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Optimizing Growth Conditions and Polyhydroxybutyrate Production in *Spirulina platensis* and *Haematococcus pluvialis* for Sustainable Bioplastic Development

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ABSTRACT

Bioplastics are increasingly being recognized as a viable substitute for conventional plastics across a broad spectrum of applications. Microalgae, such as *Spirulina platensis* (Geitler) and *Haematococcus pluvialis* (Flotow), are emerging as sustainable sources for bioplastic production, aligning with the principles of a circular bioeconomy. This study employed response surface methodology (RSM) to optimize growth conditions for bioplastic-producing microalgae, *Spirulina platensis* and *Haematococcus pluvialis*, with the aim of maximizing polyhydroxybutyrate (PHB) accumulation. The results showed that aerated Zarrouk's medium optimized *S. platensis* growth at 20°C, pH 8, 5000 lux, and 0.025 M salinity, while medium growth was optimized at 30°C, pH 9, 5000 lux, and 0.3 M salinity. In contrast, aerated BG11 medium optimized *H. pluvialis* growth at 26.5°C, pH 7.9, 6500 lux, and 0 M salinity, with medium growth optimized at 27.5°C, pH 5.5, 3500 lux, and 0.2 M salinity. Notably, *H. pluvialis* under medium growth conditions yielded the highest PHB content (61.04±0.3mg/ g), whereas *S. platensis* under maximum growth conditions yielded the lowest PHB content (20.43±0.56mg/ g). Furthermore, PHB production was significantly enhanced for *H. pluvialis* by the addition of sodium acetate in phosphorus-deficient media, reaching a maximum of 197.58±0.6mg/ g with 2g/ L acetate.

INTRODUCTION

Plastics have become an integral part of our daily lives, with their versatility and widespread availability leading to their extensive use in various industries, including consumer goods, food packaging, medical supplies, agriculture, construction, and automotive parts **(Chinthapalli *et al.*, 2021; Mohamed *et al.*, 2024)**. However, plastics also have negative attributes, such as their resistance to biodegradation **(Reddy *et al.*, 2003)**, and their toxicity when incinerated **(Zeller *et al.*, 2013)** and high waste accumulation in landfills and marine environments, making plastic pollution one of the most pressing environmental challenges of our time. Bioplastics, derived from natural

sources, offer a promising alternative to traditional plastics due to their biodegradable properties. Since they are more environmentally friendly than petroleum-based polymers, bioplastic made from renewable resources has become essential in recent years. Microalgal biomass, either directly or through its secondary metabolites, can be utilized in bioplastic production **(Becker, 2007; Rahman & Miller, 2017; Abd El-Sadek & Ahmed, 2022)**. Polyhydroxyalkanoates (PHAs) are particularly promising biodegradable polymers that exhibit properties similar to conventional plastics **(Steinbu¨chel & Fuchtenbusch, 1998; Siddiq *et al.*, 2020)**. PHAs are a class of biodegradable polyesters synthesized and stored by microorganisms in response to nutrient deprivation. Among the approximately 155 types of PHAs, polyhydroxybutyrate (PHB) is one of the most extensively studied due to its unique properties.

The growing concern over non-degradable plastic waste has fueled a surge in interest for biodegradable polymers. This has led to numerous efforts focused on producing PHB, a bioplastic with promising properties. Notably, a wide range of algae naturally synthesize PHB as a natural energy storage compound **(Falcone, 2004)**, which accumulates as insoluble cytoplasmic granules, serving as a carbon and energy storage reserve **(Stal, 1992)**.

The PHB yield of algal biomass is improved from the recyclability of plastic and reduces the reliance on fossil fuel -based sources. PHB possesses several unique properties, including piezoelectricity, biocompatibility, non-toxicity, and elasticity, along with a high degree of polymerization **(Hocking & Marchessault, 1994)**. These properties make PHB a versatile material with applications in agriculture, environmental science, and biomedicine **(Philip *et al.*, 2007)**. PHB's piezoelectric property enables its use in the manufacturing of various sensors for detecting light pressure and sound. It also finds applications in the development of audio equipment **(Aguilar & San Rom, 2014; Mohammed & Aburas, 2016; Zaher & Ibrahim, 2023)**.

PHB plays a crucial role in various medical procedures, where it is utilized in the production of scaffolds, bone plates, drug delivery systems, and surgical sutures **(Steinbüchel & Füchtenbusch, 1998)**. Blending PHB with inorganic materials can create bioactive composites with enhanced properties, making them suitable for tissue engineering techniques for medical treatments **(Chen, 2009)**. As result of all these advantages of PHB, , this study aimed to explore the feasibility of using algal biomass as a source for polyhydroxybutyrate (PHB) production. By investigating the potential of algal biomass for PHB production, this study seeks to contribute to the development of sustainable and environmentally friendly bioplastic solution.

MATERIALS AND METHODS

Algal strains and growth conditions.

Microalgae species were gained from the Algal Culture Collection at Al-Azhar University (ACCAZ), Cairo, Egypt. Also, from Culture Collection of Hydrobiology Lab, Water Pollution Research Department, National Research Center. The algal species used were *Spirulina platensis* (blue green algae) and *Haematococcus pluvialis* (green algae). Different growth media were used for the growing of the selected algal species, Zarrouk's media (**Zarrouk, 1966)** for culturing of *Spirulina platensis*, while BG11 medium **(Allen, 1968)** was used for the culture of *Haematococcus pluvialis*. The growth of algal biomass was assessed by optical density of selected strains at 760nm **(Adhikary, 1983)**.

CCD-based statistical optimization.

Statistical optimization was carried out by using different factorial designs available in the response surface methodology (RSM). The central composite design (CCD) is the most widely used and efficient RSM technique. CCD a powerful statistical tool, was employed to investigate the relationships between process variables (temperature, pH, light intensity, and salinity) and responses (optical density and algal growth). By utilizing cube points, axial points, and replicated center points, a comprehensive model was developed to assess linear, interaction, and quadratic effects. The resulting mathematical and graphical outputs enabled the generation of optimization curves, which were used to predict optimal settings for maximizing algal growth. The final step involved validating these predicted settings through experimental verification; by evaluating how good the setting is maximizing the response. The validity was assessed by comparing the predicted response with the response values obtained from the confirmatory experimental runs **(Myers *et al*., 2016)**. All the experimental data of RSM models were processed and analyzed with Minitab® version 18 extended with statistical and graphical software packages.

Design of experiments (DOE) models

The DOE differs from one species to another as follows: Thirty - one flasks packed with 300ml of algal culture, then inoculated with 100ml of algal suspension, these flasks were exposed to five levels of temperatures (20, 25, 30, 35, and 40°C), five levels of pH (5, 7, 9,11 and 13), five levels of light intensity (500, 2000, 3500, 5000 and 6500 lux), and five levels of salinity (0, 0.15, 0.27, 0.4 and 0.52 M) for *Spirulina platensis*. On the other hand, *Haematococcus pluvialis*, the condition were temperatures at 12.5, 20, 27.5, 35, and 42.5°C, pH at 0.5, 3, 5.5,8 and 10.5, light intensity at 500, 2000, 3500, 5000 and 6500 lux, and salinity at 0, 0.1, 0.2, 0.3 and 0.4 M. All flasks were filled with air using an air pump. The run of the experiment continued for many days according to the algae growth rate. All flasks were processed into a dark/light cycle system 12/12h.

The optical density was measured at the end of the experiment. Analysis of variance (ANOVA) was employed to evaluate the statistical significance of linear, quadratic, and interaction effects.

PHB quantification in micro algal strains

A 0.01g of commercial PHB powder was added to 10ml of chloroform, the mixture was heated in a water bath at 65-70°C with gentle shaking until the PHB was completely dissolved. The solution was cooled to room temperature and the tube was tightly capped. One mL of the 1mg/ mL PHB stock solution was added to 9mL of chloroform in test tube, resulting in a 100µg/ mL PHB stock solution. Then, 10mL of conc. H_2SO_4 was added to 1mL of the 100µg/ mL of PHB stock solution. The tube was capped tightly and heated in a boiling water bath at 94-96°C for 20 minutes. This acid hydrolysis converts PHB into crotonic acid. The hydrolyzed sample was let to cool in room temperature. After that 1mL of the cooled sample was transferred to silica cuvette. UV-visible spectrophotometer was used to measure the absorbance of the sample at a wavelength of 235nm, concentrated H_2SO_4 was used as a blank. A standard curve was generated by plotting the absorbance at 235nm against the corresponding weight of PHB **(Bonarteseva & Myshkina, 1985)**.

Extraction of PHB from algal biomass

Algal biomass (0.01g) was added to 2ml of 2 N HCl and heated in a water bath for 2 hours. The resulting suspension was centrifuged at 6000rpm for 20 minutes. Subsequently, 5ml of chloroform was added to the pellet, and the mixture was shaken overnight at 28°C and 150rpm. After shaking, the sample was centrifuged again at 2000 rpm for 20 minutes, and the chloroform extract was collected and dried at 40°C. The dried residue was treated with 5ml of concentrated sulfuric acid and heated at 100°C for 20 minutes to convert PHB crystals into crotonic acid. PHB content was quantified spectrophotometrically at 235nm, using sulfuric acid as a blank. Finally, the percentage of PHB was calculated based on cell dry weight following the method described by **Bonarteseva and Myshkina (1985)**.

Enhancement for PHB concentration in selected algal species

To further enhance PHB productivity, the highest polyhydroxybutyrate concentration in *Haematococcus pluvialis* under optimal growth conditions was identified using statistical data. The PHB production was then optimized by testing five concentrations of sodium acetate (0.0, 0.5, 1, 1.5, and 2g/ L) under four different media conditions: normal media, nitrogen-free media, phosphorus-free media, and media lacking both nitrogen and phosphorus. The cells were initially grown under normal photoautotrophic conditions for 12 days to reach maximum biomass production.

Subsequently, the cells were exposed to different acetate concentrations, and PHB content was measured in mg/g.

RESULTS

The central composite design for *Spirulina platensis*

The CCD was employed to optimize the growth conditions for both species *Spirulina platensis* and *Haematococcus pluvialis*, focusing on maximizing biomass production, as measured by optical density (OD). Four factors-temperature, pH, light intensity, and salinity-were investigated based on preliminary experiments. The 31 experimental runs proposed by the CCD were conducted, and statistical tools, including analysis of variance (ANOVA), Pareto charts, normal probability plots, and optimization curves, were used to assess the significance of the factors and their interactions.

Based on the 31 experimental runs evaluating the growth conditions of *Spirulina platensis* (Table 1), the most promising conditions for maximizing biomass production measured by OD was Run 5: with 0.40 OD, 25°C temperature, pH 8, light intensity 5000 lux, and salinity 0.15 M. Also, Table (1) showed the most promising conditions for maximizing biomass production of *H. pluvialis* was Run 22: with 0.70 OD, 27.5°C temperature, pH 5.5, light intensity 6500 lux, and salinity 0.2 M. These conditions yielded the highest optical density, suggesting they are the most favorable for algal growth.

The main (linear) and square (non-linear) effects

The results (Table 2 & Fig. 1A, B) reveal that light intensity has no significant effect on optical density value at low and high levels for *S. platensis*. Meanwhile, temperature has no crucial influence on the optical density with *P* value=0.34 for *H. pluvialis*. However, there are significant effects at low and high levels of the other factors including temperature, pH and salinity have crucial influence on the optical density for data obtained by *S. platensis*, while the results achieved by *H. pluvialis* revealed that pH, light intensity, and salinity significantly affected optical density value at low and high levels.

The interaction effects

An interaction is effective when the change in the response from low to high levels of a factor is dependent on the levels of a second factor. The interaction effects were also tested by the ANOVA model. For the *S. platensis* optical density, the two-way interaction between temperature and salinity was considerably influencing the OD with *P*-value=0.001 (Table 2, Fig. 1A). Where, *H. pluvialis* did not reveal a two-way interaction.

The Pareto chart and normal probability plots

Pareto chart for both species (Fig. 2A, B) assess the significance of main, quadratic, and interaction effects. The t-value of 2.06 for optical density, exceeding the reference line, indicated a significant effect on OD. Normal probability plots (Fig. 2C

and D) for both species determined the direction of effects, points near the fitted line (zero effect) represented insignificant factors, while distant points indicated significant factors. Positive effects meant increased levels that increased the response, and vice versa.

Based on the Pareto chart and normal probability plot, the key factors affecting the optical density of *S. platensis* and *H. pluvialis* were analyzed. Here's a breakdown using the numbers from the ANOVA and Pareto chart:

For *S. platensis* temperature had the most significant impact on OD, with (*P*-value = 0.000), indicating a highly significant effect. The Pareto chart also showed that the effect of pH exceeded the reference line, highlighting its importance. Normal probability plot showed a negative effect on OD. Followed by squared effect (BB) of pH was a significant factor with a *P*-value = 0.000 in the ANOVA. The Pareto chart confirmed its significant effect, and the normal probability plot indicated a negative effect on OD. The Pareto chart and normal probability plot demonstrated that the main effect of pH (B) indicated a negative effect on OD. Also, salinity (D) played a significant role with a *P*-value = 0.000. The Pareto chart and normal probability plot demonstrated that its squared effect (DD) was also influential. Salinity showed a negative effect on OD in certain cases. Its interaction with temperature Temp. X Salinity was significant, with a *P*-value = 0.001. The normal probability plot indicated a positive effect of temperature on OD. Finally, squared effect of Temperature (AA) had a lower but significant influence with a *P*-value = 0.006. The normal probability plot indicated a negative effect of temperature on OD (Fig. 2A, C).

On the other hand, for *H. pluvialis* squared effect of light intensity (CC) had the most significant impact on OD, with *P*-value = 0.000, indicating a highly significant effect. The Pareto chart also showed that the effect of pH exceeded the reference line, highlighting its importance. Meanwhile, pH (B) followed as a significant factor with a *P*-value = 0.000 in the ANOVA. The Pareto chart confirmed its significant effect, and the normal probability plot indicated a positive effect on OD, meaning higher pH increases OD. Also, salinity (D) played a significant role with *P*-value = 0.001. The Pareto chart and normal probability plot demonstrated was also influential. Salinity showed a negative effect on OD in certain cases. Where, squared effect of temperature had a lower but significant influence with *P*-value = 0.000. The normal probability plot indicated a negative effect squared effect of temperature on OD. The squared effect of pH (BB) had low significant influence with *P*-value = 0.002, and it indicated a negative effect (Squared effect) of pH on OD. Finally, light intensity (C) showed significant impact on OD, with *P*-value = 0.006, indicating a significant effect. The Pareto chart also showed that the effect of pH exceeded the OD value (Fig. 2B, D).

Table 1. Design matrix of 25 full factorial CCD and results of both species *S. platensis* and *Haematococcus pluvialis*, OD (nm) in response to all combinations of low and high levels of interacted factors

Species	Design points		Factors						Response and error estimation*		
	Std Order	Run Order	Pt Type	Blocks	Temp.	pH	LI	Salinity	OD	Fits	Residual
Spirulina platensis	1	1	1	1	25	7	2000	0.150	0.32	0.332548	-0.0125481
	2	2	1	1	35	7	2000	0.150	0.16	0.135881	0.0241186
	3	3	1	1	25	11	2000	0.150	0.17	0.234215	-0.0642147
	4	4	1	1	35	11	2000	0.150	0.00	0.037548	-0.0375481
	5	5	1	1	25	7	5000	0.150	0.40	0.332548	0.0674519
	6	6	1	1	35	7	5000	0.150	0.11	0.135881	-0.0258814
	7	7	1	1	25	11	5000	0.150	0.23	0.234215	-0.0042147
	8	8	1	1	35	11	5000	0.150	0.00	0.037548	-0.0375481
	9	9	1	1	25	7	2000	0.400	0.22	0.162548	0.0574519
	10	10	1	1	35	7	2000	0.400	0.11	0.135881	-0.0258814
	11	11	1	1	25	11	2000	0.400	0.06	0.064215	-0.0042147
	12	12	1	1	35	11	2000	0.400	0.04	0.037548	0.0024519
	13	13	1	1	25	7	5000	0.400	0.12	0.162548	-0.0425481
	14	14	1	1	35	7	5000	0.400	0.16	0.135881	0.0241186
	15	15	1	1	25	11	5000	0.400	0.08	0.064215	0.0157853
	16	16	1	1	35	11	5000	0.400	0.00	0.037548	-0.0375481
	17	17	-1	1	20	9	3500	0.275	0.21	0.229071	-0.0190705
	18	18	-1	1	40	9	3500	0.275	0.05	0.005737	0.0442628
	19	19	-1	1	30	5	3500	0.275	0.08	0.125737	-0.0457372
	20	20	-1	1	30	13	3500	0.275	0.00	-0.070929	0.0709295
	21	21	-1	1	30	9	500	0.275	0.20	0.212692	-0.0126923
	22	22	-1	1	30	9	6500	0.275	0.23	0.212692	0.0173077
	23	23	-1	1	30	9	3500	0.025	0.34	0.297692	0.0423077
	24	24	-1	1	30	9	3500	0.525	0.13	0.127692	0.0023077
	25	25	0	1	30	9	3500	0.275	0.21	0.212692	-0.0026923
	26	26	0	1	30	9	3500	0.275	0.22	0.212692	0.0073077
	27	27	0	1	30	9	3500	0.275	0.14	0.212692	-0.0726923
	28	28	0	1	30	9	3500	0.275	0.19	0.212692	-0.0226923
	29	29	0	1	30	9	3500	0.275	0.27	0.212692	0.0573077
	30	30	0	1	30	9	3500	0.275	0.25	0.212692	0.0373077
	31	31	0	1	30	9	3500	0.275	0.21	0.212692	-0.0026923

Haematococcus pluvialis	1	1	1	1	20.0	3.0	2000	0.1	0.18	0.192890	-0.012890
	2	2	-1	1	35.0	8.0	2000	0.1	0.20	0.171223	0.028777
	3	3	-1	1	20.0	8.0	2000	0.1	0.34	0.332890	0.007110
	4	4	-1	1	35.0	3.0	2000	0.1	0.30	0.311223	0.058777
	5	5	-1	1	20.0	3.0	5000	0.1	0.26	0.261223	-0.001223
	6	6	-1	1	35.0	8.0	5000	0.1	0.32	0.239557	0.080443
	7	7	-1	1	20.0	8.0	5000	0.1	0.33	0.401223	-0.071223
	8	8	-1	1	35.0	3.0	5000	0.1	0.35	0.379557	-0.029557
	9	9	-1	1	20.0	3.0	2000	0.3	0.01	0.067890	-0.057890
	10	10	0	1	35.0	8.0	2000	0.3	0.01	0.046223	-0.036223
	11	11	0	1	20.0	8.0	2000	0.3	0.29	0.207890	0.082110
	12	12	0	1	35.0	3.0	2000	0.3	0.14	0.186223	-0.046223
	13	13	0	1	20.0	3.0	5000	0.3	0.06	0.136223	-0.076223
	14	14	0	1	35.0	8.0	5000	0.3	0.05	0.114557	-0.064557
	15	15	0	1	20.0	8.0	5000	0.3	0.29	0.276223	0.013777
	16	16	0	1	35.0	5.5	5000	0.3	0.20	0.254557	-0.054557
	17	17	1	1	12.5	5.5	3500	0.2	0.07	0.034220	0.035780
	18	18	-1	1	42.5	0.5	3500	0.2	0.00	-0.009113	0.009113
	19	19	-1	1	27.5	10.5	3500	0.2	0.00	-0.047447	0.047447
	20	20	-1	1	27.5	5.5	3500	0.2	0.23	0.232553	-0.002553
	21	21	-1	1	27.5	5.5	500	0.2	0.45	0.484220	-0.034220
	22	22	-1	1	27.5	5.5	6500	0.2	0.70	0.620887	0.079113
	23	23	-1	1	27.5	5.5	3500	0.0	0.26	0.362234	-0.102234
	24	24	-1	1	27.5	5.5	3500	0.4	0.16	0.112234	0.047766
	25	25	-1	1	27.5	5.5	3500	0.2	0.24	0.237234	0.002766
	26	26	0	1	27.5	5.5	3500	0.2	0.26	0.237234	0.022766
	27	27	0	1	27.5	5.5	3500	0.2	0.24	0.237234	0.002766
	28	28	0	1	27.5	5.5	3500	0.2	0.25	0.237234	0.012766
	29	29	0	1	27.5	5.5	3500	0.2	0.26	0.237234	0.022766
	30	30	0	1	27.5	5.5	3500	0.2	0.27	0.237234	0.032766
	31	31	0	1	27.5	3.0	3500	0.2	0.24	0.237234	0.002766

*OD (nm), the response; Fits, are the value of point estimates of the mean optical density for given values of the factors; Residuals, are the difference between the observed value and its corresponding fitted value.

Table 2. Results of ANOVA model to test for differences in *S. platensis* and *H. pluvialis* as OD in response to different factors

Spirulina platensis						***Haematococcus pluvialis***					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**	**Source**	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Model	6	0.27921	0.046535	25.85	0.000	**Model**	7	0.583393	0.083342	27.51	0.000
Linear	3	0.17618	0.058728	32.62	0.000	**Linear**	4	0.242183	0.060546	19.98	0.000
Temp	1	0.07482	0.074817	41.56	0.000	**Temp.**	1	0.002817	0.002817	0.93	0.345
pH	1	0.05802	0.058017	32.22	0.000	**pH**	1	0.117600	0.117600	38.81	0.000
Salinity	1	0.04335	0.043350	24.08	0.000	**LI**	1	0.028017	0.028017	9.25	0.006
Square	2	0.07413	0.037063	20.59	0.000	**Salinity**	1	0.093750	0.093750	30.94	0.000
Temp.*Temp.	1	0.01657	0.016567	9.20	0.006	**Square**	3	0.341209	0.113736	37.54	0.000
pH*pH	1	0.06264	0.062641	34.79	0.000	**Temp.*Temp.**	1	0.091255	0.091255	30.12	0.000
2-Way Interaction	1	0.02890	0.028900	16.05	0.001	**pH*pH**	1	0.037840	0.037840	12.49	0.002
Temp.*Salinity	1	0.02890	0.028900	16.05	0.001	**LI*LI**	1	0.179732	0.179732	59.32	0.000
Error	24	0.04321	0.001800	-	-	**Error**	23	0.069685	0.003030	-	-
Lack-of-Fit	18	0.03267	0.001815	1.03	0.526	**Lack-of-Fit**	17	0.068799	0.004047	27.42	0.000
Pure Error	6	0.01054	0.001757	-	-	**Pure Error**	6	0.000886	0.000148	-	-
Total	30	0.32242	-	-	-	**Total**	30	0.653077	-	-	-

Significant differences are denoted as $R^2 = 86.66\%$ and $R^2 = 89.33\%$, respectively.

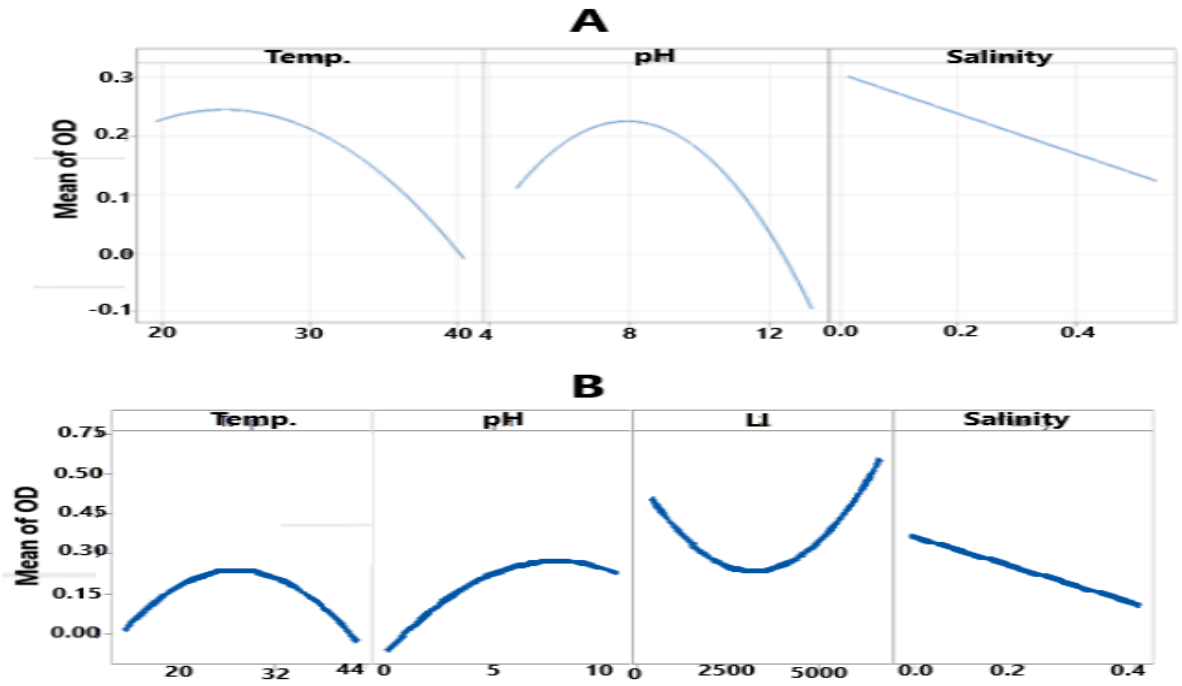

Fig. 1A and B. Main effects plots explain the changes in *S. platensis* and *H. pluvialis* OD between low and high levels of each factor

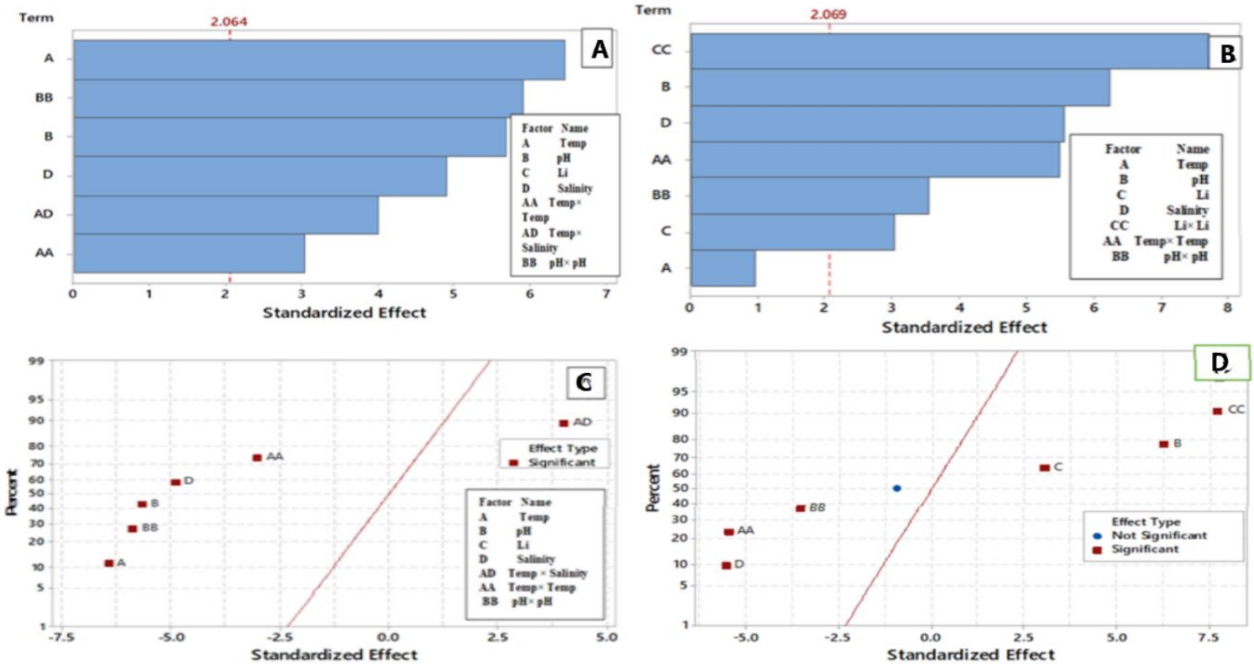

Fig. 2. Pareto chart (A and B) and Normal probability plot (C and D) of standardized effects on *S. platensis* and *H. pluvialis*, respectively, OD (nm)

The optimization curves

The response optimizer tool was used to generate the optimization curves (Figs. 3A, B and C & 4A, B, and C) to determine the final optimal settings of the factors that showed the three cases of growth (maximum, medium and minimum) (Table 3) of *S. platensis* and *H. pluvialis* according to the value of optical density as the way of growth determination.

Table 3. The optimal settings for optimization curves of both species *S. platensis* and *H. pluvialis*

Factors	Optimal Settings					
	S. platensis			***H. pluvialis***		
	Maximum Growth	**Medium Growth**	**Minimum Growth**	**Maximum Growth**	**Medium Growth**	**Minimum Growth**
Temperature	20 °C	30 °C	40 °C	26.7 °C	40.4 °C	12.5 °C
pH	7.9	9	13	7.8	8.7	5
Salinity	0.025 M	0.025 M	0.025 M	0 M	0.15 M	0 M
Light Intensity	-	-	-	6500 Lux	1136.9 Lux	500 Lux

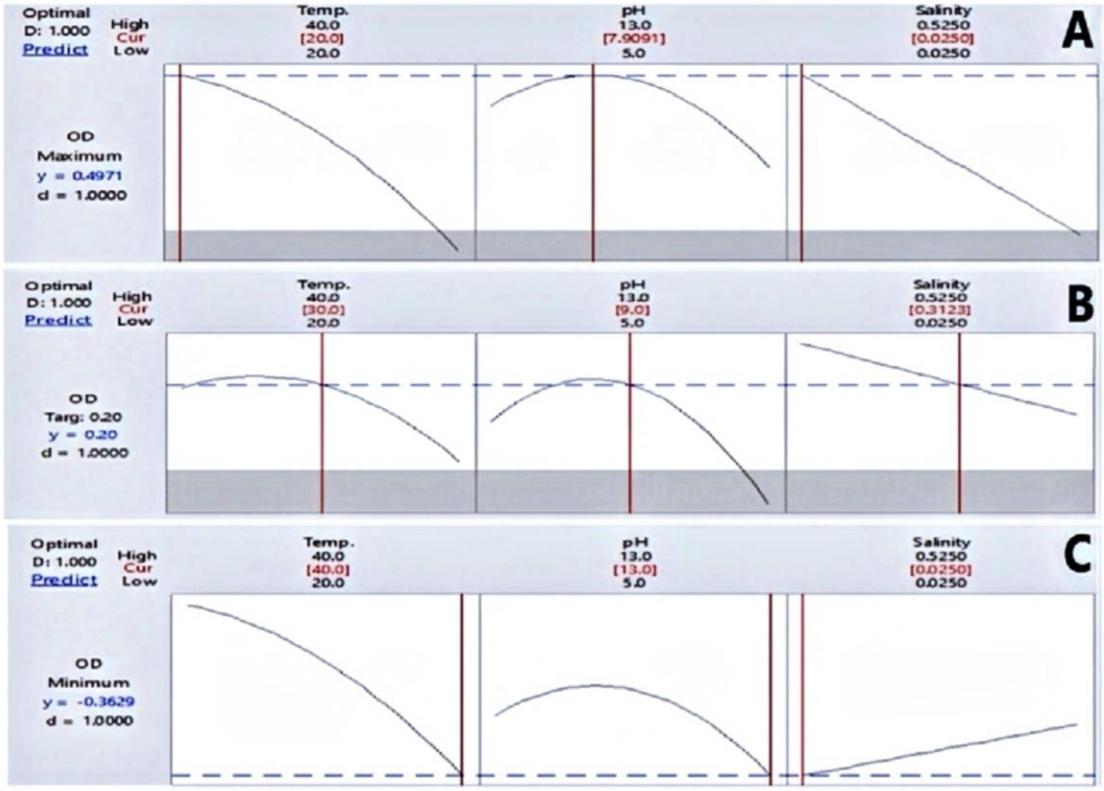

Fig. 3. The optimization curves of *S. platensis* show how the factors affect the predicted responses (y) including A- Maximum, B- Medium and C- Minimum OD, the optimum factor settings (Cur) were predicted with composite desirability (D) = 1.0 (100%)

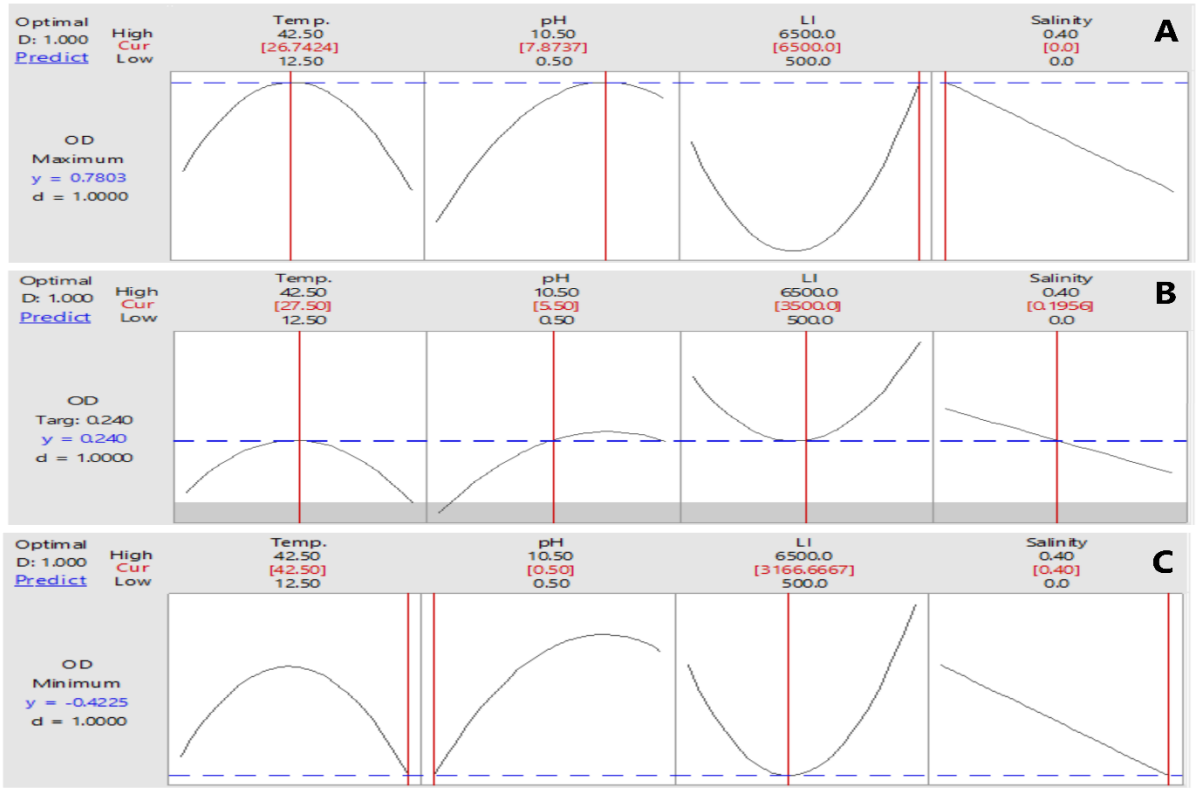

Fig. 4. The optimization curves of *H. pluvialis* show how the factors affect the predicted responses (y) including A- Maximum, B- Medium and C- Minimum OD, the optimum factor settings (Cur) were predicted with composite desirability (D) = 1.0 (100%)

The predicted outcome

The optical density increased to 0.49 A. U, achieving an individual desirability of 100%, as shown in Fig. (3A). While, OD reached a medium value of 0.20 A. U, achieving individual desirability of 99%, as shown in Fig. (3B). Moreover, the optical density decreased to 0 A.U, achieving an individual desirability of 100%, as shown in Fig. (3C) for *S. platensis*. Whereas, for *H. pluvialis* the optical density increased to 0.78 A. U, achieving the maximum growth with an individual desirability of 100%, as shown in Fig. (4A). Meanwhile, OD reached a medium value of 0.24 A. U, achieving an individual desirability of 99%, as shown in Fig. (4B). Finally, the optical density decreased to 0 A.U, achieving minimum growth with an individual desirability of 100%, as shown in Fig. (4C).

The results showed that maximum growth, indicated by a composite desirability (D) of 1.0 (100%), was achieved in *S. platensis* by increasing the light intensity and pH,

decreasing the temperature, and eliminating salinity. In contrast, maximum growth in *H. pluvialis* was attributed to an interaction among several factors: moderate temperature (26.7°C), increased light intensity (6500 lux), slightly alkaline medium (pH 7.8), and a salt-free environment.

Moderate growth of *S. platensis* occurred at a temperature of 30°C and a pH of 9. This moderate performance may be attributed to the elevated temperature (30°C), leading to a slight decrease in the OD value. For *H. pluvialis*, medium growth, reflected by moderate composite desirability, was achieved under suboptimal conditions characterized by a lower pH (5.5) and reduced light intensity.

Furthermore, the results highlighted the negative impact of certain suboptimal settings, demonstrating minimum growth despite a high composite desirability (D = 100%), due to the combination of high temperature and high pH.

Table 4. Results of polyhydroxybutyrate concentration in different microalgae strains

Samples	Growth condition	PHB concentration (mg/g)
Spirulina platensis	**Maximum growth**	**20.43 ± 0.56**
	Medium growth	**36.14 ± 0.37**
Haematococcus pluvialis	**Maximum growth**	**55.29 ± 0.31**
	Medium growth	**61.04 ± 0.3**

The results showed that PHB is high in the medium growth conditions in both algal strains. The highest value was recorded in *H. pluvialis* (61.04 ± 0.3mg/ g) in the case of medium growth condition, while the lowest value was recorded in *Spirulina platensis* in the maximum growth condition (20.43 ± 0.56mg /g).

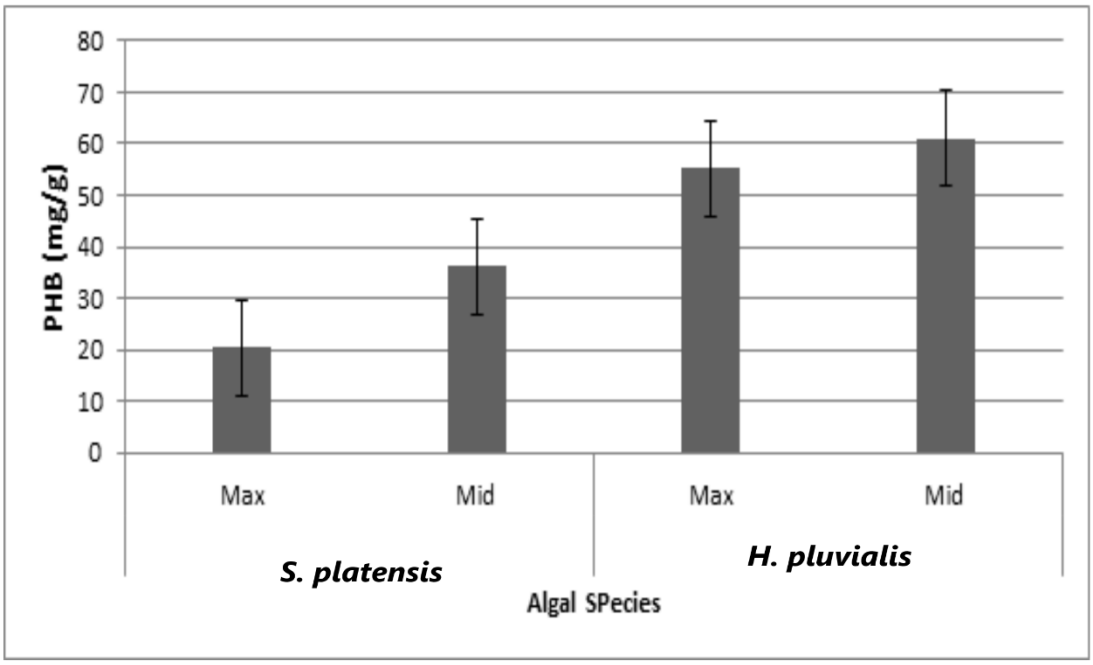

Fig. 5. Polyhydroxybutyrate concentration in different microalgae strains

Enhancement of polyhydroxybutyrate concentration

The results of the enhancement of Polyhydroxybutyrate concentration for *H. pluvialis* are shown in Table (5) and Fig (6). The results showed that nitrogen and phosphorus deficiency led to higher PHB concentrations compared to normal media, also the increase in acetate concentration generally enhanced PHB production across all treatments. A maximum PHB content (197.58 ± 0.6mg/ g) was achieved in cells cultured in phosphorus-limited media containing 2g/ L acetate. Nitrogen and phosphorus, at an acetate concentration of 1.5–2g/ L, resulted in a decrease in PHB concentration compared to the control media at the same acetate levels. The recorded PHB concentrations were 81.44 ± 1.2mg/ g and 50.76 ± 1.8mg/ g, respectively.

Table 5. The PHB content of *H. pluvialis* under different nutrient condition

Nutrient Condition	Acetate concentration (g/L)	OD	Concentration of PHB (µg/ml)	Concentration of PHB (mg/g)	Mean Concentration of PHB (mg/g)
Normal	0	1.30	607.47	60.74	61.04±0.3
		13.1	612.21	61.22	
		13.1	612.21	61.22	
	0.5	2.20	1081.15	108.64	108.40±0.2
		2.21	1086.42	108.64	
		2.21	1086.42	108.64	
	1	2.44	1207.47	120.74	121.09±0.6
		2.46	1218.00	121.8	
		2.44	1207.47	120.74	
	1.5	2.71	1349.57	134.95	134.6± 1.09
		2.72	1354.84	135.48	
		2.68	1333.78	133.37	
	2	3.01	1507.47	150.74	151.79±0.9
		3.04	1523.26	152.32	
		3.04	1523.26	152.32	
- N	0	2.09	1023.26	102.32	102.67±0.6
		2.09	1023.26	102.32	
		2.11	1033.78	103.37	
	0.5	2.49	1233.78	123.37	124.07±0.6
		2.51	1244.31	124.43	
		2.51	1244.31	124.43	
	1	2.75	1370.63	137.06	136.88±0.3
		2.75	1370.63	137.06	
		2.74	1365.36	135.53	
	1.5	2.98	1491.68	149.16	148.81±0.3
		2.97	1486.42	148.64	
		2.97	1486.42	148.64	
	2	3.13	1570.63	157.06	157.04±0.02
		3.13	1570.63	157.06	
		3.11	1560.10	156.01	

-P	0	2.55	1239.05	123.90	123.70±1.3
		2.57	1249.57	124.95	
		2.54	1223.26	122.32	
	0.5	3.04	1523.26	152.36	153.02±0.8
		3.07	1539.05	153.90	
		3.05	1528.52	152.85	
	1	3.17	1591.68	159.16	159.48±0.2
		3.18	1596.94	159.69	
		3.17	1591.60	159.16	
	1.5	3.30	1660.10	166.01	166.70±0.7
		3.31	1675.89	167.58	
		3.33	1665.36	166.53	
	2	3.88	1965.36	196.53	197.58±0.6
		3.90	1975.89	197.58	
		3.90	1975.89	197.58	
-N, - P	0	2.34	1154.80	115.48	114.79±0.2
		2.33	1149.57	114.95	
		2.33	1149.57	114.95	
	0.5	2.55	1265.36	126.53	126.35±0.3
		2.55	1265.36	126.53	
		2.54	1260.10	126.01	
	1	2.80	1396.94	139.69	140.21±0.9
		2.83	1412.73	141.27	
		2.80	1396.94	139.69	
	1.5	1.72	828.52	82.85	81.44±1.2
		1.68	807.47	80.74	
		1.68	807.47	80.74	
	2	1.07	486.40	48.64	50.76±1.8
		1.13	518.00	51.80	
		1.13	518.00	51.80	

The cells of *H. pluvialis* was cultured in normal nutrient **(**Normal**)**, nitrogen limiting media **(**-N**)**, phosphorus limiting media **(**-P), nitrogen and phosphorus limiting media (-N, -P), with the addition of various acetate concentration (0, 0.5, 1, 1.5, 2 g/L). Data are the average ± SD from three independent culture.

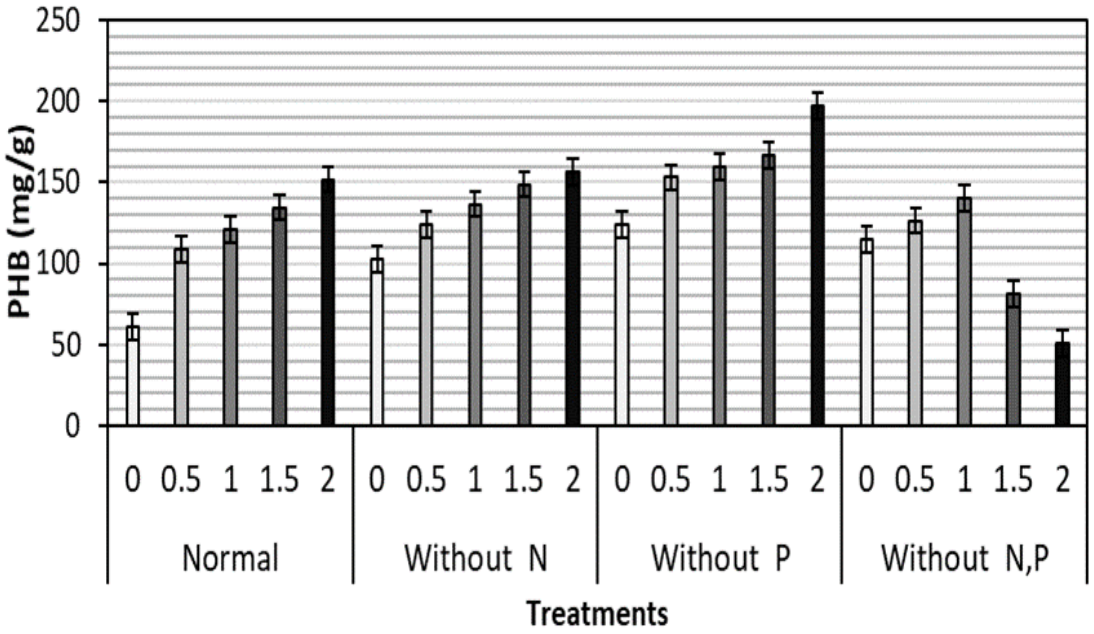

Fig. 6. Polyhydroxybutyrate concentration of *Haematococcus pluvialis* under different nutrient conditions

DISCUSSION

Polyhydroxyalkanoates (PHAs), particularly polyhydroxybutyrate (PHB), offer a promising alternative to traditional plastics due to their biodegradable nature and diverse biotechnological applications. The economic feasibility of PHA production is closely tied to the productivity of the producing organism, which is influenced by factors like growth rate and PHA accumulation in the biomass. To minimize the overall production cost of PHAs, it is essential to achieve high yields and enhanced productivity **(Kumar *et al.*, 2015)**. Therefore, this study aimed to identify optimal conditions for microalgal growth to maximize biomass production and to explore strategies that increase the accumulation of PHB in microalgal cells.

To optimize the growth conditions for both *Spirulina platensis* and *Haematococcus pluvialis*, with a focus on maximizing biomass production, 31 experimental runs were conducted to evaluate various growth parameters, as shown in Table (1). The results indicate that *S. platensis* as well as *H. pluvialis* performs best growth under a combination of moderate to high light intensity, low salinity, temperature with range 20-35 and 20-30°C, respectively, and a pH range of 7-8 and 5-8, respectively. These results agree with those of **Singh and Singh (2015)**, who found an optimal algal growth between 20-30°C, moreover **Liu *et al.* (2016)** reported pH 7-9 to be the most suitable for most algae. On the other hand, our results are in harmony with **Hana *et al.* (2013)**. They

reported that *Haematococcus* generally prefers a pH between 4.0 and 9.0, and temperatures between 20 and 30°C.

The predicted outcome obtained from optimization curves for both *S. platensis* and *H. pluvialis* showed that maximum growth in *S. platensis* was achieved by increasing light intensity and pH, decreasing temperature, and eliminating salinity. In contrast, *H. pluvialis* achieved the maximum growth through the interaction of various factors, including moderate temperature, increased light intensity, slightly alkaline media, and salt-free medium. **Borowitzka *et al*. (1984)** found that the low salinity maximized biomass in freshwater microalgae. Additionally, **Bialevich *et al*. (2022)** linked the increased light intensity to larger cell size and more daughter cells.

Moderate growth observed in *S. platensis* may be attributed to the increase in temperature to 30°C, which led to a slight decrease in the OD value. In contrast, *H. pluvialis* exhibited medium growth, with the reduction in growth below optimal levels attributed to the combined effects of decreased pH (to 5.5) and reduced light intensity. The results also demonstrated the negative impact of certain environmental conditions on OD, with minimum growth occurring due to the combination of high temperature and high pH. This aligns with **Machida *et al*. (2024)**, who reported synergistic negative effects of high temperature. High temperatures create physiological stress for aquatic organisms, this stress arises because warmer water holds less oxygen, while at the same time, the metabolic demands of these organisms increase, requiring more oxygen **(Mohammed *et al*., 2018)**. Additionally, **Cai *et al.* (2011)** demonstrated that extreme pH levels, whether low or high, inhibit algal growth and can lead to cell death. The negative impact of these suboptimal conditions on OD is attributed to the combination of high temperature (42°C) and extremely acidic pH (0.5). This finding is consistent with **Singh and Singh (2015)**, who reported that temperatures up to 35°C are lethal for certain algal species, particularly green microalgae.

The relationship between algal growth conditions and the production of PHB, a biodegradable plastic, was clearly observed in the results. PHB levels were higher under moderate growth conditions in both algal strains. The optimization experiments for algal growth and PHB production indicated that limited growth conditions led to increased PHB accumulation. This finding aligns with **Perez-Rivero *et al.* (2019)**, who reported that PHAs are synthesized by various microalgae species under environmental stress or nutrient-limited conditions.

The highest PHB concentration was recorded in *H. pluvialis* (61.04 ± 0.3mg/ g), while the lowest was observed in *Spirulina platensis* under optimal growth conditions (20.43 ± 0.56mg/ g). This result is consistent with **Ansari and Fatma (2016)**, who reported that among the tested strains, *Arthrospira platensis* produced the lowest amount of PHB.

The results of the enhancement of Polyhydroxybutyrate concentration for *H. pluvialis* showed that nitrogen and phosphorus deficiency led to higher PHB concentrations compared to normal media. These results agree with those of **De Philippis *et al*. (1992)**, **Nishioka *et al*. (2001)**, **Panda *et al*. (2005)**, **Bhati and Mallick (2012)** and **Monshupanee *et al*. (2016)**. While increased acetate concentration generally enhanced PHB production across all treatments, these findings corroborate previous studies demonstrating the positive impact of sodium acetate on PHB accumulation **(Vincenzini *et al*., 1990; De Philippis *et al*., 1992; Maheswari & Ahilandeswari, 2011**; **Abdo & Ali, 2019)**. A maximum PHB content (197.58 ± 0.6mg/ g) was achieved in cells cultured in phosphorus-limited media containing 2g/ L acetate. This result is correlated with **Panda and Mallick (2007)** and **Monshupanee *et al*. (2016)**.

The limited production of PHB is not a result of insufficient activity of the enzymes involved in the PHB biosynthetic pathway. **McQualter *et al.* (2014)** provided evidence suggesting that the synthesis of PHB and fatty acids may not be directly competitive. However, under conditions of severe stress, the activity of acetyl-CoA carboxylase (ACCase)—a key enzyme in fatty acid biosynthesis—may be inhibited **(Page *et al.*, 1994)**. This inhibition allows competition between ACCase and thiolase for acetyl-CoA, potentially favoring PHB synthesis.

These findings help explain the low PHB concentrations observed in this study under nutrient-deprived conditions. Specifically, cells cultured in media lacking nitrogen and phosphorus, with an acetate concentration of 1.5–2g/ L, showed a decrease in PHB content compared to the control media with the same acetate concentrations. PHB levels under these conditions ranged from 81.44 ± 1.2 to 50.76 ± 1.8mg/ g.

CONCLUSION

This study successfully optimized growth conditions for *Spirulina platensis* and *Haematococcus pluvialis*, leading to maximum polyhydroxybutyrate (PHB) accumulation. The findings of this study underscore the potential of these microalgae as sustainable sources for bioplastic production, offering a promising solution for a more environmentally friendly future.

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