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Efficacy of Cellulose Degrading Microbes in Bio-ethanol Production from Estuarine Algae – *Ulva lactuca*

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Abstract
Cellulosic biomass is the good source of fermentable sugars, which can be converted to simpler sugars by simultaneous saccharification by the microorganism for bioethanol production. *Ulva lactuca* an estuarine macro algae was collected from Kirubele region of Aghanashini estuary for bioethanol production and its biomass was subjected to various macromolecular studies. Bacteria were isolated from the gut region of crab *Grapsus albolineatus* and were identified as gram –ve cocci and gram –ve bacilli and the fungal species were isolated from organic decomposition and moist soil and were identified as *Aspergillus* sp., *Trichoderma* sp., and *Rhizopus* sp. The biomass estimation reveals of 26.71% total carbohydrates and 7.64% cellulose, which was converted to simple sugar molecules through successive scarification, later monosaccharide's were converted to bio-ethanol through fermentation using *Saccharomyces cereviceae* which yielded around 16.69g/l of ethanol, whereas combination of bacteria with yeast yielded around 18.09 g/l of ethanol, combination of fugal strains and yeast yielded around 18.22- 24.98g/l of ethanol. The maximum yield of 24.98g/l with *Aspergillus* sp. followed by 19.55g/l with Trichoderma sp. Hence the results shows that fungal species are the best cellulosic degrading microbes for the estuarine seaweeds with dilute acid pre-treatment.

Keywords: *Ulva lactuca*, Cellulose Degrading, Saccharification, Bioethanol

Introduction

There has been ever-increasing demand for energy since Industrial Revolution that has been met mainly by petroleum derived fuel (pdf) as coal, crude oil and natural gas. About 86 % of 12171.4 Mtoe (million tons of oil equivalents) of the total primary energy consumption was derived from pdf. With rampant growth of low and medium-income economies and rapid increase in the world population the coal, gas and crude oil consumption are expected to increase by 26% over the next 20 years (Khandekar et al., 2005). Biomass can be the best alternative source for the production of fuel and to meet the needs of the present and future generation. Fuels derived from biomass feed stocks popularly known as biofuel have been reported to be an attractive and excellent alternative to conventional pdf as these are neutral and renewable. Biofuel is being considered as a potential liquid fuel due to limited amount of natural resources (Masami et al., 2008). These are solid, liquid, or gaseous derivatives from the biomass or the biological feedstock as crops and/or crop residues. Liquid biofuels have the potential to be used as an alternative transportation fuel and are classified as bio-ethanol and biodiesel. Bio-ethanol is used as an alternative for gasoline, that serves as an excellent alternative to traditional fossil fuel-derived energy sources, as these can be produced from abundant supplies of renewable biomass (Na Wei et al.,2013).Macro-algae have wider industrial applications from conventional food, feed and hydrocolloids to recently explored sources of fertilizer, bioactive compounds, and energy molecules (McHugh 2003). The bio-based fuels are considered carbon neutral and eventually can mitigate the negative impact on the environment arising due to excessive use of pdf. Seaweeds, benthic photoautotrophic organisms, consume a large amount of CO_2 during photosynthesis and act as a natural CO_2 sink (Chung et al., 2013). The photosynthetic capacity of the aquatic biomass (~6%–8%) is three to four times higher than terrestrial biomass (~1.8%–2.2%) signifying higher CO2 fixation and biomass production. Muraoka in 2004 reported that ~1000 tons of carbon can be sequestered by aquatic plants by the virtue of photosynthesis. A higher share of seaweed productivity over other aquatic plants thereby reflects their potential in CO2sequestration. In another study (Kaladharan et al., 2009), the seaweed biomass present all along the Indian coast was shown to utilize 9052 tons of CO2 $day^{-1}$by photosynthesis against the emission of 365 tons of CO2 day^{-1}during respiration, indicating a net carbon credit of 8687 tons day–1 Cultivated seaweed biomass removes an astounding quantity of 0.7 million tons of carbon each year (Turan and Neori 2010). Therefore, seaweed biomass is considered as a potential bio-energy crop with the advantages in reducing the CO2 debt and other benefits. Marine algae have a number of advantages over other biomass resources.They do not compete with food resources; also, given their limited lignin and cellulose contents compared with starch-based or lignocellulosic materials, their pre-treatment and saccharification is simple and easy.

Green-algae such as Ulva *spp., Enteromorpha spp*., and *Caulerpa spp.* Are composed of starch, cellulose, Ulvan and Mannan. Unlike the two other macro-algae species, it has a high cellulose content in the cell wall. Seaweed species with higher cellulose content together with higher growth rate is of great importance for sustainable bio-ethanol production. In this context green seaweed Ulva lactuca was selected for the assessing its credibility as a potential feedstock for bio-ethanol production based on its abundance and year round availability. Moreover, it is very much comparable to land plants with almost similar amounts of photosynthetic-pigment carotene and xanthophyll's (Suganya et al., 2013).Although there have been many studies reported in the literature on the production bio-ethanol, a majority of these studies are focused on raw seaweed as feedstock, that mainly involves physicochemical treatment, which is commonly used for the hydrolysis of ligno-cellulosic biomass. The production of ethanol from biomass involves saccharification of sugars, fermentation and distillation. Saccharification, involves breaking down complex polymers as cellulose into glucose that can be accomplished by two main methods i.e. enzyme hydrolysis and acid hydrolysis. Factors that affect hydrolysis include concentration of acid or enzyme, physical properties of the starting material, type of pre-treatment employed and the hydrolysis environment. Following hydrolysis of cellulose to glucose, fermentation of the glucose to ethanol can be accomplished with the help of bacteria or yeast using proven technologies (Cheung, 1995).Enzymatic based cellulosic ethanol production technology was selected as a key area for biomass technology development in the 1980's supported by the US Department of Energy (DOE). Post energy crisis in 1970's this method was used to scale up bioethanol production. The biological conversion of cellulosic biomass to fuel offers advantages as higher yields, higher selectivity, lower energy costs and milder operating conditions than chemical processes which were judged as high risk for industry to pursue at that time. However, recent application based tools in biotechnology and microbiology offers promising and significant advances that could dramatically reduce the cost and make ethanol productivity efficient (Bin Yang et al., 2011).

Kim et al.,(2001) reported production of 7.0 to 9.8 g/L bioethanol by from raw macroalgae (Laminaria japonica) via dilute-acid pre-treatment followed by simultaneous enzymatic saccharification and fermentation with *Saccharomyces cerevisae.* The low bioethanol yield obtained was because *S. cerevisae* could only consume glucose but not Mannitol, which was found to be 81% of the total sugars in the hydro lysates. Yeast, under anaerobic conditions, metabolizes glucose to ethanol primarily by way of the Embden–Meyerhof pathway (EMP Pathway).The overall net reaction involves the production of 2 mole of ethanol per mole of glucose, or 510g of ethanol per kg of glucose, giving a theoretical yield of 51%. However, their saccharification and fermentation are less commonly studied. Thus, special efforts are required to understand these processes with a view to identify the potential of such macro algae for bioethanol production(Khambhaty et al., 2012). reported various microorganism which are able to degrade cellulose, bacteria like *Trichonympha, Clostridium, Actinomycetes, Bacteroides succinogenes, Butyrivibrio fibrisolvens, Ruminococcus albus, and Methanobrevibacter ruminantium.* And fungal species like *Chaetomium*, *Fusarium*, *Myrothecium*, *Trichoderma*, *Penicillium*, and *Aspergillus* were also reported as cellulose degrading organisms. Cellulose degrading microbial taxa has significant for nutrition, degradation, biotechnology and carbon cycle metabolism, physiology and functional enzyme systems of cellulose degrading bacterial and fungal sp. that are responsible for the largest flow of carbon in the biosphere.

Cellulase enzyme is best when extracted from the gut portion of the organism which has the capability to digest the cellulose source as their feed. Gupta et al., (2011) reported the isolation of gut bacteria from insects like termites, bookworm, snails, caterpillars which was found to have a syntropic symbiotic micro flora for the degradation of cellulose. Lee et al., (2001) reported that cellulase are the inducible bio-active compounds produced by microorganisms during their growth on cellulosic matters, they were used in enzymatic hydrolysis of cellulosic substances. Although a large number of microorganisms are capable of degrading cellulose only a few of them produces significant quantities of cell-free bioactive compounds capable of completely hydrolysing crystalline cellulose in-vitro.

In consonance with the above raised issues and concerns, the present study attempts to produce bio-ethanol from marine seaweeds with the help of naturally available microorganism.

Materials and methods

Seaweeds sampling:

Seaweed Ulva lactuca, was collected from two sites Belekan located to the north of Aghanashini estuary and Kirubele located to the South (Lat 14.391 ° to 14.585° N; Long 74.304 ° to 74.516° E)(Fig-1) Protocol followed in the sample collection and bioethanol prospects analyse. Samples were thoroughly rinsed with fresh water to remove salt and foreign materials such as epiphytes, shells sand, etc. Fig-2 depicts Ulva in natural habitat. Treated seaweeds were shade dried until they had constant weight. After being ground into fine powder sample were sieved to obtain uniform sized particles and sealed in zip lock covers for further analysis. Samples at Kirubele region were adequate (biomass, etc.)

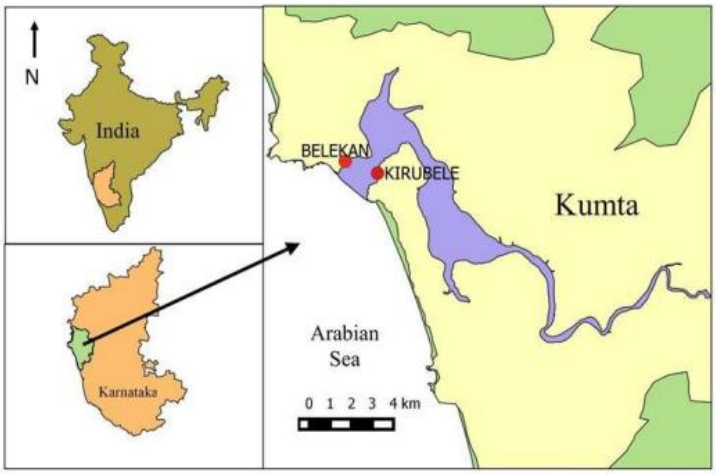

Figure 1: Sampling locations - Belekan and Kirubele

Figure 2: *Ulva lactuca* in Aghanashini estuary - natural habitat

***Bacteria Isolation*:**

The bacteria was isolated from the gut portion of brachyuran crabs(Grapsus albolineatus), which were also collected from the sampling locations (where seaweeds were collected). Gut region of these samples was dissected and macerated using phosphate buffered saline solution. Serial dilution was performed from $10^{-1}$to $10^{-7}$and plated on the Carboxy Methyl Cellulose agar medium for the isolation of cellulose degrading microorganisms. Continuous streaking was performed to obtain the pure and isolated colonies (Aneja, 2003). Biochemical test were performed using the IMViC kit (number – KB001) which was obtained from the Hi-Media (kit experiment). The pure cultures of cellulose degrading bacteria (Aneja, 2003) was isolated through catalase, oxidase and Congo red inhibition tests along with the kit experiments.

***Fungal Isolation*:**

Fungal isolates were obtained from the 2 types of soil samples – (i) soil which was used for decomposition of organic matter, (ii) normal moist soil. One of the soil sample was taken and diluted serially from $10^{-1}$to 10^{-6}, which was plated on the potato dextrose agar. The plates were incubated in room temperature for about 7 days for the proper growth, followed with continuous streaking for isolation of pure culture. These fungal species were identified based on the spore structure and colony characteristics as per the standard protocol (Aneja, 2003).

***Biochemical composition of Seaweed*:**

Estimation of Total Carbohydrate (Dubois et al., 1956) Estimation of reducing sugars by DNS method (Miller, 1972) Estimation of Cellulose (Updegroff, 1969) Estimation of protein (Lowry's method) Estimation of Lipid (Folch et al.1957).

***Bioethanol production from Seaweed*:**

a. *chemical Pre-treatment*: Sample was crushed into fine particles using mortar and subjected to acid pre-treatment (dilute 2.5N HCl). 100 mg of the sample was taken in a test tube and 5ml of the diluted acid was added, covered it with foil paper and kept in boiling water bath for 3 hrs. After hydrolysis, sample was centrifuged at 5000 rpm for 10min and supernatant was collected and made up to 100 ml for further experiments.

b. *Culturing of the organism for enzymatic treatment*: The sugars extracted from the seaweeds was taken in a sterilized conical flasks. Microorganisms were isolated, inoculated and incubated (bacteria at 37°C for

24-48 hours; fungi at room temperature for 5-7 days). After the complete growth of the organism the fungal mat was filtered and only the seaweed extract was taken, in bacterial culture it was centrifuged and the pellet was discarded and the supernatant was collected. These extract was supplemented with 3 loop-full of yeast colonies which was obtained on the potato dextrose agar medium and was fermented for 3 days in a dark chamber with regular agitation.

c. *Fermentation*: The sugars which were extracted from the seaweed sample was subjected to fermentation in an airtight container with an outlet (for the release of CO2). This process is carried out for 3 days to ensure maximum reducing sugar is converted to ethanol by yeast.

d. *Distillation of the extract*: The extract after fermentation was subjected to distillation at boiling point (78°C for ethanol). The distilled sample was taken to estimate the amount of ethanol by potassium di-chromate method through a standard graph.

Results and discussion

***Chemical composition of seaweed*:**

Figure 3 represents the average composition (based on triplicate samples) of macro molecules such as total carbohydrate, reducing sugar, cellulose, protein and lipid in Ulva. The total carbohydrate was 26.7 % as per Phenol- sulphuric acid (PSA) method, which was comparable to the earlier report of 10.63 % and 28.58 % (Anantharaman et al., 2013) and lower compared to the report of ~35.27% (Chakrabarty and Santra, 2008).The reducing sugar with 80 % ethanol treatment that is followed as a standard procedure for estimation of RS was 5.09 % whereas the dilute acid pre-treated sample yielded 6.09 % that is attributed to better cleavage of bonds at a lower H^+ ion concentration. The above results show dilute acid pre-treated of the algal sample to be efficient that has resulted in comparatively higher amount of reducing sugar.The amount of cellulose in the Ulva species was 3.2 % and the dilute acid pre-treated sample yielded 7.64 % of cellulose. The results indicate that there is almost a two fold increase in cellulose extraction with the pre-treatment (dilute acid), due to better hydrolysis and degradation of complex polymer into simpler oligomers. The estimation of protein through Bi-uret technique indicate the protein content of 15.61% in Ulva samples. This is lower compared to the earlier reports of 22.2% (Mairh, 1991), which could be due to prevailing nutrient status, physico-chemical environment, etc. (Dave and Parekh, 1975).The lipid estimation show 3.66% of lipid, which is comparable to 3.15% -5.30% reported earlier in various green marine algae (Parthiban, et al., 2013).

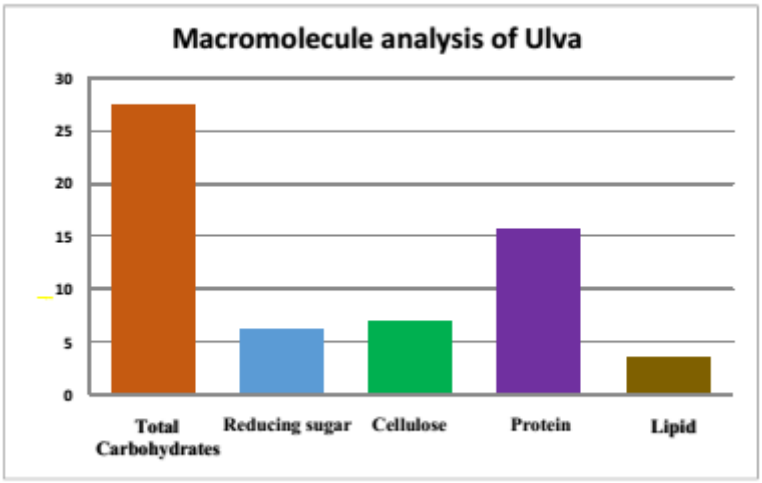

Figure-3: Biochemical composition of *Ulva* sp

***characterization of the bacterial isolates*:**

In this study, bacterial species were isolated from the gut portion of Grapsusalbo lineatus toexplore the potential of natural isolates for bio-ethanol conversion. Serial dilution was performed to get isolated colonies on the CMC agar medium. After incubation of about 37 ºC the isolates were seen in$10^{-3}$and 10^{-4}dilution. Pure cultures were obtained by streaking method (figure-4). The morphological characteristics of the bacteria is provided in table 1, based on their shape, margin, elevation, size, texture, appearance, colour, optical property, and gram's reaction. Bacteria -1 was found to be gram –ve cocci (Figure-5) and bacteria-2 was found to be gram –ve bacilli (Figure-6).The bacterial isolates were characterized using biochemical result of both bacteria 1and 2 (table-2). The cellulose degrading test (Congo red inhibition test) shows clear zone (Figure-7).

Table-1: Colony characterization of the bacterial isolates.

Morphology	Shape	Margin	Elevation	Size	Texture	Appearance	Color	Optical property	Gram's reaction
Bacteria 1	Rhizoid	Lobate	Flat	Moderate	Smooth	Glistening (shining)	Cream	Opaque	Gram -ve cocci
Bacteria 2	Irregular	Lobate	Flat	Small	Smooth	Glistening (shining)	Cream	Opaque	Gram -ve Bacilli

Figure-4 Pure culture of bacteria 1 and bacteria 2 on nutrient agar (NA) plates

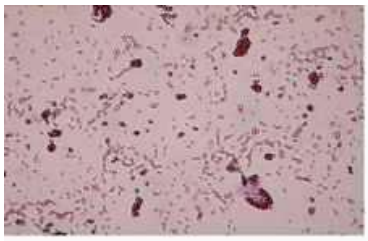

Figure-5, Bacteria-1

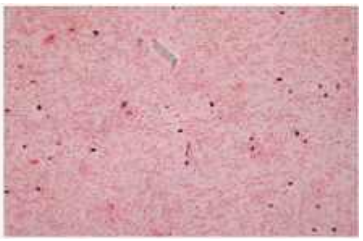

Figure-6, Bacteria-2

Table-2 Biochemical characterization of bacteria.

Sl.no	Bio-Chemical Tests	Bacteria 1	Bacteria 2
1	Indole	-	-
2	Methyl Red	+	-
3	Voges–Proskauer	-	-
4	Citrate Utilization	-	+
	Carbohydrate Fermentation		
5	Glucose utilization	+	+
6	Adonitol utilization	-	-
7	Arabinose utilization	V	V
8	Lactose utilization	-	-
9	Sorbitol utilization	-	-
10	Mannitol utilization	V	-
11	Rhammnose utilization	-	-
12	Sucrose utilization	+	+

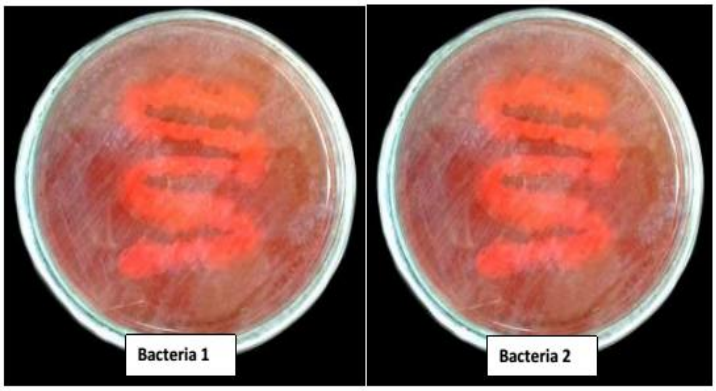

Figure-7 Congo red inhibition test for bacterial samples

***Characterization of the fungal isolates*:**

The preliminary test of fungal analysis was carried out by characterizing the fungal colony obtained (listed in table 3). The fungal isolates were pure cultured on potato dextrose agar substituted with 1% cellulose using the cork – borer method. Figure8 represents the culture plates of fungi and figure 9 represents the micrographs of the fungal spore.

Table-3 Colony characterization of fungi

Morphology	Form	Elevation	Margin	Color of the colony above	Color of the colony behind	Identification by wet mount
Fungi 1	Irregular	Raised	Curled	White to black	Creamish to yellow	*Aspergillus*
Fungi 2	Circular	Convex	Lobate	Green	Brown	*Trichoderma*
Fungi 3	Filamentous	Nmbonate	Undulate	White	White to cream	*Rhizopus*

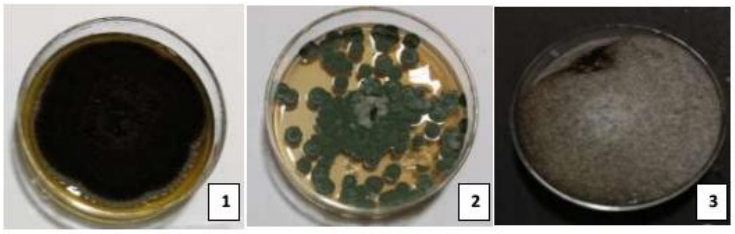

Figure-8 pure culture plates of fungi. 1 *Aspergillus sp, 2 Trichoderma,* 3 *Rhizopus*

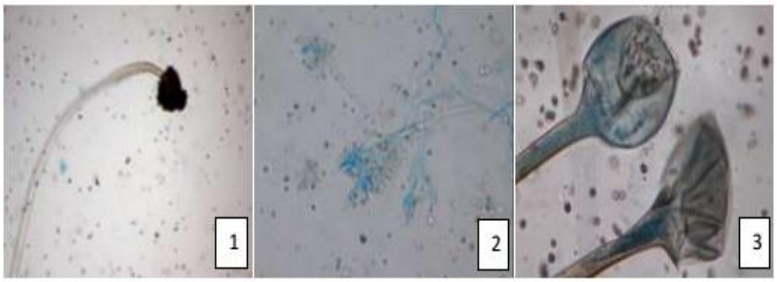

Figure-9 Micrographs of fungal spores. 1. *Aspergillus,* 2.*Trichoderma,* 3.*Rhizopus*

***Fermentation*:**

The various inoculates along with their ethanol yield is provided in table 4 Moreover, the set-up for the development of inoculums is provide in figures 10 and 11 respectively.

Table-4 Fermentation with various microbes (bacteria, fungi, yeast)

Substrate	Combination of Organisms	Incubation time (days)	Temperature (°C)	Ethanol yield (g/L)
Ulva	Yeast	3	28-30	16.69
Ulva	Bacteria 1	1	37	
	Yeast	3	28-30	18.09
Ulva	Bacteria 2	1	37	
	Yeast	3	28-30	17.71
Ulva	*Trichoderma*	3-4	28-30	
	Yeast	3	28-30	19.55
Ulva	*Aspergillus*	3-4	28-30	
	Yeast	3	28-30	24.98
Ulva	*Rhizopus*	3-4	28-30	
	Yeast	3	28-30	18.22

Fig 10

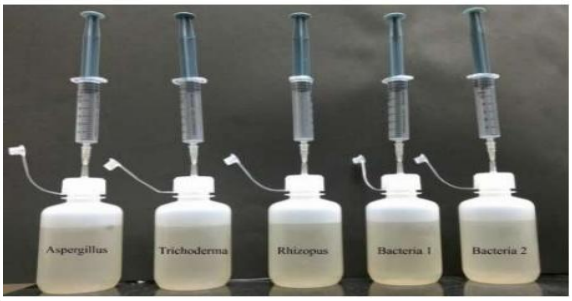

Fig 11

Figure-10 Fermentation of the seaweed sugar with *Saccharomyces cerevisae*

Figure-11 Fermentation set up for the production of ethanol using the isolated organism followed by *Saccharomyces cerevisae*.

***Yield of ethanol*:**

The combination of acid hydrolysed sample with yeast yielded 16.69 g/L of ethanol whereas with bacteria 1 and 2 yielded 18.09 g/L and 17.71 g/L of bio-ethanol respectively. Contrary to the bacterial and yeast samples, the fungal isolates *Aspergillus* has achieved the maximum yield of ethanol (24.98 g/L) followed by *Trichoderma* (19.55 g/L) and *Rhizopus* (18.22 g/L) (Figure 12). The batch fermentation studies conducted with the bacterial, fungal and yeast isolates which showed relatively high bio-ethanol concentration in Aspergillus species and a low bio-ethanol concentration in yeast sp. The bacterial isolates showed a marginal difference in bio-ethanol concentration compared to the yeast sp. High bio-ethanol production in fungal sp. (*Aspergillus* sp. followed by *Trichoderma* sp.) Is due to the presence of abundant extra-cellular cellulolytic enzymes and abilities to degrade cellulose rapidly.

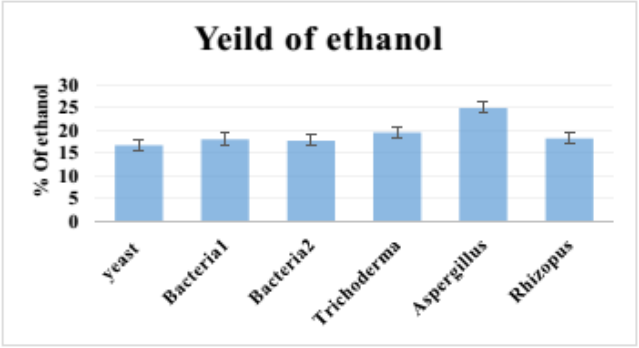

Figure-12 Yield of ethanol obtained from following organisms.

Conclusion

Biofuel is emerging as viable alternative to fossil oil resources. The current study focuses on the bioethanol prospects of estuarine macro algae – Ulva lactuca. Samples were collected from Kirubele region of Aghanashini estuary and the biomass was subjected to various macromolecular studies involving the

estimation of total carbohydrates, reducing sugars, cellulose, protein and lipids. The estimation reveals of 26.71% total carbohydrates and 7.64% cellulose in Ulva. Samples when subjected to dilute acid pre-treatment yielded 6.09% compared to 5.09% with 80% ethanol pre-treatment. This substrate of complex sugar units was converted to simple sugar molecules through successive saccharification. Finally, monosaccharides were converted to bio-ethanol through fermentation using microbes. Yeast is a ubiquitous organism present in all the forms which have been used from time immemorial for the conversion of available free sugars to ethanol. Naturally available baker's yeast was used for the fermentation of sugars which yielded around 16.69g/l of ethanol whereas, combination of bacteria with yeast yielded around 18.09 g/l of ethanol and combination of various fugal strains and yeast yielded around 18.22- 24.98g/l of ethanol and the maximum yield of 24.98g/l with *Aspergillus* sp. Followed by 19.55g/l with *Trichoderma* sp. The fungal species *Aspergillus* sp. And *Trichoderma* sp. degrades cellulose of the estuarine seaweeds with dilute acid pre-treatment better than other microbes (yeast, bacteria).

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