



Mixed cultivation of *Euglena gracilis* and *Chlorella sorokiniana*: a production method of algae biomass on a large scale.

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ABSTRACT

Objective: *Euglena gracilis* and *Chlorella sorokiniana* are single cell freshwater green micro-algae that are super food of choice for over 10 million people worldwide. Mixed cultivation of *Chlorella sorokiniana* and *Euglena gracilis* was compared with their monocultivation under photoautotrophic, mixotrophic and heterotrophic conditions.

Methodology and Results: The Cell Density was measured by cell counting using a Heamocytometer, Final Cell Concentration was determined by cell counting at the 480 hours of culture and Biomass Yield were calculated using standard method. The Cell Density, Final Cell Concentration and Yield from change in cell growth to change in substrate consumed glucose were compared. The results showed that in a mixed cultivation, there was significant increase ($p \geq 0.05$) in growth rates of *Euglena gracilis* and *Chlorella sorokiniana* compared to the values obtained in monocultures. A total Yield of 67.64×10^5 cells/mg glucose and 51.73×10^5 cell/mg glucose were obtained in mixed cultivation and monocultivation of two cell strains respectively.

Conclusion and application: This demonstrates that mixed cultivation has a very high potential as a substitute for the current monocultivation of *Euglene gracilis* and *Chlorella sorokiniana*.

Key words: Autotrophic, Heterotrophic, Mixotrophic, Monocultivation, Mixed cultivation

INTRODUCTION

The photosynthetic efficiencies of many photosynthetic microorganisms such as micro algae are much higher than those of higher plants and it has been postulated that cultivation of micro algae can supplement that of conventional agriculture for production of food and nutritional supplement (Shelef and Soeder 1980). Cultivation of micro algae for production of single cell protein as health food and animal feed has been extensively investigated in various countries, especially in Asia (Lee, 1997; Belay, 1997).

Application of micro algae biotechnology for environment purification such as removal of oxides of nitrogen (NO_x) and Sulphur oxides (SO_x) from flue gases (Negoro, *et al.* 1991; Yoshihara, *et al.* 1996) wastewater treatment (Laliberte, *et al.* 1997; Ogbonna, *et al.* 2000a), for production of metabolites such as pharmaceuticals, pigments, and various other fine chemicals such as carotenoid insecticide, pyrethrum from *Chrysanthemum*, papain, steroids, phenolics and jasmine, for food, cosmetic and other industries

(Borowitzka, 1995); (Apt and Brehrens, 1999; Shimizu, 2000) as well as for gas exchange in enclosed life support systems have also been demonstrated. Thus, efficient cultivation of micro algae and other photosynthetic microorganisms can contribute significantly in solving the world's food, energy and environment problems. However, translation of the above potentialities into reality has not been easy. In spite of the extensive research for several decades, progress has been limited and commercial cultivation of micro algae has been limited to only a very few strains. The solar light conversion efficiencies in commercial large-scale production systems are still very low compared to the theoretical value (Ogbonna et al 2003). Most of the commercial cultivation of microalgae is currently done in open cultivation ponds. Although these culture ponds are very simple and cheap to construct, their production is very low due to various problems which include the poor mixing system, the low mass transfer capacity, the difficulty in controlling the culture conditions, and the difficulty in maintaining monoculture (pure culture) for a long period of time. Consequently, only very few algae species that have selective growth condition are currently

MATERIALS AND METHOD

Euglena gracilis and *Chlorella sorokiniana* were obtained from seed culture maintained at OGB Biotechnology Research and Development Center Enugu, Nigeria. The medium consisted of glucose (Fluka Chemical Buchs; Switz) and liquid fertilizer from the CANDEL Company Ltd, Lagos and which consisted of 20% Nitrogen, 20% Phosphorous, 20% potassium; 0.1% Magnesium, 0.15% Iron EDTA, 0.0755% Manganese EDTA, 0.0755% Copper EDTA, 0.0755% Zinc EDTA; 0.0315% Boron, 0.0012% Cobalt EDTA and 0.0012% Molybdenum.

Preparation of Medium:

Medium I: 0.5g of glucose was dissolve in 250ml conical flask containing 100ml of distilled water and 0.25ml Of liquid fertilizer was added and mix thoroughly. The mixture was autoclaved at 121^o C for 15min and then cooled to 25^o c.

Medium II: 0.25ml liquid fertilizer was dissolved in 250ml conical flask capacity containing 100ml of

cultivated on commercial scale (Ogbonna et al 2000a). Many micro algae of commercial interest cannot be grown in open culture ponds because of their susceptibility to contamination, and variation in culture conditions.

Chlorella sorokiniana are adapted to grow in open systems and commercial production facilities are available. However they are, less nutritive and contain hard cell wall (Apt et al, 1999). *Euglena gracilis* is difficult to be cultivated in open systems because they are sensitive to environmental factors and easily contaminated. However they produce various vitamins such as vitamin C, E and β - carotene (Takeyama et al, 1997). Thus co-culture would enhance mixed vitamin complex (Vitamin C, E and β - carotene) production, control the relative proportion of the cells and reduce contamination. In other words, it is expected that a co-culture of the two strains would be better adapted to uncontrolled environmental conditions than mono-culture of *Euglena gracilis*. Therefore, this study was aimed at developing a co-culture system of *Euglena gracilis* and *Chlorella sorokiniana* as a method for efficient production of vitamins rich algae biomass on a large scale.

distilled water. The mixture was autoclaved at 121^o C for 15min and then cooled at 25^o c.

Subculture and Seed Culture: 0.3m Liquid fertilizer was measured into two separate 250ml conical flask containing 100ml of distilled water. It was autoclaves at 121^o C for 15 min and cooled to 25^o c. 0.5ml of each seed culture (0.5ml) obtained from OGB Biotechnology Research and Development Center, Enugu, Nigeria was used to inoculate each flask. The flasks were incubated inside a box with illumination from four (4) fluorescent lamps arranged at four corners of the box for two weeks.

Procedures for Mono Cultivation:

Autotrophic Culture: 0.1ml of each subculture was inoculated into a 100ml of medium II prepared above. The flasks were incubated at room temperature (25^o c) inside an illuminated box with four fluorescent lamps for 480 hours.

Heterotrophic Culture: 0.1ml of each subculture was inoculated into a 100ml of medium I. The flasks were

incubated at room temperature in the dark for 480 hours.

Mixotrophic Culture: 0.1ml of each subculture was inoculated into 100ml of medium I. The flask was incubated at room temperature inside a box illuminated by four fluorescent lamps for 480 hours. These were done for both *Euglena* and *Chlorella* cells.

Procedures for Mixed Cultivation: Equal volumes (0.1ml) of *Euglena gracilis* and *Chlorella sorokiniana* subcultures were inoculated into each medium and cultivated under the same conditions as described for the monocultures (in Autotrophic, Heterotrophic, Mixotrophic culture system).

Analytical Methods: Cell growth rate was determined by cell counting using a Hemacytometer with Improved Neubauer (Marienfeld, Germany) and Olympus Binocular Microscope, at 5 days (120 hours) intervals. The glucose consumed was determined by glucose peroxidase method as reported by Ogbonna *et al* (1997). The cell density and the glucose consumed

for both mono-cultivation and mixed cultivation were recorded. Final concentrations and biomass yield were calculated using method described by Ogbonna and Tanaka, (1996).

Final Cell Concentration: $X = X_f - X_0$

Where X_f = cell count at 480 hour of culture.

X_0 = cell count at zero hour.

Biomass Yield

While the Biomass Yield was calculated as follows

$$\text{Yield} = dx/ds$$

Where: dx = change in cell concentration ($X - X_0$)

Where X is final cell count and X_0 is initial cell

ds = change in substrate concentration (amount of substrate consumed) ($S_0 - S$)

Where S_0 is initial substrate and S is substrate after at last cell count.

Result and Discussion

Figure 1 and 2 shows the characteristic growth rate of monocultivation system of *Euglena gracilis* and *Chlorella sorokiniana* respectively. The results show that at 480 hours cultivation time, *Euglena* final cell concentration in autotrophic, heterotrophic and

mixotrophic condition of cultivation was 8.80×10^5 cell/ml, 16.40×10^5 cell/ml and 37.20×10^5 cell/ml respectively, while *Chlorella Sorokiniana* has 27.90×10^5 cell/ml, 33.70×10^5 cell/ml, and 44.80×10^5 cell/ml accordingly.

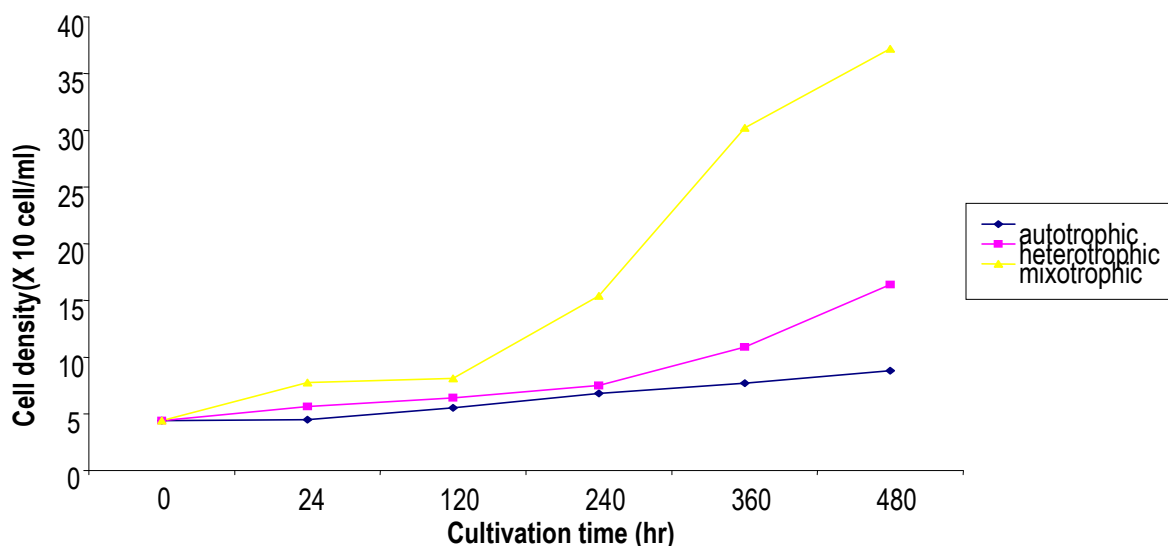


Figure 1: Characteristic growth rate of *Euglena gracilis* in autotrophic, heterotrophic and mixotrophic conditions in monoculture system

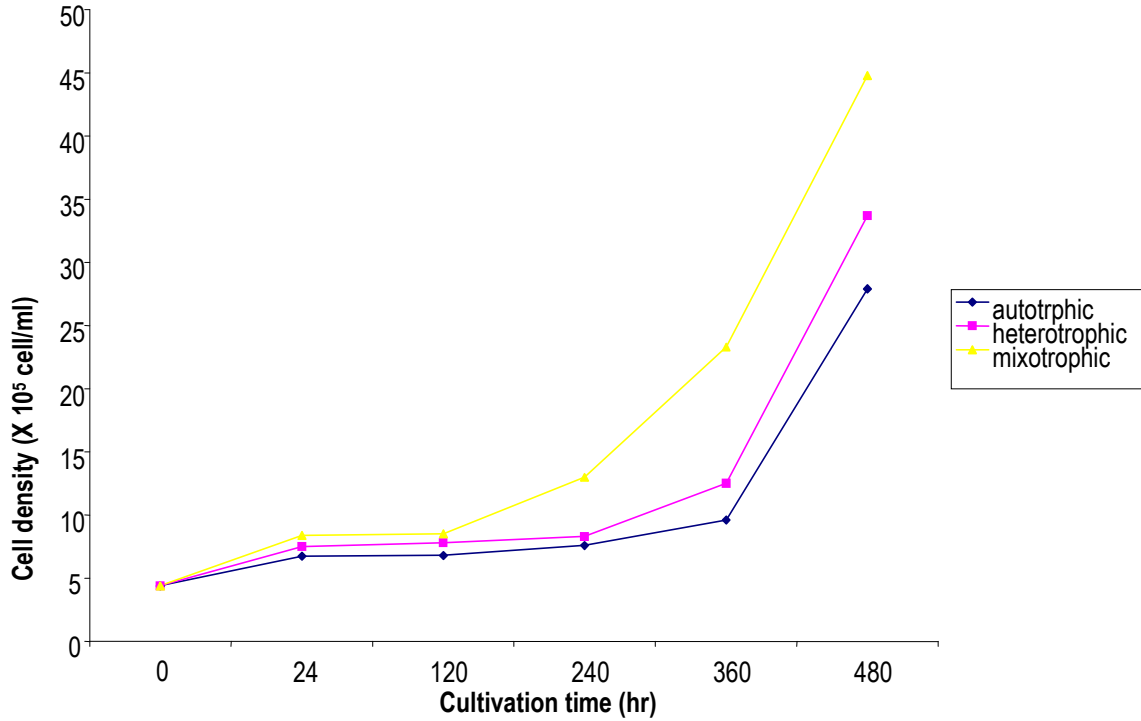


Figure 2: Characteristic growth rare of *Chlorella sorokiniana* in autotrophic, heterotrophic and mixotrophic conditions in monoculture system

Figure 3 and 4 shows the characteristic growth rate in mixed cultivation systems of *Euglena gracilis* and *Chlorella sorokiniana* respectively. In the result *Euglena gracilis* final cell concentration was 16.10 x 10⁵ cell/ml in autotrophic, 27.30 x 10⁵ cell/ml in heterotrophic, and

47.70 x 10⁵ cell/ml in mixotrophic, while *Chlorella sorokiniana* final cell concentration at 480 hour cultivation time of 39.4 x 10⁵ cell/ml in autotrophic, 50.7 x 10⁵ cell/ml in heterotrophic and 61 x 05 x 10⁵ cell/ml in mixotrophic condition.

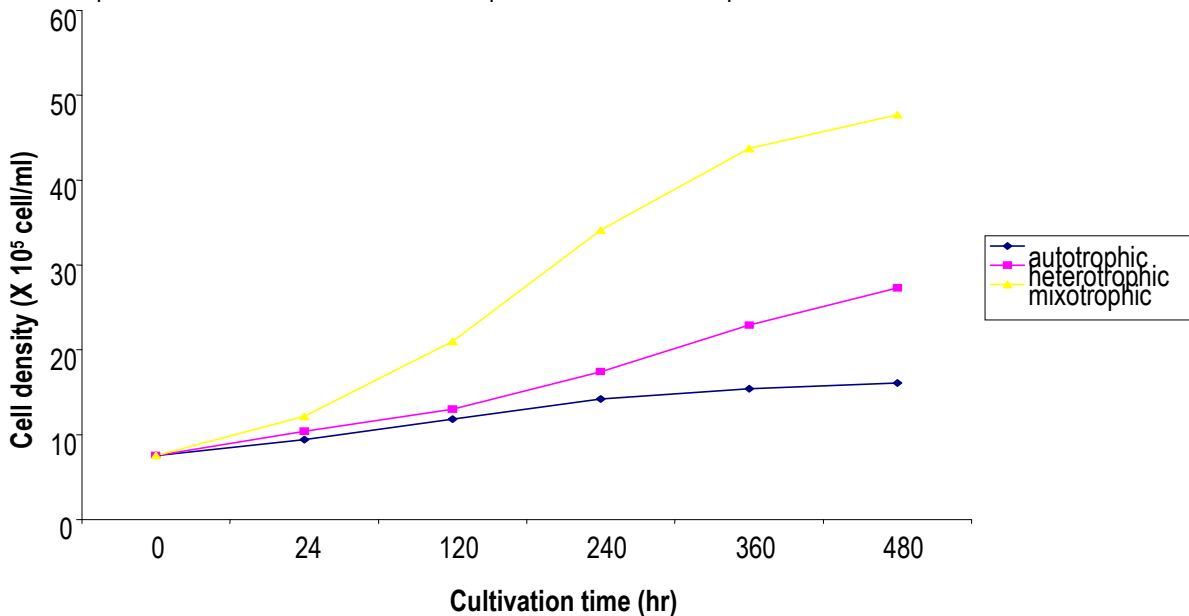


Figure 3: Characteristic growth rate of *Euglena gracilis* in Autotrophic, Heterotrophic and Mixotrophic condition in mixed cultivation system.

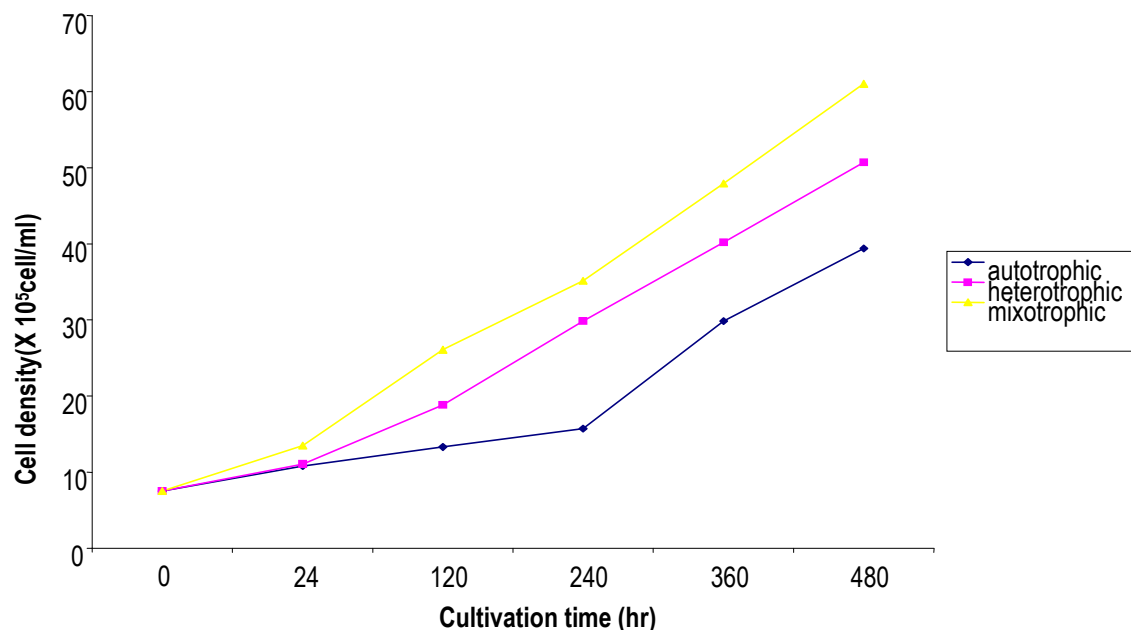


Figure 4: Characteristic growth rate of *Chlorella sorokiniana* in Autotrophic, Heterotrophic, and Mixotrophic condition in mixed cultivation system.

The growth characteristics of *Euglena* and *Chlorella* showed that, the cells grew better in mixotrophic followed by heterotrophic, while photoautotrophic culture gave the lowest cell growth. This may be as a result of light and an organic carbon (glucose) supplied simultaneously to the mixotrophic culture (Ogbonna et al 2000a & b). Another possible reason as reported by Ogbonna *et al.* (1997) is that at high cell concentrations, light becomes limiting and the autotrophic growth rate is very low in comparison with the heterotrophic growth rate. Thus in large scale systems, the cell growth rate and final cell concentration are expected to be much higher if the carbon concentration and light intensity are increased (Ogbonna *et al.* 1996). It was also reported that higher cell concentration can easily be obtained by using higher glucose concentration in heterotrophic phase provided efficient light is supplied to such a dense culture (Ogbonna et al. 1997). Fast growth rates and higher cell concentration recorded in mixotrophic culture as compared to other cultures is therefore expected. This is consistent with some reports of Ogbonna *et al.* (1997) which indicated that in mixotrophic both the heterotrophic and autotrophic metabolisms proceed simultaneously and independently resulting in the specific growth rates and final cell concentrations in the mixotrophic culture being

the sum of those in the photo-autotrophic and heterotrophic culture (Kobayashi et al. 1992; Endo et al. 1977).

Comparison of Mono and Mixed Cultivation: Under photo-autotrophic, heterotrophic and mixotrophic mixed culture conditions, the *Euglena* and *Chlorella* growth rates and final cell concentration were higher when compared to the values obtained in monocultures as showed in figures 1-2 & 3-4. An increased growth rate of *Chlorella* than that of *Euglena* in photoautotrophic, heterotrophic and mixotrophic cultures was also observed. In comparison of monocultivation with mixed cultivation, the results showed that cells grew faster in mixed cultures (figure 5-10). This may be as a result of glucose utilization in mixed culture, reduction in contamination in mixed culture (Ogbonna, 2000) or it may be as reason of (Endo et al. 1977), who reported that: "in heterotrophic and mixotrophic cultures, high cell concentration is achieved because the cell is capable of utilizing organic carbon source". The chlorophyll spectrum differences of the two cells may also enhance the growth rate (Kobayashi et al. 1992). Thus while *Euglena* may be affected by high light intensity, *Chlorella* may accommodate it, thus the relative presence of the two cells can control or prevent growth inhibition.

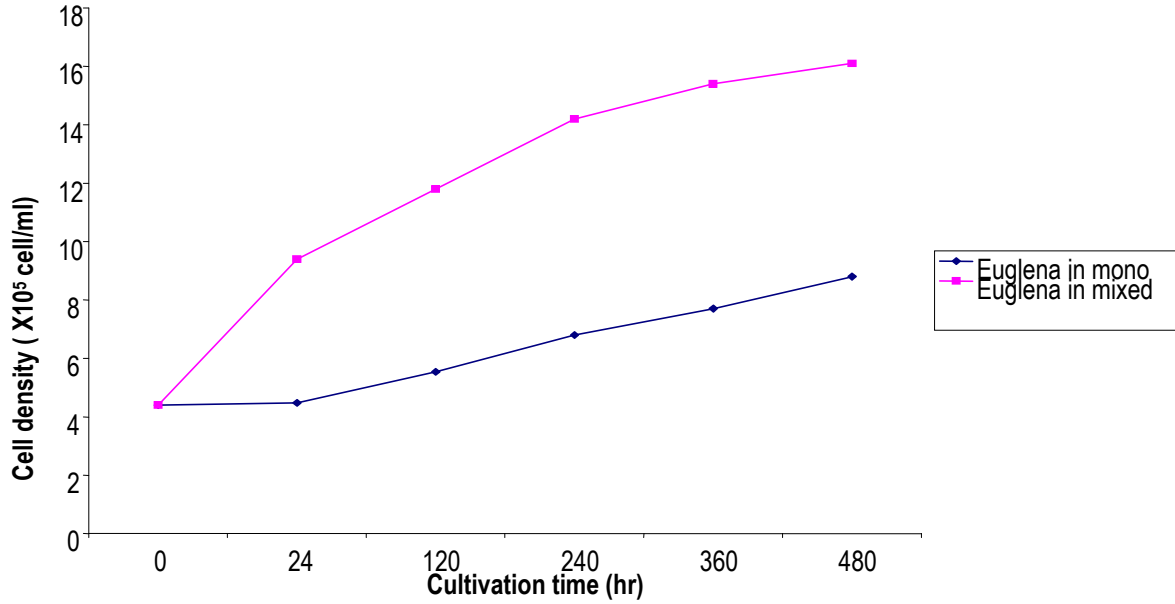


Figure 5: Comparison of growth rate of *Euglena gracilis* in autotrophic condition of monocultivation (mono) and mixed cultivation (mixed)

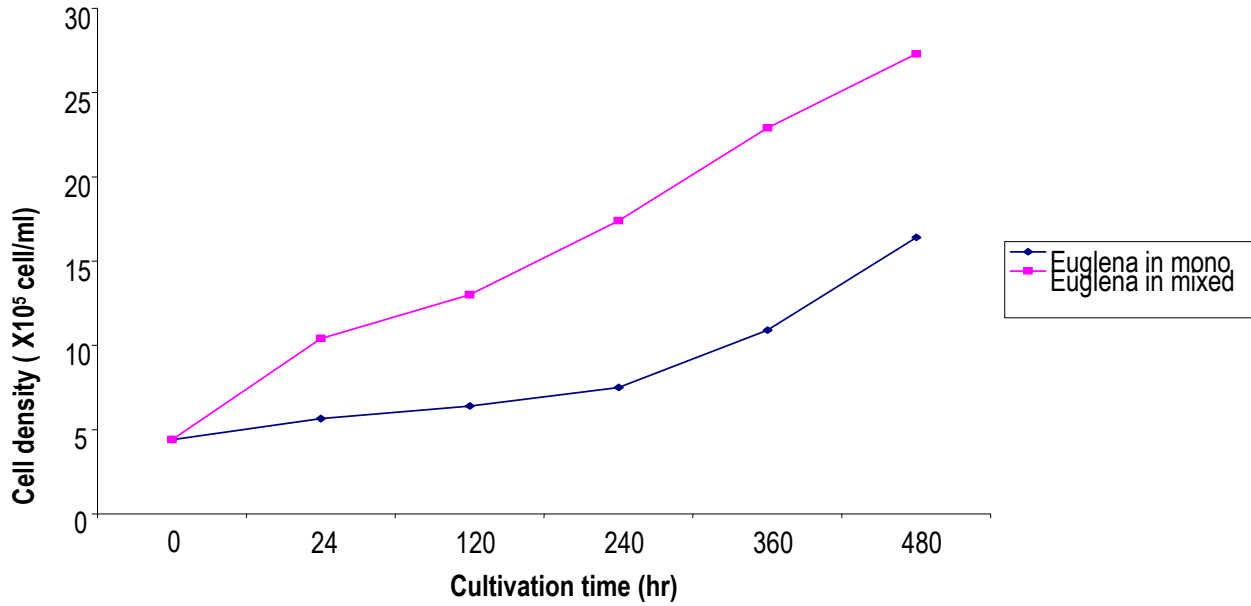


Figure 6: Comparison of growth rate of *Euglena gracilis* in Heterotrophic condition of monocultivation (mono) and mixed cultivation (mixed)

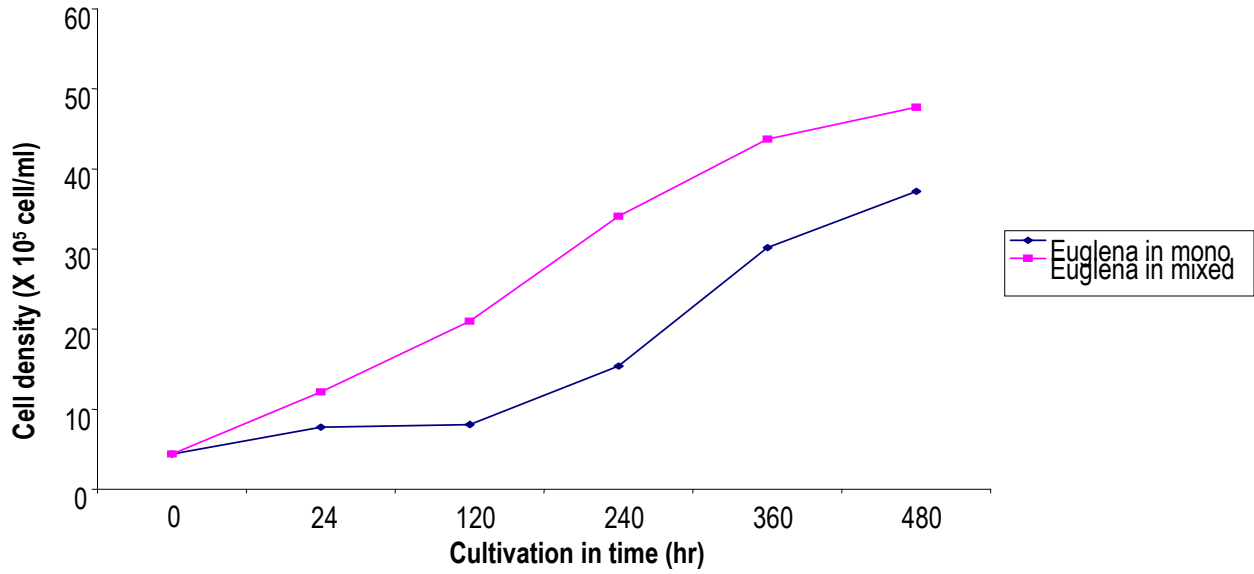


Figure 7: Comparison of growth rate of *Euglena gracilis* in mixotrophic condition of monocultivation (mono) and mixed cultivation (mixed)

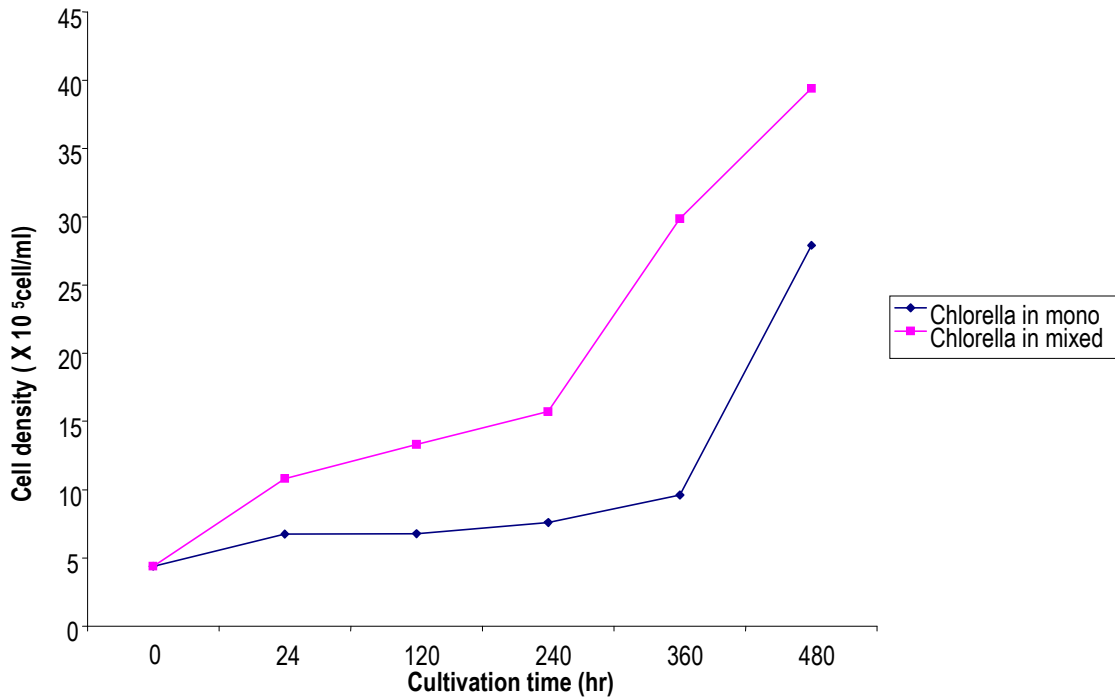


Figure 8: Comparison of growth rate of *Chlorella sorokiniana* in Autotrophic condition of monocultivation (mono) and mixed cultivation (mixed)

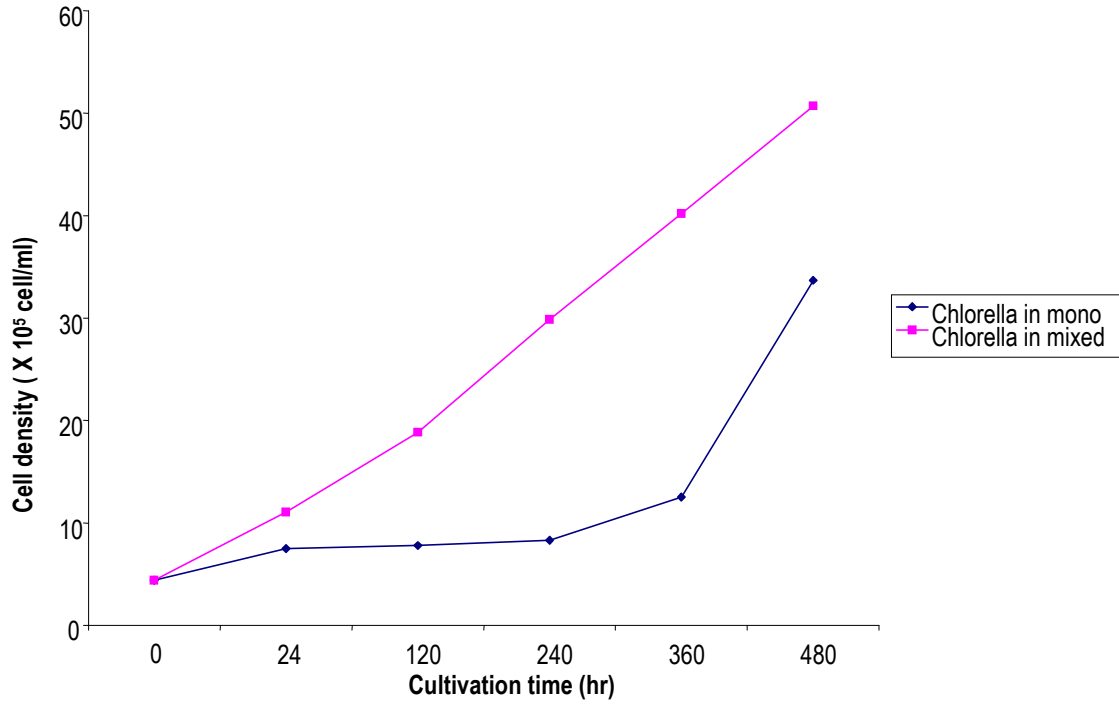


Figure 9: Comparison of growth rate of *Chlorella sorokiniana* in Heterotrophic condition of monocultivation (mono) and mixed cultivation (mixed)

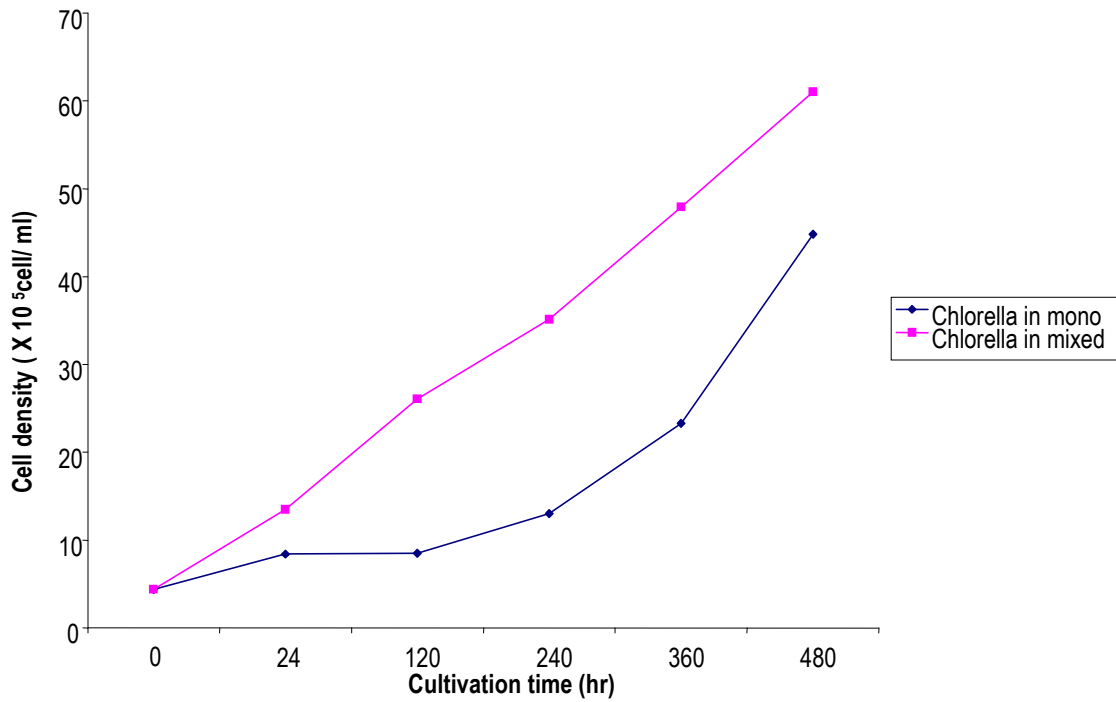


Figure 10: Comparison of growth rate of *Chlorella sorokiniana* in mixotrophic condition of monocultivation (mono) and mixed cultivation (mixed)

Effect of Mixed Culture on the Biomass Yield: Figure 11 shows the biomass yield from glucose consumed in mixed and monocultures. The results

showed that there were increased biomass yield both in heterotrophic and mixotrophic culture of mixed cultivation as compared to the yield in monocultivation.

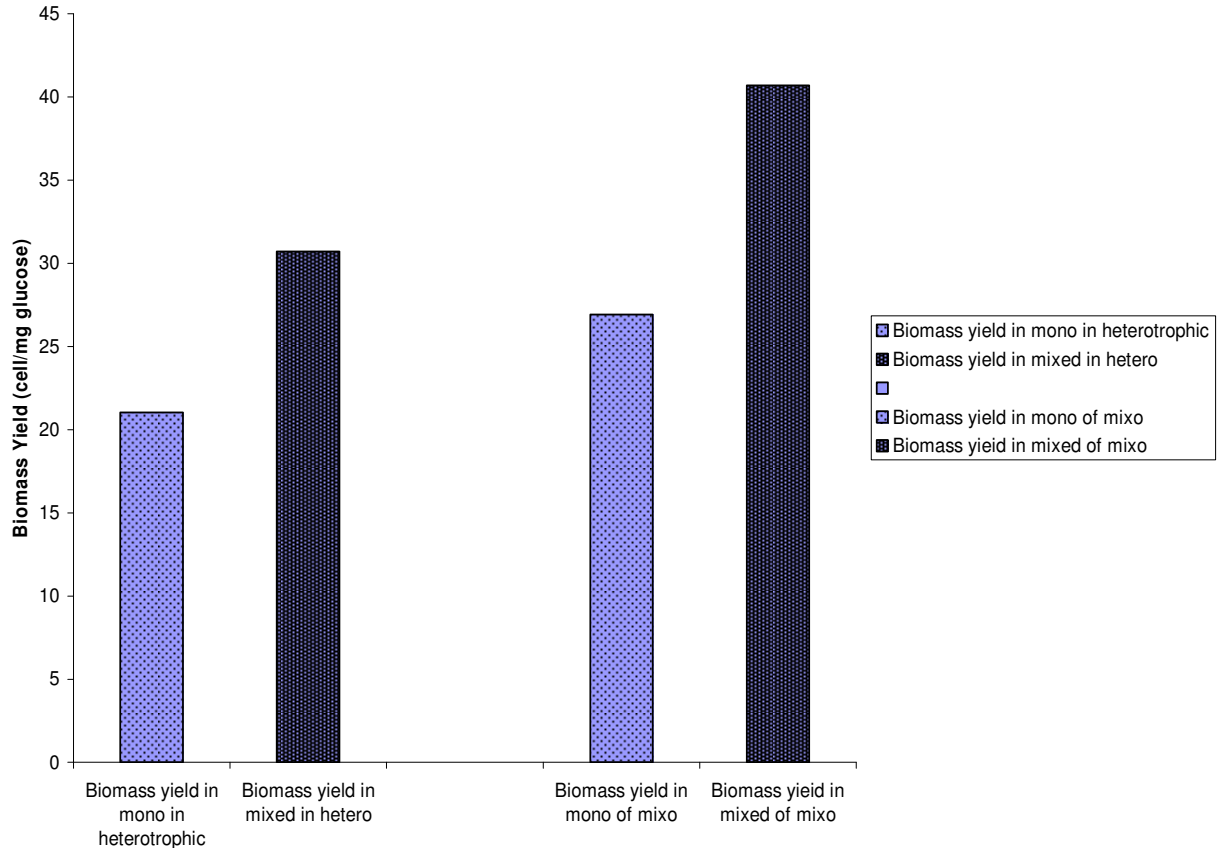


Figure 11: Biomass Yield in mixed and mono cultivation under Heterotrophic and Mixotrophic culture conditions

A total yield of 2.693×10^6 cell/mg glucose and 4.071×10^6 cell/mg glucose were obtained in heterotrophic and mixotrophic in mixed cultivation, respectively. On the other hand, a total biomass yield of 2.104×10^6 cell/mg glucose and 3.070×10^6 cell/mg glucose were recorded in monocultivation (see figure 11). Final cell concentration also showed that there was significant ($p \geq 0.05$) growth in mixed culture under all the modes of cultivation as compared to those obtained in monocultures. The results showed that biomass yield increased in the mixed culture when compared to

monocultivation in both cells. There was significant ($p \geq 0.05$) yield increase in heterotrophic mixed culture and mixotrophic mixed cultures over those obtained in monocultures.

The results above have demonstrated that, in large scale systems, the cell growth rate and thus final cell concentrations in photo-autotrophic, heterotrophic, and mixotrophic cultures are expected to be much higher if the organic carbon source concentration, and light intensity are increased and this will probably result in reduced contamination level.

CONCLUSION

From the results, it can be concluded that the growth rate and the biomass yield of *Euglena gracilis* and *Chlorella sorokiniana* improved appreciably in the mixed cultivation than monocultivation. This imply that

cultivation of *Euglena* and *Chlorella* cells together can be used to obtained optimum single cell protein/ vitamin rich algae biomass yield than when they are cultivated separately. Also, in view of the simplicity and the

economy of the methodology, the results obtained are very significant and demonstrated that mixed cultivation has a very high potential as substitute for the current monoculture systems. However, this result can be improved by optimizing the light intensity.

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