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PII: S1878-8181(24)00325-6

DOI: https://doi.org/10.1016/j.bcab.2024.103341

Reference: BCAB 103341

To appear in: *Biocatalysis and Agricultural Biotechnology*

Received Date: 26 November 2023

Revised Date: 5 July 2024

Accepted Date: 4 August 2024

Please cite this article as: Giovanni Luca, R., Langellotti, A.L., Verardo, V., Garcia, B.M., Oliviero, M., Masi, P., Sustainable Cultivation of *Porphyridium cruentum* via Agro-Industrial By-Products: A Study on Biomass and Lipid Enhancement, *Biocatalysis and Agricultural Biotechnology*, https://doi.org/10.1016/j.bcab.2024.103341.

Sustainable Cultivation of *Porphyridium cruentum* via Agro-Industrial By-Products: A Study on Biomass and Lipid Enhancement

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Abstract:

This study explored the cultivation of *Porphyridium cruentum* using beet molasses and corn steep liquor (CSL) as alternative agri-waste substrates. The objective was to enhance the biomass and lipid production of *P. cruentum*, known for its potential as an industrial EPA producer. Using a Box-Behnken Design (BBD) as part of the response surface methodology (RSM), we investigated the effects of beet molasses, CSL, and F/2 nutrients on the microalga's cultivation. The results demonstrated a significant influence of these factors on the final cell count and lipid content over an 8-day cultivation period. Specifically, optimal growth conditions were identified at approximately 1.78 g/L of molasses and 1.89 g/L of CSL, yielding a cell count of 12.1 x 10^6 cell/mL and a lipid content of 24.48%. Validation experiments reaffirmed these findings, with observed results closely aligning with predicted values. This research underscores the potential of using agro-industrial by-products for large-scale cultivation of *P. cruentum*, offering a sustainable approach for enhancing lipid production and other biotechnological applications.

Keywords: food waste; microalgae; waste valorization; response surface methodology; molasses

1. Introduction

Microalgae represent a pivotal role in high added value compounds production due to their capacity to accumulate significant amounts of lipids, omega-3 oil, pigments or carbohydrates under specific growth conditions (Giovanni L. Russo et al., 2021). Apart from high lipid yield per unit area, the rapid cultivation cycles (Adams et al., 2013), robustness against different environments such as saltwater or eutrophic waters (Herrera et al., 2021), and the creation of valuable by-products, e.g., proteins and residual biomass (Giovanni L. Russo et al., 2021), are some appealing features of microalgae.

In the field of omega-3 oil production, the availability and cost of nutrients, particularly nitrogen, phosphorous and organic carbon, play a vital role. Omega-3 fatty acids are essential nutrients renowned for their beneficial effects on human health, notably in reducing the risk of heart disease, inflammation, and neurological disorders. The traditional sources of omega-3, mainly fish oils, have been associated with sustainability challenges, such as overfishing and contamination with heavy metals (Wang et al., 2022). However, the production of algae oil requires higher production costs if compared to the fish oil.

Porphyridium cruentum (also known as *Porphyridium purpureum*), a red unicellular microalga, offers a promising alternative for the sustainable production of omega-3 oils (Di Lena et al., 2019; Kim et al., 2021). Rich in eicosapentaenoic acid (EPA) and other essential fatty acids, this microalga has proven to be an efficient producer of lipids that can be easily cultivated in various environmental conditions. The growth of *Porphyridium cruentum* not only aligns with the current trend of environmental stewardship but also introduces new avenues for industrial scalability. Understanding and optimizing the growth of *P. cruentum* is, therefore, not merely a scientific pursuit but a global imperative. In harnessing its potential, researchers and industries may unlock new pathways to address the intertwined challenges of health, sustainability, and energy (Kim et al., 2021).

The continuous search for economical and sustainable nutrient sources for microalgae growth has directed attention towards agricultural waste products. Beet molasses (BM), a by-product of sugar

production, contains sugars, vitamins, and various organic acids, which could make it suitable for microalgae growth (Piasecka et al., 2017). The global production of BM is estimated to range between 65 and 70 million metric tons annually, with a market value of approximately 7 billion US dollars (S&P global, 2023). The BM represents an easily obtainable waste in Europe, since EU (France and Germany in particular) detains the highest production of beet pulp with 13 mT beet pulp generation and about 32 mT of molasses (Diwan et al., 2018). Corn steep liquor (CSL) is another agro-industrial waste widely used as cheap substrate for bioprocessing. It is a by-product of corn wet-milling, is rich in amino acids and vitamins, making it a potential nutrient source for microalgae (Kim et al., 2020). Studies like Jung et al. (2010) have shown that these by-products can be used as substrates for bacterial fermentation (Jung et al., 2010), indicating their potential applicability for microalgae cultivation. The utilization of nutrients derived from BM and CSL represents a promising pathway. These sources contain a wealth of carbohydrates, amino acids, vitamins, and minerals essential for microalgae growth (Nakahara et al., 1996; Piasecka et al., 2017). Previous work has shown that BM can be an efficient carbon source for different microorganisms (Piasecka et al., 2020, 2017; Schmidt et al., 2005), while CSL has been used as an organic nitrogen supplement (Maddipati et al., 2011; Mohammad Mirzaie et al., 2016; Russo et al., 2023). In fact, numerous studies have explored the use of these agricultural waste materials for fermentation and microbial growth (Kim et al., 2020) but their application in microalgae cultivation remains relatively an underexplored field. The use of BM and CSL represents a sustainable approach by valorizing agricultural by-products, which are often considered waste. This not only helps in waste management but also significantly reduces the cost of cultivation media.

Earlier research on aquatic protists has shown promising results using different agricultural waste products, such as *Scenedesmus obliquus* grown in brewery wastewater (Navarro-López et al., 2020), *Chlorella vulgaris* cultivated using dairy effluent (Peter et al., 2021) and *Euglena gracilis* on spent brewery grain and CSL (Kim et al., 2020). Nevertheless, studies focusing on BM and CSL for microalgae growth, particularly regarding biomass and lipid production, are limited.

In this study, we explore for the first time the potential of BM and CSL as alternative nutrient sources for the growth of *P. cruentum*. We evaluate their effectiveness in terms of biomass yield, lipid productivity, and fatty acid composition, comparing them with conventional growth media. We also analyze the sustainability and economic advantages of these agricultural by-products. To the best of our knowledge, this is the first comprehensive study assessing the use of BM and CSL for *P. cruentum* cultivation targeting biomass and lipid production. The outcomes of this study will provide an important contribution to the field of alternative omega-3 fatty acids oil production and may lead to more environmentally friendly and cost-effective microalgae cultivation practices.

2. Materials and Methods:

2.1. Microalga and Culture Conditions

P. cruentum (RCC653) was obtained from the Roscoff Culture Collection (Roscoff, France) and pre-cultured for 10 days in sterilized seawater with modified F/2 medium containing 75 mg $NaNO_3$, 5 mg $NaH_2PO_4 \cdot H_2O$, 4.12 mg Na_2EDTA, 3.11 mg $FeCl_3 \cdot 6H_2O$, 0.02 mg $MnCl_2 \cdot 4H_2O$, 0.02 mg $ZnSO_4 \cdot 7H_2O$, 0.01 mg $CoCl_2 \cdot 6H_2O$, 0.01 mg $CuSO_4 \cdot 5H_2O$, 0.006 mg $Na_2MoO_4 \cdot 2H_2O$, 30 mg Na_2SiO_3, 0.2 mg thiamine-HCl, 0.01 mg vitamin B_{12}, and 0.1 mg biotin per liter (Kim et al., 2021). The cultures were maintained with a working volume of 200 mL in 500 mL Erlenmeyer flasks at 21 °C and 140 rpm using an orbital shaker. Continuous illumination of 150 µmol photons m^{-2} s^{-1} was provided to the flasks.

2.2 Agri-waste utilization and screening experiments

To establish the growth performance of the microalga under investigation, two types of growth screening were conducted: the first, evaluating the growth of the biomass with a supplementation of various sources of organic carbon; the second by evaluating biomass growth with various concentrations of BM, CSL, and a mix of the two.

For the first screening, the organic carbon sources evaluated were glucose (5 g/L), sucrose (4.75 g/L) and fructose (5 g/L), supplemented to the standard media with the same concentration of carbon (2 g L^{-1} of C). These organic carbon sources were selected as these are the major sugar present in BM and CSL. For the second screening, different types of medium were evaluated: A medium with 1.5 g L^{-1} of BM; media with 3 g L^{-1} BM; media with 6 g L^{-1} of BM; media with 1 g L^{-1} of CSL; media with 2 g L^{-1} of CSL; media with 3 g L^{-1} of CSL; mixed media with 1.5 g L^{-1} of BM and 1 g L^{-1} of CSL; mixed media with 3 g L^{-1} of BM and 1 g L^{-1} of CSL; media with 1.5 g L^{-1} of BM and 2 g L^{-1} of CSL; media with 3 g L^{-1} of BM and 2 g L^{-1} of CSL. The BM and CSL used in this experiment was already used and characterized in our previous work (Russo et al., 2023). BM is characterized by very

high sugar (total sugars 667 g/kg) and ash content (81.9 g/kg) while CSL was found to be the richest in terms of proteins, with a concentration of 305 g/kg, but also in lactic acid (121.2 g/kg).

All these cultivation media were obtained by adding the corresponding amount of BM or CSL to artificial seawater (ASW) without supplementation of other nutrients. The pH was adjusted at 8.0 for all the media with NaOH 5 M.

The screening trials were conducted 500 mL air-lift reactor with a working volume of 350 mL. The mixing was provided through an air bubbling system equipped with a filter of 0.22 μm in order to prevent any contamination and to provide oxygenation to the culture. The initial cell density for each experiment was 1×10^5 cells/ml. To evaluate the growth performance with the various media, the optical density was determined using a spectrophotometer (ONDA UV-30 SCAN, Torino, Italy) at a wavelength of 686 nm.

2.3 Box-Behnken Design

In this study, a Box-Behnken Design (BBD) was employed as part of the response surface methodology (RSM) to investigate the effects of BM, CSL, and F/2 nutrients on the cultivation of *Porphyridium cruentum*. This design was chosen for its efficiency and robustness in modeling the response surface with three-level factorial designs (Esua et al., 2021). The three independent variables considered were molasses concentration (g/L), CSL concentration (g/L), and F/2 nutrients concentration (%). Each of these factors was varied across three levels, determined based on preliminary studies. The exact values of these levels will be detailed in the results section.

The BBD for three factors includes a total of 15 experimental runs. Twelve of these runs were used to estimate the first-order and second-order terms in the regression model, and three additional center points were included to estimate the experimental error.

The BBD also allows for the estimation of interaction effects between the factors, providing a comprehensive understanding of the impact of these variables on the cultivation process. The experiments were conducted within a single block to avoid block effects.

The specific levels of the three independent variables used in the Box-Behnken Design are presented in Table 1.

Table 1. Levels of the independent variables used in the Box-Behnken Design for the cultivation of *P. cruentum*.

Factor	Low Level	Center Level	High Level
Molasses (g/L)	-1 (0)	0 (1.5)	+1 (3)
CSL (g/L)	-1 (0)	0 (1)	+1 (2)
F/2 (%)	-1 (0)	0 (50)	+1 (100)

The levels of the factors are coded as -1 for the low level, 0 for the center level, and +1 for the high level. The actual values corresponding to these coded levels are based on the range of feasible operation in the lab environment and preliminary experimental observations.

The Box-Behnken Design matrix for this experiment consisted of 15 runs, including 12 factorial points and 3 center points. These experimental runs were conducted in a random order to minimize the effects of uncontrolled factors.

In each run, the microalga *P. cruentum* was cultivated under the specific conditions of molasses, CSL, and F/2 nutrient levels, and the cell count and lipid content (% w/w) were measured as the response variables.

2.3 Lipid analysis and cell count procedures for data collection

The response variables in this study were cell count and lipid content of the *P. cruentum* culture. Cell count was determined using a hemocytometer (Burk chamber). The cultured sample was first diluted if necessary, and then placed onto the chamber. The cells were counted under a microscope, and the cell count was calculated based on the dilution factor and the dimensions of the counting chamber. The cell count was expressed in terms of millions of cells per milliliter ($x10^6$ cells/mL). For the lipid

evaluation, the biomass was firstly lyophilized. The lipid content of biomass was determined using a modified procedure of Bligh and Dyer method (Bligh and Dyer, 1959) (a well-established procedure for lipid extraction) using chloroform/methanol/water solution with a volume ratio of 1:2:0.8, respectively. Following the extraction, the lipids were dried and weighed, and the lipid content was expressed as a percentage of the dry weight of the algal biomass (% w/w).

All the measurements were carried out at the end of each experimental run corresponding to the BBD. Care was taken to ensure the accuracy and reproducibility of these measurements. Quality control procedures, such as repeating the counts or the extraction for some samples, were also implemented to ensure the reliability of the data (n=3).

2.4 Fatty acid methyl esters (FAMEs) analysis

The fatty acid methyl esters (FAMEs) were prepared from the total amount of previously obtained lipids by a transmethylation reaction according to a previous methodology described with certain modifications (Aued-Pimentel et al., 2004). For that purpose, 20 mg of lipid extract was mixed with 50 µL 2N KOH in methanol, 500 µL of n-hexane and 500 µL of methylnonadecanoate (Sigma, St. Louis, MO, USA) as internal standard (1mg/ mL). The mixture was shaken by using a vortex for 2 min. The upper layer supernatant (FAME extract) was collected and injected into a Gas chromatography-mass spectrometer (GC-MS). Microalgal extracts were analyzed according to the method conditions reported by Conde et al. (Conde et al., 2021). The analyses were achieved by using an Agilent 7890A Gas chromatography coupled to a Waters QUATTRO microTM mass spectrometer detector. The separation was done by using a capillary column DB-5MS (30 m × 0.25 mm; f.t. 0.25 µm) from Agilent Technologies (J&W Scientific, Folsom, CA, USA). The oven temperature was 58°C for 2 min, 25°C min^{-1} to 160°C, 2°C min^{-1} to 210°C, 30°C min^{-1} to 225°C (held for 20 min). MS detector operates with ionization energy of 70 eV and a scanning range of m/z 50–550 m/z. The conditions were helium as carrier gas at 1.4 mL min^{-1}, inlet temperature 220°C, detector temperature

230°C, 2µL of injection volume (splitless). Data were processed by using MassLynx version 4.1 (Waters, San Jose, CA, USA).

2.5 Determination of carotenoids and phycobiliproteins

The procedure for determining the carotenoid profile from the *P. cruentum* extract was already described in our prior study (Giovanni L Russo et al., 2021). The microalgal extracts were obtained using an ultrasonic bath (Bandelin, Sonorex) set with a frequency of 35 kHz. Briefly, 10 mg of microalgal sample was extracted with 1.5 mL of ethanol (with 0.1% butylated hydroxytoluene), centrifuged, and then stored at -18°C post-filtration.

For analysis, UPLC Acquity coupled with XEVO-TQ-S Triple quadrupole mass spectrometry was used. The separation of carotenoids was done in a YMC-C30 reversed-phase column. Mobile phases included methanol (with 5% water and 0.1% formic acid) and methyl tert-butyl ether. Analysis was set in a positive-ion mode, with specific mass spectrometric parameters outlined. Data was processed using MassLynx version 4.1, and carotenoids were quantified against known standards. The calibration curves have shown a good linearity with determination coefficients higher than 0.9918. The analysis method had a limit of detection between 0.02 and 2.06 µg L^{-1}, whereas the limit of quantification was 0.08-6.85 µg L^{-1}.

The phycobiliproteins were extracted following the procedure from the method presented by (Tounsi et al., 2023). Briefly, a wet sample of *P. cruentum* biomass was obtained by centrifuging 1 mL of the microalgal mixture at 8000 × g for 10 minutes. This wet residue was cleaned twice using the growth medium through centrifugation and later reintroduced in a sodium phosphate buffer solution (100 mM, pH 6.0) of 1 mL. To isolate the phycobiliproteins from the microalgal residues, a combination of freezing, thawing, and ultrasonication was utilized. This commenced with multiple freeze-thaw cycles until the cellular waste no longer showed a clear red hue. A single cycle comprised 3 hours of freezing at -20 °C and an hour of thawing in room temperature conditions (20–25 °C). Following this,

the mixture underwent ultrasonic exposure with a sonication probe, configured at 60% amplitude and a 0.5 cycle for 10 minutes. The separation of liquid from solid was achieved by another round of centrifugation at 8000 × g for 10 minutes. The resulting clear liquid was then collected, and the phycobiliprotein levels were determined using a spectrophotometer, referencing the absorbance A_{565} (phycocyanin), A_{620} (allophycocyanin), and A_{650} (B-phycoerythrin) values based on the formulas provided by Marcati et al. (2014).

2.6 Statistical Analysis

The collected data were subjected to statistical analysis to establish the relationship between the independent variables (molasses, CSL, and F/2 nutrients) and the response variables (cell count and lipid content). A response surface regression model was fitted to the data. This type of model is particularly suitable for the analysis of factorial designs and allows for the estimation of both linear and quadratic effects of the independent variables, as well as their interactions.

The model used in this study can be written as follows:

$$Y = \beta 0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 + \varepsilon$$

Where Y is the response variable (either cell count or lipid content); X1, X2, and X3 are the independent variables (molasses, CSL, and F/2 nutrients, respectively); β_0 is the intercept; β_1, β_2, and β_3 are the linear coefficients for X1, X2, and X3, respectively; β12, β13, and β23 are the interaction coefficients for X1*X2, X1*X3, and X2*X3, respectively; β11, β22, and β33 are the quadratic coefficients for $X1^2$, $X2^2$, and $X3^2$, respectively; ε is the error term.

An analysis of variance (ANOVA) was also performed to determine the statistical significance of the independent variables and their interactions. The ANOVA results provide the sum of squares, degrees of freedom, mean square, F-value, and p-value for each term in the model, which are critical for understanding the contribution of each term to the variation in the response variable.

The data were analyzed using IBM© SPSS© Statistics software Ver. 23 (SPSS, Inc., Chicago,IL, USA). RSM analysis was carried out using the Statistica 7.0 package (StatSoft, Tulsa,OK, USA). The fit of the model was checked by examining the residual plots and other diagnostic tools. The final model was chosen based on statistical significance of the terms, goodness-of-fit statistics, and the principle of parsimony.

3. Results and discussion

3.1 Growth performance using different organic carbon sources

The screening results regarding the organic carbon supplementation to standard media for *P. cruentum* are reported in **Figure 1**.

The growth curves of *P. cruentum* under different sugar supplementations reveal insightful trends in the organism's response to varying nutritional conditions. Among the sugars examined, glucose and sucrose were found to markedly enhance growth, as evidenced by the higher optical densities observed in the later stages of the monitoring period. In contrast, fructose supplementation mirrored the control condition, suggesting that it might not be as effective in promoting growth. These observations could be indicative of the organism's specific metabolic preferences and utilization pathways. In fact, while *P. cruentum* is capable of autotrophic nutrition, it can also be cultivated using certain organic carbon sources. Previous studies have provided evidence that the growth of *Porphyridium* spp. can be enhanced in the presence of specific organic carbon substrates (Kim et al., 2021; Li et al., 2019; Oh et al., 2009). This can be attributed to the efficient uptake and assimilation of glucose and sucrose through glycolysis and the subsequent tricarboxylic acid (TCA) cycle. These pathways provide essential precursors and energy (ATP and NADPH) for biomass production and lipid biosynthesis. Sucrose resulted the optimal organic carbon source for the growth of *P. cruentum*, showing a significant higher value of optical density after 8 days respect to the autotrophic control. Regarding glucose supplementation, our data are in line with those of Oh et al. (2009) which found the highest growth of *P. cruentum* when supplementing glucose (5-15 g/L) to the media (Oh et al.,

2009). The pronounced growth in the presence of glucose and sucrose may be attributed to their more efficient assimilation or the activation of specific metabolic routes that favor growth (Jiao et al., 2018). Conversely, the similarity between the fructose curve and the control (without supplementation of organic carbon) may reflect a limited ability to utilize fructose as an energy source or the requirement of specific conditions for its effective uptake. Despite the need for further investigation to understand the exact mechanism, the evidence suggests that *P. cruentum* exhibits superior growth under mixotrophic conditions compared to strictly autotrophic conditions.

3.2 Screening results with alternative agri-waste substrates

Since the organic carbon supplementation lead to a growth boost, we tested the growth performances of media composed exclusively of BM, CSL and a mix of them.

The figure presents an insightful comparison of the final cell count and lipid content in *P. cruentum* under different supplementation conditions over an 8-day cultivation period. The results highlight the significant influence of different agri-waste supplementations on both cell growth and lipid accumulation in *P. cruentum*.

The control (without additional supplementation) resulted in a baseline cell count, establishing a reference for comparison. The cell count was notably enhanced with 1.5 g/L and 3 g/L BM supplementation, reaching a peak at 3 g/L. However, further increase in molasses concentration led to a decline in cell count. A moderate increase in cell count was observed with 1 g/L and 2 g/L CSL, while 4 g/L CSL resulted in a slight decline. The combination of molasses and CSL showed a synergistic effect on cell growth, especially in the conditions of 3 g/L of BM + 1 g/L CSL and 3 g/L of BM + 2 g/L CSL. In our previous work, we observed that also the diatom *Phaeodactylum tricornutum* is capable to utilize the nutrients present in BM and CSL (Russo et al., 2023). BM was already used succesfully for the growth of heterotrophic microalgae like *Galdieria sulphuraria* (Schmidt et al., 2005). The study by Schmidt et al., (2005) pointed out an inhibitory effect of BM

296 when used in high concentrations (10 g/L), and this is in line with our findings where the highest
297 content of molasses showed a significant lower cell density respect to the control. In general, molasses
298 serves as a rich source of carbon, primarily in the form of sugars, which is a fundamental prerequisite
299 for mixotrophic microalgal metabolism (Mohammad Mirzaie et al., 2016). However, the
300 concentration of molasses in the culture medium requires careful calibration. Both deficient and
301 excessive concentrations can impede the cellular processes, leading to suboptimal growth and lipid
302 accumulation. Similarly, CSL contains an array of nutrients including amino acids, vitamins, and
303 minerals, which contribute to the overall nutritional quality of the culture medium (Kim et al., 2020;
304 Russo et al., 2023). Previous research has consistently shown that these by-products can enhance
305 microbial growth and product yield. For instance, the use of beet molasses and CSL has been
306 associated with increased biomass production and higher yields of valuable metabolites, such as lipids
307 and proteins.
308 Regarding the lipid content, the lowest amounts were observed for the control (11.58%) and for the
309 medium composed only of CSL. The lipid content of the control was higher than those of Rebolloso
310 Fuentes et al. (2000) but lower than other found in literature for the same strain (Yongmanitchai and
311 Ward, 1991). A substantial increase in lipid content was evident with molasses, peaking at 3 g/L
312 concentration. CSL supplementation also resulted in an increase in lipid content, but less pronounced
313 compared to molasses. The lipid content in mixed mediums was generally higher, indicating a
314 favorable effect on lipid accumulation. These agro-waste substrates showed also a lipid concentration
315 boost for *P. tricornutum*, which is in line to our previous study (Russo et al., 2023). Moreover, another
316 study reported that supplementation of BM to growth media increased the lipid content of *Chlorella*
317 *vulgaris* (Kendirlioğlu Şimşek and Cetin 2022). Mixotrophic mode has been widely reported to
318 increase the lipid yield of microalgae cultivation respect the classic autotrophy. The recent study of
319 Piasecka et al., (2020) investigated the role of BM in the growth of *Tetradesmus obliquus* under
320 different culture conditions. The authors reported a lipid productivity doubled respect the autotrophic
321 control which could be due to a protective mechanism in algal cells induced by exposure to nutrient

322 stress (Piasecka et al., 2020). *P. cruentum* is recognized to be an excellent candidate for the production
323 of long-chain polyunsaturated fatty acids, especially EPA (Hu et al., 2018; Giovanni L. Russo et al.,
324 2021; Yongmanitchai and Ward, 1991)
325 The results demonstrate a complex relationship between sugar supplementation and both cell growth
326 and lipid accumulation in *P. cruentum*. Specific concentrations of molasses and CSL, individually or
327 in combination, could optimize the cell count and lipid content.

328

329 **3.3 BBD results and regression analysis**

330 The cultivation of *P. cruentum* is a complex process influenced by various environmental and
331 nutritional factors. Among these, our study focused on the impacts of molasses, CSL, and F/2 nutrient
332 concentrations, which were identified as critical parameters in the cultivation process. The response
333 surface methodology (RSM), grounded in regression analysis, was employed in our study to
334 scrutinize the impact of three key factors: BM concentration; CSL concentration; and F/2 nutrient
335 concentration, on two response variables: cell count and lipid content of *P. cruentum*. This advanced
336 statistical technique facilitated a comprehensive understanding not only for the main effects but also
337 the interaction effects of the factors on the response variables.

338 The experimental runs and results of BBD are reported in Table 2.

339 **Table 2.** Box-behnken design and results of biomass growth and lipid content with supplementation
340 of beet molasses, corn steep liquor and standard nutrients (f/2).

Run	Factor Assignment			Responses (Y)	
	X_1 (g/L)	X_2 (g/L)	X3 (%)	Cell count ($x10^6$/mL)	Lipid (% w/w)
1	1.5	1	50	11.59	17.23
2	3.0	1	100	8.92	22.14
3	1.5	1	50	11.80	18.34
4	1.5	0	100	8.74	13.84
5	3.0	0	50	5.21	12.34
6	3.0	1	0	8.26	24.32
7	0.0	0	50	2.31	11.34
8	0.0	2	50	7.99	19.75
9	1.5	1	50	10.34	18.45
10	3.0	2	50	7.16	24.75
11	1.5	0	0	3.65	17.45

12	0.0	1	0	9.53	16.88
13	0.0	1	100	9.23	19.33
14	1.5	2	100	10.95	23.32
15	1.5	2	0	12.17	22.85

Coded values; X_1 Beet molasses, X_2 Corn steep liquor; X_3 f/2 nutrients.

The highest cell count was observed in Run 15 (12.171 x 10^6 cells/mL), where 1.5 g/L of BM, 2 g/L of CSL, and no f/2 nutrients were used. In contrast, the lowest cell count was seen in Run 7 (2.31 x 10^6 cells/mL) with no BM, 0 g/L of CSL, and a 50% concentration of f/2 nutrients. The highest lipid concentration was observed in Run 10 with 24.75% w/w, showcasing the same composition that gave rise to the maximum cell count. On the other hand, the minimum lipid concentration was found in Run 5 with 12.34% w/w, which had 3.0 g/L of BM, no CSL, and a 50% concentration of f/2 nutrients.

The significance of BBD was tested by analysis of variance (ANOVA) and reported in Table 3. P-value lower than 0.05 was considered significant in the analysis.

Table 3. Analysis of variance for cell count and lipid productivity from Box-Behnken Design used for *P. cruentum*.

Source	DF[a]	Adj SS[b]		Adj MS[c]		*P*-Value	
		Cell count	Lipids	Cell count	Lipids	Cell count	Lipids
Model	9	114.33	245.669	12.704	27.297	0.008	0.004
Linear	3	44.44	203.241	14.815	67.747	0.008	0.001
Molasses (g/L)	1	0.030	33.008	0.030	33.008	0.877	0.007
CSL (g/L)	1	42.17	168.361	42.177	168.361	0.002	0.001
F/2 (%)	1	2.239	1.872	2.238	1.872	0.219	0.346
Square	3	56.23	30.697	18.745	10.232	0.005	0.042
Molasses ^{2}	1	27.64	0.008	27.647	0.008	0.004	0.950
CSL ^{2}	1	29.81	3.745	29.811	3.745	0.004	0.201
F/2 ^{2}	1	0.84	25.257	0.849	25.257	0.426	0.012
2-Way Interaction	3	13.65	11.731	4.551	3.910	0.084	0.199
Molasses x CSL	1	3.49	4.000	3.495	4.000	0.139	0.189

Molasses x F/2	1	0.23	5.359	0.230	5.359	0.671	0.139
CSL x F/2	1	9.92	2.372	9.928	2.372	0.061	0.294
Error	5	5.66	8.650	1.131	1.730		
Lack-of-Fit	3	4.41	7.739	1.471	2.580	0.311	0.154
Pure Error	2	1.24	0.911	0.623	0.455		
Total	14	119.99	254.320				

R^2 = 96.28 ([a]DF, degree of freedom; [b]SS, sum of squares; [c]MS, mean squares; *P*, probability; CSL, corn steep liquor)

The regression equation for cell count obtained from the model has been shown in eq. (1)

$$\text{Cell count (x } 10^6/\text{mL)} = 0.99 + 4.152\, Molasses\left(\frac{g}{L}\right) + 10.49\, CSL\left(\frac{g}{L}\right) + 0.0181\, f/2(\%) - 1.216\, Molasses\left(\frac{g}{L}\right) x\, Molasses\left(\frac{g}{L}\right) - 2.841\, CSL\left(\frac{g}{L}\right) x\, CSL\left(\frac{g}{L}\right) + 0.000192\, \text{f/2(\%)} x\, \text{f/2 (\%)} - 0.623\, Molasses\left(\frac{g}{L}\right) x\, CSL\left(\frac{g}{L}\right) + 0.00320\, Molasses\left(\frac{g}{L}\right) x\, f/2\ (\%) - 0.0315\, CSL\ (g/L) * f/2\ (\%) \quad (1)$$

For cell count, the estimated coefficients for the factors and their interactions were obtained from the fitted model. The model was statistically significant for cell count (P=0.008). The P-value for molasses was found to be not significant (P= 0.877), indicating that variations in molasses concentration don't have a substantial impact on cell count. Similarly, f/2 nutrients percentage also didn't show any significant effects. CSL instead was significant for cell count (P=0.002) revealing its crucial role in influencing cell count. The square terms were also statistically significant, indicating that there are likely non-linear relationships between the factors and the responses. This is supported by the strong influence of the squared terms of molasses (P = 0.004) on cell count and f/2 (P = 0.012) on lipids. Quadratic effects refer to the impact of the square of the factor on the response variables. These effects are crucial to capture the potential non-linear relationships between the factors and the response variables (Harker et al., 1995). However, two-way interactions, although presenting various levels of impact, did not generally reach a significant level of 0.05. The role of f/2 nutrients in influencing cell count is multifaceted. In fact, it is supposed that an increased nutrient concentration would consistently result in heightened cell counts. However, our data contradicts this assumption. A

371 case in point is the comparison between Run 4, with a 100% f/2 nutrient concentration yielding a cell
372 count of 8.747 x 10^6, and Run 6, where the absence of f/2 nutrients resulted in a slightly elevated cell
373 count of 8.263 x 10^6. The derived regression equation for cell count (1) integrates all these factor
374 influences and can be instrumental in predicting the cell count given specific concentrations of
375 molasses, CSL, and f/2 nutrients.

376 The same approach was employed for the lipid content. In Table 2 is reported the statistical results of
377 the BBD used on lipid productivity, while in equation (2) is reported the regression equation.

$$\begin{aligned} Lipid\ (\%\ w/w) = {} & 14.14 + 1.4\ Molasses\left(\frac{g}{L}\right) + 4.83\ CSL\left(\frac{g}{L}\right) + 0.1065\ f/2(\%) + \\ & 0.02\ Molasses\left(\frac{g}{L}\right) x\ Molasses\left(\frac{g}{L}\right) - 1.007\ CSL\left(\frac{g}{L}\right) x\ CSL\left(\frac{g}{L}\right) + 0.001046\ f/2(\%) x\ \mathrm{f/2}\ (\%) + \\ & 0.667\ Molasses\left(\frac{g}{L}\right) x\ CSL\left(\frac{g}{L}\right) - 0.01543\ Molasses\left(\frac{g}{L}\right) x \frac{f}{2}(\%) + 0.0154\ CSL\ (g/L) * f/2\ (\%) \end{aligned} \quad (2)$$

378

379 The results from the BBD on *P. cruentum* lipid productivity illuminate the impacts of molasses, CSL,
380 and f/2 nutrient concentrations on the microalgal response. The model was statistically significant for
381 lipid productivity (P = 0.004). The linear effects were pronounced, especially for CSL, with a P-value
382 of 0.001, suggesting a remarkable influence on lipid productivity. The linear effect of molasses was
383 also significant (P = 0.007), indicating its importance in the lipid yield. Additionally, non-linear
384 relationships between factors and lipid content are evident, considering the significance of squared
385 terms. Specifically, f/2 squared (P = 0.012) points to a notable non-linear relationship between f/2
386 concentration and lipid content. This lack of linear significance suggests a delicate balance for this
387 nutrient concentration, which is in any case the lowest significant factor for the increase of cell count
388 and lipid productivity. In other words, the supplementation of standard nutrients to the combination
389 of BM and CSL was not useful in any of the analyzed responses. A similar results was obtained also
390 on *P. tricornutum* cultivated using cheese whey and CSL (Russo et al., 2023). In our study the
391 standard f/2 nutrients were not needed to support the biomass productivity, since those present in CSL
392 and BM were sufficient to sustain biomass growth and lipid productivity.

393 The lack-of-fit values for both cell count and lipids indicate that the model fits the data reasonably
394 well and the variations not explained by the model aren't significantly large. This supports the
395 reliability of the BBD in studying the effects of these factors on *P. cruentum* growth and lipid
396 productivity.
397 The cultivation of *P. cruentum*, as depicted by our research, is markedly influenced by BM, CSL.
398 The robustness of our model, as depicted by the high R^2 value, underscores the criticality of these
399 factors in determining both cell count and lipid productivity of the microalga. Among the individual
400 factors, CSL showcased the most significant influence on both cell count and lipid productivity. This
401 might suggest the importance of proteins, organic acids, and minerals contained in large amounts in
402 the CSL for the growth of biomass and protists (Tan et al., 2016). The nutrient stress on *P. cruentum*
403 can indeed change its biochemical composition, as reported in other studies (Hu et al., 2018). On the
404 other hand, while molasses plays a more substantial role in lipid productivity, its impact on cell count
405 is relatively muted. This could point towards the energy-rich sugars in molasses being primarily
406 channeled into lipid synthesis. In fact BM, which are rich in sugars, presented a strong influence on
407 lipid productivity. This is congruent with the idea that carbohydrate-rich substrates can often promote
408 lipid accumulation in many microalgae, given that sugars serve as carbon sources, which are then
409 transformed into lipids in the cells (Gao et al., 2019; Oh et al., 2009). This is also in line with the
410 study of Gao et al., (2019) where the organic matter in the cultivation media promoted the mixotrophic
411 growth and lipid productivity of *Chlorella* sp. G-9. In algal biorefineries, achieving a high lipid
412 content within cells is vital for ensuring the economic viability high added value compound
413 production (Russo et al., 2022). Nevertheless, cultivating these algal cells under conditions that
414 promote lipid accumulation, such as nitrogen starvation, cooler temperatures, elevated pH levels, and
415 increased salinity, often coincides with a reduction in biomass productivity (Abreu et al., 2012). This
416 compromises the overall yield of lipid production. Embracing mixotrophic cultivation methods
417 emerges as a promising strategy to address this challenge in the context of *P. cruentum* biorefineries.
418 Recent research has indicated that using these cultivation modes can concurrently boost both biomass

production and lipid accumulation in specific algae strains (Gao et al., 2019; Russo et al., 2023). However, further research is essential to fine-tune the growth conditions tailored to each specific strain, emphasizing the desired target compounds for production.

3.4 Optimization and validation of cell concentration and lipid productivity

The optimization strategy employed in this study was underpinned by the BBD. To better visualize the results of BBD and to provide insights for the optimization process, a 3-D surface plot combined with contour plot was reported for both cell concentration and lipid content (Figure 3).

The graphical interpretation of the results allows for the easy identification of trends and optimal regions. The color of the surface and the contour plot both reflect the cell count (Figure 3a) and lipids (Figure 3b), with darker colors corresponding to lower values and lighter colors corresponding to higher values. The contour plot at the bottom serves as a 'footprint' of the surface plot, providing an additional perspective on the response surface. This can aid in identifying optimal conditions for maximizing the cell count. In fact, from the figure, it can be observed that the cell count reaches a zone of maximum concentration, after which the biomass decreases at a certain rate. In contrast, the lipid content doesn't appear to reach a maximum point; instead, it shows an increasing trend as the molasses concentration increases.

To understand the optimal region, in Figure 4 is reported the overlaid contour plot between cell count and lipid content.

This overlaid plot represents the total yield (product of cell count and lipid content) as a function of the CSL and molasses concentrations, with the f/2 nutrient concentration held constant at low value (0%). The contour lines represent points of equal total yield. In Figure 4 has been highlighted the optimal area where the cell count and lipid concentration is at their maximum. The stationary point where the maximum cell count meets the maximum lipid content is approximately at 1.78 g/L of molasses and 1.89 g/L of CSL. These are the most effective concentrations for maximizing both cell

count and lipid content simultaneously in the cultivation of *P. cruentum* following the BBD. In these conditions a cell count of 12.1 x 10^6 cell/mL and a lipid content of 24.48% should be reached. To validate the model the experiment was repeated with these growth media condition. The results were the following: 11.88 ± 0.6 x 10^6 cell/mL and 23.75 ± 0.69% of lipid concentration after 8 days of cultivation in the same conditions as the BBD first experiment. The new data obtained with the optimal nutrient concentrations were not significantly different from the predicted value, validating the BBD results.

The determined optimal concentrations not only facilitate the maximization of cell count and lipid content but also potentially enhance the cost-effectiveness of the cultivation process. Particularly, the use of molasses and CSL, which are often considered waste or by-product resources, can significantly reduce the overall production costs, making the cultivation process more economically viable (Russo et al., 2022; Yan et al., 2011).

However, to make a biotechnological process economically advantageous, it is also necessary to produce molecules with a high added value (Graziani et al., 2013; Massa et al., 2019).

4. Biomass fatty acids and pigments profiles

This study's findings hold practical implications for the large-scale cultivation of *P. cruentum.* This microalga is well-known as potential industrial EPA producer and other valuable lipids (Hu et al., 2018; Oh et al., 2009).

To harness the biotechnological process centered on molasses and CSL, we analyzed and presented the fatty acids, carotenoids and phycobiliproteins of the biomass grown with optimized media in Table 4.

Table 4. Fatty acids (expressed as % of total fatty acids), carotenoids and phycobiliproteins on the extracts of *P. cruentum* grown in standard media and new optimized media.

Fatty acids	Control	Optimized media
C14:0	0.22 ± 0.03	0.2 ± 0.05
C14:1	4.43 ± 0.23	4.85 ± 0.22
C16:0	33.1 ± 1.02	34.2 ± 1.21

C16:1	4.21 ± 0.38	3.43 ± 0.52
C18:0	2.8 ± 0.25	2.6 ± 0.36
C18:1	1.71 ± 0.34	3.1 ± 0.12*
C18:2	17.8 ± 0.38	18.73 ± 0.94
C20:3	0.11 ± 0.02	0.15 ± 0.03
C20:4	15.22 ± 0.73	17.51 ± 0.34*
C22:1	0.53 ± 0.17	0.21 ± 0.04
C20:5 EPA	13.15 ± 0.44	12.43 ± 0.29
Σ PUFAs	46.2	48.7
Lipid content (% w/w)	11.84 ± 0.24	23.75 ± 0.69*
Carotenoids (µg/g dry weight)	**Control**	**Optimized media**
Fucoxanthin	660.6 ± 3.7	808.2 ± 0.9*
Violaxanthin	0.85 ± 0.01	0.70 ± 0.05
Antheraxanthin	0.33 ± 0.01	0.34 ± 0.01
Meso-zeaxanthin	0.46 ± 0.02	0.43 ± 0.05
Zeaxanthin	56.69 ± 0.08	68.75 ± 0.04*
Canthaxanthin	0.22 ± 0.01	10.7 ± 0.4*
Echinenone	<0.1	<0.1
β-Carotene	100.7 ± 0.4	340.23 ± 0.01*
Phycobiliproteins (mg/L)	**Control**	**Optimized media**
Phycoerythrin	11.21 ± 1.71	23.74 ± 2.26*
Allophycocyanin	0.89 ± 0.09	1.19 ± 0.17
Phycocyanin	2.68 ± 0.55	4.23 ± 0.62*

Values are expressed as means ± SD (n=3). PUFA= polyunsaturated fatty acids. Values followed by an asterisk (*) represents a significant difference respect to the control ($p < 0.05$).

No significant differences were observed in terms of EPA between the control and the new optimized media. However, the arachidonic acid and oleic acid was found to be significantly higher in the biomass cultured with the new media. Moreover, the lipid content of *P. cruentum* was boosted after the optimization with BBD, doubling the lipid productivity and confirming the prediction value. The EPA content among the various *Porphyridium* strains can vary greatly. In the study of Vazhappilly and Chen (1998) the fatty acids of two *Porphyridium* strains (CSIRO CS-25 and UTEX 161) were studied. It was noted that in CSIRO CS-25 strain there was an EPA content of 6.7% while in UTEX strain it was 19.7%. While our study did not exceed 14% of TFA, our EPA findings were consistent with other literature (Fábregas et al., 1998), and also higher than another study (Breuer et al., 2012), that reported 7% EPA content on TFA. Nonetheless, it's crucial to consider not just the EPA percentage but also lipid yield, especially given our evidence supporting the use and optimization of agro-industrial waste to enhance biomass concentration.

Regarding the carotenoids profile, distinct variations emerged between the control and the optimized media. Specifically, fucoxanthin concentrations showed a significant increase in the optimized media, underscoring the media's potential metabolic influence. Also, the canthaxanthin content was significantly higher than the control, suggesting a significant metabolic shift or stimulation provided by the optimized media. Concurrently, the tripling of β-carotene concentrations under optimized conditions cannot be overlooked, especially given its commercial and biochemical significance (Di Lena et al., 2019). These delineations underscore the necessity for further in-depth investigation into the constituents and conditions of the optimized media, as well as their interplay with the metabolic pathways of *P. cruentum*.

The phicobiliproteins of *P. cruentum* also showed a significant increase when cultivated in optimized media, doubling the phycoerythrin and phycocyanin (Table 4). These bioactive compounds are known for their anti-inflammatory, immunostimulant, anti-radical, and antioxidant properties (Peña-Medina et al., 2023)

Harnessing the potential of microalgae for omega-3 production and other biotechnological applications necessitates a deep understanding of the complex interplay between various cultivation parameters (Giovanni L. Russo et al., 2021). *P. cruentum* might serve as an alternative source of EPA and can be combined with DHA-rich microalgae, such as *Aurantiochytrium* sp, frequently found in supplements alongside *Ulkenia* sp. (Lee Chang et al., 2015). Studies indicate that consistent consumption of EPA and DHA can decrease the n-6:n-3 PUFA ratio, leading to increased EPA and DHA levels in the membranes of red blood cells (known as the omega-3 index) (Stiefvatter et al., 2021). This is believed to be advantageous, for instance, in reducing inflammation. Moreover, the presence of carotenoids in the obtained omega-3 rich oil, can generate additional advantages. In fact it is well known that carotenoids act as antioxidants and they might protect the oil from lipid oxidation (Yang et al., 2021; Yin et al., 2023), potentially eliminating the need for added antioxidants. Additionally, there's evidence suggesting that carotenoids might reduce oxidative stress. As such, they could potentially mitigate the oxidative stress triggered by PUFA consumption (Awada et al.,

509 2012). Moreover, the biomass obtained is rich of phycobiliproteins. Beyond their biological
510 significance, phycobiliproteins possess high economic value due to their potential applications in the
511 food, cosmetic, and pharmaceutical industries (Chini Zittelli et al., 2023). One of the advantages of
512 cultivating *P. cruentum* is the ability to separate phycobiliproteins from the lipid fraction, allowing
513 for a dual stream of revenue. The separated phycobiliproteins can be sold as high-value compounds,
514 while the lipid fraction, rich in PUFAs, can be processed further for various applications (Huang et
515 al., 2022).
516 Agro-industrial waste, often considered an environmental burden, can be repurposed as a cost-
517 effective growth medium for *P. cruentum*. Utilizing such waste not only provides a sustainable
518 solution to waste management but also enhances the economic viability of *P. cruentum* cultivation.
519 By doing so, producers can maximize the added value derived from both the phycobiliproteins and
520 the lipid fraction, making the entire process more profitable and environmentally sustainable.
521

5. Conclusion

This research underscores the promising potential of harnessing agro-industrial by-products, specifically BM and CSL, for the sustainable cultivation of *Porphyridium cruentum*. Through the application of the Box-Behnken Design, we successfully identified optimal conditions that significantly enhance both the biomass and lipid yield of *P. cruentum*. Our findings not only validate the efficacy of using alternative substrates in microalgal cultivation but also emphasize the environmental and economic advantages of such approaches. The consistency between predicted and observed results further strengthens the reliability of our methodology. As the demand for bioactive compounds, especially EPA, continues to rise, innovations like these play a pivotal role in meeting these demands sustainably. Future research can explore the scalability of these findings and delve deeper into understanding the seasonal variations agro-industrial by-products. The high biomass and lipid yields achieved in this study can support the development of integrated biorefineries where *P. cruentum* is used to produce multiple products, including biofuels, nutraceuticals, and pigments. The dual revenue stream from both lipids and valuable co-products such as carotenoids and phycobiliproteins can significantly enhance the economic sustainability of microalgae biorefineries.

Acknowledgement: Beatriz Martín García thanks the Spanish Ministry of Economy and Competitiveness (MINECO) for "Juan de la Cierva" contract (FJC2020-046307-I).

Funding: This study is part of ERA-Net SUSFOOD2 project SUSPUFA, ID 145, with funding provided by the Italian Ministry of Education, Universities, and Research (MIUR) and the Spanish Ministry of Science, Education, and Universities/Spanish State Research Agency (PCI2018-093178), as well as co-funding by the European Union's Horizon 2020 research and innovation program.

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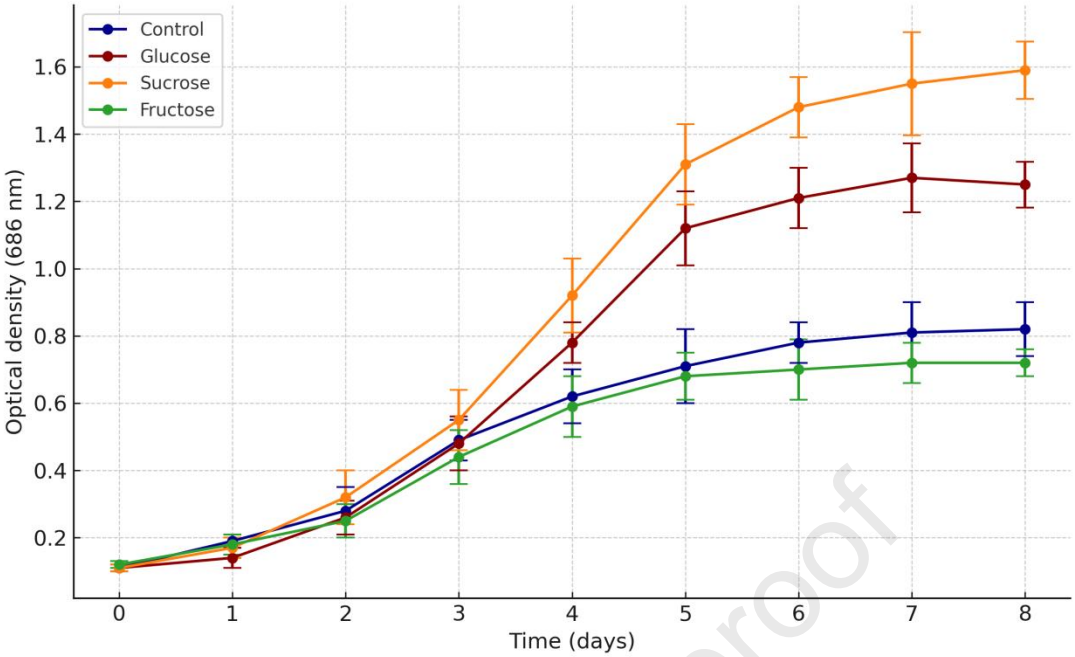


Figure 1. Screening test for different type of organic carbon source for *P. cruentum* cultivation. The basal medium used is Guillard f/2. Control refers to Guillard f/2 media without supplementation of organic carbon. Data are reported as mean (n=3) ± SD.

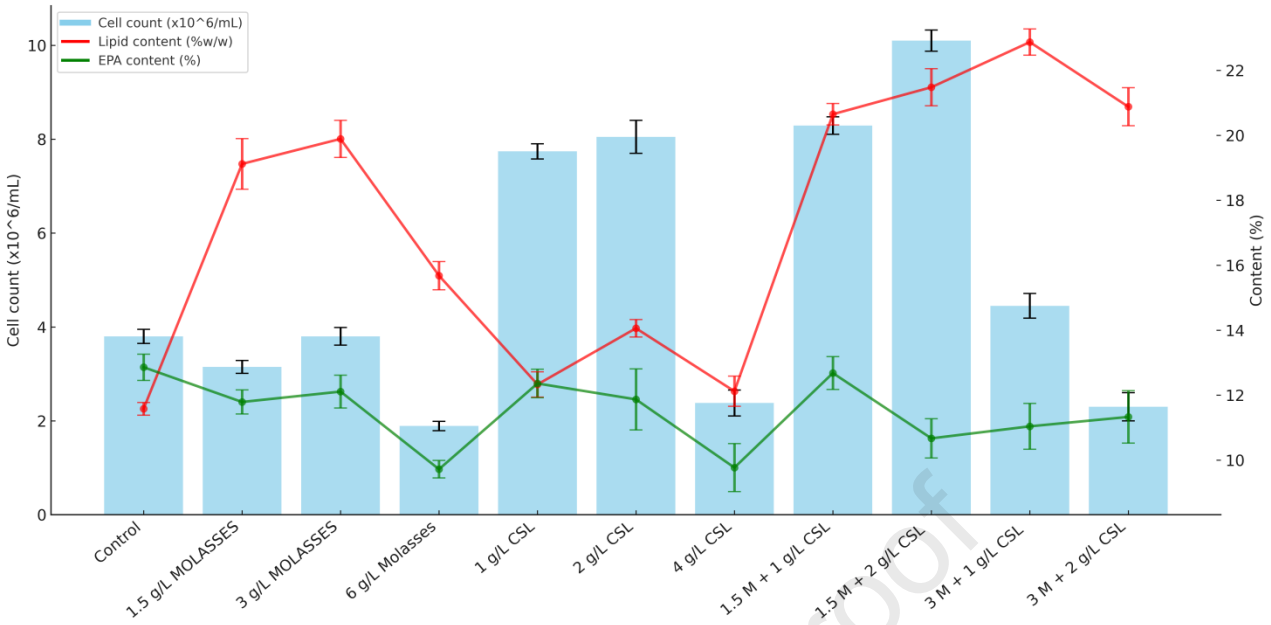
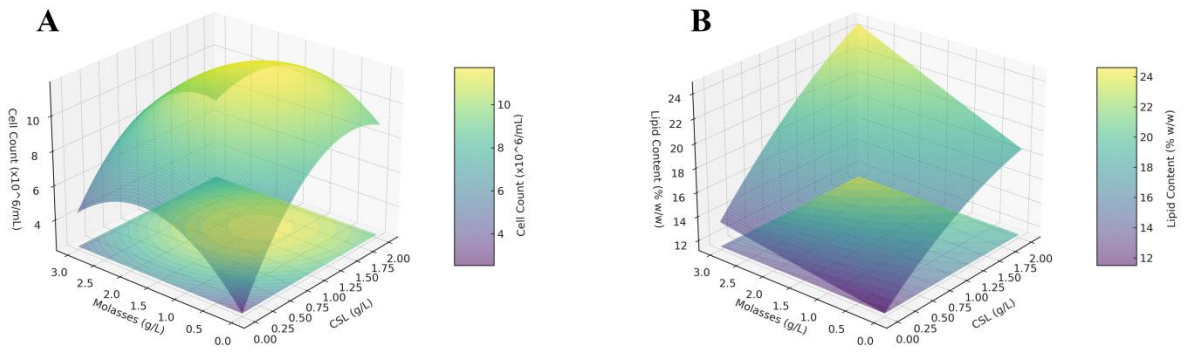


Figure 2. Comparison of the final cell count, lipid content and EPA concentration of *P. cruentum* after 8 days of cultivation under various supplementation conditions. The blue bars represent the final cell count (mean ± SD). The red line plot illustrates the lipid content (% w/w) for the corresponding conditions. The green line represents the EPA content (% of total fatty acids).



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739 **Figure 3.** Surface and contour plot for cell count (A) and total lipid content (B) of *Porphyridium*
740 *cruentum* cultivated with beet molasses and corn steep liquor through box-behnken design.

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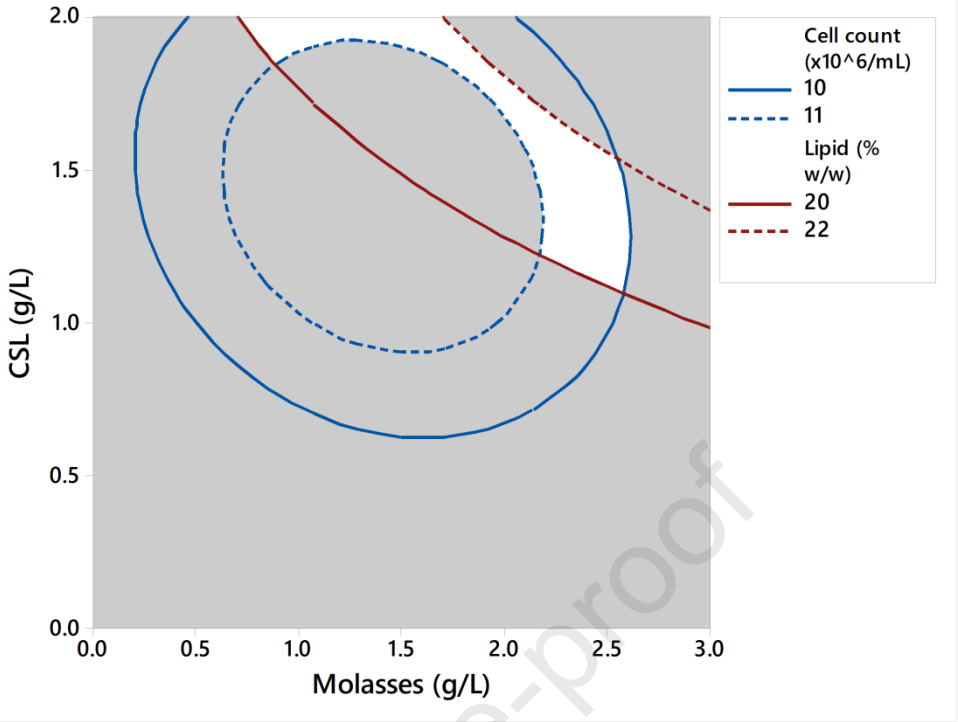

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743 **Figure 4.** Overlaid contour plot for cell concentration and lipid content in the cultivation of microalga
744 *Porphyridium cruentum*. The blue lines depict the minimum and maximum predicted cell concentrations (in
745 $\times 10^6$ cells/mL) across varying levels of beet molasses (g/L) and corn steep liquor (CSL, g/L), with f/2 nutrients
746 fixed at 0%. The overlaid red lines represent specific levels of lipid content (in % w/w). White colored region
747 is the optimal region to maximize the responses.

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Highlights

- First-time use of beet molasses and corn steep liquor for cultivating *P. cruentum*.
- Demonstrated the potential of agro-industrial by-products for sustainable microalgae cultivation.
- Optimized biomass and lipid production using Box-Behnken Design.
- Achieved 12.1 x 10^6 cell/mL & 24.48% lipid content with optimal conditions.
- Showcased *P. cruentum* as a sustainable source for omega-3 production.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: