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RESEARCH

Optimization of trace metal composition utilizing Taguchi orthogonal array enhances biomass and superoxide dismutase production in *Tetraselmis chuii* under mixotrophic condition: implications for antioxidant formulations

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Abstract

The natural ageing process in all organisms is majorly influenced by the production rate and dismutation of reactive oxygen species (ROS) within cells. Certain microalgae, such as *Tetraselmis chuii*, possess the ability to produce superoxide dismutase (SOD), a powerful antioxidant enzyme that mitigates oxidative damage caused by ROS during oxygen metabolism. This study investigated the impact of trace elements (nickel, manganese, copper, zinc, and iron) and nitrogen sources in the growth medium on both the biomass and SOD synthesis of *T. chuii* under mixotrophic conditions. Initially, the one-factor-at-a-time (OFAT) approach was employed to filter out the most significant factors in the production medium. Next, Taguchi orthogonal array method, known for its robustness in experimental design, was employed to analyse the effects of various media components on algal biomass and SOD production. Using only a few well-defined experimental sets, Taguchi's L18 orthogonal array facilitated a 1.21-fold increase in biomass yield, reaching a maximum of 0.643 g/L. Furthermore, SOD activity was enhanced from 85.28 to 91.94% following optimization. Notably, nitrogen source, nitrogen concentration, and zinc concentration emerged as significant influencers of biomass and SOD production. The Taguchi optimization thereby improved SOD yield in a cost-effective manner. The heightened antioxidation activity of SOD holds promising applications in formulating antioxidants and topical ointments in pharmaceutical and cosmeceutical industries.

Keywords *Tetraselmis chuii* · Trace elements · Superoxide dismutase (SOD) · Taguchi orthogonal array · Antioxidant

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Introduction

Antioxidant plays a pivotal role in the body's defence mechanism by scavenging free radicals and mitigating the harmful effects of reactive oxygen species (ROS) (Kumar and Ralph 2017; Francenia Santos-Sánchez et al. 2019). ROS, including the hydroxyl radical (•OH) and superoxide radical ($\bullet O_2^-$), are formed from unpaired electrons of oxygen (Zhang et al. 2021), induced by environmental stressors, leading to oxidative stress, aging, and various diseases (Chokshi et al. 2017; Warraich et al. 2020). Living organisms regulate ROS through intracellular antioxidant systems, preventing their detrimental impact (Zhang et al. 2021), with microalgae, such as *Tetraselmis chuii*, produce antioxidant enzymes like superoxide dismutase (SOD) to counteract ROS (Francenia Santos-Sánchez et al. 2019; Coulombier et al. 2021).

SOD, a primary antioxidant enzyme, converts $\bullet O_2^-$ into H_2O_2, subsequently broken down by catalase or glutathione peroxidase (Nam et al. 2024). Various forms of SOD, i.e., Cu/ZnSOD, MnSOD, FeSOD, and NiSOD, exist depending on metal cofactors at the enzyme's active site (Su et al. 2021). Due to its potent oxidative stress protection, SOD finds applications in pharmaceuticals, industries, and cosmetics (Mawardi and Yanuar 2021; Rosa et al. 2021), leading to a growing demand for its maximal production. Microorganisms, especially microalgae have been the prominent source of SOD due to their high antioxidant potential (Coulombier et al. 2021). *Tetraselmis* species, such as *Tetraselmis chuii*, are reported to demonstrate a high potential for SOD production as a secondary metabolite (López-Hernández et al. 2020), contributing to its commercialization as health supplements due to its antioxidative properties (Cocksedge et al. 2025).

However, producing SOD from microalgae is challenging due to their stringent nutrient requirements. Macrominerals and microminerals are essential for microalgae growth and enzyme production (Fox and Zimba 2018), particularly, trace elements like nickel, iron, copper, zinc, and manganese influence SOD production as metal cofactors for SOD (Awasthi et al. 2022). Additionally, nitrogen concentration also impacts enzyme production (Barman et al. 2022). Despite reports of growth inhibition at elevated concentrations (Alho et al. 2022; León-Vaz et al. 2021), nickel, manganese, and copper were selected due to their known involvement in metalloenzymes including superoxide dismutase.

The optimal medium composition to produce SOD by microalgae remains unclear, prompting the need for efficient optimization strategies. Statistical designs such as Plackett–Burman design (PBD), Box–Behnken design (BBD), central composite design (CCD), and Taguchi orthogonal array have been employed to evaluate many factors with controlled number of experimental runs (Jankovic et al. 2021). Among these, Taguchi method offers several advantages, being robust and less sensitive to variation by noise factors (Hamzaçebi et al. 2021; Wahid et al. 2020). In Taguchi method, signal-to-noise (S/N) ratios (smaller-the-better, larger-the-better, or nominal-the-better) are used to measure the effect of noise on each design and eventually reduce the noise effects to optimize the experiment (Chen et al. 2022), making it suitable for media composition optimization.

This study aims to investigate the effect of trace elements on biomass and SOD production by *T. chuii*, and further optimize the medium composition using Taguchi Orthogonal Array method. Initially, the OFAT (one-factor-at-a-time) approach was employed to screen significant factors from initial parameters, which were then used to formulate the production medium for *T. chuii* in the Taguchi experimental design. Given the focus on maximizing biomass and SOD production, the signal-to-noise (S/N) quality in Taguchi design was chosen as "larger-the-better" to maximize the SOD activity as the response variable of interest. The findings of this study are expected to contribute to the development of a more efficient and economically viable strategy for SOD production from *T. chuii*. By optimizing culture conditions, this work supports the commercial-scale application of microalgal SOD in pharmaceutical, nutraceutical, and cosmeceutical industries where high antioxidant activity is desirable.

Materials and methods

Collection and maintenance of microorganism

The microalga, *Tetraselmis chuii* was obtained from the Agricultural Research Service (ARS), ARS Culture Collection, US Department of Agriculture, MD, USA. The pure *T. chuii* was cultured in F medium enriched with sterilized seawater and maintained at a salinity of 30 ppt. The media composition was adapted and modified according to the standard F medium proposed by (Guillard and Ryther 1962) (Table 1). Aeration was provided using aerated flask connected to a gas filter using a silicon tube, and the gas filter was linked to a gas line from the gas distributor that supplied air to stimulate culture growth. The cultures were illuminated for a constant diurnal cycle of 12 h (light):12 h (dark) at 27 °C. The temperature of 27 °C was selected based on preliminary trials indicating optimal growth for *T. chuii* under these conditions, aligning with a prior study reporting *T. chuii* cultured in 25–27 °C (Bonilla-Ahumada et al. 2018). Light was supplied using 6500 K cool daylight T8 fluorescent tubes, providing an estimated irradiance of approximately 50 µmol photons m^{-2} s^{-1} at the culture

Table 1 F medium chemical composition

Components	Concentration
$NaNO_3$	150 mg/L
$NaH_2PO_4.H_2O$	10 mg/L
Trace elements	
$FeCl_3$•6(H_2O)	6.3 mg/L
Na_2(EDTA)•2H_2O	8.72 mg/L
$CuSO_4$•5(H_2O)	0.0196 mg/L
Na_2MoO_4	0.0126 mg/L
$ZnSO_4$•7(H_2O)	0.044 mg/L
$CoCl_2$•6(H_2O)	0.020 mg/L
$MnCl_2$•4(H_2O)	0.360 mg/L
Vitamins	
Thiamine•HCl (vitamin B1)	0.2 mg/L
Biotin (vitamin H)	1 µg/L
Cyanocobalamin (vitamin B_{12})	1 µg/L

Table 2 Experimental run in OFAT

Experiment run	Concentration (mg/L)				
	Ni	Mn	Cu	Zn	Fe
1	0.03	0.1	0.005	0.01	1.3
2	0.06	0.1	0.005	0.01	1.3
3	0.12	0.1	0.005	0.01	1.3
4	0.25	0.1	0.005	0.01	1.3
5	0	0.05	0.005	0.01	1.3
6	0	0.2	0.005	0.01	1.3
7	0	0.1	0.0025	0.01	1.3
8	0	0.1	0.01	0.01	1.3
9	0	0.1	0.005	0.005	1.3
10	0	0.1	0.005	0.02	1.3
11	0	0.1	0.005	0.01	0.65
12	0	0.1	0.005	0.01	2.6
13 (control)	0	0.1	0.005	0.01	1.3

surface. The cultures were agitated at 120 rpm using an incubator shaker (IS-971R, Lab Companion, Korea). Subculturing was conducted every 2 weeks to ensure the purity and health of the stock culture, which was subsequently used as the active culture for experimental work.

One-factor-at-time

To determine the tolerance range of trace elements and assess their effects on biomass and SOD production using the OFAT method, a modified F medium was prepared by supplementing it with varying concentrations of nickel (Ni), which is not included in the original F medium. For the other trace elements which existing in the F medium, including manganese (Mn), copper (Cu), zinc (Zn), and iron (Fe), their concentrations were adjusted to three levels: F (standard strength), F/2 (half strength), and 2 F (double strength). The OFAT experiment comprised a total of 13 experimental runs, with the 13 th run serving as the control using F media. The experimental setup for each run, along with the specific concentrations of each trace

Table 3 Taguchi orthogonal array experimental design

Run	Factors and their levels						
	A	B	C	D	E	F	G
1	1	1	1	1	1	1	1
2	1	1	2	2	2	2	2
3	1	1	3	3	3	3	3
4	1	2	1	1	2	2	3
5	1	2	2	2	3	3	1
6	1	2	3	3	1	1	2
7	1	3	1	2	1	3	2
8	1	3	2	3	2	1	3
9	1	3	3	1	3	2	1
10	2	1	1	3	3	2	2
11	2	1	2	1	1	3	3
12	2	1	3	2	2	1	1
13	2	2	1	2	3	1	3
14	2	2	2	3	1	2	1
15	2	2	3	1	2	3	2
16	2	3	1	3	2	3	1
17	2	3	2	1	3	1	2
18	2	3	3	2	1	2	3

Table 4 Selected variables and concentration levels in Taguchi optimization

	Factors	Level		
		1	2	3
A	Nitrogen source type	$NaNO_3$	KNO_3	-
B	Nitrogen concentration (g/L)	A*	B*	C*
C	Ni concentration (mg/L)	0.045	0.06	0.09
D	Mn concentration (mg/L)	0.025	0.05	0.075
E	Cu concentration (mg/L)	0.005	0.007	0.010
F	Zn concentration (mg/L)	0.015	0.020	0.025
G	Fe concentration (mg/L)	1.95	2.60	3.25

Levels 1, 2, and 3 correspond to A*, B*, and C*, respectively. The nitrogen concentration for A*, B*, and C* are equivalent to 0.0124 g/L, 0.0247 g/L, and 0.0494 g/L, respectively, regardless of the nitrogen source used

element, is detailed in Table 2. For inoculum preparation, 45 mL of active culture was inoculated into 300 mL of F medium in 500-mL Erlenmeyer flasks and cultivated for 21 days. Approximately 37×10^6 cells/mL from the exponential phase of the inoculum were then transferred into 250-mL sterile Erlenmeyer flasks containing 100 mL of the respective modified media for each OFAT experimental run and cultivated for 21 days. All cultivation conditions (temperature, aeration, agitation, and lighting) remained consistent with the stock culture protocol.

Taguchi orthogonal array

Taguchi orthogonal array method was employed to identify the optimal factors and levels for maximizing microalgal biomass and SOD activity. To assess the effect of different nitrogen sources on *T. chuii* growth, $NaNO_3$ (0.15 g/L) in the standard F medium was substituted with KNO_3 (0.1784 g/L) while maintaining a consistent nitrogen, N concentration of 0.0247 g/L, as per the standard F medium. The choice of nitrogen source was based on previous literature indicating its effects on cell growth and biochemical composition of *Tetraselmis* sp. (Kim et al. 2016). The nitrogen concentration range was adapted and modified from El-Sheekh et al. (2015), where levels 1, 2, and 3 correspond to F/2, F, and 2 F, respectively. Accordingly, when $NaNO_3$ is used, F/2, F and 2 F are equivalent to $NaNO_3$ concentrations of 0.075 g/L, 0.15 g/L, and 0.3 g/L, respectively, while when KNO_3 is used, F/2, F and 2 F are equivalent to KNO_3 concentrations of 0.089 g/L, 0.178 g/L, and 0.357 g/L, respectively. As a result, the nitrogen concentration for both $NaNO_3$ and KNO_3 was maintained at 0.0124 g/L (F/2), 0.0247 g/L (F), and 0.0494 g/L (2 F). Similarly, the concentration levels of Ni, Mn, Cu, Zn, and Fe were determined based on optimal levels of SOD activity observed in the OFAT study. Table 3 shows the experimental matrix, generated using Minitab software (Minitab version 21.1.1., Minitab Inc., USA). The L18 Taguchi orthogonal array was used, comprising 18 total experimental runs, with one factor having two levels while five factors having three levels (2^1, 3^5). Each column depicts an independent factor, and the experimental condition changes according to the levels assigned to each factor as outlined in Table 4. The cultivation protocol for the Taguchi experimental runs was identical to that used in the OFAT experiments to ensure consistency in culture conditions.

Analytical procedures

Determination of dry cell weight

Fifteen millilitre of culture broth was harvested in a centrifuge tube and centrifuged at 2500 rpm for 5 min (2010, Kubota, Japan). The supernatant was discarded. The cells were transferred into a pre-weighed microcentrifuge tube and washed with 0.5 M ammonium formate to remove excess sea salts, followed by distilled water. The resuspended cells were centrifuged again at 2500 rpm for 5 min (Microfuge 16, Beckman Coulter, USA). The supernatant was discarded. The tubes were dried in an oven at 70 °C overnight, cooled to room temperature in a desiccator, and weighed. The dry cell weight was calculated as shown in Eq. (1).

$$\text{Dry cell weight (g/l)} = \frac{(\text{Weight of microcentrifuge tube with dried cells} - \text{Weight of microcentrifuge tube})}{\text{Volume of sample}} \quad (1)$$

Preparation of extract for SOD, Bradford, and DPPH assay

Ten millilitre of the algal sample was collected and centrifuged at 2500 rpm for 5 min. For SOD and Bradford assay, the cell recovered were washed with 1 mL of potassium phosphate buffer (0.2 M, pH 8.0, 5 mM EDTA, 1 mM dithiothreitol) as adapted from Santiago-Morales et al. (2018) and resuspended in the same buffer and sonicated for 10 × 40 s in ice with 40% amplitude (UP100H, Hielscher, Germany), as adapted from Escosura and Gálvez (2022). After centrifugation at 5000 rpm for 10 min, the supernatant was stored frozen at − 20 °C. For DPPH assay, the cell recovered were washed with 0.5 M of ammonium formate and centrifuged again at 2500 rpm for 5 min to remove excess salts. The pellet was resuspended in methanol and sonicated under the same conditions. After centrifugation (5000 rpm, 10 min), the supernatant was stored at − 20 °C for analysis.

SOD activity assay

Superoxide dismutase (SOD) assay was carried out using SOD assay kit (Catalog No. 19160; Sigma-Aldrich Chemie, Switzerland) according to the manufacturer's protocol. The algal extract samples with the reaction mixture were incubated at 37 °C for 20 min. The absorbance was measured at 450 nm using UV/Vis spectrophotometer (Optizen POP, Mecasys, Korea). The concentration of SOD in the sample was quantified using the standard curve of known concentration of reference SOD.

Protein analysis

Protein analysis was carried out using the Bradford assay method (Bradford 1976). Twenty-five microlitre of each algal extract sample were mixed with 750 µL of Bradford reagent. The mixture was incubated for 15 min at room temperature. The absorbance was measured at 595 nm using UV/Vis spectrophotometer. The protein concentration of the extracts was calculated based on the bovine serum album (BSA) standard curve.

DPPH radical scavenging assay

The DPPH (2.2-diphenyl-1-picrylhydrazyl) radical scavenging activity of microalgal extracts was adapted from Danouche et al. (2020). A total of 200 µL of each algal extract sample prepared in methanol was mixed with 800 µL of 0.06 mM of DPPH solution. The mixture was incubated for 20 min at room temperature. The absorbance was measured at 517 nm using UV/Vis spectrophotometer. Ascorbic acid was used as the standard for comparison. The percentage of scavenging activity of DPPH radical by the sample was calculated as shown in Eq. (2).

$$\text{Scavenging activity } (\%) = \frac{(A_{\text{control}} + A_{\text{sample blank}}) - A_{\text{sample}}}{A_{\text{control}}} \times 100\% \quad (2)$$

Validation of optimized parameters

The optimized conditions with expected SOD activity and final DCW were predicted using Minitab software. S/N ratio that predicted the response and optimal levels was calculated as shown in Eq. (3).

$$S/N = -\text{loglog (MSD)} \quad (3)$$

Statistical analysis

The data collected in the OFAT experiment were analysed using one-way ANOVA (with significance $p \leq 0.05$) data analysis and the mean differences were compared using the Tukey HSD post hoc tests. All studies were conducted in triplicate, and the values were expressed as mean ± standard error. The statistical analysis for OFAT was performed using JASP statistical software (JASP version 0.16.4.0, University of Amsterdam, The Netherlands). The statistical analysis for the Taguchi experiment was performed using Minitab software.

Results and discussion

Trace elements effect on biomass, SOD activity, and DPPH scavenging activity of Tetraselmis chuii during OFAT study

In the control culture grown in standard F medium without modifications to trace metal concentrations for 21 days, a final dry cell weight (DCW) of 0.433 ± 0.007 g/L, superoxide dismutase (SOD) activity of 81.04 ± 0.44%, and DPPH scavenging activity of 51.90 ± 4.85% were recorded. Figure 1 illustrates the final DCW, SOD activity, and DPPH scavenging activity of *Tetraselmis chuii* following cultivation with varying concentrations of Ni, Mn, Cu, Zn, and Fe.

Nickel

As shown in Fig. 1a, a progressive decline in the final DCW was observed with increasing concentration of Ni. Statistical analysis revealed significant differences in the final DCW between cultures cultivated in standard F medium (0 mg/L Ni), which lacks nickel in its original formulation, against those added with 0.06, 0.12, and 0.25 mg/L Ni ($p < 0.05$). The observed reduction in final DCW with increasing Ni concentration can be attributed to the rising toxicity of Ni at higher concentrations, which inhibits the growth of *T. chuii*. This finding aligns with previous studies, such as Cameron et al. (2018), which reported a significant decrease in cell densities of *Tetraselmis marina* at any concentration of Ni compared to controls. Similarly, Martínez-Ruiz and Martínez-Jerónimo (2015) observed inhibited cellular growth in *Ankistrodesmus falcatus* over 96 h at Ni concentrations of 0.03, 0.06, and 0.12 mg/L. Nickel exerts toxic effects on cells when its levels exceed the physiological requirements of the organism (Martínez-Ruiz and Martínez-Jerónimo 2015). Specifically, nickel interacts with photosystem II, disrupting its active site and interfering with electron transport and photosynthetic pathways, ultimately reducing chlorophyll concentrations and inhibiting growth (Boisvert et al. 2007; Martínez-Ruiz and Martínez-Jerónimo 2015). The

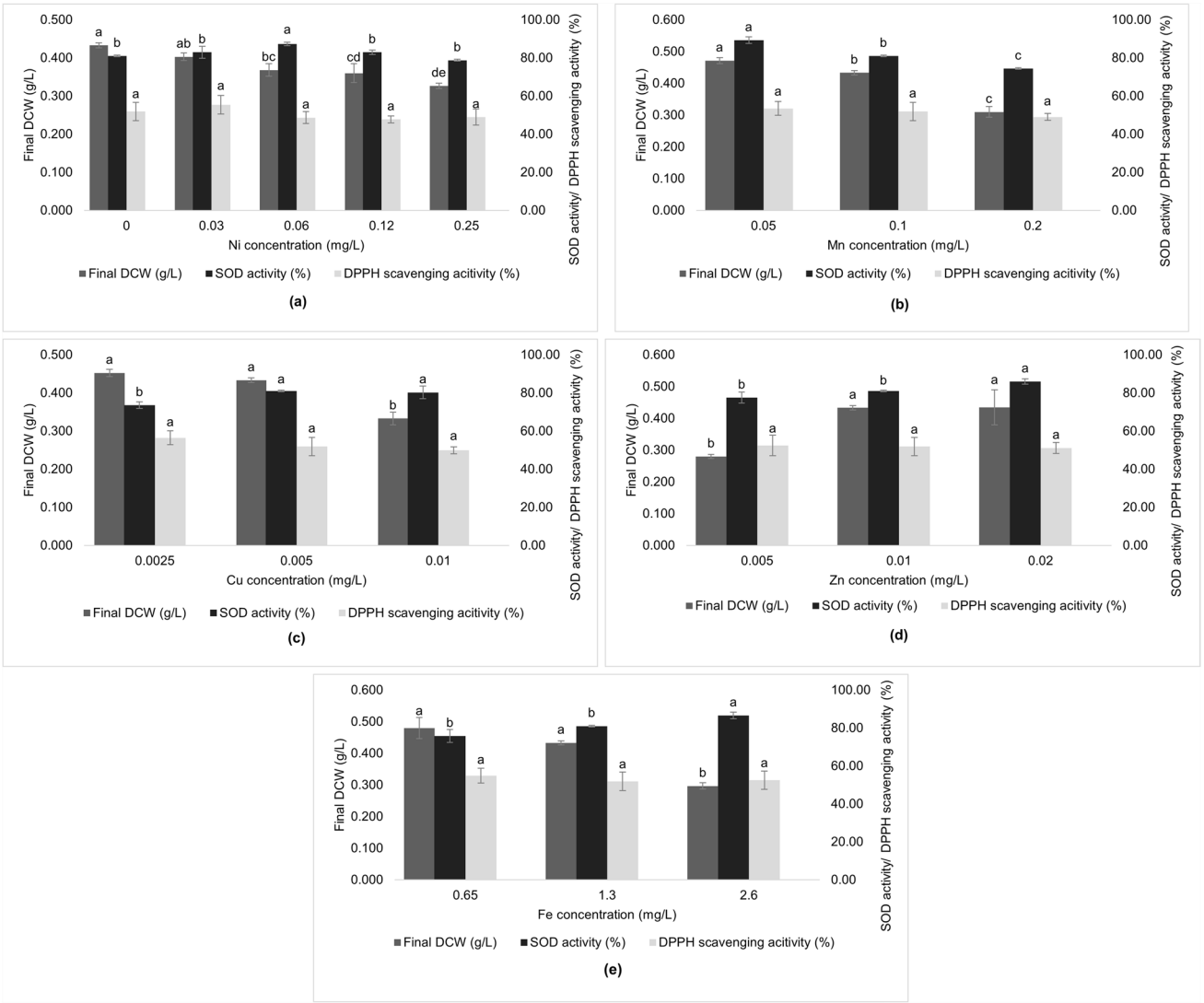

Fig. 1 Effect of different concentrations of **a** nickel, **b** manganese, **c** copper, **d** zinc, and **e** iron on final dry cell weight, SOD activity, and DPPH scavenging activity of *T. chuii*. Differences between means with the same letter are not significant ($p > 0.05$ ANOVA followed by Tukey's HSD test). Error bars indicate the standard deviation of the mean

highest SOD activity was obtained in the cultures cultivated with 0.06 mg/L of Ni, and it was significantly different ($p < 0.05$) as compared to other concentrations. This could be due to *T. chuii* tolerable to the increased in oxidative stress induced by Ni up to 0.06 mg/L. Above 0.06 mg/L, higher Ni concentrations became highly toxic to the cells, reducing cell growth, which in turn decreased the production of SOD enzymes. This result matches those observed in earlier studies, where a significant increase in SOD activity was observed in the diatom *Amphora subtropica* and green algae *Dunaliella* sp. after exposure to 300 mg/L and 500 mg/L nickel, respectively, which induced ROS scavenging mechanism in cells (Dahmen-Ben Moussa et al. 2018). Martínez-Ruiz and Martínez-Jerónimo (2015) reported decreasing SOD activity in *Ankistrodesmus falcatus* when exposed to 0.001 to 0.008 mg/L of Ni, while at highest Ni concentration (0.017 mg/L), the elevation of oxidative stress–induced cells to increase production of SOD to counteract that toxic effect. This explained the increase of SOD activity from 0.03 to 0.06 mg/L Ni due to Ni-induced oxidative stress, which increased the SOD production in microalgae.

Manganese

Likewise, Fig. 1b illustrates that the final DCW of *T. chuii* decreased with increasing manganese (Mn) concentrations. Statistical analysis revealed significant differences in final DCW across all three Mn concentrations tested ($p < 0.05$), indicating that elevating Mn levels from 0.05 to 0.2 mg/L adversely affects *T. chuii* growth. This finding is

consistent with Alho et al. (2022), who observed a significant reduction in the growth of *Raphidocelis subcapitata* after 72 h of exposure to Mn concentrations of 3.64 µM (0.2 mg/L), 7.28 µM (0.4 mg/L), and 14.56 µM (0.8 mg/L). Excessive Mn impairs microalgal chlorophyll production, thereby hindering photosynthesis (Alho et al. 2022). While Mn is essential for algal growth, elevated concentrations become toxic to the cells. Statistical analysis showed significant differences in SOD activity for all three different Mn concentrations ($p < 0.05$). The result was consistent with previous findings where there was a significant decrease in the SOD activity of *Scenedesmus quadricauda* with increasing Mn concentration, which suggested that antioxidant capacity in microalgae was altered at high Mn concentrations (Švec et al. 2016). However, the increasing concentration of $MnCl_2$ did not increase the content of ROS. Thus, higher Mn concentrations did not induce the production of SOD enzymes (Švec et al. 2016). Thus, the decrease of SOD activity with increasing concentration of Mn in the present finding may be attributed to the low quantity in final biomass.

Copper

Figure 1c shows a decrease in final DCW with increasing Cu concentrations. However, statistically, the final DCW of culture at 0.01-mg/L Cu was significantly lower ($p < 0.05$) than that at 0.0025 mg/L and 0.005 mg/L Cu. This revealed that the growth of *T. chuii* had been inhibited by 0.01 mg/L Cu. This finding is supported by previous studies; for instance, Kumar and Shin (2017) observed a slight increase in the growth of *Tetraselmis suecica* up to 2.7 µM Cu, but a decrease above 20 µM Cu. Additionally, Rodgers et al. (2010) reported that Cu concentrations between 0.01 and 0.04 mg/L significantly decreased the diversity of phytoplankton genera. While Cu is essential in low concentrations for microalgae, high levels reduce photosynthetic pigments and disrupt cell structure, impairing cellular processes and growth (Li et al. 2010; Zhou et al. 2012). Both 0.005 mg/L and 0.01 mg/L Cu concentrations showed significantly higher ($p < 0.05$) SOD activity compared to 0.0025 mg/L, indicating that increased Cu levels stimulated SOD activity in *T. chuii*. This finding matches with Wang et al. (2018), which revealed a significant increase in SOD activity in *Closterium ehrenbergii* even at low concentrations of Cu (0.1 mg/L). Qiu et al. (2022) also reported that SOD activity in *Chlorococcum* sp. had increased with increasing concentration of Cu from 0 to 200 mg/L.

Zinc

From Fig. 1d, the final culture biomass at 0.005 mg/L Zn was significantly lower ($p < 0.05$) than that at 0.01 mg/L and 0.02 mg/L Zn. This suggested that Zn is very essential for the growth of *T. chuii*. The present result supports the previous finding, which reported that culture media without zinc produced less biomass and cell density than the control medium (Alfiarty 2018). El-Agawany and Kaamoush (2023) claimed that low zinc concentrations aided microalgae growth while high zinc concentrations slowed growth and reduced cell division. The result of the present study indicates that 0.02 mg/L Zn, while it was set as the highest in the range, may be considered to be not too excessive, which induces the growth of *T. chuii.* The culture in 0.02 mg/L Zn also recorded a significantly higher ($p < 0.05$) SOD activity compared to conditions at 0.005 mg/L and 0.01 mg/L Zn. The finding suggested that higher Zn concentration increases SOD activity in *T. chuii*. Mandal et al. (2019) also reported that *Chlorella* sp. cultured in a medium supplemented with Zn induced oxidative stress because ROS production increased. Hamed et al. (2017) also reported an increased in SOD activities in *Chlorella sorokiniana* and *Scenedesmus acuminatus* after 1-week exposure to Zn. Zinc induced oxidative stress in both species and enhanced the antioxidant defences by producing SOD. The present finding shows that Zn at 0.02 mg/L had induced oxidative stress, which induced higher production of SOD.

Iron

Figure 1e then exhibits a reducing trend in the final dry cell weight with increasing concentration of Fe. However, statistical analysis shows that there was only a significant difference in final dry cell weight at 2.6-mg/L Fe ($p < 0.05$). This indicated that the concentration of Fe 2.6 mg/L was toxic to *T. chuii*, which reduced the growth of *T. chuii*. This finding is consistent with the previous finding, where there was a significant reduction in microalgal growth at a high concentration of Fe. Iriani et al. (2011) obtained a low cell number of *Chlorella* sp. with increasing Fe concentrations. Fe is highly reactive and toxic due to its ability to cause a Fenton reaction (Sevcikova et al. 2011). The present finding shows that Fe at 2.6 mg/L had affected the cell growth of *T. chuii*, which caused the reduction in growth. For SOD activity, the SOD activity of culture at 2.6-mg/L Fe was significantly higher ($p < 0.05$) than the other two. This shows that at higher Fe concentrations, the SOD activity had increased. This result is consistent with the previous finding that the SOD activities of *Chlorella* sp. NC-MKM, *Graesiella emersonii* NCM1, and *Scenedesmus acutus* NC-M2 grew in media supplemented with 90.4 µM of Fe NC-M1 (Mandal et al. 2019). This was explained by Fe was able to induce oxidative stress and enhance the production of SOD. In the present study, although 2.6 mg/L of Fe was toxic enough that it reduced the growth of *T. chuii*, the SOD activity was still increased compared to the control (1.3 mg/L Fe), indicating that concentration could be optimal for maximum SOD activity.

Statistical analysis revealed no significant differences in DPPH scavenging activity across cultures treated with varying concentrations of Ni, Mn, Cu, Zn, and Fe. This was unexpected, given the significant increase in final dry cell weight with higher metal concentrations. A possible explanation is the induction of other antioxidant systems not captured by the DPPH assay, which primarily measures the free radical scavenging capacity of low-molecular-weight antioxidants. Previous studies have shown that metal exposure can stimulate other antioxidant systems. For instance, the antioxidant activity of catalase and glutathione peroxidase in *Ankistrodesmus falcatus* was induced at higher concentrations of Ni (Martínez-Ruiz and Martínez-Jerónimo 2015), while higher Fe concentrations have been associated with elevated peroxidase activity (Mandal et al. 2019). Therefore, assessing total antioxidant capacity is complex, as multiple antioxidant enzymes may be differentially regulated by metal exposure.

Effect of trace metal factors on final biomass, SOD activity, DPPH scavenging activity, and protein concentrations by Taguchi optimization

One-factor-at-time study revealed significant effects of all five trace metals (Ni, Mn, Cu, Zn, Fe) on SOD activity and biomass accumulation in *T. chuii*. These metals were selected for further Taguchi optimization, along with nitrogen source type and concentration, based on their impact and a previous study. Table 4 presents the factors and their concentration levels in the Taguchi experiment.

Biomass production

Figure 2a shows the analysis of the signal-to-noise (S/N) plot regarding the effect of factors on biomass production. Generally, the larger the difference between a particular factor's levels, the stronger its influence. This correlates to the ranking and delta values, which classify the seven factors. Delta values indicate the effect size by comparing the highest and lowest S/N ratios of a factor. These values were used to rank the effects from greatest to least. The order in which the individual factors affect biomass production is as follows:

N source > N concentration > Ni concentration > Mn concentration > Cu concentration > Zn concentration > Fe concentration.

To determine which factors significantly affect the biomass production of *T. chuii*, ANOVA analysis was performed (data not shown). The statistical analysis showed that nitrogen source and concentrations were the only statistically

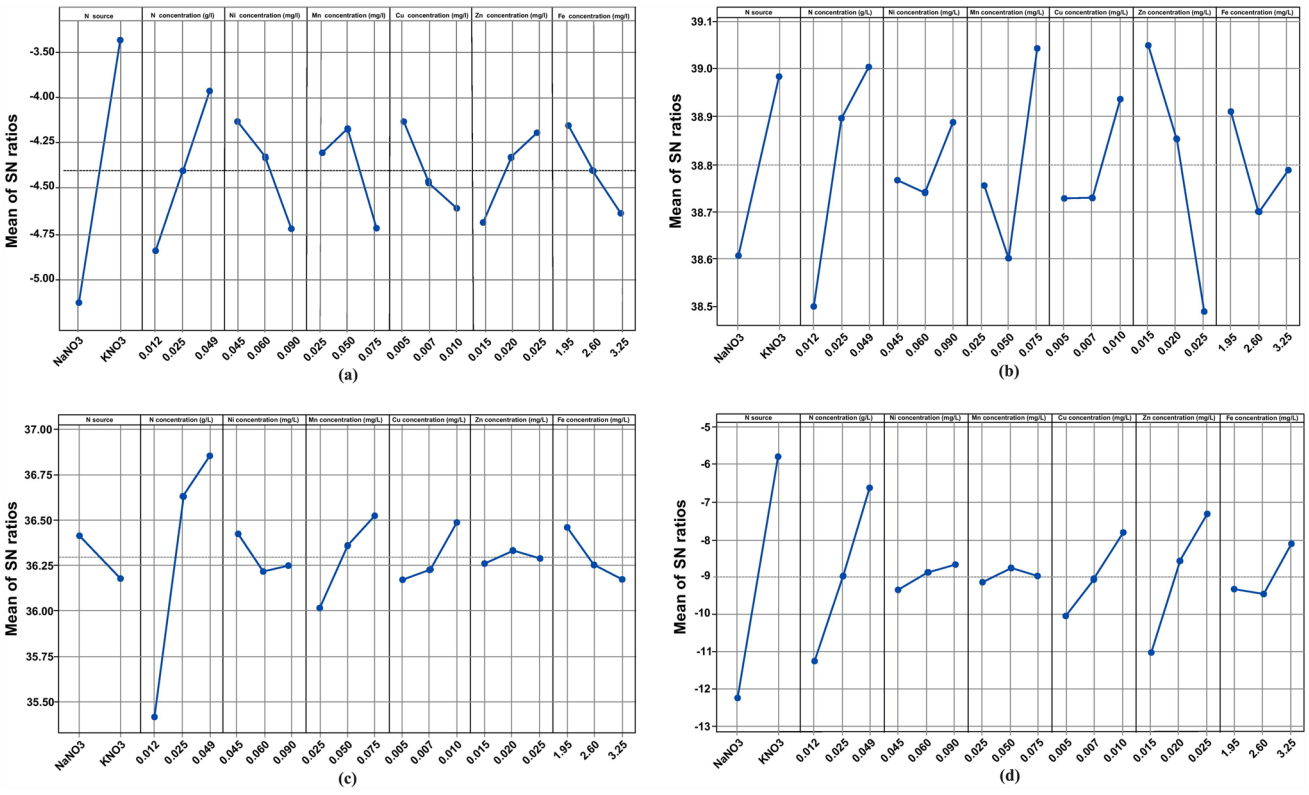

Fig. 2 Main effect plot for S/N ratio in Taguchi analysis of **a** final dry cell weight, **b** SOD activity, **c** DPPH scavenging activity, and **d** protein concentration

significant factors ($p < 0.05$). No significant change in biomass production resulted from the changes in concentrations of all trace elements. This finding was expected as nitrogen is a macronutrient in microalgae. Therefore, it has a more important role in regulating the growth, compared to trace element, which is only a micronutrient for microalgae. Nitrogen is a key nutrient that plays a crucial role in cellular growth, particularly in synthesizing nucleic acids, amino acids, proteins, and pigments (Coban et al. 2021). Among the selected factors, nitrogen source exhibited the strongest effect on biomass production. Based on Fig. 2a, a significantly higher S/N ratio was attained when KNO_3 was used as the nitrogen source to cultivate *T. chuii*. The findings observed in this study mirror those of the previous studies. Kim et al. (2016) found a higher cell concentration (1.52 g/L) of *Tetraselmis* sp. when culturing in KNO_3 compared to $NaNO_3$. Besides, Safavi et al. (2021) also observed that KNO_3 was the most proper nitrogen source for *Picochlorum* sp. growth compared to $NaNO_3$ and NH_4NO_3. Coban et al. (2021) also reported the highest growth of *Scenedesmus acutus* in the KNO_3 culture compared to other nitrogen sources ($CaNO_3$, $NaNO_3$, and NH_4NO_3). Karemore et al. (2013) also showed that *Chlorococcum* sp. produced higher biomass when culturing in KNO_3 compared to $NaNO_3$. Thus, the present study showed that KNO_3 could produce higher biomass than $NaNO_3$ as the nitrogen source in the culture medium.

Following the nitrogen source, nitrogen concentration also strongly affected *T. chuii* growth. As nitrogen is an important component for microalgae growth, the nitrogen concentration in a culture medium affects microalgae's growth rate and final biomass produced. According to Fig. 2a, higher nitrogen concentration resulted in a higher final biomass produced. This result is consistent with Dammak et al. (2017), where the highest level of $NaNO_3$ (1.76 g/L) resulted in the highest biomass production in *Tetraselmis* sp. Roy et al. (2021) found that the optimized media with a higher KNO_3 concentration (3.145 g/L) produced higher biomass of *Dunaliella* sp. compared to the KNO_3 concentration in the original basal medium (0.5 g/L). Rukminasari et al. (2019) also reported that increasing nitrate concentration tends to result in higher biomass of *Tetraselmis* sp. Besides, this result was also found in other microalgal species whereby increasing nitrogen concentration from 72 to 144 mg/L enhanced the biomass production in *Isochrysis galbana* (Zarrinmehr et al. 2019). Thus, the present observation agrees with previous findings whereby the nitrogen concentrations positively affected the biomass in *T. chuii*.

SOD activity

Figure 2b shows the S/N graphical analysis concerning the seven factors' effect on SOD activity. Based on the ranking and delta values computed which classify the seven factors, the individual factors influence SOD activity in the order as follows:

> Zn concentration > N concentration > Mn concentration > N source > Fe concentration > Cu concentration > Ni concentration.

ANOVA analysis of the Taguchi experiment showed that zinc concentration, nitrogen concentration, and nitrogen source are the only statistically significant factors ($p < 0.05$) (data not shown). No significant change in SOD activity resulted from the changes in concentrations of the remaining four factors, which were the concentrations of Ni, Mn, Fe, and Cu. This could be due to the range of concentrations tested being narrower compared to that in OFAT. Based on Fig. 2b, when KNO_3 was utilized as the nitrogen source in the media, higher SOD activity was obtained, for nitrogen concentration, higher SOD activity was attained at higher nitrogen concentrations. This finding was expected because there was higher biomass produced during KNO_3 as a nitrogen source with higher concentration, thus resulting in higher SOD activity. The most interesting finding was that the zinc concentration had a significant negative effect on the SOD activity in the Taguchi experiment. This finding was unexpected as higher zinc concentration had a positive impact on biomass production (Fig. 2a). Theoretically, higher zinc concentration improved microalgal growth and induced SOD activity (Kumar et al. 2014; Hamed et al. 2017; Mandal et al. 2019). However, since the effect of zinc concentration on the biomass production in the Taguchi experiment was not statistically significant, the decrease in SOD activity with higher zinc concentration could be due to growth inhibition by higher zinc concentration, as claimed in other findings (El-Agawany and Kaamoush 2023; Cao et al. 2015; Zhou et al. 2012). Thus, from Taguchi optimization, 0.015-mg/L Zn was the optimal concentration to improve SOD production while maintaining cell growth at the optimum level.

DPPH scavenging activity

Figure 2c shows the S/N ratio plot of the seven factors' effect on DPPH scavenging activity. Based on the ranking and delta values computed which classify the seven factors, the order of the influence of individual factors on DPPH scavenging activity is:

> N concentration > Mn concentration > Cu concentration > Fe concentration > N source > Ni concentration > Zn concentration.

Through ANOVA analysis of the Taguchi experiment (data not shown), there was no significant change ($p > 0.05$)

in DPPH scavenging activity resulting from all changes in concentrations of all factors. This finding was expected since there was no significant change in DPPH scavenging activity during the OFAT study. As there was no significant difference in DPPH scavenging activity in the Taguchi experiment, it was then speculated that each factor had only minimal influence on the total antioxidant production.

Protein concentration

Figure 2d shows the S/N ratio graphical analysis related to the effect of seven factors on protein concentration. Again, based on the ranking and delta values which classify the seven factors, the order in which the individual factors influence protein production is as follows:

> N source > N concentration > Zn concentration > Cu concentration > Fe concentration > Ni concentration > Mn concentration.

Through ANOVA analysis (data not shown), nitrogen source and nitrogen concentrations are the only statistically significant factors ($p < 0.05$). This finding was expected as nitrogen is the main component of protein, and the nitrogen concentration in the culture medium will influence the biochemical composition of microalgae. Among the selected factors, nitrogen source exhibited the strongest effect on the protein production in *T. chuii*, where KNO_3 resulted in higher protein concentration than $NaNO_3$. This result is consistent with previous findings where the protein content in *Scenedesmus acutus* was the highest when culturing in KNO_3, compared to other nitrogen sources ($CaNO_3$, $NaNO_3$, $(NH_4)_2SO_4$, and NH_4NO_3) (Coban et al. 2021). Nitrogen concentration was the second strongest factor that affected protein production in *T. chuii*, indicating nitrogen concentration positively affected protein production. This finding is consistent with the earlier study where *T. chuii* yielded the highest protein content (0.27 mg/mL) at the highest level of nitrogen concentration (El-Sheekh et al. 2015). Thus, the present finding revealed the fact that higher nitrogen concentration with KNO_3 as a nitrogen source will increase the protein production by *T. chuii*.

Validation of optimized parameters

In the Taguchi experiment, the concentrations of Ni, Mn, Cu, and Fe did not significantly influence biomass production, SOD activity, DPPH scavenging activity, and protein production of *T. chuii*. Hence, only the optimum level of nitrogen source, nitrogen concentrations, and Zn concentrations was considered to formulate optimized media. The optimized media was formulated with KNO_3 as a nitrogen source, with nitrogen concentration at a high level (0.049 g/L) and Zn concentration at a low level (0.015 mg/L). For other trace elements, including Mn, Cu, and Fe, the concentrations were maintained as in basal F medium. By using the Taguchi analysis prediction model in Minitab, the maximum biomass production, SOD activity, DPPH scavenging activity, and protein were predicted at optimized levels of nitrogen source, nitrogen concentration, and zinc concentration. The prediction model was experimentally validated using the determined optimum conditions. As shown in Table 5, the obtained result from validation experiments was close to the predicted values, such as 0.686-g/L biomass; 93.60% SOD; 68.72% DPPH, and 0.574-mg/mL protein. The obtained biomass (0.643 ± 0.024 g/L) was 20.63% or 1.21-fold higher than that of nonoptimized medium (0.533 ± 0.019 g/L) with high SOD activity (91.94 ± 0.39% vs. 85.28 ± 1.96%), DPPH scavenging activity (67.40 ± 1.16% vs. 56.58 ± 0.58%), and protein (0.590 ± 0.025 mg/mL vs. 0.472 ± 0.011 mg/mL), which were 1.08, 1.19, and 1.25-fold higher respectively than under non-optimized conditions.

Table 5 Final dry cell weight, SOD activity, DPPH scavenging activity, and protein concentration of *T. chuii* before and after optimization of variables by Taguchi orthogonal array

Variables	Before optimization	After optimization	
N source	$NaNO_3$	KNO_3	
N concentration (g/L)	0.025	0.049	
Zinc concentration (mg/L)	0.01	0.015	
Responses		Predicted	Experimental
Final dry cell weight (g/L)	0.533 ± 0.019	0.686	0.643 ± 0.024
SOD activity (%)	85.28 ± 1.96 (24.40 ± 3.55 U/mg protein)	93.60	91.94 ± 0.39 (39.76 ± 3.90 U/mg protein)
DPPH scavenging activity (%)	56.58 ± 0.58 (12.76 ± 0.25 mg AAE/mL/g biomass)	68.72	67.40 ± 1.16 (13.82 ± 0.85 mg AAE/mL/g biomass)
Protein concentration (mg/mL)	0.472 ± 0.011	0.574	0.590 ± 0.025

Limitations and future directions

While this study provides valuable insights into the effects of trace elements on the biomass and SOD production of *T. chuii*, several aspects were not covered. Specifically, biochemical estimations such as chlorophyll content, changes in cell size, and the effects on the cell wall were not included. These analyses could have further strengthened the findings and provided a more comprehensive understanding of the physiological responses of *T. chuii* to varying trace element concentrations. Additionally, measurements of residual element concentrations in the media post-cultivation were not conducted. Such data would offer valuable insights into the feed versus consumption dynamics of the trace elements and their relationship to the observed biomass and SOD production. Moreover, growth curve analysis was not conducted in this study. Instead, the focus was on evaluating the impact of trace element addition on final biomass yield after 21 days of cultivation. Incorporating growth curve monitoring in future work would help elucidate how trace elements influence different growth phases over time. These limitations highlight key areas for future research to enhance the comprehensiveness and robustness of findings related to trace element utilization in *T. chuii* cultivation.

Conclusions

Through Taguchi optimization, the optimal conditions for biomass and SOD production were identified as follows: nitrogen source (KNO_3), nitrogen concentration (0.049 g/L), and zinc concentration (0.015 g/L). This optimization resulted in a 20.63% increase in biomass and an 8% increase in SOD production. The Taguchi orthogonal array method efficiently optimized the medium with a minimal number of well-defined experiments, significantly reducing both time and costs. Moreover, the high robustness of the Taguchi design is crucial for scaling up microalgae cultivation and SOD production. Future studies exploring *T. chuii* tolerance to a broader range of trace element concentrations, including values beyond those tested in this study, may further enhance biomass and SOD yields, leading to more impactful outcomes.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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