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Evaluation of Nutrient Stress (Nitrogen, Phosphorus Regimes) on Physio-Biochemical Parameters of Oleaginous Micro algal Strains and SEM Study under Nutrient Stress

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Abstract

The objective of this study was to investigate the accumulation of lipid, biomass, photosynthetic pigment, protein and carbohydrates from various micro algal strains. Algal cultures were grown in BG-11 medium inoculated into nitrogen (N) and phosphorus (P) en rich and stress medium. Three different treatments were set up: N+P+ (control group); (N-P+); (N+P-) respectively for algal growth evaluation in the form of lipid accumulation, biomass yield, protein, carbohydrate and total chlorophyll contents. When the cells were grown in BG-medium under nutrient stress (N,P) for 12 days, in (N+P+) nutrient regime, significant (P≤0.05) higher biomass yield 1.129±0.036 gL-1 and 1.115±0.021gL-1 have been reported in Nannochloropsis oculata, Chlorella pyrenoidosa respectively. Interestingly, nitrogen deficiency condition promoted (P≤0.05) significant higher lipid accumulation 25.75%, 23.78% and 20.26% in Nannochloropsis oculata, Chlorella pyrenoidosa and Scenedesmus obliquus respectively as comparison was made with other nutrient stress. On the other hand, (nitrogen+ phosphorus) condition promoted higher chlorophyll, carbohydrates and protein content in almost all algal species.SEM study also conducted under normal and stress condition for these micro algal strains (Chlorella, Scenedesmus, Nannochloropsis and Chlorococcum). Results showed, cells wall of algal species smooth under normal condition, while under stress condition distorted cell morphology. Thus, this native micro alga strain could be a potent candidate for feed, food or bio fuel production.

Keywords: Microalgae; Nutrient Stress; Biomass; Lipid; Sem; Oleaginous Algae; Nitrogen; Phosphorus Deficiency; Biomass; Lipid; Scanning Electron Microscopy

Introduction

As global population and consequently energy demand increase over time the introduction and commercialization of renewable sources of energy becomes a critical issue. Microalgal biomass as feedstock for bio-energy production is an attractive alternative to bio-energy derived from terrestrial plant utilization [1,2]. Micro algal biotechnology has gained increasing attention over the last few decades as a next-generation driver for obtaining food, feed and bio fuels and to carry out bioremediation of effluents and CO_2 mitigation [3]. Oleaginous microalgae are well known as promising candidates for renewable energy production mainly because of high biomass productivity and lipid content Chisti [4,5]. Microalgae, cultivated under specific stress conditions, can accumulate, along with the lipids and carbohydrates, considerable amount of secondary metabolites, whose industrial exploitation strongly enhances a bio-based economy [6].

Nitrogen and phosphorus, as the two main nutrients, are hypothesized to influence the attachment efficiency and growth of microalgae [7,8]. Similarly, phosphorus is an essential nutrient for the growth of microalgae as it plays a significant role in cellular metabolic processes related to energy transfer, signal transduction, photosynthesis and respiration. Studies have shown that the phosphorous deprived conditions responsible for significant lipid accumulation in Chlorella spp. Chaetoceros spp. Phaeodactylum tricornutum, Isochrysis galbana and Pavlovalutheri [9-13]. In addition to these factors, nitrogen deficiency severely affects protein synthesis and reduces photosynthetic rates which result in metabolic flux towards lipid biosynthesis [14,15]. High lipid accumulation was reported under nitrogen deprived conditions in microalgal species viz., Neochloris oleoabundans, Nannochloris sp., Chlorella muelleri and Scenedesmus sp. [16-19]. According to Li, et al. [20] phosphorus strongly influenced Chlorella vulgaris growth

but has little influence on lipid accumulation. Anand, et al. [21] revealed that the 2.27-fold increase in lipid yield (226 mg/L) was observed in nitrogen-depleted condition when compared to nitrogen rich condition (99.33 mgl^{-1}). The present study aimed at to evaluate the nutrient stress of Nitrogen and phosphorus, simultaneously scanning electron microscopy study of various micro algal species viz. Chlorella, Nannochloropsis, Chlorococcum, Scenedesmus sp. Under nutrient stress.

Materials and methods

Collection of Water Samples Having Algal Growth

The water samples having algal growth were collected in pre cleaned sterilized plastic containers from different fresh water bodies located in Haryana, Punjab, Rajasthan and Uttarakhand. Marine water samples were collected from Mumbai, Maharashtra.

Isolation and Molecular Characterization

The freshwater micro algal species was isolated from the freshwater pond at Shahidawaali village, Dist. Sirsa (Haryana) India. Genomic DNA from micro algal sample was extracted by using cetyl tri methyl ammonium bromide (CTAB) method Scott & Bendich [22]. Polymerase chain reaction (PCR) was analyzed to amplify 18S rRNA gene of microalgae using forward (5"GGGACCCGTTACCGTAGGTGAACCTGC-3") and reverse primers (5"-GGGATCCATATGCTTACGTTCCGCGGAT-3"). The purified PCR products were sequenced by Amnion Biosciences Pvt. Ltd. (Bangalore, India). Comparisons of nucleotide sequences and statistical significance of matches were carried out with the National Centre for Biotechnology Information (NCBI) nucleotide BLAST program.

Analytical Methods for Physio-Biochemical Parameters

Bligh and Dyer Lipid Extraction Method: The extraction total lipid were carried out by mixing methanol-chloroform (2:1.5 v/v) with the algal samples using slightly modified version of Bligh and Dyer's method Bligh & Dyer [23]. According to Suganya and Renganatha [24] (the oil extraction yield (%w/w) was determined by the following formula:

$$Oil\ extraction\ yield\ (dcw\ \%) = \frac{\text{Weight of extracted oil}}{\text{Weight of biomass}} * 100$$

Dry Biomass estimation: Dry cell biomass was measured as the cell density (dcw) at OD625 of an 11 day old culture at dilutions ranging from 0.2 to 1.0. The dry biomass was calculated using the regression equation as the linear relationship [25].

$$y = 0.137x + 0.1766,\ R^2 = 0.9859$$

Extraction and Determination of Photosynthetic Pigment: Chlorophyll content of the algae was estimated spectrophotometrically at 650 and 665 nm. The concentration of chlorophyll was calculated using the formula:

$$Total\ chlorophyll\left(mgmL^{-1}\right) = 2.55\times10^{-2}E650 + 0.4\times10^{-2}E665\times10^{3}$$

Extraction and Determination of Total Soluble Carbohydrate by Anthrone Reagent: Glucose was determined at 625 nm using Anthrone reagent method by Dubois et al. [26]. The sugar content was calibrated against standard curve prepared by using graded conc. of glucose dilution ranging from 0.2 to 1 and expressed in terms of mg ml^{-1}

$$y = 0.636x + 0.0592,\ R^2 = 0.9595$$

where y, concentration of glucose, x optical density.

Total Protein Estimation by Lowry Method: The protein content was estimated using Lowry's method. Protein concentration was calculated from the standard curve prepared with bovine serum albumin (BSA) [27].

$$y = 0.1097x - 0.0005,\ R^2 = 0.9989$$

Effect of nitrogen and phosphorus stress on physio-biochemical parameters of screened algal strains

To evaluate the ability of screened algal strains to accumulate lipid under phototrophic conditions, screened algal cultures were grown in BG-11 medium inoculated into nitrogen (N) and phosphorus (P) enrich and stress medium. Three different treatments were set up: N+P+ (control group); (N-P+); (N+P-) respectively for algal growth evaluation in the form of lipid accumulation, biomass yield, protein, carbohydrate and total chlorophyll contents. All the experiments were conducted in triplicate over a cultivation period of 12 days.

Scanning Electron Microscopy (SEM)

In the present study, morphological features and other cellular details of screened algal under nutrient stress (nitrate and phosphate deficient condition) were studied with the help of Scanning Electron Microscope (Carl Zeiss, Model no. SMT EVO 50SEM) as method described by Fowke et al. [28]. Bacterial and algal broths were centrifuged and washed the pellets with phosphate buffer saline for three times and collected the pellet by centrifugation. The fundamental steps for SEM sample preparation are fixing of samples in 0.25% buffered glutaraldehyde (in Sodium phosphate having pH 7.2) and incubated at room temperature for 30 minutes, then freeze dried for 24 hrs, after that fixing is done using tetra oxide of osmium, samples dehydration by different ethanol grading starting; 30%, 50%, 70%, 80%, 90% and 100% and for each ethanol volume incubate for 10 minutes then incubation in 100% ethanol for 1 hour, drying with air dryer, placed in desiccators until constant weight attain, mounting it on stubs using double sided sticky tape coated with carbon. Preparation of SEM stub by applying the adhesive tape and then adding the dried bacterial and algal samples on the tap. The exposed surface was coated with gold with the help of sputter coater device and then the inner surface was scanned at 20 kV potential and various magnifications.

Results and Discussion

Morphological and Molecular Identification of Micro Algal Isolates

Purified algal species were preliminary identified with the help of algal identification guide on the basis of morphological

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features by using Olympus (CX41) light microscope equipped with digital camera. Microscopic images of these algal strains under (100 x) magnification are depicted in (Figure 1) 18S rRNA sequences of screened algal strains were aligned with global sequence available in Gen bank (NCBI) using the standard nucleotide -nucleotide basic local alignment search tool (BLAST) programme. Sequences alignment outcomes revealed that screened algal strains were exhibiting 100% homology with Chlorococcum aquaticum (Accession No. KT961379), Scenedesmus obliquus (Accession No. KT983434), Nannochloropsis oculata (Accession No. KU160538), Chlorella pyrenoidosa (Accession No. KU236002).

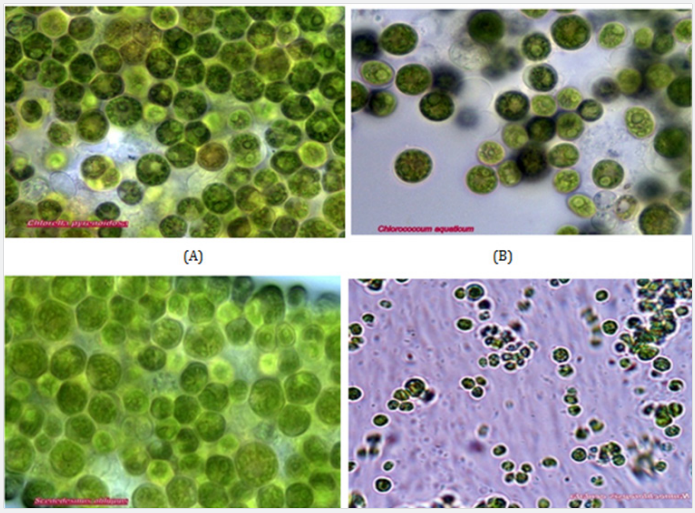

Figure 1: Microscopic images of microalgal strains under (100 x) magnification (A) *Chlorella pyrenoidosa* (B) *Chlorococcum aquaticum* (C) *Scenedesmus obliquus* (D) *Nannochloropsis oculata*.

Effects of Nutrient Stress (Nitrogen, Phosphorus Regimes) on Physio-Biochemical Parameters of Algal Strains

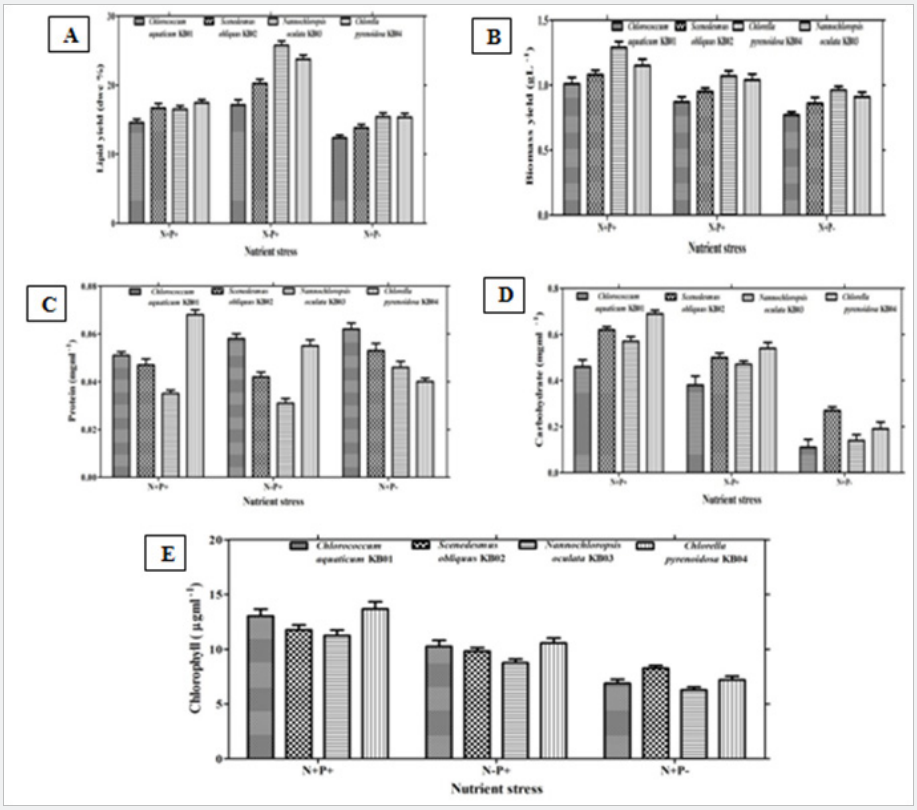

Figure 2: Effect of nutrient stress on Lipid (A) Biomass (B) Protein (C) Carbohydrate (D) and Chlorophyll (E) content in screened algal strains.

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To evaluate the ability of screened algal strains to accumulate lipids under nutrient stress conditions, algal cultures were grown in full BG-11 medium were inoculated into nitrogen (N) and phosphorus (P) enrich medium, respectively, with significant (P≤0.05) higher biomass, protein, carbohydrate and total chlorophyll as given in (Figures 2A-2E). Three different treatments were set up: N+P+ (control group); N-P+ (nitrogen deficiency); N+P- (phosphate deficiency). Anova table suggested that significant (P≤0.05) higher lipid content was observed in nitrogen deficiency condition in four algal strains as shown in (Figure 2A). In (N+P+) nutrient regime, significant (P≤0.05) higher biomass yield 1.129±0.036 gL^{-1} and 1.115±0.021 gL^{-1} have been reported in *Nannochloropsis oculata, Chlorella pyrenoidosa* respectively as compared to *Scenedesmus obliquus, Chlorococcum aquaticum* (Figure 2 B).

The lipid percentage also slightly increases in (N+P+) condition in comparison to other nutrient stress condition in four algal strains. Interestingly, nitrogen deficiency condition promoted (P≤0.05) significant higher lipid accumulation 25.75%, 23.78% and 20.26% in *Nannochloropsis oculata, Chlorella pyrenoidosa,* and *Scenedesmus obliquus* respectively as comparison was made with other nutrient stress. Similar to biomass yield, (N+P+) regimes condition also responsible for significant (P≤0.05) higher protein content in *Nannochloropsis oculata* (0.062±0.005 $mgmL^{-1}$) and *Chlorella pyrenoidosa* (0.068±0.003 $mgmL^{-1}$) respectively (Figure 2D) illustrated that *Scenedesmus obliquus, Chlorella pyrenoidosa* showed significant (P≤0.05) higher carbohydrate content in (N+P+) condition as compared to nitrogen and phosphorus deficient media. In case of total chlorophyll content, nitrogen + phosphorus condition promoted higher chlorophyll content in *Chlorococcum aqauticum* (13.02±0.037 μgmL^{-1}) *Chlorella pyrenoidosa* (13.68±0.029 μgmL^{-1}) as compared to *Nannochloropsis oculata, Scenedesmus obliquus* as shown in (Figure 2E).

Anand, et al. [21] revealed that the 2.27-fold increase in lipid yield (226 mg/L) was observed in nitrogen-depleted condition when compared to nitrogen rich condition (99.33mgL^{-1}). In this study, it was validated that four algal strains were able to accumulate large quantity of lipid and reached the highest lipid content (25.75%) in *Nannochloropsis oculata* under N deficiency, which was in agreement with previous report that N-deficiency was an efficient prompted to induce lipid accumulation (particularly triacylglycerols) in many microalgae [29]. It is known that the different nitrogen sources and levels were effective on the growth of microalgae and biochemical composition [30-34]. Our study also showed that P-deficiency was a suitable condition for lipid accumulation in screened experimental cultures as well. This observation is similar to prior reports [35] who proposed that lipid storage in *Monodus subterraneus* can be increased by P deficiency. Similarly Feng, et al. [36] found that the lipid contents of *Chlorella zofingiensis* grown in media deficient of nitrogen (65.1%) or phosphate (44.7%) were both higher than that obtained from cells grown in full medium (33.5%).

Usually, the nitrogen deficiency would result in more metabolic flux and to lipid accumulation in algae cells as the synthetic rate of essential cell structures including proteins and nucleic acids lowered Li et al. [37] Kirrolia et al. [38]. According to Li et al., phosphorus strongly influenced *Chlorella vulgaris* growth but has little influence on lipid accumulation as we found in our study. Higher protein content reported in our study with (N+P+) condition, similar finding has been revealed by Mutlu et al. [39] and found significantly higher protein content in *Chlorella vulgaris* in nitrogen and phosphorus rich condition. Dortch et al. [40] also observed that the proteins associated with the chlorophyll-protein complex decreased in nitrogen starved cultures. Smit et al. [41] reported a positive relationship between protein and chlorophyll a. Chlorophyll a is one of the most important nitrogen pools in algae: the pigment may reduce nitrogen limitation. Similar to our study, observed that chl a content of *Chlorella vulgaris* decreased and also, a yellowish colour was recorded under N-starvation condition.

Scanning Electron Microscopy

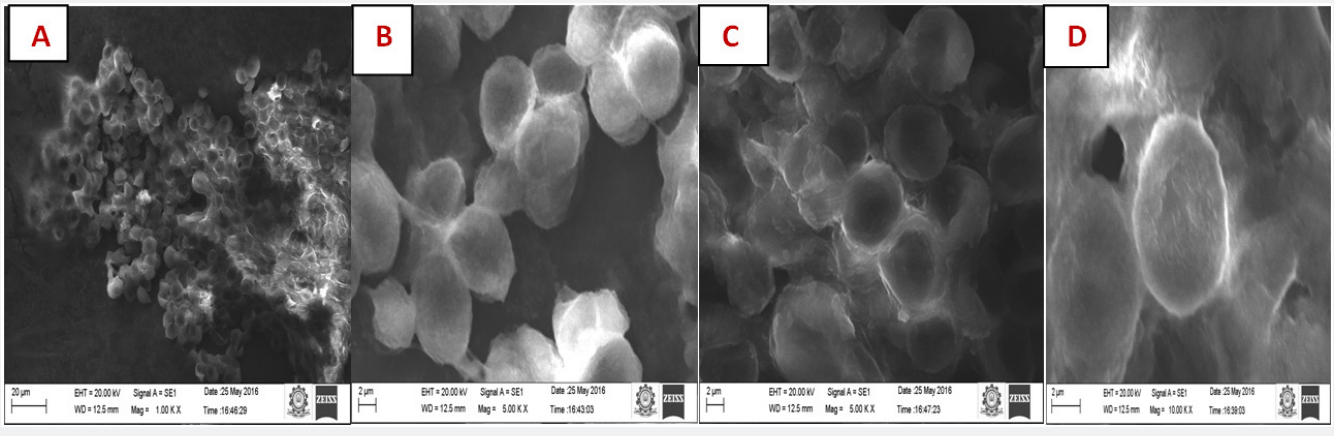

Figure 3: Scanning electron micrographs of *Chlorella pyrenoidosa* (A) (1KX), (B) (5KX-under normal condition; (C) (5KX), (D)-10KX-under nutrient stress conditions.

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Scanning electron micrographs of four algal species of *Chlorella pyrenoidosa, Chlorococcum aquaticum, Nannochloropsis oculata* and *Scenedesmus obliquus* under normal as well as stress conditions were taken at potential of 20 kV and under various magnifications. Scanning electron micrographs of *Chlorella pyrenoidosa* having cell size 2µm revealed that cells of *Chlorella pyrenoidosa* in normal stage was smooth and compacted as well as covered with irregular network of subtle ribs (Figure 3 A,B) whereas in nutrient stress conditions cells become dispersed with rough cell wall (Figures 3C & 3D). Under nutrient stress no longer smooth surface of algal cells walls and outer region was irregular and cell wall roughly folded. Similar to our present work Kirrolia also found striking changes in cell morphology in *Chlorella sp*. under nutrient stress condition. Similar to our findings, [42] observed smooth cell wall in scanning electron micrographs of *Chlorella sp*. in normal condition but cell wall of *Chlorella species* no longer remained smooth after absorption of metal ions Cu^{+2} and Ni^{+2}. Similarly Scanning electron micrographs of Scenedesmus obliquus under normal and stress condition showed characteristics colonies of cells, usually round in shape with prominent nucleus. Under normal conditions, *Scenedesmus obliquus* cells are compactly arranged in two or four cells and are non-fragmented wheareas, under stress conditions there is fragmentation and separation of *Scenedesmus* cells (Figure 4 A-D). Kirrolia [43] also observed distorted morphology under stress condition in *Scenedesmus quadricauda. Chlorococcum aquaticum* is green microalgae having cell size 2 µm round elongated shape with smooth lines over cells walls in normal condition, whereas in stress conditions cell walls distorted with no smooth coverage of fine lines on cell wall (Figures 5A-5D).

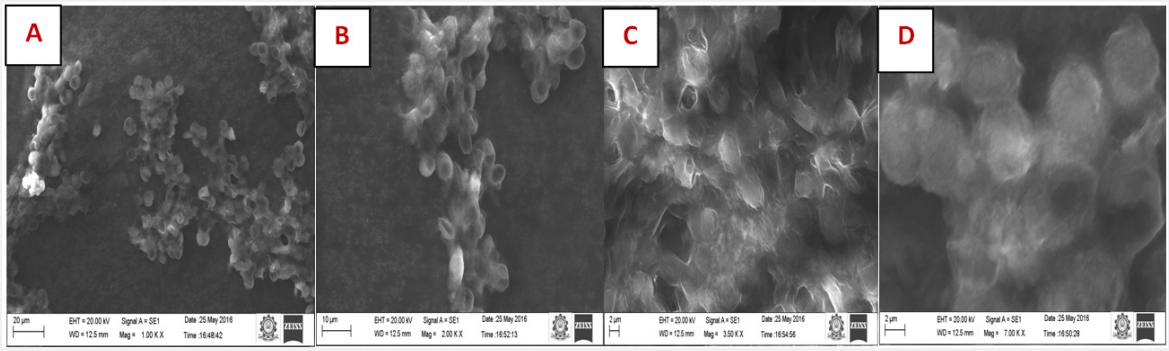

Figure 4: Scanning electron micrographs of *Scenedesmus obliquus* (A) (1kx), (B) (2kx)- under normal condition; (C) (3.5kx); (D) (7.0kx) – under stress condition.

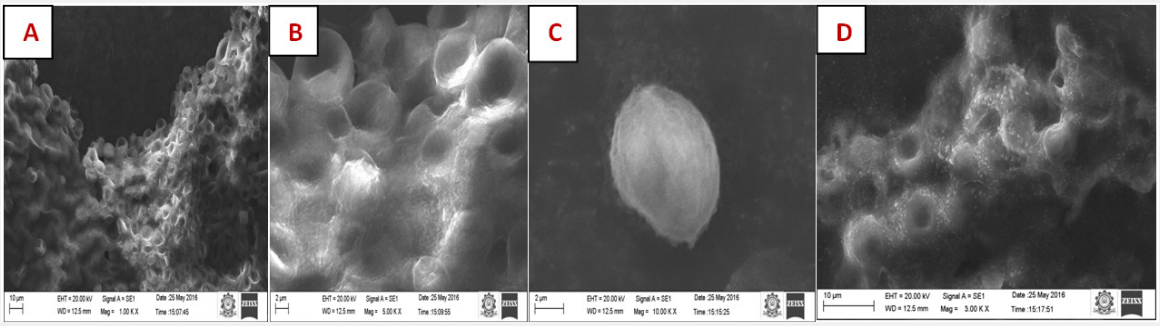

Figure 5: Scanning electron micrographs of *Chlorococcum aquaticum* (A) (1kx), (B) (5kx) (C) (10 kx) –under normal condition; (D) (3kx)- under stress condition.

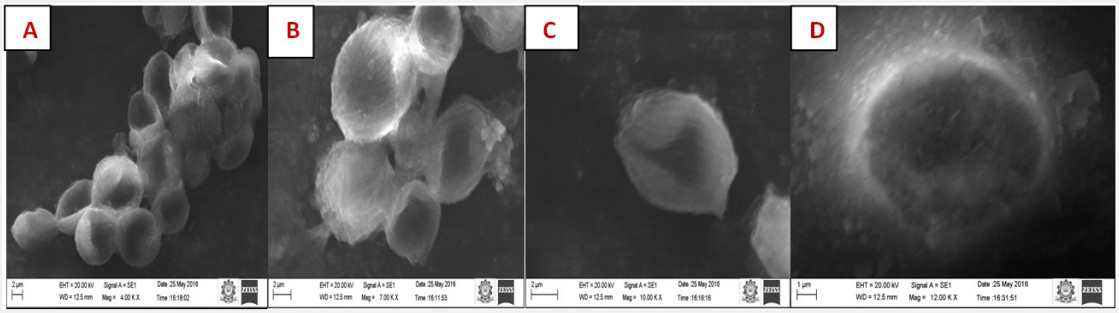

Figure 6: Scanning electron micrographs of *Nannochloropsis oculata* (A) (4kx), (B) (7kx), (C) (10kx) - under normal conditions; (D) (12kx) under stress conditions.

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Nannochloropsis oculata showed intact structure with no cell lyses. Whereas in stress condition in normal condition, disrupted morphology of micro algal structure and appeared completely broken cells under Scanning Electron Microscope (Figures 6A-6D). Similar results have been found in Nannochloropsis oculata in normal condition. In addition, acid treatment 1M HCl totally disrupted the morphology of micro algal structure appearing completely broken cells under Scanning Electron Microscope Surendhiran and Vijay [44-46].

Conclusion

Nutrient stress variables for enhancement of micro algal performance towards sustainable biodiesel synthesis could be effectively optimized in (N+P+) nutrient regime; significant ($P \leq 0.05$) higher biomass yield 1.129±0.036 gL^{-1} and 1.115±0.021gL^{-1} have been reported in *Nannochloropsis oculata, Chlorella pyrenoidosa* respectively. Interestingly, nitrogen deficiency condition promoted ($P \leq 0.05$) significant higher lipid accumulation 25.75%, 23.78% and 20.26% in *Nannochloropsis oculata, Chlorella pyrenoidosa* and *Scenedesmus obliquus* respectively as comparison was made with other nutrient stress. On the other hand, (nitrogen+phosphorus) condition promoted higher chlorophyll, carbohydrates and protein content in almost all algal species. It is meaningful to examine the cellular morphology to further understand the cell disruption under nutrient stress. Scanning electron micrographs (SEM) was found to be efficient tool for characterization of change in cell morphology under normal and stress condition in selected indigenous algal strains. The cellular morphology of micro algal strains was investigated by scanning electron microscope (SEM) which certified that the cells damage was caused by both nitrogen and phosphorus stress.

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