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Original Article

Macromolecular characterization and growth kinetics of *Arthrospira platensis* and *Chlorella Vulgaris* as affected by different media (synthetic and Anaerobic Digestor Effluent) - A comparative study.

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Abstract

Background: Global concern about the availability of irrefragable, nutritious food to the masses to eliminate malnutrition had raised interest in microalgae-based dietary supplements. Furthermore, improper disposal of industrial wastewater is contaminating freshwater resources around the world, which is increasing the demand for eco-friendly water recycling strategies. The focus has been shifted to the use of microalgae because of their ability to treat wastewater and their biotechnological applications. Among them, *Chlorella Vulgaris* and *Arthrospira* platensis are attracting considerable attention for their effective growth on wastewater and their nutritional value and use in biofuel production.

Methodology: Cultures of *C. Vulgaris* and *A. platensis* were subjected to different growth conditions to analyze their growth kinetics and biomass production in their respective synthetic media and Anaerobic Digestor Effluent (ADE). The biomass produced was further analyzed for its protein (Lowry method), carbohydrate (phenol-sulfuric method), and lipid (Bligh and Dyer method) content.

Results: The results obtained through the growth kinetics study showed that *C. Vulgaris* and *A. platensis* display a better growth rate on ADE at higher light intensities as compared to cultures growing on synthetic media. Significantly different levels of protein, carbohydrate, and lipid were also reported. *C. Vulgaris* grown on ADE had 78.5 % protein content compared to 62 % achieved in F/2 medium. Similarly, 7.6 % of carbohydrate content was achieved in ADE compared to 6 % in F/2. Lipid content however was much higher in F/2 (22 %) compared to ADE (13 %). The culture of *A. platensis* grown on ADE had 84 % protein content compared to 71 % achieved in Zarrouk's medium. Similarly, 11.7 % of carbohydrate content was achieved in ADE compared to 16.6 % in Zarrouk's. Lipid content in *A. platensis* was found to be very low (3 %).

Conclusion: The present study builds an understanding of the relationship between different factors contributing to economical microalgae production and macromolecular characterization of the targeted microalgae for analyzing its potential applications and uses. The study further suggests the added advantage of cost-effective production of algal biomass with an added advantage of phycoremediation using ADE as growth media.

Keywords

Phycoremediation, Micro-Algae, *Chlorella Vulgaris, Arthrospira Platensis*, Anaerobic Digestor Effluent.

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Introduction

The Industrial Revolution of the 19th century brought amazing technological advances in the industrial sector but at the cost of endangering our flora and fauna, coupled with the contamination of freshwater resources leading to water scarcity. Various physiochemical methods are being employed for the removal of toxic elements from the wastewater before its discharge; however, they call for huge investments, utilize harmful chemicals, and require high energy input. Nowadays researchers are focusing more on a better and natural alternative by using phycoremediation-biotransformation of waste material that may contain harmful chemicals and xenobiotics using photosynthetic microorganisms. Among these microalgae, *Arthrospira platensis* and *Chlorella Vulgaris* appear to be the potential candidates for pollutant removal from industrial wastewater[1]. The dramatic fluctuation in wastewater quality makes it harder for other microalgae to survive but *A. platensis* and *C. Vulgaris* can thrive even in absence of nutrients, providing evidence of their versatile metabolism[2]. The cell wall of *A. platensis* is a porous macromolecular network, capable of bio-sorption passive cation binding by dead or living biomass. The process could be employed effectively even in a concentration range below 100 mg/l, where other techniques are ineffective or costly. Similarly, for *C. Vulgaris* species, previous studies reported removal of about 50% nitrogen and 85.7% phosphorus with an increase in COD removal efficiency (around 83%) from industrial wastewater[3]. It not only removes pollutants but can also absorb heavy metals and remove dyes from the textile industry effluent thus reducing the treatment cost of wastewater[1]. Detailed review and analysis have been conducted on various methods involving microalgae to treat textile effluent[1], domestic wastewater[4], and agro-based industrial wastewater[5] such as distillery-anaerobic digestor effluent (ADE), which will be the focus of this study.

On the other hand, there has been a huge demand for commercial production of microalgae, however, high production cost still limits its use on a commercial scale. Guillard's and Zarrouk's media are the two standard growth media for *C. Vulgaris* and *A. platensis*[6,7], however, despite yielding optimal biomass production, these are not suitable for commercial setups because of relatively expensive ingredients needed for formulating the medium.

Anaerobic digestor effluent is the cost-effective alternative for the industrial-scale production of these microalgae, where microalgae are expected to simultaneously treat ADE. ADE is the distillery effluent that goes through a process of anaerobic digestion, a treatment procedure where microorganisms decompose organic waste and produce biogas[8]. Compared with typical effluents, ADE has relatively lower carbon content because digestion by methanogens converts carbon to methane[9]. The ADE being used in this study has nitrogen mainly in the form of ammonium, and dilution of ADE is usually needed before feeding to algae to avoid the potential inhibition of algal growth due to high ammonium concentration and turbidity[9]. Distillery wastewater is a rich source of organic nutrients such as reduced sugars, polysaccharides, proteins, lignin, and waxes leads to high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) concentrations, and inorganic compounds including sodium, potassium, sulfate, carbonate, and ammonium contributing to its dark brown color[10]. This nutrient-rich composition allows microalgae and cyanobacteria to sequester inorganic nutrients from the effluent which are then exploited by them to fulfill their minimum requirements of growth[5].

Considered a rich source of protein, essential amino acids, and natural pigments[11], microalgae are now widely being considered as a natural, non-perishable food that is nutritionally competent, maintains a long shelf-life, and can be consumed in various forms. More than 50 % of the microalgae consumed as a portion of food is Spirulina, which consists of dried powder of *A. platensis* and *Arthrospira maxima*[12], with *C. Vulgaris* being a close second[13]. When compared

to traditional malnutrition supplements as well as other microalgae species, they contain the most abundant protein content, 50–70 % of its dry weight[13]. Several studies have been published in which *Spirulina* is recommended as a therapeutic agent to counter malnutrition- both in children and adults. When fed to nutrition-deficient children, made them gain 25 g/day of weight as compared to 15 g/day of the placebo group, with no side effects[14]. While calories and fats in *A. platensis* are relatively low, it is approximately 10 % higher in proteins. It is suitable as a dietary supplement for obese and athletes. The lipoproteins of A. platensis consist of medicative ɣ-linolenic acid (GLA) comprising 49 %, rendering it the richest source (10 mg/g) of GLA after breast milk[15]. *C. Vulgaris*, on the other hand, is reported to be rich in pro-vitamin A, Riboflavin, dietary nucleic acids, and certain minerals[16].

This study analyzes the growth kinetics of *A. platensis* and *C. Vulgaris* and the effect of different conditions on their growth and biomass production. The study further analyzes the protein, carbohydrate, and lipid content of *A. platensis* and *C. Vulgaris* biomass intending to assess their potential applications and utilization. The biomass can be used as a whole for fish feed, poultry feed, and cattle fodder can be purified to extract agents bearing medicinal and industrial importance, can be used for biofuel production, or can be used as a dietary supplement for human consumption[12].

Methodology

Microalgal culture and growth media

Chlorella Vulgaris, used in this study was kindly provided by Seed Production Unit Hawksbay (SPUH), Karachi while *Arthrospira platensis* was purchased from a commercial culture bank (Algae Research Supply, US). To evaluate the growth of microalgae cultures in wastewater, a sterilized synthetic medium was used for comparison. Sea-water based F/2 or Guillard's medium[6] for *C. Vulgaris* consisted of three fractions, (a) nutrient solution (per liter) was composed of 75 g $NaNO_3$, 5 g $NaH_2PO_4.H_2O$ and 30 g Na_2CO_3 (b) The trace metal solution consists of (per liter) 22 g $ZnSO_4.7H_2O$, 0.217 g $MnCl_4.4H_2O$, 6.3 g $Na_2MoO_4.2H_2O$, 180 g $CoCl_4.7H_2O$, 3.15 g $FeCl_3.6H_2O$ (0.65 mg/L as Fe), and 4.4 g $Na_2EDTA.2H_2O$ and (c) vitamin solution consists of (per liter) 0.0675 g vitamin B12, 0.55 g vitamin B1, and 0.012 g biotin.

Zarrouk's media (per liter)[7] for Arthrospira was composed of 16.8 g $NaHCO^3$, 2.5 g $NaNO_3$, 1 g NaCl, 1 g K_2SO_4, 0.5 g K_2HSO_4, 0.2 g $MgSO_4.7H_2O$, 0.04 g $CaCl_2$, 0.01 g $FeSO_4.7H_2O$, 0.08 g EDTA. For the comparative analysis, Anaerobic Digestor Effluent (5 % ADE) was prepared by diluting settled effluent obtained from the distillery, Pak Ethanol Private Limited. Sharing the composition of the ADE provided by industry was not permitted, however, the composition and properties of a similar ADE are shown in Table 1.

Table 1: Characteristics of ADE, adapted from Sankaran et al., 2014.

Parameters	Concentration Range
Color	Dark brown
Temperature	35-40°C
pH	7.5-8.0
Conductivity	31-36 mS/cm
Turbidity (NTU)	40
TDS	35,000-45,000 ppm
TSS	22,000-34,000 ppm
COD	25,000-40,000 ppm
Alkalinity	17,000-24,000 ppm
BOD	7,000-10,000 ppm

Total hardness as CaCO3	3,100-3,200 ppm
Calcium hardness as CaCO3	600 ppm
Magnesium hardness as MgCO3	2,500-2,600 ppm
Ammoniacal Nitrogen as N	1,000-1,100 ppm
Dissolved Phosphate as P	400 ppm
Chlorides as Cl-	8,400-8,600 ppm
Sulphates as SO4-	4,000-4,500 ppm
Fluorides as F-	5 ppm
Nitrates as NO3-	350-400 ppm
Sodium as Na	250-300 ppm
Total Iron as Fe	10 ppm
Oil & Grease	30 ppm
Potassium as K	12,800 ppm
Total silica	60-65 ppm
Reactive silica (Dissolved silica)	50-55 ppm
Bicarbonate	12,800-12,850 ppm

Culture plates were placed under LED lights (1500-2200 lux) at 25-30 °C with a 16:8 hour light/dark photoperiod. Distinct and isolated colonies were observed under a light microscope (CX21 LED, Olympus, Japan) at 100 x magnification (immersion oil) for confirmation of purity and were transferred to 1.5 ml micro-centrifuge tube using a sterile wire loop. Both C. Vulgaris and A. platensis were adapted to various physical parameters including light intensity (lux), temperature, and aeration in different combinations to find the conditions that result in the maximum microalgal biomass production, and the ideal conditions were used for further growth kinetics analysis. All the flasks were provided with autoclaved cotton plugs and submerged silicon pipes connected to an aeration pump for a continuous supply of air and were incubated under the LED light. All experiments were done in triplicates or stated otherwise. Percent growth rate[17] of each of the culture for the cell count was calculated using the formula;

$$\% \text{ Growth rate} = \frac{(V_{Presesnt} - V_{Past})/ V_{Past} * 100}{\text{Time (Days)}}$$

Here, $V_{Present}$ is cell count at the end time, V_{Past} is the starting count and Time is the total number of days. Generation time was also calculated as an indication of doubling time of cells using the formula;

$$\text{Generation time} = \frac{\text{Time (days)}}{3.3 * \log (V_{Present} / V_{Past})}$$

Here, $V_{Present}$ is cell count at the end time, V_{Past} is the starting count and Time is the total number of days.

Growth kinetics

For growth kinetics, optical density and cell count of culture samples were performed every day in triplicates under sterile conditions to characterize and compare the growth of *C. Vulgaris* and *A. platensis* in synthetic media and ADE. The optical density of the cultures was measured using a spectrophotometer (Spectroquant Pharo300, Merck, US), *Chlorella* at 680 nm[18], and *Arthrospira* at 720 nm of wavelength[19]. Microalgal cells were counted using a hemocytometer (Marienfeld, Germany).

Both cultures of *C. Vulgaris* and *A. platensis* were grown on 5 % ADE and their respective synthetic media. The growth kinetics analysis was divided into two phases. In phase I, 100 ml culture of each microalga was diluted in 200 ml of respective media (ADE and synthetic media). Flasks were set in duplicates. ADE cultures were kept in a shaking incubator under LED lights (7000-7500 lux, 25°C) and those on F/2 and Zarrouk's media were kept under LED light (1500-2200 lux) at room temperature (25-30 °C). Baseline OD_{680} for *Chlorella* and OD_{720} for *A. platensis* was maintained the same for all the cultures. OD and cell count were recorded in triplicates every day till the cultures reached a consistent stationary phase. Observations from each day were recorded and graphs of OD against time and cell count against time were plotted. Phase I culture was sub-cultured in their respective media and cultures was set again for phase II of growth kinetics. Cell count and OD readings from each day were recorded again. Samples for biomass extraction and macromolecular composition were extracted at the late log phase.

Biomass Estimation

For biomass estimation, a volume of 50 ml of *C. Vulgaris* culture on F/2 medium and *A. platensis* culture on Zarrouk's medium were taken from 5 different cultures in their different growth phases with different ODs. Cells of *C. Vulgaris* were centrifuged (2366 K, HERMLE, Germany) at 2320 g for 20 mins at 4 °C while *A. platensis* cells were centrifuged at 11770 g for 15 minutes at 4 °C. Pellets obtained were washed with water and then resuspended in 10 ml distilled water. Mixtures were vortexed and then poured separately in pre-weighed dry Petri plates. Plates were left in a drying oven at 85 °C for 1 hour. After drying, plates were weighed again to obtain dry cell weight (DCW)[20]. Biomass was also determined indirectly through optical density measurement using a spectrophotometer[21].

Estimation of Macromolecules

For protein and carbohydrate estimation, Bligh, and Dyer method[22] was used with slight modifications. 10 ml of late-log phase culture in each media (F/2, Zarrouk's, and ADE) was taken and 4 ml of 5 % trichloroacetic acid (TCA) was added, vortexed for 30sec, heated in the water bath at 90 °C, cooled in an ice bath for 30mins and then centrifuged at 1500 g for 20mins leaving behind protein pellet and the supernatant containing extracted carbohydrates.

Carbohydrate Estimation

To quantify the amount of carbohydrate extracted, spectrophotometric determination of carbohydrates using modified phenol sulfuric acid method was followed. 1 ml of 5 % aqueous solution of phenol was added to 2 ml of the carbohydrate sample, 5 ml of conc. H_2SO_4 was then added rapidly and left for 10mins. It was then placed in a water bath (30 °C) for 20 mins. Standard glucose solutions were processed parallel to test samples. To plot the standard curve, the glucose stock solution of 1 mg/ml was diluted in a range of 100-400 µg/ml. A 20 % glucose solution was also made to be used as a positive control. Light absorption recorded at 490 nm was plotted against concentration to quantify the amount of carbohydrate extracted from both cultures of *C. Vulgaris* and *A. platensis*.

Protein Estimation

The pellet collected from Bligh and Dyer method was treated with 3 ml of 0.5 M NaOH, vortex for 30 secs, heat at 90 °C for 30 mins, left overnight at room temperature, and then it was centrifuged at 1500 g for 10 mins. A supernatant was collected to quantify the amount of protein extracted. Spectrophotometric determination of proteins

was done by following the Lowry method of total protein estimation[23]. 19.2 ml of alkaline copper sulfate reagent was mixed with 0.2 ml of the protein samples from both media (F/2 and ADE), incubated for 10 mins followed by the addition of 0.2 ml of diluted Folin reagent. Bovine Serum Albumin (BSA) standard solutions at concentrations from 50 to 1000 µg/ml (R2>0.96) were also used as a positive control. Given 30 mins of the reaction time, absorbance recorded at 600 nm was plotted against concentration to quantify the amount of protein extracted from both cultures.

Lipid Estimation

For the lipid estimation, Bligh, and Dyer method[22] was used with slight modifications. 50 ml of microbial inoculums grown in each of the media (F/2, Zarrouk's, and ADE) were taken out in their respective late-log phase. Cultures were centrifuged as before. The supernatant was discarded, and the pellet was washed with 10 ml of distilled H_2O. The falcons were centrifuged again at 1300 g for 5 min. The pellets were ultra-sonicated for 1 min at 100 W and 20 kHz in BAC spreader™ 1100. Once the cells were disrupted, chloroform, methanol, and distilled H_2O were added in the ratio 2:4:1.6. Biomass along with the mixture of solutions was vortexed for 30 sec intermittently for 20 mins. 2 ml of chloroform and water were added again. The microfuge tubes were then centrifuged for 5 min at 4400 g. As the three phases were formed, the upper H_2O layer was discarded while the lower chloroform layer was extracted to be placed in a pre-weighed Petri-plate. This was done twice and chloroform layers were pooled together. Lastly, the plates were left for 24 hours at RT to allow complete evaporation. The gravimetrical determination of the extracted lipids was done by subtracting the actual weight of the Petri-plates.

Results

Growth Kinetics

Cultures of Chlorella Vulgaris and Arthrospira platensis were subjected to various physical parameters including light intensity (lux), temperature, and aeration in different combinations, and ideal conditions were selected for further studies. Cultures were sub-cultured in their respective synthetic medium (Guillard's (F/2) and Zarrouk's) and ADE, before the growth kinetic study, and were adapted to the conditions.

For F/2 based *C. Vulgaris* cultures, baseline OD_{680} and cell count were set at 0.77 and 127.9×10^5 cells.ml^{-1} respectively. Cultures were grown for 16 days and the late log phase appeared on the 13th day of inoculation as shown in Figure 1, having OD_{680} 3.25 and cell count 466.3×10^5 cells.ml^{-1}. For ADE cultures, baseline OD_{680} and cell count were set at 0.86 and 111.9×10^5 cells.ml^{-1} respectively. Late log phase of ADE-based cultures appeared on the 13th day of inoculation as shown in Figure 1 having OD_{680} 4.70 and cell count 858.5×10^5 cells.ml^{-1}.

For Zarrouk's based *A. platensis* culture, baseline OD_{720} and cell count were set at 0.58 and 3.1×10^5 cells.ml^{-1} respectively. Cultures were grown for 17 days and the late log phase appeared on the 17th day of inoculation as shown in Figure 1 with OD_{720} 2.38 and cell count 20.6×10^5 cells.ml^{-1}. For ADE culture, baseline OD_{720} and cell count were set at 0.46 and 3.6×105 cells.ml^{-1} respectively. The late log phase for ADE-based cultures appeared on the 17th day of inoculation as shown in Figure 1 with OD_{720} 2.95 and cell count 24.4×10^5 cells.ml^{-1}.

The growth pattern of both cultures was compared using generation time and percentage growth rate using data recorded during the growth kinetic study. For *C. Vulgaris* grown on ADE, the generation time was reduced to 4.8 days as compared to 7.6 days when the cultures were grown on F/2 media. Also, the percentage growth rate was increased from 20.8 % to 57.9 % when the cultures were grown in F/2 and ADE respectively as shown in Table 2. For *A. platensis* grown on ADE, the generation time was comparable to cultures grown on Zarrouk's media with 6.2 days in ADE compared to 6.3 days in Zarrouk's media. Similarly, the percentage growth rate was also similar in both cultures, 34 % on ADE, and 33.2 % in Zarrouk's (Table 2). To establish a relationship between OD and cell

count of growth kinetics, a graph of OD against cell count was plotted (Figure 2).

Biomass Estimation

To compare the biomass production of the microalgae in their respective synthetic media and ADE, biomass was determined by measuring optical density correlated to dry cell weight of the two cultures in their late-log phase. 76 % higher biomass was obtained from *C. Vulgaris* grown in ADE with 3.59 g/L as compared to the F/2 medium which was 2.04 g/L. Similarly, 12.4 % higher biomass was obtained from *A. platensis* growing in ADE with 15.18 g/L as compared to Zarrouk's medium which was 13.50 g/L (Table 2).

Estimation of macromolecules

The cultures were taken out in their late-log phase to obtain a maximum concentration of macromolecules. Both cultures of *A. platensis* were taken out on the 17th day of inoculation while both *C. Vulgaris* cultures were obtained on the 13th day of inoculation. Keeping the volume, the same for each of the dilutions and the unknown samples (2 ml), the concentration of glucose and proteins made by *A. platensis* and *C. Vulgaris* in their synthetic media and ADE was directly calculated from the least-squares line of the standard curve.

The absorbance of glucose was taken at 490nm and the standard curve plotted (correlation 0.99). The carbohydrate content was 67 g/Kg of *A. platensis* in Zarrouk's medium while in ADE medium it was 46 g/Kg. The carbohydrate content in *C. Vulgaris* was 78 g/Kg in the F/2 medium while 86 g/Kg in the ADE medium. The carbohydrate percentage in *A. platensis* was 16.6 % in Zarrouk's medium while 11.7 % in the ADE medium. The carbohydrate percentage in *C. Vulgaris* was 6 % in the F/2 medium while 7.6 % in the ADE medium.

The absorbance of BSA was taken at 600 nm and the standard curve plotted (correlation 0.96). The protein content in *A. platensis* was 287 g/Kg in Zarrouk's medium while 331 g/Kg in the ADE medium. The protein content was 305 g/Kg of *C. Vulgaris* in F/2 medium while in ADE medium it was 881.4 g/Kg. The protein percentage in *A. platensis* was 71 % in Zarrouk's medium while 84 % in the ADE medium. The protein percentage in *C. Vulgaris* was 62 % in the F/2 medium while 78.5 % in the ADE medium.

Cells disrupted through ultra-sonication resulted in a successful subsequent lipid extraction. The lipids were dried and weighed gravimetrically. The amount of lipid in *A. platensis* was 49 g/Kg in Zarrouk's medium while 13.2 g/Kg in the ADE medium. The lipid content in *C. Vulgaris* was 107 g/Kg in the F/2 medium while 154 g/Kg in the ADE medium. The lipid percentage in *A. platensis* was 12 % in Zarrouk's medium while 3 % in the ADE medium. The lipid percentage in *C. Vulgaris* was 22 % in the F/2 medium while 13 % in the ADE medium.

Table 2: Comparison of growth and biomass of microalgae on different media. Generation time, percentage growth and DCW (dry cell weight) was calculated on 17th day for *A. platensis* and on 13th day for *C. vulgaris*.

Micro-algal culture	Generation time	Percentage growth rate	DCW g/L
A. platensis ZK	6.3	33.2%	13.50
A. platensis ADE	6.2	34%	15.18
C. vulgaris F/2	7.6	20.8%	2.04
C. vulgaris ADE	4.8	57.9%	3.59

ZK: Zarrouk's medium. ADE: Anaerobic Digestor Effluent

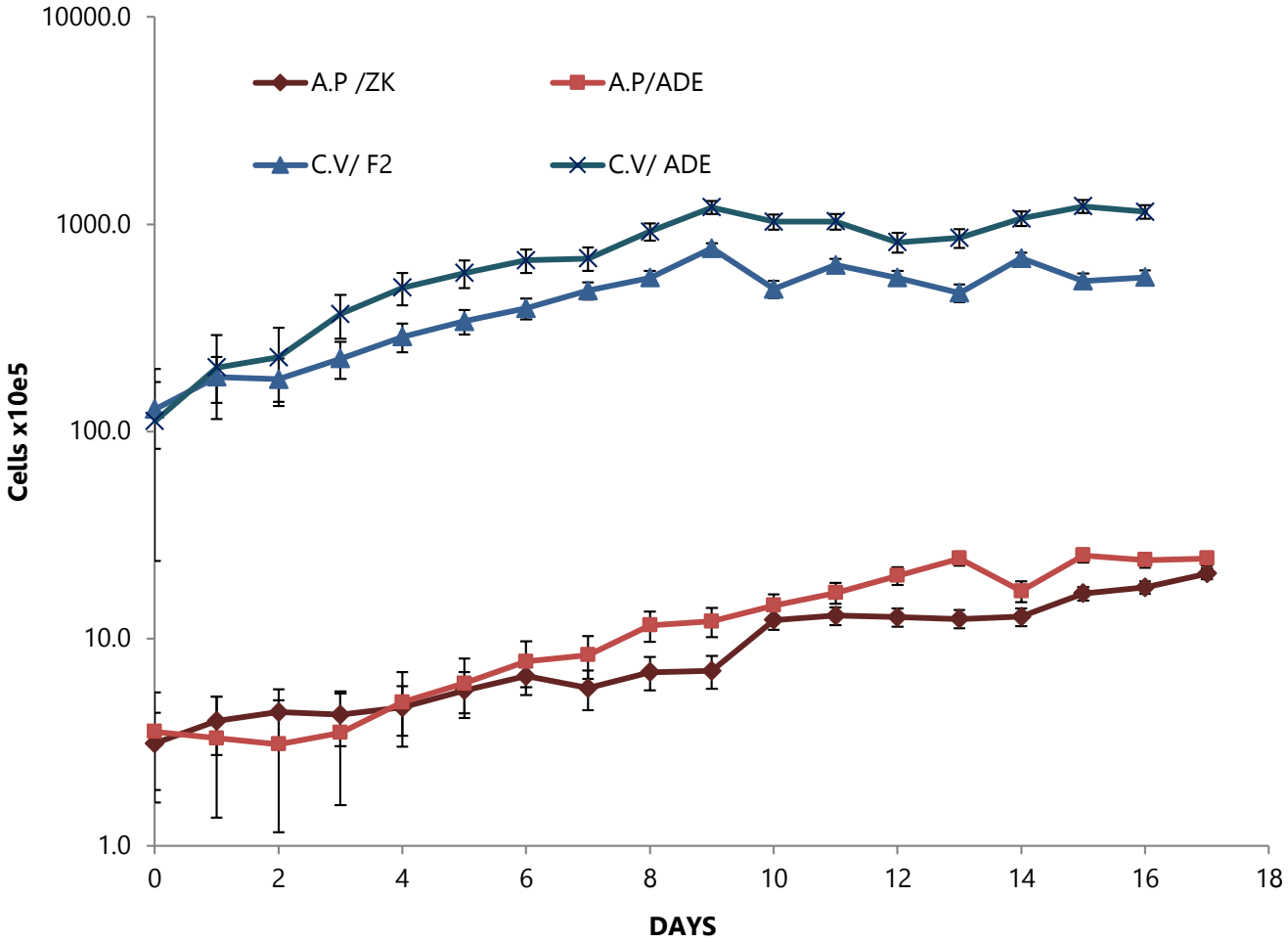

Figure 1: Growth kinetics of *C. vulgaris* and *A. platensis*. Comparison of growth kinetics of *C. vulgaris* on F/2 and ADE and *A. platensis* on Zarrouk's media and ADE. *C. vulgaris* and *A. platensis* were counted every 24 hours for 16 and 17 days respectively.

Table 3: Carbohydrate, Protein and Lipid concentration (g/Kg) of microalgae.

Micro-algal culture	A. platensis		C. vulgaris	
Medium	ZK	ADE	F/2	ADE
Carbohydrate Conc.	67	46	78	86
Protein Conc.	287	331	305	881.4
Lipid Conc.	49	13.2	107	154

ZK: Zarrouk's medium. ADE: Anaerobic Digestor Effluent

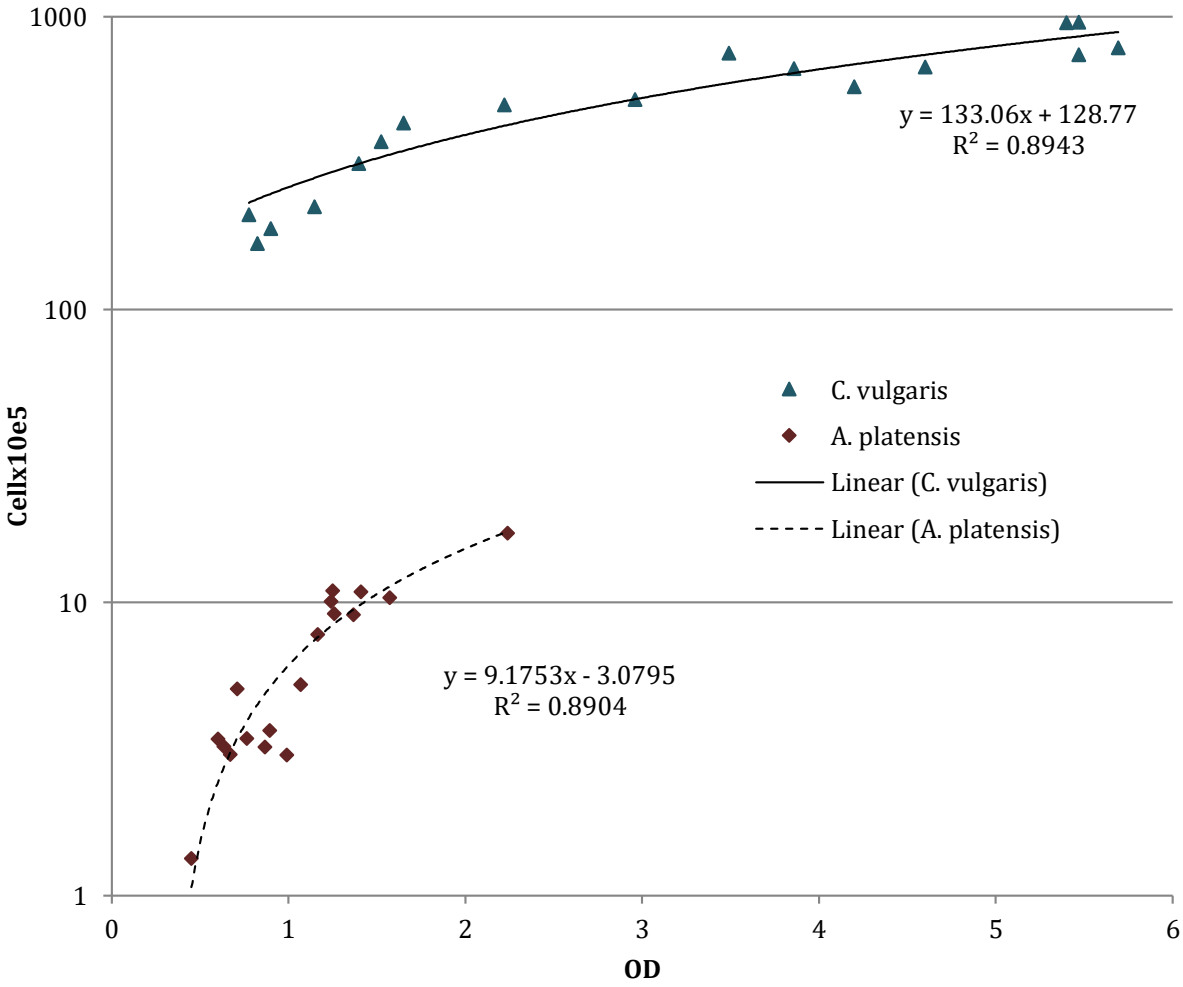

Figure 2: Correlation between Cell count and OD. OD of *C. vulgaris* and *A. paltensis* were measure at 680nm and 720nm wavelength respectively. The correlation for *C. vulgaris* was found to be 0.8943 indicating a strong correlation. The regression equation for *C. vulgaris* was found to be y = 133.06x + 128.77. The correlation for *A. platensis* was found to be 0.8904 indicating a strong correlation. The regression equation for *A. platensis* was found to be y = 9.1753x - 3.0795

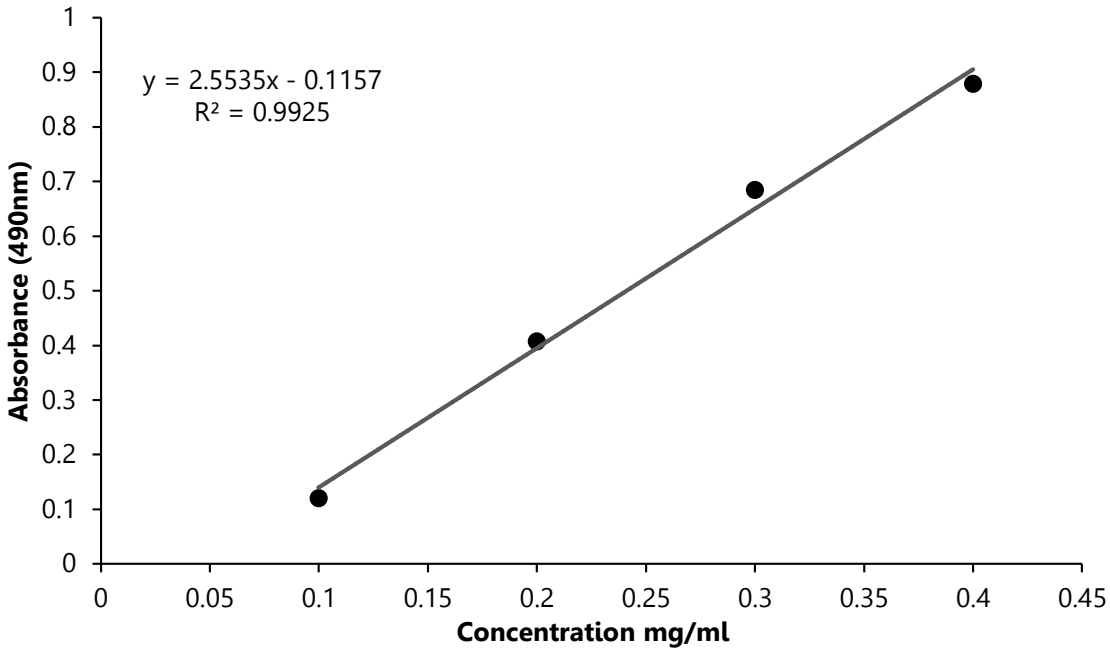

Figure 3: Graph of absorbance of glucose at 490nm against concentration. The correlation was found to be 0.99 indicating a strong correlation. The regression equation was found to be y = 2.5535x - 0.1157. Samples were run in duplicate.

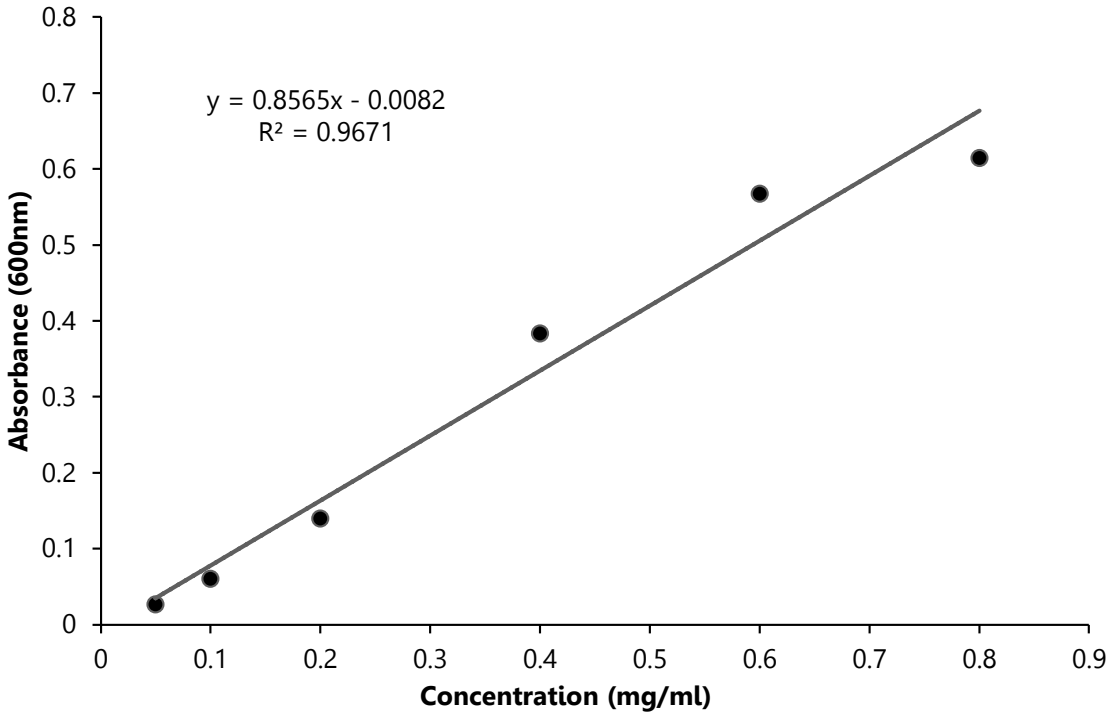

Figure 4: Graph of absorbance of BSA against its concentration at 600nm. The correlation was found to be 0.96 indicating a strong correlation. The regression equation was found to be y = 0.8565x - 0.0082. Samples were run in duplicate.

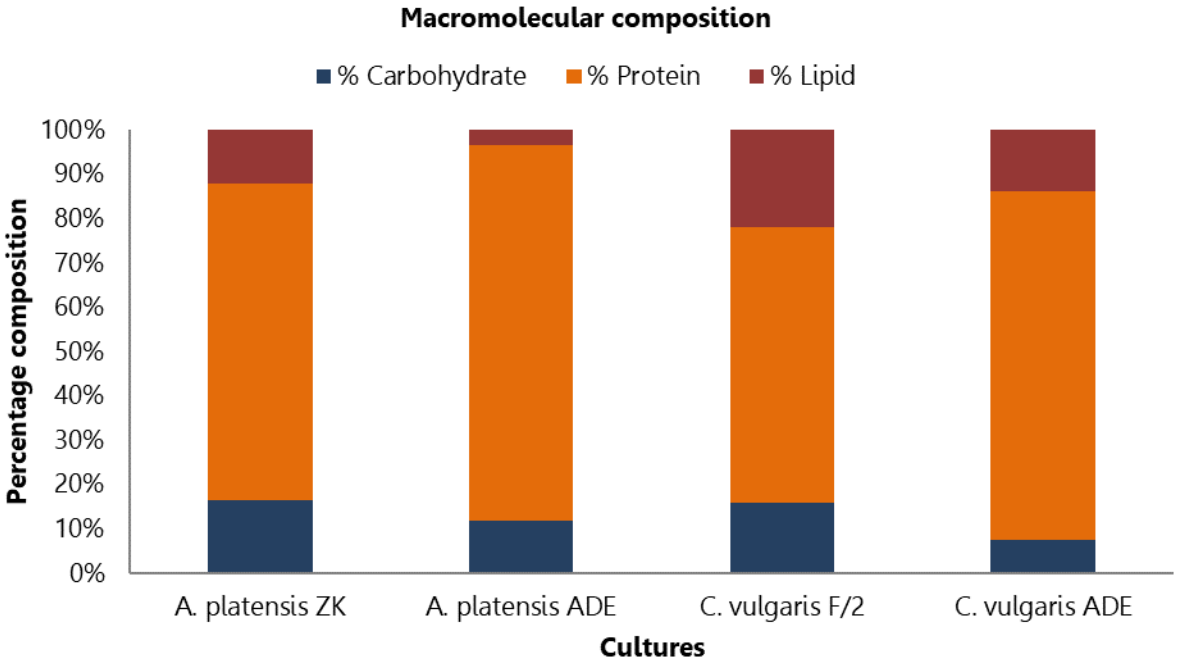

Figure 5: Macromolecular composition of C. vulgaris and A. platensis in ADE and respective synthetic media

Discussion

It was observed during the study that increasing light intensities result in a higher growth rate. Similar data were reported, which observed that growth rate increases with the increasing light. However, synthetic medium based cultures were de-colorized by the degradation of pigments due to stress caused by high light intensity and eventually resulted in inhibiting photosynthesis[24] by changing its color from green to yellow. ADE cultures showed no such effect; this may be due to the shading effect provided by the dark brown effluent as it is denser than a synthetic medium that allowed limited penetration of light thus ensuring better growth. This led us to the selection of higher light intensities for ADE based cultures and lesser light intensities for synthetic medium based cultures for growth kinetics analysis.

Growth kinetics analysis through optical density is more feasible compared to the cell counting method. To establish a relation between the two, a linear correlation was obtained. The correlation factor for *C. Vulgaris* was found to be 0.894 indicating a strong relation. Using the forecast formula, it was also estimated that 1.0 OD_{680} equals approximately $26x10^6$ cells. The correlation factor for A. platensis was found to be 0.890 and using the forecast formula it was also estimated that 1.0 OD_{720} equals approximately $6.09x10^5$ cells. It was also found that growth curve based on cell count is more precise and reliable than optical density as cell count only consists of the viable microalgal cells while optical density includes dead cell's debris and interference by the turbidity of the medium, like in the case of ADE. A positive correlation between OD and cell count has also been reported by Osundeko et al. 2013[25].

Previous studies[26] also reported that higher light intensity contributes to higher biomass production due to increased light utilization for photosynthesis. Since the cultures grown on ADE media were given higher light than the F/2 medium, it could be the reason for higher biomass. Another reason for increased biomass is

the presence of a higher concentration of nitrates and phosphates in ADE[10] which leads to faster and higher growth[27]. Consequently, ADE, a cheap medium can be used as a large-scale production media, reducing the media cost, and subsequently the overall cost for biomass production given that it accounts for 15-25 % of total production[28,29]. It is also reported[30] that cultures with algal biomass greater than 1.5 g/L accelerates inorganic nutrients removal as compared to cultures with l and are termed as hyper-concentrated cultures.

Figure 5 shows a significant difference between the concentrations of carbohydrate estimated in *A. platensis* cultured on Zarrouk's media (67 g/Kg) as compared to *A. platensis* grown in ADE (46 g/Kg), where the former proved to exceed in carbohydrates by more than 45 %. The percentage carbohydrate content (16.6 % and 11.7 %) was within the reported carbohydrate range of 10-20 %[31]. *C. Vulgaris* cultures growing on F/2 (15.9 %) produced twice the amount of carbohydrate than ADE based culture (7.6 %) (Figure 5), which compared to the reported range of 12-18 % is expected[32].

The protein content of both cultures of *A. platensis* was similar to each other (Figure 5) with 71 % in Zarrouk's based culture and 84 % in ADE based culture, just slightly higher than reported 50-70. It was also observed during the study that the extraction of proteins from the cells of *A. platensis* using the Bligh and Dyer extraction method was limited. This might be due to the multiple layers of the obstinate cell wall structure of microalgae[33], that make complete protein extraction difficult. Further studies need to evaluate other extraction methods and confirm the protein content of *A. platensis* in these conditions. *C. Vulgaris* (Figure 5), however, showed higher protein concentration in ADE based culture (78 %) than F/2 based culture (62 %). Protein accumulation might be caused by higher nitrogen and phosphorous levels in ADE that help the cells in producing more protein for growth[28].

Table 3 shows that lipid content of A. platensis from both media is very low as expected[34], with 12 % in Zarrouk's medium and 3 % in ADE based culture. On the other hand, in the case of *C. Vulgaris*, a 76.5 % increase in lipid content was found in F/2 based cultures (22 %) when compared to ADE based cultures (13.2 %). This is due to nitrogen limitation in F/2 based culture that shifts the metabolic pathway to accumulate more lipid compared to ADE that had abundant nitrogen sources available and a culture has grown in it is without stress.

Since ADE based cultures showed relatively higher growth rate and greater biomass than cultures based on synthetic media, this must be the reason for lesser lipid content as cells are expected to use the available carbon source for photosynthesis to support growth instead of accumulating lipids. To use ADE media for the production of lipid-enriched biomass, techniques such as nitrogen-phosphorous limitation can be used for lipid accumulation.

The study investigates that ADE proves to be a comparable medium for the cultivation of nutritionally important microalgae. In addition to no requirement of investment, growing cultures on ADE is a way to minimize the toxicity of this wastewater. In turn, it gives us potentially useful biomass. According to the results of the study, *C. Vulgaris* grown on ADE is richer in proteins but has lower lipid and carbohydrate content compared to F/2 media-based biomass. This guides to its possible applications as a food source, in aquaculture, cattle feed, or human dietary supplements. If treated further to nitrogen-phosphorous limitation, the lipid content of *C. Vulgaris* will improve as well, and the biomass can be used for biodiesel production. Similarly, a further increase in carbohydrate content can establish it as a source of bioethanol production. On the other hand, *A. platensis* is a low-lipid but high protein source and can be used as an ideal food source.

Further studies to evaluate macromolecule composition in ADE based cultures using different

extraction methods is also required. The method used in this study was a limitation, especially in the case of *A. platensis*. Further toxicity studies and downstream processes are required to establish *A. platensis* and *C. Vulgaris* as sustainable dietary supplements.

Conclusion

Although commercially formulated media is widely used for the cultivation of microalgae, the present study demonstrates the use of ADE as a suitable alternate. Since the use of ADE was evaluated by growth kinetics as well as the production of macromolecules, in comparison to synthetic media, macromolecules are produced at incomparably higher levels when grown in ADE. This indicates the significance of nutrient composition for the growth and nutritional profile of microalgae. Microalgae have been declared as a superfood by the WHO and the Food and Agriculture Organization (FAO) and a comparable nutritional profile of *A. platensis* and *C. Vulgaris*. The growth of Microalgae on ADE provides an added advantage of phycoremediation with adequate levels of macromolecules that can be used for various applications.

Conflicts of Interest

None.

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