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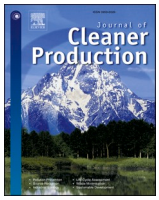

# Manipulating the biomass composition of *Arthrospira platensis* through high frequency acoustic treatments

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ABSTRACT

Photosynthetic microorganisms such as *Arthrospira platensis* are considered a more sustainable source of bioactive compounds. Moreover, their biomass composition can be altered by subjecting these cultures to physical or chemical stresses, at the expense of reduced growth rates. Alternative approaches such as external fields stimuli could enhance biomass composition while minimizing the negative impact on its growth. This study explores the impact of mild acoustic radiation on the compositions of *A. platensis*. Acoustic treatment variables such as power (ranging from 25% to 75%), frequency (in the range of 578–1148 kHz), duration (from 5 to 15 min) and culture age (from 4 to 12 days) were investigated using a custom multifactorial Design of Experiments approach. As a result, maximum increase in protein, lipids and carbohydrate concentrations of 10%, 96% and 88% respectively were observed with negligible impact on growth rates. This increase was achieved under different optimized acoustic treatment conditions. These findings could specifically maximize the production of certain compounds without sacrificing growth rates, fine-tunning the *A. platensis* biomass for various applications.

## 1. Introduction

The search for alternative biobased sources of active ingredients is underway (Wang, 2022). Proteins, fatty acids, and complex carbohydrates represent essential functional components used in food, nutraceuticals, pharmaceutical cosmetics and other value-added applications (Mujwar et al., 2022). Biobased components sourced from cyanobacteria and microalgae represent an interesting source for these components (Arrieta Payares et al., 2023). One of the most interesting sources is *Arthrospira platensis*, or more commonly known as *Spirulina*. This species is regarded as a nutritional supplement rich in proteins and other functional components such as fatty acids, carbohydrates and phycobiliproteins, with GRAS status by the Food and Drug Administration (Carlson, 2011). *A. platensis* is a blue-green multicellular and spiral-shaped cyanobacteria, belonging to the *Oscillatoriaceae* family (Sahin and Akpinar Bayizit, 2022). It is mostly found in alkaline water where solar radiation is low (Asmaz and Seyidoglu, 2022). *Spirulina* is used as a source of protein, vitamin supplements, and health supplements. Rich in nutrients, it contains 25 different types of vitamins and minerals for health and longevity, regulation of metabolism, elimination of toxins, and strengthening of the immune system (Lupatini et al., 2017). These tiny organisms also hold great potential for sustainable energy production as evidenced by the development of innovative microbial fuel cells using *Spirulina platensis* nanoparticles, generating high cell potentials of up to 1.0 V (Sallam et al., 2021, 2022).

Presently, two main strategies are considered for manipulating the biomass composition of photosynthetic microorganisms such as cyanobacteria and microalgae. Most of these approaches consist in stressing the cells during their culture through physical (high temperatures and/or intense light conditions) or chemical (nutrient starvation, extreme pH and high salinity) treatments (Han et al., 2016a). However, most of these treatments also result in decreased cell proliferation rates (Pereira et al., 2023). More recently, alternative strategies such as mild external fields have been explored to both enhance the growth of these microorganisms as well as promoting the bioaccumulation of certain compounds such as lipids (Han et al., 2016b). Among these, low frequency

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sonication (30–80 kHz) and nanosecond (100 ns) pulsed electric field (PEF) represent two of the most promising technologies that have been evaluated for such applications (Buchmann et al., 2019). Static electric fields (SEF) and moderate electric fields (MEF) could also represent suitable treatment options (Pereira et al., 2023). However, these strategies often lead to the incidental inactivation of the treated cells, impacting the overall biomass production. Thus, novel, milder approaches are required. Higher frequency acoustic treatments generate substantially less cavitation bubbles, heat and free radicals (Boboescu et al., 2022). Moreover, these improve mass transfers, temporarily permeabilize cell membranes and impact reaction pathways (Singh et al., 2019). Although these treatments produce lower levels of reactive oxygen species (ROS), even small amounts can induce oxidative stress in algal cells, potentially damaging lipids, proteins, and DNA. However, the reduced free radical production is less likely to overwhelm the algae's antioxidant defenses. Interestingly, low levels of ROS can stimulate growth and enhance secondary metabolite production in some algal species. The overall impact depends on the specific algal species and their antioxidant capacities; resilient species may benefit from mild oxidative stress, while more sensitive species could experience damage. Thus, higher frequency treatments appear to be less disruptive to algal cells than lower frequency treatments, but further research is needed to fully understand their effects on algal physiology and productivity.

Initial studies on the application of ultrasound for stimulating cell growth and the production of certain compounds focused on the physical and chemical effects of cavitation since most of the existing research focused on the low (10–30 Hz) and medium frequency (30–80 kHz) ultrasonic treatments. For instance, subjecting *Scenedesmus* sp. to low-power and frequency ultrasound resulted in increased lipid content (Singh et al., 2019). Similar observations were made when treating *Anabaena variabilis* and *Scenedesmus quadricauda* with medium power sonication (Han et al., 2016a, 2016b). However, low frequency acoustic treatments generate shear forces, heat and free radicals which lead to cell disruption, as well as require considerable energy (Boboescu et al., 2022). The generated forces and their effect on the processed cells depend on the nature of the acoustic field (frequency and power), as well as the nature of the cell wall, with more complex and denser ones being more resistant to these treatments. Moreover, it is not clear if the benefits of increasing the concentration of a certain compound outweigh these shortcomings. Even though in general sonication is used to hinder the growth of microalgae and cyanobacteria and disrupt their cells in downstream processing applications, several studies showed that using milder conditions could lead to the opposite. For instance, *Chlorella ellipsoidea* and *Picochlorum oklahomensis* experienced increased growth rates when stimulated with mild low and medium power sonication (Cai et al., 2016; Theerapisit et al., 2023). However, in most of these studies, prolonged ultrasonic irradiation significantly inhibited cell growth and lipid accumulation. Thus, a milder cell-stressing strategy could increase even more the production of certain compounds while preserving the cellular integrity as well as further reducing energy requirements. High frequency (0.5–5 MHz) acoustic treatments could provide this alternative as the generation of cavitation bubbles, shear forces and free radicals under these conditions is limited (Ahn et al., 2003; Li et al., 2021; Oleszek and Krzemińska, 2021; Thomas et al., 1989). Under these higher frequencies, small oscillating and stable bubbles are being formed, which transfer the kinetic energy to nearby cells, effectively providing a direct mechanical stimulus. Under harsher conditions (long power and exposure time), these vibrations can lead to the inactivation and even complete burst of the cells (Boboescu et al., 2022). However, to the best of our knowledge, to date there are no reports exploring this novel biomass tuning avenue.

The acoustic treatment of biomass is a complex process being influenced by a series of factors such as treatment intensity, frequency, time, growth stage of the treated microorganisms, etc. Thus, to adequately elucidate the impact of these factors and their interactions on the biomass composition and growth rate of these microorganisms, a statistical Design of Experiments (DOE) approach is recommended (Fisher R. A., 1936). This strategy, unlike the classical "One Factor at a Time" (OFAT) approach, allows the simultaneous investigation of multiple variables, and when coupled with a Response Surface Methodology (RSM), the modeling and prediction of various outcomes based on the inputs considered (Czitrom, 1999; Mathews, 2004; (Abdelhay et al., 2021). Thus, a DOE can result in estimating the effect of each influencing factor, the systematic estimation of their interactions, while requiring less resources (Anderson and Whitcomb, 2010; Boboescu et al., 2018; Montgomery, 2017).

The current work explores the possibility of manipulating the biomass composition of *Arthrospira platensis* by using high frequency acoustic fields. These are being deployed at different stages during the growth phase of these microorganisms and various frequencies, intensities, and treatment times. A multifactorial DOE couped to an RSM is used to investigate these different variables as well as to generate a quadratic model. This allows the prediction of conditions in which the investigated responses (proteins, lipids, carbohydrates, growth rate and incidental cell disruption) are being maximized or minimized. To the best of our knowledge, this is the first time that high frequency acoustic fields have been investigated for this purpose, generating such a comprehensive understanding of these phenomena.

## 2. Material and methods

### 2.1. Culture and growth conditions

The *AlgaePARC* pilot facility of Wageningen University provided the *Arthrospira platensis* inoculum used in the present study. The biomass was cultivated as described by Aiba et al. (Aiba and Ogawa, 1977). All the chemicals were purchased from Sigma Aldrich. To adapt the cells to the experimental environment, a preculture lasting two weeks was conducted prior to both preliminary and main tests. To initiate the main cultivation phase, the optical density (OD) was adjusted to 0.1. The cells were cultured in 500 mL shake flasks with 100 mL culture volume in Infors HT Multitron Pro incubators at 120 RPM $min^{-1}$ and 0.2 % $CO_2$. In the preliminary tests, microalgae growth occurred at 25 °C while during the main experimental work the growth conditions were maintained at 30 °C to be closer to optimal growth conditions. The light intensity and the day-night cycle were 150 μmol/$m^2$/s and 16:8, respectively.

### 2.2. Experimental set-up

#### 2.2.1. Ultrasound treatment set-up

The experiments in this study were performed in cylindrical glass reactors (Meinhardt Ultrasonics) with a capacity of 0.5 L and an internal diameter of 6.925 cm, operating in a fed-batch mode (Fig. 1). Each reactor featured a double-jacketed vessel filled with water for temperature control. Ultrasound waves were generated by a piezoelectric ceramic transducer (Meinhardt Ultrasonics) positioned at the base of the reactor. This transducer was capable of operating at three different frequencies (578, 865, and 1148 kHz) using an ultrasonic multi-frequency generator (Meinhardt Ultrasonics) in continuous mode. Integrated sensors were used to monitor the temperatures of both the transducer and the generator throughout the experiments.

#### 2.2.2. Screening procedure

The *Spirulina* biomass underwent a 14-day cultivation period during the initial screening experiments. These were performed to determine the appropriate levels for the influencing factors which were subsequently investigated during the DOE phase. Each culture received a single high frequency ultrasound treatment for 5 min at an intensity of 30%. This was administered at various times for each individual culture during their growth phase, ensuring mild treatment and minimizing cell damage. Untreated control cultures were cultivated in parallel to establish the benchmark biomass growth and composition. The

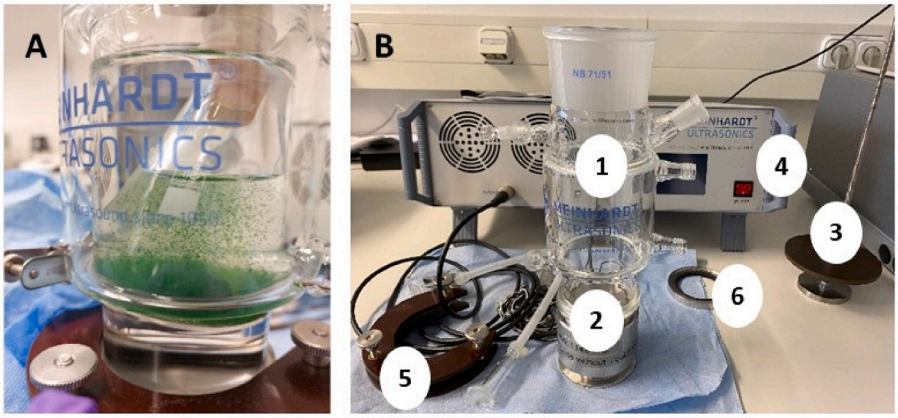


**Fig. 1.** Ultrasound treatment setup. A: Closeup of the *A. platensis* being treated in the sonication vessel. B: 1) glass reactor; 2) transducer; 3) metal reflector cap; 4) wave generator; 5) attachment piece and connection cables; 6) sealing ring.

screening phase focused on two key parameters: ultrasound frequency (578 kHz, 865 kHz, 1148 kHz) and specific points during their growth phase (on the 4th, 7th and 10th day). It is important to note that there is currently a lack of systematic studies on the effects of high-frequency ultrasound on the growth and biomass composition of microalgae. The primary objective of this study is to investigate the impact of high-frequency ultrasound on the growth and composition of *Arthrospira platensis*. To achieve this, we utilized three specific frequencies: 578 kHz, 865 kHz, and 1148 kHz. These provide a sufficient range under relevant frequencies to be able to detect statistically significant changes throughout the exposure to these treatments. In future work, we plan to expand our investigation to include a broader range of frequencies and additional micro-organisms.

Optical density (OD) measurements performed every two days monitored biomass growth and response to treatments throughout cultivation. This systematic approach provided a comprehensive evaluation of the impact of the sonication treatments on *Spirulina* growth under varied conditions. After completing the cultivation phase, the biomass was freeze-dried and stored at −20 °C for subsequent analysis. The screening was done in triplicate and the day-night cycle, light intensity and temperature did not change during the cultivation period.

#### 2.2.3. Design of experiment (DOE) optimization

During the previous screening phase, parameters like intensity, duration, and culture time were systematically explored to identify conditions that produced the most significant responses. Based on these findings, the subsequent optimization phase examined these parameters over a broader range for a more comprehensive analysis. Acoustic treatment frequencies were chosen as described earlier, with a power range of 25–75% to stimulate cellular responses without causing damage (Sivaramakrishnan and Incharoensakdi, 2019). Treatment durations of 5–15 min minimized stress while allowing metabolic changes (Liu et al., 2022; Vintila et al., 2023).

A multifactorial design was then used to identify optimal conditions for modifying *A. platensis* biomass composition without harming cell viability. A DOE optimization strategy was carried out using Design-Expert 13 (StatEase Inc., USA) software. The impact of ultrasound on protein, lipid and carbohydrate production, as well as their interactions have been investigated through a Central Composite multifactorial design (CCD) coupled to a Response Surface methodology (RSM). This can be further used to predict the region of maximum response for the investigated responses under different process constrains. A total of 27 experimental runs were generated and performed in order to fit a quadratic CCD-RSM. The DOE optimization focused on four important parameters such as sonication frequency (from 578 to 1148 kHz), intensity (from 25% to 75%) and administration time (from 5 to 15 min), as well as the specific stage throughout the culture growth (from the 4th to the 12th day) (Table 1). Each of these were investigated on three levels, coded as −1, 0 and + 1. The process responses were related to biomass growth rates and cell disruption, as well as biomass composition, including total proteins, lipids and carbohydrates. The distinct experiments with different treatment conditions were performed in individual shake flasks and kept in the same incubator as described above. Samples were collected throughout the experimental period (each condition done in triplicate) and at the end of the cultivation run (day 14th), freeze-dried, and stored at −20 °C for subsequent analysis.

### 2.3. Analytical methods

#### 2.3.1. Biomass growth determination

Biomass growth was assessed throughout the cultivation period by measuring the optical density (OD) at 670 nm every two days. This provided a quantitative assessment of the culture's density. The recorded OD values served as indicators for evaluating the growth performance of the microorganisms over the course of the experiment. In addition, dry weight measurements were performed regularly. In brief, to measure the dry weight of microalgae, a known volume of the culture was harvested via centrifugation and rinsed with distilled water to remove impurities and centrifuged again. The biomass pellet was then freeze-dried to remove water content while preserving heat-sensitive compounds. After freeze drying, the biomass was weighed to determine its dry weight. Sterile conditions were maintained throughout, and replicates were conducted for accuracy. Growth rate (day $^{-1}$) is calculated by:

Growth rate ($\mu$)= (ln ($OD_t$)−ln ($OD_0$))/$t$ (1), where $OD_t$ is the biomass $OD$ at time t, $OD_0$ is initial biomass $OD$, and $t$ is cultivation time (day).

#### 2.3.2. Total carbohydrates

Total carbohydrates were quantified using the Dubois method. In brief, this method relies on hydrolyzing polysaccharides into monosaccharides which generate an orange-yellow color change when subsequently exposed to phenol and concentrated sulfuric acid (Sadasivam and Manickam, 1996). For each sample, 1 mg of finely ground dried biomass is prepared in duplicate and placed into small bead beating tubes. Subsequently, 1 mL of MilliQ water is added to each tube. The tubes undergo three cycles of bead beating using a Bertin Technologies system, each consisting of three 60-s runs with a 120-s pause in between, at 5000 rpm. Following this mechanical disruption of the cells, the tubes are centrifuged at 1200 g for 10 min. After centrifugation, 50 µl of the supernatant is carefully extracted from each tube and transferred to fresh glass tubes. An additional 450 µl of MilliQ water is added to these tubes, resulting in a total volume of 500 µl for all samples, controls, and

**Table 1**
The influencing factors and their levels investigated in the developed quadratic CCD-RSM.

| | Name | Units | Type | Subtype | Minimum | Maximum | Low level | Medium level | High level |
|---|---|---|---|---|---|---|---|---|---|
| A | Frequency | kHz | Numeric | Continuous | 578.00 | 1148.00 | 578.00 | 863.00 | 1148.00 |
| B | Treatment stage | Day | Numeric | Continuous | 4.00 | 12.00 | 4.00 | 8.00 | 12.00 |
| C | Treatment duration | Minutes | Numeric | Continuous | 5.00 | 15.00 | 5.00 | 10.00 | 15.00 |
| D | Treatment intensity | % | Numeric | Continuous | 25.00 | 75.00 | 25.00 | 50.00 | 75.00 |

glucose calibration samples. Glucose calibration samples using increased concentrations are prepared using a glucose stock solution with a concentration of 1 g/L in MilliQ water.

After adding 500 μL of a 5% phenol solution, 2.5 mL of concentrated sulfuric acid is introduced directly onto the liquid's surface. The mixture is then allowed to incubate at room temperature for 10 min. Afterward, the samples are transferred to a 35 °C water bath and maintained for 30 min, ensuring thorough mixing by vortexing every 5 min during this incubation period. Finally, the absorbance is measured at a wavelength of 483 nm.

### 2.3.3. Total lipids

The lipid extraction and analysis are based on the Folch method (Breuer et al., 2013). In brief, 5–10 mg of finely ground dried biomass was placed in individual bead beater tubes. In each of these tubes, 1 mL of a 1:2 (v/v) $CHCl_3$: MeOH solution was added. For the extraction phase, the bead beater tubes undergo three cycles of bead beating, with each cycle consisting of three 60-s runs and a 120-s pause in between at 5000 rpm. The subsequent step involves transferring the solution, along with the glass beads from the bead beater tubes to fresh heat-resistant 10 mL glass tubes. To these glass tubes, 2.75 mL of a 1:2 (v/v) $CHCl_3$: MeOH solution was added. Additionally, 1.25 mL of a 20 mM $KH_2PO_4$ solution (pH 7.0) is included, and thorough mixing is ensured through vortexing. Next, 1.25 mL of $CHCl_3$ is added, and the mixture is vortexed once more. The samples are then subjected to centrifugation at 1000 RPM in a tabletop centrifuge for 5 min at room temperature, resulting in a two-phase system with an aqueous top layer and an organic bottom layer. The organic bottom phase is carefully harvested and placed inside pre-weighed tubes, which are left to dry under a flow of $N_2$ overnight. Finally, the weight of the tubes is measured after drying overnight.

### 2.3.4. Total soluble proteins

The Lowry Bio-Rad DC protein assay was employed to determine the concentration of total soluble proteins in the different cultures. The assay is based on the protein's reaction with an alkaline copper tartrate solution and Folin reagent. The color development involves two steps: the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein (LOWRY et al., 1951). Color development primarily results from the amino acids tyrosine and tryptophan, and to a lesser extent, cysteine, cysteine, and histidine producing one or more reduced species with a characteristic blue color, having maximum absorbance at 750 nm and minimum absorbance at 405 nm (Krohn, 2005; LOWRY et al., 1951; Peterson, 1979).

1 mg of ground dry biomass was mixed with 1 mL of 0.4M NaOH and incubated for 30 min at 100 °C using a heating block. After centrifugation for 10 min at 3500 rpm the supernatant was diluted with 0.4M NaOH to achieve a total protein concentration of around 1.4 mg/mL. A BSA standard of 2 mg/mL was prepared in 0.4M NaOH and a BSA concentration range between x and y was made. Subsequently, 10 μL of BSA standard as well as the microalgal extracts were loaded into a 96-well flat-bottom transparent Greiner Bio-one microplate. Reagent S (Bio-Rad Dc protein assay kit) was mixed with Reagent A (Bio-Rad Dc protein assay kit) at a ratio of 20:1 to create Reagent A', which in turn was added to each sample, together with 200 μL of Reagent B (Bio-Rad Dc protein assay kit). The plate was covered and incubated for 30 min at room temperature before measuring the absorbance at 750 nm using the Tecan M200 Plate Reader.

### 2.3.5. Cell disruption

Flow cytometry (BD Accuri C6 Plus) was utilized to determine the cell disruption efficiency throughout the different treatment conditions. The recording volume for each sample was set at 35 μl, resulting in a variable number of recorded events due to differences in the number of cells present in the same volume of different samples (Figueiredo et al., 2022). To evaluate cell disruption, a 2D dot graph was utilized, specifically SSC (side scatter) versus FSC (forward scatter), employing a logarithmic approach for the height. Gating in the control sample the main cluster of intact cells allowed the determination of the percentage of cell disruption. This was achieved by comparing the events within the gated region with the events outside it and closer to the origin point. The latter ones representing the generated cell debris (Günerken et al., 2017). The gating of intact cells was established using untreated control samples. The distinction between smaller cell debris and larger intact cells is revealed as a factor of SSC and FSC. In the samples where cell disruption has occurred, as confirmed by microscopic observations as well, a population of events exhibiting lower SSC intensities was detected, representing the formation of cell debris. A simultaneous decrease in number of events withing the intact cells gate was observed in these samples. Cell disruption efficiency is calculated by Eq. (2):

$$\text{Cell disruption is} = (\text{Number of total events} - \text{number of live cells})/\text{Number of total Events} \quad (2)$$

It is worth noting that, in flow cytometry, "total events" refers to the total number of individual particles detected and recorded by the flow cytometer during an experiment, with each event corresponding to a single particle analyzed for its characteristics.

## 3. Result and discussion

The impact of high-frequency acoustic waves on the biomass composition of *Arthrospira platensis* was investigated. Preliminary screening experiments were conducted to identify the influence of acoustic frequency and timing of treatment during the culture growth, on both the cell proliferation and protein accumulation. Subsequently, a CCD-RSM statistical DOE was developed and implemented to study the influence of four key parameters such as acoustic frequency, intensity, and duration, as well as treatment stage, on the biomass growth and composition. This revealed both the direct impact of these individual variables as well as their second level interactions. This, in turn, provided an understanding of this process, allowing predictions on what specific conditions are required to precisely manipulate the biomass composition of *Spirulina* using solely high frequency acoustic forces.

### 3.1. Impact of acoustic treatment strategies on protein production

*A. platensis* is well known for the high concentration of proteins. Thus, emphasis was first directed on the biomass production and accumulation of total proteins through higher frequency acoustic stimulation. Therefore, the biomass was subjected to three different acoustic frequencies (578 kHz, 865 kHz and 1148 kHz) on three different time points during the growth stage (day 4, day 7 and day 10). The impact of these treatment strategies on biomass and protein production was monitored throughout the growth stages of this microorganism.

In general, all treatments follow a similar OD pattern to the one observed in the untreated cultures (Fig. 2). This suggests minimal cell disruption. However, when the acoustic treatments were performed on 4-day old cultures, a slight decrease in the biomass production was observed for all the investigated frequencies. In contrast, latter treatments such as the ones performed on the 7th and 10th day of cultivation, show a relatively consistent growth rate, comparable to the untreated cultures. Only when applying 578 kHz frequency to 7-day old cultures resulted in a slight decrease in biomass production. Regarding the total protein production, there seems to be a connection between their accumulation and acoustic treatment frequency as well as with the timing of the application, i.e. culture age. As the acoustic frequency decreased, the total protein concentration increased in all treatment stages, reaching around 70% at 578 kHz when applied on the 4th and 10th day of cultivation, approximately 20% more than the untreated cultures (Fig. 3). Regarding the application stage, lower general protein concentrations were observed when the acoustic treatments were performed on the 7th day of cultivation, with at least a 15% decrease in total protein content as compared to the untreated cultures.

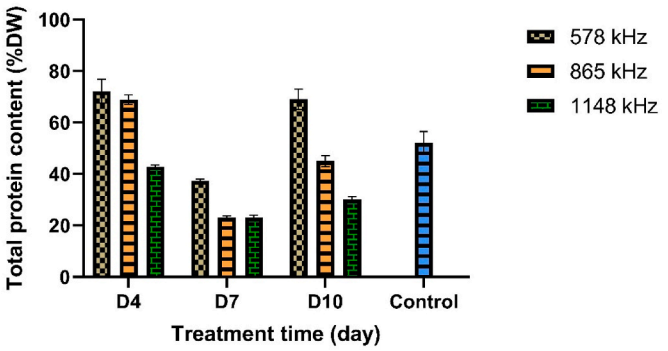


**Fig. 3.** Impact of acoustic treatment strategies on the total protein content of *Arthrospira platensis*. The acoustic treatments were performed on day 4 (D4), day 7 (D7) and day 10 (D10). The untreated control is shown for comparison. In all cases, the treatment was performed for 5 min at 30% intensity and three different frequencies. The measurements were performed for all samples at the end of cultivation period, on day 14.

While the combination of duration and frequencies used is generally

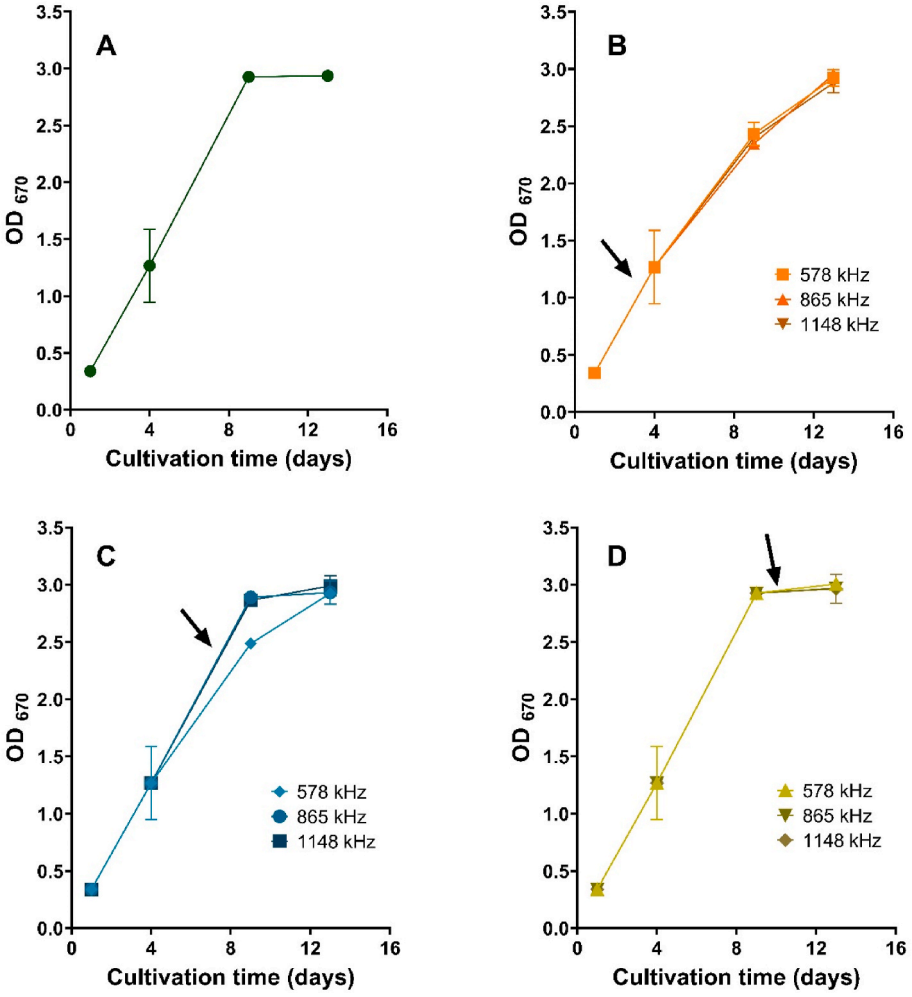


**Fig. 2.** Growth curves of *A. platensis* throughout the acoustic treatment strategies. A: control (no acoustic treatment); B: acoustic treatment on day 4; C: acoustic treatment on day 7; D: acoustic treatment on day 10. In all cases, the treatment was performed for 5 min at 30% intensity and three different frequencies. Black arrows mark the treatment points.

non-disruptive for the *A. platensis* cells, the critical factor lies in the specific day of cultivation when this treatment is administered. Analysis of the results during the final stages of cultivation (day 10) reveals that applying the sonication treatments at this later stage does not significantly alter the biomass production. When younger cultures are however subjected to acoustic treatment (day 7), particularly at the lower frequency of 578 kHz, and the early stages of cultivation (day 4) on all frequencies tested, show a slightly lower growth rate compared to the untreated cultures. The literature indicates a common use of low-frequency ultrasound (20–40 kHz) in microalgae inactivation and higher frequencies (1–5 MHz) in biomass pre-treatment (Pereira et al., 2023). Research indicates that low-frequency ultrasound produces shock waves and cavitation bubbles that rupture cell membranes, effectively inactivating microalgae such as *Microcystis aeruginosa* (Liu et al., 2022; Tan et al., 2018). In contrast, higher ultrasound frequencies are more effective for extracting valuable compounds, as they promote the release of bioactive substances without causing extensive cell damage (Ferreira et al., 2016; Huang et al., 2021). Cell disruptions in microalgae growth are influenced by ultrasound parameters, including frequency, duration, and cell wall characteristics. Ultrasound frequency and duration impact cell disruption by regulating cavitation intensity and the length of exposure, with lower frequencies and longer durations generally leading to greater damage (Liu et al., 2022; Rahman et al., 2022). The characteristics of the cell wall also play a role, as thicker and more rigid walls offer more resistance to ultrasonic forces, necessitating more energy to break them down (Rahman et al., 2022). Some studies demonstrated however that early applications of lower power acoustic fields were more effective for increasing biomass production, while treatments during the stationary phase impacted the production of various secondary metabolites and biomass production (Han et al., 2016a; Singh et al., 2019). Although the exact mechanisms are not fully understood, it is believed that these sound waves might affect cellular processes or nutrient absorption. Consequently, more research is needed to clarify how ultrasound influences different growth phases and to support these findings.

The current screening experiments, however, showed an inverse trend in terms of biomass and protein production. Increasing the frequency led to an overall decreased protein content (Fig. 3). Applying ultrasound in the early stages triggers more protein production and thus appears more effective than in latter stages. The outlier is the treatment of the 10-day old cultures at 578 kHz frequency which resulted in protein production levels similar with the ones observed in the case of the 4-day old cultures treated at the two lowest frequencies. The differences between the current screening experiments and some of the previously reported literature could be explained by the fact that the frequencies investigated are significantly higher. Lower frequencies of around 30 kHz–80 kHz generate harsher phenomena such as acoustic cavitation, heat and free radicals, while higher frequencies do not trigger these phenomena to the same extent but are hypothesized to align with the resonance frequency of vacuoles or even whole-cell resonance (Ahn et al., 2003; Rajasekhar et al., 2012; Tang et al., 2004). Thus, the employed frequencies affect these cells differently and could explain the differences observed both in biomass as well as protein production. Moreover, the interactions among these forces and the cells are influenced by the characteristics of these organisms such as cell wall structure and biomass composition. *A. platensis* does not have a cell wall as rigid as some of the investigated microalgal species (Martínez-Sanz et al., 2020). The treatment needs to be harsh enough to trigger stress and subsequently protein production response but not too harsh to dramatically impact their growth and cell disruption. Thus, there is a specific range in which the higher frequency acoustic treatment is the most efficient in promoting the accumulation of certain compounds without leading to significant biomass production losses.

In summary, the effect of acoustic treatments on growth rate and total protein content varies based on the specific day of cultivation and chosen frequency. Applying ultrasound in the early stage adversely affects cell growth but positively influences total protein content, especially at the lowest frequencies. These findings suggest the existence of interactions among these variables as well as an optimum where maximum protein production can be achieved without negatively impacting the overall growth rates. The mechanisms of action could be related to DNA replication, mRNA formation, and consequently, higher protein production, as demonstrated by (Jeamton et al., 2008) who investigated temperature and other stress responses in *Spirulina platensis*. These mechanisms—DNA replication, mRNA formation, and protein synthesis—play a crucial role in achieving maximum protein production without compromising overall growth rates. Efficient DNA replication ensures that genetic material is accurately passed on during cell division, while enhanced transcription of mRNA allows for increased availability of templates for protein synthesis. Additionally, optimized translation processes enable rapid production of proteins, facilitating cellular functions. By manipulating acoustic treatment conditions, particularly the frequency and timing of ultrasound application, it is possible to strike a balance that maximizes protein output while supporting healthy growth rates in cultures such as *Spirulina platensis*. Thus, further investigations are required to explore the impact of acoustic frequency, intensity, treatment time and growth stage application on biomass production and overall composition.

### 3.2. Impact of acoustic treatment on total biomass composition

Complex systems are often influenced by multiple variables as well as their interactions. In order to identify these effects, a statistical CCD-RSM was developed and implemented (Table 2). A randomized quadratic model was developed containing 27 experimental runs. This included several variables related to the acoustic field properties, such as frequency and intensity, as well as the timing of the treatment application throughout the growth stages of *A. platensis*. To thoroughly characterize the impact of these influencing factors, the main biomass components such as proteins, lipids carbohydrates and pigments were analyzed as process responses. Moreover, biomass production and cell disruption were investigated.

All the analyzed responses showed statistically significant quadratic model terms and not significant lack of fit (Table 2). No significant changes in the overall trends were observed when the models were reduced to decrease their p-values. Thus, even values slightly above a sequential p-value of 0.05 were still considered significant. This suggests the resolution of the developed model and the data fitting are sufficient to determine not only the direct effect of the investigated individual factors on the chosen responses, but also their first level interactions and squared effects. For instance, a strong interaction has been determined among the treatment intensity and stage in the case of protein accumulation in *A. platensis* biomass (Fig. 4, Panel A). When applied in latter culture stages, the different intensities of the acoustic treatment result in similar protein production. When, however, this is performed on younger cultures, a lower treatment intensity resulted in higher protein concentrations. On the other hand, when various acoustic treatment times were performed on younger cultures, similar lipid concentrations were detected in the *A. platensis* biomass, while higher concentrations were observed under shorter treatment times when stressing older

**Table 2**
Analysis of variance (ANOVA) test to determine the statistical significance of the model and lack of fit for each of the analyzed responses. Significant terms are marked with one asterisk and not significant ones with two asterisks.

| | Model | Sequential p-value | Lack of fit p-value |
|---|---|---|---|
| Protein (%DW) | Quadratic | 0.0652* | 0.8376** |
| Total lipids (%DW) | Quadratic | 0.0491* | 0.0879** |
| Carbohydrates (%DW) | Quadratic | 0.0670* | 0.1529** |
| Biomass growth rate (μ) | Quadratic | 0.0720* | 0.9655** |
| Cell disruption (%) | Quadratic | 0.0445* | 0.9466** |

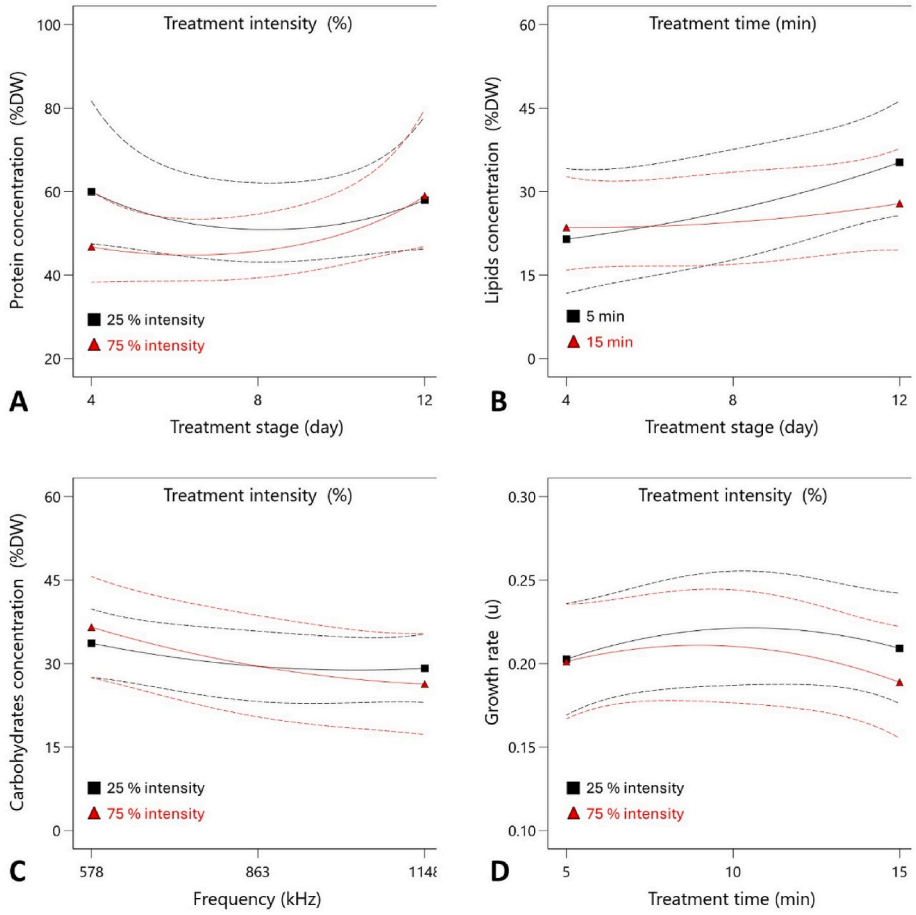


**Fig. 4.** Two-factor interactions and their effect on the biomass composition and production of *A. platensis*. A. Protein concentration (% DW) impacted by treatment intensity (%) and treatment stage (day); B. lipid concentration (% DW) impacted by treatment time (min) and treatment stage (day); C. carbohydrate concentration (% DW) impacted by treatment intensity (%) and frequency (kHz); D. Growth rate (μ, [$day^{-1}$]) impacted by treatment intensity (%) and treatment time (min). The dotted lines represent the 95% confidence intervals.

cultures (Fig. 4, Panel B). With regards to the carbohydrate production by *A. platensis*, slightly lower concentrations were observed when using a lower intensity at the lowest investigated frequency, while the opposite was observed at the highest frequency (Fig. 4, Panel C). Finally, the different treatment intensities seem to have no impact on the cell growth rates under short treatment times. However, when extending this time, lower intensities lead to overall higher growth rates (Fig. 4, Panel D).

Increasing the acoustic treatment time as well as the culture stage in which it is applied led to measured protein concentrations of up to 52% DW. However, the predictions suggest a potential increase to 63% DW of the total biomass content (Fig. 5, Panel A). Moreover, higher acoustic treatment intensities combined with lower treatment times resulted in measured lipid concentrations of up to 44% DW (Fig. 5, Panel B). Similar observations have been made when analyzing the carbohydrate concentrations in *A. platensis*, where higher treatment intensities triggered higher carbohydrate production when combined with lower acoustic frequencies (Fig. 5, Panel C). Measured values reached a maximum carbohydrate concentration of 33% DW. The biomass growth rates were significantly influenced especially by the acoustic treatment intensity and time, with the highest observed rates being at around their mid-levels (Fig. 5, Panel D). A minor cell disruption effect of the acoustic treatments has been observed in some of the cases, with the highest percentages being identified when the cells were stressed in the earlier culture stages and lower acoustic frequencies (Fig. 5, Panel E). When comparing these results with the untreated biomass, the concentrations of proteins, lipids and carbohydrates in treated cultures was significantly higher (Fig. 5, Panel F).

Strong interactions were identified among some of the investigated variables and the observed responses (Fig. 4). The biomass composition varied significantly based on the combination of acoustic treatment conditions (frequency, intensity, and duration), as well as the exact exposure stage during the growth phase of *A. platensis*. Interestingly enough however, various combinations of these treatment conditions trigger different responses in the biomass composition. For instance, lower treatment harshness during the earlier *A. platensis* growth stage (day 4) results in higher protein concentrations, while similar treatment conditions, especially later in the growth stage (day 8 and 12) triggered higher accumulation of lipids. Moreover, lower acoustic frequencies require slightly higher treatment intensities in order to generate the highest carbohydrate concentration within these cultures, while at the highest employed frequencies the opposite was observed. These observations, together with the determined interactions regarding the growth rate, confirm our hypothesis that these cells require just enough exposure to these high frequency treatments to trigger their stress response, but not sufficient to destroy their structural integrity. Previous studies observed microorganism adaptive responses to environmental stressors (Ahn et al., 2003). Studies on ultrasonic radiation effects on Microcystis aeruginosa UTEX 2388 showed growth inhibition and minimal lipid peroxidation, indicating its potential for controlling cyanobacterial blooms (Rajasekhar et al., 2012). Similarly, laboratory-scale sonication experiments displayed immediate population reduction and varied impacts on microcystin release, suggesting sonication's promise in managing cyanobacteria (Tang et al., 2004).

The RSM provides further insights into these phenomena. For

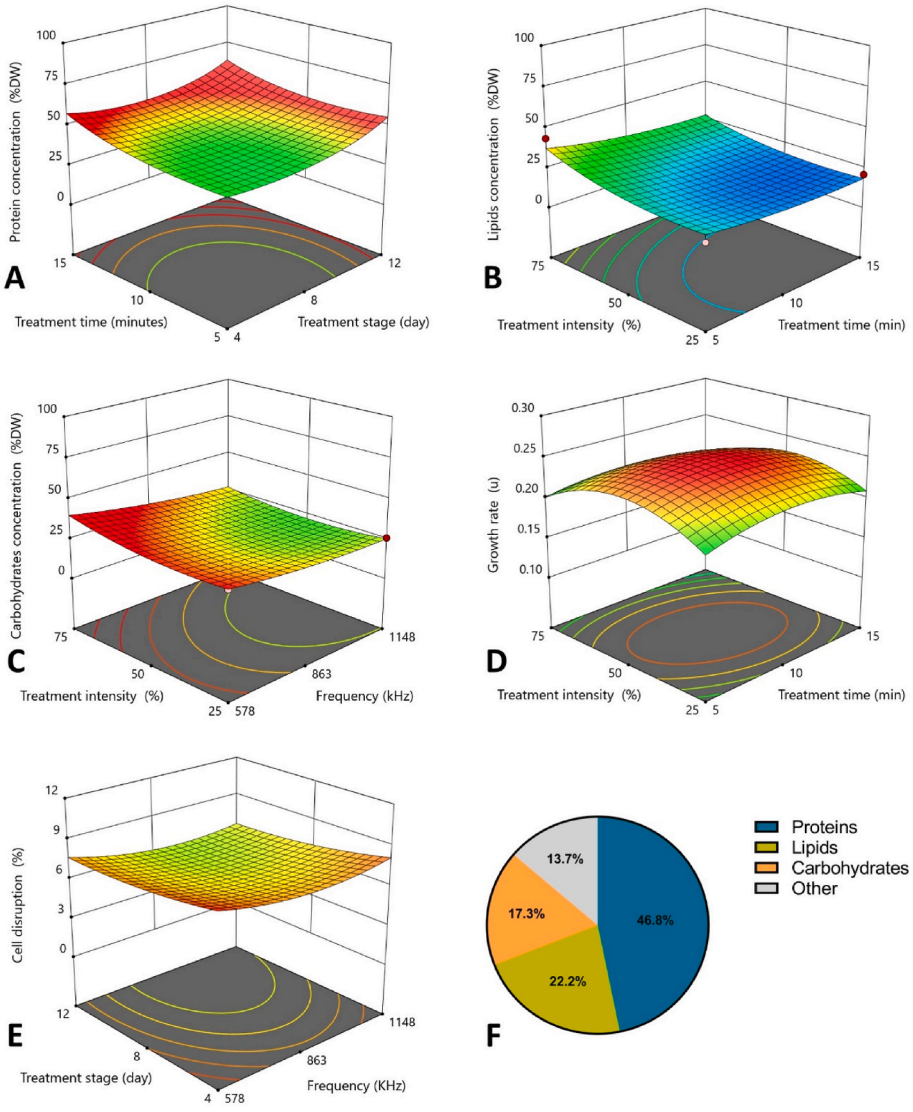


**Fig. 5.** Impact of various acoustic treatment strategies on the biomass composition and production of *A. platensis*. A. Protein concentration (% DW) impacted by treatment time (min) and treatment stage (day); B. lipid concentration (% DW) impacted by treatment intensity (%) and treatment stage (day); C. carbohydrate concentration (% DW) impacted by treatment time (min) and frequency (kHz); D. Growth rate (μ) impacted by treatment intensity (%) and treatment time (min).); E. Cell disruption (%) impacted by treatment stage (day) and frequency (kHz). ; F. Biomass composition of untreated *Arthrospira platensis*: proteins 46.8%, lipids 22.2 %, carbohydrates 17.3 %, other 13.7 %.

instance, looking at the impact of other influencing factors on biomass composition and cell proliferation, the regions of highest and lowest responses were identified. The highest protein, lipid and carbohydrate accumulations within the *A. platensis* biomass were observed at different combinations of all the investigated influencing factors. This further confirms the importance of these variables on biomass composition, with some of them being more relevant for certain compounds, than other. For instance, while protein production in *A. platensis* is greatly influenced by the acoustic treatment time and stage, the lipid accumulation is mostly impacted by the treatment time and intensity, and carbohydrate production by the intensity and frequency of the acoustic treatment.

Ultrasound treatment significantly impacts microalgae growth, cell composition, and membrane permeability, affecting nutrient uptake and metabolite production. However, microorganisms respond differently to these stimuli (Pereira et al., 2023). For instance, *Dunaliella salina* and *Chlamydomonas concordia* experienced a decrease in biomass productivities, while *Nannochloropsis oculata* exhibited increased cell concentration when subjected to 20 kHz sonication for 16 min (Joyce et al., 2014). Greenly and Tester observed 50% cell viability loss in *Chlamydomonas reinhardtii* after 2 s and *Nannochloropsis* sp. after 1 min of ultrasound treatment at frequencies between 20 and 30 kHz, suggesting response differences tied to cell wall structure and size (Greenly and Tester, 2015). In the present study, a reduced treatment intensity seems to promote to a certain extent cell proliferation by providing just enough mechanical stimuli. When treating these cultures earlier during their growth stage however, slight cell disruption phenomena were observed, albeit to a relatively low extent. This could be explained by the more accelerated cell division taking place during the earlier culture stages, making them more vulnerable to mechanical shear forces. Some previous studies showed the significance of timing in ultrasonic treatment. For instance, exposing *Microcystis aeruginosa* UTEX 2388 to acoustic radiation towards the end of the light period, coinciding with increased cell division activity, has been observed to be more effective in reducing their growth rate (Ahn et al., 2003). Low-intensity and low-frequency ultrasound can also induce stress responses that enhance accumulation of certain compounds and biomass productivity (Han et al., 2016b). However, at increased intensities and treatment durations, the opposite

can be observed (Vintila et al., 2023). These findings further support the need to explore higher frequency acoustic radiation to specifically alter cell composition without negatively impacting their growth rates.

Subjecting *A. platensis* to high frequency acoustic treatments triggered different responses in the biomass composition and proliferation. Thus, this could provide a new tool to precisely tune the ratio between proteins, lipids, and carbohydrates, as well as achieve significantly higher concentrations of these compounds as compared to untreated cultures. Moreover, the cell proliferation rate can be increased as well to a certain extent. These new insights can be used to make predictions on what are the specific acoustic treatment conditions to maximize or minimize the production of certain compounds as well as the whole biomass.

### 3.3. Developing and optimizing an acoustic multiproduct process

Manipulating the biomass composition and production of *A. platensis* through high frequency acoustic stress is strongly influenced by each of the process variables as well as their interactions. It is thus imperative to identify the optimal combination of these variables and their levels for obtaining the desired responses. Thus, various optimization scenarios have been developed to maximize outputs such as the concentration of main biomass compounds like proteins, lipids and carbohydrates, as well as their growth rate while at the same time minimizing cell death. Moreover, the inclusion of certain process restrictions such as reducing treatment time and intensity was investigated to further optimize the overall approach and increase feasibility.

The first optimization scenario focused on simultaneously maximizing *A. platensis* biomass production as well as the concentration of its main compounds. Among the different solutions identified, the most promising one consisted of utilizing lower frequencies in combination with medium treatment times and higher intensities to treat late-stage *A. platensis* cultures (Fig. 6). This resulted in predictions of a biomass consisting mostly of proteins (52%), followed by lipids and carbohydrates both at concentrations of around 27%. These concentrations are slightly overestimated. Nevertheless, it identifies the optimal conditions required to simultaneously maximize these responses. Moreover, these conditions kept the cell disruption at below 5% and a medium cell growth rate. In the second optimization scenario additional restrictions were included, such as reducing the acoustic treatment time and intensity. These represent some of the most energy-intensive steps of this treatment. In this scenario, the predicted protein concentrations were kept at 52% (Fig. 7). However, the model predicts slightly lower lipids and carbohydrates concentrations, at 23% and 20% respectively. Both the growth rates and cell death are predicted to slightly increase in comparison with the first optimization scenario.

Both optimization scenarios predicted as optimal similar acoustic frequencies and culture treatment stage. Once the restrictions were set in place when developing the second optimization scenario, the acoustic intensity and treatment time were significantly decreased. In both scenarios, however, the protein concentration in *A. platensis* was similar. This is because the highest weight of the optimization parameters was put on proteins, followed by lipids and carbohydrates. These optimization scenarios, however, predict values for the main biomass components below the highest levels measured throughout the experiments. This is because these optimized conditions targeted the increase of several responses simultaneously. Moreover, the second scenario included additional restrictions. This resulted in compromised solutions with desirability levels below 1. However, this strategy is in line with developing multiproduct processes focused on exploiting most of the biomass components, while also enabling high biomass production rates. The restrictions were put in place to further bring down the treatment costs by reducing some of the most energy-intensive steps such as the time and intensity of the biomass exposure to the acoustic field. The economic feasibility and scalability of the prototype bioreactor used for high-frequency acoustic treatments in cultivating *Arthrospira platensis* are critical for practical applications. Currently, several companies are developing and even commercializing production-scale acoustic treatment systems for various applications ranging from acoustic mixing and extraction (e.g. Industrial Sono-Mechanics®, Hielscher Ultrasonics and Resodyn™), to antifouling systems designed for shipping and pipes (Sonihull). The controlled growth conditions, including a two-week preculture and optimal shaking and temperature, support significant increases in valuable bioactive compounds, with enhancements of up to 96% for lipids and 88% for carbohydrates. Although the optimized conditions yield lower values for some components compared to maximum levels observed, they still enhance overall biomass productivity and thus their potential market value. This scalable technology allows for adjustments in treatment parameters suitable for larger-scale applications, promoting uniform growth and efficient nutrient distribution.

The different optimization scenarios developed as a result of the CCD-RSM experimental strategy suggest a potential new biotechnological process able to precisely tune the biomass composition of *A. platensis*

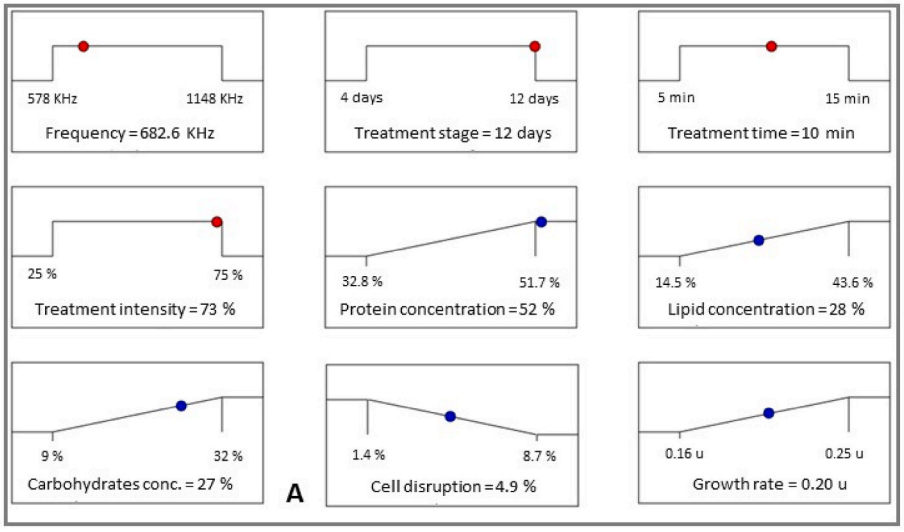


**Fig. 6.** Optimization scenario for maximizing the main target biomass components and growth rate of *Arthrospira platensis* while minimizing cell disruption. The influencing factors have been kept in range, while the 5 responses such as, proteins, lipids, carbohydrates, biomass production and growth rates have been maximized while cell death minimized.

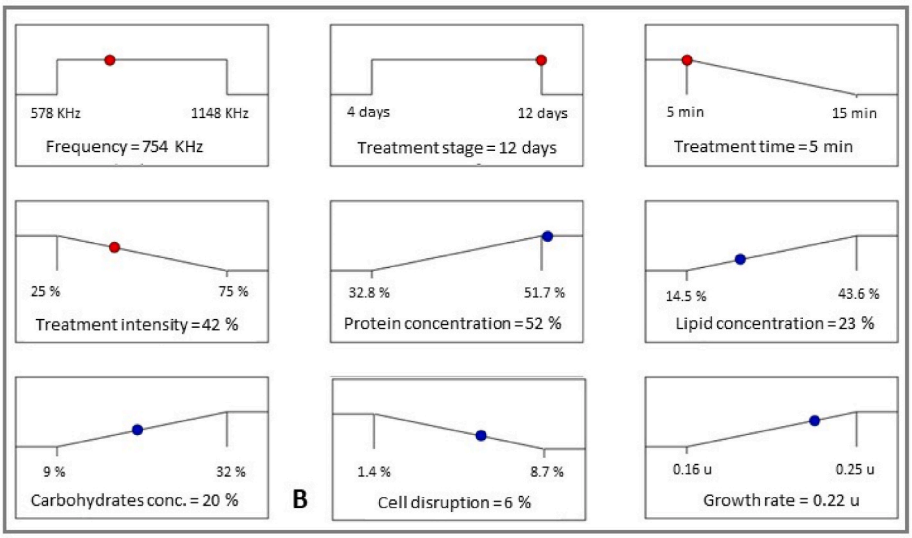


**Fig. 7.** Optimization scenario for maximizing the target main biomass components and growth rate of *Arthrospira platensis* while minimizing cell disruption and restricting some of the most energy-intensive treatment steps. The acoustic frequency and treatment stage have been kept in range, while the acoustic treatment time and intensity have been minimized. All 5 responses such as, proteins, lipids, carbohydrates, biomass production and growth rates have been maximized, while cell death minimized.

by varying only the frequency, intensity, treatment time and culture stage. These novel chemical-free and non-nutrient-limitation strategies have the advantage of being more precise on what specific compound is increased in the biomass and not impact the biomass productivity, but even stimulate it. Even in the scenarios where restrictions have been incorporated, the biomass and compound productions are above the non-treated cultures. Considering the short treatment times (once for 5–10 min within 12 days) and high frequencies, the additional energy requirements should be negligible. These promising results could open the way towards developing more sustainable and feasible acoustic-based multiproduct biotechnological processes.

## 4. Conclusions

The present study explored the manipulation of biomass composition in *A. platensis* by exposing it to short, high-frequency acoustic treatments. The initial part of the study investigated the direct impact of high acoustic frequencies and point of exposure during 14 days of cultivation on biomass and protein production. A subsequent custom multifactorial CCD-RSM approach elucidated the impact of key variables such as acoustic treatment frequency, intensity, duration and application time, on *A. platensis* biomass production and composition. Several optimization strategies were explored, resulting in increased productions of up to 11%, 26% and 56% for proteins, lipids and carbohydrates respectively, when maximizing all these compounds simultaneously. The current study provides a first glimpse into employing acoustic processes for precise manipulation of biomass composition and growth. These, however, require further confirmation experiments under a wider range of frequencies as well as expansion to other types of microorganisms.

## CRediT authorship contribution statement

**Shirin Marsousi:** Writing – original draft, Investigation, Data curation, Conceptualization. **Javad Karimi Sabet:** Writing – original draft, Visualization, Supervision. **Michel H.M. Eppink:** Writing – review & editing, Supervision. **Maria J. Barbosa:** Writing – review & editing, Supervision. **Rene H. Wijffels:** Writing – review & editing, Supervision, Funding acquisition. **Mohammad Ali Moosavian:** Writing – review & editing, Supervision, Funding acquisition. **Iulian Z. Boboescu:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

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## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Iulian Z. Boboescu reports financial support was provided by the University of Tehran, Iran and the Dutch Research Council NWO, The Netherlands. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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