Effect of Ultrasonication on Growth of Chlorella sp. and Determination of Nitrate and Phosphorus Concentration in Culture Supernatant

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ABSTRACT

Aims: The study aims to investigate the effect of ultrasonic treatment of different durations which was given to the *Chlorella* sp. culture to analyze biomass, biomass productivity, chlorophyll and macronutrients such as nitrate and phosphate which was given to the *Chlorella* sp. culture.
Studydesign: In this study, the duration of exposure to ultrasonication treatment on live microalgal culture was optimized to estimate economically important biomass and pigments. Furthermore, using the optimized duration of ultrasonication treatment, residual nitrate and phosphate in the medium were also analyzed, which provides key insights into the nutrient consumption of *Chlorella* sp. under ultrasonication stress.

PlaceandDurationofStudy: The present investigation was carried out for 36 days to estimates effect of ultrasonication on biomass, chlorophyll, nitrate and phosphorous concentration in C*hlorella* culture at laboratory of Synthetic Ecology and Environment Biotechnology, Department of Environmental Science, School of Earth Sciences, Central University of Rajasthan, Bandarsindri, Ajmer

Methodology: In this study, the effects of ultrasonication on biomass and chlorophyll content were evaluated by treating *Chlorella* sp. cultures for 5, 10, and 15 minutes, respectively, in addition to an untreated control group.Based on the results, the optimized ultrasonication duration of 5 minutes was selected for further analysis. In this phase, the nitrate and phosphorus content in the culture medium were measured to investigate the influence of ultrasonication on these nutrient levels. All treatments were conducted in triplicates to ensure statistical reliability and reproducibility of results.

Results: The biomass and biomass productivity concentration increased from 29.9 ± 0.00240 mg/L (control) to 66.8 ± 0.01485 mg/L, 1.275 ± 0.5033 (control) to 3.71 ± 0.4163 mg/L/day respectively. The highest total chlorophyll concentration was observed in the exponential phase (9 day of culture) which was 3.7772 ± 0.0500. There is a decrease in the concentration of Nitrate and phosphate throughout the culture period, with total nitrate consumption of 38.5% and total phosphate consumption of 24% observed.

Conclusion: Chlorophyll and biomass concentration was maximized with 5 minutes of ultrasonic treatment but there is declined in the concentration with longer exposure. Nutrient analysis revealed a significant decrease in nitrate and phosphate concentrations over time, consistent with nutrient uptake by the growing cells and the subsequent accumulation of metabolites

Keywords:Chlorella, Microalgae, Ultrasonication, Biomass, Cell biovolume, Macronutrients

1.INTRODUCTION

Microalgae are considered as highly innovative and a promising source of the biomass for biorefinery applications especially for producing biofuels like biodiesel from microalgal oil[1].Beyond biofuels, microalgae are a rich source of valuable biproducts[2], such as pigments, proteins, carbohydrates etc.[3]. These products have significant applications in the food and feed industries, which shows the versatility of microalgae in the various biotechnological applications[4]. During the growth cycle of microalgae, it possesses incredible capacity to accumulate a variety of different macromolecules. Due to this unique characteristic which endows them in versatility in the composition which rarely seen in the other organism[5,6,7]. The growth conditions in the microalgae plays an important role in determining the macromolecular profile of the microalgae for example during nutrient starvation conditions in the microalgae its shifts its metabolism and enhances the accumulation of carbohydrates which are used as energy reserves in the late phase.

Chlorella is an important nutritional source for human consumption and contains high levels of proteins and other important nutrients[8]. The cell wall of *Chlorella* is very compact; its disruption requires a greater amount of energy. The proximate composition of microalgae may alter significantly during the stationary phase, as nitrate starvation causes an increase in carbohydrate levels[9]. A major challenge for utilizing micro algal biomass in biorefinery facilities is the requirement for downstream processes that are sufficiently effective to extract important chemicals in a sustainable manner. Disruption of the micro-algal cell wall is an important step in increasing product recovery in the later stages of algal biorefineries, which aim to use algal biomass as a sustainable resource. To disrupt the algal cell wall, various physical-mechanical and bio- chemical technologies have been applied to increase the intracellular microalgae content[10]. Ultrasonic treatment ruptures the microalgal cell wall, allowing the extraction of internal chemicals directly in water for commercial purposes[11]. During ultrasonic treatment, there was an increase in the surface area of the algal cells, which indicated the breakdown of the cell wall and its disintegration. There is a change in the spherical shape of *Chlorella* to an asymmetrical shape under the revelation of ultrasonic energy and intracellular components released in the surrounding medium[12].

Several studies have been conducted in the past to enhance the extraction yield of microalgal products using ultrasonication, but no study has reported the accumulation of value-added products using ultrasonication on live microalgal cultures. In this study, the duration of exposure to ultrasonication treatment on live microalgal culture was optimized to estimate economically important biomass and pigments. Furthermore, using the optimized duration of ultrasonication treatment, residual nitrate and phosphate in the medium were also analyzed, which provides key insights into the nutrient consumption of *Chlorella* sp. under ultrasonication stress.

2.MATERIALANDMETHODS

2.1 Micro algae cultivation and medium composition

Microalgal samples (*Chlorella* sp.) were collected from the Laboratory of Synthetic Ecology and Environment Biotechnology, Department of Environmental Science, School of Earth Sciences, Central University of Rajasthan, Bandarsindri, Ajmer. In this study, microalgae samples were cultivated in bold basal medium (BBM). The initial pH of the BBM medium was 6.8-7.2. Microalgae cultivation was carried out using a 1 L flask and kept at room temperature (27 ± 0ºC). The culture was maintained at 24:0 (Day: Night) under light conditions until it reached its stationary phase.

2.2 Biomass Harvesting

Ultrasonic treatment was applied to the cultures for 1-, 5- and 10-minutes excluding control and each treatment was conducted in set a of triplicates. The process of centrifugation was used for biomass harvesting at 4500 rpm for 15 minutes and dry cell weight was calculated.

2.3 Chlorophyll estimation

Chlorophyll concentration was estimated using 80% acetone method. The ultrasonic treatment was applied for the duration of 1,5,10 minutes prior to the estimation of chlorophyll content. Chlorophyll a and b was calculated by using the equation provided by Jeffrey and Humprey (1975)[13]:

Chlorophyll a = (11.93*A664) -(1.93*A647)

Chlorophyll b = (20.36*A647) -(5.50*A664)

Total Chlorophyll= (Chlorophyll A + Chlorophyll B)

2.4 Macronutrients analysis

Total and Particulate nitrate was analyzed by using Salicylate method (TRI- reagent method). Total and particulate phosphorous was analyzed by using Ascorbic acid method (APHA,2007)[14].

3.RESULTSANDDISCUSSION

3.1 Biomass concentration

The concentration of biomass in the control group was 29.9 ± 0.00240mg/L. There was an increase in biomass concentration when the culture was treated by ultrasonication for 1 min at 47.3 ± 0.00164 mg/l. The maximum biomass (66.8 ± 0.01485 mg/L) was observed after 5 min of ultrasonic treatment. However, when ultrasonication was applied for > 5 min, there was a slight decrease in the biomass. After 10 min of ultrasonic treatment, the biomass content was 56.3 ± 0.00566 mg/L. The highest biomass productivity was observed after 5 min of ultrasonication treatment with a total biomass of 66.8 mg/L which was 2.91 folds more than the control (22.9 mg/L). In the ultrasonic pre-treatment for biomass extraction, it was observed that the biomass concentration increased when ultrasonic pre-treatment was applied for 1 and 5 min. However, with an increase in the duration of ultrasonic pre-treatment (10 min), the biomass concentration decreased. One-minute ultrasonic pretreatment resulted in a short exposure, which caused a slight interruption that resulted in a small increase in the biomass. Five minutes of ultrasonication treatment was more extensive and caused more cell disruption, which increased the mass transfer of the intracellular components present in the medium[15].

Table:1Effect of ultrasonic treatments on Biomass (Mean SD N=4)

Treatments	**Biomass (mg/l)**	**Biomass Productivity (mg/L/day)**
Control	22.9 ± 0.0024	1.275 ± 0.5033
1 minute	47.3 ± 0.0016	2.62 ± 0.2886
5 minutes	66.8 ± 0.0148	3.71 ± 0.4163

10 minutes	56.3 ± 0.0056	3.310 ± 0.6557

**Ultrasonic treatment of different duration to analyze Biomass concentration*

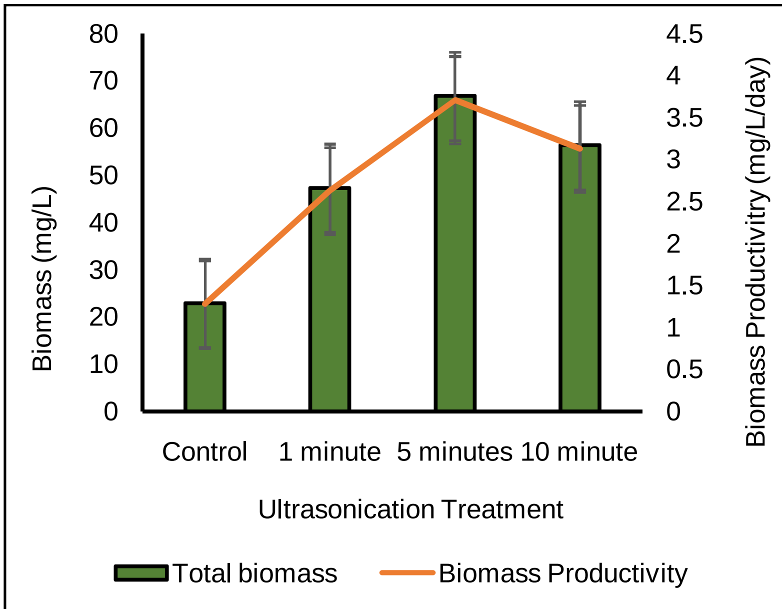

Fig.1 Effect of ultrasonic treatment on biomass and biomass productivity

3.2 Chlorophyll estimation

The Chlorophyll concentration was increased when the culture was optimized for 1 and 5 minutes. The more concentration of chlorophyll was observed in 5 minutes of ultrasonic optimization from the induction phase to stationary phase. It was observed that the chlorophyll concentration was decreased when the culture is optimized for 10 minutes. Table 2 and figure 2 illustrated that in the exponential phase, it was observed that the chlorophyll b concentration is more than chlorophyll a on the 9 and 12 day of the culture which was 1.9593 ± 0.00433, 1.7764 ± 0.03126 respectively. The highest total chlorophyll concentration was observed in the exponential phase (9 day of culture) which was 3.7772 ± 0.0500. As the culture aged there is decrement in the chlorophyll content in the stationary phase due to the less availability of the nutrients as in the logarithmic phase the chlorophyll content was more because of the availability of nutrients[16]. Chlorophyll a decrease with increase in the concentration of the nitrate in the medium. The availability of the nutrients affects the chlorophyll content in microalgal species.

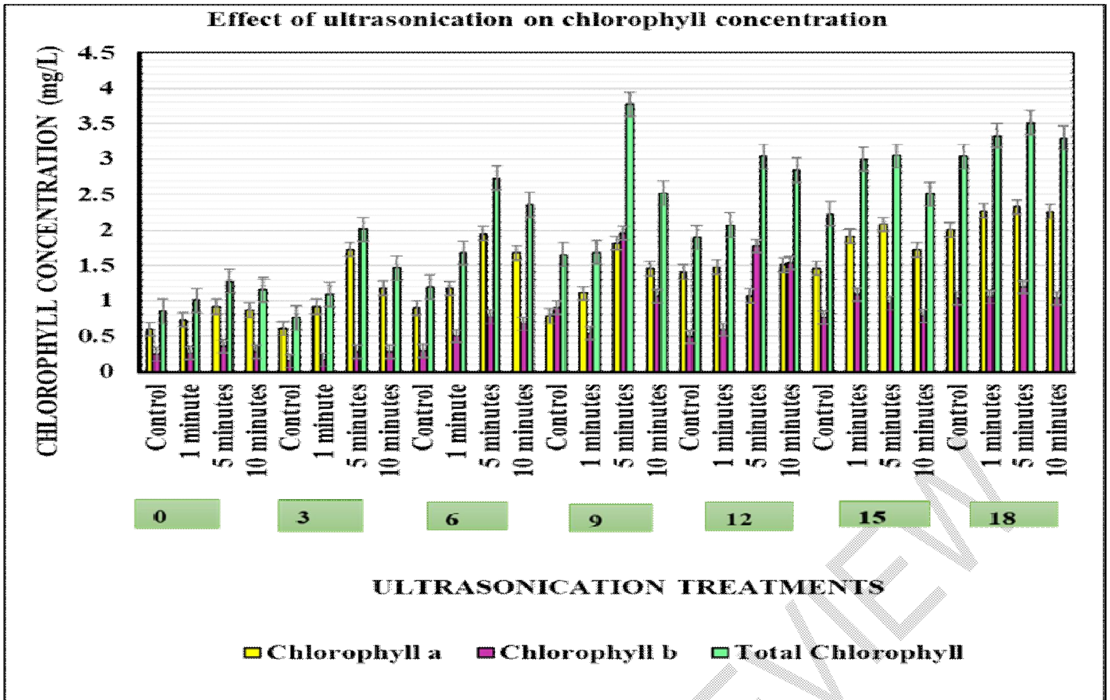

Fig.2 Chlorophyll concentration in different ultrasonic treatments

Table: 2 Chlorophyll concentration in growing culture of *Chlorella* sp.

Days of culture	**Treatments**	**Chlorophyll a**	**Chlorophyll b**	**Total Chlorophyll**
0 day	Control	0.6028 ±0.135	0.2521 ± 0.006	0.8548 ± 0.129
	1 minute	0.7371 ±0.044	0.2663 ± 0.0076	1.0033 ± 0.051
	5 minutes	0.9174 ±0.002	0.3598 ± 0.062	1.2771 ± 0.064
	10 minutes	0.8685 ±0.027	0.289 ± 0.041	1.15841 ± 0.014
3 days	Control	0.6123 ± 0.012	0.1527 ± 0.072	0.7650 ± 0.084
	1 minute	0.9166 ± 0.084	0.1721 ± 0.058	1.0887 ± 0.142
	5 minutes	1.7258 ± 0.302	0.2876 ± 0.046	2.0135 ± 0.348
	10 minutes	1.1790 ± 0.204	0.2870 ±0.192	1.4660 ± 0.397

6 days	Control	0.8999 ±0.007	0.2919 ± 0.005	1.1918 ± 0.001
	1 minute	1.1751 ± 0.005	0.5058 ± 0.057	1.6808 ± 0.063
	5 minutes	1.9546 ± 0.050	0.7792 ± 0.069	2.7338 ± 0.019
	10 minutes	1.6821 ± 0.285	0.6809 ±0.154	2.3630 ±0.440
9 days	Control	0.7901 ± 0.050	0.9006 ± 0.021	1.6536 ± 0.054
	1 minutes	1.1080 ± 0.003	0.5455 ± 0.057	1.6907 ± 0.071
	5 minutes	1.8179 ± 0.045	1.9593 ± 0.004	3.7772 ± 0.050
	10 minutes	1.4586 ± 0.035	1.0667 ± 0.013	2.5252 ±0.021
12 days	Control	1.4083 ± 0.068	0.4932± 0.033	1.9015 ± 0.101
	1 minutes	1.4789 ± 0.032	0.5962 ± 0.069	2.0751 ± 0.102
	5 minutes	1.0695 ± 0.052	1.7764 ± 0.031	3.0411 ± 0.073
	10 minutes	1.5090 ± 0.032	1.5321 ± 0.041	2.8459 ± 0.084
15 days	Control	1.4601 ± 0.028	0.7680 ± 0.054	2.2281 ±0.025
	1 minutes	1.9153 ± 0.096	1.0854 ± 0.060	3.0007 ± 0.096
	5 minutes	2.0823 ± 0.014	0.9666 ± 0.149	3.0489 ± 0.163
	10 minutes	1.7209 ± 0.017	0.7896 ± 0.118	2.5104 ± 0.136
18 Days	Control	2.0067± 0.000	1.0352 ± 0.272	3.0419 ± 0.271
	1 minutes	2.2751± 0.022	1.0578 ± 0.746	3.3329 ± 0.768

	5 minutes	2.3263± 0.014	1.1890 ± 0.523	3.5153 ± 0.538
	10 minutes	2.2656 ± 0.017	1.0329± 0.013	3.2985 ± 0.004

Concentration of chlorophyll a, b and total chlorophyll in Chlorella sp. culture

3.3 Nitrate and Phosphorous concentration

The initial concentration of the total nitrate in the media before inoculation was 49.97 mg/L and after inoculation the total nitrate concentration was 53.6 ± 0.3214 mg/L. The total nitrate consumption without ultrasonic optimization in the *Chlorella* culture was 38.5 % from the lag phase to the stationary phase. (Table 3, 4 and figure 3, 4 illustrate the data of total nitrate and particulate nitrate concentration without ultrasonic optimization and with ultrasonic optimization). It was observed that there is fluctuation in the total nitrate concentration during the exponential phase and particulate nitrate concentration was decrease from lag phase to stationary phase. Similarly, the total phosphate concentration in media before inoculation was 30 mg/L. There was a significant decrease in the concentration of the phosphate from lag phase to stationary phase in the culture of the *Chlorella* sp. The total phosphate consumption was 24% in the *Chlorella* sp. culture. There was also a decrease in the concentration of the total and particulate phosphate from the induction phase to the stationary phase of growth. (Table5,6 and Figure 5,6 illustrate the data of total and Particulate Phosphate concentration in the culture supernatant without ultrasonic optimization and with ultrasonic optimization.). In present study it was observed that there was decrease in the concentration of the nitrate and phosphate in the culture because of nutrients are uptake by the cells for their growth. It was also observed that there is decrease in the nutrient concentration inside the cell also because nitrate and phosphate are essential nutrients which are utilized by the microalgae for various metabolic activities, protein synthesis, nucleic acid synthesis and for energy production. Nitrogen is an important element which is required for synthesis of the nucleic acids, proteins, amino acid (including enzymes, coenzymes), chlorophyll and metabolic activities. The reduction in the concentration of the nitrogen lead to slow growth and produce stress conditions, which trigger the microalgae to accumulate more lipid and carbohydrate. Like Nitrate, Phosphorous is also an essential constituent of the nucleic acid, cell energy carriers and bio membrane system. Limitation of phosphorous also leads to the stress conditions which allow the microalgae to accumulate energy storage metabolites[17,18].

Table: 3. Total nitrate concentration (mg/L) (Mean SDN=4)

Days of Culture	Total Nitrate without optimization (mg/L)	Total nitrate with ultrasonic optimization (mg/L)
0 day	53.6 ± 0.3214	48.2 ± 0.4582
3 days	43.3± 0.2516	45.3 ± 0.1527
6 days	33.3±0.2645	38.4 ± 0.2086
9 days	38.06 ± 0.6082	29.1 ± 0.2081
12 days	29.3 ± 0.4256	23.3 ± 0.2516
15 days	18.8 ±0.3511	22.2 ± 0.1905

18 days	15.1 ± 0.3785	18.8 ± 0.0577

Total nitrate concentration was estimated for 18 days (every 3 day) with and without ultrasonic optimization

Table: 4. Particulate Nitrate concentration (mg/L)

Days of culture	**Particulate Nitrate without ultrasonic optimization (mg/L)**	**Particulate Nitrate with ultrasonic optimization (mg/L)**
0 day	14.5 ± 0.2081	16.6 ± 0.1527
3 days	12.10 ± 0.1732	15.3 ± 0.2645
6 days	10.4 ± 0.1527	11.2 ±0.0577
9 days	5.73± 0.2086	6.33 ±0.1527
12 days	4.87±0.1154	6.03±0.0577
15 days	3.17 ±0.0577	5.43±0.3214
18 days	2.13± 0.0580	4.8 ± 0.200

Particulate nitrate concentration was estimated for 18 days (every 3 day) with and without ultrasonic optimization

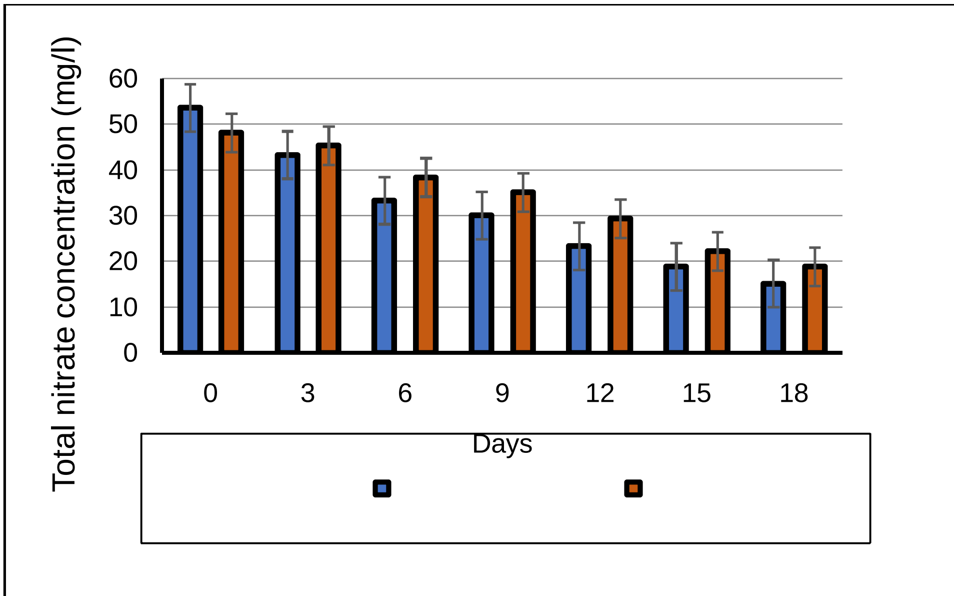

Fig.3 Total nitrate concentration was analyzed with and without ultrasonic treatment in culture supernatant

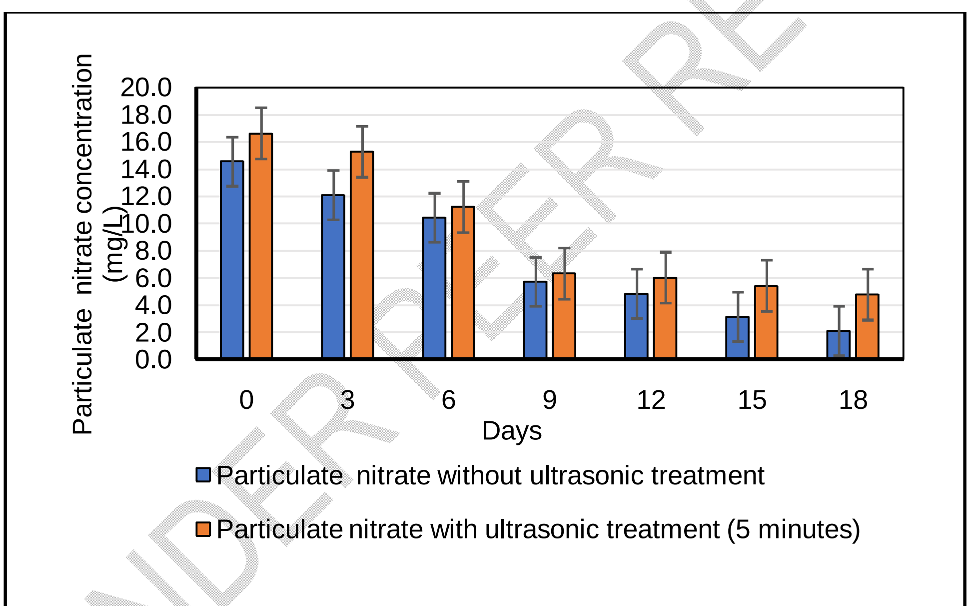

Fig.4 Particulate nitrate concentration in culture supernatant with and without ultrasonic treatment

Days of culture	Total Phosphate without optimization (mg/L)	Total Phosphate with ultrasonic optimization (mg/L)
0 day	27.2 ± 0.2645	29.5 ± 0.2309

3 days	19.7 ± 0.5777	22.2 ± 0.0577
6 days	17.6 ± 0.5507	20.1 ± 0.1527
9 days	15 ± 0.3464	19.6 ± 0.1537
12 days	5.58 ± 0.8036	10.1 ± 0.2081
15 days	4.13 ± 0.1154	6.7 ± 0.1732
18 days	2.96 ± 0.1527	3.3 ± 0.1527

Table:5Particulate Phosphate Concentration in culture supernatant (mg/L) (Mean SDN =4)

**TotalPhosphate concentration was estimated for 18 days (every 3 day) with and without ultrasonic optimization*

Table: 6**.** Particulate Phosphate Concentration in culture supernatant (mg/L) (Mean SDN =4)

Daysof culture	Particulate Phosphate without optimization (mg/L)	Particulate Phosphate with ultrasonic optimization (mg/L)
0 day	16.7 ± 0.5196	16 ± 0.5032
3 days	10.1 ± 0.3214	10.8 ± 0.2516
6 days	8.8 ± 0.2516	10.7 ± 0.2886

9 days	7 ± 0.5033	9.7 ± 0.4163
12 days	6.2 ± 0.2645	9.1 ± 0.6557
15 days	5.2 ± 0.2081	7.1 ± 0.1154
18 days	4.4 ± 0.2645	6.3 ± 0.1527

**ParticulatePhosphate concentration was estimated for 18 days (every 3 day) with and without ultrasonic optimization*

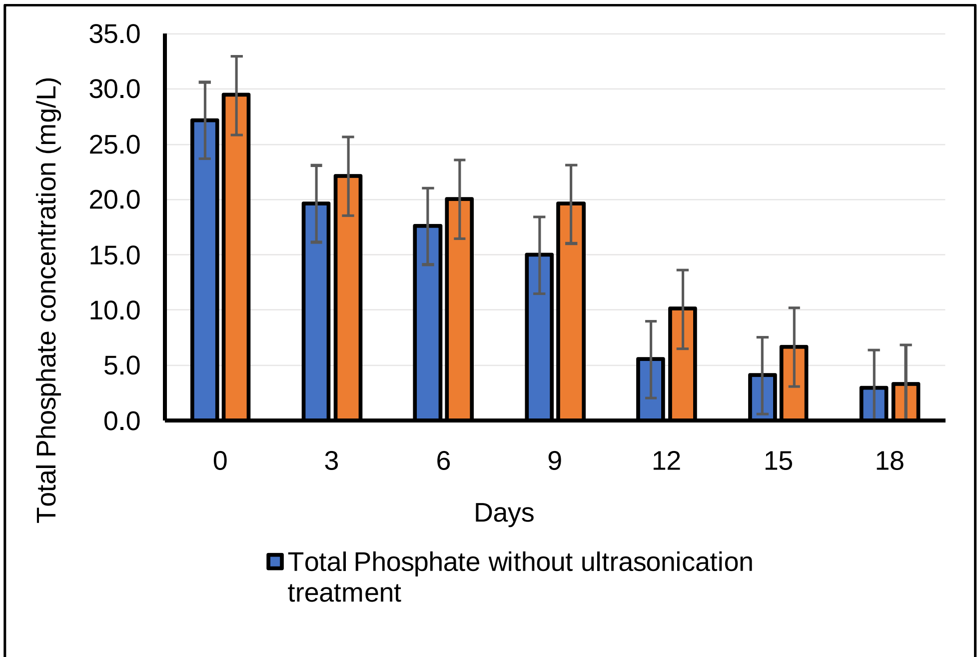

Fig. 5 Total Phosphate concentration in culture supernatant

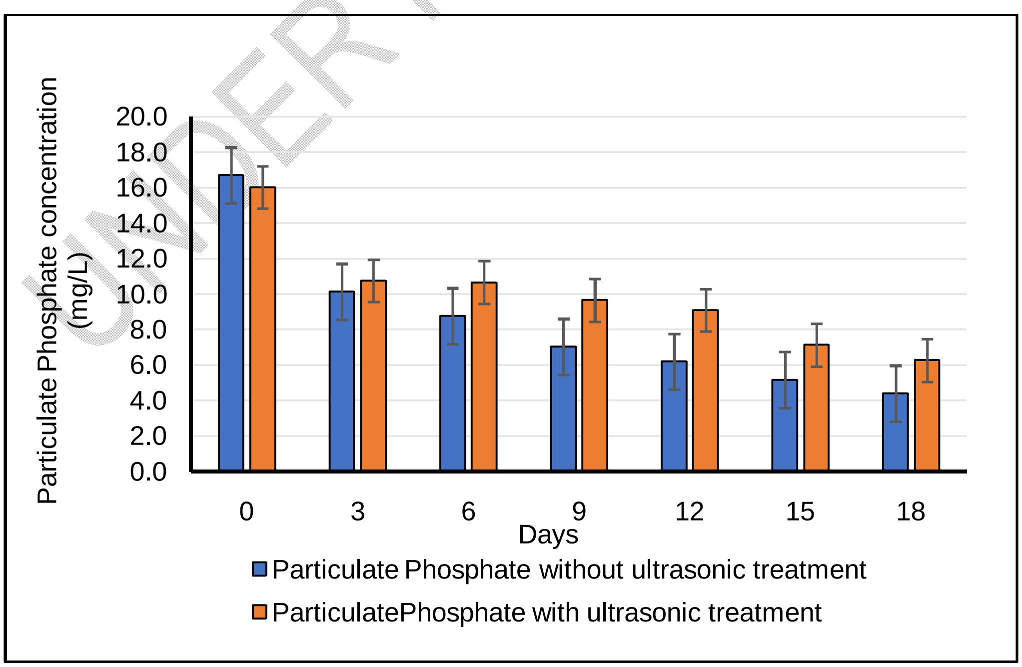

Fig.6 Particulate Phosphate concentration in culture supernatant

4.CONCLUSION

Ultrasonication induces stress in *Chlorella* cultures, which enhances the accumulation of secondary metabolites. This study found that ultrasonic treatment had a significant effect on major physiological and biochemical parameters in *Chlorella* sp. Chlorophyll concentration was maximized with 5 minutes of ultrasonic treatment but there is declined in the concentration of chlorophyll with longer exposure, which indicates a potential trade-off between cell disruption and pigment preservation. Nutrient analysis revealed a significant decrease in nitrate and phosphate concentrations over time, consistent with nutrient uptake by the growing cells and the subsequent accumulation of metabolites. Overall, ultrasonic treatment effectively enhances biomass productivity and nutrient utilization in microalgal cultures, offering a valuable approach for optimizing algal biomass production and metabolic profile in various biotechnological applications. Further studies could explore the long-term effects and optimize treatment parameters for improved industrial scalability

REFERENCES

1. Dolganyuk, V., Belova, D., Babich, O., Prosekov, A., Ivanova, S., Katserov, D., Patyukov, N., & Sukhikh, S. Microalgae: A Promising Source of Valuable Bioproducts. Biomolecules, (2020) 10 (8), 11531
2. Zhang, S., Zhang, L., Xu, G., Li, F., & Li, X. A review on biodiesel production from microalgae: Influencing parameters and recent advanced technologies. Frontiers in Microbiology, (2022) 13, 970028.
3. Nair, A., Ahirwar, A., Singh, S., Lodhi, R., Lodhi, A., Rai, A., Jadhav, DA., Harish, Varjani, S., Singh, G. Astaxanthin as a King of Ketocarotenoids: Structure, Synthesis, Accumulation, Bioavailability and Antioxidant Properties. Marine Drugs, (2023) 21(3), 176
4. Geada, P., Moreira, C., Silva, M., Nunes, R., Madureira, L., Rocha, C. M., ... & Teixeira, J. A. Algal proteins: Production strategies and nutritional and functional properties. Bioresource Technology, (2021) 332, 125125
5. Foley, P. M., Beach, E. S., & Zimmerman, JB. Algae as a source of renewable chemicals: opportunities and challenges. Green Chemistry, (2011) 13(6), 1399-1405.
6. Yang, L., Chen, J., Qin, S., Zeng, M., Jiang, Y., Hu, L. & Wang, J. Growth and lipid accumulation by different nutrients in the microalga Chlamydomonas reinhardtii. Biotechnology for biofuels, (2018) 11, 1-12
7. Mourya, M., Khan, M. J., Ahirwar, A., Schoefs, B., Marchand, J., Rai, A., ... & Vinayak, V. Latest trends and developments in microalgae as potential source for biofuels: The case of diatoms. Fuel, (2022) 314, 122738
8. Lorenzo K, Santocildes G, Torrella JR, Magalhães J, Pagès T, Viscor G, Torres JL, Ramos-Romero S. Bioactivity of Macronutrients from Chlorella in Physical Exercise. Nutrients. 2023; 15(9):2168
9. Ambati, RR. Nitrogen, Phosphorus and Microalgal: In Encyclopedia (2021). https://encyclopedia.pub/entry/7701

10. Di Caprio, F., Altimari, P., & Pagnanelli, F.. Ultrasound-assisted extraction of carbohydrates from microalgae. Chemical Engineering Transactions, (2021) 86, 25-30.
11. Chemat, F., Rombaut, N., Meullemiestre, A., Turk, M., Perino, S., Fabiano-Tixier, AS & Abert-Vian, M.. Review of green food processing techniques. Preservation, transformation, and extraction. Innovative Food Science & Emerging Technologies, (2017) 41, 357-377.
12. Huang, Y., Yang, L., You, S., Gan, W., Kainer, KU., & Hort, N. Unexpected formation of hydrides in heavy rare earth containing magnesium alloys. Journal of Magnesium and Alloys, (2016) 4(3), 173–180.
13. Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. BiochemPhysiolPflanzen 167:191–194
14. https://r.search.yahoo.com/_ylt=Awr984mc8OZm0v8K3NpXNyoA;_ylu=Y29sbwNncTEEcG9zAzMEdnRpZAMEc2VjA3Ny/RV=2/RE=1727620509/RO=10/RU=https%3a%2f%2farchive.org%2fdetails%2fgov.law.apha.method.2320.1992/RK=2/RS=n6oQf0rttSEH2WzzZqJo5IyTPPw-
15. Naveena, B., Armshaw, P. & Tony Pembroke, J. Ultrasonic intensification as a tool for enhanced microbial biofuel yields. *Biotechnol Biofuels* **8**, 140 (2015). https://doi.org/10.1186/s13068-015-0321-0
16. Fiz Da Costa, Fabienne Le Grand, Claudie Quere, Gael Bougaran, Jean Paul Cadoret, et al., Effects of growth phase and nitrogen limitation on biochemical composition of two strains Tisochrysis lutea. Algal Research - Biomass, Biofuels and Bioproducts, 2017, 27, pp.177-189
17. Ratomski P, Hawrot-Paw M. Influence of Nutrient-Stress Conditions on *Chlorella vulgaris* Biomass Production and Lipid Content. *Catalysts*. 2021; 11(5):573. https://doi.org/10.3390/catal11050573
18. Estevam, B.R., Pinto, L.F.R., Filho, R.M. *et al.* Growth and Metabolite Production in *Chlorella* sp.: Analysis of Cultivation System and Nutrient Reduction. *Bioenerg. Res.* **16**, 1829–1840 (2023). https://doi.org/10.1007/s12155-022-10532-z