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Sugarcane Molasses as a Cost-effective Carbon Source on *Arthrospira maxima* Growth by Taguchi Technique

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ABSTRACT

In this research, a new cost-effective carbon source of medium was provided in terms of high-efficiency growth from *Arthrospira maxima*. Sugarcane molasses was used in two different modes (alternative and additive) at four different concentrations (0, 0.5, 1.0 and 1.5 gL^{-1}) to determine the effect of new carbon source versus its standard carbon source of Zarrouk's medium ($NaHCO_3$). The experimental results were analyzed by Taguchi L8 method as a statistical technique. The highest biomass production obtained when sugarcane molasses was added as an alternative source, which was 5.31 times higher than the usual Zarrouk's media. Furthermore, final biomass concentration increased with increasing molasses concentration from 0 to 1.5 gL^{-1} in this group. At highest concentration, phycocyanin (at 0.11 and 0.12 gL^{-1}), allophycocyanin (at 0.13 and 0.12 gL^{-1}), carotenoids (at 2340 and 2535 mgL^{-1}), chlorophyll a (at 23.83 and 24.83 mgL^{-1}), and chlorophyll b (at 0.343 and 2.99 mgL^{-1}) obtained when molasses were added as an additive and alternative sources, respectively. Finally, the replacement of standard carbon sources of medium with sugarcane molasses had the potential possibility in order to reduce the production costs of *Arthrospira maxima* growth.

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NOMENCLATURE

A	Absorbance at different wavelength	DT	Doubling time
APC	Allophycocyanin	μ_{max}	Maximum specific growth rate
$C_{w.d}$	Concentration of *Arthrospira maxima* by dry-weight	P	Productivity
$C_{w.dt}$	Biomass concentration (gL^{-1}) at time $t_{cd.w}$ (days)	PC	Phycocyanin
$C_{w.d0}$	The initial biomass concentration (gL^{-1}) at the time t_0	R^2	Interpret R Squared
C_a	Chlorophyll a	t_0	Initial time by day
C_b	Chlorophyll b	$t_{cw.d}$	Selected time in the day
C_{x+c}	Carotenoid		

1. INTRODUCTION

Microalgae are a group of eukaryotic algae and prokaryotic cyanobacteria which do the photosynthesis [1]. Among the commercial species of microalgae, *Spirulina* is one of the most essential microalgae for a wide range of applications in various industries [2-5]. as a source of vitamins [6]. Recently, there is a massive demand of natural pigment extraction from *Spirulina* due to their non-toxic, non-allergic, and antimicrobial effects (FDA has banned the use of synthetic colorants) [7-9].

Furthermore, *Spirulina* contains the most important sources of pigments [10]. The phycocyanin and allophycocyanin have considerably been noticed world.

Biomass growth of microalgae depends on the environmental situations such as lighting [11], temperature [12] and pH [13]. Furthermore, aeration plays a significant role on the production of biomass by

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increasing the dissolved oxygen content of culture [14]. In fact, light intensity can affect the biomass and pigments production [15-17]. Nutrient availability is one of the major promising strategies to change and control the microalgae growth and the production of pigments [18]. The essential nutrients are an organic or inorganic carbon source as well as nitrogen, and other micronutrient during the cultivation [19]. Nowadays, feasibility of *Spirulina* growth in several nutritional conditions has been encouraged to enhance biomass and pigment production [18]. Therefore, various media can be used for the growth of Spirulina like Zarrouk[20], modified Zarrouk [21], CFTRI & JPJM [22] and Bangladesh one [23]. However, there are different applications on algae such as wastewater treatment through their produced biomasses [24, 25] but, their nutrition should be an important issue.

Carbon source is one of the most cost-effective factors in the production of biomasses and pigments [26]. The replacement of basic carbon sources of media ($NaHCO_3$) with cost-effective materials such as glucose and molasses has previously been reported in the cultivation of Spirulina platensis [27-29]. Since, there were no published works on the impacts of sugarcane molasses on the cultivation of *Arthrospira maxima*, the current research was conduct on this area. Moreover, sugarcane molasses as an alternative and additive carbon source of Zarrouk's media was prepared at four different concentrations (0, 0.5, 1.0 and 1.5 gL^{-1}) in this research.

2. EXPERIMENTS

2. 1. Inoculums Preparation and Cultivation Method Axenic Arthrospira maxima CIB79 strain was obtained from National Polytechnic Institute (IPN), (Mexico City, Mexico), which was grown in a batch culture. The cultivation process using Zarrouk's media was carried out at laboratory temperature ranging from 28-32 °C under a white fluorescent with an illumination of 1350±100 lux light intensity. The treatments continuously aerated by adjusting a fixed aeration with an air pump [AC-9602 (RESUN, Mexico)] during 7 days of cultivation. Furthermore, the measurement of culture pH was daily carried out using a pH-meter (HANNA, pH21 pH/mV meter, US). Zarrouk's media with $NaHCO_3$ 16.8 gL^{-1}, $NaNO_3$ 2.5 gL^{-1}, K_2HPO_4 0.5 gL^{-1}, K_2SO_4 1.0 gL^{-1}, NaCl 1.0 gL^{-1}, $MgSO_4.7H_2O$ 0.2 gL^{-1}, EDTA-$Na_2.2H_2O$ 0.08 gL^{-1}, $CaCl_2.2H_2O$ 0.04 gL^{-1}, and $FeSO_4.2H_2O$ 0.01 gL^{-1}, micronutrient elements solution (H_3BO_3 2.86 gL^{-1}, $MnCl_2.4H_2O$ 1.81 gL^{-1}, $ZnSO_4.7H_2O$ 0.222 gL^{-1}, MoO_3 0.01 gL^{-1}, $CoCl_2.6H_2O$ 0.01 gL^{-1}, $CuSO_4.5H_2O$ 0.079 gL^{-1}) 1.00 mLL^{-1} was used as a cultivation media. All chemicals were purchased from Merck Company (Darmstadt, Germany). The media preparation was carried out according to the literature [30]. Treatments were performed in a 125 mL Erlenmeyer flask containing 25 mL of Arthrospira maxima inoculum with an initial biomass concentration of 1.08 gL^{-1}. During the process of growth, double-distilled water was daily added to keep the media in a constant level. The cultivation environment was prepared either with (additive) or without (alternative) basic carbon source of Zarrouk's media. Then, sugarcane molasses as a cheap by-pass product [at different concentrations (0.5, 1 and 1.5 gL^{-1})] were added into the media in the mixotrophic culture to determine the effect of new carbon source versus standard carbon source of Zarrouk's media. The biomass growth and pigment production were recorded during the cultivation. all experiments were repeated three times and the data reproducibility were carefully checked. The data collections were performed during 2019-2020 in September.

2. 2. Analysis and Pigments Measurement The biomass concentration ($C_{w.d}$, gL^{-1}) by dry-weight was daily recorded for each treatment by measuring optical density at wavelength of 674 nm using a spectrophotometer (Thermo Scientific, England) based on the validation curve. The maximum specific growth rate (μ_{max}, day^{-1}) and doubling time (DT, day) at the end of each run was calculated based on literature [31]:

$$\mu_{max} = \frac{\ln(C_{w.d_t}) - \ln(C_{w.d_0})}{t_{C_{w.d}} - t_0}, DT = \frac{0.693}{\mu} \quad (1)$$

Phycobiliproteins concentration was determined using repeated freezing-thawing cycles [18]. The concentrations of phycocyanin (PC) and allophycocyanin (APC) were measured using the following equations at wavelengths of 620 and 652 nm, respectively [32]:

$$PC\ (gL^{-1}) = \frac{A_{620} - 0.474\ A_{652}}{5.34}, APC\ (gL^{-1}) = \frac{A_{652} - 0.208\ A_{620}}{5.09} \quad (2)$$

The pellet collected from the previous step homogenized with 0.4 mL of acetone and chloroform solvent (7:3 v/v) and refrigerated for few days until no color could be seen in the pellet. Then, it was centrifuged at 13300 rpm for 5 min and then the green supernatant was collected. Its absorption was determined at wavelengths of 470, 645 and 662 nm by spectrophotometer. The total content of chlorophyll (C_a) and carotenoid ($C_{(X+C)}$) were calculated using the following equations [33]:

$$C_a = 11.24 \times A_{662} - 2.04 \times A_{645}, C_b = 20.13 \times A_{645} - 4.19 \times A_{662}\ (mgL^{-1}) \quad (3)$$

$$C_{(x+C)} = (1000 \times A_{470}) - (1.09C_a - 63.14C_b)/214\ (mgL^{-1}) \quad (4)$$

All pigments extraction processes were carried out under the dim light to protect them from degradation.

2. 3. Experimental Design The experiments were designed using Taguchi L8 method by Minitab software (2019). The parameters and their levels were tabulated in Table 1.

The experimental design represents eight treatments evaluated by Taguchi L8 approach (four two-level parameters) as shown in Tables 2. Evaluation of experimental data was based on signal-to-noise ratio (S/N ratio) and mean ratio.

All graphic designs and pigment calculations in this study were performed using the Graph Pad Prism 8 software.

3. RESULTS AND DISCUSSION

3. 1. Calibration Curve for Culture Media The maximum absorption wavelength (674 nm) was calculated by measuring the absorption spectra in the wavelengths from 300 to 800 nm, which was in good agreement with the literature [33]. The relationship between concentration of *Arthrospira maxima* by cell dry-weight ($C_{w.d}$) and corresponding absorbance results at 674 nm were estimated by the straight-line equation as follows:

$$A_{674} = 0.5800 \times (C_{w.d}) + 0.02010, (R^2 = 0.9971) \quad (5)$$

TABLE 1. Experimental parameters and their level

Parameters	Level No.	Value of each Level
Sugarcane molasses concentration	4	0, 0.5, 1.0, 1.5
Adding method of molasses	2	1 (additive carbon source), 2 (alternative carbon source)

TABLE 2. Orthogonal array of Taguchi L8 (4^1 2^1)

Treatments No.	Concentration	Adding method
1a	0	1
2	0.5	1
3	1	1
4	1.5	1
5	0	2
6	0.5	2
7	1	2
8	1.5	2

[a] treatment that was prepared in control culture media (Zarrouk's media). Number 1 represents molasses was added as an additive nitrogen source. Number 2 represents molasses was added as an alternative nitrogen source.

3. 2. Effect of Molasses Concentrations on Growth Parameters

3. 2. 1. Biomass Dried Weight Figure. 1 estimates healthy cells and illustrates $C_{w.d}$ affected by changes in the molasses concentration. The lag-phase of most cultures was 2 days. The highest cell concentration (4.49 gL^{-1}) was obtained at the maximum concentration of molasses (1.5 gL^{-1}) with bicarbonate-free media, which was almost 1.5 times higher than molasses-based media with bicarbonate (Fig. 1b). Furthermore, molasses concentration increment from 0.5 to 1.5 gL^{-1} led to a significant enhancement in the biomass accumulation. The result was in agreement with a similar work on Spirulina platensis [27]. Other results showed an increase of $C_{w.d}$ between 0.495 gL^{-1} and 0.609 gL^{-1} after 7 day of cultivation when culture media was supplemented by 0.1% and 1% v/v of sugarcane vinasse.

In additive-molasses group, treatment 4 had the highest molasses concentration and produced the highest $C_{w.d}$ although it was not high enough to be effective compared with the alternative group. In treatment 2 the cell growth was higher than that treatment 1 and also higher than that encountered by Andrade and Costa [34] during mixotrophic growth ($C_{w.d}$= 1.14 gL^{-1}) of *Spirulina platensis* with molasses (0.75 gL^{-1}) at the media light intensity (45.5 µmol $m^{-2}s^{-1}$) although they used *Spirulina platensis* microalgae for cultivation period of 11 days; while *Arthrospira maxima* microalgae during 7 days of cultivation was used in the current research [35]. Moreover, the $C_{w.d}$of the highest molasses concentration in both groups (additive and alternative) was higher than that of *Spirulina platensis* growth stimulated in media supplemented with glucose ($C_{w.d}$=2.52 gL^{-1}) during mixotrophic condition [36]. However, light and organic carbon source are the two most important factors on the growth rate of Arthrospira maxima in the mixotrophic condition; but, the light could not effectively penetrate in a high-concentrated media. It causes turbidity after 4 days. According to the literature, this phenomenon inhibited photosynthetic activity of Spirulina platensis[37]. On the other hand, the cultivation conditions such as light and organic carbon source in heterotrophic condition can inhibit growth process [38].

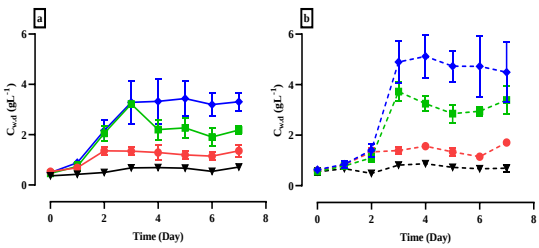

Figure 1. Change in biomass concentration during the period of day: additive (panel a) and alternative (panel b) molasses. (▼: 0 gL^{-1}, ●: 0.5 gL^{-1}, ■: 1 gL^{-1},♦: 1.5 gL^{-1})

3. 2. 2. Culture Media PH The pH was gradually increased in molasses additive-based media as bicarbonate dissolved in the media that releases CO_2 and OH^- (as data shown in Fig. 2). Then, the pH increased with respect to time during cultivation according to the following equations [39]:

$$NaHCO_3^- \rightarrow Na^+(micronutrientforgrowingalgae) + HCO_3^- \quad (6)$$

$$HCO_3^- \xrightarrow[\text{Carbonic anhydrase enzyme}]{} CO_2(carbonsource) + OH^-(alkaline) \quad (7)$$

In both molasses-based media, the pH decreased 2 days after inoculation and then increased due to the activity of bacteria in the media. Additional amounts of bacteria were contained in the media at high concentration of molasses. Based on Oswald principle, the organic compounds of wastewater were converted into CO_2 by oxidation bacteria in the media at the beginning of cultivation. Then, carbonate was formed by chemical reaction of CO_2 and water. Carbonate then was used by microalgae throughout photosynthetic process. OH^- ions were released and pH of the solution has increased [40]. Therefore, Arthrospira maxima utilized organic carbon such as molasses for producing CO_2 through respiration (the heterotrophic growth) while the growth should be photoautotrophic when Arthrospira maxima utilized light as an energy source and carbon dioxide as a carbon source during the photosynthetic process (pH media will remain high due to photoautotrophic growth). According to this research, the $C_{w.d}$ reached its maximum value when the pH value was in minimum (9.10±0.3). This result is properly justified by a similar work reported in literature [41].

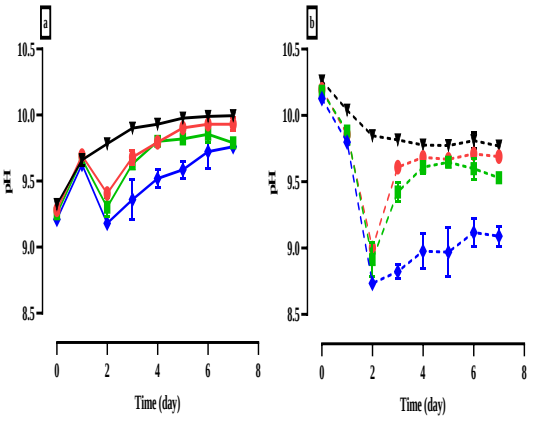

Figure 2. Changes in pH during the growth phase of *Arthrospira maxima*: additive (panel a) and alternative (panel b) molasses. (▼: 0 gL^{-1}, ●: 0.5 gL^{-1}, ■: 1 gL^{-1},♦: 1.5 gL^{-1})

3. 2. 3. Growth Parameters Figure. 3 shows the effect of various concentration ns of molasses-containing media on μ_{max} and DT at the end of each run (7 days). Moreover, μ_{max} and the minimum DT (0.3 day^{-1}, 2.3 day) occurred at concentration of 1.5 gL^{-1} in the media when molasses was added as an alternative carbon source. Furthermore, sum of the μ_{max} values of autotrophic and heterotrophic cultures corresponded to μ_{max} of the mixotrophic culture [36]. These results revealed that the lowest DT (2.55 day) is in fact at the highest μ_{max} (0.27) at concentration of 1.5 gL^{-1} when molasses is added as an additional carbon source. Moreover, μ_{max} decreased from 0.09 to 0.3 day^{-1} when the initial concentration increased from 0.05 to 1.5 gL^{-1}.

3. 3. Effect of Molasses Concentrations on the Pigments Production

3. 3. 1. Phycocyanin and Allophycocyanin Contents According to Figures 4(a) and 4(b), the value of PC increased with time and concentration of molasses increment. The formation of PC pigment in the highest concentration remained consistent in a high level after 4 days of cultivation. That is probably due to the turbidity of media after 4 days (rapid growth of biomass during the first few days of cultivation) [37]. Furthermore, Arthrospira maxima requires high light intensity to cover turbidity problem after 4 days of cultivation at high concentrations of carbon source. Changes in APC content in molasses-containing media as an additive and alternative are shown in Figures 4(c) and 4(d).

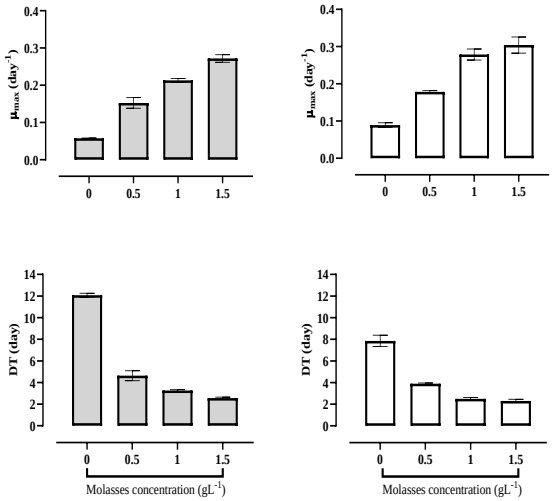

Figure 3. Effect of different concentrations of molasses-based media on μ_{max} specific growth rate (panel a & b) and doubling time (panel c & d) whereas molasses was added as an additive (gray box) and alternative (white box) carbon sources

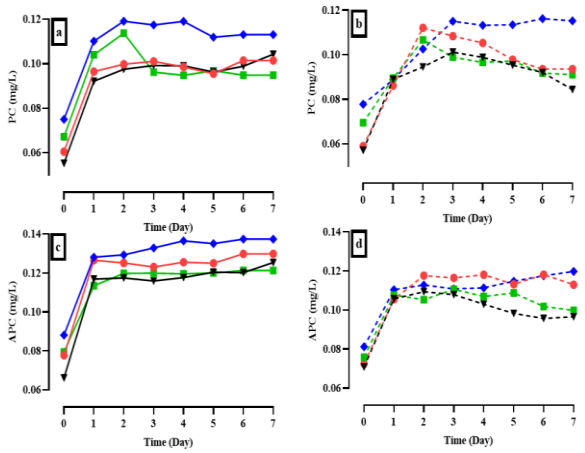

Figure 4. Changes in phycocyanin (panel a & b) and allophycocyanin (panel c & d) content in molasses-containing media. (▼: 0 gL^{-1}, ●: 0.5 gL^{-1}, ■: 1 gL^{-1},♦: 1.5 gL^{-1})

At the highest concentration, the APC and PC contents increased from 0.08 to 0.13 mgL^{-1} and from 0.07 to 0.12 mgL^{-1}, respectively. In photoautotrophic culture, PC and APC did not grow as fast as the mixotrophic culture while photoautotrophic culture cannot be satisfied by biomass growth in clash with the mixotrophic culture. On the other hand, the results showed that the mount of PC and APC slightly increased with time in the control treatment. Therefore, it was concluded that carbon source and its concentration as well as light intensity during the cultivation were the significant factors. Moreover, the amounts of PC and APC pigment in the carbon-free conditions were declining. Therefore, carbon source was a vital source for *Arthrospira maxima* growth.

3. 3. 2. Photosynthetic Content Table 3 shows the amount of photosynthetic pigments (C_a, C_b and $C_{(x+c)}$) for each treatment on the final day of cultivation. It was observed that molasses was not able to produce the highest content of photosynthesis pigment throughout the course of cultivation due to the lack of light penetration [42]. The C_a, C_b and $C_{(X+C)}$ of *Arthrospira maxima* were in maximum at maximum pH value [43]. According to the control treatment, the highest C_a, C_b and $C_{(X+C)}$ growth data were at 29.13, 10.25 and 2672 mgL^{-1}, respectively. This amount was similar to treatment 5 (without any carbon source). In addition, the amount of photosynthetic pigments decreased with an increase in molasses concentration in the both methods.

Furthermore, molasses concentration increment may disrupt microalgae breath and consequently reduce the pigment content. The current study shows a good efficiency compared with the control treatment in terms of C_a. It almost was 2.5 times higher than that of the *Spirulina platensis* growth in additive molasses-based Zarrouk's media [27].

TABLE 3. Estimation of photosynthetic pigments in Arthrospira maxima

Treatment No	Molasses addition method	C_a(mgL^{-1})	C_b(mgL^{-1})	$C_{(X+C)}$ (mgL^{-1})
1[a]	Additive	29.13	10.25	2672
2		27.23	2.89	2542
3		26.58	1.89	2582
4		23.83	0.343	2340
5	Alternative	28.59	10.34	2620
6		28.05	3.44	2553
7		24.43	2.17	2540
8		24.83	2.99	2535

[a] *treatment that was prepared in control culture media (Zarrouk's media)*

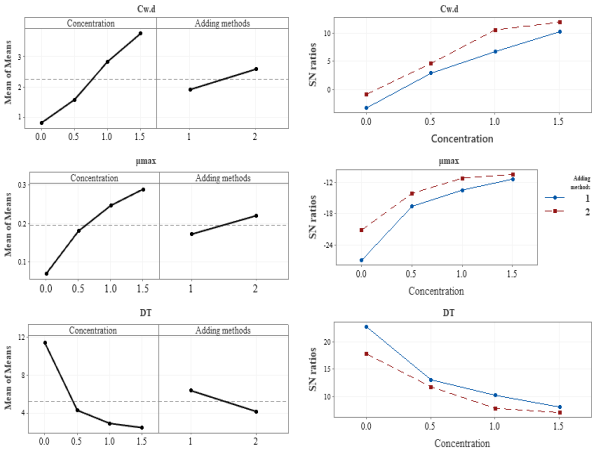

Figure 5. Mean graph (vertical diagram on the left side) and S/N ratio graph (diagram on the right side) corresponding to biomass concentration (gL^{-1}), specific growth rate and doubling time.

3. 3. 3. Experimental Analysis and Design Figure. 5 shows Mean and S/N ratio graph corresponding to the growth parameters.

The maximum value of S/N ratio plot of $C_{w.d}$ and μ_{max} described the optimum level for a particular parameter. On the other hand, smaller S/N ratio was the best factor for the DT. Furthermore, molasses as an alternative carbon source (adding methods 2) at a concentration of 1.5 $gL^{-1}$was chosen as the best parameter.

4. CONCLUSIONS

Arthrospira maxima cultivation requires the best carbon source instead of bicarbonate source of Zarrouk's media as well as sufficient concentration for the enhanced

biomass growth. $C_{w.d}$and μ_{max} amounts almost increased with an increase in the molasses concentration from 0.5 to 1.5 gL^{-1} during the cultivation. However, adding molasses as an additive and alternative carbon source should be useful but, molasses consumption (as an additive carbon source) did not show an excellent output on $C_{w.d}$in comparison with the alternative sources. Moreover, the availability of organic carbon source in media and sufficient light during the first few days of cultivation increased the penetration of light and carbon-fixation route into the media. The formation of PC pigment simultaneously decreased or consistently remained high throughout most part of the cultivation period. Its reason is due to the effect of restricted light dispersion (biomass accumulation). Furthermore, the intensity of light could increase after the first few days of cultivation in order to avoid the growth rate inhibition.

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Persian Abstract

چکیده

در این تحقیق، از منبع کربن مقرون به صرفه جدیدی با راندمان تولید بالا *Arthrospira maxima* استفاده شد. از ملاس نیشکر در دو حالت (جایگزین و افزودنی) و چهار غلظت مختلف (0، 0/5، 1 و 1/5 گرم در لیتر) برای تعیین تأثیر منبع کربن جدید در برابر منبع کربن استاندارد محیط زاروک استفاده شد. نتایج تجربی با استفاده از روش تاگوچیL8 به عنوان یک تکنیک آماری مورد تجزیه و تحلیل قرار گرفت. بیشترین تولید زیست توده زمانی حاصل شد که ملاس نیشکر به عنوان منبع جایگزین اضافه شد که تقریبا 5/31 برابر بیشتر از محیط استاندارد زاروک بود. علاوه بر این، غلظت زیست توده نهایی با افزایش غلظت ملاس از 0 تا 1/5 گرم در لیتر در این گروه افزایشیافت. در بالاترین غلظت ملاس، فیکوسیانین (در 0/11 و 0/12 گرم در لیتر)، آلوفیکوسیانین (در 0/13 و 0/12 گرم در لیتر)، کاروتنوئیدها (در 2340 و 2535 میلی گرم در لیتر)، کلروفیلa (در 23/83 و 24 میلیگرم در لیتر)، و کلروفیلb (در 0/343 و 2/99 میلی‌گرم در لیتر) به‌ترتیب زمانیکه ملاس بعنوان منبع افزودنی و جایگزین اضافه شد، به‌دست می‌آید. در نهایت، جایگزینی منبع کربن استاندارد محیط کشت زاروک با ملاس نیشکر امکان بالقوه ای را برای کاهش هزینه های تولید دارد.