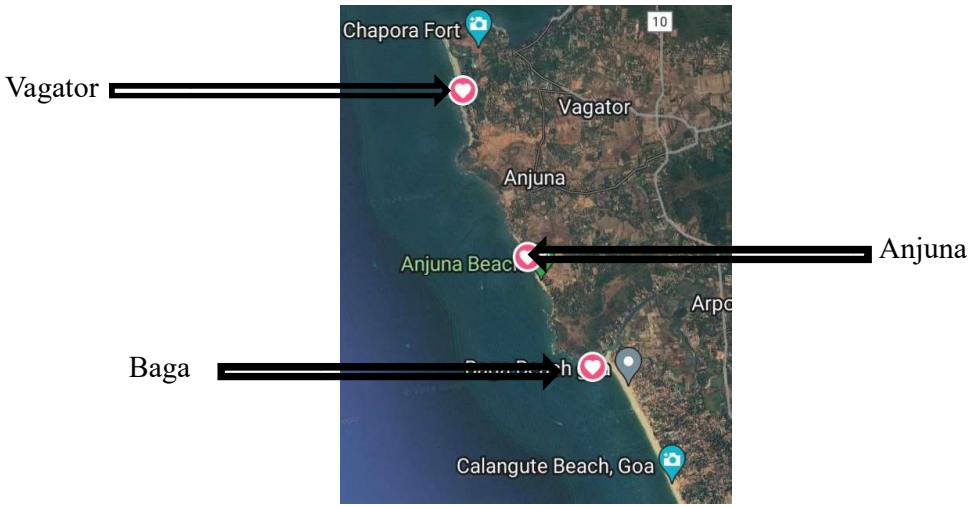


Fig. 1: Map showing three different sampling site Anjuna, Baga and Vagator

3.2 In-vitro cultivation of seaweed

Seaweed samples after collection were brought to lab and cleaned using forceps to remove sand particles, marine organisms, and rinse with seawater (collected from sampling site) multiple times. Seawater was filtered using muslin cloth and autoclaved at 121 ˚C for 20 minutes (15 psi). In a culture vessel of different volumes (Fig. 2), clean seaweed samples

were cultivated at the density of 5 g/L initially, and later changed to 2.5 g/L. Salinity of seawater was measured using handheld refractometer and adjusted to optimum salinity of 25 PSU.

(i) (ii) (iii)

Fig. 2: Cultivation of seaweed in three different culture vessels; i) 2 litres, ii) 5 litres, iii) 10 litres

As a nutrient source of phosphorus and nitrogen, Monosodium phosphate (NaH_2PO_4) and ammonium chloride (NH_4Cl) were given on alternate days at the concentrations of 0.057 mM/L and 0.59 mM/L respectively. During the process of one month cultivation, seawater was replaced every week and temperature was maintained at 24 ±2˚C, with continuous aeration. Besides, tubelights were used as a light source during daytime. Light intensity was measured by lux meter app (Light meter version 1.7) at three different times of day i.e., 10 am, 1 pm, and 6 pm respectively. The intensity was found to be 1429.33 lux, 5300.33 lux, and 28842.66 lux.

3.3. Seaweed Sample Processing

Ulva samples were harvested after one month of cultivation. These samples were first dried on blotting paper and then in spinner to remove excess moisture content. About 38 g of sample were kept for drying in Hot air oven for 24 hours at 55℃ until constant weight

obtained. This sample was referred to as B1 (Steinbruch et al., 2020). After drying, it was crushed with mortar and pestle using liquid nitrogen into fine powder and stored at room temperature in a falcon tube tightly sealed with paraffin.

3.4 Starch Extraction from *Ulva*

Starch granules from *Ulva* was extracted as per (Prabhu et al., 2019). Freshly harvested biomass was used for starch extraction. It was washed thrice with distilled water to remove surface salts. The biomass was homogenized in distilled water (1:20 (w/v)) to fine particulate suspension using blender at full speed. The homogenate obtained was filtered through filter membrane of pore size 100µm. The residue remained was spread on petri plate or parchment paper, dried at 60°C and then crushed into fine powder which was referred to as B2 (biomass after starch extraction). Filtrate was kept settling at 4˚C, after 24 hours the top green extract was carefully discarded so that bottom slurry is retained. The slurry was washed with absolute ethanol several times till the green color is removed. The pellet after ethanol washes was resuspended in distilled water and sequentially passed through 10 µm and then through 5 µm pore size nylon filters. The final filtrate obtained was centrifuged at 9000 rpm, 4˚C for 10 minutes. Final wash of absolute ethanol was given to the pellet and the starch pellet was air dried at 4˚C.

3.5 Physicochemical composition of seaweed samples

3.5.1. Estimation of Total Solids, Volatile Solids, Ash content and Moisture Content

The total solids of both biomass samples were assessed by drying 0.5 g of each sample (W2) at 105°C in a pre-weighed crucible (W1) until a constant weight (W3) was achieved. The

ash content was determined by subjecting the dried samples (from 105℃) to 550℃ using a furnace (catalogue no. 12001, serial no. 792 Pathak Electrical works) for 3 hours and then re-weighing the samples (W4). Volatile solids and moisture content were calculated using the respective formulas.

Total solids (%) = (W3-W1/W2) *100

W1= weight of the crucible (g)

W2 = weight of sample

W3 = Dry weight (g)

Ash content (%) = (W4-W1/W2) *100

W4 = Weight of crucible and sample after 550℃ (g)

Volatile Solids (%) = 100- Ash content (%)

Moisture content (%) = 100 - Total solids (%)

3.6 Chemical composition of *Ulva* samples

3.6.1. Estimation of Proteins

The determination of total protein concentration in *Ulva* was done by Folin's Lowry method (Lowry et al., 1951). A working standard solution was prepared using a 1 mg/ml concentration of Bovine Serum Albumin (BSA) stock solution. Next, 10 mg of each finely ground sample (both B1 and B2) were weighed into 2 ml centrifuge tubes in triplicates, followed by the addition of 1 ml of 0.25 N NaOH. The samples were homogenized using a bead beater (Benchmark Beadbug^{TM} Mini Homogenizer model D1030 (E)) for 2 minutes with 30-second intervals each and then refrigerated overnight. Subsequently, the samples were centrifuged (Lab ndia C series Microtable High speed refrigerated centrifuge) at 3000 rpm for 10 minutes, and the supernatant was collected for protein estimation. From each sample, 1 ml of supernatant was transferred into test tubes, to which 5 ml of Reagent C was added, and the contents were mixed using a vortex. After allowing the tubes to stand for 10

minutes at room temperature, 0.5 ml of Reagent D was added immediately with continuous mixing. The tubes were then incubated in the dark at room temperature for 30 minutes, and the absorbance was measured at 660 nm using SHIMADZU™ UV- Visible spectrophotometer.

3.6.2. Estimation of Total Carbohydrate

The Anthrone test was employed to determine the Total Carbohydrate content (Niemi et al., 2024). Each powdered sample (B1 and B2), weighing 10 mg, was placed into separate test tubes, and subjected to hydrolysis by boiling in a water bath for 3 hours with 5 ml of 2.5N HCl. After cooling to room temperature, solid sodium carbonate (Na_2CO_3) was added to neutralize the mixture until effervescence ceased. The samples were then diluted to a final volume of 10 ml and centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected, and 1 ml was extracted for analysis. To each sample, 4 ml of Anthrone reagent was added and the mixture was heated again in a boiling water bath for 10 minutes. After rapid cooling, the absorbance was measured at 620 nm. A standard glucose (1 mg/ml) curve with varying concentrations was prepared for comparison.

3.6.3. Estimation of lipid

The total lipid content in *Ulva* biomass (B1) and biomass after starch extraction (B2) was determined by Bligh and Dyer method (Bligh & Dyer, 1959). Finely ground sample (0.2g) were placed into separate Falcon tubes and sonicated using a Chloroform and Methanol mixture in a 1:2 ratio for 2 minutes using an Ultrasonicator (MRC Ultrasonic processor SONIC-650WT-V2). The samples were subsequently incubated for 24 hours at room temperature. Afterward, 5 ml of chloroform was added to the mixture and sonicated for an

additional minute. Then, 5 ml of distilled water was added, followed by another minute of sonication. Upon allowing the mixture to separate, the lower solvent phase was decanted and filtered through Whatman filter paper no. 1. The filtrate was then left to separate in the Falcon tube, and the volume of the chloroform layer was measured. Finally, the filtrate was transferred to pre-weighed petri plates, where the samples were left to evaporate overnight in a fume hood before being reweighed.

3.6.4. Estimation of reducing sugar in B1 and B2

The reducing sugar in both biomass was determined by DNSA method (Miller, 1959). Standards of glucose were prepared by diluting 1 mg/ml of stock solution in 0.1, 0.2, 0.3, 0.4, 0.5 mg/ml concentrations. Each finely powdered biomass of 0.5 g was taken into test tubes in duplicates and diluted with 1 ml of distilled water. To each test tubes 1 ml of DNSA was added and kept on boiling water bath for 10 minutes. Tubes were kept on ice for 1 min immediately after removing from water bath. Optical density of standards and samples were recorded using spectrophotometer at 450 nm.

3.6.5. Estimation of Starch content in *Ulva*

Total Starch (AA/AMG) Assay Kit (Megazyme K-TSTA-50A / K-TSTA-100A Assay kit) was used for the determination of starch content in *Ulva.* Finely ground powder of each sample weighed 10 mg was taken into 2 ml of Eppendorf tubes and was treated with 0.2 ml of KOH, kept on shaker at 150 rpm for 30 mins at 37℃ (Rivotek Incubator shaker) meanwhile sample was mixed at every 10 minutes by vortexing. Immediately after incubation, tubes were heated in boiling water bath for 1 minute to completely dissolve the starch in KOH. Then 0.8 ml of 1.2 M sodium acetate buffer (pH 3.8) was added to the tubes

followed by addition of 0.01 ml amylase and 0.01 ml amyloglucosidase (AMG). Again, incubated at 50℃ for 90 minutes (mix every 10 minutes). The tubes were then centrifuged at 3000 rpm for 10 minutes. In another 2 ml Eppendorf tubes, 0.01 ml of this supernatant was taken and 0.3 ml of GODPOD reagent was added into it. Tubes were further incubated at 50℃ (i=therm Hot air oven AI-7981) for 20 minutes and the absorbance was measured at 510 nm. Standard of glucose (100 mg/ml) and reaction blank was prepared. The total starch concentration (%) was calculated using given formula:

Concentration of starch = "ΔA × F × (D/Sample weight) × final Volume × 0.90"

where,

ΔA = absorbance of sample against blank,

F = factor to convert absorbance values to mg glucose (100 mg glucose divided by the GOPOD absorbance value obtained for 100 mg of glucose),

D = dilution factor,

0.90 = factor to convert from free glucose, as determined, to anhydroglucose, as occurs in starch.

3.7 Elemental Analysis

Elemental analysis of both Biomass and Residual Biomass was performed by Elementar® Vario Micro Cube analyser. It was done to know the content of Carbon, hydrogen, Nitrogen and Sulphur in a sample.

3.8 Saccharification

3.8.1. Pretreatment of *Ulva* sample

About 2.5 g of each powder (B1 and B2) was taken into 250ml Screwcap Bottle containing 25 ml of 1% v/v H_2SO_4 and autoclaved at 121 ℃ for 30 minutes. The pH was adjusted to

5.0 using pH strip and then the flask was kept on shaker incubator at 30 ℃ (Hally shaker incubator) for 1 hour, 150 rpm (Trivedi et al., 2013).

3.8.2. Enzymatic hydrolysis of Pretreated biomass

After pretreatment, the hydrolysate obtained was used for enzymatic hydrolysis. The enzyme used in this study was Viscozyme L (cocktail of enzymes) purchased from Sigma Aldrich. Hydrolysates were treated with 2% v/v of Viscozyme L in 25 ml of 1 M sodium acetate buffer (pH 4.8). Flasks were kept on incubator shaker for 36 hours at 45 ℃ (REMI Cis-24 plus shaker incubator), 150 rpm (Trivedi et al., 2013). The reducing sugar was estimated post hydrolysis using DNSA method.

3.9 Fermentation of B1 and B2 hydrolysate

Fermentation of both sample hydrolysate was carried out using commercial turbo yeast (*Saccharomyces cerevisiae*) purchased from Arishtam probiotics, Uttrakhand. A loopful of dry yeast was inoculated in sterile YEPD (1% yeast extract, 2% peptone, 2% dextrose) broth, followed by incubation at 28±2 ℃ for 2 days, on shaker with a speed of 120 rpm. Pure culture of yeast was maintained on YEPD slants and stored at 4 ℃.

For the fermentation, 250 ml screw cap bottle containing seaweed hydrolysate was inoculated with 10% of yeast broth having optical density 1.25 and all the samples were kept in static condition at room temperature for 72 hours. At every 12 hours, 1 ml of sample was withdrawn and analyzed for leftover reducing sugar and ethanol yield by spectrophotometer.

3.9.1. Ethanol estimation

The quantification of total ethanol produced after fermentation of seaweed hydrolysate was done by Megazyme Ethanol kit (K-ETOH 05/21 kit assay, Megazyme, Bray, Ireland). For

ethanol yield the broth was withdrawn and centrifuged at 10,000 rpm, 4 ℃. In 3 ml eppendorf tube, 2 ml of distilled water was added and mixed with 100 µl of supernatant. To that mixture, 200 µl of kit buffer (pH 9.0, 0.02% w/v sodium azide) and 200 µl of NAD^+ was added. After this, first enzyme aldehyde dehydrogenase (50 µl) was pipetted. The tube containing solution were vortexed and optical density of solution was read after 2 minutes using spectrophotometer at 340 nm (A_1). The reaction was started by addition of 20 µl of second enzyme alcohol dehydrogenase. Solutions were again mixed using vortex and after approx. 5 minutes optical density was measured at 340 nm spectrophotometrically (A_2). The absorbance was read at 1 minute interval until it gets constant. The concentration of ethanol was calculated using the formula:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 2} \times \Delta A \ (g/L)$$

where ;

V = final volume (2.57 ml)

MW = Molecular weight of ethanol (46.07 g/mol)

ε = extinction coefficient of NADH at 340 nm

= 6300 ($1\times mol^{-1} \times cm^{-1}$)

d = light path (0.2 cm)

v = sample volume (0.10 ml)

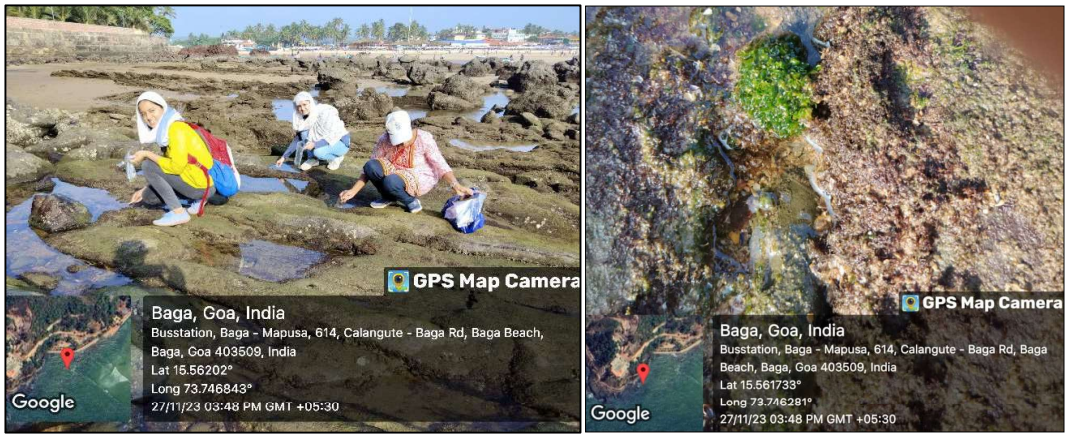
2 = 2 moles of NADH produced for each mole of ethanol

Chapter - 4

RESULT AND DISCUSSIONS

4.1. Collection of seaweed samples

The seaweed samples were collected from rocky shores during low tides, when the intertidal zone experience significant exposure. The time and day for sampling was predicted by Tide forecast website. Collection was started 1 hour before the low tide as it facilitates enough time for collection and observation of different seaweeds in their natural habitat. The seaweeds collected were in juvenile stage and most of which were in clusters shape (Fig. 3b), showed leaf like structure, and exhibited bright dark green colour thalli. Based on these morphological traits, it was identified as *Ulva* sp.



(a) (b)

Fig. 3: Collection of seaweed samples: a) from Baga beach using forceps; b) seaweeds clusters attached to rocky shores.

4.2. In-vitro cultivation

In-vitro cultivation of seaweed was done in Seaweed Cultivation Facility at Biotechnology Laboratory, Goa University (Fig. 4). After 1 month of cultivation, 80 gram of biomass was harvested, showing healthy thalli which was grown fully in size, measuring up to 8-10 cm (Fig. 5-iii). During the period of cultivation, seaweed showed phenomenon growth and

development. The salinity, temperature, nutrients and light both significantly facilitates the growth. Biomass was weighed every week and the increase in biomass was shown in table below:

Fig. 4: Seaweed cultivation setup at Biotechnology laboratory, Goa University

i) ii) iii)

Fig. 5: i) harvested biomass, ii) biomass before cultivation, iii) biomass after cultivation

Table.1. Growth rate of *Ulva* biomass per day.

Days	Weight of biomass (g)	Growth rate/day (% d^{-1})
0 day	7.5	0
7 days	12.76 ± 0.10	7.67 ± 3.79
14 days	17.83 ± 0.55	4.36 ± 2.16
21 days	20.13 ± 1.12	1.20 ± 0.59
28 days	22.41 ± 1.92	0.86 ± 0.42

The growth of biomass over a period of four weeks increased from 7.5 g to 25.12 ± 1.92 g on the last day. The growth rate on first week was 7.67 ± 3.79% /day, 4.36 ± 2.1% d^{-1} in second week whereas 1.20 ± 0.59% d^{-1} and 0.86 ± 0.42% d^{-1} in the last two week (Table 1). At the end there was 134% of increase in biomass was observed. According to (Balina et al., 2017), the growth rate was found 7.13 ±3.44%/d under optimum condition. There was significant decrease in growth rate in last two weeks, attributed to the fact that space was less, and density should be lesser.

4.3. Seaweed sample Processing

Biomass after drying had shrunken, crunched, and reduced in size due to loss of water. Residual Biomass became rough and hard to crush after drying (Fig. 6).

(i) (ii) (iii) (iv)

Fig. 6: Seaweed sample processing: (i) B1 after drying, (ii) B2 spread on parchment paper, (iii) B2 after drying, (iv) Powdered *Ulva* Biomass (B2)

4.4. Physicochemical Composition of *Ulva* biomass

Seaweed contains huge amount of moisture content about 90% of fresh weight as compared to terrestrial biomass (Fasahati et al., 2022). The moisture content of B2 was found to be almost similar with B1 which was 17.02% and 16.73% respectively. The ash content

represents inorganic materials like silicates, oxalates etc. It was found to be 10.48% and 18.3% in B2 and B1 whereas total solid content of B2 was 82.9% and of B1, it was 83.2%. Volatile solids were also calculated, and it was reported as 81.6% for B1 and 89.5% for B2 (Table 2).

Table.2: Physicochemical analysis of *Ulva* Samples B1 – before extraction, B2 after extraction

Ulva Sample	Moisture content	Ash content	Total solids	Volatile solids
B1	16.73%	18.3%	83.2%	81.6%
B2	17.02%	10.48%	82.9%	89.5%

According to literature, the moisture content of both dry biomass before oil extraction and after oil extraction was about 13% (Pardilhó et al., 2021), and in present study also both biomass had almost similar moisture. Besides the obtained result of this study corresponds to previous studies, there can be a difference in sample processing steps.

The previous studies revealed that ash content of residual biomass was lesser (15.39 ± 0.06%) than biomass (18.26 ± 0.09%) as ash content depends on washing with seawater having presence of salts (Baghel et al., 2020). Present studies agreed with literature having higher ash content in biomass. Since these values were found to be higher than other seaweeds except *Ulva lactuca* as shown in table 3.

Table.3. Comparison of ash content in different green seaweed

Seaweed	Ash content (%)	References
Ulva lactuca	19.59 ± 0.51	(Yaich et al., 2011)
Ulva prolifera	16.3 ± 0.3	(Li et al., 2016)
Ulva ohnoi	1.51	(Prabhu et al., 2019)
Chaetomorpha linum	14.83 ± 0.36	(Neifar et al., 2016)

4.5. Characterization of *Ulva* Biomass

4.5.1 Estimation of Proteins in B1 and B2

Protein content was estimated by Folin's Lowry method showed significantly higher in B2 (20.16 ±0.03%) than B1 (8.4 ±0.005%) (Fig. 7). This study agreed with the previous literature, where it was found that residual biomass after lipid extraction has higher protein content than biomass before extraction (Lee et al., 2015).

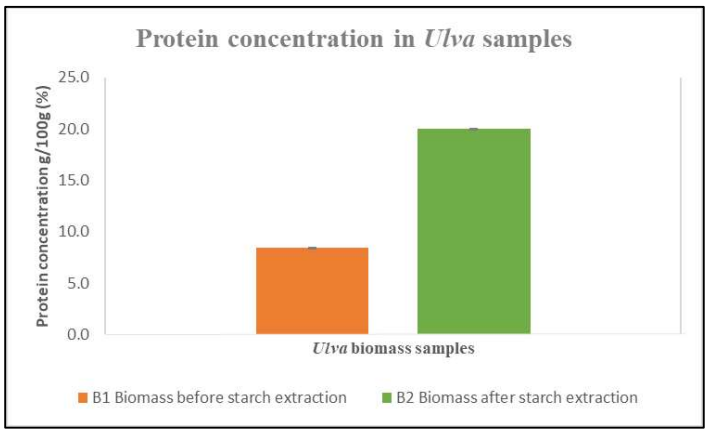


Fig. 7: Estimation of Proteins in *Ulva* biomass B1 before starch extraction and B2 after starch extraction

4.5.2. Estimation of carbohydrates in B1 and B2

The carbohydrate content determined by Anthrone test, showed that B2 had higher (59.8 ±0.14%) concentration of carbohydrates than B1 (48.8 ±0.16%) (Fig. 8). The carbohydrate content in residual biomass after lipid extraction was found to be 49.7% and in biomass it was 36.1% (Lee et al., 2015). Similarly, after oil extraction residual biomass from seaweeds had higher carbohydrate then biomass before extraction reported by (Pardilhó et al., 2021).

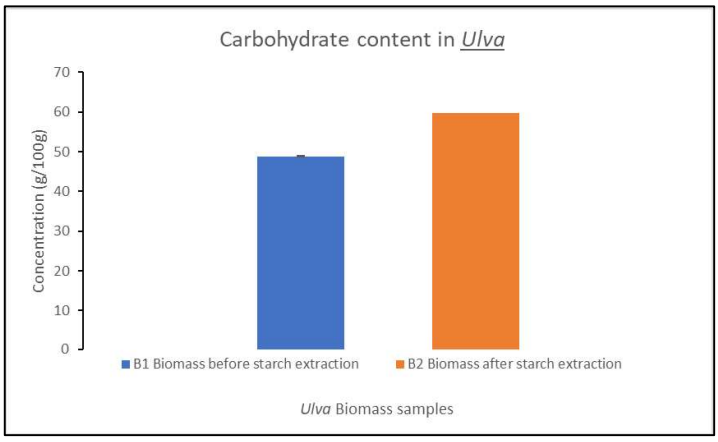


Fig. 8: Estimation of carbohydrate in *Ulva* biomass B1 and B2

4.5.3. Estimation of reducing sugar

The reducing sugar obtained in sample was found to be 0.17±0.002 g/L in B1 and 0.30 ±0.001 g/L in B2. The content of reducing sugar was found to be higher in B2 biomass after starch extraction.

4.5.3. Estimation of lipid

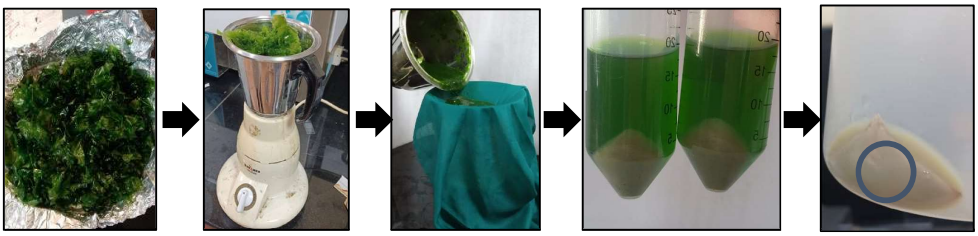
The total lipid content of B1 and B2 was calculated using this formula:

$$\textbf{Total lipids}(\%) = \frac{\textbf{weight of lipid in aliquot} \times \textbf{volume of chloroform layer}}{\textbf{volume of aliquot}}$$

It was found out that B1 had 1.11 % of lipid content whereas B2 had little higher content of lipid about 2.15%. As compared to literature, the content of lipid in seaweed was very low (Mendis & Kim, 2011). This recent study agreed with literature reported by (Li et al., 2016), where the lipid content in *Ulva prolifera* was 1.4 ±0.1%. Exceptionally, the highest content of lipid was found in *Ulva lactuca* i.e. 6.2% as reported by (Offei et al., 2018).

4.5.4. Estimation and extraction of starch content

After extraction of starch, residual biomass was dried, crushed and stored in falcon tubes for further analysis. Brownish white pellet of starch was found after extraction procedure as shown in (Fig. 9). These starches were processed further for applicative purposes.



(i) (ii) (iii) (iv) (v)

Fig. 9: Extraction of starch: i) fresh biomass, ii) biomass disruption using grinder, iii) filtration of homogenate using 100 µm nylon cloth, iv) centrifuge filtrate after ethanol wash v) starch pellet in centrifuge tube after washing.

Starch estimation in each biomass was done by total starch assay kit (Megazyme). It was found out that B1 (3.73%) before extraction had higher starch content than B2 (2.64%) (Fig. 10).

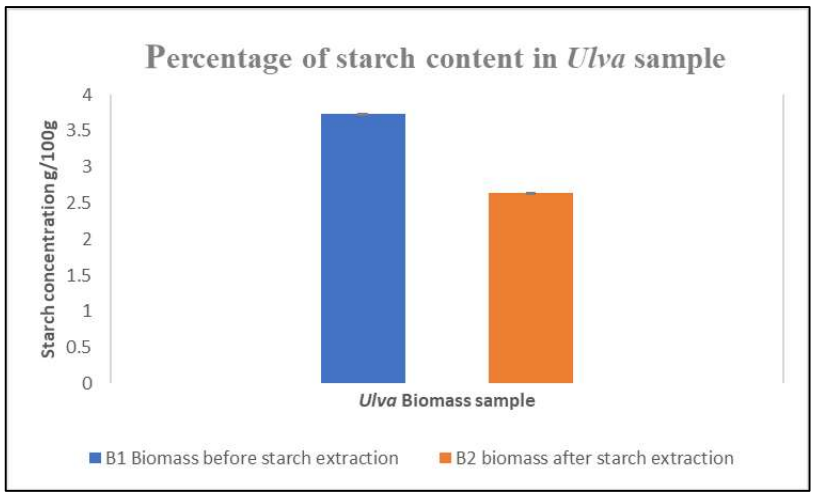


Fig. 10: Starch content (%) in both *Ulva* biomass B1 and B2

4.6. Elemental analysis of B1 and B2

The CHNS analysis carried out to know the Carbon, Nitrogen, and hydrogen content of seaweed biomasses, showed that the Carbon content was higher in B2 (33.54 ±0.54%) than B1 (25.81 ±0.18%). According to literature, the carbon content is found to be in range of 30-54.9% (Pardilhó et al., 2021), in this study the B1 biomass (before starch extraction) shows significantly lower carbon content indicating less carbon uptake.

Further the nitrogen content of both the biomass was found to be almost similar which was around 3.2 ±0.07% and 3.5 ±0.05% respectively. This study showed similar nitrogen content as mentioned in literature (Pardilhó et al., 2021). Hydrogen content of biomass was 5.57 ±0.04% whereas of residual biomass it was found to be little higher 6.1 ±0.06%. Moreover, the sulphur content of B1 biomass was 4.6 ±0.01% and, B2 biomass was 2.93 ±0.04%. The lower amount of sulphur content in B2 biomass could be because of lower amount of soluble sulphated polysaccharides (ulvan), which could have lost during the washing and homogenization of the biomass for starch extraction.

Overall, higher concentration of carbon, nitrogen and hydrogen in B2 was due to lesser ash content which got washed off during pretreatment of biomass for starch extraction. The loss of ash (salts and minerals) during washing and homogenization from the biomass is responsible for the proportionate increase in organic content in the biomass. Such observations are also reported in the literature (Pardilhó et al., 2021). This was also true for physicochemical characteristics such as carbohydrates, lipids, and proteins.

4.7. Saccharification

The reducing sugar concentration in B1 and B2 biomass hydrolysate was and 10.9 ±0.002 g/L biomass and 14.46 ±0.001 g/L biomass respectively (Fig. 12). This shows that the sugar

concentration obtained was higher in B2 biomass hydrolysate compared to B1 biomass hydrolysate. This shows that the sugar yield per gram of biomass obtained after hydrolysis increased significantly as shown in the table no 4.

Table .4. Reducing sugar g/L of biomass hy before and after hydrolysis

Sample	Biomass sugar Pre hydrolysis (g/L)	Biomass sugar Post hydrolysis (g/L)
B1 (biomass before starch extraction)	0.17±0.002	10.9 ±0.002
B2 (biomass after starch extraction)	0.30 ±0.001	14.46 ±0.001

By comparing the sugar concertation obtained in the hydrolysate to that of the initial B1 and B2 biomass was 0.17 ±0.002 g/L and 0.30 ±0.001 g/L respectively. It was observed that reducing sugar in both the biomass increased after hydrolysis but it was very less as compared to what was reported in the literature (Trivedi et al., 2013).

Another report by (Lee et al., 2015), showed that after lipid extraction, whole biomass and lipid extracted biomass underwent saccharificaion with pectinex enzyme, which resulted in higher (19.1%) reducing sugar in lipid extracted biomass as compared to whole biomass before extraction (4.9%), due to its pretreatment during extraction procedure.

Fig. 11. Image showing hydrolysates obtained from B1 and B2.

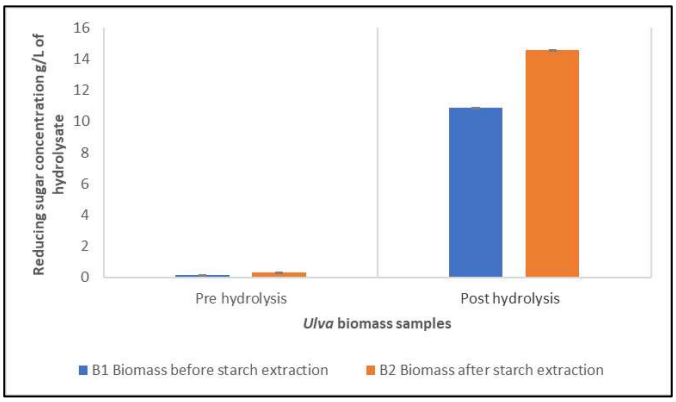


Fig. 12. Estimation of reducing sugar in biomass B1 and B2 before and after hydrolysis.

4.8. Fermentation

The hydrolysate obtained from Enzymatic saccharification of 2.5 g dry *Ulva* biomass B1 and B2 in 50 ml each was used as a substrate for fermentation by turbo yeast. The fermentation of this hydrolysate over intervals ranging from 12 to 72 hours, with increments of 12 hours each time, resulted in varying ethanol yields. Around 12 hours of fermentation period, the reducing sugar decreased to 9.7 ± 0.001 g/L from initial 10.9 ±0.002 g/L in B1 and in B2 it gets reduced to 12.32 ± 0.002 g/L from 14.46 ±0.001 g/L and the maximum utilization of sugars by turbo yeast was shown between 12-24 hours in both biomass i.e. 5.27 ±0.005 g/L (B1) and 0.89 ±0.002 g/L (B2) as shown in Fig. 13.

Accordingly, the fermentation efficiency of turbo yeast shown higher ethanol yield at 36 hours in both the biomass i.e. 273.67 ±0.29 mg/L of hydrolysate(B1) and 215.41 ±0.22 mg/L of hydrolysate (B2) (Fig. 13). The ethanol yield in B2 was 21% less than B1. After 36 hours the ethanol yield was becoming constant till 60 hours and again there was some increments observed (Fig. 13). At 48 hours, 60 hours, and 72 hours the yield was 245.01 ±0.26 mg/L, 250.88 ±0.26 mg/L and 215.65 ±0.23 mg/L respectively in B2 where as in B1 it was 222.46 ±0.23 mg/L, 215.88 ±0.23 mg/L and 262 ±0.28 mg/L respectively.

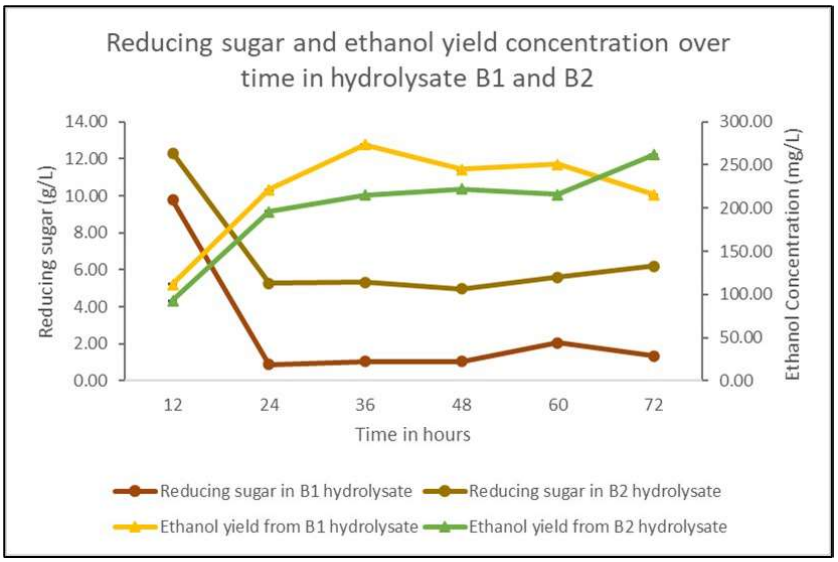


Fig. 13: Concentration of reducing sugar and ethanol over a period of 72 hours.

The higher ethanol yield was obtained in B1 (before starch extraction) as compared to B2 (after starch extraction) due to maximum utilization of sugars by yeast. Also, temperature and static condition facilitates the ethanol yield over time.

Biomass having starch, contributes more towards ethanol yield but in recent study it was lesser than what was reported in previous studies i.e. 1.28 g of ethanol yield (Trivedi et al., 2013).

As per the literature, residual biomass after lipid extraction gave 5.9 g/l of ethanol, which was too higher than ethanol yield in present study. (Sudhakar et al., 2020) studied different pH and temperature effect on ethanol yield from spent seaweed biomass and found out that spent seaweed fermentation gave 4.85 % of ethanol yield. In one of the study, residual biomass was hydrolysed enzymatically after ulvan extraction and fermented using *Saccharomyeces cerevisiae* yield 440 mg ethanol / g of reducing sugar (Trivedi et al., 2016).

Conclusion

This research determined the potential of seaweed *Ulva* sp. as a marine feedstock for bioethanol production. In vitro cultivation of seaweed revealed 7.66% biomass growth per day. Chemical composition analysis showed that residual biomass had higher content of protein, carbohydrates, lipids, and ash compared to initial biomass. Optimized condition for pretreatment and enzymatic saccharification was employed. It was observed that reducing sugar obtained after hydrolysis significantly increased in both B1 and B2 biomass. In this study, Separate hydrolysis and fermentation technique was used, which gave ethanol yield of 273.67 ±0.29 mg/L in B1 hydrolysate and 215.41 ±0.22 mg/L in B2 hydrolysate. Despite of the lower yield of ethanol in B2 biomass by 21% compared to B1, result suggest that valuable intermediate product, such as starch, can be extracted from seaweed biomass and the residual biomass can still be used as a potential feedstock for bioethanol production.

REFERENCES

1. Adams, J. M. M., Toop, T. A., Donnison, I. S., & Gallagher, J. A. (2011). Seasonal variation in *Laminaria digitata* and its impact on biochemical conversion routes to biofuels. *Bioresource Technology*, *102*(21), 9976–9984. https://doi.org/10.1016/j.biortech.2011.08.032
2. Baghel, R. S., Suthar, P., Gajaria, T. K., Bhattacharya, S., Anil, A., & Reddy, C. R. K. (2020). Seaweed biorefinery: A sustainable process for valorising the biomass of brown seaweed. *Journal of Cleaner Production*, *263*, 121359. https://doi.org/10.1016/j.jclepro.2020.121359
3. Balina, K., Lika, A., Romagnoli, F., & Blumberga, D. (2017). Seaweed Cultivation Laboratory Testing: Effects of Nutrients on Growth Rate of *Ulva intestinalis*. *Energy Procedia*, *113*, 454–459. https://doi.org/10.1016/j.egypro.2017.04.038
4. Bligh, E. G., & Dyer, W. J. (1959). A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917. https://doi.org/10.1139/o59-099
5. Buijs, N. A., Siewers, V., & Nielsen, J. (2013). Advanced biofuel production by the yeast *Saccharomyces cerevisiae*. *Current Opinion in Chemical Biology*, *17*(3), 480–488. https://doi.org/10.1016/j.cbpa.2013.03.036
6. Bušić, A., Marđetko, N., University of Zagreb, Faculty of Food Technology and Biotechnology, Pierottijeva 6, 10000 Zagreb, Croatia, Kundas, S., Belarussian National Technical University, Power Plant Construction and Engineering Services Faculty, Nezavisimosti Ave (2018). Bioethanol Production from Renewable Raw Materials and its Separation and Purification: A Review. *Food Technology and Biotechnology*, *56*(3). https://doi.org/10.17113/ftb.56.03.18.5546
7. Demirbas, A. (2008). Biofuels sources, biofuel policy, biofuel economy and global biofuel projections. *Energy Conversion and Management*, *49*(8), 2106–2116. https://doi.org/10.1016/j.enconman.2008.02.020
8. Dismukes, G. C., Carrieri, D., Bennette, N., Ananyev, G. M., & Posewitz, M. C. (2008). Aquatic phototrophs: Efficient alternatives to land-based crops for biofuels. *Current Opinion in Biotechnology*, *19*(3), 235–240. https://doi.org/10.1016/j.copbio.2008.05.007

9. El-Said, G. F., & El-Sikaily, A. (2013). Chemical composition of some seaweed from Mediterranean Sea coast, Egypt. *Environmental Monitoring and Assessment*, *185*(7), 6089–6099. https://doi.org/10.1007/s10661-012-3009-y
10. Fasahati, P., Dickson, R., Saffron, C. M., Woo, H. C., & Liu, J. J. (2022). Seaweeds as a sustainable source of bioenergy: Techno-economic and life cycle analyses of its biochemical conversion pathways. *Renewable and Sustainable Energy Reviews*, *157*, 112011. https://doi.org/10.1016/j.rser.2021.112011
11. Gengiah, K., Rajendran, N., Al-Ghanim, K. A., Govindarajan, M., & Gurunathan, B. (2023). Process and technoeconomic analysis of bioethanol production from residual biomass of marine macroalgae *Ulva lactuca*. *Science of The Total Environment*, *868*, 161661. https://doi.org/10.1016/j.scitotenv.2023.161661
12. Herrmann, C., FitzGerald, J., O'Shea, R., Xia, A., O'Kiely, P., & Murphy, J. D. (2015). Ensiling of seaweed for a seaweed biofuel industry. *Bioresource Technology*, *196*, 301–313. https://doi.org/10.1016/j.biortech.2015.07.098
13. Jambo, S. A., Abdulla, R., Mohd Azhar, S. H., Marbawi, H., Gansau, J. A., & Ravindra, P. (2016). A review on third generation bioethanol feedstock. *Renewable and Sustainable Energy Reviews*, *65*, 756–769. https://doi.org/10.1016/j.rser.2016.07.064
14. John, R. P., Anisha, G. S., Nampoothiri, K. M., & Pandey, A. (2011). Micro and macroalgal biomass: A renewable source for bioethanol. *Bioresource Technology*, *102*(1), 186–193. https://doi.org/10.1016/j.biortech.2010.06.139
15. Khambhaty, Y., Mody, K., Gandhi, M. R., Thampy, S., Maiti, P., Brahmbhatt, H., Eswaran, K., & Ghosh, P. K. (2012). *Kappaphycus alvarezii* as a source of bioethanol. *Bioresource Technology*, *103*(1), 180–185. https://doi.org/10.1016/j.biortech.2011.10.015
16. Kim, H. M., Oh, C. H., & Bae, H.-J. (2017). Comparison of red microalgae (*Porphyridium cruentum*) culture conditions for bioethanol production. *Bioresource Technology*, *233*, 44–50. https://doi.org/10.1016/j.biortech.2017.02.040
17. Klnc, B., Cirik, S., Turan, G., Tekogul, H., & Koru, E. (2013). Seaweeds for Food and Industrial Applications. In I. Muzzalupo (Ed.), *Food Industry*. InTech. https://doi.org/10.5772/53172
18. Korzen, L., Pulidindi, I. N., Israel, A., Abelson, A., & Gedanken, A. (2015). Marine integrated culture of carbohydrate rich *Ulva rigida* for enhanced production of bioethanol. *RSC Advances*, *5*(73), 59251–59256. https://doi.org/10.1039/C5RA09037G

19. Kraan, S. (2013). Mass-cultivation of carbohydrate rich macroalgae, a possible solution for sustainable biofuel production. *Mitigation and Adaptation Strategies for Global Change*, *18*(1), 27–46. https://doi.org/10.1007/s11027-010-9275-5
20. Kumar, A., Kumar, K., Kaushik, N., Sharma, S., & Mishra, S. (2010). Renewable energy in India: Current status and future potentials. *Renewable and Sustainable Energy Reviews*, *14*(8), 2434–2442. https://doi.org/10.1016/j.rser.2010.04.003
21. Lee, J. Y., Li, P., Lee, J., Ryu, H. J., & Oh, K. K. (2013). Ethanol production from *Saccharina japonica* using an optimized extremely low acid pretreatment followed by simultaneous saccharification and fermentation. *Bioresource Technology*, *127*, 119–125. https://doi.org/10.1016/j.biortech.2012.09.122
22. Lee, O. K., Oh, Y.-K., & Lee, E. Y. (2015). Bioethanol production from carbohydrate-enriched residual biomass obtained after lipid extraction of *Chlorella* sp. KR-1. *Bioresource Technology*, *196*, 22–27. https://doi.org/10.1016/j.biortech.2015.07.040
23. Lee, S. Y., Chang, J. H., & Lee, S. B. (2014). Chemical composition, saccharification yield, and the potential of the green seaweed *Ulva pertusa. Biotechnology and Bioprocess Engineering*, *19*(6), 1022–1033. https://doi.org/10.1007/s12257-014-0654-8
24. Li, Y., Cui, J., Zhang, G., Liu, Z., Guan, H., Hwang, H., Aker, W. G., & Wang, P. (2016). Optimization study on the hydrogen peroxide pretreatment and production of bioethanol from seaweed *Ulva prolifera* biomass. *Bioresource Technology*, *214*, 144–149. https://doi.org/10.1016/j.biortech.2016.04.090
25. Poland, Robak, K., Balcerek, M., & Lodz University of Technology, Faculty of Biotechnology and Food Sciences, Institute of Fermentation Technology and Microbiology, Department of Spirit and Yeast Technology Wolczanska 171/173, PL-90-924 Lodz, Poland. (2018). Review of Second-Generation Bioethanol Production from Residual Biomass. *Food Technology and Biotechnology*, *56*(2). https://doi.org/10.17113/ftb.56.02.18.5428
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, *193*(1), 265–275.
27. Mendis, E., & Kim, S.-K. (2011). Present and Future Prospects of Seaweeds in Developing Functional Foods. In *Advances in Food and Nutrition Research* (Vol. 64, pp. 1–15). Elsevier. https://doi.org/10.1016/B978-0-12-387669-0.00001-6

28. Milledge, J., Smith, B., Dyer, P., & Harvey, P. (2014). Macroalgae-Derived Biofuel: A Review of Methods of Energy Extraction from Seaweed Biomass. *Energies*, *7*(11), 7194–7222. https://doi.org/10.3390/en7117194
29. Miller, G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry*, *31*(3), 426–428. https://doi.org/10.1021/ac60147a030
30. Neifar, M., Chatter, R., Chouchane, H., Genouiz, R., Jaouani, A., Slaheddine Masmoudi, A., Cherif, A., & 1 University of Manouba, ISBST, BVBGR-LR11ES31, Biotechpole Sidi Thabet, 2020 Ariana, Tunisia. (2016). Optimization of enzymatic saccharification of *Chaetomorpha linum* biomass for the production of macroalgae-based third generation bioethanol. *AIMS Bioengineering*, *3*(3), 400–411. https://doi.org/10.3934/bioeng.2016.3.400
31. Niemi, C., Takahashi, J., Gorzsás, A., & Gentili, F. G. (2024). Quantitative and qualitative saccharide analysis of North Atlantic brown seaweed by gas chromatography/mass spectrometry and infrared spectroscopy. *International Journal of Biological Macromolecules*, *254*, 127870. https://doi.org/10.1016/j.ijbiomac.2023.127870
32. Offei, F., Mensah, M., Thygesen, A., & Kemausuor, F. (2018). Seaweed Bioethanol Production: A Process Selection Review on Hydrolysis and Fermentation. *Fermentation*, *4*(4), 99. https://doi.org/10.3390/fermentation4040099
33. Pardilhó, S., Costa, E., Melo, D., Machado, S., Espírito Santo, L., Oliveira, M. B., & Maia Dias, J. (2021). Comprehensive characterisation of marine macroalgae waste and impact of oil extraction, focusing on the biomass recovery potential. *Algal Research*, *58*, 102416. https://doi.org/10.1016/j.algal.2021.102416
34. Prabhu, M., Chemodanov, A., Gottlieb, R., Kazir, M., Nahor, O., Gozin, M., Israel, A., Livney, Y. D., & Golberg, A. (2019). Starch from the sea: The green macroalga *Ulva ohnoi* as a potential source for sustainable starch production in the marine biorefinery. *Algal Research*, *37*, 215–227. https://doi.org/10.1016/j.algal.2018.11.007
35. Ramachandra, T. V., Bajpai, V., Kulkarni, G., Aithal, B. H., & Han, S. S. (2017). Economic disparity and CO 2 emissions: The domestic energy sector in Greater Bangalore, India. *Renewable and Sustainable Energy Reviews*, *67*, 1331–1344. https://doi.org/10.1016/j.rser.2016.09.038

36. Sarris, D., & Papanikolaou, S. (2016). Biotechnological production of ethanol: Biochemistry, processes and technologies. *Engineering in Life Sciences*, *16*(4), 307–329. https://doi.org/10.1002/elsc.201400199
37. Singh, A., Singhania, R. R., Soam, S., Chen, C.-W., Haldar, D., Varjani, S., Chang, J.-S., Dong, C.-D., & Patel, A. K. (2022). Production of bioethanol from food waste: Status and perspectives. *Bioresource Technology*, *360*, 127651. https://doi.org/10.1016/j.biortech.2022.127651
38. Somma, D., Lobkowicz, H., & Deason, J. P. (2010). Growing America's fuel: An analysis of corn and cellulosic ethanol feasibility in the United States. *Clean Technologies and Environmental Policy*, *12*(4), 373–380. https://doi.org/10.1007/s10098-009-0234-3
39. Steinbruch, E., Drabik, D., Epstein, M., Ghosh, S., Prabhu, M. S., Gozin, M., Kribus, A., & Golberg, A. (2020). Hydrothermal processing of a green seaweed *Ulva* sp. For the production of monosaccharides, polyhydroxyalkanoates, and hydrochar. *Bioresource Technology*, *318*, 124263. https://doi.org/10.1016/j.biortech.2020.124263
40. Subhadra, B., & Edwards, M. (2010). An integrated renewable energy park approach for algal biofuel production in United States. *Energy Policy*, *38*(9), 4897–4902. https://doi.org/10.1016/j.enpol.2010.04.036
41. Sudhakar, M. P., Arunkumar, K., & Perumal, K. (2020). Pretreatment and process optimization of spent seaweed biomass (SSB) for bioethanol production using yeast (Saccharomyces cerevisiae). *Renewable Energy*, *153*, 456–471. https://doi.org/10.1016/j.renene.2020.02.032
42. Tan, I. S., Lam, M. K., Foo, H. C. Y., Lim, S., & Lee, K. T. (2020). Advances of macroalgae biomass for the third generation of bioethanol production. *Chinese Journal of Chemical Engineering*, *28*(2), 502–517. https://doi.org/10.1016/j.cjche.2019.05.012
43. Trivedi, N., Baghel, R. S., Bothwell, J., Gupta, V., Reddy, C. R. K., Lali, A. M., & Jha, B. (2016). An integrated process for the extraction of fuel and chemicals from marine macroalgal biomass. *Scientific Reports*, *6*(1), 30728. https://doi.org/10.1038/srep30728
44. Trivedi, N., Gupta, V., Reddy, C. R. K., & Jha, B. (2013). Enzymatic hydrolysis and production of bioethanol from common macrophytic green alga *Ulva fasciata Delile*. *Bioresource Technology*, *150*, 106–112. https://doi.org/10.1016/j.biortech.2013.09.103
45. Vo Hoang Nhat, P., Ngo, H. H., Guo, W. S., Chang, S. W., Nguyen, D. D., Nguyen, P. D., Bui, X. T., Zhang, X. B., & Guo, J. B. (2018). Can algae-based technologies be an

affordable green process for biofuel production and wastewater remediation? *Bioresource Technology*, *256*, 491–501. https://doi.org/10.1016/j.biortech.2018.02.031

46. Wei, N., Quarterman, J., & Jin, Y.-S. (2013). Marine macroalgae: An untapped resource for producing fuels and chemicals. *Trends in Biotechnology*, *31*(2), 70–77. https://doi.org/10.1016/j.tibtech.2012.10.009
47. Yaich, H., Garna, H., Besbes, S., Paquot, M., Blecker, C., & Attia, H. (2011). Chemical composition and functional properties of *Ulva lactuca* seaweed collected in Tunisia. *Food Chemistry*, *128*(4), 895–901. https://doi.org/10.1016/j.foodchem.2011.03.114

Appendix

1. Instruments

- UV-Vis spectrophotometer mini 1240
- Lab India Micro table high speed refrigerated centrifuge (C-series)
- MRC Ultrasonic processor (SONIC-650WT-V2)
- Remi cis-24 plus incubator shaker
- Hally instruments incubator shaker
- Rivotek incubator shaker
- Benchmark Beadbug^{TM} Mini Homogenizer Model D1030 (E)
- Thermo fisher medifuge small benchtop centrifuge
- Erma Hand Refractometer

2. Reagents for protein estimation by Folin's Lowry

Reagent A: 2% sodium carbonate in 0.1N sodium hydroxide

Reagent B: 0.5% copper sulphate in 1% potassium sodium tartarate. Prepare freshly by mixing stock solutions.

Reagent C (Alkaline copper solution): Add 50 ml of Reagent A and 1 ml of Reagent B prior to use.

Folin's reagent (Reagent D): Dilute Folin-Ciocalteau with an equal volume of 0.1 N NaOH

Standard: 1 mg/ml of BSA

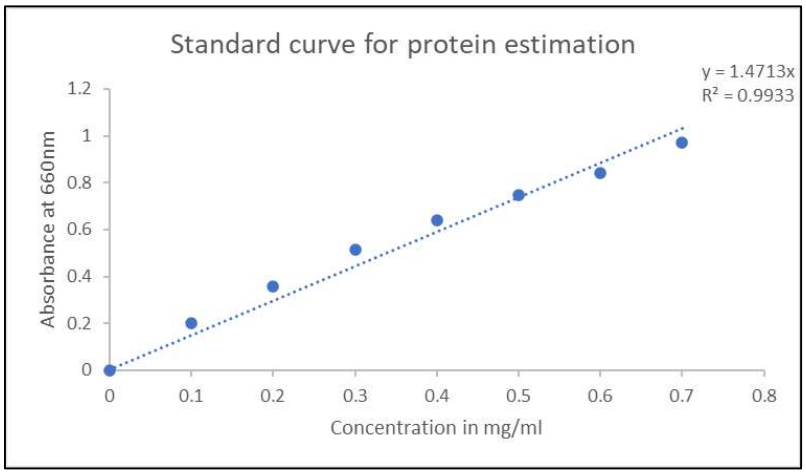


Fig. 15: Standard graph of BSA of concentration vs absorbance

3. Reagents for Carbohydrate estimation

Anthrone reagent: 0.2 g of anthrone powder in 100 ml of concentrated H_2SO_4. Use freshly prepared reagent and store in amber colour bottle.

Glucose as standard: 1mg/ml

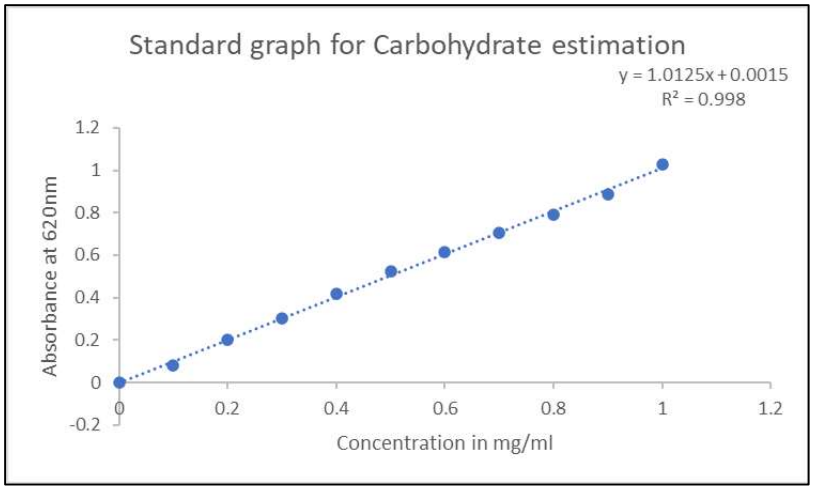


Fig. 16: Concentration vs absorbance graph of glucose

4. Reagents for DNSA

Sodium potassium tartrate: Dissolve 30 gms of sodium potassium tartrate in 50 mL of distilled water.

3,5-DNS solution: Dissolve 1 gm of DNS reagent in 20 mL of 2 M NaOH with help of magnetic stirrer.

2 molar NaOH: 1.6 g of NaOH in 20 ml of distilled water

DNS reagent: Prepare fresh by mixing the reagents (1) and (2) make up the volume to mL with water.

Glucose as standard: 1 mg/ml

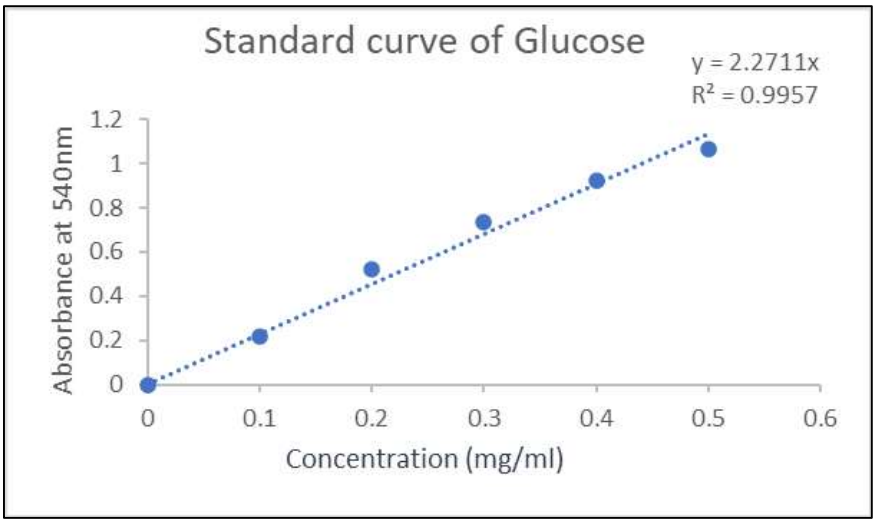


Fig. 17: Standard graph of glucose for DNSA

5. **Sodium Acetate buffer solution (pH 4.8)**

Sodium acetate solution: Dissolve 0.82 g of sodium acetate in 100 ml distilled water.

Acetic acid solution: Add 0.58 ml of acetic acid to 100 ml of distilled water.

Buffer: Add 30 ml of sodium acetate solution to 20 ml of acetic acid solution. Adjust the final volume to 100ml with distilled water and adjust the pH to 4.8.