Identification and Characterization of Kenyan Marine Microalgae Strains towards Bio-fuel Production

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Abstract

The rising global energy demand in both developed and developing countries is heavily straining the limited energy reservoir. The increased use of fossil fuels result in large Green House Gases (GHS) emissions, that are usually considered the main cause of global warming. Microalgae are projected to be the source for the third generation biofuel. Microalgae are one of the most abundant organisms present on the planet and seem to have received more attention due to their lipid reserving capacity. The purpose of this study was to seek, identify and characterize microalgae from the Indian Ocean and its environs which included; polluted sites; tenable for biofuel production. Six stations (Mikindani, Moroto, Fort Jesus, Kenya Meat Commission, Coast General Hospital, Technical University of Mombasa) were selected based on their; physic-chemical parameters. Sixty four (64) strains of microalgae were obtained in five stations through morphological techniques. On further isolation, 19 strains were independently secluded and their *in vitro* cultures established. The microalgae were cultured in the laboratory conditions in three media namely Walne, F/2 and TAP. Algal growth parameters i.e., pH, optical density (OD), dry and chlorophyll-a (Ch-a), were measured at zero-time and at the end of the experimental period. The results revealed that media preference for overall microalgae growth was 38% in F/2 media, 31% in Walne and 30% in TAP media. The *Oscillatoria* genera from all the stations were found to exhibit 100% growth in all the media. From the results, the suitable candidate strains recommended for mass cultivation for mass biodiesel production exploitation include *Oscillatoria* genera because of their fast growth and ability to withstand varying physicochemical conditions of *in vitro* culture.

Keywords—Biodiesel, Biofuel, Microalgae, Oscillatoria.

INTRODUCTION

Biofuel production from renewable sources is widely considered a sustainable energy alternative source compared to petroleum. Microalgae provide a viable means of environmental and economic sustainability (Dragone et al. 2010). Microalgae currently hold promise as an ideal third generation biofuel feedstock. This is supported by their rapid growth rate, carbon dioxide fixation ability and high lipid production capacity. They also do not compete with food crops for arable land and water resources for irrigation. They can potentially be produced on non-arable land (Dragone et al., 2010). Some microalgae strains are capable of generating 70% weight by weight (w/w) lipids in their biomass (Alcain, Anna, & Kushiga, 2010).However; they may yield significant lipid content under stress conditions. Some of the stress causing factors include; nutrients, light, salinity among others. Utilization of microalgae for biofuel production is meritorious over higher plants since they synthesize and accumulate large quantities of neutral lipids; they are also capable of all year round production yielding higher oil quantities (Abubakar & Mutie, 2012).Further, Chisti (2007) advances that microalgae need less water than higher crops, hence reducing the load on freshwater sources.

Microalgae are reported to exhibit faster growth rates besides expressing the ability to grow under saline conditions that are unsuitable for agriculture (Alcain, Anna, & Kushiga, 2010).Adoption of biodiesel will support environmental conservation, agricultural and economic developmental goals. However, a successful and economically viable algal based oil industry depends on the selection of appropriate algal strain. Species of microalgae should therefore be bio prospected to determine suitability for oil production (Araujo, Matos, Gonçalves, Fernandes, & Farias, 2011). In the last decade, Deoxyribonucleic Acid (DNA) sequencing and genomics have brought substantial changes to microalgae taxonomy. Both the subunit ribosomal DNA and its genes have been used in studies for species identification because they include the highly conserved regions at the species level.

MATERIALS AND METHODS

Description of Study Site

Sampling was done three times at the Tudor Creek. Tudor Creek is located along Latitude of 4.0000°, Longitude 39.6500°. It is 15.5km away from Mombasa town to the North. The study site was accessed by a fibre glass boat at the Tudor Water sports entrance. Sampling was done in triplicates; one set was preserved with 5% lugol's iodine for quantitative diversity studies. The second set was isolated and cultured, while the third set was incubated at 28^{0}C for fourteen days. Temperature, pH, total suspended solids and salinity measurements for all the sampled stations were recorded.

Sampling of microalgal strains

Water samples were collected in triplicate at the sampling stations for analysis. Surface water samples were collected using a bucket. 40 litres of water was passed through 20µm mesh-size

plankton net for concentration to 50ml. The resultant concentrated plankton was transferred to sample bottles labeled with date and sampling station, and preserved in 5% Lugol's solution. Qualitative samples were stored in a cooler box after collection and transported to the laboratory for further analysis.

Water Quality Parameter

Surface water quality parameters were measured *in situ* at each station; pH was measured using an electronic pH probe, temperature was measured using the YSI Model 550A, salinity was measured using a hand-held refractometer.

Quantitative Sample Analysis

In the Laboratory, 1ml aliquots of samples preserved in Lugol's iodine solution were mounted on slides and observed under an inverted microscope and the counts of all seen phytoplankton recorded. The algal species were identified using the identification manual of marine microalgae by Hasle & Syvertsen (1977).

Isolation and Purification

The algal samples for qualitative analysis were subjected to purification by serial dilution, addition of antibiotics, and addition of enrichment media followed by culturing them in TAP, F/2 and Walne media for microalgae and incubated at 25 ± 1°C under 1.2+0.2 k lux intensity with 16:8 hours light photoperiod. The purity of the culture was ensured by repeated sub culturing in fresh media and regular observation under the inverted microscope.

Identification of Microalgal Strains

The purified monoalgal samples were observed under the inverted microscope and the morphological properties of the isolates identified based on the manuals.

Culture Maintenance

Unialgal cultures of the microalgae strains were maintained in the F/2 and Walne culture media. A drop of the respective culture was inoculated aseptically using a sterile micropipette into 50 ml of the medium in sterile 50 ml conical flasks. The cultures were incubated in an algal growth room with constant illumination at 110 µmol.m-2/s at 25°C. This procedure was performed on weekly a basis.

Isolation and Purification of Microalgal Cultures

This was done through isolation of the microalgae and culturing them on solid and the three liquid media namely; TAP, Walne and F/2

Measurement of Growth Rate

The growth rate of algae was measured by optical density at 660nm, 680nm, 760nm and 780nm for 4weeks. Daily measurements were also taken for 7 days.

Identification of Lipid Producing Microalgae Strains

Nile red staining was conducted to detect intracellular lipid droplets. Microalgae cells (0.5 ml) were collected by centrifugation at 1,500 rpm for 10 minutes and washed with physiological saline solution (0.5 ml) several times. Thereafter, the collected cells were resuspended in the same solution (0.5 ml). Nile red solution (0.1 mg/ml in acetone) was added to cell suspensions (1:100 v/v) and incubated for 10mins. After washing once, stained microalgae cells were observed by Fluorescent microscopy. Microscopic photographs were taken with a Nikon E600microscope

Determination of Oil Content Microalgae

One ml of growing algae in F/2 media was collected in 3 replicates in 1.5ml tubes and centrifuged at 13000rpm for 5 minutes. The pellets were frozen in liquid nitrogen and stored at -80^{0}C. Thereafter the pellets were measured to determine the weight. The lipids were extracted as follows; in the frozen pellets, 200µl of a mixture of chloroform: isopropanol (1:1) was added and vortexed vigorously for 3 minutes. They were then centrifuged using an electronic centrifuge(model 80-1) at 13,000rpm for 5minutes .The supernatant was transferred to a new tube, and then the pellets were re-extracted with 500µl of hexane and; vortexed vigorously for 3 minutes. The samples will then be centrifuged and the supernatants combined. The supernatants were dried by an evaporator and the amount of lipids measured gravimetrically .The Bligh and Dyer method of extraction using chloroform and methanol was also used (Bligh & Dyer, 1959).

RESULTS AND DISCUSSIONS

Physico-chemical Characteristics

The mean surface water temperature was 27.75^{0}C with a standard deviation of 0.5 and standard error of 0.25. The highest average temperature for the Creek was 31^0 C while the lowest average temperature was 21^{0}C (Table 1). Analysis of variance reveals that there was no statistically significant variation in Water and Temperature between sampling stations and between the three months of sampling at (P>0.05) For the pH was observed during the study period, with the highest average value of 8 and a lowest of 7.8. The mean ± SD for pH is 7.25 ± 0.23 (Table 1). This observed variation was statistically significant. The surface Water Temperature ranged from 27^{0}C to 28^0 C. The highest temperature was recorded at Coast General Hospital station and Technical University while the lowest temperature was recorded at Mkomani station. The pH was observed during the study period, with the highest average value of 8.0 and a lowest of 7.8. The salinity ranged from 34 - 36.3 PSU. With the highest concentration at Mkomani station. Total Suspended solids ranged from 0.037-0.162g/l with the highest concentration being recorded at Fort Jesus.

Table 1: Physico-chemical parameters of the sampled stations

Parameter (Units)	TSS (g/l)	Salinity (psu)	Temp (°C)	Ph
Mean	0.07	35	27.75	7.85
Standard Error	0.03	0.48	0.25	0.05
Median	0.05	34.85	28	7.8
Standard Deviation	0.06	0.96	0.5	0.1
Variance	0.003	0.92	0.25	0.01
Range	0.125	2.3	1	0.2
Maximum	0.162	36.3	28	8
Minimum	0.037	34	27	7.8

Quantitative Sample Analysis

This was determined by observing the samples preserved in lugol's iodine under the Leica Inverted microscope.

Species Present

The species were identified morphologically using conventional means by use of the phytoplankton guides. The species observed are shown in table 2. The species present in all the seven stations were; *Oscillatoria* spp., *Thallasiosira* sp., *Coscinodiscus* sp., and *Nitzschia*. The species present in only one station were; Monidiscus sp, *Coscinodiscus* sp, *Protoperidinium* sp, Dictyliosolenia sp, Bleaklayer sp., Protocentrum sp., Hemidiscus sp, Anabaena sp., *Dactyliosolen fragilissimus*, *Cylindrotheca closterium*, *Gonyaulax spinifera*, Spatulodinium sp., *Nitsia longissima*, *Anacustis nidulans*.

The most abundant phytoplanktons are in the group of Bacilliarophyta (diatoms) which occupied 60% followed by the Cynanophyta (cyanobacteria) which occupied 30% and chlorophyta occupying 10%. Bacillariophyta have been reported by many authors to be dorminat in the phytoplankton composition as it is in the present study (Polat & Aka, 2007). Chlorophyta was the second group after Bacillariophyta in the number of identified species, these result also agree with study on Grand River in Oklahoma by (Pfiester et al.,1980). It is also in agreement with other studies in Iraq (Al-Handal, Al-Assa, & Al-Mukthar, 1989). The maximum occurrence of phytoplankton was in October 2014 and thereafter it decreased. This may be due to available nutrients and other physical and chemical factors which promote growth of phytoplankton. While the minimum total number of phytoplankton species was recorded at Moroto which might be due to domestic discharge and effluents from run-off that empty into the river. This corresponds to the work of (Hassan et al.,2008).The differences in number of taxa and number of individuals between sampling stations for each class of phytoplankton may be due to differences in temperatures and pH. According to Wilhm and Dorris, (1966),species obtained at different pH and temperatures have suggest a relationship between species diversity and pollution status of aquatic system and classified as follows; > 3 = Clean water, 1-3 = moderately-polluted < 1 = Heavily polluted. Water pollution levels could also be accurately identified by analyzing the species abundance, physiological, biological responses and residue contents (Chen et al 2011). However, algae may not be only significant for biomonitoring studies but could also be a useful phytoremediation technology to restore water quality due to their high bioaccumulation ability

Shannon Wiener Diversity Index

Computation of diversity index was also done. Resultant graphs were drawn as Shown in Fig.1. From the results shown;

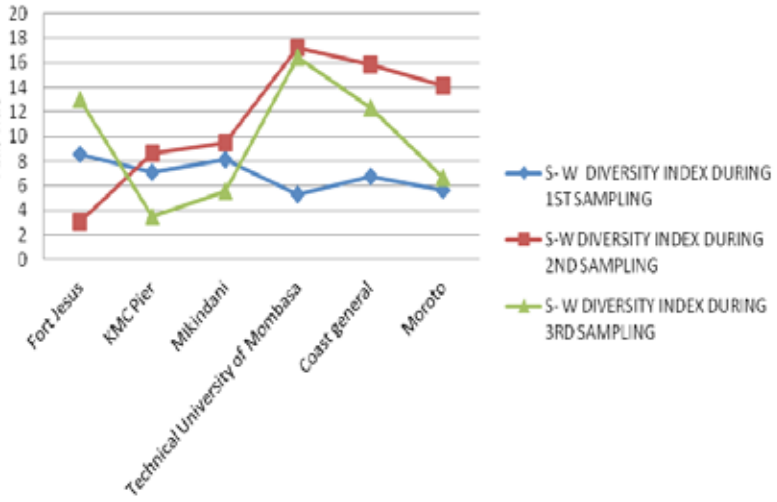

Fig. 1: Comparative diversity indices of the sampled stations

Phytoplankton Abundance and Diversity

Diversity indices reveal that TUM station had the highest index of 16.47. For all the diversity was stable. This indicated a large abundance of phytoplankton in the marine ecosystem sampled.

The highest diversity occurred during the second sampling which indicates very high diversity indices from the graphs shown.

Liquid Media Preferences

Species occurrence was highly dependent on the media, the media used were TAP, Walne and F/2. Plots of media and the species obtained are shown in Fig.2

Solid Media Preferences

The three media namely TAP; Walne and F/2 were prepared and solidified with Agar. Samples from all the four stations were inoculated. Incubation was done at 27°C for two weeks with 12:12 light /dark .

Streak Plate Method

Two weeks after initial incubation, most of the plates showed colonial formations. Some plates also showed strains growing beneath the surface. For subsequent sub culturing rounds, microorganisms showed growth after only seven days in the agar

surface, probably due to acclimatization in the new conditions. Even though single colony picking and subculturing was done with extreme care, many of the new plates still showed mixtures of two organisms. After approximately three subculturing rounds, most plates showed organism uniformit In few cases, however, it was not possible to get rid of a round, colonial, transparent microorganism that appeared in the agar two or three weeks after incubation

Serial Dillution

The following microalgae were isolated and cultured through serial dilution.They were observed under the digital microscope (Motic DM 111) and their photographs taken.

Culture of Microalgae in Liquid Media

The isolated microalgae were then cultured in test tubes and conical flasks of 250 ml with the suitable media being supplied to each of them. Continuous and vigorous aeration was provided to culture which keeps the culture in suspension. In addition continuous aeration is also helpful for uniform distribution of nutrients.

From the graph it shows tha F/2 and Walne Media are the most prefered media for growth of most microalga

Total Isolates Obtained

At the end of the isolation process, there were various species obtained. Isolation was done based on stations, and species. The methods used for isolation included; serial dilution, culturing in liquid and solid media. The isolation protocol developed in-house became an efficient method for the microalgae isolation and transfer from the natural environment in to laboratory conditions. The streak plate method for microalgae enrichment, although slow, proved to be an excellent approach for the isolation of green phototrophic microorganisms. With regards to the physical properties of the water samples, they remained fairly constant all throughout the different depths assessed as well as in the different locations.

Temperatures fluctuated between 20.5 and 23.5^{0}C, pH between 6.9 and 8.1, and salinity between 32 and 37.8 psu.

Regardless of the stations the microalgae colour appearance on the solid media included; 15% green, 16% brown, 8% pink, 23% cream. These results corresponds with the study from (Wilhm & Dorris, 1966).Isolation procedures using solid media allowed for the selection of microorganisms with colors besides green; culture attempts of the same in liquid media were utterly unsuccessful. None of the solid or liquid media were prepared with an external carbon source, with the purpose of selecting phototrophic organisms. However, solid media was gellied with agar, which is a polymer of the sugar galactose.

However, certain marine organisms have the capacity of producing agarose and liquefy solid media efficiently (Chen, Sommerfeld, & Hu, 2011).These enzymes are responsible for allowing them to use agar as their primary carbon source and enables their ability to thrive in the ocean .In this case, it might have happened that the organisms isolated, other than the green ones, had this innate ability to metabolize agar and thus developed efficiently in solid media; interestingly, all of them showed growth close to the agar surface.

As for the liquid Media preference, F/2 Media with silicate seemed to be the most preferred because there was a large growth of the species. TAP media was the least preferred probably due to the fact that it forms a cloudy appearance after a short while. These results are consistent with observations made by (Pfiester, Lynch, & Wright, 1980) and many others who noticed the zonation of pigments on marine organisms depending on depth.

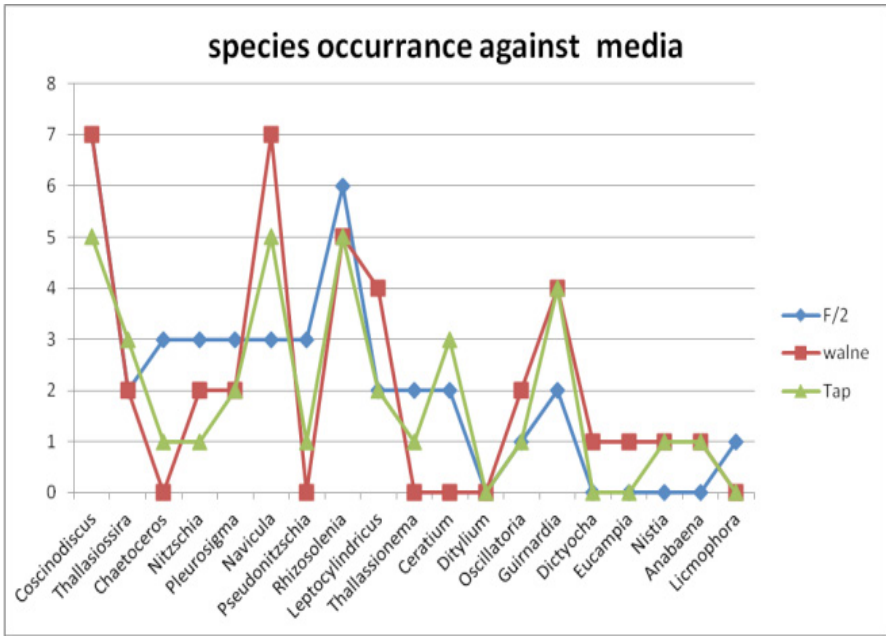

Fig. 2: Line graph showing a graph of media against media

Measurement of Growth

Optical density was taken at intervals of one week. The absorbance readings were taken at three optical densities of; 620nm, 650nm and 750 nm. The results are shown in Fig.3 below.

Total Lipid Content of Selected Isolates using Conventional Extraction Methods.

The results revealed that *Oscllatoria spp*.had the highest percentage of total lipid of 16$\pm$0.5 these results were in harmony with the results of (Christi, 2010)

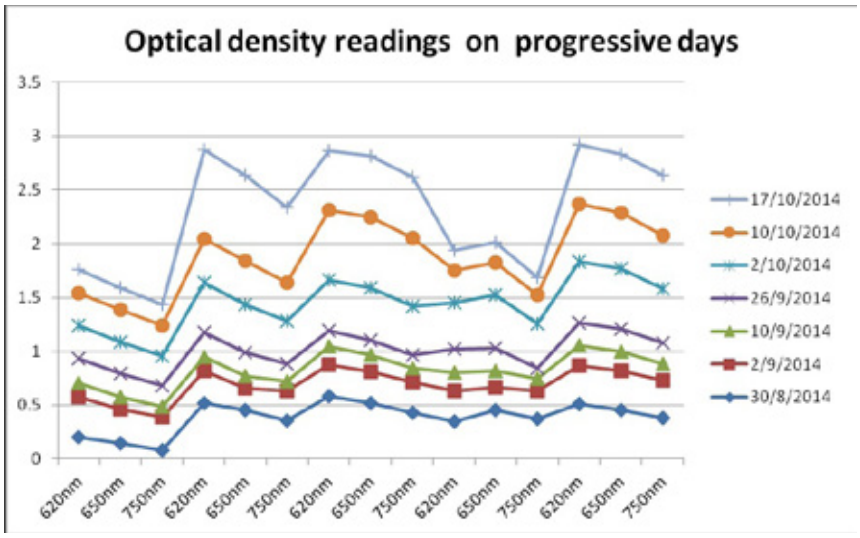

Fig. 3: Graph showing Optical densities on progressive days

who stated that oscillatoria has total lipid content of 16.5%. Two diatoms species were investigated in this study *Nitzschia linearis* and *Navicula cuspidata*. The two species showed relatively low percentage of oil 6.1% and 10.2% for *Nitzschia linearis* and *Navicula cuspidata* respectively. *Nitzschia sp*. was investigated by (Chisti, 2007) and found to have lipid percentage of 45-47%. On the other hand (Sayeda et al., 2014) found that *Nitzschia frustulum* has a total lipid percentage of 13.9%. This percentage near to that presented in this study in table 2

Table 2: Percentages of total oil content in the microalgae studied

Microalgae isolate	Total lipid content %
Oscillatoria sp 1	11± 0.4
Oscillatoria sp.2	16±0.5
Nitzschia lineaus	6 ± 0.1
Navicula cuspulata	10± 0.2

Intracellular lipid droplets of were observed by Nile Red staining under fluorescent microscope with excitation at 450–490-nm and emission at 515-nm (Matsunaga et al., 2009).Neutral lipid or triglycerides appeared as yellow dots, whereas polar lipid and chlorophyll were stained in red colour cells were observed by Nile Red staining under fluorescent microscope with excitation at 450–490-nm and emission at 515-nm.

Nile red staining: Nile red (9-(Diethylamino) -5H benzo [α] phenoxazin- 5-one) staining is specifically used to identify and confirm the intracellular lipid droplets from the biological samples (Greenspan, Mayer, & Fowler, 1985).

The results indicated that not all algal species could be affected by Nile red staining since oil droplets were not clear and the whole cells were stained in red. This was the situation with green isolates, while certain blue green isolates were affected by the dye *where* the yellow stained parts were clear. Referring to *Oscillatoria sp 1* and *Oscillatoria sp 2* the cells showed yellow florescent color under florescent microscope even without adding the dye. So this gives false results when referring to lipid content. Diatoms were stained well with the dye and the oil drops were clear. Since the staining method may not be accurate The florescent method has been applied successfully to the determination of lipids in certain microalgae, but has been unsuccessful in many others, particularly those with thick, rigid cell walls that prevent the penetration of the dye (Held, 2011).Since Nile red method was not accurate in determining lipid content in microalgal cells so lipid content was determined using conventional extraction method using two organic solvents.

CONCLUSION

Results show that physico-chemical parameters play a major role to play in species occurrence. The method of lipid extraction, type of media and level of axenicity of the microalgal species determines the percentage of lipid extracted . *Oscillatoria* species seems to be a hardy species and therefore still survives when subjected to stress. It is therefore recommended for large scale cultivation in photobioreactors for biofuel production. Stress conditions such as nutrient stress results to production of more lipids from the microalgae. Nile red staining is not so accurate therefore should be supplemented by other methods of lipid determnination. The choice of algal strain, the method used for culture and the location of sampling highly determines the amount of lipid produced by microalgae.

RECOMMENDATIONS

The strains used for large-scale algal biofuel production need to be improved through selection and genetic approaches. Breakthroughs and innovations in areas such as increasing the capability of algae to use nutrients efficiently or engineering designs to reduce processing requirements have the potential to greatly improve the energy balance and enhance the overall sustainability of algal biofuels.

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