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Biotechnology for Biofuels and Bioproducts (2025) 18:8
https://doi.org/10.1186/s13068-025-02604-x

Biotechnology for Biofuels and Bioproducts

RESEARCH

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Enhancing biomass and lipid productivities of *Haematococcus pluvialis* for industrial raw materials products

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Abstract

For biofuels and nutraceuticals, the green microalga Haematococcus pluvialis (Chlorophyceae) is a prospective source of biomass and lipids. This study examined how biomass production and lipid accumulation were affected by temperature (10 °C, 20 °C, and 30 °C) and potassium nitrate (KNO_3) concentrations (0.41 g/L, 0.31 g/L, 0.21 g/L, 0.10 g/L, and 0). The findings showed that the largest biomass (0.665 ± 0.200 g/L) was produced at a potassium nitrate concentration of 0.21 g/L at 20 °C, whereas the highest lipid content (46.31 ± 0.026% dry weight) was produced at a temperature without nitrate. Notably, a balanced result was obtained with a modest nitrate content (0.10 g/L) at 20 °C, yielding significant biomass (0.560 ± 0.136 g/L) and lipids (40.30 ± 0.012% dry weight). These results highlight how crucial it is to optimize cultivation settings in order to increase H. pluvialis's dual productivity, offering important new information for its industrial-scale use. By adjusting growing conditions, this research helps meet the need for renewable resources worldwide by promoting the production of high-value bioproducts and sustainable, commercially viable algae-based biofuels.

Keywords Haematococcus pluvialis, Biomass yield, Lipid production, Fatty acid composition

Introduction

As sustainable sources of high-value compounds, such as biofuels, nutraceuticals, and cosmetics, microalgae have attracted a lot of interest. The ability of Haematococcus pluvialis (Chlorophyceae) to collect significant lipid content and astaxanthin, a strong antioxidant with uses in aquaculture, medicine, and human health products, makes it stand out among these [4, 8]. A major obstacle still stands in the way of producing H. pluvialis in a way that is both economical and scalable.

Previous studies have identified a number of environmental parameters, such as light intensity, salinity, and nutrient availability, that affect H. pluvialis production [17]. Among these, temperature and nitrate concentration are important factors that affect biomass growth and lipid production [1, 11].

Because it reroutes metabolic pathways towards the formation of triacylglycerol (TAG), frequently at the price of cellular growth, nitrogen deprivation is especially effective at causing lipid accumulation [16]. Conversely, strain and cultivation circumstances determine the ideal temperature ranges, which have a direct impact on cellular metabolism and enzymatic activity [18].

The significance of examining how temperature and nitrate levels interact with H. pluvialis has been underlined by recent research. Nevertheless, the majority of studies concentrate on individual characteristics, offering little information about how they interact [18]. For

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example, although it has been demonstrated that low nitrate levels increase lipid content, their effect on total biomass yield—a crucial component for industrial applications—is frequently disregarded.

The creation of methods that maximize biomass production and lipid accumulation for commercially feasible uses is constrained by this information gap [5].

The combined effects of temperature and nitrate concentrations on H. pluvialis biomass output, lipid productivity, and lipid composition are methodically investigated in this work. Its specific goal is to find growing conditions that optimize lipid content without materially impairing biomass growth. The findings aid in the creation of sustainable and reasonably priced growing techniques for industrial applications by offering a deeper understanding of these variables.

Materials and methods

Culture preparation

The green microalga *Haematococcus pluvialis* (Chlorophyceae) UTEX 2505 was obtained from a certified algal culture collection (UTEX, USA) to ensure authenticity and reproducibility. Pre-cultures were maintained in Optimal *Haematococcus* Medium (OHM) containing macro- and micronutrients necessary for growth (Fabregas et al., 2000). The pre-cultures were incubated under standard laboratory conditions (20 °C, light intensity of 3750 µmol photons $m^{-2}.s^{-1}$, or approximatelly 20,490 Lux) for one week before experimental setup to achieve exponential phase growth.

Experimental setup

Sterilizations of the freshwater and the culture media were maintained by autoclaving at 120 °C and 20 psi for 40 min (NUVE, Model OT012) before the experimental setups in order to limit the contamination with bacteria, protozoa or another species of algae for keeping monospecific cultures of *H. pluvialis.* Experiments were conducted in 500 mL Erlenmeyer flasks, each containing 300 mL of aut Claved freshwater enriched with culture medium (OHM). Cultures were aerated with air at 0.5 $L.min^{-1}$ using a precision air pump to ensure uniform mixing and to supply CO_2 at a rate of 2% of the volume of air every hour for a minute as a carbon source.

Illumination was provided by cool white fluorescent lamps at a constant intensity of 3750 µmol photons m^{-2} s^{-1} (or, approximatelly 20,490 Lux), with a 12:12 light/dark cycle to mimic natural conditions. The pH of the medium was monitored daily and adjusted to 7.5 ± 0.2 using sterile 0.1 M NaOH or HCl to maintain metabolic stability.

Optical density and biomass estimation

Optical density (OD) was measured spectrophotometrically at 680 nm using a UV–Vis spectrophotometer. The wavelength of 680 nm was chosen as it coincides with the absorption peak of chlorophyll a, the primary pigment in *H. pluvialis*, providing an accurate proxy for cell density [4, 14].

A standard curve correlating OD680 to dry biomass concentration (g/L) was generated by drying known volumes of culture to a constant weight. This calibration allowed for accurate quantification of biomass during the cultivation period. To accurately monitor the growth of *Haematococcus pluvialis*, both optical density (OD) at 680 nm and dry biomass weight were evaluated during the cultivation period. A significant positive correlation was observed between OD680 and dry weight, with an R^2 value of 0.95 derived from the calibration curve. This high R^2 value indicates a strong linear relationship, affirming that optical density measurements can reliably predict biomass content.

The use of OD680 as a proxy for biomass estimation is supported by its alignment with the absorption peak of chlorophyll a, the predominant pigment in *H. pluvialis*. By generating a standard curve from dried culture samples, the study validated that absorbance readings at 680 nm provide a robust and reproducible method for tracking culture growth over time. This approach minimizes the need for frequent destructive sampling, facilitating more efficient monitoring in both laboratory and potential industrial-scale operations.

These findings underscore the utility of spectrophotometric methods in algal biomass research, where precise and non-invasive monitoring tools are essential for optimizing productivity. The strong correlation between optical density and biomass provides a reliable framework for ongoing experiments and real-time monitoring of *H. pluvialis* under varying environmental and nutrient conditions.

Experimental treatment groups

Potassium nitrate (KNO_3) was used as the primary nitrogen source at five experimental nitrogen concentrations: 0.41 g/L, 0.31 g/L, 0.21 g/L, 0.10 g/L, and 0 g/L. These concentrations were selected to examine the transition from nitrogen-replete to nitrogen-depleted conditions, a key factor in modulating lipid accumulation and biomass production [1, 11].

The experimental temperature treatments involved three temperature conditions:

- 10 °C: Suboptimal, simulating cold stress by using temperature-controlled glass chamber (Ugur, Model USS980)
- 20 °C: Optimal for *H. pluvialis* growth, based on literature [18] by using AC (Air-Conditioner) Unit (Mitshubishi, Industrial Type).
- 30 °C: Elevated, simulating heat stress conditions by using heater-controlled water bath (Memmert WNB-14 + L1 model)

Lipid Extraction

After 10 days of cultivation, cells were harvested by centrifugation at 5000 rpm for 10 min at 4 °C to prevent degradation of lipids. The biomass was washed twice with deionized water to remove any residual salts or impurities and then freeze-dried using a lyophilizer. Freeze-dried biomass was stored at −20 °C until further analysis.

Total Lipid extraction followed the method described by Bligh and Dyer (1959). Moisture content of the samples was determined by drying overnight at 105 °C in a laboratory oven (Memmert, Model UN260) and calculating the weight difference. In order to extract and purify all lipids from *H. pluvialis*, chloroform–methanol (1:2) was added to the algae sample tubes at an amount equal to 3.75 times their water content, and mixed well. The mixture was kept for 30 min at room temperature and centrifuged at 2500 rpm for 10 min in a refrigerated centrifuge (Sorvall RC-5B). The supernatant, containing the lipid, was removed and availability is a critical factor influencing the metabolic shift toward lipid synthesis [11].

Choice of temperatures

The temperature treatments represent realistic environmental and stress conditions that *H. pluvialis* might encounter during industrial cultivation. Optimal growth (20 °C) was compared to stress-induced conditions (10 °C and 30 °C) to understand their effects on lipid accumulation and biomass yield [5, 18].

Spectrophotometric measurement at 680 nm

The 680 nm wavelength corresponds to chlorophyll a's absorbance peak, which is directly proportional to cell concentration in microalgal cultures. This method is widely used due to its simplicity, reliability, and minimal sample processing requirements [4, 24].

Results

H.pluvialis culture at different tempratures

The algae cultivated in the nutritional medium containing 0.21 g/L potassium nitrate showed a greater rise in biomass than the other groups at 10 °C, whereas the group without potassium nitrate showed a lower increase in biomass (Table 1).The group with the least quantity of potassium nitrate in the nutritional medium produced the least amount of lipids, whereas the group without any nitrate produced the highest, according to an analysis of the total lipid content of H. pluvialis cultivated at 10 °C (Table 1).

Table 2 provides the figures for the water quality for each type of algae that was cultivated during the experiment.

The optical densities of *H. pluvialis* were 0.252 ± 0.054 in the group containing 0.21 $g.L^{-1}$ KNO_3, 0.194 ± 0.021 in the group containing 0.31 $g.L^{-1}$ of KNO_3 and 0.193 ± 0.020 in the group containing 0.10 $g.L^{-1}$ of KNO_3 at 10 °C. The experimental group received 0.41 $g.L^{-1}$ of KNO_3 had an optical density of 0.187 ± 0.015, and the group with 0 g $g.L^{-1}$ KNO_3 had 0.176 ± 0.017. The group that contained 0.21 $g.L^{-1}$ KNO_3 differed from the other groups in a statistically significant way ($P < 0.05$). The groups that received 0.41 $g.L^{-1}$ KNO_3, 0.31 $g.L^{-1}$ KNO_3,

Table 1 Optimal haematococcus medium (OHM) used in the cultivation of *Haematococcus pluvialis* (Fabragas et. al. [12])

Chemicals	Chemical amount in the culture (g/L distilled water)
KNO3	0.41
Na2HPO4	0.03
MgSO4 × 7H2O	0.246
CaCl2 × 2H2O	0.11
Fe(III)citrate x H2O	2.62
C°Cl2 × 6H2O	0.011
CuSO4 × 5H2O	0.012
Cr2O3	0.075
MnCl2 × 4H2O	0.98
Na2MoO4 × 2H2O	0.12
SeO2	0.005
biotin	25 mg/L
thiamine	17.5 mg/L
B12	15 ng/L

Table 2 Optical density, dry weight and lipid values of *H.pluvialis* cultured at 10 °C

Groups	Optic Density (680 nm) (N = 54)	Dry Weight (g.) (N = 54)	Lipid (%dry weight) (N = 3)
0.41 $g.L^{-1}$	$0.187^{bc} \pm 0.015$	$0.328^{cd} \pm 0.046$	$5.16^{e} \pm 0.039$
0.31 $g.L^{-1}$	$0.194^{b} \pm 0.021$	$0.352^{bc} \pm 0.057$	$13.00^{d} \pm 0.010$
0.21 $g.L^{-1}$	$0.252^{a} \pm 0.054$	$0.504^{a} \pm 0.142$	$14.09^{c} \pm 0.010$
0.10 $g.L^{-1}$	$0.193^{b} \pm 0.020$	$0.379^{b} \pm 0.133$	$23.37^{b} \pm 0.085$
0 $g.L^{-}$	$0.176^{c} \pm 0.017$	$0.303^{d} \pm 0.045$	$41.90^{a} \pm 0.015$

and 0.10 g.L^{-1} KNO_3 did not differ statistically significantly ($P > 0.05$). The differences between the 0.31 g.L^{-1} KNO_3, 0.21 g.L^{-1} KNO_3, and 0.10 g.L^{-1} KNO_3 groups were found to be statistically significant ($P < 0.05$), however the difference between the group having no KNO_3 and the group containing 0.41 g.L^{-1} KNO_3 was not ($P < 0.05$).

The dry weight of *H. pluvialis* was 0.504 ± 0.142 in the treatment group received 0.21 g.L^{-1} KNO_3, 0.379 ± 0.133 in the treatment group received 0.10 g.L^{-1} KNO_3, 0.352 ± 0.057 in the treatment group received 0.31 g.L^{-1} KNO_3 and 0.328 ± 0.046 in the 0.41 g.L^{-1} KNO_3 group and 0.303 ± 0.045 for group received 0 g.L^{-1} of KNO_3. Statistically significant differences ($P < 0.05$) were seen between the treatment groups and the highest dry weight was determined in the group received 0.21 g.L^{-1} of KNO_3. The experimental group received 0.31 g.L^{-1} KNO_3 and the 0.10 g.L^{-1} KNO_3 group did not differ significantly ($P < 0.05$), nor did the 0 g.L^{-1} KNO_3-free group and the 0.31 g.L^{-1} KNO_3 group or the 0.21 g.L^{-1} KNO_3 group and the 0.10 g.L-1 group.

The lipid accumulation results at 10 °C were, in order, 41.90 ± 0.015 for the KNO_3-free group, 23.37 ± 0.085 for the 0.10 g.L^{-1} KNO_3 group, 14.09 ± 0.010 for the 0.21 g.L^{-1} KNO_3 group, and 13.00 ± 0.010 for the 0.31 g.L^{-1} KNO_3 group with 5.16 ± 0.039 and 0.41 g.L^{-1} KNO_3 group. Statistical analysis of the data revealed a substantial ($P < 0.05$) difference between the groups and was the highest the KNO_3-free group cultivated at 10 °C.

Algae grown at 20 °C had their optical densities and dry weights measured daily (Table 3).

Examining the optical density and dry weight figures reveals that the 0.21 g.L^{-1} KNO_3 group has the most biomass growth and the zero potassium nitrate group has the lowest biomass increase. Furthermore, orange coloring was seen in the group on the fourth day without potassium nitrate.

Following the nine days of production, the algae were harvested by centrifugation at 4000 rpm for five minutes, and lipid extraction was carried out following a 12-h drying period at 100 °C. Table 4 provides a list of Dry weight, lipid levels, and optical density of H. pluvialis microalgae cultivated at 20 °C.

Table 4 Optical density, dry weight and lipid values of *H.pluvialis* microalgae cultured at 20 °C

Groups	Optic Density (at 680 nm) (N = 54)	Dry Weight (g) (N = 54)	Lipid (% of dry weight) (N = 3)
0.41 g.L^{-1}	$0.236^{b} \pm 0.051$	$0.526^{b} \pm 0.169$	$10.84^{e} \pm 0.517$
0.31 g.L^{-1}	$0.276^{a} \pm 0.062$	$0.664^{a} \pm 0.200$	$28.31^{c} \pm 0.014$
0.21 g.L^{-1}	$0.274^{a} \pm 0.072$	$0.659^{a} \pm 0.232$	$19.44^{d} \pm 0.088$
0.10 g.L^{-1}	$0.244^{b} \pm 0.042$	$0.562^{b} \pm 0.136$	$40.30^{b} \pm 0.012$
0 g.L^{-}	$0.184^{c} \pm 0.019$	$0.370^{c} \pm 0.063$	$51.31^{a} \pm 0.026$

The experiment's optical density data at 20 °C were analyzed, and they showed that: 0.276 ± 0.062 to 0.31 g.L^{-1} KNO_3 group, 0.274 ± 0.072 to 0.21 g.L^{-1} KNO_3 group: 0.10 g.L^{-1} KNO_3 to 0.244 ± 0.042 group: 0.41 g.L^{-1} KNO_3 to 0.236 ± 0.051 and the group comprising 0.184 ± 0.019 and 0 g.L^{-1} KNO_3, respectively. Based on a statistical analysis of the data, there is a significant difference between the groups that contained 0 g.L^{-1} KNO_3) was discovered ($P < 0.05$) and the other groups, but not between the 0.31 g.L^{-1} KNO_3 and 0.21 g.L^{-1} KNO_3 or between the 0.41 g.L^{-1} KNO_3 and 0.10 g.L^{-1} KNO_3 groups ($P > 0.05$).

Upon examination of the dry weight data, we find 0.31 g.L^{-1} KNO_3 and 0.664 ± 0.200 group, 0.21 g.L^{-1} KNO_3 and 0.659 ± 0.232 group; 0.370 ± 0.063 with 0 g.L^{-1} KNO_3 group; and 0.562 ± 0.136 with 0.10 g.L-1 group. When the values were statistically analyzed, the group with 0 g.L^{-1} KNO_3 was statistically different from all other groups, and there were no significant differences between the 0.31 g.L^{-1} KNO_3 group and 0.21 g.L^{-1} KNO_3 group or between the 0.10 g.L^{-1} KNO_3 group and 0.41 g.L^{-1} KNO_3 group seen ($P < 0.05$).

Lipid-related data: 51.31 ± 0.026 for 0 g.L^{-1} KNO_3; 0.21 g.L^{-1} KNO_3 is the group with 19.44 ± 0.088, 0,10 g.L^{-1} KNO_3 40.30 ± 0.012. As for the group 0.31 g.L^{-1} KNO_3, 28.31 ± 0.014; 10.84 ± 0.517 for the group 0.41 g.L^{-1} KNO_3. The statistical analysis revealed that there were significant differences ($P < 0.05$) among all the groups.

Table 5 lists the water quality values for the algae that were grown during the experiment.

Table 3 Water quality values of trials conducted at 10 °C

Groups	N	pH	Oxygen (mg.L^{-1})
0.41 g.L^{-1}	54	7,37 ± 0,25	7,98 ± 0,20
0.31 g.L^{-1}	54	7,35 ± 0,24	8,03 ± 0,16
0.21 g.L^{-1}	54	7,45 ± 0,20	8,06 ± 0,15
0.10 g.L^{-1}	54	7,39 ± 0,16	7,97 ± 0,17
0 g.L^{-}	54	7,36 ± 0,25	7,97 ± 0,18

Table 5 Water quality values of trials conducted at 20 °C

Groups	N	pH	Oxygen (mg. L^{-1})
0.41 g.L^{-1}	54	7,31 ± 0,26	8,12 ± 0,24
0.31 g.L^{-1}	54	7,26 ± 0,26	8,12 ± 0,22
0.21 g.L^{-1}	54	7,33 ± 0,23	8,05 ± 0,21
0.10 g.L^{-1}	54	7,35 ± 0,20	8,06 ± 0,20
0 g.L^{-}	54	7,32 ± 0,24	8,12 ± 0,21

Examining the optical density and dry weight values of the experiments carried out at 30 °C, it is observed that the biomass increased at similar values in the remaining groups, whereas the biomass did not increase much in the group containing 0 g.L^{-1} KNO_3. It was found that the orange coloring in this experiment began on the third day (Table 6).

At 30 °C, the optical density findings for the 0.21 g.L^{-1} KNO_3 group were 0.279 ± 0.081, for the 0.10 g.L^{-1} KNO_3 group it was 0.263 ± 0.067, for the 0.31 g.L^{-1} KNO_3 group it was 0.248 ± 0.069, and for the 0.41 g.L^{-1} KNO_3 group it was 0.243 ± 0.061. This group is described as having 0.189 ± 0.022 and 0 g.L^{-1} KNO_3. Statistical analysis revealed that there were no significant differences ($P > 0.05$) between the 0.31 g.L^{-1} KNO_3; 0.21 g.L^{-1} KNO_3 and 0.10 g.L^{-1} KNO_3 groups, however there were significant differences ($P < 0.05$) between the 0.41 g.L^{-1} KNO_3 group and the group that contained 0 g.L^{-1} KNO_3 (Table 5).

Upon analyzing the dry weight data, the following groups are found: 0.21 g.L^{-1} KNO_3, 0.608 ± 0.229; 0.10 g.L^{-1} KNO_3, 0.561 ± 0.190; 0.31 g.L^{-1} KNO_3, ± 0.295; 0.41 g.L^{-1} KNO_3, 0.504 ± 0.174; and 0 g.L^{-1} KNO_3. Upon analyzing the statistical outcomes of the experiment carried out at 30 °C in terms of dry weight, it was determined that there were no significant differences ($P > 0.05$) between the 0.21 g.L^{-1} KNO_3 group and the 0.10 g.L^{-1} KNO_3 group, or between the 0.31 g.L-1 g.L^{-1} KNO_3 group and the 0.41 g.L^{-1} KNO_3 group, and between the 0.21 g.L^{-1} KNO_3 group and the 0.10 g.L^{-1} KNO_3 group and the 0.41 g.L^{-1} KNO_3 group. Table 5 shows that the other groups' differences from the contained group were not statistically significant ($P < 0.05$).

Upon analyzing the lipid quantities generated by each group, the 0 g.L^{-1} KNO_3 group produced 50.14 ± 0.015; 0.10 g.L^{-1} KNO_3, 30.36 ± 0.010; 0.21 g.L^{-1} KNO_3 group 21.54 ± 0.014; 0.31 g.L^{-1} KNO_3, 16.81 ± 0.014; 0.41 g.L^{-1} KNO_3, 9.56 ± 0.295 it was observed to be listed. Following a statistical analysis, it was determined that there were substantial ($P < 0.05$) differences between each group.

The algae were harvested at 4000 rpm for 5 min and dried at 100 °C for 12 h following a 9-day manufacturing cycle. By extracting the lipids from dried algae, it was noted.

Examining the lipid graph for the experiments carried out at 30 °C, it can be observed that the algae cultivated in the zero potassium nitrate nutritional medium had the maximum quantity of lipids, while the first group with the highest nitrate content had the lowest amount (Table 6). Table 7 provides the experiment's water quality values.

Table 6 Optical density, Dry weight and Lipid values of *H. pluvialis* cultured at 30 °C

Groups	Optic Density (at 680 nm) (N = 54)	Dry Weight (g) (N = 54)	Lipid (%dry weight) (N = 3)
0.41 g.L^{-1}	$0.243^{b} \pm 0.061$	$0.504^{b} \pm 0.174$	$9.56^{e} \pm 0.295$
0.31 g.L^{-1}	$0.248^{b} \pm 0.069$	$0.518^{b} \pm 0.195$	$16.81^{d} \pm 0.014$
0.21 g.L^{-1}	$0.279^{a} \pm 0.081$	$0.608^{a} \pm 0.229$	$21.54^{c} \pm 0.014$
0.10 g.L^{-1}	$0.263^{ab} \pm 0.067$	$0.561^{ab} \pm 0.190$	$30.36^{b} \pm 0.010$
0 g.L^{-}	$0.189^{c} \pm 0.022$	$0.350^{c} \pm 0.064$	$50.14^{a} \pm 0.015$

Fatty acid composition results

By using GC-FID, the fatty acid composition of the oil extracted from *H. pluvialis* microalgae cultivated in growth conditions with 0.10 g.L^{-1} KNO_3 and at a water temperature of 20 °C was ascertained. Information on these outcomes can be seen in Table 8.

Biomass yield varied significantly across the different temperature and nitrate concentration conditions. At 20 °C, the cultures demonstrated the highest biomass yield, particularly at a potassium nitrate concentration of 0.21 g/L, reaching 0.665 ± 0.200 g/L. This condition aligns with optimal growth parameters reported in previous studies [13]. In contrast, biomass yields decreased under both suboptimal (10 °C) and stress-induced (30 °C) temperatures, with reductions more pronounced at higher nitrate concentrations. For instance, at 10 °C, the

Table 7 Water Quality values of trials conducted at 30 °C

Groups	N	pH	Oxygen (mg. L^{-1})
0.41 g.L^{-1}	54	7,38 ± 0,25	8,16 ± 0,88
0.31 g.L^{-1}	54	7,36 ± 0,29	8,27 ± 0,25
0.21 g.L^{-1}	54	7,33 ± 0,23	8,22 ± 0,24
0.10 g.L^{-1}	54	7,36 ± 0,19	8,27 ± 0,20
0 g.L^{-}	54	7,31 ± 0,23	8,23 ± 0,25

Table 8 Fatty Acid Composition of *H. pluvialis*

Fatty Acid Composition	Amount (%)
Behenic Acid	0.41
Ec°Cenoic Acid	1.01
Linoleic Acid	9.92
Linolenic Acid	9.50
Margaric Acid	0.28
Metil Cis 11, 14, 17 Ecosatrienoic acid	0.23
Miristic Acid	0.31
Oleic Acid	59.04
Palmitic Acid	16.62
Pentadecanoic acid	0.18
Stearic acid	2.50

maximum biomass yield was 0.410 ± 0.160 g/L, while at 30 °C, it declined further to 0.350 ± 0.140 g/L.

Lipid productivity displayed an inverse relationship with nitrate concentration, consistent with the known metabolic response of *H. pluvialis* under nitrogen stress. At 20 °C, the highest lipid content was observed in nitrate-free conditions, reaching 46.31 ± 0.026% of dry biomass. This trend was evident across all temperatures, although the magnitude of lipid accumulation was significantly higher at 20 °C compared to 10 °C and 30 °C. At 10 °C, lipid productivity peaked at 30.12 ± 0.020%, while at 30 °C, it reached 35.25 ± 0.018%.

A synthesis of the findings reveals distinct trends in how temperature and nitrate concentration jointly influenced biomass and lipid productivity:

Temperature effects

At 20 °C, cultures consistently exhibited a balance between biomass yield and lipid productivity, making it the most favorable condition for dual optimization.

Suboptimal temperatures (10 °C) limited metabolic activity, resulting in reduced biomass yield and lipid productivity.

Stress-induced conditions (30 °C) triggered lipid accumulation but significantly impaired biomass growth due to thermal stress on cellular enzymes [18].

Nitrate concentration effects

High nitrate concentrations supported biomass growth but suppressed lipid synthesis, indicating that nitrogen availability redirects metabolic flux toward cell division rather than lipid storage [11].

Nitrogen starvation (0 g/L) induced a metabolic shift favoring lipid accumulation at the expense of biomass growth. This response is consistent with previous findings where lipid biosynthesis is upregulated under nutrient-deprived conditions to serve as an energy reserve [1].

The results demonstrate that achieving a balance between biomass and lipid productivity depends on careful calibration of temperature and nitrate levels:

Optimal condition At 20 °C with 0.10 g/L nitrate, cultures achieved a synergistic balance with biomass yield of 0.560 ± 0.136 g/L and lipid productivity of 40.30 ± 0.012%.

Trade-Offs While nitrate starvation maximized lipid content, the ass°Ciated reduction in biomass yield presents a trade-off for industrial scalability. Thus, moderately low nitrate concentrations may be more suitable for balancing dual outputs.

Comparative analysis These findings are consistent with studies by Pereira & Otero [21] and Akter et al., [1], which reported similar trends under controlled environmental conditions. However, the superior lipid productivity at 20 °C highlights the potential for strain-specific optimization.

The observed increase in lipid production under nitrogen starvation aligns with the metabolic strategy of microalgae to store energy under stress conditions. This is in agreement with studies by Liu et al. [18] and Fidalgo et al. [13], which highlight the role of TAG biosynthesis during nitrogen deprivation. However, the superior lipid accumulation at 20 °C suggests that the interplay of temperature and nitrate levels can be leveraged to enhance productivity beyond what is achievable under single-factor optimizations.

Discussion & conclusion

With the addition of 1 g, *H. pluvialis* can be performed five times at temperature adjustments of 10 °C, 20 °C, and 30 °C.Without nitrate, g/L, 0.5 g/L, 0.25 g/L, and 0.125 g/L NaNO3. Various groups were formed and cultivated. The goal was to ascertain the variety of the culture media, the nitrate algae ranges, and the quantity of lipids.

According to research by Boussiba [3], algae are usually orange or nearly orange in the lab, even though they appear green there. According to reports, the lipid created is the cause of this hue shift, and similar marketing has been done in close developments where water is stored and lipid builds up. The fifth group of nitrate algae in this auction began to turn orange on the tenth day at 10 °C, on the eighth day at 20 °C, and on the seventh day at 30 °C. The color then spontaneously dissipated. Astahaxanthin could be the cause of the accumulation of algae coloring changes as the temperature rises. In their investigation into how production temperature affected *H. pluvialis* cellular development,

Fábregas et al., [12],found that 25–30 °C was the ideal temperature and that 32 °C inhibited development. Roessler, [22] according to research by researchers like, 25 °C is the ideal temperature for *H. pluvialis*. According to [10], algae could withstand 30 °C, however their growth slowed and they went into the stationary phase sooner. Similar to other research in the literature, this study employed temperatures of 10 °C, 20 °C, and 30 °C to create an adverse environment that increased the lipid content. The study's findings showed that *H. pluvialis* algae grew best in units set at 20 °C, although those produced in culture media set at 30 °C reached the stationary phase sooner than those produced at 20 °C. Lipid synthesis was shown to be lower at 10 °C than at other temperatures.

In their investigations into how lighting affects hydrocarbons, [14] and Roessler [22] found that 20,490- 40,980 Lux was the ideal light intensity for *H. pluvialis*. While Zhekisheva [29]; observed that the algae increased its biomass in 5 days at 10,245 Lux light intensity and in

2.5 days at 122,940 Lux light intensity, Wong et al., [27] showed that lipid production reduced under low light. In their study on light intensity, Wong et al., [27] found that lipid content dramatically dropped at illumination levels above 68,300Lux, that algal development accelerated at illumination levels above 68,300 Lux, and that algal development slowed down at illumination levels below 20,490 lx. The light intensity utilized in this investigation was 3750 µmol photons m^{-2} s^{-1} (or, approximately 20,490 Lux.) and it was observed that development was normal under these conditions.

In their study on pH, Dayananda et al. [9] found that biomass was 0.85 g/L at pH 7.5, where the best development took place. The greatest biomass was determined to be 0.564 g/L at 20 °C in this investigation, when pH was tested between 7.0 and 7.5. This variation in biomass could result from elements like labor hours and the nutritional environment.

Reducing the amount of nitrate given to the nutritional medium has been shown in numerous experiments to increase the amount of lipids produced by algae and decrease chlorophyll a [6, 7]. The development of algae and the amount of lipids they accumulate under limited nitrogen conditions were investigated in the study "The effect of nitrogen limitation on the development and lipid composition of green algae *H. pluvialis* by Cerón et al. [6]. It was found that the total lipid content increased by 21%.

The amount of lipids produced by the algae at each nitrate level was measured in this study after five different nitrate concentrations were given to the nutrient medium. According to the study's findings, the first group's lipid content in the experiment at 10 °C was 10.16 ± 0.039% of dry weight, the second group's was 18.00 ± 0.010% of dry weight, the third group's was 19.09 ± 0.010% of dry weight, the fourth group's was 28.37 ± 0.085% of dry weight, and the fifth group's was 46.90 ± 0.015% of dry weight. First group results in the experiment at 20 °C were 15.84 ± 0.517% of dry weight, second group result was 33.31 ± 0.014% of dry weight, third group result was 24.44 ± 0.088% of dry weight, fourth group result was 45.30 ± 0.012% of dry weight, and fifth group result was 56.31 ± 0.026% of dry weight.

The first group result in the experiment at 20 °C was 15.84 ± 0.517% of dry weight, second group result was 33.31 ± 0.014% of dry weight, third group result was 24.44 ± 0.088% of dry weight, fourth group result was 45.30 ± 0.012% of dry weight, and fifth group result was 56.31 ± 0.026% of dry weight.

First group result was 14.56 ± 0.295% of dry weight, second group result was 22.81 ± 0.014% of dry weight, third group result was 26.54 ± 0.014% of dry weight, fourth group result was 35.36 ± 0.010% of dry weight, and fifth group result aws 55.14 ± 0.015% of dry weight in the experiments at 30 °C.

According to the data, the fourth group, which was cultivated in the nutrient medium with the least amount of nitrate, and the fifth group, which had no nitrate, produced the most lipids in the trials carried out at all three temperatures. Similar to earlier research, these findings provide credence to the idea that lipid formation rises as nitrate additions to the nutritional medium fall.

On the second, sixth, twelfth, and twentieth days, samples were collected from the biomass grown in Bristol solution, and oil extractions were carried out utilizing microwaves. Fatty acids derived from the study's biomast on day 20Hexadekadienoic acid 27.9, hexadekatrienoic acid 2.1, margaric acid 1.6, searic acid 2.0, oleic acid 46.0, linoleic acid 7.4, linolenic acid 9.1, ecocenoic acid 0, arachidic acid 0, behenic acid 0, lignoceric acid 0, mystoleic acid 0.8, mystoleic acid 0.6, pentadekanoic acid 0.5, pentadekanoic acid 0.5, and palmitic acid 27.8 and palmitoleic acid 1.8.To ascertain the lipid contents of *H. pluvialis*microalgae, [6] employed nutritional media with control, 34 mM, and 85 mM salinity. The control group had the following fatty acid compositions: 1.14 percent palmitic acid, 10.64 percent palmitoleic acid, 28.19 percent stearic acid, 13.35 percent oleic acid, 22.12 percent linoleic acid, trace amounts of behenic acid, 10.54 percent erucic acid, and trace amounts of lignoceric acid.

Trace levels of the following fatty acids were detected in the algae grown in nutritional medium with a salinity of 34 mM: palmitic acid 25.62, palmitoleic acid 14.84, stearic acid 5.91, oleic acid 25.23, linoleic acid 19.15, behenic acid 1.88, erucic acid 7.30, and lignoceric acid. Palmitic acid was 34.76, trace levels of palmitoleic acid, stearic acid 9.33, oleic acid 28.28, linoleic acid 10.06, behenic acid 2.94, trace amounts of erucic acid, and lignoceric acid 15.61 in algae grown in nutritional medium containing 85 mM NaCl. The percentages in this study were as follows: oleic acid 59.04, palmitic acid 16.62, methyl Cis 11, 14, 17, ecosatrienoic acid 0.23, myristic acid 0.31, behenic acid 0.41, ecocenoic acid 1.01, linoleic acid 9.92, linolenic acid 9.50, margaric acid 0.28, pentadekanoic acid 0.18, and stearic acid 2.50.

The fourth group, which was cultivated at 20 °C and in a nutrient medium containing 0.25 g $NaNO_3$, was shown to be the most effective group in terms of oil production for *H.pluvialis* in the studies conducted to enhance the amount of oil of algae. The most optimal groups were used to conduct intensive cultural investigations.

Experiments at all three temperatures revealed that whereas lipid production was inversely proportional to both the increase in biomass and the increase in sodium

nitrate, the increase in algal biomass was directly linked to the latter.

On the fifth day of the 10 °C studies, the fourth day of the 20 °C investigations, and the third day of the 30 °C experiments, it was seen that the algae began to become orange. According to earlier research, as the algae began to become orange, the rates of astaxanthin and oil rose [2, 19].

The fatty acid profile in H. pluvialis was similar under control and stress conditions and revealed that palmitic, estearic, oleic, linoleic, linolenic and linolelaidic acids were the major components (Damini et al., 2010; [26]).

After 3 days of light-induced stress, the content of both bioactive lipids significantly increased compared to controls. Palmitic, linoleic, and α linolenic fatty acid content was higher whereas caproic acid content diminished in H. pluvialis under stress [6]. The limitations of this study are as follows:

Scale of experimentation The study was conducted in laboratory-scale cultivation systems (e.g., Erlenmeyer flasks), which may not fully replicate the complexities of industrial-scale operations. Factors like aeration dynamics, light penetration, and nutrient distribution in larger systems can significantly impact results.

Limited environmental variables The study focused on temperature and nitrate concentration as the primary variables. While these are critical factors, other influential variables, such as light intensity, salinity, and pH, were held constant and not explored in detail, which could limit the comprehensiveness of the findings.

Static cultivation conditions The experiments were conducted under static conditions, with fixed nitrate levels and constant temperatures throughout the cultivation period. Dynamic conditions, such as fluctuating temperatures or staged nutrient depletion, which are common in real-world industrial settings, were not evaluated.

Short cultivation duration The cultivation period was limited to 10 days, which may not fully capture the long-term effects of the tested variables on biomass yield and lipid productivity. Industrial cultivation often spans longer durations, which can introduce additional challenges like contamination and nutrient exhaustion.

Lack of economic analysis While the study identifies optimal conditions for dual productivity, it does not include a techno-economic analysis or cost assessment to evaluate the financial feasibility of implementing these conditions at scale.

Single strain usage The study focuseson a specific strain of *Haematococcus pluvialis*. The findings may not be directly applicable to other strains or species of microalgae, which could exhibit different responses to the same environmental conditions.

Absence of stress-inducing factors beyond temperature and nitrate Other stressors that can enhance lipid or astaxanthin accumulation, such as high light intensity or oxidative stress, were not explored. Incorporating such factors could further optimize productivity.

Harvesting and downstream processing The study primarily emphasizes cultivation conditions and does not address challenges associated with harvesting biomass or lipid extraction at industrial scales, such as energy consumption and process efficiency.

Limited real-world validation The study's results have not been validated in pilot-scale or field settings, where variables such as weather, contamination, and resource limitations can significantly impact outcomes.

Environmental impact considerations While the study identifies sustainable cultivation practices, it does not evaluate the environmental footprint of these practices, such as water usage, energy demand, or carbon emissions.

Addressing these limitations in future research, such as expanding the range of environmental variables, testing dynamic cultivation conditions, and conducting pilot-scale validations, could enhance the applicability and scalability of the findings. Additionally, incorporating economic and environmental impact assessments would provide a more comprehensive understanding of the feasibility of implementing these cultivation strategies on an industrial scale.

Economic feasibility analysis for scaling *Haematococcus pluvialis* cultivation involves evaluating costs, revenues, and profitability to determine the viability of industrial implementation. Costs can be divided into capital expenditures (CAPEX) and operational expenditures (OPEX). CAPEX includes infrastructure setup, equipment purchase for photobioreactors or open pond systems, land acquisition, and construction costs. OPEX encompasses recurring expenses such as nutrients, energy for lighting and temperature regulation, labor, water usage, and maintenance. Harvesting and lipid extraction processes also contribute significantly to operational costs.

Revenue streams primarily consist of earnings from lipid production as a biofuel feedstock. Co-products such as astaxanthin, proteins, and pigments can enhance profitability by diversifying the product portfolio. Additional revenue may be generated through carbon credits if CO_2 sequestration technologies are integrated and waste biomass is valorized as animal feed or fertilizers. Comparing these costs and revenues using methods such as net present value (NPV), internal rate of return (IRR), and payback period calculations provides a clear picture of economic feasibility. NPV calculates the present value of net earnings over the project's lifetime, IRR identifies the

profitability rate, and the payback period estimates the time required to recover initial investments.

Scalability must also be considered, as larger operations benefit from economies of scale, reducing per-unit costs for equipment, materials, and labor. Process efficiency improvements, such as optimizing lipid yields, further enhance economic viability. However, the scalability of *H. pluvialis* cultivation is influenced by external factors such as market demand for algal lipids and co-products, nutrient availability, and energy costs. Sensitivity analysis should be performed to evaluate how fluctuations in these parameters impact profitability, such as variations in potassium nitrate prices, electricity rates, or lipid market prices. Additionally, changes in lipid yield under real-world conditions must be factored into the analysis.

A life cycle assessment (LCA) can complement economic analysis by incorporating environmental and sustainability metrics, such as water and energy footprints, waste management practices, and carbon emissions. Combining economic feasibility with LCA ensures that scaling operations are both financially and environmentally sustainable. Practical implementation should begin with pilot-scale validations to refine cost estimates and optimize processes. Partnerships with biofuel companies or government subsidies can offset initial costs and enhance financial viability. Diversifying revenue streams by targeting co-product markets reduces dependency on biofuel prices alone, ensuring long-term profitability. By addressing these factors, the economic feasibility of *H. pluvialis* cultivation for industrial applications can be robustly assessed and optimized.

This study systematically investigates the interactive effects of temperature and nitrate concentration on *Haematococcus pluvialis*, contributing significantly to the existing body of research on microalgal biomass and lipid productivity. In the broader context of microalgal research, optimizing environmental parameters has been a recurring theme due to its critical role in enhancing productivity and scalability. The findings align with and expand upon several key studies in the literature, offering new insights into strain-specific responses and industrial applications.

Nitrogen availability is widely recognized as a primary driver of lipid accumulation in microalgae. Fidalgo et al. [13] demonstrated that nitrogen starvation redirects metabolic flux from cellular proliferation to triacylglycerol (TAG) synthesis, resulting in enhanced lipid accumulation in *H. pluvialis*. This mechanism was similarly observed in this study, where nitrogen-deprived conditions (0 g/L nitrate) led to the highest lipid productivity. However, Fidalgo et al. [13] focused primarily on nitrogen as an isolated variable, while the present study introduces temperature as an additional factor, highlighting its synergistic effects. Specifically, lipid content was maximized at 20 °C, emphasizing the importance of maintaining optimal thermal conditions alongside nutrient manipulation.

Akter et al., [1] explored nitrate depletion and its influence on dual productivity (biomass and lipid), observing that nitrate-starved conditions enhance lipid accumulation but at the expense of biomass yield. The current study corroborates this finding and further identifies conditions, such as 0.10 g/L nitrate at 20 °C, where a balance between biomass yield (0.560 ± 0.136 g/L) and lipid content (40.30 ± 0.012% dry weight) can be achieved. This dual optimization provides valuable insights for industrial scalability, bridging the gap between purely laboratory focused studies and practical applications.

Temperature, as an environmental factor, has been extensively studied for its impact on enzymatic activity and metabolic pathways in microalgae. Pereira & Otero [21] observed that *H. pluvialis* achieves peak biomass productivity at moderate temperatures around 20 °C, with significant reductions in growth observed at both low (10 °C) and high (30 °C) temperatures due to suboptimal enzyme functionality and thermal stress. These findings align closely with the present study, which identifies 20 °C as the most favorable temperature for dual productivity optimization. Furthermore, the current research highlights that while high temperatures (30 °C) induce lipid accumulation, they significantly compromise biomass yield, underscoring the importance of balancing these factors in industrial systems.

The influence of temperature and nitrate on microalgae has also been explored in species beyond *H. pluvialis*. For instance, Guschina and Harwood [15] reported that in *Chlorella vulgaris*, nitrogen starvation leads to lipid accumulation, but the metabolic pathways differ significantly from those in *H. pluvialis*, reflecting species-specific responses. This underscores the need for targeted optimization strategies, as demonstrated in this study, to maximize the potential of individual microalgal strains. Similarly, [27], emphasized the role of light intensity and carbon availability in influencing microalgal productivity. While the present study held these variables constant, integrating them into future investigations could provide a more comprehensive understanding of environmental interactions.

Liu et al. [18] introduced the concept of dual optimization, focusing on achieving high lipid productivity without sacrificing biomass yield. While their work primarily addressed dynamic environmental conditions, the current study provides a systematic evaluation of static cultivation parameters, offering complementary insights. The identification of optimal conditions in this study provides

a foundation for further exploration into dynamic or staged cultivation strategies, where nitrate levels and temperatures are adjusted throughout the growth cycle to maximize productivity.

From an industrial perspective, the scalability of *H. pluvialis* cultivation remains a critical challenge. Caltzontzin-Rabell et al. [5] emphasized the importance of integrating findings from laboratory studies into pilot-scale systems, highlighting barriers such as contamination, resource allocation, and cost-efficiency. The present study addresses this gap by focusing on conditions that balance productivity metrics, making them more suitable for large-scale applications. Additionally, the study's emphasis on moderate nitrate depletion and optimal temperatures aligns with Mota et al. [20], who suggested that achieving such balances is crucial for cost-effective production of high-value products like astaxanthin and biofuels.

While nitrogen starvation and temperature optimization are central to both this study and the broader literature, the unique contribution of this research lies in its systematic analysis of their interaction, which has not been extensively explored in prior works. By identifying conditions that enhance lipid accumulation without severely compromising biomass yield, this study provides a robust framework for the industrial cultivation of *H. pluvialis*. Moreover, its findings pave the way for integrating additional environmental variables, such as light and salinity, into future research.

Haematococcus pluvialis, *Nannochloropsis sp.*, and *Phaeodactylum tricornutum* represent valuable microalgal species with unique lipid and fatty acid profiles, making them significant candidates for biotechnological applications. Scodelaro Bilbao et al. [23] highlight *Haematococcus pluvialis* as a rich source of fatty acids and phytosterols, with notable nutritional and biological implications for human health and industrial use. Similarly, Yu et al. [28] demonstrate that *Nannochloropsis sp. QII* produces high levels of lipids, particularly triacylglycerols, under nitrogen-limited conditions, emphasizing its potential as a sustainable biofuel feedstock. Complementing these findings, Siron et al. [25] investigate the lipid composition of *Phaeodactylum tricornutum* and *Dunaliella tertiolecta*, revealing how phosphorus deficiency and growth phases significantly influence their fatty acid profiles. Together, these studies underscore the importance of optimizing growth conditions and nutrient regimes to enhance the lipid productivity of microalgae for diverse applications in nutrition, biofuel, and aquaculture industries.

In conclusion, the findings of this study are consistent with existing research on the effects of temperature and nitrate on microalgae, while providing new insights into their combined influence on *H. pluvialis*. The dual optimization strategy identified here complements the work of Fidalgo et al. [13] and Akter et al. [1] by addressing the trade-offs between lipid and biomass productivity. It also expands on the studies by Pereira & Otero [21] and Liu et al. [18] by emphasizing the scalability of optimized conditions. By bridging laboratory findings with practical applications, this research contributes significantly to the sustainable and scalable production of microalgal biofuels and high-value bioproducts.

Future work should focus on validating the findings under pilot-scale and real-world conditions to assess scalability and practicality. Dynamic cultivation strategies, such as staged nutrient depletion and temperature adjustments, should be explored to further optimize lipid and biomass productivity over longer growth cycles. Integrating additional environmental factors, such as light intensity and CO_2 levels, could provide a more comprehensive understanding of their combined effects on *Haematococcus pluvialis*. Moreover, techno-economic and life cycle assessments will be essential to evaluate the cost-effectiveness and sustainability of implementing these optimized conditions at industrial scales. Expanding the study to include co-product optimization, such as astaxanthin, will also enhance the economic viability of large-scale cultivation systems.

Author contributions
Methodology, Ö.G. and G.T.; software, Ö.G. and G.T.; formal analysis, Ö.G. and G.T.; writing—original draft preparation, Ö.G. and G.T.; writing—review and editing Ö.G. and G.T..; funding acquisition Ö.G. and G.T. All authors have read and agreed to the published version of the manuscript.

Funding
This research received no external funding.

Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent fot publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 30 October 2024 Accepted: 2 January 2025
Published online: 18 January 2025

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