Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.47750/jptcp.2023.30.06.032

Dertermination of the Parameters Kinetic Growth to the Culture Algal Biomass from Locumba River in Peru for its future use in the Pharmaceutical Industry

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Submitted: 14 February 2023; Accepted: 21 March 2023; Published: 04 April 2023

ABSTRACT

Microalgae form the basis of the trophic chain of the aquatic environment and they have diverse applications in the pharmaceutical industry. We collect samples of local microalgae from Locumba River, in Tacna, Perú. They were isolated, morphologically described as Ankistrodesmus sp, Tetraselmis striata and Stichococcus sp. Then, they were adapted to the culture technology of the Aquaculture Research Laboratory of IMARPE-Ilo up to 20 L in controlled environment, at 21°C ± 1°C, pH 7.7, 2126 Lux, dissolved oxygen 6.04 mg/L, salinity 35146 PSU, and applying two kinds of nutrient F/2 Guillard and Bayfolan. Specific growth rate (μ), doubling time (tD) and cell density (C.D.) have measured and analysed. The results of one-way ANOVA showed that there was no significant difference ($p > 0.05$) between the two fertilizers. Bayfolan proved to be more efficient than F/2 Guillard to produce 1000 L of local microalgae to obtain wet biomass that favors the subsequent extraction of secondary metabolites.

Keywords: *Microalgae, foliar fertilizer, liquid biomass, secondary metabolites*

INTRODUCTION

The aquatic environment and especially the marine is one of the settings where the microalgae constitute the main component of the phytoplankton that supports the trophic chain (Cordero et al., 2012) and represent 50% of the global fixation of organic carbon (León et al., 2004). This heterogeneous group presents a broad historical fossil record (Ali, 2010).

Seaweed produces a wide variety of remarkable natural compounds, such as small biomolecules that has considered non-essential during the life of the producing organism called secondary metabolites (Agostini et al 2012 in Breitling et al., 2013). They have carried out by biochemical reactions to synthesize complementary molecules of the vital functions in diverse organisms, as intermediate products have obtained highly

bioactive metabolites. It is constituting by-products that are synthesized depending on external conditions (Paliwal et al., 2017) such as attacks of pathogens, predators, thermal or light changes, nutritional deficiencies or the presence of other organisms (Payyavula et al., 2012).

MATERIALS AND METHODS

Collection of samples

Samples of water were collected in the last section of the Locumba River, included from the Boca Toma de Agua (LS 17°44'47.9" and LO 70°53'11.2") until before the Land Irrigation bridge Eriazas - ITE (LS 17°54'22.4" and LO 70°57'28.6"). This is located in the district called ITE, situated in the Jorge Basadre Province, 90 km north of the city of Tacna (Figure 1 and Table 1). The surface temperature of the river (°C), salinity (PSU), dissolved oxygen (mg/l) (Carpenter 1965), pH by colorimetric scale (Boyd et al., 2011), nutrients for the determination of phosphate were recorded at each sampling point. Following the methodology of Strickland and Parson (1972), silicate and nitrate under the method of Patey et al. (2008), we collected plankton water without fixative for later analysis in the laboratory (UNESCO, 1981).

FIGURE 1: Sample of station

(Source: Google Earth.)

TABLE 1: Location of sampling stations

Station	Bocatoma	Bajada Dakar	Before Pte. ITE
Latitude	17°44’47.9”	17°48’00.0”	17°54’22.4”
Lenght	70°53’11.2”	70°55’09.9”	70°57’28.6”
Observations		Tailing dragged	pH high

Source: Self-made.

The Culture mediums and its composition

We have used as culture medium traditional The F/2 Guillard. Its composition is:

Solution A: Nitrate and Phosphate stock solution (1 L)

Nutrient	Amount
$NaNO_3$	84.15 g
$Na_2HPO_4 \cdot H_2O$	6.0 g
$FeCl_3 \cdot 6H_2O$	2.90 g
$Na_2EDTA \cdot 2H_2O$	10.0 g

Solution B: Silicate stock solution (1 L)

Nutrient	Amount
$Na_2SiO_3 \cdot 9H_2O$	33.0 g

Solution C: Trace metal stock solution (1 L)

Nutrient	Amount
$CuSO_4 \cdot 5H_2O$	1.96 g
$ZnSO_4 \cdot 7H_2O$	4.40 g
$Na_2MoO_4 \cdot 2H_2O$	1.26 g
$MnCl_2 \cdot 4H_2O$	36.0 g
$CoCl_2 \cdot 6H_2O$	2.0 g

Solution D: Vitamin stock solution (1 L)

Nutrients	Amount
Vitamin B1	0.4 g
Vitamin B12	0.002 mg
Biotin	0.10 mg

FIGURE 2: Composition of the culture medium F/2 Guillard.

(Source:https://www.researchgate.net/publication/272723760_Comparison_between_Airlift_Photobioreactor_and_Bubble_Column_for_Skeletonema_Costatum_Cultivation/link/550698200cf24cee3a058da5/download)

In addition, we have used as culture medium alternative the fertilizing foliar Bayfolan. Its composition is:

Macronutrients: Nitrogen (110g/L), Phosphoric Anhydride (80 g/L), Potassium Oxide (60 g/L).

Micronutrients: Iron (190 mg/L), Manganese (162 mg/L), Boron (102 mg/L), Copper (81 mg/L), Zinc (61 mg/L), Molybdenum (9 mg/L), Cobalt (3.5 mg/L).

Vitamins B1

Growth Hormones (4ppm).

Isolation of microalgae and obtaining pure culture for scale up

The technique of the micropipette has used to obtain the selected species and isolate a monospecific strain from a water sample and subsequently produce clonal cultures was applied (Gonzales 2000). The technique consisted in aspirating a small drop and transferring it to a new multi-purpose plate, diluting it in the sterile medium until obtaining a completely isolated cell to inoculate it in a test tube with liquid medium F/2 Guillard (1975) and place it in an environment with favourable conditions for the culture.

The technique of successive washing of cells of the microalgae of interest have used, and later the content was inoculated gradually increasing the volume until reaching 20 l of cell culture.

The method of cultivation, according to its nature, was intensive, type batch, and monospecific culture according to its purity. The nutritious meals were the F/2 of Guillard and Bayfolan. The air-conditioned room has kept at 21°C ± 1°C, the average intensity of 2126 lux (fluorescent 40 W), pH 7.7, dissolved oxygen average of 6.04 mg/L and average salinity of 35146 UPS in the case of the saline medium. The inoculums have transferred upwards from 250 mL to 20 L flasks with constant air supply, as described in the following protocol (Figure 3)

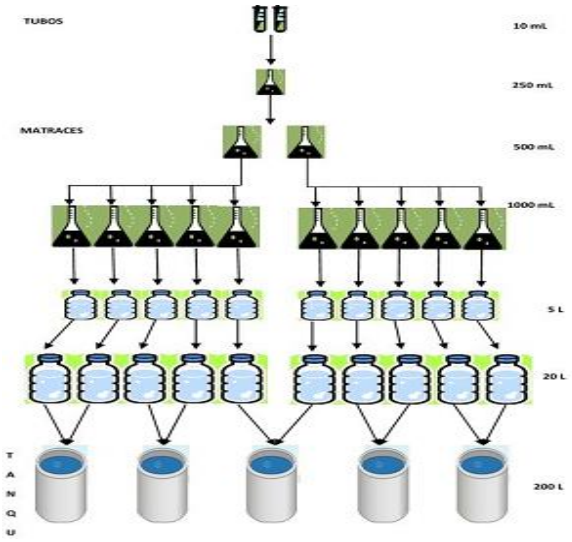

FIGURE. 3: Microalgae culture flow.

(Source: Internal Report microalgae Culture Technology of the Aquaculture Research Laboratory)

Culture Protocol

A. The phytoplankton samples have collected from the natural environment using a 20 µm network.

B. They transferred to the unpreserved laboratory and analysed immediately.

C. The microalgae of interest were isolated using the technique of successive washing, considering for its selection mainly the size, and adaptation to the cultivation system.

D. We scaled up the culture to 20 L, requiring:

• To sterilize the glass material with neutral soap diluted in potable water (1 mL: 1 L) and dry heat using a stove.

• To treat seawater using sand filters, diatomaceous earth filter, stamp microfilters, UV sterilizer, vacuum filtration and autoclave.

• To enrich the sterile seawater supplying F/2 Guillard composed of macronutrients, micronutrients, and vitamins.

• To sow the container with the inoculum of microalgae from the lower volume, considering the crop stage (initial, intermediate, and massive), and the flow of culture.

• Inject air from an air regenerator (blower) into the containers from 500 mL. Smaller volumes are manually agitated.

• To keep the ambient temperature constant in a range of 18°C - 20°C with the help of ducted air conditioning.

• Distribute flasks and bottles inoculated in shelves with artificial lighting from fluorescents (50 W) to promote photosynthesis and crop development.

• Each carafe of 20 L have seeded with 7 L of microalga during the term of the exponential phase and the beginning of the stationary phase.

E. Daily counting of samples from containers have carried out of microalgae of the different cultivated species, in some six pseudo-repetitions, calculating the average of the cell density for determination of the kinetics growth parameters.

Specific Growth Rate (µ)

For the determination of the specific rate of growth (µ) was used, the daily cellular density recorded and subsequently adjusted an exponential regression in the growth phase of each crop (Paniagua, 1986). The specific rate of population growth has calculated with the following formula:

$$\mu = \frac{\ln N_f - \ln N_0}{t_f - t_o}$$

(1)

Where Nf = cellular density in tf, No = cellular density in t0, t0 = initial time of the exponential phase and tf = final time of the exponential phase.

The initial and final times of the exponential phase corresponded respectively to the first positive value and the maximum value of the average finite growth rates ($\Delta N/\Delta t$).

RESULTS

Isolation of marine microalgae from the southern part of Peru

The microalgae of interest were isolated using the technique of successive washing, considering for its selection mainly the size and adaptation to the cultivation system.

Ankistrodesmus sp

The cells live alone or can be grouped forming bundles, some have a relatively curved elongated body in their central part (in the form of Crescent) and sharp ends in the form of a spindle, inside it, is possible to find an elongated chloroplast parietal that Remains parallel to the cell wall. It measures between 17 and 20 microns in length and has developed in properly sterilized fresh water (Figure 4).

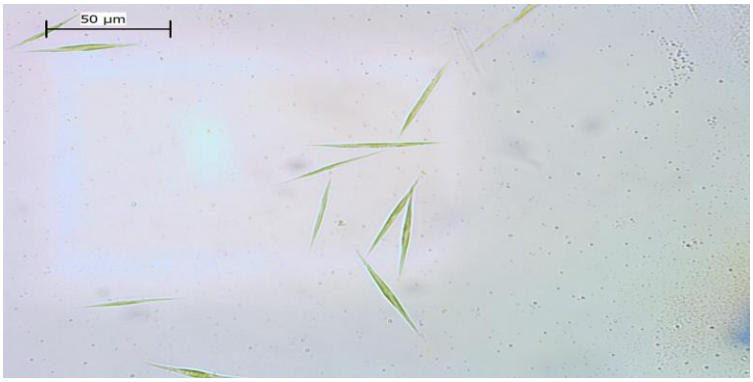

FIGURE 4: Morphology of Ankistrodesmus

(Source: Own Image)

Tetraselmis striata

Several species of Tetraselmis are economically important, as they are ideal for mass cultivation due to their Euryhaline and Euriterma nature (Butcher 1952, Fábregas et al. 1984). The genus is widely used in aquaculture facilities as food for juvenile mollusks, shrimp larvae, and rotifers (Brow 1999, Azma et al. 2011). In addition, strains with high lipid content have the potential has been used in the production of biofuels (Loong 2014)

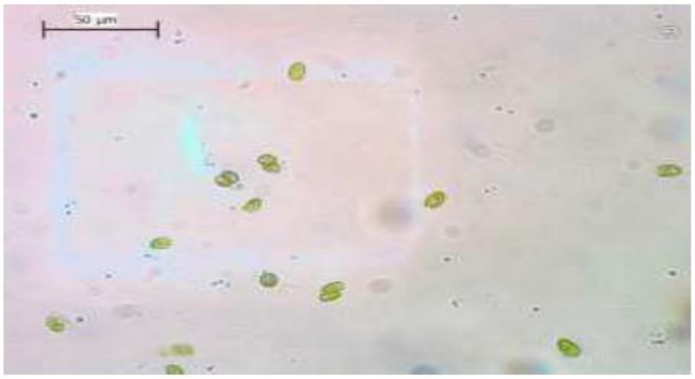

FIGURE 5: Morphology of Tetraselmis striata.

(Source: Own Image)

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The application of Methanol + chloroform (1:1) in the extract of Tetraselmis suecica shows significant antimicrobial activity and therefore have an excellent potential for the solvent to extract bioactive compounds from the natural sources of importance Clinical and Pharmaceutical (Dooslin & Krishnakumar 2013).

Stichococcus sp

Green microalgae characterized by having cylindrical shaped cells and oval edges; they contain parietals chloroplasts with a simple pyrenoid, its reproduction is asexual by binary fission. It measures between 5 to 9 microns long and 2 to 3 microns in diameter. These cells usually grouped, forming filaments, they develop in fresh water.

They lack a cell wall and can be aquatic and marine (Butcher 1952), with a cosmopolitan distribution (Ettl & Gärtner 1995). They have found in a wide range of substrates, such as soil, stonewalls, shingles, tree trunks, glass, polyethylene, and plastic; Sometimes they float freely in small masses of water (John 2002) (Figure 5).

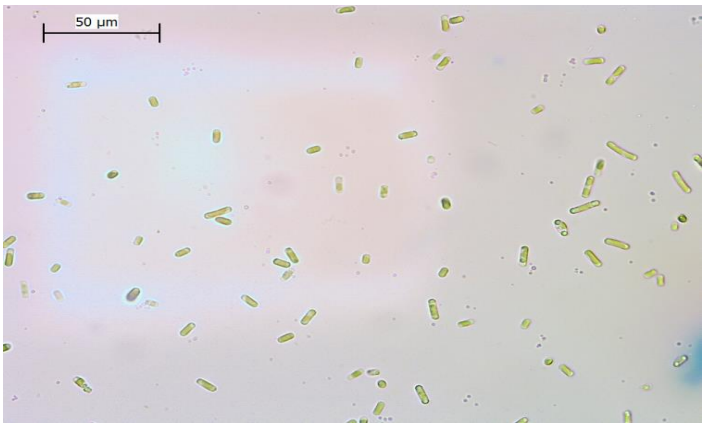

FIGURE 6: Morphology of Stichococcus

(Source: Own Image)

Recently, Stichococcus bacillaris naegeli has been propoused as a potential candidate for use in fuel production (Gargano et al. 2013). S. bacillaris is an oilseed green microalga that include about 14 species (Neustupa et al. 2007). The cells are approximately 2 to 3 µm in diameter, their filamentous state or single-cell structure depends on salinity. So it can grow in both types of water, sweet and marine with different kinetic parameters (Lataa 1991), it is tolerant to high salinities (Hayward 1974). S. Bacillaris is quite abundant around the world, can remove cadmium from some solutes (Shang and Majidi 1993), and could minimize pollution or improve water quality (Sivakumar et al. 2014).

Determination of the growth Curve

The cultivation of Ankistrodesmus sp. with the culture medium traditional, F/2 Guillard, during the adaptation phase showed a cell density of 6.99 x 105 cel/mL, while then, the culture medium alternative, Bayfolan (foliar fertilizing), shown a cell density of 7.08 x 105 cel/mL. In the exponential phase, the culture medium traditional (F/2 Guillard), reached a level of 2.69 x 106 cel/mL. The culture medium alternative (Bayfolan) shown a cell density of 5.55 x 106 cel/ml. Concerning the stationary phase, the densities for both mediums, the traditional (F/2 Guillard) and alternative (the fertilizing Bayfolan) showed values of 1.97 x 106 cel/mL (Figure 7).The cell density remained constant during the stationary period.

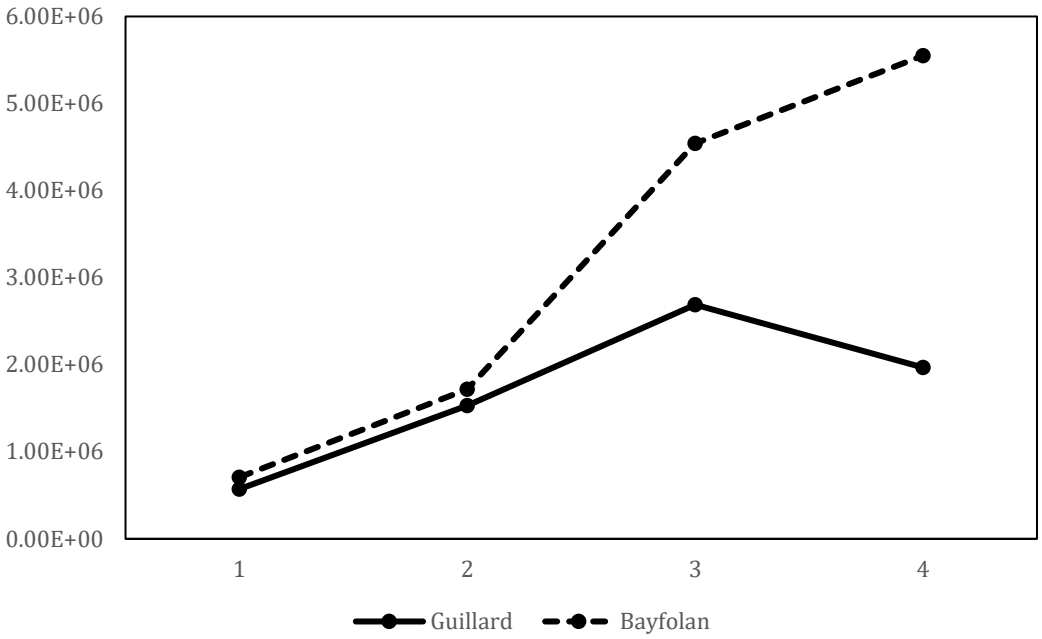

FIGURE 7: Ankistrodesmus sp. Growth Curve

(Source: Own Elaboration)

The cultivation of Tetraselmis striata with F/2 Guillard (culture medium traditional) during the adaptation phase reached a cell density of 9.81 x 105 cel/mL. When cultured with Bayfolan (culture medium alternative), the cell density during the adaptation stage was 1.85 x 105 cel/mL. Then, in the exponential phase, F/2 Guillard showed a cell density of 4.68 x 106 cel/mL and Bayfolan a cell density of 7.85 x 106 cel/mL. (Figure 8).

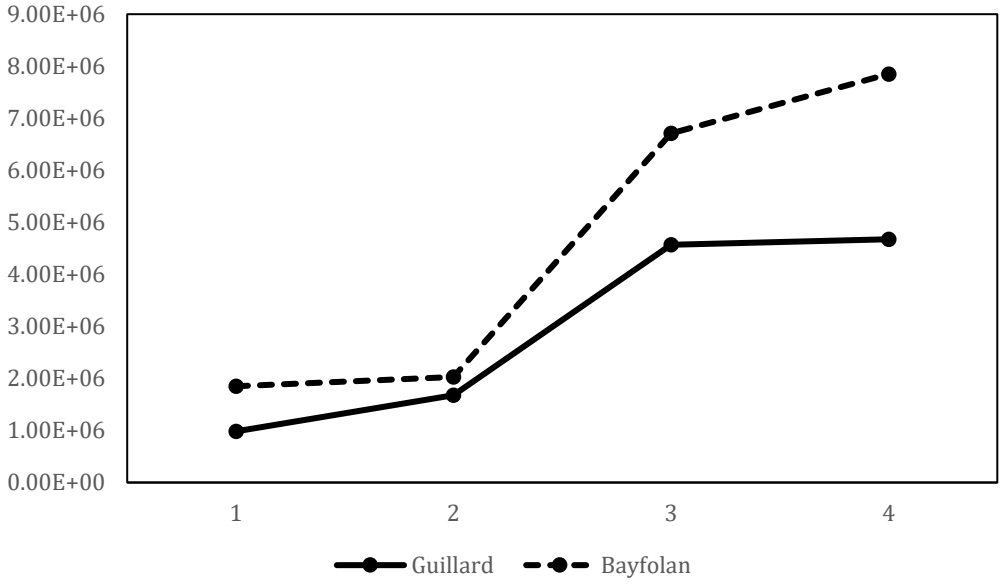

FIGURE 8: Tetraselmis striata Growth Curve

(Source: Own Elaboration)

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The cultivation of Stichococcus sp. with the medium F/2 Guillard during the adaptation phase showed a cell density of 4.56 x 105 cel/mL. When it cultured with Bayfolan, the cell density during the adaptation stage was 4.31 x 105 cel/mL. While in the exponential phase F/2 Guillard reached a cell density of 5.82 x 106 cel/mL and Bayfolan a cell density of 6.35 x 106 cel/mL. Concerning the stationary phase, the cell density for F/2 Guillard and Bayfolan were 4.19 x 106 cel/mL and 3.79 x 106 cel/mL, respectively (Figure 9).

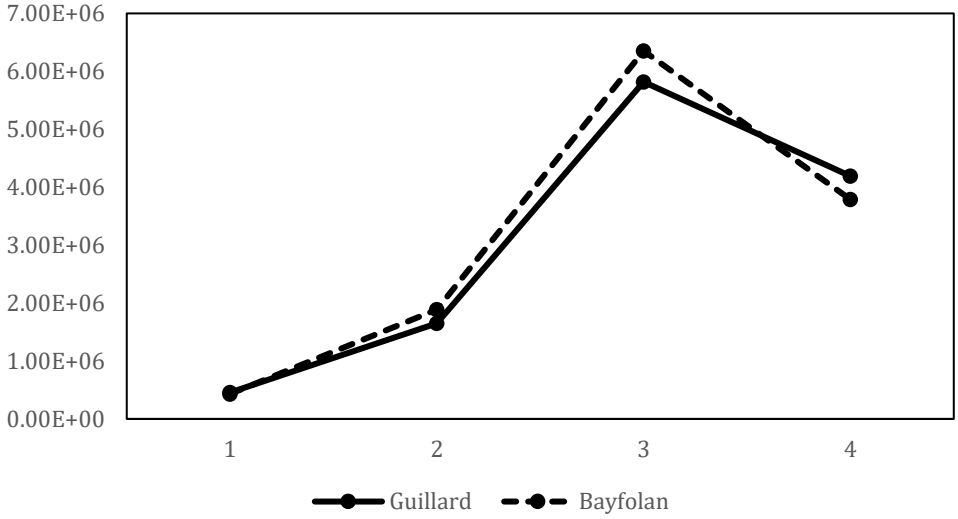

FIGURE 9: Stichococcus sp. Growth Curve

(Source: Own Elaboration)

Determination of cell density

The culture of Ankistrodesmus sp. has had the best developed with Bayfolan fertilizer, showing a maximum cell density with a value of 5.55 x 106 cel/mL during the exponential phase, compared with the maximum cell density of 2.69 x 106 cel/mL reached with the traditional nutrient Guillard (Figure 10).

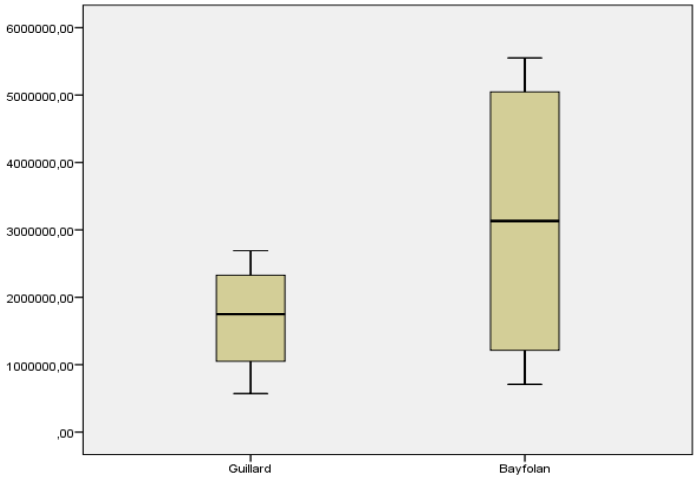

FIGURE 10: Ankistrodesmus sp. box-and-whisker plot

(Source: Own Elaboration)

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According one-factor analysis of variance, the maximum cell density of Ankistrodesmus sp. with the two foliar nutrients Guillard and Bayfolan did not present significant statistical differences ($p = 0.285 > 0.05$) (Table 3).

TABLE 3: One-way analysis of variance for the cultivation of Ankistrodesmus sp. applying Guillard and Bayfolan

	Sum of squares	fd	Mean quadratic	F	Sig.
Inter-groups	4149878832450	1	4149878832450	1.379	0.285
Intra-groups	18051551628774	6	3008591938129		
Total	22201430461224	7			

(Source: Own Elaboration)

The cultivation of Tetraselmis striata obtained better results with the fertilizer Bayfolan; since it showed higher maximum cell density with a value of 7.85 x 106 cel/mL during the exponential phase, compared with the maximum cell density of 4.68 x 106 cel/mL reached with the traditional nutrient Guillard (Figure 10).

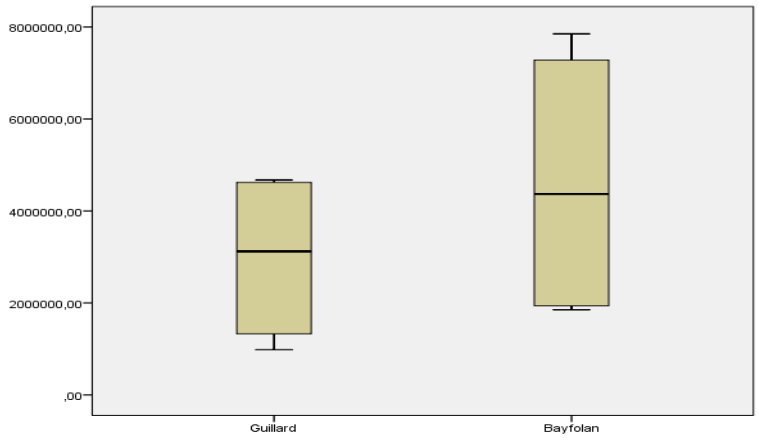

FIGURE 11: Tetraselmis striata box-and-whisker plot

(Source: Own Elaboration)

According to the one-factor analysis of variance, the maximum cell density of Tetraselmis striata with the two foliar nutrients Guillard and Bayfolan did not present significant statistical differences ($p = 0.407 > 0.05$) (Table 4)

TABLE 4: One-way analysis of variance for the cultivation of Tetraselmis striata applying Guillard and Bayfolan

	Sum of squares	**fd**	**Mean quadratic**	**F**	**Sig.**
Inter-groups	5339699582113	1	5339699582112.5	0.795	0.407
Intra-groups	40291216770453	6	6715202795075.5		
Total	45630916352566	7			

(Source: Own Elaboration)

The cultivation of Stichococcus sp. showed higher maximum cell density with the fertilizer Bayfolan with a value of 6.35 x 106 cel/ mL during the exponential phase, compared with the maximum cell density of 5.82 x106 cel/mL reached with the traditional nutrient Guillard (Figure 12).

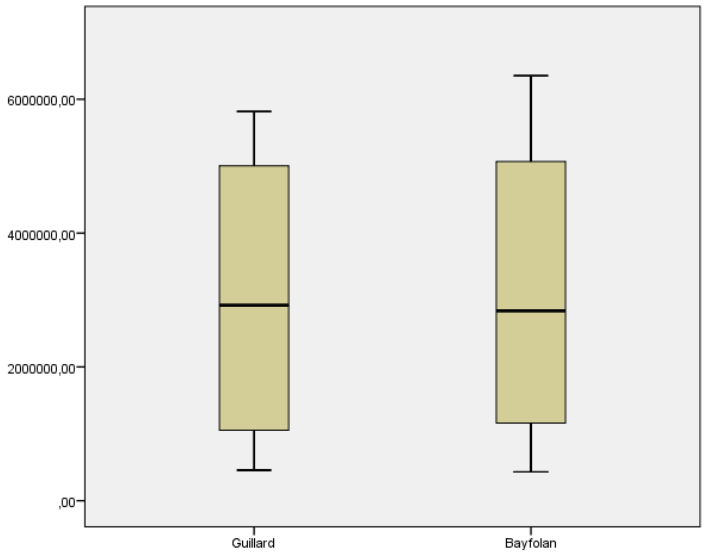

FIGURE 12: Stichococcus sp. box-and-whisker plot

(Source: Own Elaboration)

According to the analysis of variance of a factor of variability, the maximum cell density of Stichococcus sp. with the two foliar nutrients Guillard and Bayfolan did not present significant statistical differences (p = 0.963> 0.05) (Table 5).

TABLE 5: One-way analysis of variance for the cultivation of Stichococcus sp. applying Guillard and Bayfolan

	Sum of squares	**fd**	**Mean quadratic**	**F**	**Sig.**
Inter-groups	14727570313	1	14727570313	0.002	0.963
Intra-groups	37296121984375	6	6216020330729		
Total	37310849554688	7			

(Source: Own Elaboration)

Determination of the specific growth rate

The constant growth rate of microalgae supplying the nutritive means Guillard and Bayfolan (Table 6) show that the cultivation of Ankistrodesmus sp. presented better specific rate of growth with the Bayfolan medium than with the Guillard medium with values of 0.18 days-1 and 0.30 days-1, respectively. In the case of Tetraselmis striata, presented the highest specific growth rate with the Guillard medium than with the Bayfolan medium with values of 0.23 days-1 and 0.21 days-1, respectively. For the cultivation of Stichococcus sp., showed the highest standards of growth with the Guillard medium that with Bayfolan medium with values of 0.32 days-1 and 0.31 days-1, respectively (Figure 13).

TABLE 6: Specific growth Rate

Specie	Guillard	Bayfolan
Ankistrodesmus sp.	0.18	0.30
Tetraselmis striata	0.23	0.21
Stichococcus sp.	0.32	0.31

(Source: Self-elaboration)

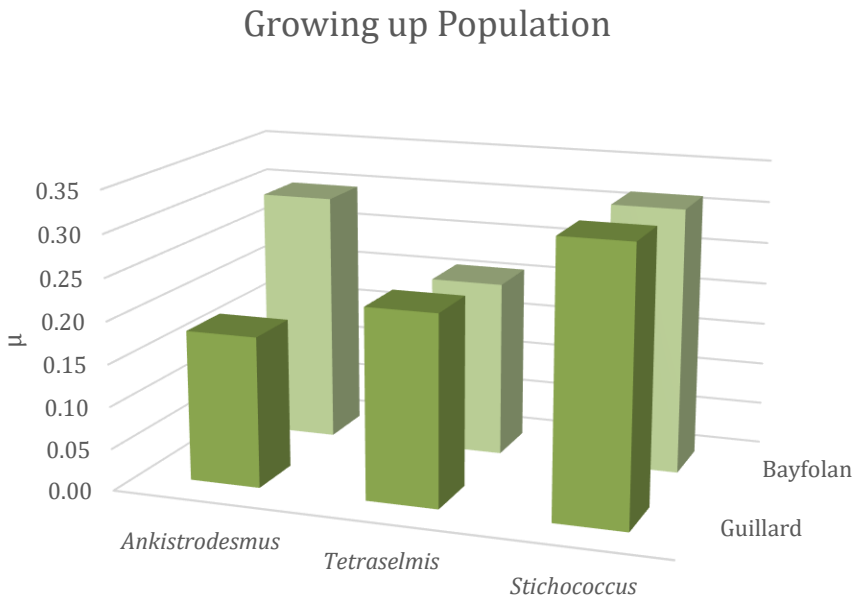

FIGURE 13: Specific growth rate of crop species

(Source: Own elaboration)

Comparing the values of the specific growth rate obtained during the exponential phase of the crops, the one-way analysis of variance showed that there were no significant differences ($p = 0.579 > 0.05$) between the application of the mediums culture used (Table 6).

TABLE 6: One-factor ANOVA for the specific growth rate

	Sum of squares	fd	Mean quadratic	F	Sig.
Inter-groups	0,002	1	0.002	0.364	0.579
Intra-groups	0,017	4	0.004		
Total	0,018	5			

(Source: Self-elaboration)

DISCUSSION

This research shows results of biomass production of controlled cultivation of microalgae Ankistrodesmus sp., Tetraselmis striata and Stichococcus sp. for the proper obtaining of the biomass of microalgae, which constitutes a requirement for the Commercial production of metabolites, as mentioned by Molina et al. (2002). There are several cultivation

systems, and their efficient development is relevant for the various industrial applications (Matsunaga et al. 2005). In this experience, we used an intensive cultivation system, batch and monospecific according to its purity.

The cell density for the microalgae cultivated with the culture medium Bayfolan showed higher cell densities in similar or reduced periods compared to the use of The F/2 Guillard medium.

The species tested are a renewable resource, as they exist in nature, and it was possible to mass their cultivation in a controlled environment, Valenzuela and Valenzuela (2014). Similarly, we agree with Álvarez and Gallardo (1989), since in this work has been managed to develop the technology of cultivation and scaling up to 20L, using the inoculum of the species of interest in appropriate vessels of ascending capacity, highlighted as factors of growth by MAGRAMA (2013) the culture mediums, the lighting and an adequate aeration.

The specific growth rate and cell density of microalgae strains are affecting by crop parameters, such as light, temperature, pH, and nutritional status. The increase of its density implies the decrease of the sun and depletion of nutrients received by each cell, influencing negatively the obtaining of biomass (Matsunaga et al., 2005). In this experience, Ankistrodesmus sp. presented a higher specific growth rate with Bayfolan than with F/2 Guillard, while Tetraselmis striata and showed similar specific growth rates with both nutrients.

Regarding the cell division of microalgae tested according to the nutrients used, Ankistrodesmus sp. presented less duplication time with Bayfolan than with F/2 Guillard. Compared with the doubling times of Tetraselmis striata and Stichococcus sp., which were not statistically different with both nutrients. Ratifying the aforementioned by Vonshak (1990) who defines the algae as microorganisms that present a single cycle of cell division of a few hours, favouring its development and production process much faster in contrast to crops.

The cultivation conditions established in the microalgae culture protocol were associated with an average temperature of 21°C ± 1°C, pH 7.7, average light intensity 2126 lux, dissolved oxygen average of 6.04 mg/L and average salinity of 35146 PSU (only in the Case of Tetraselmis striata). We support the version of Laing (1991), who noted that the growing conditions necessary for the growth of a population of microalgae respond to the mutual interaction of biological, physical, and chemical factors.

Therefore, it is necessary to emphasize that the changes in the manipulation of the conditions of cultivation as well as of the nutrients, could generate an influence in its metabolism, as indicated Greque et al. (2015), who suggest that microalgae metabolism reacts against changes in the external environment, by the accumulation of metabolites in their metabolic pathways. Biosynthesis of metabolites can be triggered by changes in temperature, salinity, UV radiation, and nutrient deprivation (Paliwal et al. 2017).

The fact of submitting the crops to different nutritious means combined with the exponential phase characterized by the assimilation of nutrients supplied to each cell in the culture and active asexual reproduction, it is possible to accumulate the highest cell density of the life cycle of each species of microalgae, directly influencing the biochemical composition. Ratifying what was pointed out by Brown et al. (1997), who support that depending on the phase of the life cycle in which they are located, the proximal composition of the microalgae can be modified, and thus generate important secondary metabolites due to the physiological changes to which they are subjected.

Even though the production of proteins has been the first industrial application developed of the microalgae because they reach up to more than 50% of the dry biomass, emphasizing Spirulina, Chlorella, Scenedesmus, and Dunaliella because of their high concentration of proteins (Camacho et al. 1988). There is extensive literature on biocomposites that microalgae can synthesize and may constitute alternative sources of obtaining essential fatty acids (Ferreira et al. 2013), to promote new pharmacological, chemical agents (Faulkner 1984). Secondary metabolites (Iwamoto et al. 1989, Shimizu 1996) with several biological actions as allelopathies

compounds by inhibitory effect against competitors or predators (Ten 2007), defense mechanisms to predation, herbivory, and competition for space (De Lara-Isassi et al. 2000; De Nys et al. 1998).

There is an infinity of research and microalgae species to be researching. For the moment, Ankistrodesmus sp. is adapted to the technology of cultivation of microalgae and required to improve its gardening for the nutrition and practical studies in different fields (Tavares and Pereira, 2008). Tetraselmis striata is the most used in aquaculture as food for larvae, juvenile bivalve mollusks, rotifers for feeding larvae of crustaceans and marine fish (Borowitzka 1997; Pauw et al. 1984), considering that the extraction by aquaculture is surpassing fishing, with the purpose of eliminating the hunger, to promote the health and to reduce poverty (FAO 2016). Stichococcus sp. presents appropriate characteristics for the accumulation of fatty acids for use in the production of fuel (Gargano et al., 2013) and the capture of greenhouse gases (Sivakumar 2014).

They are potential species to execute experiences in our environment, provided if high biomass has ensured by implementing larger scale and optimal cultivation systems, such as photobioreactors (Debowski et al. 2012; Avalos 2004).

CONCLUSIONS

From the results obtained, it has concluded that the hypothesis of the research work has accepted, considering the following:

The culture of the microalgae Ankistrodermus sp, Tetraselmis striata and Stichococcus sp. that have carried out with a foliar fertilizing (Bayfolan) compared to the culture with a traditional medium (F/2 Guillard), they shows an increase in the cell density of 100%, 68% and 9%, respectively.

The microalgae Ankistrodermus sp., Tetraselmis striata and Stichococcus sp. of fluvial origin (Locumba River, Jorge Basadre province, Tacna department) were isolated and incorporated into the IMARPE ceparium, Ilo headquarters.

The cultivated microalgae are a source of secondary metabolites, which have extracted for their subsequent application or use in the pharmacological industry.

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