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Gibberellic Acids Promotes Growth and Exopolysaccharides Production in Tetraselmis Suecica Under Reciprocal Nitrogen Concentration: An Assessment on Antioxidant Properties and Nutrients Removal Efficacy of Immobilized Iron-magnetic Nanoparticles

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Abstract

The present study was aimed to assess the effect of gibberellic acids to enhance the growth, biomass, pigment, and exopolysaccharides production in *Tetraselmis suecica* under reciprocal nitrogen concentrations. For this study, the seven types of experimental media (N-P, NL-P/2GA3, N0-P/2GA3, NL-P/4GA3, N0-P/4GA3, NL-P/6GA3, N0-P/6GA3) were prepared include the addition of gibberellic acids under various nitrogen concentrations. The experiment was lasted for 15 days and the cell density, biomass, chlorophyll 'a', and exopolysaccharides (EPS) concentration of *T. suecica* were estimated for every three days. Then the EPS was subjected to the analyses of chemical (carbohydrate, protein, sulfate, and uronic acid), and antioxidant activity. In addition nutrient removal efficiency was evaluated by using different concentration of EPS. The highest DPPH (86.7±0.95 %) and hydroxyl radical activity (85.7±2.48 %) were observed in 2.5 and 1.2 mg/mL of EPS concentration. The immobilized magnetic Fe_3O_4-EPS nanoparticles (5.0 and 10.0 g/L) have efficiently removed the excessive phosphate (89.5±1.65 %) and nitrate (73.5±1.72 %) from the *Litopenaeus vannamei* cultured wastewater. Thus, applying gibberellic acids combined with limited nitrogen concentration could produce higher EPS that could exhibit excellent antioxidant activity, and nutrient removal efficacy in the form of Fe_3O_4-EPS magnetic nanoparticles.

Highlights

- NL-P/4GA3 media composition influenced the highest growth and EPS production.
- The obtained EPS showed 86.7±0.95 % of DPPH 85.7±2.48 % of hydroxyl radical activity.
- The highest carbohydrate and uranic acid were observed from N-P EPS (control).
- The Fe_3O_4-EPS magnetic nanoparticles efficiently eliminated the < 90% nutrients.

Introduction

The microalgae being single cell organisms, it is tough task for them to convert the inorganic compounds like CO_2, N_2 and phosphate in to valuable biomass of high value products like pigments, lipids, and EPS, through autotrophic process. An innovative research-approach on microalgae resulted in day to day increase in conversion rate with high biomass and compounds like pigments, exopolysaccharides (EPS) with the help of CO_2, N, and P (Villay et al., 2013; Reshma *et al*., 2021). The ratio of EPS in microalgae is being dominant extracellular than the other content. The EPS are tightly linked with the algal cell wall and is constructed by biological macromolecules which is secreted by microalgae. Mostly, they are protecting algal cells from the toxic substances of the surrounding habitats (Xiao and Zheng, 2016). Also, their concentration may affect the other properties like surface charge, structure, and viscosity of the algal cells (Parker, 2013). Being natural candidate and nontoxic compound, the EPS have major uses like immunomodulatory, antitumor, anti-inflammatory, and anti-cancer properties (Yim et al., 2004).

The presence of EPS have been reported from both the micro-flora and macro-plants and they have applications in various industries like chemical, thickener, film former and such other daily utilities

(Subudhi et al., 2016). And, the application and efficiency of EPS may vary based on their structures which is different from species to species (Gopinath et al., 2018). The limiting of nutrients in the algal culture medium is a common method to enhance the biomass and lipid production in microalgae and many researchers have investigated such culture-method and obtained a notable results (Kamalanathan et al., 2016, Dinesh Kumar et al., 2018). Regarding the biomass, lipid and other value added pigments accumulation in algal cells, it has been commonly agreed that the environmental stress like nutrient starvation is largly responsible for the high protection of metabolites (Breuer et al., 2012, Dinesh Kumar et al., 2020) and till date there is no attempt has been made on the influence of nitrogen on EPS production by *Tetraselmis suecica*.

The method of manipulation of only the physico-chemical characteristics like pH, temperature, salinity, and irradiance for growth enhancement of algae, is quite unsuitable in case of large scale culture (Medipally et al. 2015). The application of phytohormones like cytokine, gibberellic acid (GA3), ethylene, and auxin are commonly practiced methods to enhance the algal-growth and for altering the physiological activities of plants and algae (Parsaeimehr et al., 2017). The phytohormones may promote the cell growth, cell division, and even in the enlargement of cell-size enlarging the cell size in microalgae as well as in higher plants (Hussain et al., 2019). Hence, the use of phytohormones is first choice for the enhancement, large-scale algal production on by the research community showing in the last decades (Han et al., 2019). Pan et al. (2008) and Mousavi et al. (2016) have stated that the GA3 can be potentially useful for algal culture in enhancing the lipid and pigments accumulations, nutrients adsorptions from the culture medium. Likewise, Park et al. (2013) have studied the *Chlamydomonas reinhardtii* for carotenoids and monosaccharides enhancement, and Pan et al. (2008) on *Microcystis aeruginosa* for protein, Du *et al*. (2015) on *Chlorella pyrenoidosa* for lipid, Madani et al. (2021) on *Isochrysis galbana* have tried with the help of GA3 and obtained very good results.

The chemical composition such as molecular weight, and functional group of microalgae are reported to be interconnected with biological activities of EPS (Qi and Kim, 2017). Among the many EPS producers, the group microalgae is considered to be an important one because their applications, functions, and compositions (Mishra and Jha, 2009). For the removal of nutrients from wastewaters the commonly followed methods include absorption (Dinesh Kumar et al., 2015), adsorption (Dinesh Kumar et al., 2013), ion exchange (Ye et al., 2019), and precipitation (Wang et al., 2006). However, efforts are on to standardize a more efficient, eco-friendly method. In this regard, Fe_3O_4 nanoparticles is getting increased attention because of their high nutrient removal and eco-friendly uses. Su et al. (2016) have stated that EPS derived from both the algae, bacteria possess good nutrient removal potential, since they show net-like structures through the denitrification, nitrifications and ammonification processes. Wei et al. (2018) suggested that the EPS when combined with Fe_3O_4 provide a multiple profits than through the individual use. The present study was aimed to investigate the effect of gibberellic acid (GA3) on the growth, biomass, pigments, and exopolysaccharides yield of the marine microalga, *Tetraselmis suecica* under reciprocal nitrogen concentration and also the analyses of chemical properties, and utilization of EPS for the in-vitro antioxidant activity and for nutrient removal through Fe_3O_4 nanoparticles.

Materials And Methods

Algae culture conditions and experimental methodologies

The microalga, *Tetraselmis suecica* used in the present experiment was prepared from the microalgae culture collection of Marine Planktonology & Aquaculture Laboratory, Bharathidasan University, India. The pure axenic strain was maintained in indoor under the following optimized conditions (Perumal et al., 2015). The stock culture was maintained in 250 mL round bottom conical flasks with 175 mL of filtered seawater (0.45 µM) fertilized with conway's medium (Walne, 1975), 150 µ mol m^{-2} S^{-1} light intensity, 12:12 hours L:D. The details of composition of culture medium are provided in Table-1. For the further experiment, the *T. suecica* cells were harvested during the exponential phase (approximately 7^{th} day of culture). All the experiments were conducted in 5 litre round bottom conical flasks containing 3.5 litre of the sterilized seawater under above said culture conditions (which was maintained for stock culture). 0.50 g L^{-1} initial population density was employed as an inoculum and was inoculated in to the filtered seawater for all the experiments. The experimental approach was designed and implemented with seven different media composition (Table-1). The experimental flasks were subjected to vigorous aeration using aquarium air pump in order to avoid settling the algae at the bottom. During the experimental period (15 days), the cell density, biomass, chlorophyll 'a', and EPS production were estimated at every three days. The final obtained EPS were used for chemical compositions analyses, in vitro antioxidant activity assays (DPPH free radicals and Hydroxyl radical scavenging assays), and for the evaluation of nutrients removal from shrimp culture-wastes efficacy with ferric oxide nanoparticles.

Table- 1. The experimental media composition

Components	N-P	NL-P/ 2GA3	N0-P/ 2GA3	NL-P/ 4GA3	N0-P/ 4GA3	NL-P/ 6GA3	N0-P/ 6GA3
Solution – A							
KNO_3 (g)	100	50	0	50	0	50	0
NaH_2PO_4 $2H_2O$ (g)	20	20	20	20	20	20	20
EDTA (g)	45	45	45	45	45	45	45
H_3BO_3 (g)	33.6	33.6	33.6	33.6	33.6	33.6	33.6
$MnCl_2$ (g)	0.36	0.36	0.36	0.36	0.36	0.36	0.36
$FeCl_3$ (g)	1.3	1.3	1.3	1.3	1.3	1.3	1.3
Dissolve 1000 ml of DDW							
Solution - B							
$ZnCl_2$ (g)	4.2	4.2	4.2	4.2	4.2	4.2	4.2
$CoCl_2$ $6H_2O$ (g)	4.0	4.0	4.0	4.0	4.0	4.0	4.0
$(NH_4)6Mo_7O_{24}$ $4H_2O$ (g)	1.8	1.8	1.8	1.8	1.8	1.8	1.8
$CuSO_4$ $5H_2O$ (g)	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Dissolve 1000 ml of DDW							
Solution - C							
Cyanocobalamin (g)	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Thiamine HCl (g)	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Biotin (g)	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002
Dissolve 100 ml of DDW							
Gibberellic acid (GA3) (mg/L)	0	2	2	4	4	6	6

Note: N-P combinations consider as control and the same media composition was used for indoor stock cultivations.

Analytical methods

To estimate the growth of *T. suecica*, the final multiplied cells were assessed by counting algal cells using haemocytometer under the light microscope. To estimate the biomass productions, the slightly modified gravimetric method was adopted (Richmond *et al.*, 2003). In brief, pre-weighed (W0) glass fibre filter

paper (GF/C, pore size 0.45µm) was used to filter the 10 mL of algal sample collected from experimental flasks. Then the filter paper was incorporated to drying using hot air oven under 100 ºC for 24 h. After drying, the weight of empty filter paper and filter paper with algal cells (W1) were noted and the biomass production of *T. suecica* was calculated using the following formula:

$$\text{Biomass (g L}^{-1}) = \frac{W1 - W0}{10/1000}$$

To estimate the chlorophyll 'a' concentrations in *T. suecica* cells, the collected algae were centrifuged for 15 minutes at 5000 rpm. Then the pellet was homogenized with addition of acetone using mortar and pestle. Further, the supernatant was subjected to centrifugation for 10 minutes at 3000 rpm. Finally, the supernatant was collected and absorbance was measured at 645 nm and 661.5 nm using UV-Spectrophotometer (Shimadzu 160A, Japan). The concentration of chlorophyll a' was calculated using the following equation derived by Lichtenthaler (1987), and Strickland and Parsons (1972).

$$\text{Chl 'a' (µg/ml)} = 11.24 \times \text{OD661.5} - 2.04 \times \text{OD654.0}$$

To extract and estimate the EPS production, the slightly modified methodology of Wu *et al*. (2018) was adopted. In short, the cells of *T. suecica* were collected and centrifuged for 5 minutes at 6000 rpm. The collected pellet was subjected to heating at 60°C for 15-30 minutes with addition of 0.075% sodium chloride. Then the suspension was centrifuged for 10 minutes at 5000 rpm. This mixture was found to consist of tightly found EPS which is considered as EPS.

The carbohydrate content was estimated by H_2SO_4-phenol method with glucose as standard (Dubois *et al*., 1956). The Folin-phenol method was used to estimate the protein content with help of DC protein assay kit (Lowry *et al*., 1956). The barium chloride gelatin method was employed to estimate the sulfate content of EPS with K_2SO_4 as standard (Dodgson and Price, 1962). The sulfamate/m-hydroxydiphenyl method was used to estimate the uronic acid content in EPS with $C_6H_{10}O_7$ (glucuronic acid) as standard (Fillsetti-Cozzi and Carpita, 1991).

The in-vitro antioxidant activity assays such as DPPH free radicals scavenging assay and hydroxyl radical scavenging assay (HRSA) in EPS were carried out as per the methodologies described by Chen *et al*. (2018) and Sies (1993) respectively. In short, 2.0 mL of sample was mixed in to the 2.0 mL of DPPH solution (which was dissolved in 0.1 µmol/L ethanol). Then the mixture was incubated for 30 minutes at room temperature followed by measurement of absorbance at 517 nm using UV-spectrophotometer. Fenton's reaction method was applied for HRSA. In brief, the mixture was prepared (including 3% hydrogen peroxide 150µL, 0.5 mM ferric sulfate 200 µL, 0.435 mM brilliant green 100 µL, and 100 µL of EPS) and dissolved in double distilled water followed by incubation for one hour at 37°C. Then the mixture was centrifuged for 5 minutes at 4696 rpm, then 200 µL of mixture was collected and measured absorbance at 624 nm using microplate reader. The HRSA was estimated using following formula.

$$\text{HRSA (\%)} = \frac{A0-A1}{A2-A1} \times 100$$

Note: A0, and A1 is the absorbance of sample and blank respectively. A2 is the background absorbance which means absence of both sample and Fenton reaction system.

During the nutrients removal experiment, the EPS were mixed with ferric oxide (Fe_3O_4) nanoparticles. The preparation of Fe_3O_4 nanoparticles was done as per the method of Liu *et al*. (2008). In short, 4.2 g of $FeSO_4$ and 6.1 g of $FeCl_3$ were dissolved in 100 mL of double distilled water and heated up to 70-90°C for 15 minutes. Then the 10 mL of 25% NH□OH was added and mixed with the help of magnetic stirrer at 70-90°C for 30 minutes. Further, the mixture was allowed to cool until the formation of black precipitates, which indicates nanoparticles fromation. Then the precipitates were passed through Whatman No.1 filter paper followed by washing with double distilled water for further preparation of magnetic nanoparticles. The preparation of EPS magnetic nanoparticles was done as per the protocol of Wei *et al*. (2018), with minor modification. In brief, in 500 mL of distilled water 0.5 g of Fe_3O_4 nanoparticles were mixed and sonicated. In addition, 0.05 g of $Na_2S_2O_8$ and 0.5 g of EPS were added in to the mixture. Then the mixture was kept in to the ice water for 5 hours with continuous mixing using magnetic stirrer for production Fe_3O_4-EPS which is being as black precipitate.

Table-2. Initial concentration of physico-chemical properties of shrimp cultured wastewater

Parameters	pH	Salinity (psu)	DO (mg/L)	Phosphate (µmol/L)	Nitrate (µmol/L)
Concentration	7.81	30.5	5.45	0.46	3.50

The nutrients removal efficacy of Fe_3O_4-EPS was conducted in shrimp cultured wastewater (120 days) (collected from nearby shrimp farm) and analyzed their initial physic-chemical properties (Table-2). In short, 150 mL of shrimp cultured wastewater was filled individually in 250 mL of round bottom conical flasks with different concentration (0.5, 1, 2, 5, and 10 g/L) of Fe_3O_4-EPS nano-mixture were added. Then the conical flasks were kept in shaker for 24 hours at a speed of 200 rpm. During the incubation the 25 mL of wastewater sample was collected at different time interval such as 3, 6, 12, 18, and 24 hours. Then the sample was filtered through the 0.45 µM whatman filter paper. The filtered wastewater was used for the analyses of the phosphate and nitrate contents as per the procedures of Strickland and Parsons (1972), Jenkins and Medsker (1964) using UV-spectrophotometer. The percentages of nutrients removal were calculated using the following formula:

$$\text{Nutrients removal (\%)} = \frac{N0-N1}{N0} \times 100$$

Note: N0 and N1 are the intitial and final concentration of nutients in the wastetwarer respectivly.

Statistical analysis

All the experiments were conducted with triplicates and the obtained values are given as means ± standard deviation. All the obtained data were used for the ANOVA (One-way) analyses with the help of SPSS version 18.

Results And Discussion

Effect of gibberellic acid on the growth of *T. suecica*

The obtained final cell densities of marine microalga, *T. suecica* to be varied under the various media are shown in Fig-1a. The multiplication and growth rate of alga was found under different media compositions viz., 172.90±2.25 × 10^4 cells mL^{-1} (N-P), 153.87±5.23 × 10^4 cells mL^{-1} (NL-P2), 105.34±3.84 × 10^4 cells mL^{-1} (N0-P2), 229.68±4.82 × 10^4 cells mL^{-1} (NL-P4), 114.36±3.32 × 10^4 cells mL^{-1} (N0-P4), 190.87±5.15 × 10^{a4} cells mL^{-1} (NL-P6), and 119.24±3.93 × 10^4 cells mL^{-1} (N0-P6). The highest rate of cell replication/biomass was found in the medium; NL-P/4GA3 (nitrogen limited with sufficient phosphorus combined with 4 mg/L of gibberellic acid). The present results demonstrated that the, nitrogen limited condition could enhance the growth, once the cell multiplication is started even in the absence of nitrogen. But the cell multiplication was quite low with the absence of nitrogen concentration, as reported earlier by Go *et al*. (2012) and Kim *et al*. (2016). Li *et al*. (2008) found that, most of the microalgal cells possess an efficiency to utilize their own intracellular nitrogen storage, pigments, nucleic acid and cell wall materials to sustain their growth and this must have been the reason behind the presently recorded maximum growth in nitrogen limited combination with sufficient phosphorus concentration combined with 4 mg/L of gibberellic acid. Plant growth harmones did not cause any positive output when there was increased in their dose in higher plants. It might be due to their cytokinin signal transduction pathway in which binding of two-component receptor system viz., cytokinin receptor and CR2 receptor takes place (Inoue *et al*., 2001). Sheen (2001) stated that the activation of regulators is an important factor for increasing the cell divisions. The Table-3 shows the comparative values of growth, biomass, chlorophyll, and exopolysaccharides production by various microalgal strains with the influence of gibberellic acid (based on the report of earlier researchers). Our present study exhibited higher values than the previous reports in terms of number of folds increase in relation to control.

Effect of gibberellic acid on the biomass production of *T. suecica*

The Figure-1b shows the biomass accumulation in *T. suecica* under seven media composition, where the highest biomass (2.03±0.05 g L^{-1}) accumulation was observed in the NL-P/4GA3 followed by NL-P/2GA3 (1.71±0.03 g L^{-1}), NL-P/6GA3 (1.55±0.05 g L^{-1}), N0-P/4GA3 (1.42±0.02 g L^{-1}), N-P (1.10±0.02 g L^{-1}), N0-P/6GA3 (1.07±0.02 g L^{-1}), N0-P/2GA3 (0.78±0.02 g L^{-1}). From this results it is clearly understood that, 4 mg/L of GA3 being an optimum concentration and nitrogen limited combination combined with sufficient phosphorus supply could be favorable conditions. Among the seven media compositions, the nitrogen depleted (NL) experimental flasks with all combination GA3 revealed higher biomass concentration (than the nil nitrogen (N0-P/2GA3, N0/4GA3, N0/6GA3) and sufficient nitrogen (N-P) concentration). This result is contrary to the earlier findings of Chu *et al*. (2013) and Dinesh Kumar *et al*. (2018, 2020) who have

obtained higher biomass production in sufficient nitrogen concentration (than the with depleted nitrogen) in *Scenedesmus obliquus* and *Tetraselmis* sp. respectively. However, the present findings are similar to the report Yao *et al*. (2012), who investigated the *Tetraselmis subcordiformis* which produced higher biomass concentration on the lower nitrogen concentration in the medium. Ozioko *et al*. (2015) stated that the concentration of phytohormones (indole acetic acid, indole butyric acid, gibelleric acid, and kinetin) was negatively correlated with biomass production and cell size of *Chlorella sorokiniana IAM* C212. Also they have stated that the triggering efficiency of GA3 was low on biomass production (than the cell size enhancement).

Effect of gibberellic acid on the chlorophyll 'a' production of *T. suecica*

The concentration of chlorophyll 'a' was estimated under reciprocal nitrogen concentration with addition of different gibelleric acid concentration as shown in Figure-2a. The highest concentration of chlorophyll 'a' (1.98±0.06 µg/mL) was observed in NL-P/4GA3 combination and the lowest (0.85±0.03 µg/mL) in N0-P/2GA3 combination at the end of the culture period (15th day). Earlier, Song *et al*. (2016) and Dinesh Kumar *et al*. (2018) have obtained the higher quantity of chlorophyll under sufficient concentration of nitrogen (than through the replicated/depleted concentration of nitrogen) when they combined with initial population density as a co-factor. In this study, nitrogen depleted condition revealed higher concentration of chlorophyll 'a' than under the nil (NO) and sufficient (N) concentration of nitrogen. Among the seven media compositions, sufficient nitrogen (N-P) and depleted nitrogen combination (NL-P/2GA3, 4GA3, and 6GA3) with GA3 resulted in higher pigments production (than through nil nitrogen combinations like N0-P/2GA3, N0-P/4GA3 and N0-P/6GA3). The synthesis of chlorophyll mainly depends on the nitrogen availability in the culture medium. If the nitrogen concentration is low or nil then the pigments synthesis could be zero, due to their in-ability to utilize their own intracellular nitrogen. In this case, their pigments rate was too low (than the other combinations) (Song *et al*., 2016). Demming-Adams and Adams (2002) have stated that nitrogen can be considered as key factor for algal cell multiplications and their pigments accumulations including chlorophyll 'a' via photosynthesis. The chlorophyll 'a' is working as an agent for photosynthesis; being as light-absorption sensor and receptor, if chlorophyll rate is low in algal cells the entire photosynthesis process will be getting affected. Ozioko *et al*. (2015) stated that the increasing GA3 concentration (from 5 to 20 mg/L) were positively correlated with chlorophyll 'a' accumulation in *Chlorella sorokiniana* IAM C212. Likewise, the present study obtained the higher chlorophyll accumulation in moderate GA3 (4GA3) concentration than through the lower (2GA3) and higher (6GA3) GA3 concentrations. It is well know that every consumer products is partially or fully covered by chlorophyll contents and hence their requirements may increase in future due to their variety of applications like cosmetics, pharmaceutical, food colorants, and treating agent for chronic ulcer disease (Hosikian *et al*., 2010). Finding optimal concentration of phytohormone is essential for producing higher biomass and pigments which may be a partially or complete contributed to world economy through their various biotechnological applications.

Effect of gibberellic acid on the EPS production of *T. suecica*

In view of multiple applications, the bio-products like EPS are getting much interest from industrialists (Sharma *et al*., 2017). The production of EPS from the cultured algae is a promising filed and presently the algal culture was tried in the medium with addition of GA3 under reciprocal nitrogen concentration. The EPS production under various experimental conditions are shown in Fig. 2b. The highest EPS production was obtained in the culture medium that contained the combination of NL-P/4GA3 followed by NL-P/2GA3, NL-P/6GA3, N0-P/6GA3, N0-P/4GA3, N-P, N0-P/2GA3 and the recorded concentrations were 140.57±5.03 mg/L, 117.88±3.47 mg/L, 116.47±3.90 mg/L, 115.49±3.63 mg/L, 111.31±3.47 mg/L, 103.22±2.94 mg/L, and 75.64±1.89 mg/L respectively. Among the seven media compositions tested, the highest EPS concentration was found in moderate GA3 concentration (4 mg/L) under depleted nitrogen concentration (NL-P) followed by lowest GA3 (2 mg/L) and highest GA3 concentration (6 mg/L). But, the depleted nitrogen concentration (NL-P) was most preferable combination for higher EPS production (than the nil (N0-P) and sufficient nitrogen concentration (N-P)). The rate of production of EPS is related to the specie type of algae, nature of ecological factors besides the compositions of the culture medium (Rabha *et al*., 2012). Determining the optimal culture conditions is essential to achieve the maximum production of EPS. Microalga is one of the major contributors of EPS production but only limited research related to enhancement of EPS production has been carried out so far. The stress on the microbes and microalgae were positively correlated with EPS production and the rate of EPS production was thus decreased when there was no stress (Naseem *et al*., 2018). Roberson and Firestone (1992) stated that the EPS production on the bacteria are with drought stress were higher than the without stress conditions. Similarly in the present study, the highest EPS concentration was observed in GA3 containing media than the without GA3 (control). This study confirms that, addition of GA3 with reciprocal nitrogen concentration on the culture media was given positive output in terms of growth, biomass, pigments and EPS production on *T. suecica*.

Table-3. The growth, biomass, chlorophyll, and EPS production of some microalgae with the influence of gibberellic acid conducted by various early researchers

S. No	Species	Combined with	(↑) Folds of increase from control strains				References
			Growth	Biomass	Chlorophyll	EPS	
1.	*Chlorella vulgaris*	Cadmium, Lead	0.30		0.9	1.0	Falkowska et al. (2011)
1.	*Chlorella sorokiniana*	-	0.20	0.1	1.0	-	Ozioko et al. (2015)
1.	*Isochrysis galbana*	-	0.80	1.0	0.15	-	Madani et al. (2020)
1.	*Nannochloropsis oceanica*	Kinetin	-	0.14	-	-	Udayan et al. (2018)
1.	*Acutodesmus obliquus*	Zeatin, indole acetic acid	-	0.49	-	-	Renuka et al. (2018)
1.	*Micractinium reisseri*	Flashing light	0.93	-	-	-	Choi et al. (2017)
1.	*Tetraselmis suecica*	Nitrogen	0.32	0.85	0.92	0.36	Present study

Note: (↑) denotes the X number of times greater than the control strain

Chemical compositions of exopolysaccharides under various culture conditions

Table-4 shows the chemical composition of exopolysaccharides extracted (from *T. suecica*) under various experimental media. Among the four chemical components (carbohydrate, protein, sulphate, and uronic acid) tested, the carbohydrate was found to be the dominant followed by protein, uronic acid, and sulphate. The highest carbohydrate (72.35±1.35 %) and uronic acid (3.89±0.48 %) contents were observed in sufficient phosphorus and sufficient nitrogen revealed (N-P) combination and the lowest (59.34±2.17 %, 0.98±011 %) was found in N0-P/2GA3 combination. However, the protein (12.84±1.23 %) and sulphate (2.98±0.35 %) contents were high in NL-P/4GA3 combination followed by N-P combination (11.58±0.68 %, 2.18±0.15 %).

Table-4. Chemical components of exopolysaccharides extracted from *T. suecica,* cultivated under various culture condition with the combination of gibberellic acid.

Component (%)	N-P	NL-P/ 2GA3	N0-P/ 2GA3	NL-P/ 4GA3	N0-P/ 4GA3	NL-P/ 6GA3	N0-P/ 6GA3
Carbohydrate	72.35± 1.35^{c}	64.18± 2.15^{c}	59.34± 2.17^{c}	72.18± 3.75^{c}	62.39± 2.71^{c}	67.38± 1.58^{c}	63.21± 3.78^{c}
Protein	11.58± 0.68^{b}	9.38± 1.14^{a}	7.35± 0.96^{a}	12.84± 1.23^{b}	8.35± 0.89^{a}	9.81± 1.14^{a}	9.11± 0.98^{a}
Sulfate	2.18± 0.15^{a}	1.35± 0.11^{a}	1.08± 0.24^{a}	2.98± 0.35^{a}	1.45± 0.11^{a}	2.11± 0.12^{a}	1.58± 0.16^{a}
Uronic acid	3.89± 0.48^{a,b}	2.14± 0.24^{a}	0.98± 0.11^{a}	3.57± 0.17^{a}	1.86± 0.09^{a}	2.61± 0.18^{a}	1.97± 0.13^{a}

Note: Values shown are averages of three triplicates ± standard deviation, and the standard deviations were calculated from three repetitions. Mean values within the same column sharing the different superscript are significantly different ($P > 0.05$)

Earlier, Suárez *et al*. (2005) and Qi and Kim (2017) have obtained similar highest quantity of carbohydrate content in *Chlorella pyrenoidosa*. The highest protein content was obtained in sufficient nitrogen limited combination with 4GA3 (than through the other combinations tested) and from this we can understand that the GA3 plays a significant role when the nitrogen concentration is reduced. However, Dinesh Kumar et al. (2020) have obtained different results on protein and carbohydrate accumulation in *Tetraselmis* sp. under various nitrogen concentrations with the combination of reciprocal salinity level. Dinesh Kumar et al. (2020) stated that the carbohydrate and protein accumulation were found to be triggered under higher nitrogen (2N) concentration when the salinity was moderate (20 psu). The accumulation of protein was found to be vary from species to species, cultivable environment and which also depends on the chemical components like glucose, mannose, galactose, and rhamnose (Qi and Kim, 2017). Mostly, the exopolysaccharides consist variety of monosaccharides like glucose with other sugar contents. Similarly, *C. ellipsoidea* consisting least quantity of arabinose and galactose with glucose as large quantity (Kojima et al., 1973). Suárez et al. (2008) and Tabarsa et al (2015) concluded that, glucose was major monosaccharide procured by many *Chlorella* species (than other saccharides accumulate in the algae). Takeda et al. (1978) studied the *C. ellipsoidea* and they have obtained variety of hemicellulose contents with sugar as prime candidate. Likewise, many early researchers have stated that the chemical composition of EPS may depends on many influencing factors like species, culture conditions, nutrients loads in the medium as well as extraction methods (Qi and Kim, 2017).

Antioxidant activity of EPS

The oxidation in substrates consist three continuous processes viz., initiation, propagation and termination (Melo-Silveira et al., 2014). Hence, in this study we have made attempt on the evaluation of EPS on the scavenging properties of DPPH radical (initiation) and scavenging of hydroxyl radical (termination). Nugud et al. (2018) opined that, the disease is related to excessive radical and is in direct proportionate to radical levels and their level may get reduced using scavengers. The results of scavenging of DPPH radical by EPS are shown in Figure-3a. The nitrogen free radical can be devastated by scavengers when the DPPH as stable radical (Zhang et al., 2019). Hence, the seven media composition-cultured *T. suecica* EPS exhibited maximum scavenging activity (86.7±0.95 %) on 2.5 mg/mL followed by 3.0 mg/mL concentration of EPS and the scavenging activity was 82.4±0.90 % which was extracted from NL-P/4GA3 combination. The control EPS (N-P) exhibited highest scavenging activity (82.7±2.72 %) at the highest EPS concentration (3.0 mg/mL). The increased EPS concentration was significantly increased (reached maximum) with the scavenging DPPH radicals up to certain limit (2.5mg/mL), then the scavenging DPPH radicals was getting decreased. From these results we clearly understand that the lowest concentration of EPS could be extracted from nitrogen limited with combination of 4 mg/L of gibberellic acid (NL-P/4GA3) which shows highest scavenging activity against DPPH radicals compared to control EPS (N-P). Similar findings were earlier obtained by Zhang et al. (2019) who studied the *Chlorella pyrenoidosa, Scenedesmus* sp., and *Chlorococcum* sp. and they stated that the scavenging DPPH radicals were higher in moderate EPS concentration (than with the higher EPS concentration).

There were seven types of EPS (with six different concentrations) that were incorporated to asses the hydroxyl radical (which is considered to be most harmful such as affecting cell, DNA and protein). The highest hydroxyl radical scavenging activities (85.7±2.48 %) was observed in 1.2 mg/mL which was being a highest EPS concentration from NL-P/4GA3 culture combinations (Fig. 3b). Likewise, all other culture combinations extracts were shown highest hydroxyl radical scavenging activity, when EPS concentration was high (1.2 mg/mL) (than the other lowest concentration of EPS) and the percentage of activities were: 82.70±2.73 (N-P), 69.20±2.49 (NL-P/2GA3), 57.90±1.97 (N0-P/2GA3), 66.20±2.65 (N0-P/4GA3), 74.50±1.42 (NL-P/6GA3), 70.10±2.38 (N0-P/6GA3). During, the present study better hydroxyl radical scavenging was recorded than through the other previous studies on *Chlorella pyrenoidosa* (63.1%), *Scenedesmus* sp. (77.5%), *Chlorococcum* sp. (72.4%), *Lactobacillus gasseri* (36.47%), *Streptococcus thermophiles* (51.37%) (Zhang et al., 2019; Rani et al., 2018; Zhang et al., 2016). But, the present rate is lower (than the resulted rate of activity of Chen et al. (2018) who obtained 93.2% hydroxyl radical scavenging from *Chlorella pyrenoidosa.* Qiao et al. (2009) reported that, strong positive correlation was found observed between antioxidant activity and their reducing capacity of radicals and the present study confirms the efficiency of DPPH and hydroxyl radical scavenging activity of EPS. The antioxidant activity and concentration of EPS are directly proportional up to certain limits as reported by Hussein et al. (2015). Table-5 shows the production and applications of EPS extracts from various microalgae as reported by earlier researchers.

Nutrients removal efficacy of Fe_3O_4-EPS

The results of nutrients (phosphate and nitrate) removal efficiency of Fe_3O_4-EPS magnetic nanoparticles are shown in Fig. 4a-b. The highest rates of removal of phosphate (89.5±1.65 %) and nitrate (73.5±1.72 %) were observed at the 24 hours of retention time in 5 g/L and 10 g/L of Fe_3O_4-EPS concentrations respectively. The slowest increasing trend of nutrients removal was found from 3 hours to 24 hours. However, the maximum rate of removal was observed at 3 hours to 6 hours (than the 6 hours to 24 hours). The total removal of phosphate was high (than nitrate) by Fe_3O_4-EPS magnetic nanoparticles and this results clearly indicates that the binding efficiency is likely high in phosphate. Zhou et al. (2012) stated that the water pH is significantly affect the phosphate removal and similar findings were observed earlier by Govarthanan et al. (2020), who have made attempt on nutrients (phosphate and nitrate) removal from wastewater using Fe_3O_4-EPS (extracted from *Chlorella vulgaris*). Their removal rate was high in phosphate (67.3±1.2 %) and low in nitrate (60.5±0.9 %). Likewise, the maximum phosphate removal was observed from wastewater with 7 pH by using hybrid polymeric anion exchanged iron nanoparticles, as a remediating agent (Wiriyathamcharoen et al., 2020). Matheickal et al. (1999) opined that the functional groups of algae and wastewater pH consist of strong bonding among them and their results were varied based on the surface charges of the beads. The maximum rate of nutrients-removal (5 g/L-phosphate; 10 g/L-nitrate) was observed in beads containing Fe_3O_4-EPS, as reported earlier by Dinesh Kumar et al. (2018) and Govarthan et al. (2020), who opined that the highest cell EPS densities could provide more surface area to bind the nutrients. Our results dissimilar to Lau et al. (1997) and Abdel Hameed (2007) who have stated that higher cell densities could not make any significant enhancement of nutrients removal due to leakage of algal cells. Similarly, Jin et al. (2012) have found highest arsenate removal with the lowest concentration (0.1 mg/L) by using etyltrimethylammonium bromide modified magnetic nanoparticles (than through the highest concentration (1.0 mg/L). However, the removal rate of nutrients was increased when the Fe_3O_4-EPS magnetic nanoparticles concentration was increased due to their surface charging area.

Table-5. The production and application of EPS extracted from microalgae conducted by early researchers

S. No.	Strain	Stimulator used	Applications	References
1.	*Chlorella ellipsoidea*		immunomodulatory activity	Qi and Kim, (2017)
1.	*Synechocystis sp.* PCC 6803	Mutant ΔsigF	Antitumor activity	Flores et al. (2019)
1.	*Chlorella pyrenoidosa, Scenedesmus* sp., *Chlorococcum* sp.,	-	Antioxidant and antitumor activity	Zhang et al. (2019a)
1.	*Chlorella Zofingiensis Chlorella vulgaris*	-	Anti-colorectal cancer activity	Zhang et al. (2019b)
1.	*Chlorella vulgaris*	-	Antiinflammatory activity	Barboríková et al. (2019)
1.	*Chlorella vulgaris*	-	Nutrients removal	Govarthanan et al. (2020)
1.	*Nostoc* sp.	-	Immunomodulatory activity	Uhliariková et al. (2020)
1.	*Spirulina platensis*	*Virgibacillus salarius*	Antioxidant, emulsifying activity	Gomaa and Yousef, (2020)
1.	*Chlorella vulgaris*	-	anti-inflammatory and anti-remodelling activity	Capek et al. (2020)
1.	*Tetraselmis suecica*	Gibberellic acid, Nitrogen	Antioxidant activity, Nutrient removal	Present study

Conclusion

Presently, the utilization of gibberellic acids was found to be successful for the promotion of growth, biomass, chlorophyll 'a', and exopolysaccharides production in *T. suecica* under reciprocal nitrogen concentrations. Of the seven different media, the combination; NL-P/4GA3 promoted the maximum cell density (229.68±4.82 × 10^4 cells mL^{-1}), biomass (2.03±0.05 g L^{-1}), chlorophyll 'a' (1.98±0.06 µg/mL), and EPS (140.57±5.03 mg/L). During the DPPH assays, the 2.5 and 1.2 mg/mL concentration of EPS exerted the highest DPPH (86.7±0.95 %) and hydroxyl radical (85.7±2.48 %) activity. In the case of nutrient removal, Fe_3O_4-EPS immobilized magnetic nanoparticles with moderate concentration (5.0 g/L) resulted in the highest phosphate removal (89.5±1.65 %) from the *L. vannamei* cultured wastewater. From this study it is clearly understood that the EPS produced from the modified medium (addition of GA3)

exhibited excellent antioxidant activities and nutrients removal efficiency when immobilized with Fe_3O_4-EPS and hence this method can be considered as eco-friendly and low cost remediant in the pilot scale wastewater treatment plants.

Declarations

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Conflict of Interest

The authors declare that they have no conflict of interest

Ethical approval

This article does not contain any studies with animals performed by any of the authors.

Credit authorship contribution statement

A. Prathipa: Formal analysis. **G. Manikandan:** Writing - review & editing. **S. Dinesh Kumar**: Conceptualization, Methodology, Resources, Writing - original draft. **P. Santhanam**: Conceptualization, Methodology, Writing - review & editing. **P. Perumal**: Writing - review & editing. **N. Krishnaveni:** Formal analysis. **K. Nanthini Devi:** Formal analysis**.**
S. Vijayalakshmi: Formal analysis.

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Figures

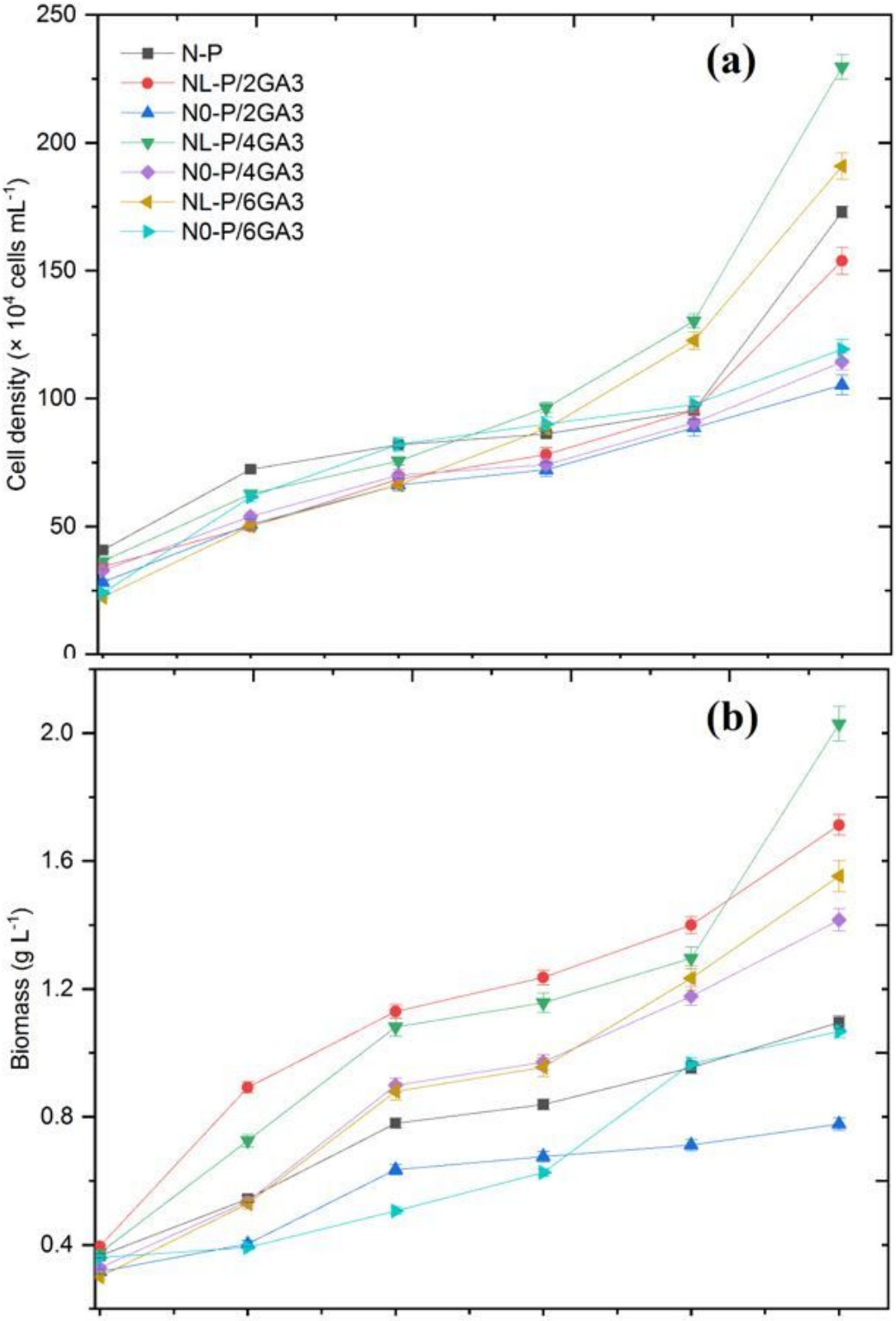

Figure 1

Effect of gibberellic acid on the cell density (a), and biomass production (b) of T. suecica under reciprocal nitrogen concentration. Values shown are averages of three triplicates ± standard deviation, and the standard deviations were calculated from three repetitions

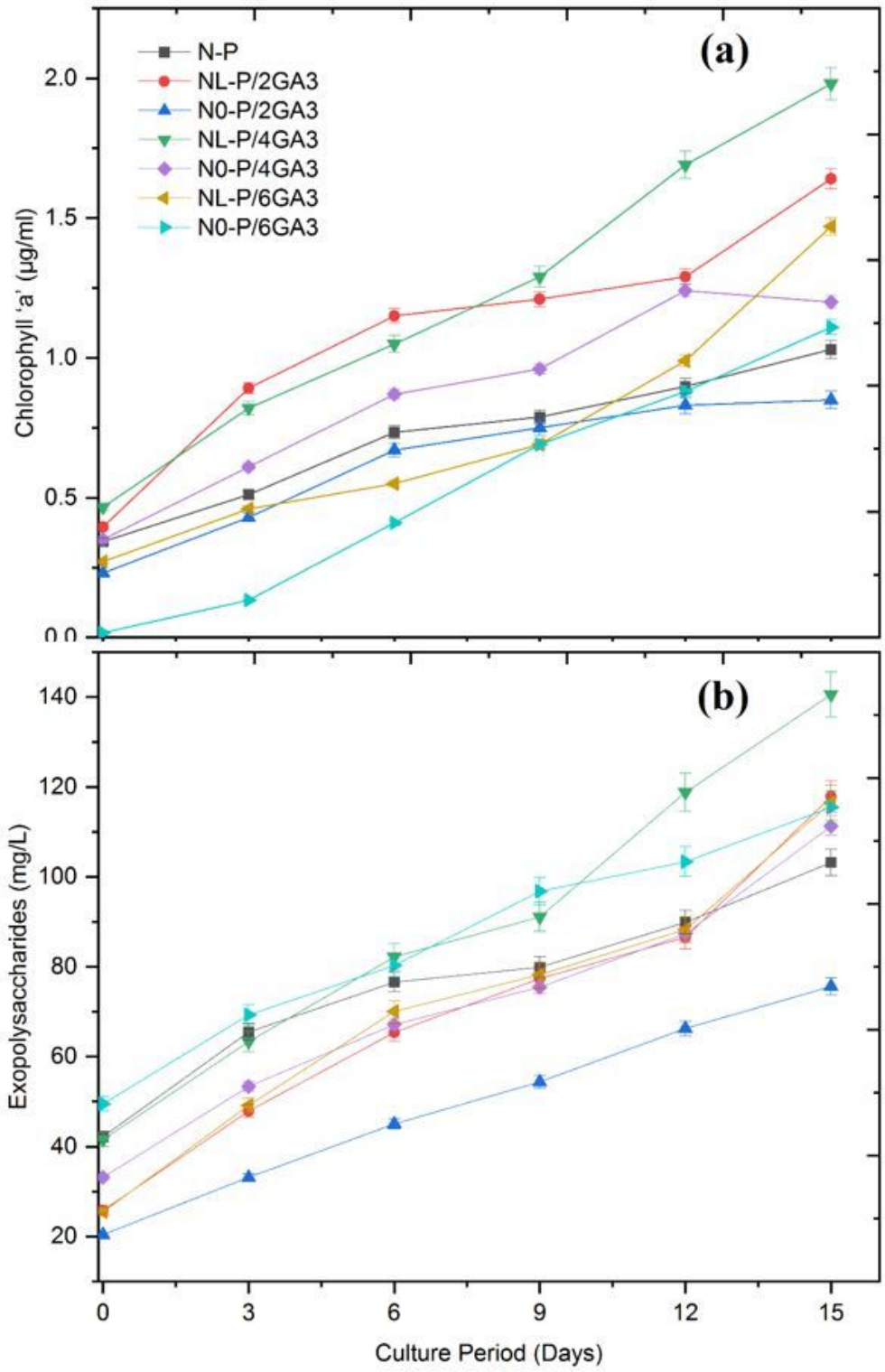

Figure 2

Effect of gibberellic acid on the chlorophyll 'a' production (a), and exopolysaccharides production (b) of T. suecica under reciprocal nitrogen concentration. Values shown are averages of three triplicates ± standard deviation, and the standard deviations were calculated from three repetitions

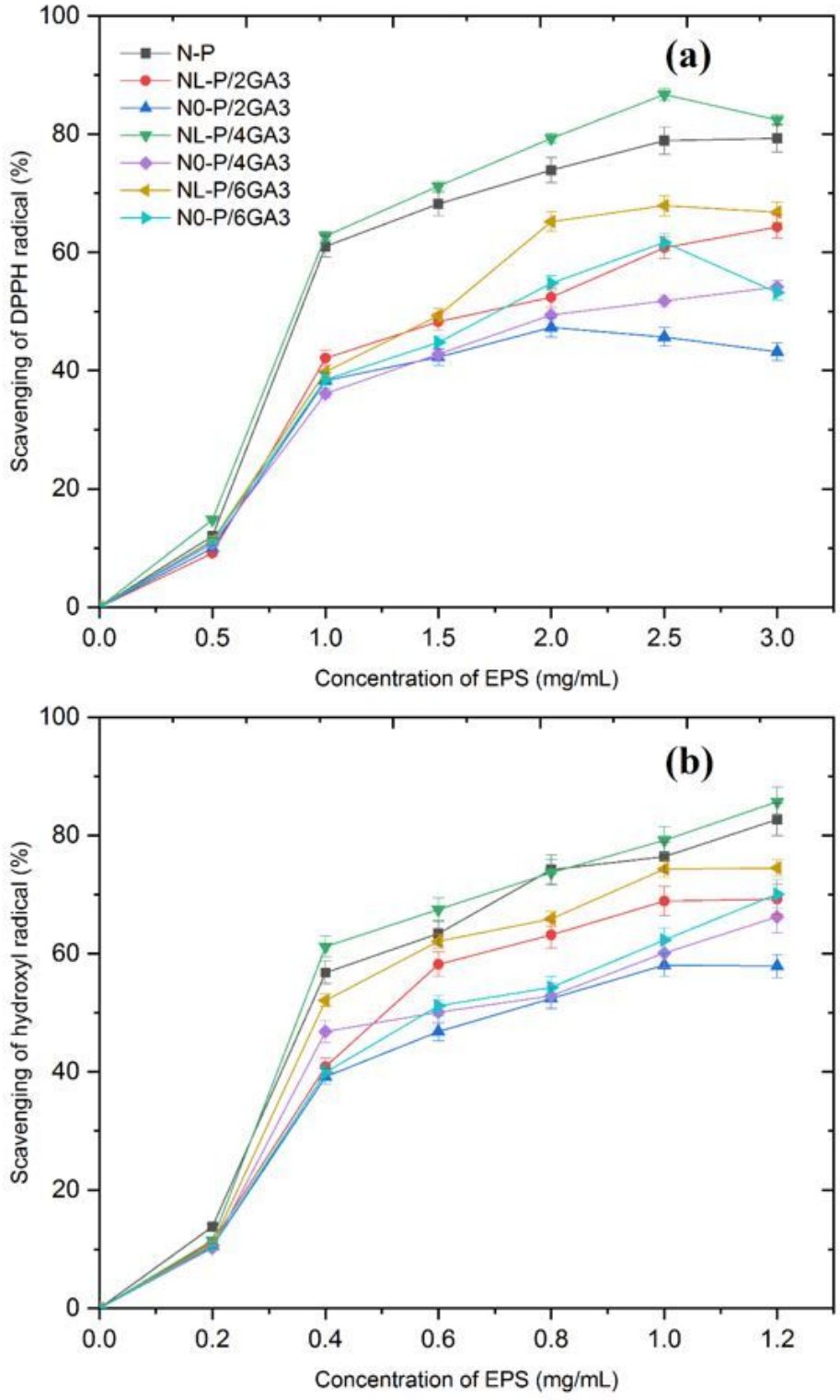

Figure 3

Antioxidant activities of exopolysaccharides extracted from T. suecica cultured under different concentrations of gibberellic acid and nitrogen. A. Scavenging of DPPH radicals, B. Scavenging of hydroxyl radicals. Values shown are averages of three triplicates ± standard deviation, and the standard deviations were calculated from three repetitions

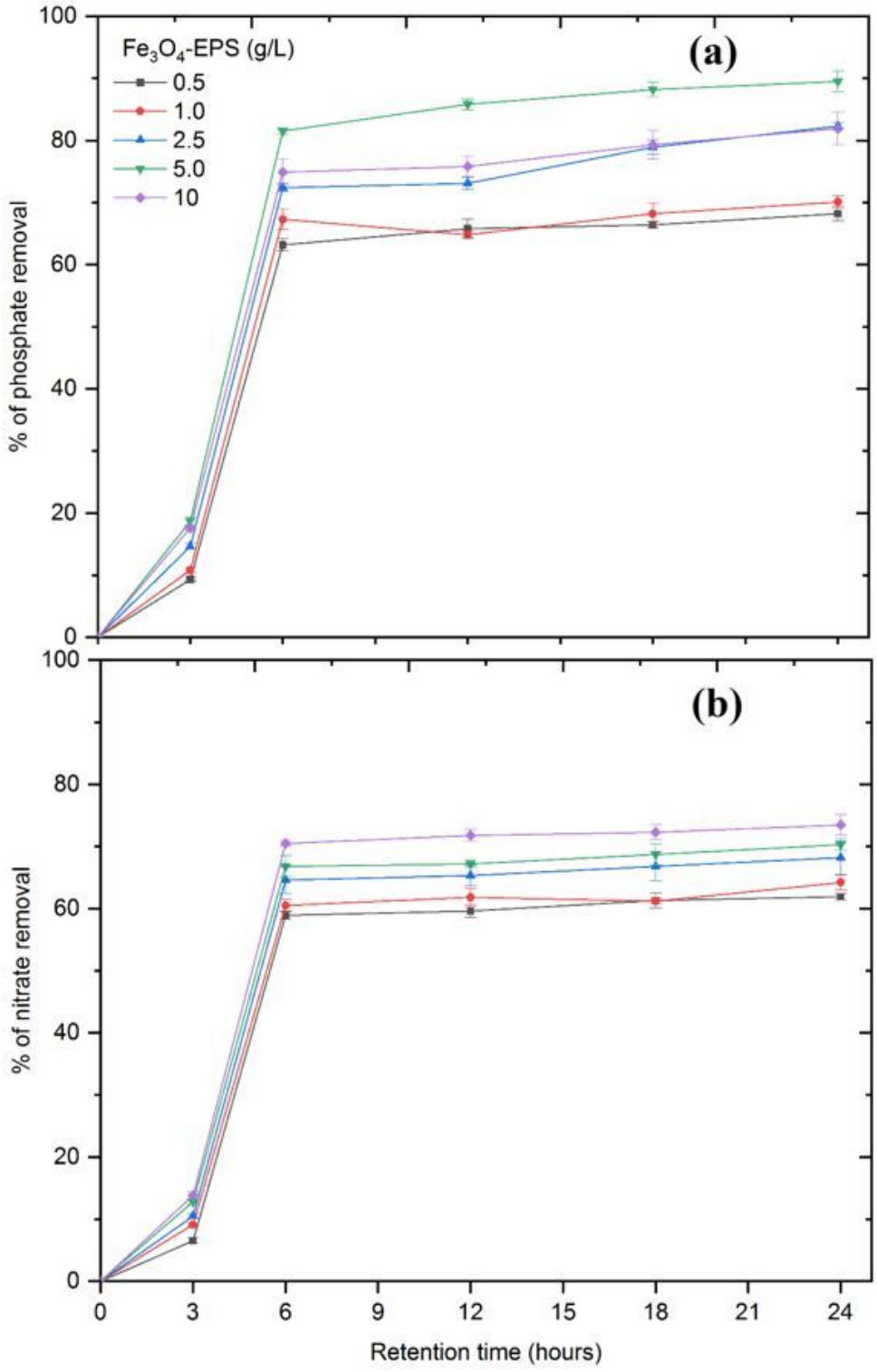

Figure 4

Effect of Fe3O4-EPS immobilized magnetic nanoparticles on the phosphate (a), and nitrate removal (b) from L. vannamei cultured (120 days) wastewater. EPS extracted from T. suecica cultured with addition of gibberellic acids under reciprocal nitrogen concentration. Values shown are averages of three triplicates ± standard deviation, and the standard deviations were calculated from three repetitions.