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Effective production of ethanol-from-cellulose (EFC) from cheap sources sawdust and seaweed *Gracilaria edulis*

Thoufik Rahuman Amanullah[1], Deepu Krishnan[1], Senthil Balaji D.[1], Karthikeyan Ganesan[1*], Bibin. G. Anand[3], Infant Santhose Barthelomai[2], Balaji Ravichandran[2] and Sathish Kumar Chinnasamy[2]

[1]*Department of Biotechnology, PSR College of Engineering and Technology, Sivakasi, Tamilnadu, India*
[2]*Robust Mushrooms Private Limited, Sivagangai, Tamilnadu, India*
[3]*Department of Biotechnology, Mepco Schlenk Engineering College, Sivakasi, Tamil Nadu, India*

ABSTRACT

Ethanol-from-cellulose (EFC) holds great potential due to the widespread availability, abundance, and relatively low cost of cellulosic materials. Ethanol can be made synthetically from petroleum or by microbial conversion of biomass materials through fermentation. In early years the ethanol was produced from fermentation of starch materials. Due to the demand of bio-fuels, there is an increase in search for an alternative source for ethanol production. Now days sources such as corn steep, rice husk, saw dust, sewage waste, sea weeds etc., were under research for bio fuels. There is higher demand for alternative source for bio-fuels, in our investigation we opted saw dust and seaweed namely Glacilaria edulis as source for ethanol since it is cheaply available. Saw dust is rich in cellulose, a polymer of glucose which is fermented to ethanol. Sea weeds are rich in agar, a polymer of galactose which is fermented to ethanol using Saccharomyces cerevisiae. For the effective conversion, pre-treatment process and time played a major role and analyzed. Production yield was enhanced with the enzymatic hydrolysis with Aspergillus niger and the obtained ethanol was analyzed with GC-MS.

Key words: *Gracilaria edulis*, *Saccharomyces cerevisiae*, *Aspergillus niger*, Saw dust.

INTRODUCTION

Over the last few decades, the negative impacts of fossil fuel on the environment and consequent global warming, progressive demand for energy, inevitable depletion of the world's energy supply, and the unstable oil market (such as the energy crisis of the 1970s) have renewed the interest of society in searching for alternative fuels (1; 2). The alternative fuels are expected to satisfy several requirements including substantial reduction of greenhouse gas emission, worldwide availability of raw materials, and capability of being produced from renewable feedstocks (3). Production of fuel ethanol from biomass seems to be an interesting alternative to traditional fossil fuel, which can be utilized as a sole fuel in cars with dedicated engines or in fuel blends.

Ethanol is currently produced from sugars, starches and cellulosic materials. The first two groups of raw materials are currently the main resources for ethanol production, but concomitant growth in demand for human feed similar to energy could make them potentially less competitive and perhaps expensive feedstocks in the near future, leaving the cellulosic materials as the only potential feedstock for production of ethanol (4). Cellulosic materials obtained from wood and agricultural residuals, municipal solid wastes and energy crops represent the most abundant global source of biomass (5). These facts have motivated extensive research toward making an efficient conversion of lignocelluloses into sugar monomers for further fermentation to ethanol. The use of ethanol as an alternative motor fuel has been steadily increasing around the world for a number of reasons.

Ethanol, unlike gasoline, is an oxygenated fuel that contains 35% oxygen, which reduces particulate and NO_x emissions from combustion. Ethanol can be made synthetically from petroleum or by microbial conversion of biomass materials through fermentation. The fermentation method generally involves three steps: (1) the formation of a solution of fermentable sugars, (2) the fermentation of these sugars to ethanol, and (3) the separation and purification of the ethanol, usually by distillation. Although ethanol can be produced various sources seaweed and cellulose are cheap and easily available sources. (6)

Mariculture of the agar-yielding, red seaweed, *Gracilaria edulis* (Rhodophyta) was conducted in Minicoy lagoon during 1990–1992. Experiments were conducted on dry matter accumulation, effects of planting density, depth of water above culture nets, periodic cleaning for removal of epiphytes, grazing and the yield as well as regeneration of *G. edulis* after successive harvests to understand the optimum requirements of this seaweed (7). Cellulosic substrates produce xylose, arabinose, glucose, mannose and galactose in various amounts (8).

Extracts from brown seaweeds could possibly be fermented to ethanol, particularly seaweeds harvested in the autumn (9). *Gracilaria have the* Carbohydrate content ~ 45% of dry wt. *Glacilaria:* Total species: 15 species Harvesting: 1500 -2000 tons (dry wt.) year. A large amount of harvested *Gracilaria* is being exported to Russia, Japan and recently China. A smaller portion is used for domestic agar processing and also commonly food vegetable and jellies (10).

Aspergillus species has been since long preferred for the production of enzymes for a number of reasons; they are capable of secreting high level of proteins; they have good fermentation properties allowing large scale production of the enzymes; several products from these fungi have "GRAS" status, allowing them to be used in food and feed applications. A very important aspect of the *Aspergillus* is that they produce a wide range of both endo and exo-acting enzymes that are involved in degradation of plant cell wall polysaccharides (11).

Traditionally, baker's yeast (*Saccharomyces cerevisiae*), has long been used in the brewery industry to produce ethanol from hexoses (6-carbon sugar). Due to the complex nature of the carbohydrates present in lignocellulosic biomass, a significant amount of xylose and arabinose (5-carbon sugars derived from the hemicellulose portion of the lignocellulose) is also present in the hydrolysate. Recently, engineered yeasts have been described efficiently fermenting xylose and arabinose, and even both together (12).

The present work is aimed at establishing potential of *Aspergillus niger* and *Saccharomyces cerevisiae* for ethanol production from cheap sources sawdust and seaweed *Gracilaria edulis.*

MATERIALS AND METHODS

Seaweed and saw dust are taken as two different sources for ethanol production. *Gracilaria edulis*, seaweed is chosen as the source for ethanol production because it is rich in carbohydrate specially agar. Saw dust is the wastes obtain from saw mill. It is easily available. They contain cellulose and lignin. The enzyme or acid hydrolysis of cellulose release reducing sugars, this can be converted to ethanol by fermentation.

Collection of raw materials:
Gracilaria edulis was collected from the sea shore of Rameswaram at early morning (Fig: 2), they were clean to make free from sand, stone and dust by washing it twice in water. They were dried in sun light for a while to drain out the water, and then they were dried in hot air oven and crushed to powder. The powder was stored. Saw dust was collected from the saw mill. They were sieved to get the fine powder of saw dust. Before starting the experiment they were washed with water.

Isolation of microorganism:
Aspergillus niger and *Saccharomyces cerevisiae* were used throughout the study. The coconut was allowed to decay in anaerobic condition to favor the microorganism growth. After three days black colonies of microorganism were grown. That black colony were swapped and made a streak in potato dextrose medium and kept it for incubation at 30^{o}c for three days. Then they were subculture in slant containing potato dextrose medium. Black colonies were grown, confirmed microscopically and stored.

Inoculum development:
0.1% Tween 80 was added to the sterile water. Spores from slant were scrapped off and suspended in 100ml of sterile water. The spore suspension was shaken thoroughly to breakup any aggregates. The spore count was determined microscopically using 1ml of the above suspension after making the serial dilutions. 5% v/v spore

suspension containing $30x10^9$ spores per ml was used as the inoculum in 100ml of moistening medium in 500ml Erlenmeyer flask. The cultures were incubated at 30°C on a rotary shaker for two days.

Enzymatic hydrolysis:
In enzymatic hydrolysis we have used *Aspergillus niger* to produce enzyme such as cellulase, α-amylase, glucoamylase, lipase, pectinase etc, based on the substrate it utilize. These enzymes have different optimal pH, cellulose has 4.8, pectinase have 4.5, α-amylase have 5.5 and glucoamylase have 4.5. So we have maintained 4.8±5.

Lab scale study:
Eight 250 ml conical flask were taken. Four of the flask is added with 10g of saw dust and another 4 flask were added with 10g sea weeds. To the conical flask containing saw dust 50ml of distilled water is added and to the flask containing sea weeds 100ml of distilled water is added and they were sterilized. They were marked with from number 1 to 8 (1 to 4 for saw dust and 5 to 8 for sea weeds) and leave as such for one day. To the flask no.1 & 2 were added with 25ml of 0.5M H_2SO_4 and 3 & 4 were added with 1M H_2SO_4. Likewise 5 & 6 were added with 0.5M and 7 & 8 were added with 1M H_2SO_4. The flask no. 1, 3, 5 and 7 were kept as such and the flask no. 2, 4, 6 and 8 were kept in the water bath at 70°C for 2 hours and then placed as such for two days. After two days all the flasks were added with 25ml sterile water and then neutralized with 6M NaOH to the around pH 4.7 to 5.2. Then they were sterilized again and 20ml of moistening medium containing *Aspergillus niger* were added. They were kept as such for 6 days and the concentration of glucose was estimated before adding *A.niger* and after the addition. They were periodically estimated by Anthrone quantitative analysis.

Bulk production for seaweeds:
An air tight container was taken and added with 150 g of seaweeds, 1500 ml of water were added to the seaweeds in the container and the whole setup was sterilized. Then the container was kept as such for one night. After that it was added with 1M sulphuric acid of 375 ml and the sample was mixed well for one minute and kept in a boiling water bath for two hours around 70°c. After 72 hours, 375 ml of sterile water were added to the container and neutralized to the pH around 4.7-5.2. Then the container was sterilized and 375ml (15%) of the moistening medium containing *Aspergillus niger* was added to flasks. The sample in the container was mixed well continuously for 3-5 minutes and then kept it as such for 48 hours and nutrient broth containing *Saccharomyces cerevisiae* of 450ml (20%) were added to the sample in the container. Then the sample was mixed well, after 96 hours the sample was filtered using the white cotton cloth, then subjected to microfilteration and the filtrate were centrifuged with the addition of methanol at 1000rpm for ten minutes. Then the supernatant were collected for the Gas Chromatographic (GC) analysis. Some of the filtrate was distilled and the sample were collected and stored.

Tests for alcohol:
Jones Oxidation for Primary and Secondary Alcohols and Lucas Test for Secondary and Tertiary Alcohols were followed.

GC Analysis:
The filtered seaweed fermentation extract was centrifuged and the supernatant was analyzed using Gas Chromatography. GC was taken by the following conditions, Column Type: Capillary Column Elite-5ms (5%Phenyl 95% dimethylpolysiloxane), Column length: 30m, Column id: 250µm, 50ºC (5min) @10 ºC/min to 150 ºC (5 min), Injector temp. : 180ºC, Carrier gas: He @ 1ml/min, Mass Range: 15-200amu,MS: Source temperature: 200 ºC, Inlet line temperature: 2200 ºC.HS: Oven temperature:80 ºC, Needle temperature: 100 ºC, Transfer line temperature: 110 ºC, Pressurizing time: 3 min, Thermostat time: 30 min, Injection time: 0.05min, Withdrawal time: 0.5 min, GC cycle time: 21 + 5 = 26 min.

RESULTS AND DISCUSSION

Isolation of *Aspergillus niger:*
Aspergillus niger was isolated from decayed coconut by using PDA medium (Fig: 3 & 4).

Morphological test:
Lacto phenol cotton blue test:
The white coloured hypae and the black coloured conidiospores were observed from the sample. It was confirmed as *Aspergillus niger*.

Confirmatory tests:
Fehling's test:
The reddish brown precipitate was obtained from the sample. Hence the presence of reducing sugar is confirmed.

Iodine test:
No change of colour was observed from the sample. Thus it is confirmed that there was no starch present in the sample.

Bials test:
No green colour formation was observed from the sample. Hence the absence of pentose is confirmed.

Optimization for bulk production:
The standard graph was obtained for sugar using anthrone reagent (Fig: 1 & Table:1). The concentration of sugar was estimated for sample at each interval and it is noted to be increased, occupied a peak and then decreased. The variation of concentration for the sample which treated with sulphuric acid (1M & 0.5M) was also noted. Based upon the graph the optimum time for the enzymatic hydrolysis was estimated at 3 days and the molarity of sulphuric acid obtained as 1M (Table: 2, 3 & 4). On comparison to the sawdust, seaweeds show more reducing sugar concentration on graph. So seaweeds are chosen for the bulk production.

Bulk production:
The process was effectively done in air tight container without any agitation. After 10 days of process the sample kept for filtration and distillation.

Micro filtration:
The filtration process was carried out and the micro particles are separated effectively, clear samples with minimum turbidity were collected from the equipment (Fig: 5).

Distillation:
Distillation is carried out using simple distillation process. The condensation process was observed in the equipment. White clear samples were collected in a conical flask as distillate (Fig: 6).

Confirmatory test for alcohol:
Lucas test:
The test was carried out as per the procedure explained earlier. It is observed that no reaction occurred during the test. This shows the absence of tertiary and secondary alcohol.

Jones oxidation test:
It was observed that the production of an opaque suspension with a green to blue color. From this it was confirmed that the sample containing primary alcohol.

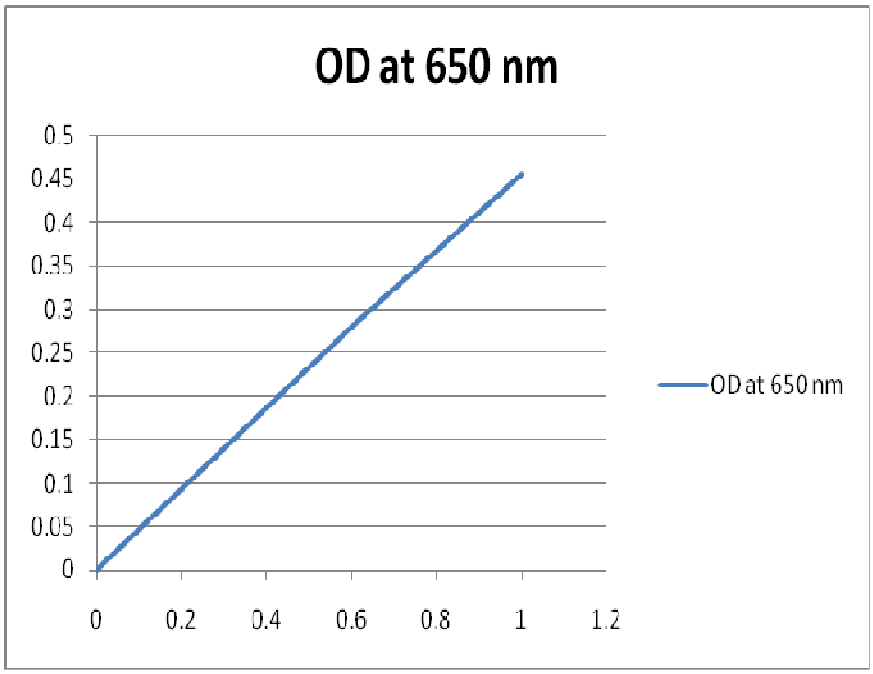
GC analysis:
The sample was analyzed using Gas Chromatographic method. The results are shown in the figures 7, 8 & 9. The fragmentation pattern obtained for the seaweed fermentation extract is similar to the standard fragmentation pattern of ethanol. So it shows the presences of ethanol in the seaweed fermentation extract.

The earlier studies indicated about the problems accompanied with the pretreatment of the source. We undergone effective pretreatment by applying high temperature (121 degree C) steam at high pressure (15lbs) for a prolonged time (1hr), and then followed by mild acid treatment with 1M sulphuric acid. Thus more polysaccharides released into the sample from the source. The process in bulk was conducted without agitation, the earlier methods were done with continuous agitation and stirring. Here we tried a method with no such usage of power and it is also a cost effective method of ethanol production. So it can be extended to mass scale which can be a alternative source for the depleting fossil fuel.

Standard value for glucose using Anthrone Table: 1

S. No	Concentration of glucose(mg/ml)	O.D at 650nm
1	0.2	0.05
2	0.4	0.21
3	0.6	0.28
4	0.8	0.40
5	1.0	0.46

Standard graph:



Concentration of glucose Vs O.D value Fig: 1

Absorbance value for sugar on day 2 Table: 2

S. No	Volume of sample(ml)	O.D at 650nm	Concentration of sugar(mg/ml)
1 (0.5M SD)	0.2	0.37	0.82
2 (0.5M SD-B)	0.2	0.44	0.98
3 (1M SD)	0.2	0.47	1.40
4 (1M SD-B)	0.2	0.34	0.76
5 (0.5M SW)	0.2	1.09	2.40
6 (0.5M SW-B)	0.2	1.15	2.52
7 (1M SW)	0.2	1.24	2.68
8 (1M SW-B)	0.2	1.16	2.56

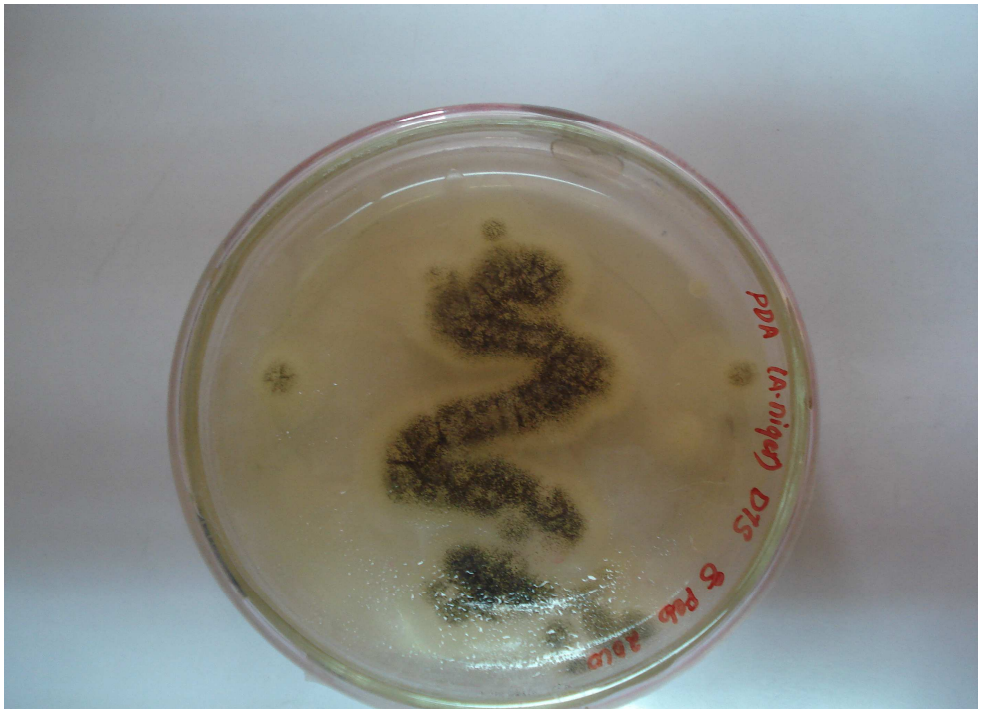
Absorbance value for sugar on day 3 Table: 3

Samples	Volume of sample(ml)	O.D at 650nm	Concentration of sugar(mg/ml)
1 (0.5M SD)	0.2	1.11	2.42
2 (0.5M SD-B)	0.2	1.07	2.36
3 (1M SD)	0.2	1.14	2.50
4 (1M SD-B)	0.2	1.12	2.46
5 (0.5M SW)	0.2	1.94	2.9
6 (0.5M SW-B)	0.2	2.45	3.2
7 (1M SW)	0.2	2.7	3.6
8 (1M SW-B)	0.2	2.89	3.9

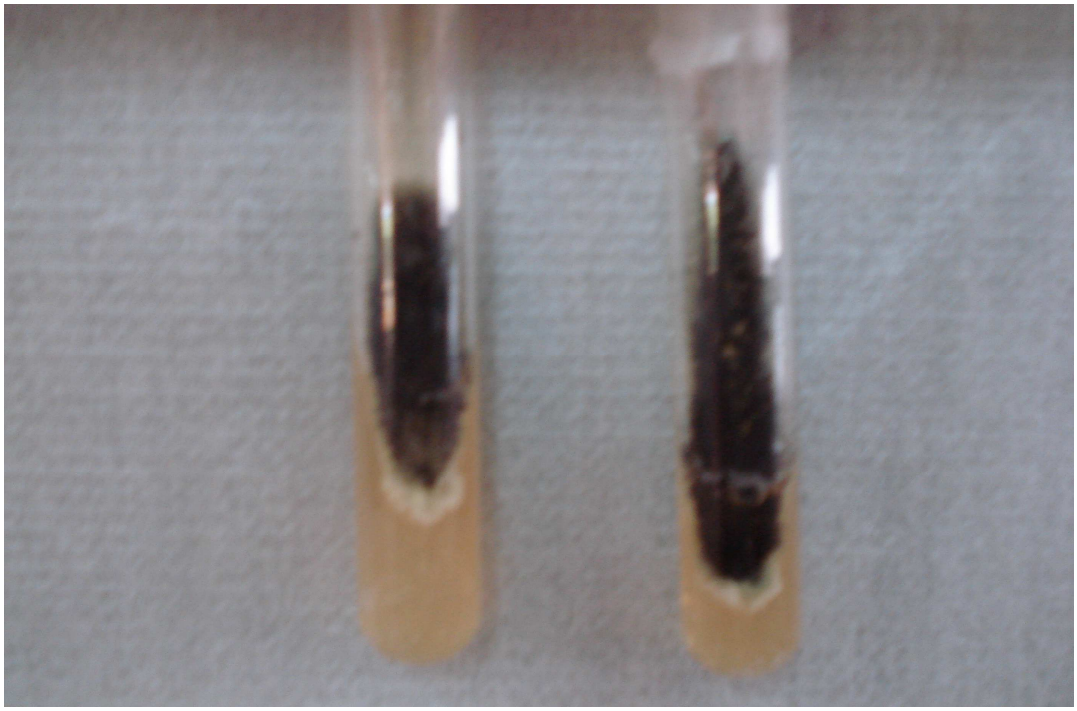
Absorbance values of sugar on day 4 Table: 4

Samples	Volume of sample(ml)	O.D at 650nm	Concentration of sugar(mg/ml)
1 (0.5M SD)	0.2	0.94	2.06
2 (0.5M SD-B)	0.2	0.88	1.95
3 (1M SD)	0.2	0.89	1.96
4 (1M SD-B)	0.2	0.91	2.01
5 (0.5M SW)	0.2	1.78	2.81
6 (0.5M SW-B)	0.2	1.97	2.92
7 (1M SW)	0.2	2.32	3.24
8 (1M SW-B)	0.2	2.48	3.54

***Gracillaria edulis* Fig: 2**



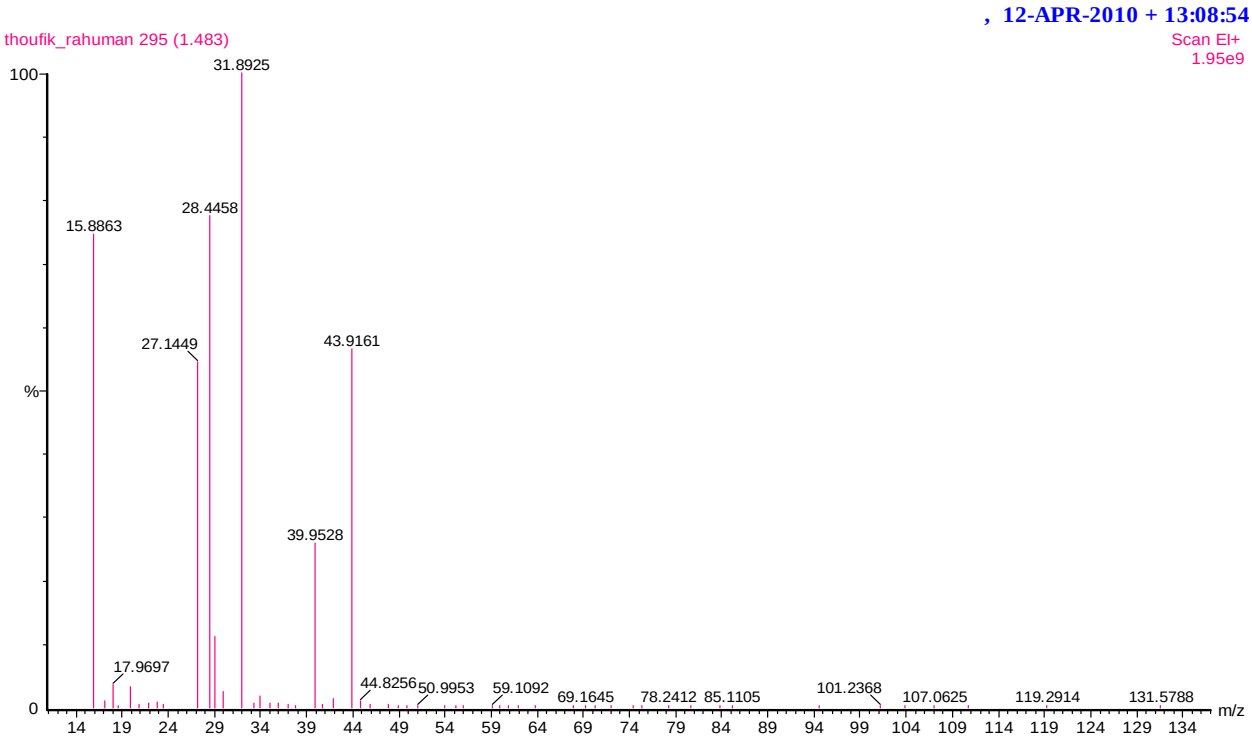
***Aspergillus niger* culture in PDA media Fig: 3**



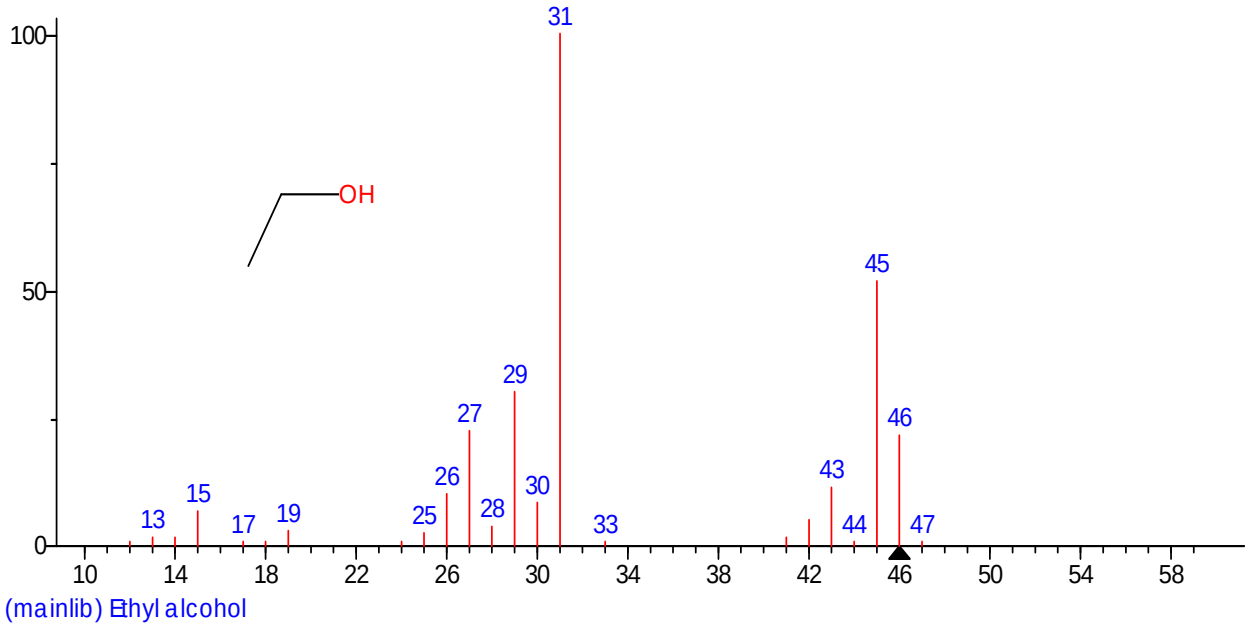
Aspergillus niger **slant preparation Fig: 4**

Micro filtration unit Fig: 5

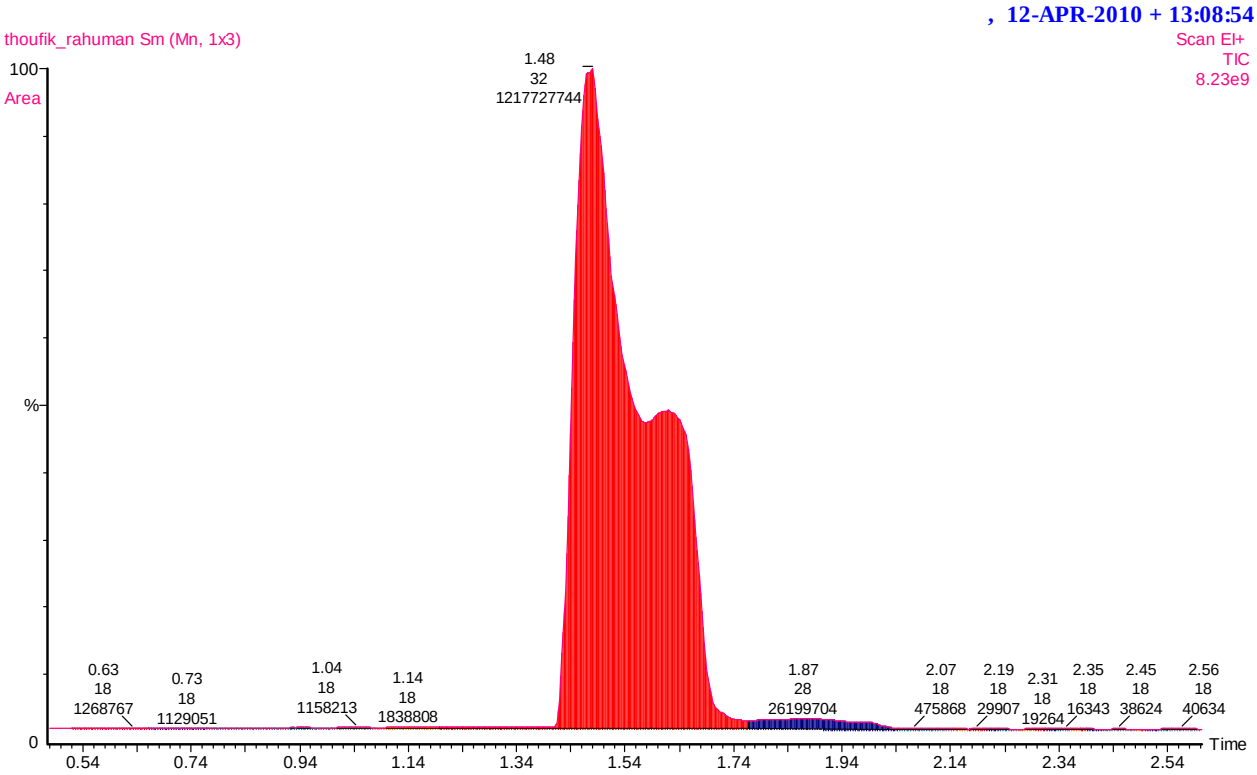
Simple distillation unit Fig: 6



Chromatogram for the seaweeds fermentation extract Fig: 7



Standard Fragmentation pattern for ethanol Fig: 8



Chromatogram showing the retention time for ethanol Fig: 9

CONCLUSION

Saw dust and seaweeds were chosen as the alternative source for production of ethanol because of its availability. Seaweed produce more reducing sugar than the saw dust, so seaweeds were step up to bulk production. The test for sugars confirms the presence of reducing sugar and Anthrone quantitative analysis shows their concentration. GC analysis of seaweed fermentation extract confirms the presence of ethanol. This process was carried out in ten days without agitation; further this will improve by agitation. Domestic production and use of ethanol for fuel can decrease dependence on foreign oil, reduce trade deficits, create jobs in rural areas, reduce air pollution, and reduce global climate change carbon dioxide buildup.

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