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Synthesis of Bioethanol by Dark Fermentation Using Marine Seaweed *Acanthophora spicifera* (Vahl.) Borgesen as a Cheap Substrate

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Abstract

The Indian subcontinent constitutes about 7516.6 km of Coastal cover on the three borders and thus rich in marine biodiversity. More than 200 species of seaweeds have been reported to exist in this region attached to the surface of the rocks. Many seaweed based industries have been laid in these coastal zones and they utilize seaweed as raw materials for the production of agar and alginate. Several research studies reveal that the seaweed biomasses are rich in simple and complex sugars and can be utilized as a cost effective substrate for the commercial production of bioethanol. In many parts of the world the commercial exploitation and evaluation of bioethanol production from seaweeds are being practiced. However, India is rich in marine bio resources for the cultivation of seaweeds and can be directly utilized for the biochemical conversion of sugars into bioethanol. The present research study is an initial step for the commercial exploitation of marine seaweed *Acanthophora spicifera* (Vahl.) Borgesen for the bioethanol production. In which, the bioethanol synthesis was analyzed between both the raw substrate (Powdered seaweed biomass) and raw substrate with banana fruit supplement. The accumulation of bioethanol was almost similar in both the substrate when growing baker's yeast. And from the results, it has been revealed that approximately 6 % of bioethanol yield was obtained from the raw seaweed substrate. Therefore, this present small scale pilot study hugely supports the commercial exploitation of marine seaweeds for the bioethanol production.

Keywords: *Acanthophora spicifera*; Fermentation; Bioethanol; GC-MS; FT-IR

Introduction

Fossil fuel depletion is the major crisis in the modern world due to its unsustainable and nonrenewable nature. Therefore, need of the hour task is to search for alternative, sustainable and renewable source of energy. Bioenergy is one of the renewable energy attained from the biomass which is nothing but a compendium of energy synthesized by the producers of an ecosystem. Dried raw wood is a very good source of renewable energy used to burn for heat energy and still today 15% of the global energy production depends on the biomass in the developing countries [1]. Ethanol (C_2H_6O) is a high octane number alcohol, broader in flammability limits, with high flame speeds and high heat vaporization than gasoline. These unique features allow it to a high compression ratio with short burning time and slimmer engine paves to the potential advantages over gasoline of engine and the popular blend is E85 constitutes 85% of ethanol and 15% of gasoline [2]. Bioethanol fuel synthesized from sugarcane and blended with gasoline about 24% of total gasoline called as gasohol was exploited well in the Brazil [3]. Ocean covers about two third among the world surface area and thus the biomass production is high when compared to the land surface area. The seaweed is a primary producer in the ocean ecosystem which can also be utilized by human as food, fodder and fertilizer etc. The seaweeds are primitive marine aquatic plants proliferate adequately including estuaries and back waters.

The seaweeds have a specialized structure at its base called holdfast to attach to the substrate to defend from ocean wave current. Approximately 8 million tonnes of wet seaweed were harvested annually and yield about 6 billion US dollars [4]. The seaweeds are simply distinguished based on the pigmentation: Phaeophyceae, Rhodophyceae and Chlorophyceae with respect to brown, red and green. The seaweeds constitutes a complex chemical composition and the complete degradation of such substrates lies within the microbial strain. India has a highly diversified seaweed flora of the tropical belt. In which, 25 species are green seaweeds, 90 species belongs brown seaweeds and 350 species are red seaweeds [5].

The biomass of seaweeds is rich in polysaccharides with low amount of lignin which can be a very cheap substrate for bioethanol production by fermentation. Methane and ethanol are the end products of anaerobic digestion [6], by microbes. Thus, the biosynthesis of ethanol need controlled conditions to hamper contamination. The biosynthesis of ethanol was reported from the mannitol and laminarin of the brown seaweed *Laminaria hyperborean* [6]. From which, mannitol was utilized by *Zymobacter palmae* and both were consumed by *Pichia angophorae* [6]. *Acanthophora spicifera* (Vahl.) Borgesen is one among the red seaweeds found in the most tropical and subtropical region rich in bioactive compounds with potent antitumor and antibacterial activity including anticancer activity [7].

The major aim of the present study is the evaluation of biological production of ethanol from a cheap substrate (seaweed biomass) from East Coast of India using a Baker's yeast.

Materials and Methods

The glass wares were cleanly washed with soap solution and soaked in Chromic acid for overnight, washed with tap water, rinsed with

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distilled water and oven dried using hot air oven. The liquid broth medium contains powdered seaweed biomass were steam sterilized in an autoclave at 121°C, 15 psi for 20 min.

Collection of seaweed

The biomass of seaweed was collected from the seashore Mandapam, a place in Ramanathapuram District, Tamil Nadu, India during the month of April 2015. The average temperature on the day of collection was between 27°C and 29°C with average humidity of 55%. The satellite map images of the collection site were taken by using the Google Earth software tool.

Processing of seaweed biomass and preparation of substrate medium

The collected wet seaweed biomass of the macroalga (Vahl.) Borgesen was shade dried for a week to eliminate moisture content. Then the biomass bereft of moisture was homogenized completely with a mortar and pestle. The fine powdered seaweed biomass was weighed approximately 300 g and about 100 g among that was taken in each of the two sterilized 5 L conical flasks. Then the fine powdered biomass was dissolved completely with 2 *L* of distilled water (5%) in both the flasks. The negative control (200 ml) without inoculum was prepared based on the same percentage (5%) of biomass in distilled water in a 500 ml conical flask for spectrophotometric analysis. Among the two 5 L conical flasks, one was named as N and the other was named as N+N, where 35 g of Banana fruit was supplemented to the latter one and all the flasks were sterilized.

In vitro bioethanol production from seaweed

The substrate medium after steam sterilization was cooled down to room temperature and 1% of Baker's yeast (inoculum) was inoculated into both the 5 L conical flasks and kept incubated under dark condition (Dark fermentation). The rate of bioethanol production was indirectly measured using based on the growth curve measurement of yeast. The light absorbance with different wavelengths 435 nm, 465 nm, 495 nm, 525 nm and 585 nm were implemented to determine the rate of bioethanol production in both the N (raw seaweed) and N+N (raw seaweed with banana fruit supplement) for up to 13 days of dark fermentation using a UV-visible spectrophotometer (Hitachi U-2900).

Quantitative biochemical estimation of bioethanol from seaweed

The quantitative rate of bioethanol production was estimated for all the 13 days of dark fermentation of both the N (raw seaweed) and N+N (raw seaweed with banana fruit supplement) samples based on [8] method. The estimation method constituted the major ingredient called Potassium dichromate. The main principle of this method is the complete oxidation of ethanol by potassium dichromate in the occurrence of concentrated Sulphuric acid results in the final product acetic acid.

Potassium dichromate reagent: A 34 g of potassium dichromate was dissolved in 325 ml of concentrated Sulphuric acid with care. And the whole mixture was made up to 500 ml using distilled water and stored in light bereft condition.

Preparation of 2M Sodium hydroxide solution: About 80 g of Sodium hydroxide (NaOH) was dissolved in 1 L of distilled water.

Procedure: The standard graph for the estimation of bioethanol was developed from the known concentration of absolute ethanol ranged from 10 µl to 100 µl. The whole reaction mixture constitutes of 1 ml of potassium dichromate reagent followed by 500 µl of extract for unknown sample and 2 ml of sodium hydroxide solution. The whole reaction mixture was then incubated for 30 minutes and the absorbance values were recorded using a UV-visible spectrophotometer (Hitachi U-2900) at 600 nm.

Gas chromatography and mass spectrometry analysis (GC-MS)

The synthesized bioethanol was extracted from both of the N (raw seaweed) and N+N (seaweed with banana fruit supplement) samples using 1:1 ratio of Isoamyl alcohol and was subjected to GC-MS analysis equipped with flame ionization detector (FID) (Perkin Elmer, USA). A SP-2560 column (100 m × 0.25 mm I. D., 0.20 µm) (Sigma, Germany) was employed. About 5 µl of the sample was injected and the GC conditions were injector temperature: 260°C; Column temperature: 140°C and detector temperature: 260°C. Helium was used as a carrier gas with the flow rate of 1 ml/min. The unknown bioethanol synthesized during dark fermentation was determined in comparison with the retention times of the standard absolute ethanol using mass spectra from NIST library.

Fourier transform infra-red (FT-IR) spectrometrical analysis of the extract

The extracted bioethanol samples from both the N (raw seaweed) and N+N (seaweed with banana fruit supplement) were analyzed in Infra-Red spectrometer (Perkin Elmer model spectrum – I PC). The FT-IR spectra with the resolution of 4 cm^{-1}, Scan Number: 3 were performed after the evaporation of the fraction on Thallium bromide tablets. The FT-IR spectrums of both the samples were obtained as a percentage of transmission ranged from 450 cm^{-1} to 4000 cm^{-1}.

Results

Collection of seaweed from East Coast of India

The (Figure 1) shows the map image where the seaweed biomass were collected from the seashore of Mandapam, Ramanathapuram District, Tamil Nadu, India with Latitude 9°16'44.61"N and Longitude 79°0'56.71"E. The collected seaweed was morphologically identified as *Acanthophora spicifera* (Vahl.) Borgesen.

Systematic position:

Division	:	Rhodophyta
Class	:	Rhodophyceae
Order	:	Ceramiales
Family	:	Rhodomeliaceae
Genus	:	*Acanthophora*
Species	:	*Spicifera* (Vahl.) Borgesen

In vitro bioethanol production from seaweed

The growth curve of both the N (raw seaweed) and N+N raw seaweed with banana fruit supplement were analyzed during dark fermentation at different wavelengths (Figure 2). The absorbance rates were high and growth curve were maximum at a wavelength of 585 nm and this shows the growth curve was elevated while increase in the ascending order of wavelengths in both the N and N+N samples for all the 13 days of dark fermentation (Figures 3 and 4). The growth

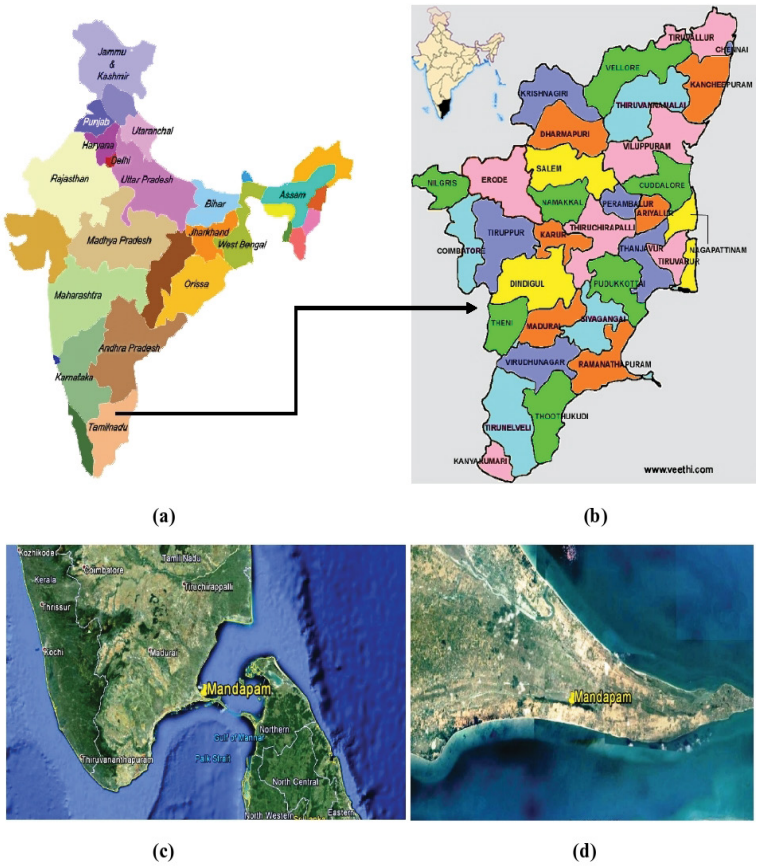

Figure 1: Place where the seaweed biomass was collected a) Map showing the state of province Tamil Nadu in the South Eastern part of the India; b) The state map of Tamil Nadu showing Ramanathapuram District; c) The satellite map image showing the place Mandapam and d) The satellite map image showing the site Mandapam in a close up view.

Figure 2: *In vitro* bioethanol production from seaweed, where N (seaweed biomass) and N+N (seaweed with banana fruit supplement) were shown.

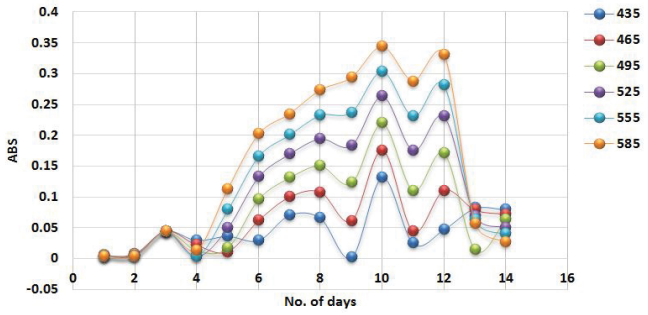

Figure 3: The growth curve of N (seaweed biomass) fermented using yeast in dark.

curve was maximum at the 10th day of incubation in dark fermentation and which was slightly high in the N+N (seaweed with banana fruit supplement) when compared to N (seaweed biomass). Thus, indirectly the fermentation and bioethanol production obtained maximum at the 10th day of incubation.

Quantitative biochemical estimation of bioethanol from seaweed

The quantitative rate of biosynthesis of ethanol from seaweed using

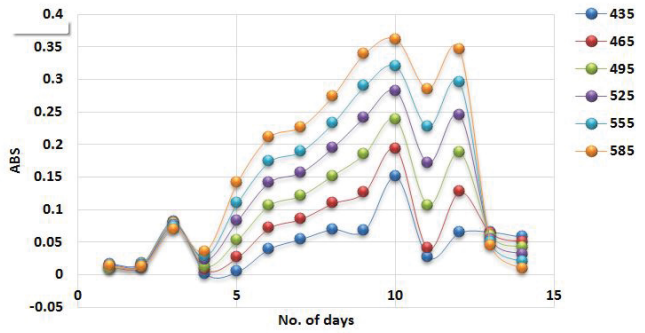

Figure 4: The growth curve of N+N (seaweed biomass with banana fruit supplement) fermented using yeast in dark.

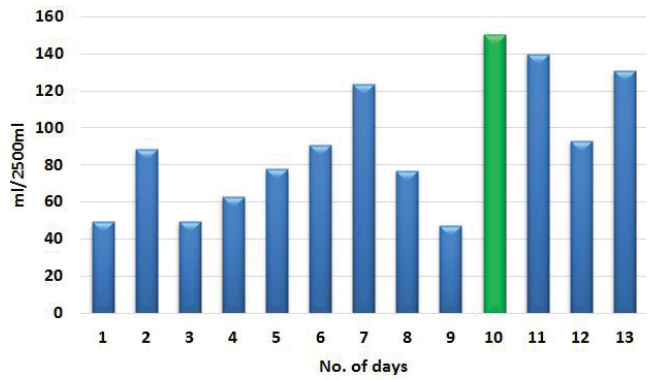

Figure 5: The histogram shows the production rate of bioethanol during dark fermentation of N (seaweed biomass) at different days of incubation from 1 to 13 days. The green color at 10th day of incubation represents quantitatively highest accumulation of bioethanol.

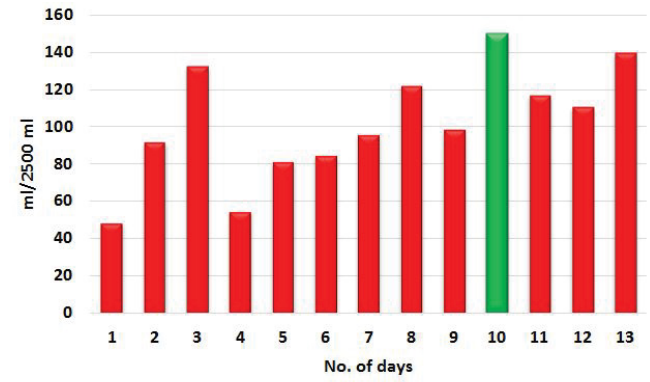

Figure 6: The histogram shows the production rate of bioethanol during dark fermentation of N+N (seaweed biomass with banana fruit supplement) at different days of incubation from 1 to 13 days. The green color at 10th day of incubation represents quantitatively highest accumulation of bioethanol.

a key microbe yeast by dark fermentation was estimated and evaluated biochemically based on the potassium dichromate oxidation method. The bioethanol production rate was yielded high at the 10th day of dark fermentation in both the N (seaweed biomass) and N+N (seaweed with banana fruit supplement). The rate of bioethanol production was almost similar in both the N and N+N samples with 150.67 ml from 2500 ml of substrate (Figures 5 and 6). From these results, it has been obviously resulted that, the yield of bioethanol from the liquefied substrate was approximately 6%.

Gas chromatography and mass spectrometry analysis (GC-MS) of the extract

Three samples (3rd, 6th and 9th days) were chosen from both the N (seaweed biomass) and N+N (seaweed biomass with banana fruit supplement) for the analysis of bioethanol using GC-MS. The gas chromatography-Mass Spectrum (GC and MS) spectrum of all the six samples had shown unique peaks at the retention times (RT) 2.1, 2.75 and 3.12 of sample N (seaweed biomass) followed by 2.5, 2.43 and 2.7 of sample N+N (seaweed biomass with banana fruit supplement) for 3rd, 6th and 9th days of dark fermentation respectively (Figures 7-20). The bioethanol content was found increasing during dark fermentation in both the N (seaweed biomass) and N+N (seaweed biomass with banana fruit supplement) samples based on the mass spectrum analysis (Figures 19 and 20). The GC and MS results revealed that the bioethanol production was elevated by increasing the duration of the dark fermentation.

Fourier transform infra-red (FT-IR) spectrometrical analysis of the extract

The presence of O-H stretch (3296 cm^{-1}) in all the samples confirms the occurrence of bioethanol in both the N (seaweed biomass) and N+N (seaweed biomass with banana fruit supplement) samples. Followed by the presence of C-H functional groups (3073 to 2873 cm^{-1}) in all the analyzed samples of N and N+N extracts. The presence of C-C aromatic groups (1543 and 1401 cm^{-1}) confirms the presence of C-C group found in the structure of ethanol. The presence of alcoholic group C-O (1310 cm^{-1}) majorly confirms the presence of ethanol from all the samples of N and N+N. Therefore, the bioethanol production was confirmed from both the N and N+N fermentation (Figures 21-26 and Table 1).

Discussion

The seaweeds (macroalgae) are the most auspicious biomass feedstock for the synthesis of third generation biofuel i.e., bioethanol which constitutes high carbohydrate content with low or without lignin in abundant. Bioethanol production from sugar-based biomass feedstock fermentation process paves an efficient way for the energy-rich transportation fuels [9]. Both corn and sugarcane have been already used as first generation feedstock for bioethanol production [10] and which has been practiced in the USA and Brazil. In recent decades, macroalgae have attracted many scientists as a valuable feedstock biomass for the production of bioethanol as an alternate for terrestrial crop plants [11]. The seaweeds are feasible for the microbial fermentation than the land plants. In this present study, the collected macroalga *Acanthophora spicifera* a red alga belongs to the family Rhodophyceae from the sea shore (East Coast of India) site of Mandapam, Ramanathapuram District, Tamil Nadu, India was employed as a cheap substrate for the *in vitro* bioethanol production.

Laminarin and mannitol were used as a substrate for the production of bioethanol from two brown seaweeds *Laminaria hyperborea* and *Ascophyllum nodosum* in Norway [12]. Conversion methods are there to release monosaccharides from various seaweed sources and structures and the choice of microorganisms for bioethanol production from seaweed are necessary pre-requisites for the development of a viable process utilising seaweed as a feedstock. The microbial strains like *Candida* sp. and *Pichia* sp. have the potency to ferment C5 sugars

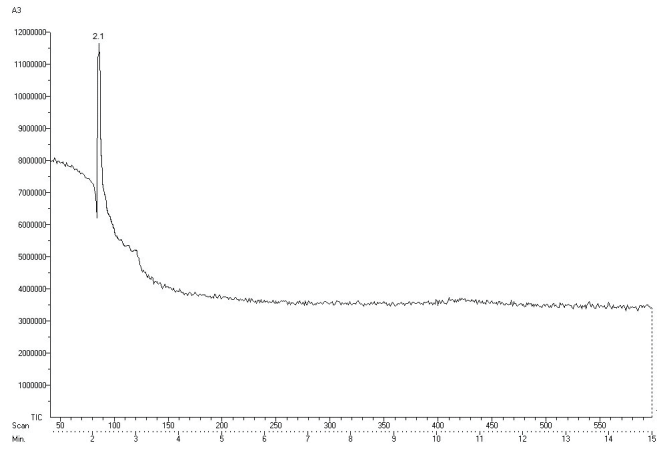

Figure 7: Gas chromatography spectrum of N (seaweed biomass) on 3rd day of dark fermentation.

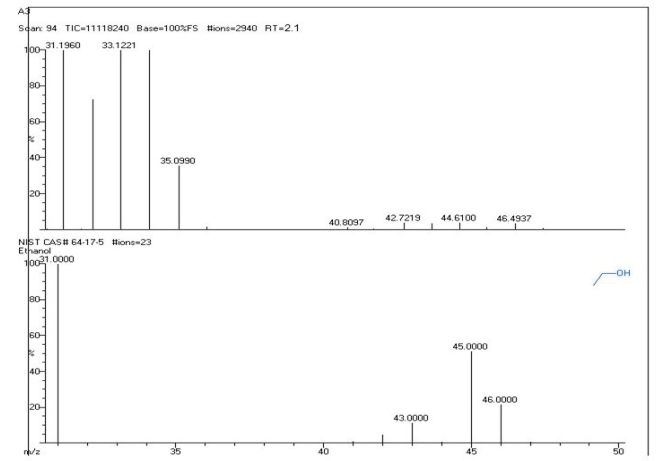

Figure 8: GC-Mass spectrum of N (seaweed biomass) on 3rd day of dark fermentation.

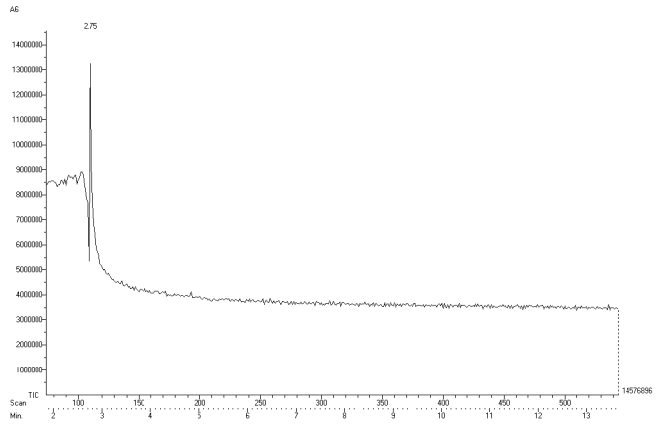

Figure 9: Gas chromatography spectrum of N (seaweed biomass) on 6th day of dark fermentation.

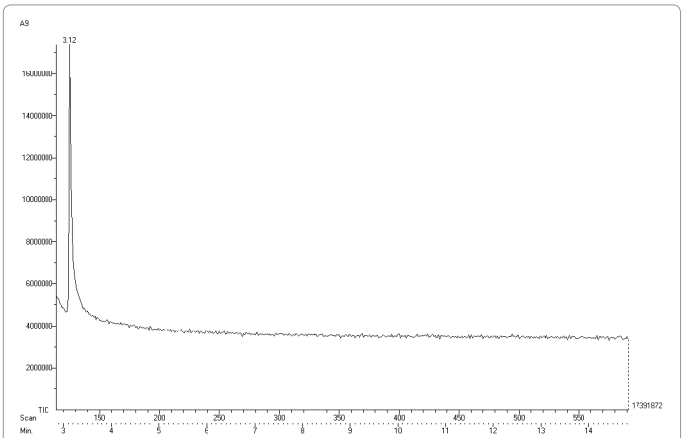

Figure 10: GC-Mass spectrum of N (seaweed biomass) on 6th day of dark fermentation.

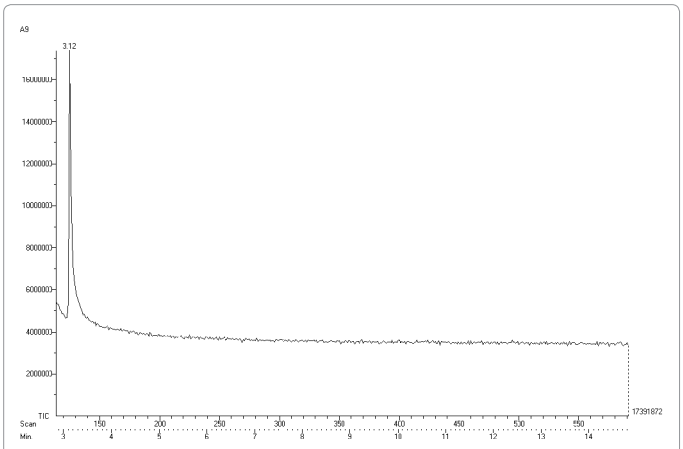

Figure 11: Gas chromatography spectrum of N (seaweed biomass) on 9th day of dark fermentation.

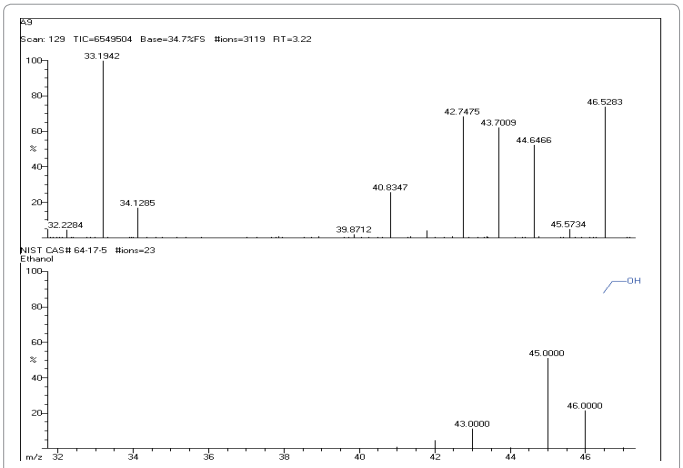

Figure 12: GC-Mass spectrum of N (seaweed biomass) on 9th day of dark fermentation.

including xylose found in the seaweeds [13]. *Saccharomyces cerevisiae* spp. are the microbes used as a traditional fermenter to produce commercial bioethanol in brewery and confectionaries [14]. The present study employed the Baker's yeast *Saccharomyces cerevisiae* as a key microorganism for the fermentation and production of bioethanol. The statistical analysis of growth of the yeast were well studied by Richards [15] which results in an asymmetrical S-shape. In this current research study, the growth curve was maximum at the 10th day of incubation in dark and which was slightly high in the fruit supplemented seaweed

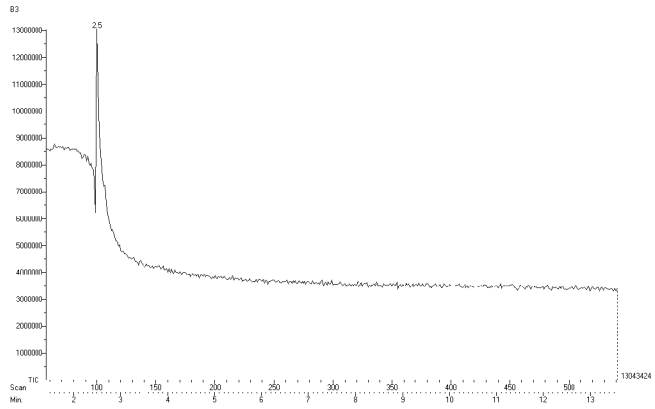

Figure 13: Gas chromatography spectrum of N+N (seaweed biomass with banana fruit supplement) on 3rd day of dark fermentation.

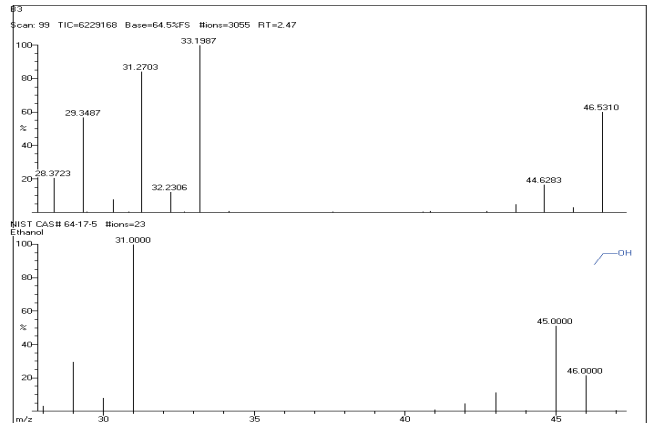

Figure 14: GC-Mass spectrum of N+N (seaweed biomass with banana fruit supplement) on 3rd day of dark fermentation.

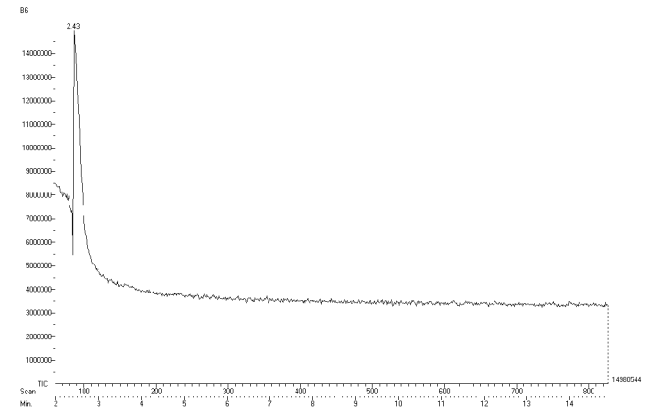

Figure 15: Gas chromatography spectrum of N+N (seaweed biomass with banana fruit supplement) on 6th day of dark fermentation.

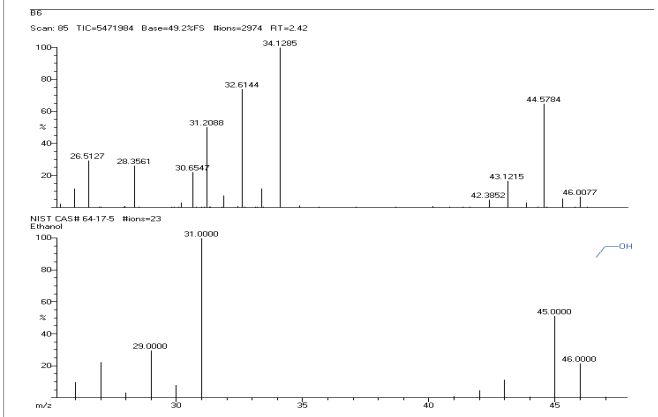

Figure 16: GC-Mass spectrum of N+N (seaweed biomass with banana fruit supplement) on 6th day of dark fermentation.

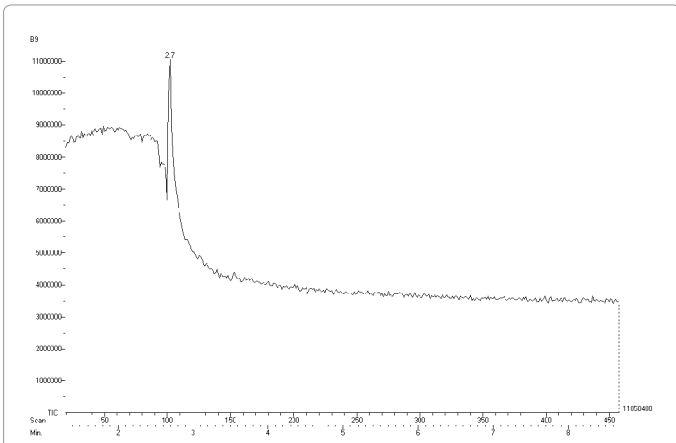

Figure 17: Gas chromatography spectrum of N+N (seaweed biomass with banana fruit supplement) on 9th day of dark fermentation.

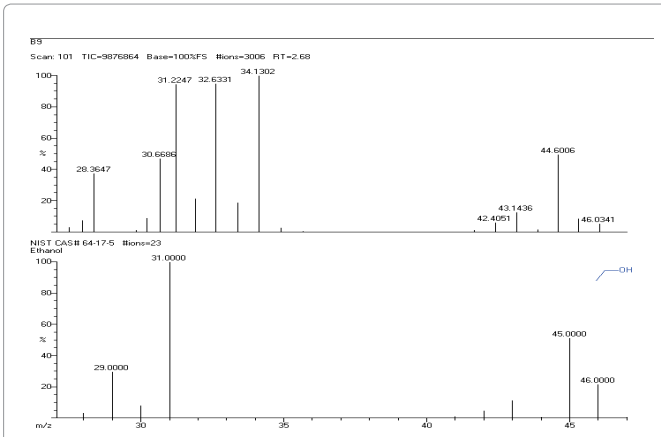

Figure 18: GC-Mass spectrum of N+N (seaweed biomass with banana fruit supplement) on 9th day of dark fermentation.

fermentation (N+N) when compared to raw seaweed fermentation (N). Thus, indirectly the fermentation rate was maximum at the 10th day of incubation. From the present investigation, the bioethanol produced from both the raw seaweed (N) and seaweed with fruit supplement fermentation (N+N) was quantitatively estimated as about 150.67 ml per 2500 ml of substrate at the 10th day of dark fermentation which was 6% of bioethanol produced from the substrate. Gas chromatography

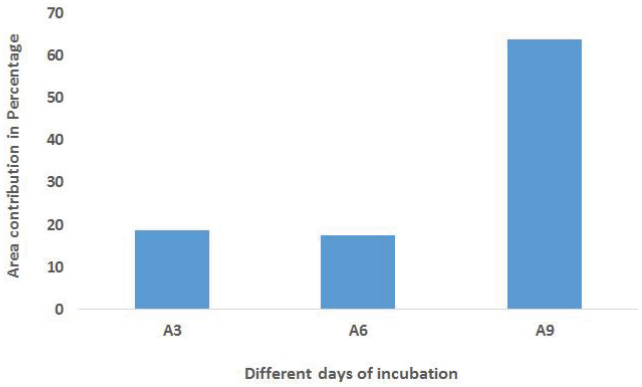

Figure 19: The bar graph shows the area percentage contribution of bioethanol for different days especially 3rd, 6th and 9th days respectively from gas chromatography of N (seaweed biomass).

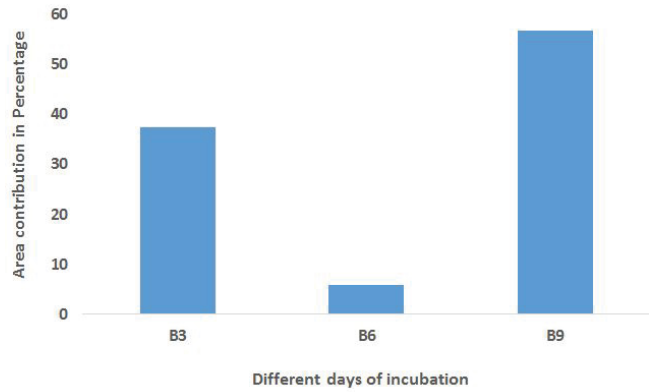

Figure 20: The bar graph shows the area percentage contribution of bioethanol for different days especially 3rd, 6th and 9th days respectively from gas chromatography of N+N (seaweed biomass with banana fruit supplement).

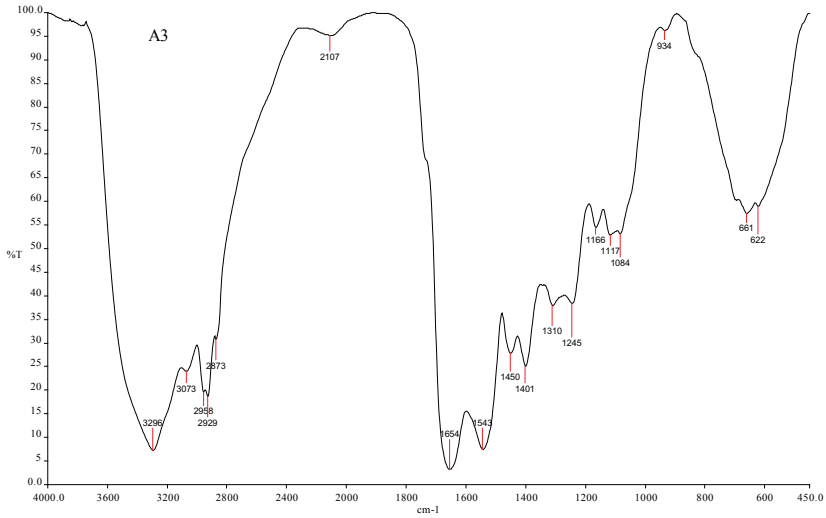

Figure 21: FT-IR spectrum of N (seaweed biomass) on 3rd day of fermentation.

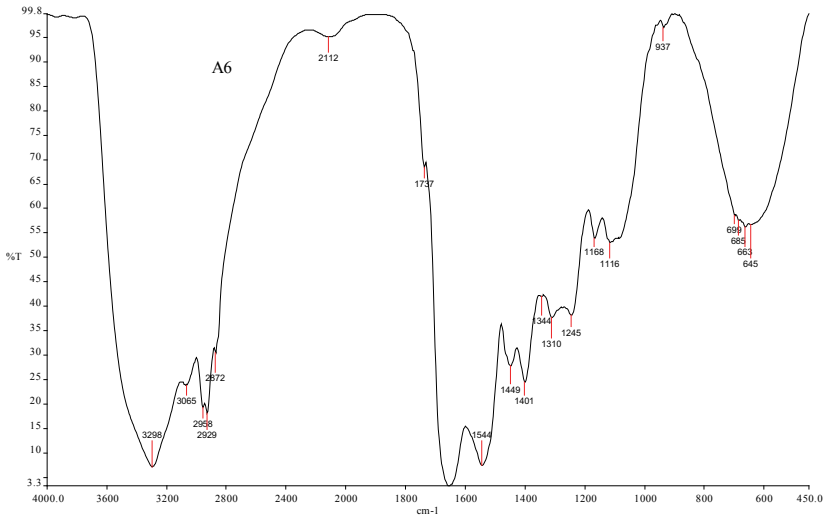

Figure 22: FT-IR spectrum of N (seaweed biomass) on 6th day of fermentation.

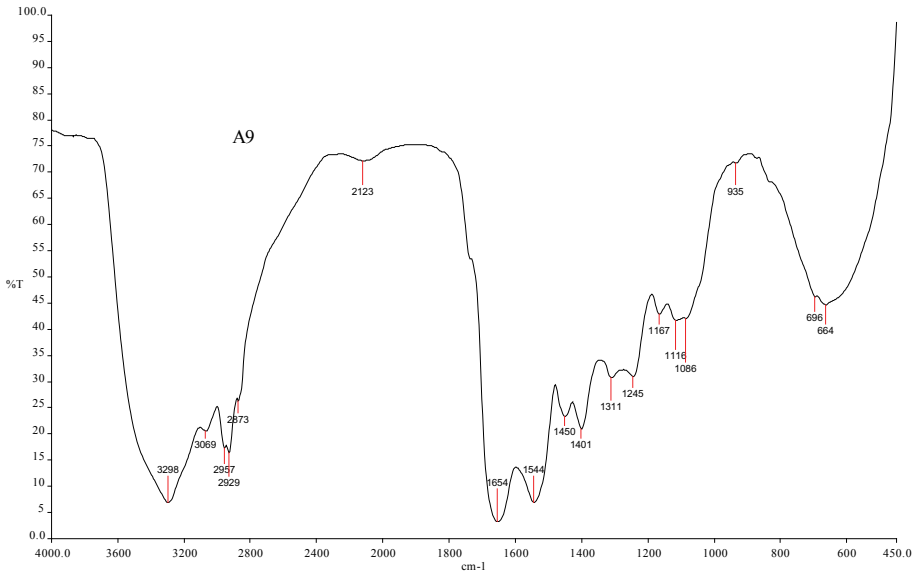

Figure 23: FT-IR spectrum of N (seaweed biomass) on 9th day of dark fermentation.

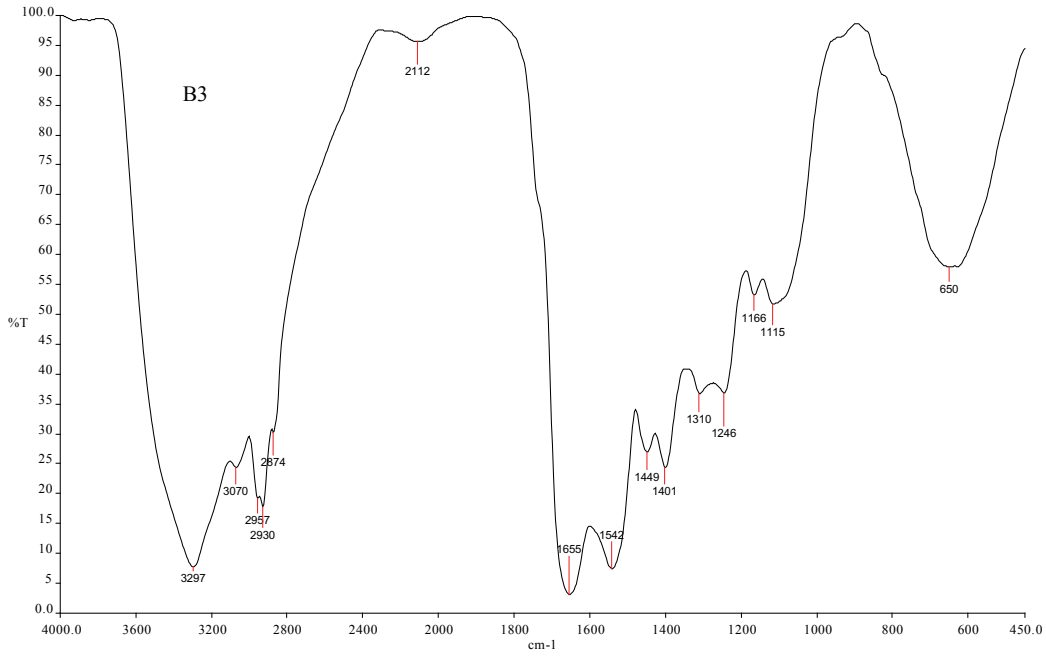

Figure 24: FT-IR spectrum of N+N (seaweed biomass with banana fruit supplement) on 3rd day of dark fermentation.

has advantages over potassium dichromate method as it is rapid, sensitive and accurate.

Potassium dichromate method is preliminary method for quantification of ethanol. More amount of ethanol was estimated by potassium dichromate method than by using a gas chromatograph as the chances of error are more in potassium dichromate method. Thus, amount of ethanol estimated by using gas chromatograph was considered more appropriate Koshy et al. [16]. Bouthilet et al. [17] had developed a packed GC method to analyze ethanol contents in alcoholic beverages. This method required at least 100 mL of sample and distillation as the pretreatment process. GC methods are currently available for determining ethanol contents in alcoholic beverages are US official methods, AOAC 968.09, 984.14, and 986.12 [18], and Taiwan official method, CNS N6181 (CNS, 1983[19]). When large numbers of samples are analyzed, advantages include short analysis time per sample and potential for extensive automation [20].

The biochemical estimation of bioethanol were done for all the 13 days of fermentation for both the raw seaweed fermentation (N) and

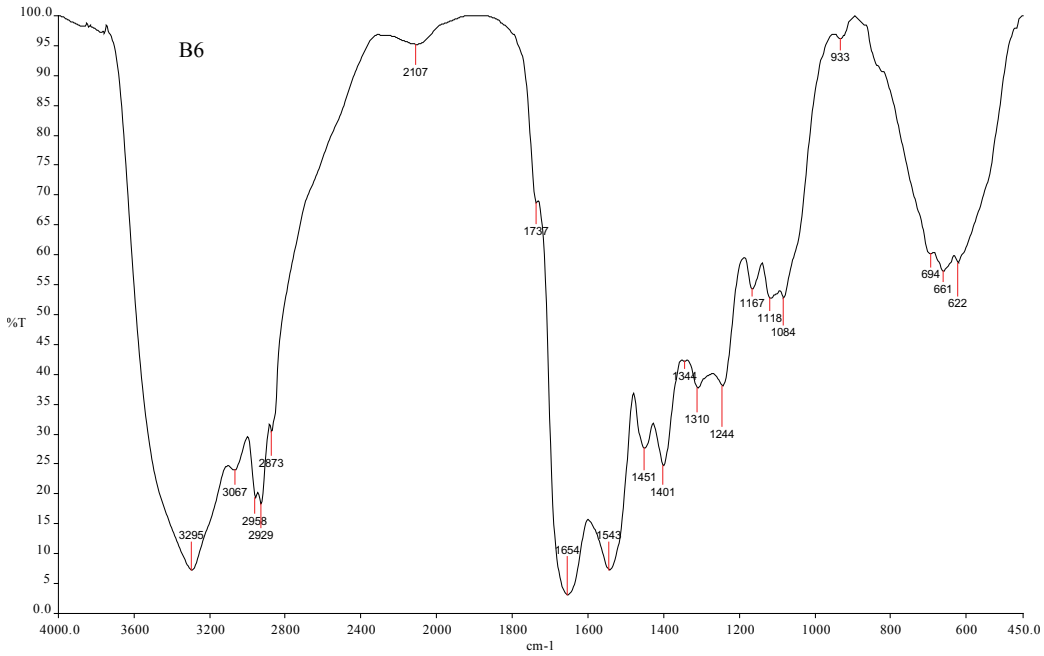

Figure 25: FT-IR spectrum of N+N (seaweed biomass with banana fruit supplement) on 6th day of dark fermentation.

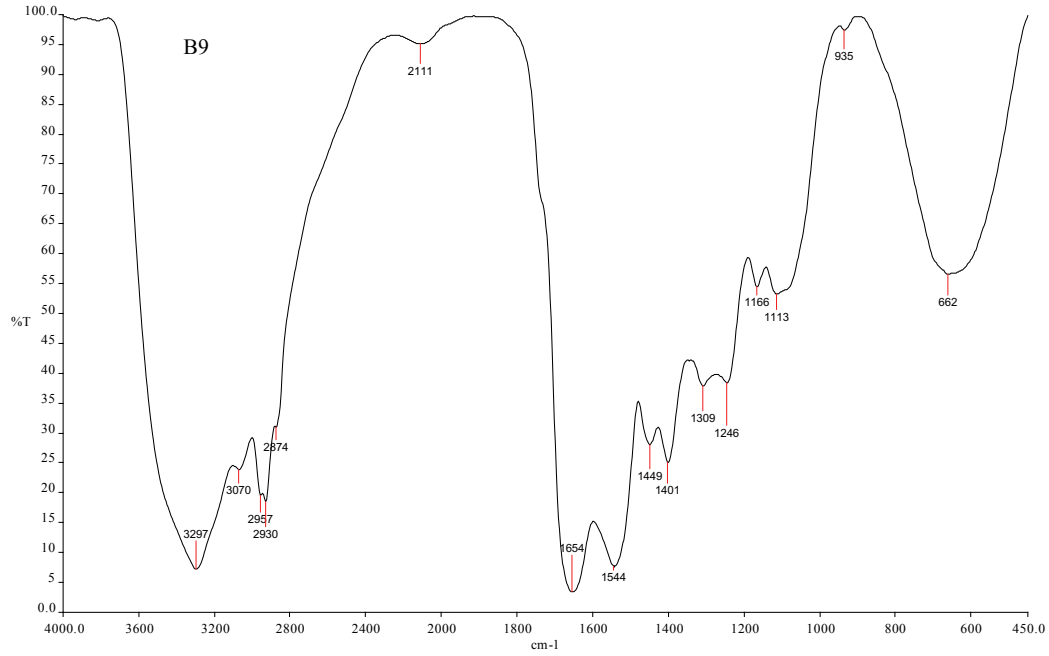

Figure 26: FT-IR spectrum of N+N (seaweed biomass with banana fruit supplement) on 9th day of dark fermentation.

seaweed with fruit supplement fermentation (N+N) followed by the gas chromatography mass spectrum analysis. Three samples (3rd day, 6th day and 9th day) were selected from both the N and N+N for the GC-MS analysis. The GC spectrum of all the samples shows a distinct peak for the presence of ethanol at retention time (RT) 2.1, 2.75 and 3.12 for N and 2.5, 2.43 and 2.7 for N+N. The presence of O-H stretch (3296 cm^{-2}) in all the samples confirms the occurrence of bioethanol production in both the N and N+N samples. Followed by the presence of C-H functional groups (3073 cm^{-1} to 2873 cm^{-1}) in all the analyzed samples of N and N+N extracts. The presence of C-C aromatic groups (1543 and 1401 cm^{-1}) confirms the presence of C-C group found in the structure of ethanol. The presence of alcoholic group C-O (1310 cm^{-1})

Functional groups	Stretch	N (A3)	N (A6)	N (A9)	N+N (B3)	N+N (B6)	N+N (B9)
Alcohols	**O-H**	**3296**	**3298**	**3298**	**3297**	**3295**	**3297**
Alkanes	**C-H**	**3073**	**3065**	**3096**	**3070**	**3067**	**3070**
Alkanes	**C-H**	**2958**	**2958**	**2957**	**2957**	**2958**	**2957**
Alkanes	**C-H**	**2929**	**2929**	**-**	**2930**	**2929**	**2930**
Alkanes	**C-H**	**2873**	**2872**	**2873**	**2874**	**2873**	**2874**
Alkynes	-C≡C-	2107	2112	2123	2112	2107	2111
Esters	C=O	-	1737	-	-	1737	-
Esters	C=O	1654	1654	1654	1655	1654	1654
Aromatics	**C-C**	**1543**	**1544**	**1544**	**1542**	**1543**	**1544**
Alkanes	C-H	1450	1449	1450	1449	1451	1449
Aromatics	**C-C**	**1401**	**1401**	**1401**	**1401**	**1401**	**1401**
Alkanes	C-H rock	-	1344	-	-	1344	-
Alcohols	**C-O**	**1310**	**1310**	**1311**	**1310**	**1310**	**1309**
Aliphatic amines	C-N	1245	1245	1245	1246	1244	1246
Aliphatic amines	C-N	1166	1168	1167	1166	1167	1166
Aliphatic amines	C-N	1117	1116	1116	1115	1118	1113
Alkenes	=C-H	1084	-	1086	-	1084	-
Carboxylic acids	O-H	934	937	935		933	935
Alkynes	C-H	-	699	696	-	694	-
Alkyl halides	C-Br	-	685	-	-	-	-
Alkyl halides	C-Br	661	663	664	-	661	662
Alkyl halides	C-Br	-	645	-	650	-	-
Alkyl halides	C-Br	622	-	-	-	622	-

Table 1: The FT-IR analysis of N and N+N samples showing the presence of different functional groups.

majorly confirms the presence of ethanol from all the samples of N and N+N. Hence, the bioethanol production was confirmed from both the N and N+N fermentation.

Energy production from seaweeds will only be economic if the harvesting costs are low. It may be noted that wastes from the alginate industry may be considered a non-cost raw material for bioenergy production (Horn, 2008).

Conclusion

This present research study is a primary instigation for the biological production of bioethanol from a marine red seaweed *Acanthophora spicifera* (Vahl.) Borgesen as an alternative fuel source to reduce the combustion of fossil fuel for energy in India. As a result, there is not much difference in the production of bioethanol in both the raw seaweed and raw seaweed supplemented with banana fruit biological treatment. The synthesized bioethanol content was estimated about 6% from the utilized substrate. Therefore, this small scale research study largely supports the commercial exploitation of seaweed for the bioethanol production.

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