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Experimental Identifications of Fresh Water Microalgae Species and Investigating the Media and PH Effect on the Productions of Microalgae

Tadele Assefa Aragaw&*[1], Abraham M. Asmare[2]

1- Faculty of Chemical and Food Engineering, Bahir Dar Institute of Technology, Bahir Dar University, Ethiopia
2- Institute of disaster Risk management and Food Security Studies, Bahir Dar University, Ethiopia

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Abstract

Identifying predominant species around marine system is essential to propose a project for harvesting those species which can be used for colored waste treatment, biofuel production, for cosmetics and carbon sequestration. The objective of this study was to investigate the co-culture microalgae species and identifying the predominant fresh water microalgae species. The production of microalgae (mixed culture) in photobioreactor configurations was investigated using two different media formulations in batch operation. The predominant co-cultured freshwater microalgae species Scenedesmus sp., Chlorella sp., Synedra sp. and Achnanthidium sp. were investigated in batch culturing media and Effect of culturing media(BB Medium and BG-11 Medium) for effective algal growth were determined. The Bold basalt medium has somehow high optical density value than the BG-11 medium and the maximum biomass concentration was found 0.608 g/L in the Bolt basalt medium and 0.5624 g/L in BG-11 medium for 15 day cultivation time. The study revealed that amount of time required to adapt the culturing environment is not differed significantly in both growth medium type. Also PH range has an effect on the mass productions of algae. The optimum PH for high productions of mixed culture microalgae were investigated at pH 8.

Keyword: Identification, Microalgae, Mixed culture, media, photobioreactor, phycology

1 Introduction

Microalgae are microscopic photosynthetic microorganisms. They are eukaryotic cell and they contain similar organelles such as chloroplasts, nucleus, etc.

Microalgae are generally more efficient than land based plants in utilizing sun light, CO_2 water and other nutrients rates. They can also grow in a variety of aquatic environments the use of fertilizers and pesticides which results in less waste and pollution [1, 2].

Different approaches can be taken when looking to grow microalgae in large volumes. Mainly outdoor ponds, with light supplied by the sun, and photo-bioreactors, which can be outdoor or indoor and light, can be supplied by electric lights. Species control is better achieved under closed conditions that are very common in laboratory setting. Closed systems provide a better opportunity meet specific demands and in the control and optimization cell growth parameters. In the case of photo-bioreactors, many design considerations need to be made depending on what the end goal is. There are basic design features that should be considered regardless of the configuration; like the materials used for the set-up, source of light, how the algae will circulate through the reactor, how to provide CO_2, and how to control other parameters such as pH, temperature and nutrient concentration in the media [3, 4]. A study on *Chlorella vulgaris* by [5] found that the most adequate growth was under indoor conditions and utilizing inorganic nutrient media.

Algae are most commonly cultivated in batch cultures and harvesting is done before the next batch is started. It is easier to control the environmental conditions when the algae are grown in batches. The algal culture is mixed by shaking or impeller mixing to promote the nutrient and gaseous exchange. Carbon dioxide gas is supplied to the culture based on the pH. Light is provided to the algae cultures by either natural or artificial light sources [2].

Ideal growth conditions for microalgal cultures are strain specific, and the biomass productivity depends upon many factors. These include abiotic factors like temperature, pH, water quality, minerals, carbon dioxide, light cycle and intensity, and biotic factors like cell fragility and cell density. Mechanical factors include mixing, gas bubble size and distribution, and mass transfer; these are of particular concern in photo bioreactors. Algae are grown heterotrophically using organic compounds and autotrophically with CO_2 supply. Culture productivity increases with optimal mineral nutrition. Nitrogen and phosphorus are essential nutrients in all algae growth Medias [6].

The two most important factors that affect algae biomass productivity are light and temperature. The energy

Corresponding author: Tadele Assefa Aragaw: Faculty of Chemical and Food Engineering, Bahir Dar Institute of Technology, Bahir Dar University, Ethiopia; E-mail: taaaad82@gmail.com.

for growing algae is provided by light through photosynthesis. Light energy must be effectively utilized to achieve higher biomass productivity. Temperature influences the rates of all chemical reactions related to algal growth and metabolism [7].

To estimate growth rates, one must have a series of measurements, at different times, that will permit the calculation of the rate of change in biomass concentration. Cell number should be counted, either through a direct method, as through light microscopy with a hemacytometer, or indirectly through biomass concentration (as dry weight) or optical density, as long as this measurements correlate linearly with the number of cells [8] .Under a typical homogenous batch regime (in a closed system).

Identifying predominant species around marine system is essential to propose a project for harvesting those species which can be used for colored waste treatment, biofuel production, for cosmotics and carbon sequestration. The objective of this study was to investigate the co-culture microalgae species and identifying the predominant fresh water co-cultured microalgae species

2 Materials and Methods

2.1 Experimental Setup

250 ml, 500 ml and 1000 ml conical flask photobioreactor were used as batch reactors in temperature incubation chamber and sealed with cap stoppers and glass tubes through which air fed, exhausted and screwed with plastic covers. Continuous Air flow (with 2.0 L/min volumetric air flow rate) from the air pump through polyvinylchloride pipes connected with the glass tubes on the top of photobioreactor. The air feed tube immersed at the bottom of the growth container to allow mixing, to prevent sedimentations of the algae, to ensure that all cell of the populations are equally exposed to light and nutrients, and to improve gas exchange between the culture medium and the air.

Two red, two blue and one cool white fluorescent lamps were employed as the light source for growth microalgae with an average light intensity of 1,450 Lux with 12/12 light-dark photoperiod with negligible external light interference. The temperature incubation chamber was monitored 25±2 ^{0}c to optimize the growth conditions of microalgae. The carbon dioxide feed were used several times per day as a carbon source of the species.

Growth Media and Algae Harvesting: Algal biomass: Water sample was collected from Lake Tana, with different sampling location. All the chemicals used were of analytical grade for the media stock solutions preparation as shown from table 2.1 and 2.2 and stored in the refrigerator at approximately -4ºC until the final media preparation. There are numerous recipes available for the preparation of algal nutrients. The one used depends on nutrient levels in local, ambient water and the algal species cultured. Routine cultivation was carried out at 25± 2°C under incubation chambers and light intensity of 1,300 lux for 12 days. Many fresh water algae grow well with Bold Basal Medium (BBM) in order to obtain stock algal cultures to be used in the experiments and the PH was adjusted (7.5 ±0.5**)** with 0.1N sodium hydroxide and hydrochloric acid [9, 10]. BBM and BG-11 macronutrients and micronutrients are used at the rate of 10 mLs and 1mL for each liter of algae cultured respectively. Algal growth could be increased by bubbling in a mixture of air and carbon dioxide instead of air alone. Fast growing cultures could be enhanced with carbon dioxide pulsed in several times a day, keeping the pH below 10.

Algae were cultured first with 250 ml conical flask photobioreactor (PBR) in BBM and BG-11 media without inoculums and sterilization with continuous air bubble feeding and with several times a day CO_2 flashing (this keeping the pH below 10) until the medium turns green, signaling adequate algae growth. Once maximum density is attained, the predominant species were identified according to its morphology and microscopic observations as prominent genera using digital camera equipped microscopy (Olympus DP 73).

Once obtained the small volume growth algae, by scale up several new cultures were used progressively for larger vessels for enough culture for mass production tanks and some of were kept as stocks (All the media compositions were sterilized using autoclave at121ºC for 15 minutes at 15 psi). Harvesting with large vessel were taken to a batch PBR (500ml and then 1000ml) conical flask medium used for higher biomass production with 50ml and 100ml inoculums from the previously cultured microalgae respectively.

Under favorable conditions, a newly inoculated culture in 1 L bottle takes 10-14 days to reach peak density. Once maximum density is attained, healthy algae bottle cultures are stable for two additional weeks.

For sampling in the case of all batch and semi-continuous runs, first, previously sterilized water was added to make up for any evaporation loss, the PBR was left undisturbed for about 5 to 10 minutes, then the sample was taken.

2.2 Algae Cell Density Determination

Monitoring algal growth in mass tank cultures is essential for successful production. The cell density of a given sample can be measured with the different methods: wet weight, dry weight, optical density, direct cell counting and successive dilutions followed by plating. For this study, the optical density method was used.

Standard routines to estimate algal concentration include direct cell counts, chlorophyll content measurement, and absorbance or turbidity numerical correlations. When spectrophotometrical absorbance is the chosen method, a reading wavelength of 750 nm is usually recommended, although values of 680 and 687 nm have also been used [12, 13, 14, and 15]. These values are correlated to the light absorbance of chlorophyll, which could be best determined at wavelength around 625 nm (PerkinElmer, Lambda 35).

2.3 Pigments production and Biomass concentrations

The analysis of profile pigments produced by microalga cells after culture on nutrient medium showed two maximum peaks obtained at corresponding to the Peaks of absorbance were between 400-500 nm and 600-700 nm. This showed that chlorophyll a and chlorophyll b, respectively. Since the maximum absorption peaks and

clear band width were observed between the ranges of 600-700 nm, 625nm, the subsequent analysis for growth determination was 625nm throughout the experiment.

Data of the cell density using optical density versus culture times were plotted and submitted to polynomial adjusts. The polynomial equations obtained were used to calculate the maximum optical density. The maximum specific growth rate (max d^{-1}) was obtained through exponential adjustment in the logarithmic phase of growth. Therefore, the growth of algae and biomass concentration was monitored by measuring optical density at a wavelength 625 nm. The optical density of the sample was measured at 625 nm using a spectrophotometer and optical path length of 10 cm. Algae biomass concentration was estimated using the following equation (eq. 1)

$$B = 0.38 * OD625 \quad (1)$$

where, OD625 is the optical density at 625 nm and B is the algae biomass concentrations (g/L).

Table 2.1Bold's Basal Medium (BBM) (Bischoff and Bold, 1963)

Component	*Stock Solution* ($g \cdot L^{-1}$ dH_2O)	*Quantity Used*
Macronutrients		
$NaNO_3$	25.00	10mL
$CaCl_2 \cdot 2H_2O$	2.50	10mL
$MgSO_4 \cdot 7H_2O$	7.50	10mL
K_2HPO_4	7.50	10mL
KH_2PO_4	17.50	10mL
NaCl	2.50	10mL
Alkaline EDTA Solution		1mL
EDTA	50.00	
KOH	31.00	
Acidified Iron Solution		1mL
$FeSO_4 \cdot 7H_2O$	4.98	
H_2SO_4		1mL
Boron Solution		1mL
H_3BO_3	11.42	
Trace Metals Solution		1mL
$ZnSO_4 \cdot 7H_2O$	8.82	
$MnCl_2 \cdot 4H_2O$	1.44	
MoO_3	0.71	
$CuSO_4 \cdot 5H_2O$	1.57	
$Co(NO_3)_2 \cdot 6H_2O$	0.49	

3 Results and Discussion

3.1 Effect of Culture Medium on the Growth Curve and Biomass Concentration

As can be seen from Figure 3.1, the zero day cultivation time was simply clear fresh water, but after five day the culture becomes a little green color. Finally, as can be seen cultivation were in deep green color (algae). The daily OD of microalgae was recorded by absorbance at 625 nm with PerkinElmer, Lambda 35 UV/VIS spectrophotometer. The OD value was determined in both media (Figure 3.2).

As shown from Figure 3.2, the different media have different optical density values but the statistical analysis from table 3 shows there is no significance difference with in the two media for the optical density value.

The amount of time required to adapt the environment differed somehow for the two growth medium type. The lag phase for BG-11 medium is longer than BBM. BB medium was devised to provide a defined freshwater medium suitable for culturing microalgae. This is due to Concentrations and type of nutrients of the major elements in BB medium are suitable for culturing of these microalgae species.

The results revealed that microalgal species had approximately 4 days lag period and reached the exponential phase within 12 days in BBM medium, but 8 days lag period and reached rapidly the exponential phase within 12 days. Microalgae cells achieved stationary phase within 10 days and after that cells growth was very slow as shown in Figure 3.2. As each microalgal species shows different biochemical composition (e.g., protein, amino acids, carbohydrate, lipid, fatty acids, chlorophyll and carotenoids) in different media which is reflected by different optical density (OD) of samples.

Table 2.2.BG-11 (Allen, 1968; Allen and Stanier, 1968; Rippkaetal, 1979)

Component	*Stock Solution* ($g \cdot L^{-1}$ dH_2O)	*Quantity Used*
Ferric Citrate solution		
		1mL
Citricacid	6	1mL
Ferric ammonium citrate	6	1mL
$NaNO_3$	—	1.5g
$K_2HPO_4 \cdot 3H_2O$	40	1mL
$MgSO_4 \cdot 7H_2O$	75	1mL
$CaCl_2 \cdot 2H_2O$	36	1mL
Na_2CO_3	20	1mL
$MgNa_2EDTA \cdot H_2O$	1.0	1mL
Trace metals solution	(See following recipe)	1mL
		2.86g
H_3BO_3	----	1.810 g
$MnCl_2 \cdot 4H_2O$	-----	0.220 g
$ZnSO_4 \cdot 7H_2O$	----	1 ml
$ZnSO_4 \cdot 7H_2O$	79	0.39 g
$Na_2MoO_4 \cdot 2H_2O$	49.4	1 ml
$Co(NO_3)_2 \cdot 6H_2O$		

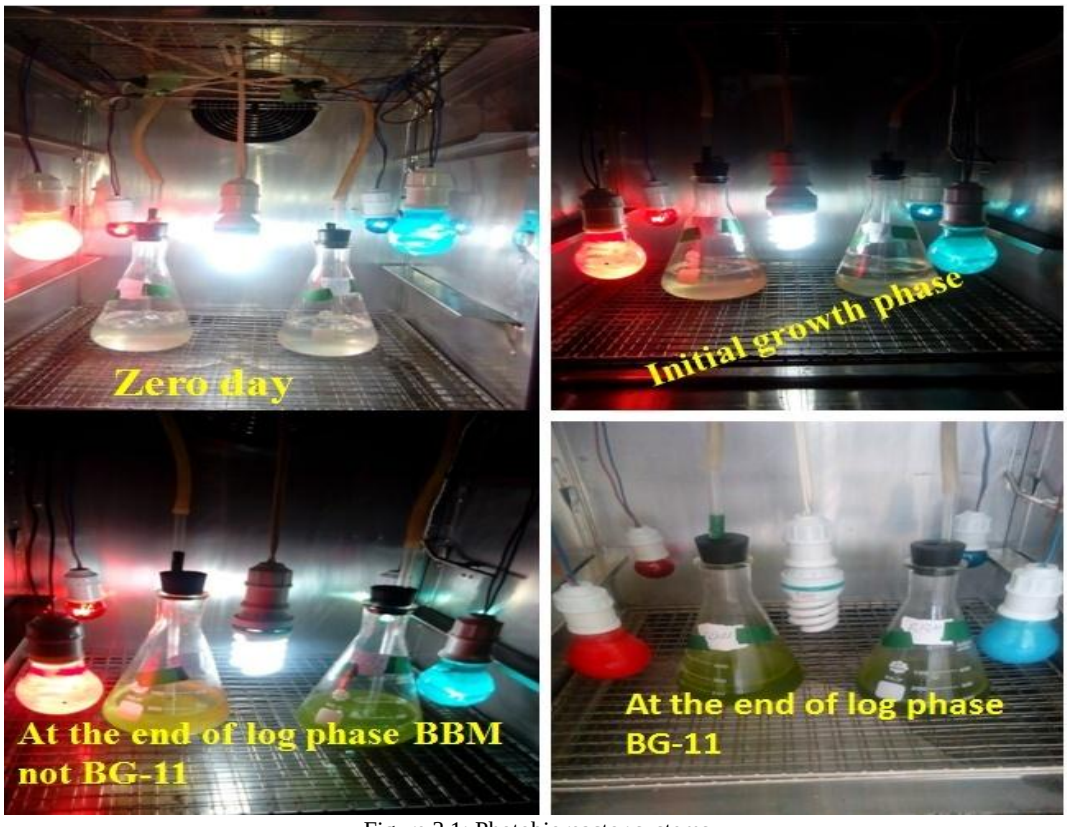
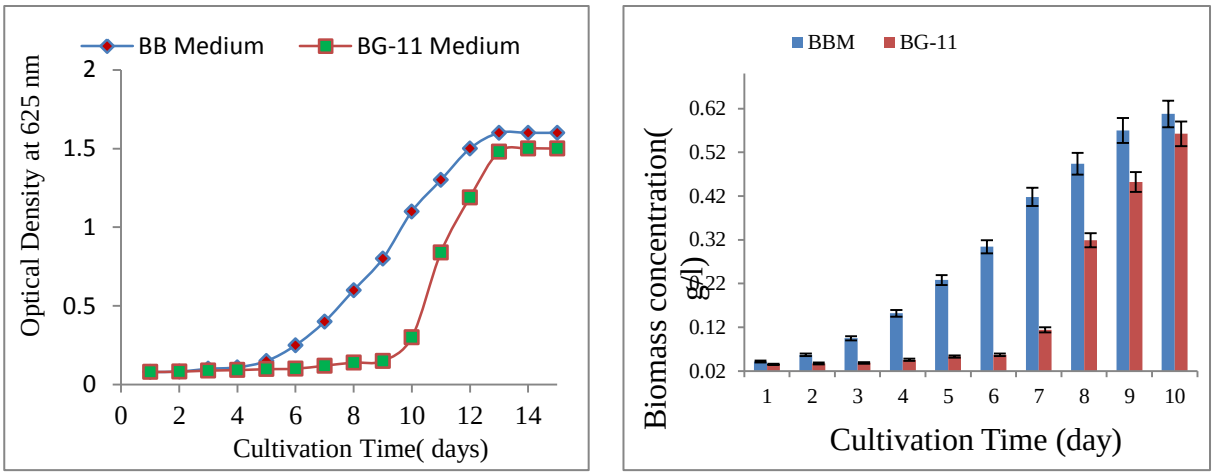


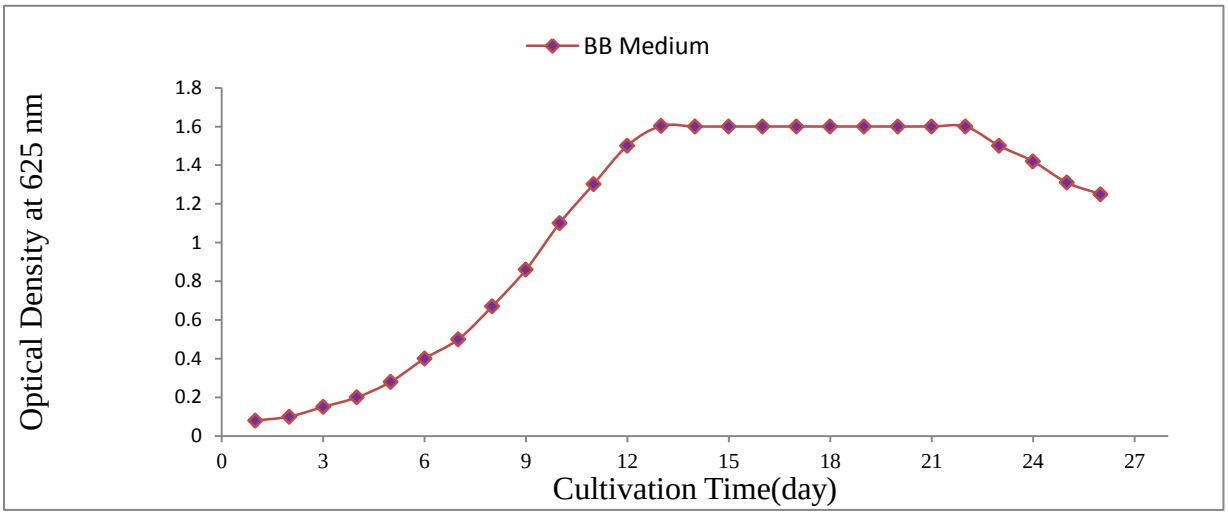
Figure 3.1: Photobioreactor systems

(a)

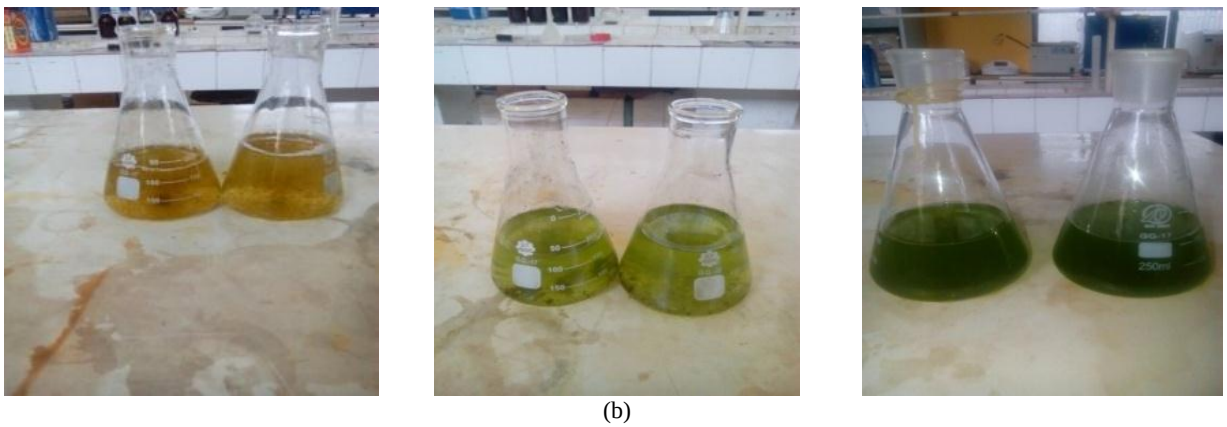


(a) (b)

Figure 3.2: (a) Effect of two growths media in the same cultivation date (b) microalgal biomass concentration.



(a)



(b)

Figure 3.3: (a) BB medium growth curve with 10% inoculums by previously cultured species (b) colour indications of microalgae growth curve.

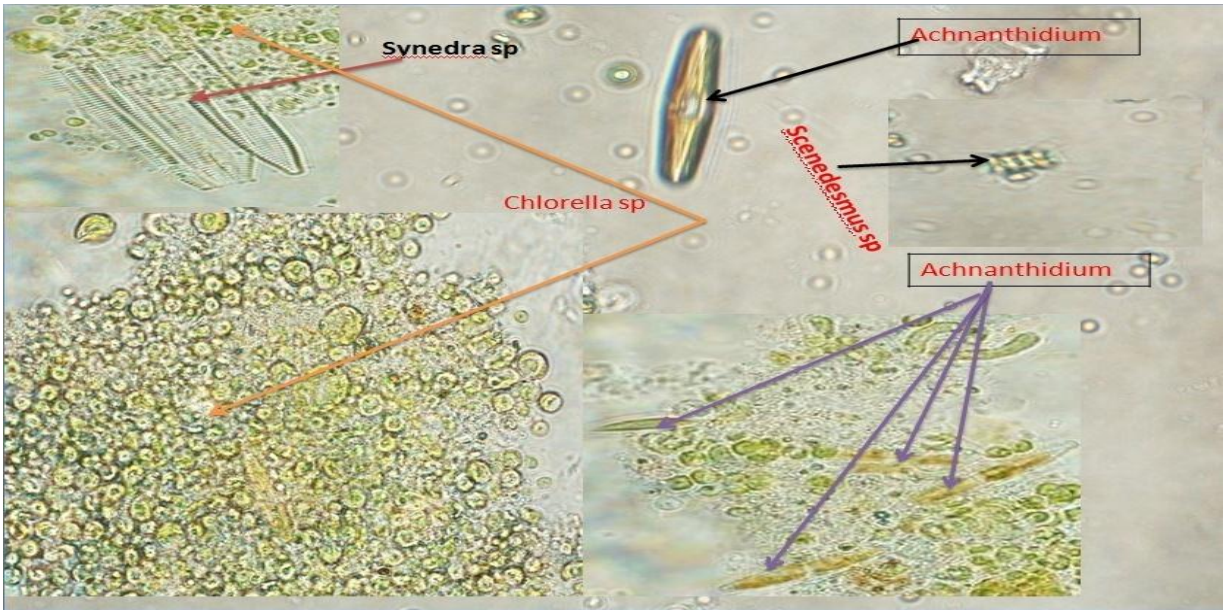
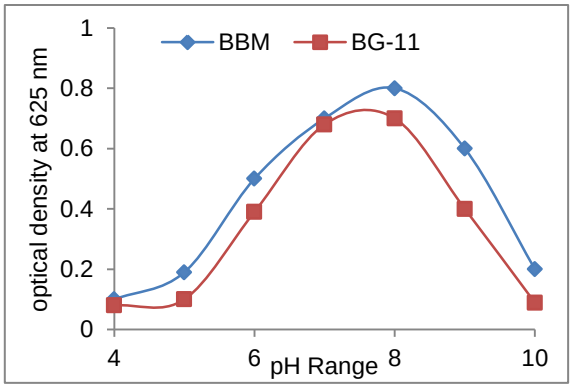
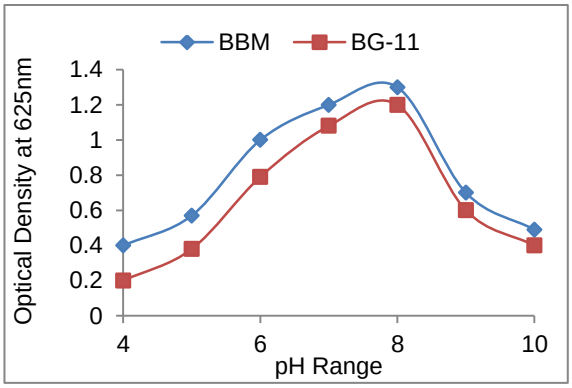


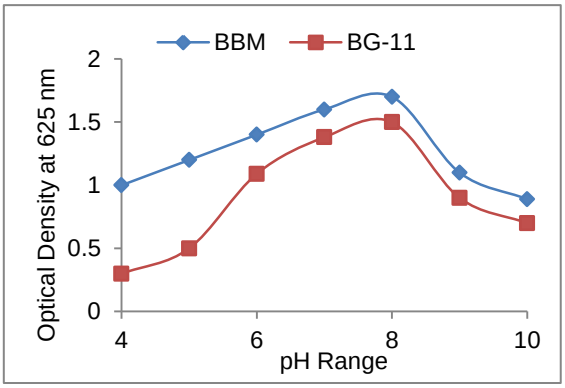
Figure 3.4: Microscopic photogram of co-cultured microalgae species



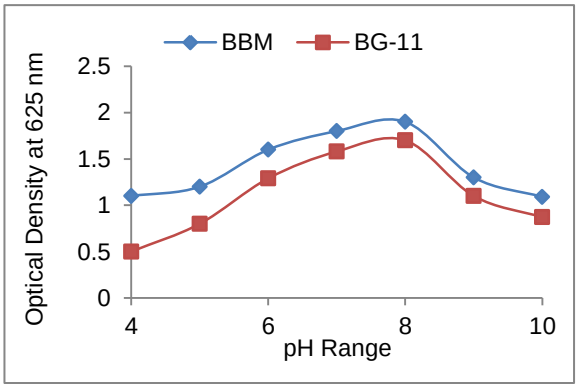
(a)



(b)



(c)



(d)

Figure 3.5 PH Effect on the algal growth (a) three day, (b) six day, (c) nine day and (d) twelve day cultivation time

Table 3 independent t-test analysis of the two growth media for optical density value (BB media and BG-11 media).

		Levene's Test for Equality of Variances		T-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Absorbance	Equal variances assumed	0.216	0.646	1.040	28	**0.307**	0.234060	0.224983	-0.226796	0.694916
	Equal variances not assumed			1.040	27.897	**0.307**	0.234060	0.224983	-0.226873	0.694993

3.2 Algal biomass concentration

The algal biomass concentration was determined by the equation (g/l): $B=0.38OD_{625}$.

As can be seen from figure 3.3 (a), exponential phase, stationary phase and dead phase. This was done to confirm that how long already harvested micro algae can maintain. As a result, the corresponding algae color for each phase has been shown from figure 3.3 (b). This reveal that once the exponential growth phase finished, the species can maintain for 9-10 days in conditioned environment

3.3 Identify the predominant fresh water co-cultured species

Microalgae species was identified from the untreated co-cultured water sample collected in Lake Tana. The specific characteristics found for algal strains under light microscopy were assessed and compared with Wehr and Sheath (2002) to find the respective algal genera. The obtained results were also confirmed on online algal database called "Micrographia" available at (http://www.micrographia.com/index.html**).**

Alga cells cultivated on nutrient medium gave different species. The size of the microalgal species was different with circular shapes, road shapes and spherical shapes. The observation by microscopy confirmed that the predominant species was dominantly green algae. The identification of the species was done on the basis of morphology of microalgae.

The Predominant consortium of microalgae species was found in this study was: *Scenedesmus sp., Chlorella sp., Synedra sp., Achnanthidium sp.*

As can be seen from Figure 3.4, *chlorela sp.* dominantly grown but *Synedra sp.* and *Achnanthidium sp*. was found in small amount. This revealed that, mostly the fresh water microalgae species are green algae.

3.4 Effect of pH on the productions of microalgal species

As can be seen from figure 3.5, the optimum growth of the fresh water algae was at PH 8 for both media. But still the absorbance of bold basalt medium is higher than the BG-11 medium. From this it can be deduce that the growth of microalgae is effected at far basic and acidic condition.

3.5 ANOVA Analysis

Assume that the population variances are not equal, so the non-pooled t-test ("Equal variances not assumed") can be used. From the independent t-test table 3, with 27.897 degrees of freedom, P-value = Sig. (2-tailed) = 0.307. Since p-value = 0.435> 0.05. This shows that, at 0.05 level of significance, there is no significant difference between the two growth media types, that is the two growth media performance of optical density means are almost the same (both media type have the same optical density value.

4 Conclusion

The findings revealed that the two culturing media types are significantly different for the growth of microalgae species in a mixed culture system due to the comparatively high productivity of biomass at 25 ± 2^0C, free air volumetric flow rate 2.0 L/min, and under continuous light intensity of1,300 Lux with 12/12 light-dark photoperiod with negligible external light interference. For this condition, both media types were devised to provide a defined freshwater medium suitable for culturing microalgae. The identification with microscopy confirmed that the predominant species was dominantly green algae.
The optimum growth of microalgae at the sample were taken is at PH 8

Abbreviations

pH: numerical scale used to specify the acidity or basicity; ^{0}C: degree centigrade ;%:Percentage ;UV: Ultra violet ;BBM:bold basal medium; BG-11: Green algae medium; PBR: photobioreactor; B :algae biomass concentration; nm: nanometer; OD625: optical density at 625nm; cm:centimeter; λmax: lambda max.

Authors' contribution Statement

Tadele Assefa Aragaw carried out the completion of harvesting, identification, microagal biomass analysis spectroscopic data collection and interpretation and completed the manuscript drafting and final writing. Abraham M Asmare participated in the data analysis and identification of the species experiments and assisted in the manuscript drafting.

Authors' information

Tadele Assefa aragaw is a master's holder in Environmental Engineering and a researcher at the

Department of Chemical Engineering Bahir Dar University, Bahir Dar Institute of Technology.
Dr. Abraham .M Asmare is a Ph.D. in Environmental Engineering at the Department of disaster Risk management and Food Security Studies Bahir Dar University, Institute of disaster Risk management and Food Security Studies and a director in the Institute of disaster Risk management and Food Security Studies

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Since we are from Ethiopia which is categorized under low-income countries list, we need to kindly request the waiver.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: this article does not contain any studies with human participants or animals performed by any of the authors.

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