Research article

Effects of Phosphorus Removal and pH Changes in the Culture Medium of *Spirulina* sp. on the Production Rate of Polyhydroxybutyrate

Azita Dorri, Akbar Norastehnia and Masoumeh Abedini

University of Guilan, Faculty of Sciences, Department of Biology, Rasht, Iran

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Abstract

Keywords

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phosphorus deficiency;
polyhydroxybutyrate;
Spirulina

The aim of this study was to investigate the production of polyhydroxybutyrate (PHB) by *Spirulina* sp., a type of cyanobacterium recognized for its capacity to produce this biopolymer. PHB accumulation takes place in *Spirulina* when it undergoes nitrogen and phosphorus limitation, and carbon abundance, acting as a vital reserve material for the microorganism. The study was conducted under autotrophic conditions, phosphorous deficiency, and varying acidity levels to confirm how the interplay between phosphorus deficiency and pH can affect PHB production in *Spirulina* without reducing microalgal biomass. Microalgal cultures in the phosphorus-free treatments at pH 8, 10, and 12 were performed, and their growth quality including levels of photosynthetic pigments, malondialdehyde, anthocyanin, phenol, and flavonoid were measured. PHB was extracted and analyzed using Fourier Transform Infrared (FTIR) spectroscopy for qualitative analysis and gas chromatography (GC) for quantitative evaluation. The amounts of anthocyanins, phenols, and flavonoids, which are important constituents of the antioxidant defense system, were highest in the treatment with a pH of 10, which also had the lowest levels of the stress indicator malondialdehyde. The extracted polyhydroxybutyrate amounts in the control treatment and treatments with phosphorus deficiency at pH levels of 8, 10, and 12 were 6.82%, 3.54%, 7.04%, and 4.23% of cell dry weight, respectively, according to GC. Based on the results, altering the acidity of the *Spirulina* culture medium had a limited effect on increasing in polyhydroxybutyrate accumulation compared to optimal acidity conditions and simultaneous phosphorus removal, which was partly due to the consumption of some polyhydroxybutyrate produced under phosphorus deficiency stress.

*Corresponding author: Tel.: (+98) 131 33333647
E-mail: norasteh@guilan.ac.ir

1. Introduction

Biodegradable polymers are an excellent substitute for petroleum-derived plastics due to their suitable physical properties and biodegradability [1]. In the production of biodegradable polymers, various sources of different biomass, including proteins (from animal and plant sources), lipids, and polysaccharides (such as starch and cellulose), can be used. Using these resources, microorganisms produce biopolymers from the group of polyhydroxyalkanoates (PHAs) [2]. 3-Hydroxybutyric acid (PHB) homopolymer is a pseudo-lipid compound and the first polymer from the PHA family discovered in microorganisms. Among the 150 types of PHAs identified so far, the PHB homopolymer is widespread in different groups of prokaryotes, including cyanobacteria [1]. Like other PHAs, PHB has thermoplastic properties, is biodegradability and optical purity, and possesses good moisture resistance [2].

The accumulation of PHB using CO_2 as a carbon source for many cyanobacterial species such as *Nostoc muscorum* [3], *Oscillatoria okeni* TISTR 8549 [4], *Scytonema geitleri* [5] and *Synechocystis* sp. PCC 6803 [6] has been reported. Cyanobacteria are capable of intracellular accumulation of PHAs (which function as carbon and energy storage sources) under conditions of stress caused by the limitation of essential nutrients, such as nitrogen or phosphorus [7, 8]. It has been suggested that phosphorus depletion could increase the amount of enzymes in the PHA synthesis pathway, which causes an increase in polyhydroxyalkanoate content in cyanobacteria. The content of total ATP is reduced by phosphorus deficiency [9], nevertheless, the production of NADPH continues. Intracellular increase of NADPH elevates the PHA synthesis as it increases the availability of acetyl-CoA for ketothiolase resulting in PHA accumulation [10].

Arthrospira (*Spirulina*) is a filamentous cyanobacterium that can grow in the presence of a suitable carbon source and produces a wide range of products [11]. Due to their metabolic versatility, ease of harvesting, high chemical complexity, and inclusion of proteins [12], carotenoids [13], and essential fatty acids [14], these cyanobacteria can be used as a bioindicator among other types of cyanobacteria.

According to previous research and studies, cyanobacterium *Spirulina* sp. can produce polyhydroxybutyrate (PHB), but the amount of this substance produced is low under normal culture conditions [15]. Several research studies demonstrated that exposing microalgae to thermal stress or nutrient limitation led to an increase in PHA content [16-18]. However, while the importance of pH in promoting microalgal growth and lipid accumulation is well established, its influence on PHA production has not yet been thoroughly studied [19]. This research was aimed to optimize the culture medium for the cyanobacterium *Spirulina* sp., which was isolated from the water reservoirs of Guilan province, by altering the pH and phosphorus content of the autotrophic culture medium. The objective was to increase the production of polyhydroxybutyrate while maintaining the biomass under laboratory conditions.

2. Materials and Methods

2.1 Preparation and cultivation of *Spirulina* sp.

Spirulina sp. isolated from the water reservoirs of Guilan province was obtained from the International Sturgeon Research Institute in Rasht. For purification, the *Spirulina* stock subculture was done first on a solid culture medium [20] and then several times [3, 4] on a liquid culture medium. To remove microbial and fungal contamination, the culture medium was sterilized using an autoclave at a pressure of 1 atmosphere and a temperature of 121°C for 1 h. Preparing the solid and liquid culture medium was done according to the method of Wegmann [21]. This medium was

prepared by aseptic technique, and it was transferred to a sterile test tube next to a flame before cooling, and then the microalgae strain was cultured on it. After cultivation, the test tube lid was closed with foil and parafilm to prevent contamination and evaporation. Then the test tube was transferred to the culture chamber under basic conditions (light intensity of 2500 lux with 16/8 h alternate light/dark cycles at 25±2°C).

2.2 Preparation of treatments

For this study, a control sample was prepared containing all elements and materials of the Zarrouk culture medium, including phosphorus (with a pH of 10), along with three treatments lacking phosphorus at pH levels of 8, 10, and 12. To apply phosphorus deficiency conditions, K_2HPO_4 was removed from the Zarrouk culture medium, and the decrease in potassium content was compensated with additional K_2SO_4 [22]. The pH of the control sample was measured and recorded during the experiment (24 days). The prepared culture medium was sterilized by autoclave and cooled in the laboratory environment. Then, 150 mL of the initial stock solution of *Spirulina* sp. (in the logarithmic phase of growth) was taken and added to 150 mL of culture medium using an aseptic technique (next to the flame). The measured pH values for the control sample were in the optimal range and between 9.5 and 10. During the study, the pH of the culture media was measured once every two days and adjusted by adding 2 N hydrochloric acid (HCl) and 2 M sodium hydroxide (NaOH). The microalgae were harvested after 24 days, using a centrifuge for 20 min at 3000 rpm and a temperature of 4°C. The biomass was placed in the refrigerator to extract and measure polyhydroxybutyrate content and subsequent analyses were performed. Some of the biomass was kept in a freezer at -70°C after shock freezing in liquid nitrogen to measure total protein and malondialdehyde contents. All chemicals in this study were prepared from Sigma-Aldrich described by Eijckelhoff and Dekker [23].

2.3 Measuring the growth of *Spirulina* sp.

To measure the growth of microalgae *Spirulina* sp., two methods were used. Firstly, the density of *Spirulina* sp. measured as the optical density (OD) by a spectrophotometer at the wavelength of 560 nm [24, 25] was determined. Second, dry weight (DW) was measured by the method of Leganés *et al.* [26].

2.4 Measurement of biological factors

Several biological factors were investigated to compare the development status of cultivated *Spirulina* and its effectiveness in different treatments. Furthermore, the intensity of stress created under the influence of these treatments was evaluated with the lipid peroxidation index by measuring malondialdehyde [27].

2.4.1 Measurement of photosynthetic pigments

To determine the concentration of chlorophyll a, chlorophyll b, and beta-carotene, at the end of the experiment (24 days), 3 mL of algal suspension was taken from each treatment, and its photosynthetic pigments were extracted according to the method of Eijckelhoff and Dekker [23]. The absorbance of the extracted solution was read at wavelengths 647, 663, 480, 460, 431, and 412 nm using a spectrophotometer (to prevent the degradation of pigments, the samples were kept in cold and dark). Using the formulas in Table 1, the amount of chlorophyll a, chlorophyll b, and beta-carotene was calculated in terms of micrograms per milliliter. Total chlorophyll was calculated as the sum of chlorophyll a and chlorophyll b.

Table 1. The formulas for calculating the amount of chlorophyll a, b, total chlorophyll, and beta-carotene

Chla	= ((12.25×OD 663 nm)-(2.79× OD 647 nm)) ×Dilution factor
Chlb	= ((21.5× OD 647 nm)-(5.1×OD 663 nm)) ×Dilution factor
T-Chl	= Chla + Chlb
β- Car	= ((-0.43×OD 412+0.251×OD431)-(4.376×OD460+13.12×OD480)×536/1000))× Dilution Factor

2.4.2 Measurement of total anthocyanin content

To measure anthocyanin, first, 0.5 g of the fresh cells of each sample was homogenized in 3 mL of acidic methanol (99:1 of methanol: acetic acid). Then, each extract was centrifuged for 15 min at 10,000 rpm and 4°C. The absorbance of each supernatant was read at a wavelength of 550 nm. The following formula was used to calculate anthocyanin concentration, and it was calculated in terms of μg g^{-1} fresh weight [28]:

$$A = \varepsilon bc \quad (1)$$

A = absorption rate; b = the width of the cuvette, which is equal to 1 cm; c = anthocyanin concentration obtained in terms of micromoles per gram of fresh weight; ε= extinction coefficient 33000 mM^{-1} cm^{-1}

2.4.3 Measurement of phenols and flavonoids

To prepare the extract, 0.1 g of the fresh cell mass (separated from the *Spirulina* cell culture) from each sample was homogenized with 2 mL of 80% methanol (the samples were placed in liquid nitrogen before adding methanol). The samples were then centrifuged at 10,000 rpm for 10 min at 4°C and the resulting supernatant was used to measure total phenols and flavonoids. Total phenols and flavonoids were assayed based on the standard curve equation for gallic acid as described by Slinkard and Singleton [29] and the standard curve equation for quercetin as described by Miliauskas *et al*. [30].

2.4.4 Measurement of lipid peroxidation

To measure the peroxidation of membrane lipids, the concentration of malondialdehyde (MDA) and other aldehydes resulting from this reaction was measured according to the method of Heath and Packer [31]. To calculate the concentration of malondialdehyde, an extinction coefficient equal to 155 mM^{-1} cm^{-1} was used, and finally, the amount of malondialdehyde, which is a product of lipid peroxidation, was calculated in terms of μM g^{-1} of fresh weight.

2.5 Analysis of polyhydroxybutyrate

Control and treated samples were exposed to the treatment conditions for 24 days. The collected samples were lyophilized and then weighed. PHB was prepared and extracted according to Hondo *et al*. [32]. These samples were used for qualitative and quantitative analysis. To confirm the presence of polyhydroxybutyrate, the pellet obtained after extraction was mixed with powdered potassium bromide and crushed into tablets. It was then subjected to Fourier Transform Infrared (FTIR) spectroscopy (Galaxy series 5000, KBr disc), and the corresponding

peaks were analyzed [33]. For isolating and quantitatively determining polyhydroxybutyrate, its methyl ester is typically utilized due to its lower boiling point. Polyhydroxybutyrate methyl ester was synthesized using the method suggested by Leung *et al*. [34]. To methylate the extracted PHB sediment, 7 mL of hexane and 2 mL of 2 M methanolic potassium hydroxide solution were added to a tube with a sandblasted lid and vortexed. Subsequently, the sample was placed in a Bain-Marie at 55°C for 20 min and vortexed for 30 s every 5 min. Following this process, two phases formed, and the upper phase containing the PHB methyl ester was utilized for injection into the gas chromatograph. The gas chromatograph model was Perkin Elmer (Clarus 580) and was manufactured in England. Test conditions include injection temperature at 150°C, carrier gas, nitrogen at 2 mL min^{-1}, and column temperature (CLP type) at 120°C (retention time one min), with increase of temperature at a gradient of 5°C/min up to 280°C and 5 min at 280°C. The detector was of the FID type with a temperature of 200°C. Standard PHB (a polymer with an average molecular weight: M_n 10.000, Sigma-Aldrich) was used as a standard sample.

2.6 Statistical analysis

All the experiments in this research were done in 3 repetitions and the average results of the experiments and the standard errors were calculated. To analyze the data, one-way ANOVA and Duncan's test were used at the probability level of $P<0.05$ with SPSS software (Statistics 17.0), and Excel 2010 software was used to draw graphs.

3. Results and Discussion

3.1 Cell dry weight and density of *Spirulina* sp.

According to Figures (1-a) and (1-b), the microalgae in each of the treatments entered the logarithmic phase after 4-6 days of the lag phase, and growth continued until the 24th day. The highest growth occurred in the control sample and the lowest growth occurred in the treatment with pH 8. Cell dry weight and density of *Spirulina* sp. in samples at pH 10 remained completely equal to the control while they moved away from control in two other treatments at the end of treatment period.

Studies show that PHB produced in *Spirulina* sp. has desirable properties, but its accumulation amount is low [35]. However, the accumulation of PHB can be increased by applying nutrient limitations, pH changes, or other stresses in the culture medium [1]. The limitation of nitrogen and phosphorus in the cell causes the synthesis of storage products, including carbohydrates and hydrocarbons. The ratio of nitrogen to phosphorus in the cultivation environment is considered a determining factor for the growth and production of a group of metabolites [22]. Due to a decrease in phosphorus, cellular components such as nucleic acids are not regularly synthesized, as a result, the biosynthesis of proteins and enzymes are limited and a serious obstacle to the growth of cells is created [36]. Biomass production is also somewhat dependent on the acidity of the environment. Çelekli and Yavuzatmaca [37] showed that by reducing the concentration of phosphorus to below 1 g/L at pH 10.5, the biomass of *Spirulina* sp. was more than the amount of biomass produced under the same conditions but with pH 9.5. Algae have a clear dependence on the pH of the environment and different species react differently to pH [38]. In the present study, it seems that phosphorus deficiency affected the biosynthesis of nucleic acids, proteins, phospholipids, and other essential compounds for growth, and changes in pH also affected the availability of nutrients in the environment. As a result, growth and biomass were affected by both factors, which caused a

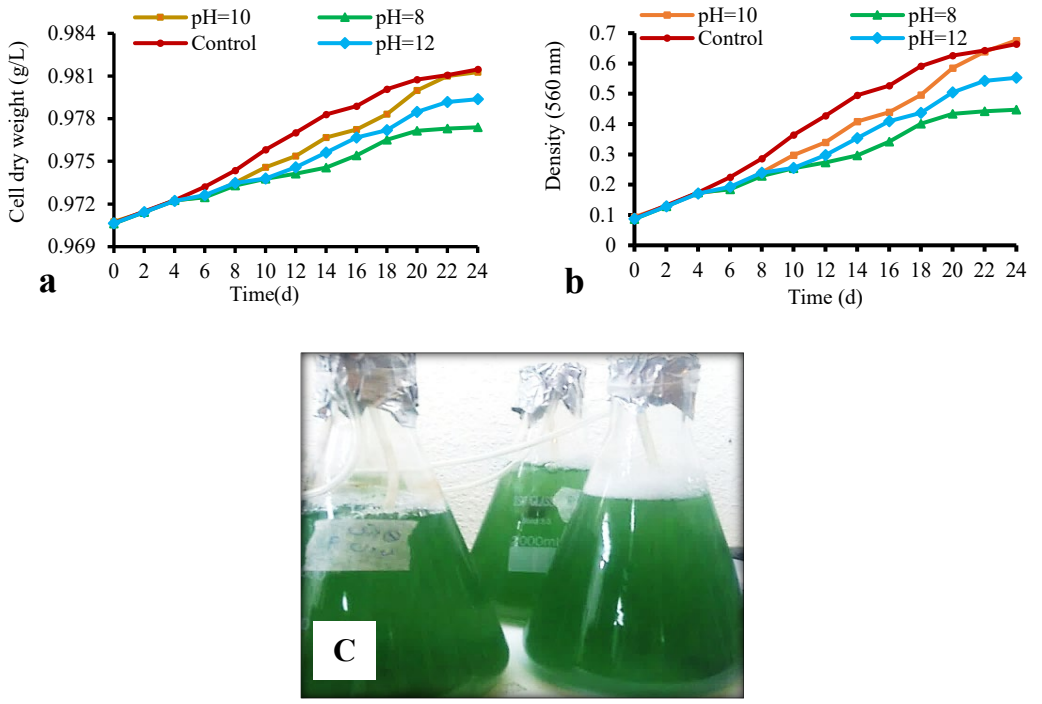

Figure 1. Cell dry weight (a), density (b) and (c) picture of cultivation media of *Spirulina* sp. including control culture medium and phosphorus-free culture medium with pH 8, 10, and 12 in the basic conditions of the culture chamber with a temperature of 25±2°C and a light intensity of 2500 lux with light/dark (12/12) cycle in aeration conditions

decrease in growth and production of biomass at pHs of 8, 10 and 12 compared to the control sample. However, the highest PHB production was obtained under phosphorus deficiency conditions and pH 10 (Figure 4-f). Moreover, high level of PHB production occurred without a decrease in biomass.

3.2 Contents of photosynthetic pigments, anthocyanin, phenol, flavonoid and malondialdehyde

The content of photosynthetic pigments, anthocyanin, phenol, flavonoid, and malondialdehyde in the cyanobacterium *Spirulina* sp. are shown in Figure 2.

3.2.1 Photosynthetic pigments

The levels of chlorophyll a, b, total chlorophyll, and beta-carotene of *Spirulina* sp. are illustrated in Figure 2a. Their amount was higher in the control sample than in the other samples, and it was decreased in the treatments with pH 10, 12, and 8, respectively. Changes in the chlorophylls and carotenoids of cyanobacteria under phosphate restriction conditions have been demonstrated [39-41]. The pH has a significant impact on the amount of photosynthetic pigments, especially chlorophylls a and b, and the optimal acidity for this growth is pH = 9 [42]. It appears that the stress associated with a lack of phosphate and pH fluctuations resulted in the reduction of photosynthetic pigments at pH 10, 12, and 8, respectively. The largest decrease occurred when the microalgae lacked P and at pH 8, from which we can conclude that this treatment caused the most stress.

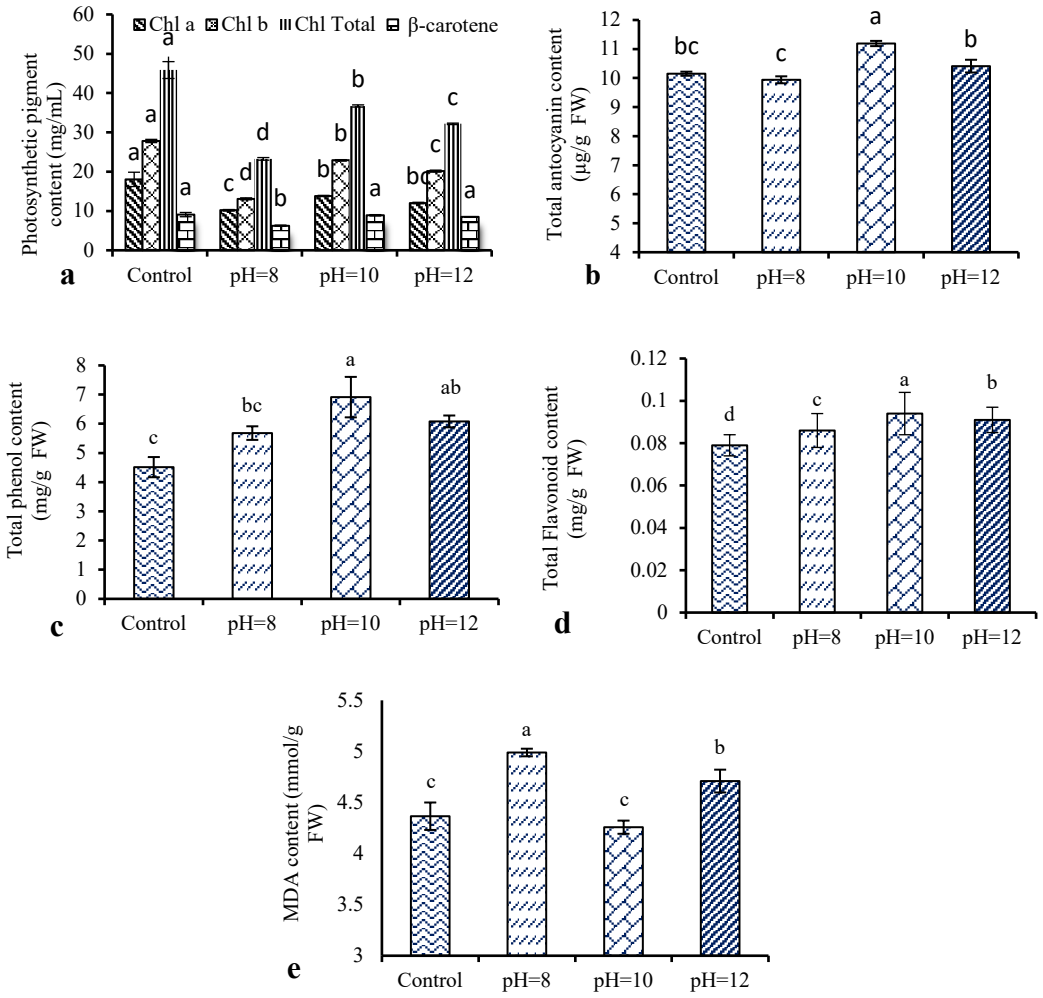

Figure 2. Changes in the content of photosynthetic pigments (a), anthocyanin (b), phenol (c), total flavonoid (d), and malondialdehyde (e) in the cyanobacterium *Spirulina* sp. in the control culture medium and phosphorus-free culture medium with pH 8, 10 and 12. The data are the average of three replicates±standard error (SE). Different letters indicate a significant difference and similar letters indicate no difference between the samples based on the comparison of means using Duncan's test at the probability level of $P<0.05$.

3.2.2 Anthocyanin content

It has been reported that anthocyanin content increases under stress conditions. This increase is due to the photoprotective role of anthocyanins, which directly eliminate reactive oxygen species during oxidative stress [43]. According to Figure 2b, the amount of anthocyanin increased significantly at pH 10. The amount of anthocyanins in the control treatment, and at pH 8 and pH 12 showed no significant difference. When the pH of the solution increases from 6 to 8, the anthocyanin content changes from purple to violet [44] with no alteration in content, thus maintaining a low level of anthocyanin content in control samples. In samples exposed to P-free media, phosphorus deficiency

results in elevated anthocyanin levels [45]. On the other hand, *Spirulina* showed more favorable growth at pH 10 and pH 12, resulting in higher anthocyanin synthesis and lower stress and MDA content compared to the samples with pH 8 and without P. Nevertheless, anthocyanin synthesis was restricted at pH 12 due to the conflict between the effects of P and pH on anthocyanin synthesis. This may likely be because a pH value >8 makes the structure of anthocyanins more unstable and susceptible to degradation [44]. Therefore, it can be concluded that the application of phosphorus deficit stress and changes in pH caused a more effective induction of anthocyanin production at pH 10. This means that the synthesis path of anthocyanin may be particularly sensitive to changes in the alkalinity of the environment. As a result, an increase in the amounts of these compounds can be obtained exclusively at pH of close to 10 which is the optimal pH condition.

3.2.3 Phenol and flavonoid content

The role of phenolic compounds and flavonoids is related to their antioxidant properties, which play an important role in absorbing and neutralizing free radicals, quenching active oxygens, and decomposing peroxidases [46]. An imbalance between antioxidants and free radicals causes oxidative stress and may lead to cell damage [47]. From the present research results, the amounts of phenol and flavonoids were at their maximum at pH 10 followed by at pHs of 12 and 8 and the control sample, respectively (Figures 2c and 2d). Considering that the growth conditions in the applied treatments were far from optimal due to the unfavorable pH as well as the lack or absence of absorbable phosphorus, the amounts of total phenols and flavonoids in *Spirulina* sp. increased for all treatments significantly compared to the control sample. Nevertheless, like anthocyanin, the best response was shown to the stress created at pH 10, which is a confirmation of this proposed hypothesis, in which some defense mechanisms are strongly limited or switched off under the influence of pH change.

3.2.4 Malondialdehyde content

Newly produced radicals can speed up lipid oxidation reactions. It was reported in previous studies that PHB production was elevated under environmental stresses such as pH change and nutrient deficiency [48]. To evaluate the level of induced stress and lipid peroxidation as its main result, malondialdehyde levels were measured. Malondialdehyde is considered a suitable indicator for membrane lipid peroxidation [27]. As can be seen in Figure 2e, the amount of MDA was maximal at pH 8 followed by pH 12. There was no significant difference between the amount in the control sample and pH 10. The similarity of the stress level at pH 10 compared to the control samples was consistent with the appropriate and significant increase of non-enzymatic antioxidants investigated in this study and indicates that at this level of pH, the stress was inhibited with higher efficiency. The better growth of samples of this treatment compared to other treatments is the result of this stress control. These findings show that one of the objectives of this study, which was to create significant stress for *Spirulina sp.*, was met. Phosphorus deficiency in conditions far from optimal pH, which adds extra tension, can be more effective than phosphorus deficiency alone. But the range of tension should be chosen in such a way that the possibility of the best defensive processes is also available.

3.3 Qualitative investigation of polyhydroxybutyrate extracted from *Spirulina* sp. using FTIR

According to the standard FTIR polyhydroxybutyrate spectrum and the FTIR polyhydroxybutyrate spectra of the samples extracted from microalgae under different culture conditions, the peak identified at 1727 cm^{-1} related to the carbonyl group (C=O), the peak at 2877 cm^{-1} related to the CH group, the peaks at1286 and 1184 cm^{-1} corresponded to the ether group (C-O) and specific peaks

below 3000 cm^{-1} represented CH_2 and CH_3 groups. The absorption peaks obtained at 1381, 1455, 2978-2877 and 3439 cm^{-1} were attributable to the -CH_3, -CH_2, -C-H, and O-H moieties, respectively. According to the obtained spectra of isolated PHB from *Spirulina* sp., the peaks indicated the presence of the same chemical structure as standard PHB (Figures 3a-3e). The strength of an absorption peak in FTIR analysis is directly proportional to the quantity of the particular functional group or bond in the sample. Thus, a greater absorption peak signifies a higher concentration or increased presence of the corresponding functional group or bond in the sample under examination [49].

The absorption spectrum of PHB isolated from microalgae *Spirulina* sp. and standard PHB showed almost the same chemical structure (Figures 3a-3e). However, due to impurities (functional groups which were added by solvent), the peaks for the extracted PHB are slightly wider. The presence of the carbonyl group (C=O) is a common feature in the structure of the standard sample and all PHBs extracted in this study, therefore it was used as an indicator to confirm the similarity of the structure of the standard PHB sample and the PHB found in the study extracts. Similarly, Ansari and Fatma [50] and García *et al*. [10], used FTIR spectra to confirm the presence of PHB extracted from microalgae, all of which had similar characteristics, and it is close to the construction characteristics of the standard sample used in this study. In these investigations, it was reported that the peak near 1728 cm^{-1} was related to the carbonyl bond (C=O), while the peaks observed between 1200 and 1000 cm^{-1} were related to methyl (CH_3), methylene (CH_2), carbon-oxygen bond (C-O), which were probably collectively indicative of amorphous PHB [10]. Moreover, the highest peak near 3000 cm^{-1} was related to carbon-hydrogen (C-H) bonds. In our study, the significant peaks in all obtained spectra indicate the correct and acceptable extraction and purification of polyhydroxybutyrate.

3.4 Quantitative investigation of polyhydroxybutyrate extracted from *Spirulina* sp. using GC

After injecting the samples into the GC, the peaks appearing in the chromatogram of the extracted sample (Figures 4b-4e) corresponded to the peaks appearing in the chromatogram of the standard PHB (Figure 4a). The spectrum of the polymer obtained from *Spirulina* sp. and the standard PHB were drawn using the GC and compared. The results confirmed the presence of polyhydroxybutyrate. The standard PHB peak appeared at 36.05 min, and the PHB peak produced in the control sample at 35.08 min. In the pH 8 treatment, the peak appeared at 35.26 min, and in the pH 10 and 12 treatments, it appeared at 35.31 min. The area under the peak indicated the concentration of the PHB sample. The concentrations of PHB extracted from microalgae in the control sample and in the pH 8, 10, and 12 samples were 6.82, 3.54, 7.04, and 4.23% of cell dry weight, respectively. It can be seen that the amount of PHB extracted from the pH 10 treatment was greater than those from other environments (Figure 4f).

PHB was produced in the control sample at 6.82% (w/w), and in the experimental samples as follows: pH 8 at 3.54% (w/w), pH 10 at 7.04% (w/w), and pH 12 at 4.23% (w /w). From these results, it can be concluded that the phosphorus-free treatment at pH 10 when compared to the other three environments in terms of the amount of PHB production, and when considering the cultivation conditions and the amount of biomass obtained during 24 days of cultivation, was the optimal treatment, although its PHB level did not seem to be much higher that that of the control. It appears that the pH level impacts the activity of the PHA synthesis enzymes [51]. Additionally, Suzuki *et al*. [52] suggested that a higher PHA fraction at an increased pH value helps an organism to endure challenging conditions. Similar findings were reported by Sharma and Mallick [53], who observed the highest PHA productivity for the diazotrophic cyanobacterium *Nostoc muscorum* at pH levels above the optimal pH for growth. This provides further evidence of the role of PHA biosynthesis in microbial resilience to stress induced by pH.

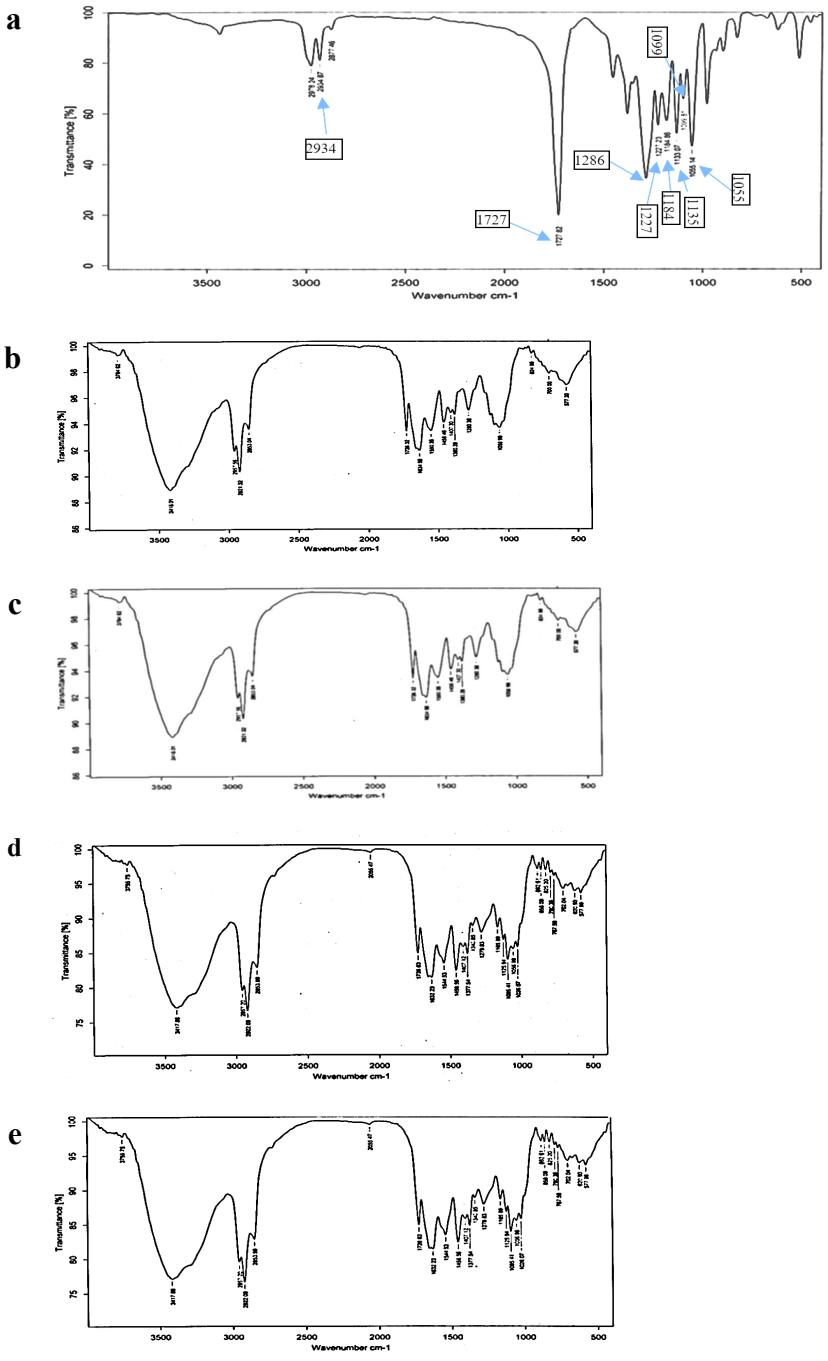

Figure 3. FTIR spectrum of polyhydroxybutyrate for the (a) standard sample (b) control sample (c) treatment without phosphorus and pH=8 (d) treatment without phosphorus and pH=10 (e) the treatment without phosphorus and pH = 12. Arrows in (a) showed the indices peaks.

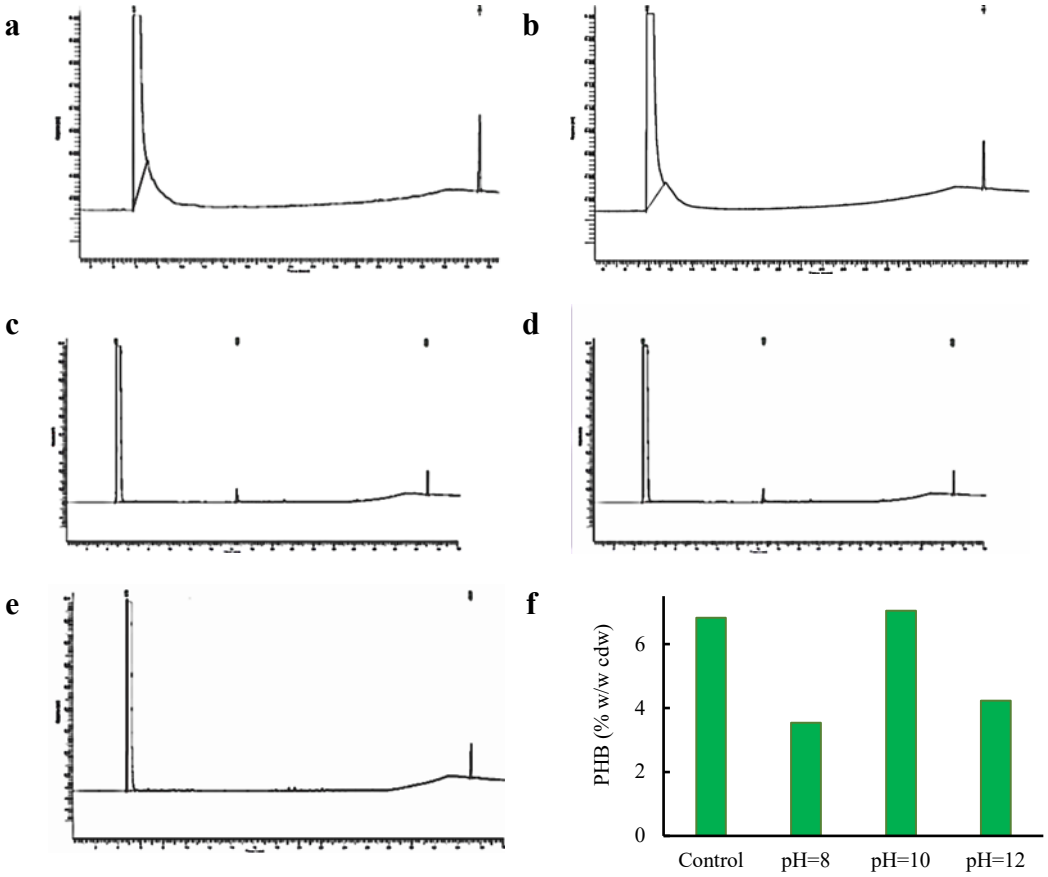

Figure 4. Chromatograms of polyhydroxybutyrate for standard sample (a), control (b), phosphorus-free treatment at pH = 8 (c), phosphorus-free treatment at pH = 10 (d), phosphorus-free treatment at pH = 12 (e) and the concentration graph of polyhydroxybutyrate [% w/w cell dry weight (cdw)] extracted from microalgae in the control sample and phosphorus-free culture medium with pHs 8, 10 and 12 (f). X and Y axis are related to time (min) from 0 to 40, and response (mV) from 4.80 to 5.30.

Previous studies showed that the lack of nutrients such as nitrogen [8, 54, 55], phosphorus [56] and combined glycerol and phosphorus [57] in microalgae cultivation led to an increase in biopolymer production. PHB was detected in different algae such as *Spirulina* sp. to the extent of 3.5% of the dry weight of the cell in an environment lacking phosphorus [58], to the extent of 46% of the dry weight of the cell in an environment lacking nitrogen at pH = 12 [59], and in *Scenedesmus* sp. in a wide range of concentrations (0.83-29.92%, w/w DW), the highest of which was found in a phosphorous-deficient medium containing a normal concentration of iron [10]. Moreover, the accumulation of PHB under phosphorus limitation in the cyanobacterium *Synechocystis* sp. PCC 6803 increased to 11.2% on day 11, compared to 3.4% under control conditions [60]. The findings of Arias *et al*. [61] also confirmed that phosphate limitation in cyanobacteria was a stimulus for PHB accumulation. The increase in PHB content under phosphate limitation may be due to excessive energy depletion, even if ATP production is limited by phosphorus depletion, NADPH production continues through photosynthetic acyclic electron flow [62]. Increasing the

accumulation of PHB in the cell has a direct correlation with the performance of photosynthesis. The increase of this polymer inside the cell is associated with the inhibition of the rate of electron transfer in the membrane, which increases the harmful compounds (free radicals) in the cell. Therefore, it can be expected that an increase in PHB concentration leads to an increase in algae photosensitivity, cell death, and a decrease in the maximum density of algae cells in the culture medium. PHB dynamics is also related to cell energy storage. Under low energy conditions in the cell, the depolymerization of PHB occurs due to the production of 3-hydroxybutyrate coenzyme A, which provides energy to the cell [53]. Therefore, it seems that the decrease in PHB (due to the consumption of PHB) in phosphorus-free treatments at pHs of 8 and 12 may be due to the reduction in the level of energy carriers, and the decomposition of this polymer and its conversion to acetyl coenzyme A. The only study we found on the combination of pH alteration and nutrient deficiency was by Montiel-Corona and Buitrón [63]. They did not find a positive relationship between alkaline pH and certain nutrient deficiencies, including phosphorous. Our results focus on stress induction caused by the combined treatment of phosphorus deficiency and pH changes, and there were no other publications in which we could compare our results.

4. Conclusions

In this study, phosphorus deficiency stress combined with pH changes, was applied as a new treatment over 24 days to microalgae medium *Spirulina* sp. In summary, the results showed that the application of this stress led to a decrease in the growth and production of biomass and photosynthetic pigments compared to the control treatment, but the amount of growth and biomass production in the treatment without phosphorus and at pH10 was closer to the control sample. The amount of PHB produced in the treatment without phosphorus and at pH 10 was closer to the control treatment and even higher. According to the obtained results, the stress caused by changes in the amount of phosphorus at pH 10 and slightly higher than the optimal pH can be considered as a method to increase the accumulation of PHB in microalgae *Spirulina* sp., although these changes should be examined more carefully and in detail.

5. Acknowledgments

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