Journal of Anatolian Environmental and Animal Sciences
(*Anadolu Çevre ve Hayvancılık Bilimleri Dergisi*)
DOI: https://doi.org/10.35229/jaes.1468898

JAES
Year: 9, No: 3, 2024 (299-306)
AÇEH
Yıl: 9, Sayı: 3, 2024 (299-306)

ARAŞTIRMA MAKALESİ RESEARCH PAPER

Living in Geothermal Energy Plant Emergency Pond: Isolation and Cultivation of *Arthrospira platensis* to Stressful High Temperature in the Geothermal Ponds

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Received: *16.04.2024* ***Accepted:*** *11.06.2024* ***Published:*** *30.09.2024*

How to cite: **Girgin, B., Balcı, K., Solmaz M. & Durmaz, Y. (2024).** Living in geothermal energy plant emergency pond: isolation and cultivation of *arthrospira platensis* to stressful high temperature in the geothermal ponds. *J. Anatolian Env. and Anim. Sciences,* ***9***(3), 299-306. https://doi.org/10.35229/jaes.1468898

Atıf yapmak için: **Girgin, B., Balcı, K., Solmaz M. & Durmaz, Y. (2024).** Jeotermal Enerji Santrali Acil Durum Göletinde Yaşam: Jeotermal Havuzlarda Arthrospira Platensis'in Stresli Yüksek Sıcaklığa Karşı İzolasyonu ve Kültürü. *Anadolu Çev. ve Hay. Dergisi*, ***9***(3), 299-306. https://doi.org/10.35229/jaes.1468898

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Abstract: Geothermal waters are waters of rich minerals because of high pressure and temperature deep underground, and they reach the earth for the first time. This work was to evaluate the phytoplankton inhabiting geothermal emergency pond water. Therefore, microalgae species in the future will focus on large-scale cultivation in geothermal emergency ponds and the results obtained in terms of technology optimization use of geothermal fluids. The growth performance of *Arthrospira platensis* isolated from geothermal inert water in different nutrient media which was geothermal water (control group), Spirulina medium (SP), Zarrouk medium (ZM) and Blue Green medium (BG11), was investigated. Optical density and the number of cells in the culture are the leading indicators for algae growth. It has been observed that Zarrouk Medium is more effective in this type of algae compared to other nutrient media. The highest cell number in *A. platensis* culture was 35.43x10^4 cells mL^{-1} in the Zarrouk medium. These species' culture cell numbers were detected in the Spirulina medium, control group, and BG11 medium as 23.75 x10^4, 6.25 x10^4, and 6.25 x10^4 cells mL^{-1}, respectively. We can deduce that these environments better provide the nutrients necessary for algae's photosynthesis and cell growth. In particular, the total hardness of water decreased from 21.4±1.04 ppm to 1.5±0.14 ppm, the amount of sulphate from 44±0.49 ppm to 0 ppm, alkalinity from 3213±43.5 ppm to 716±58.9 ppm and the iron content from 20.3±2.23 ppm to 4.1±0.15 ppm. In this study, because of the production of *A. platensis* species in the water in an emergency pond, the production of species containing a maximum of about 43% protein was realized.

Keywords: *Arthrospira platensis*, culture, geothermal emergency pond water, geothermal energy plant.

Jeotermal Enerji Santrali Acil Durum Göletinde Yaşam: Jeotermal Havuzlarda Arthrospira Platensis'in Stresli Yüksek Sıcaklığa Karşı İzolasyonu ve Kültürü

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Öz: Jeotermal sular yerin derinliklerinde yüksek basınç ve sıcaklıktan dolayı zengin mineraller içeren ve yeryüzüne ilk kez ulaşan sulardır. Bu nedenle, gelecekte mikroalg türlerinin, jeotermal acil durum havuzlarında büyük ölçekli yetiştiriciliği ve jeotermal akışkanların teknoloji optimizasyonu kullanımı açısından elde edilen sonuçlara odaklanacaktır. Jeotermal atıl sudan izole edilen *Arthrospira platensis*'in jeotermal su (kontrol grubu), Spirulina ortamı (SP), Zarrouk ortamı (ZM) ve Mavi Yeşil ortam (BG11) olmak üzere farklı besin ortamlarında büyüme performansı araştırıldı. Optik yoğunluk ve kültürdeki hücre sayısı alglerin büyümesinin ana göstergeleridir. Zarrouk ortamı bu tür alglerde hem optik yoğunluk hem de hücre sayısı açısından diğer besin ortamlarına göre daha etkili olduğu gözlenmiştir. *A. platensis* kültüründe en yüksek hücre sayısı Zarrouk besin ortamında 35,43 x10^4 hücre mL^{-1} olarak ölçülmüştür. Bu tür kültür hücre sayıları Spirulina ortamında, kontrol grubunda ve BG11'de sırasıyla 23,75 x10^4, 6,25 x10^4 ve 6,25 x10^4 hücre mL^{-1} olarak tespit edildi. Bu ortamların alglerin fotosentezi ve hücre büyümesi için gerekli besinleri daha iyi sağladığı düşünülebilir. Özellikle suyun toplam sertliği 21,4±1,04 ppm'den 1,5±0,14 ppm'e, sülfat miktarı 44±0,49 ppm'den 0 ppm'ye, alkalinitesi 3213±43,5 ppm'den 716±58,9 ppm'e ve demir içeriği 20,3±2,23 ppm'den 4,1±0,15 ppm'e düşmüştür. Bu çalışmada jeotermal santralinin acil durum havuzu suyunda *A. platensis* türlerinin üretimi nedeniyle maksimum %43 civarında protein içeren türlerin üretimi gerçekleştirilmiştir.

Anahtar kelimeler: *Arthrospira platensis*, jeotermal acil durum havuz suyu, jeotermal enerji santrali, kültür

INTRODUCTION

Geothermal resources are formed by heating groundwater where volcanic activity under the ground approaches the surface. In addition to volcanic activities, due to the thinning of the earth's crust, which is opened due to tectonism, magma is coming to the earth's surface, and geothermal resources are being formed by heating the surface waters. The geothermal systems developed in Büyük Menderes Graben and Gediz Graben in Turkey can be shown as an example. The geothermal resources in these regions, especially the temperatures above 150°C, have intensified the investments of power plants in the area. Geothermal waters are waters of rich minerals that have reached the earth for the first time due to high pressure and temperature deep underground. Besides energy production, geothermal resources are used in many areas, such as agriculture, greenhouses, residential heating, industrial drying, and cooling. Many new studies have been initiated in geothermal mineral mining and agricultural fields, especially recently due to their rich mineral content.

Maspo Energy geothermal power plants is an enterprise operating in Alaşehir region of Manisa with two geothermal power plants with a total power of 44 MWe. These plants utilize the heat extracted from geothermal fluids for energy production, after which the cooled fluids are redirected back into the earth via re-injection wells using pumps. In all geothermal power plants, when electricity production is interrupted, the geothermal fluid from the wells is collected in emergency ponds (mud pits) for a certain period. Emergency pools with different water-holding capacities are covered with thick, waterproof tarpaulins. Upon resuming operations, the fluid is pumped back into the re-injection wells from these ponds. A residual amount of water typically remains at the bottom of these ponds, gradually cooling down if the production process continues.

Furthermore, emergency pools are constructed at sites where production wells are drilled to extract fluid. These pools are intended to contain the water discharged during the initial flow aimed at heating the well, a process that occurs during the early stages of commissioning and persists even after the completion of the wells. After the wells are operational, the water in these pools begins to cool, with the cooling rate varying based on the duration the water remains stored (as illustrated in Figure 1).

Research on microalgal taxonomy and ecology of the aquatic environment has mostly focused on more extensive and permanent water bodies such as rivers, lakes, and oceans (Arguelles, 2019a,b; Zafaralla 1998). Recent studies show that precursor microorganisms such as microalgae in aquatic microcosms produce essential substances such as oxygen, nutrients, and polysaccharides consumed by other organisms (Brouard et al., 2011; Ramos & Moura, 2019). Water resources derived from geothermal fluid provide an excellent habitat for microalgae growth and proliferation. However, studies on the taxonomy and diversity of this group of microorganisms are still limited (Arguelles, 2021a,b; Arguelles, 2020; Poniewozik et al., 2020; Ramos et al., 2018).

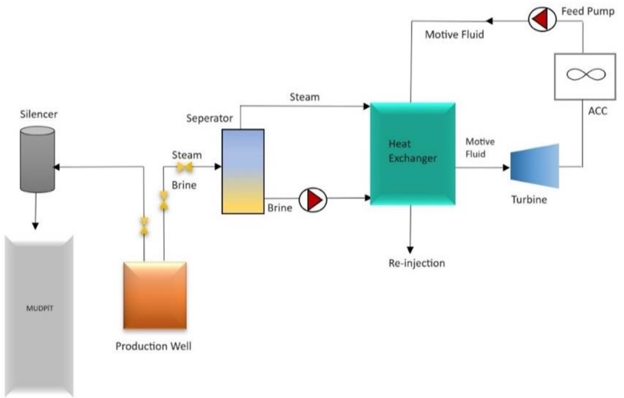

Figure 1. Maspo Energy Geothermal Energy Plant Diagram.

Consequently, the geothermal emergency pond constitutes a natural laboratory for the analysis of adaptation by phytoplankters to highly stressful conditions. To distinguish between the pre-selective or post-selective origin of adaptation processes allowing the existence of phytoplankters in the pond, isolated from extreme waters from the geothermal emergency pond was used as a selective factor. However, in the case of thermophilic micro-algae and cyanobacteria inhabiting geothermal emergency ponds, a question remains: How has it adapted to survive and proliferate under stressful conditions? Suppose the stressful conditions do not exceed the limits of the physiological tolerance of algae. In that case, the survival results from physiological adaptation (i.e., acclimation) supported by gene expression modifications (Bradshaw & Hardwick, 1989). Various adaptive mutations or related phenomena have also been recognized in yeast (Heidenreich, 2008). However, as far as we know, they have not been reported in other microorganisms such as cyanobacteria and microalgae.

Algae species main requirements for growth are nutrients, CO_2, and water. Microalgae lower carbon footprints by capturing CO_2 during photosynthesis (Righelato & Spracklen 2007). Another advantage of algae over land crops is that utilizing algae could be utilized for wastewater treatment results in minimizing usage of fresh water as a growth medium. Microalgae can be cultivated using variable climates and non-arable land, including marginal areas that are inappropriate for farming purposes, non-potable water, and wastewaters (Campbell, 1997; Chisti, 2007, 2008). Since shortage of drinking water is a rising challenge for the world and the amount of freshwater on the planet has remained fairly constant over time, there is a crucial need to have beneficial use of non-fresh water, such as geothermal water. Geothermal water often contains high concentrations of ions, including some nutrients that which are believed to be vital for algae growth such as K^+, HCO_3^-, and PO_4^{3-} (Rastegary, et al., 2013). Geothermal water was being used to grow algae in the Group Field of Bulgaria for processing green, Chlorella and Scenedesmus, and blue-green, Spirulina, algae biomass (Bojadgieva et al., 2000). Others report specialists from the Bulgarian Academy of Sciences producing Spirulina blue-green algae in the Therma-Nigrita field in Greece (Karydaki et al., 2005).

This work aimed to evaluate, from an evolutionary point of view, the adaptation of cyanobacteria and microalgae to growth and survival in the stressful environment of an emergency pond on geothermal plants. For this purpose, first, we will carry out a survey of phytoplankton inhabiting geothermal emergency pond water. After that, isolated microalgae species will be cultured in different mediums using geothermal waters at a high temperature. Therefore, future microalgae focus on the large-scale cultivation of microalgae species in geothermal emergency ponds and the results obtained regarding the technology optimization use of geothermal fluids.

MATERIAL AND METHOD

Study Area: The emergency ponds of Maspo Energy geothermal power plants are located between 38° 26' 31.26"E longitude and 28° 23' 9.4" N latitude in Alaşehir district of Manisa Province (Figure 2). It is at an altitude of 123.0 m above sea level. The emergency ponds are 3rd in the planned shutdown of the production well (maintenance, commissioning, or deactivation of the system, etc.) and were built with the intention of using it in running again. The total volume of the emergency pond is 800 m^3 (Figure 2).

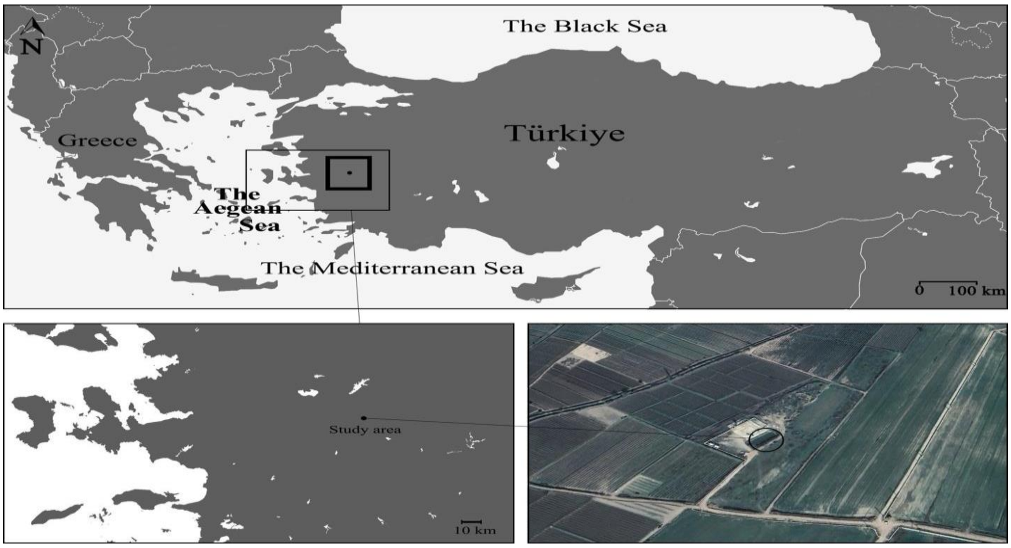

Figure 2. Study area and location of Maspo Energy Geothermal Energy Plant.

Sample Collection and Water Quality: The microalgae samples were collected from the geothermal emergency pond using plankton mesh with a pore diameter of 55 meters as vertically and every month (May 2023-November 2023). The values of pH and temperature in the emergency pond were determined by using a YSI 6820-C-M probe (Yellow Springs, OH, USA). The samples taken as 5 liters were taken into sterile dark-colored containers and transported to the laboratory. The samples were poured directly into brown 100 mL glass bottles for algal identification. In two of these three glass bottles, 30 drops of Lugol's potassium iodine solution were added for preservation, and 30 drops of 4% formalin (diluted formaldehyde) were added to one bottle. Lugol's solution changes the color of the sample, so formalin, which does not change its color, was added instead. The bottles were then placed in a refrigerator.

These samples were examined microscopically and analyzed using Zeiss Primostar 3 binocular microscope provided with a ToupTek Toupcam Xcam1080 PHD digital camera at 1000x magnification. The Neubauer counting lamp was used to determine the cell numbers in the samples.

Chemical water analyses performed in the received waters were performed using titrimetric or spectrophotometric methods according to the device methods used. Temperature, pH, conductivity, salinity, TDS and resistance: with HACH brand HQ40d model device, Total hardness, Ca and Mg hardness, chloride, m-alkalinity and p-alkalinity: BRAND brand class A precision model using automatic burette titrimetric methods, Sulfate, phosphate, iron, boron, silica, AKM: Analyses were performed using HACH brand DR3900 model spectrophotometric methods.

Identification: Identification of algae was carried out following Cox, (1996) for diatoms, Wolowski, (2002) for euglenophytes, and John and Tsarenko, (2002) for green algae. In addition, the identification of Microalgae was carried out to the lowest practical level using all possible and accessible taxonomic literature, monographs and information (Velasquez, 1962; Wehr and Sheath, 2003; Zafaralla, 1998). The color and characteristics of the sheath and cellular envelopes, the shape and size of vegetative cells, trichome characteristics and cellular filaments, the absence or presence of cellular constrictions and sheath, and morphological characteristics used in taxonomic identification, such as the number of cells in a coenobium, were considered in the taxonomic identification of different microalgae species. The taxonomic references used in the classification and identification of all observed microalgae species are listed under the taxonomic definition of each algal taxon. In addition, taxonomic names currently accepted based on the algal base (Guiry and Guiry, 2018) are used in this study. The identification of algae was made according to Kramer and Lange-Bertalot, (1991, 1999), and Huber-Pestalozzi, (1983).

Isolation and Culture Experiment: The cultured microalgae sample was isolated by diluting it with culture media at intervals and using the bulk plate method. The sample taken to culture was stored on agar (1.5%) medium, and by applying the line inoculation method to the recumbent agar (Sukatar, 2002). The purified species was incubated on sterile agar at 25°C and under continuous illumination (Wiselight brand 24W daylight).

Each sample was inoculated in plagiarized Petri dishes with different fattening places: geothermal water (control group), Spirulina medium (SP), Zarrouk medium (ZM), and Blue Green medium (BG11) and placed in an air-conditioned, illuminated cabinet. The illumination of culture was as 50 µmol m^{-2} s^{-1}photons at 30 °C.

The volume was increased so that the samples taken by scraping over purified agar were 15 mL, 50 mL, 250 mL and 1000 mL, respectively. Growth parameters were analyzed by conducting four fattening place tests on 6-liter flasks.

Analysis: Samples were taken daily to determine the cell concentration and to be able to extract growth charts. Of the 2.5 mL microalgae sample, 2 mL were counted under optical density (OD) measurement and the rest under a microscope with the help of the Neubauer counting chamber. *A. platensis* OD value was measured using SOIF UV-VIS/UV5000 branded spectrophotometer at 680 nm.

All measurements were performed in triplicate during the study. Samples were taken daily for cell count at the Neubauer hemocytometer. Specific growth rates were calculated by using the equation given below:

$$\mu = \frac{\ln(N_t) - \ln(N_0)}{t - t_0}$$

Nt is the cell number at a time (t), and No is the beginning cell number at a time to.

Statical Analysis: Two-way analysis of variance (ANOVA) was used to test the effects of the nutrient medium of culture on cell number and optical density. When differences were found in the two-way ANOVA, the Duncan multiple comparison test (HSD) of the one-way ANOVA was used to compare the mean differences by the Statistical Package for the Social Sciences (SPSS) (Version 12.0, SPSS, Chicago, IL). As such, the differences were considered significant at $p \leq 0.05$.

RESULTS

Chemical water analyses were first performed on the samples taken from the Mak-3 emergency pond (Table 1). In the light of the projection of the results obtained, ambient light and temperature optimization were provided, and microbiologically, the tendency of strain proliferation was observed in 2-liter flasks and then in 6-liter balloons with the samples taken. In this direction, species isolation has been successfully achieved in both petri dishes and tubes from the breeding strains. Scale-up operations have continued in the studies carried out.

In addition to the minerals used when algae grow, factors such as temperature, pH levels, and total hardness can also affect the algae-growing process. Algae can react to changing conditions to ensure optimal growth, leading to expected changes in mineral levels in the water. The microalgae culture process using water from the thermal power plant source shows various significant chemical and physical changes after algae cultivation (Table 1). In particular, the total hardness of water decreased from 21.4±1.04 ppm to 1.5±0.14 ppm, salinity from 6.14±021% to 2.44±0.16%, the amount of phosphate from 497.9±17.9 ppm to 415±10.12 ppm, and the iron content from 20.3±2.23 ppm to 4.1±0.15 ppm. Resetting the sulfate level in the measured water values after growing algae means a complete reduction. This shows that the algae cultivation process almost eliminates the sulfate content of water.

After the isolation process, the obtained strain was examined under the microscope (Figure 3). The size of the cells varied between diameters of 13-18 µm, filament lengths of 80-200 µm, helix diameter of 30-70 µm, microscopic filamentous structure (Figure 3). Cells were single and non-motile. Also, single-cell formation was observed without flagella. The microalgae "*A. platensis*" in the phylum of blue-green algae (Cyanobacteria) has been isolated.

Table 1. Chemical analysis of emergency pond water.

Parameters	Results	Before Culture Results	After Culture Results
pH	-	10.30 ±0.15	9.41±0.11
Conductivity	(µS/cm)	10840±145	4570±57
Total Hardness	ppm	21.4±1.04	1.5±0.14
Ca Hardness	ppm	18±0.14	0.0±0.00
Mg Hardness	ppm	3.4±0.38	1.5±0.19
Salinity	%o	6.14±0.21	2.44±0.16
SO_4^-	ppm	44±0.49	0.0±0.00
Total PO_4	ppm	497.9±17.9	415±10.12
Fe (Iron)	ppm	20.3±2.23	4.1±0.15
Boron	ppm	52.5±1.88	52 ±1.53
Alkalinity (p-alk)	ppm	3213±43.5	716±58.9
Alkalinity (m-alk)	ppm	3110±55.3	1684±34.7
AKM	ppm	321±4.7	100±2.4
TDS	g/L	5.96±0.52	2.39±0.43
Resistance	Ω.cm	91.6±6.82	218.7±12.68

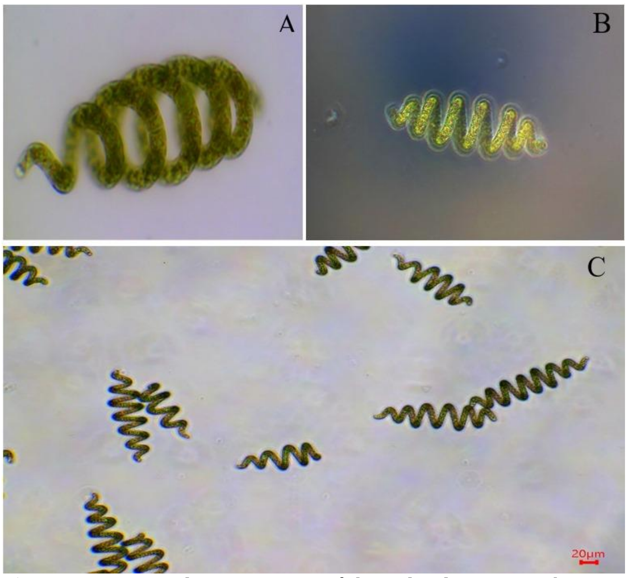

Figure 3. Images and measurements of the isolated species *Arthrospira platensis* under a microscope in the geothermal water (image; A,B,C and a- coil measurements, b- height measurement)).

Culture trials of *A. platensis* species have been conducted in four different nutrient media (Figure 4). According to the highest growth of the optical density was achieved at 6th day with Zarrouk medium, while a similar result with Zarrouk medium was obtained in the culture trial using Spirulina medium ($p<0.05$). However, low growth was recorded in the control group and BG11 medium and it was analyzed that it was statistically different from the results using other mediums ($p<0.05$).

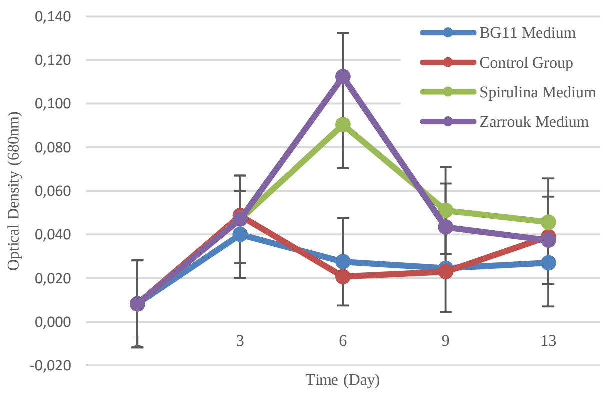

Figure 4. Optical density of *Arthrospira platensis* cultures with different nutrient medium and geothermal water.

The highest cell number in *A. platensis* culture was measured as 35.43 x 10^4 cells mL^{-1} in Zarrouk medium in the shown Figure 5 ($p<0.05$). This species culture cell numbers were detected in the Spirulina medium, control group and BG11medium as 23.75 x 10^4, 6.25 x 10^4, and 6.25 x 10^4 cells mL^{-1}, respectively. It has been determined that Zarrouk medium is the best medium for this species of culture.

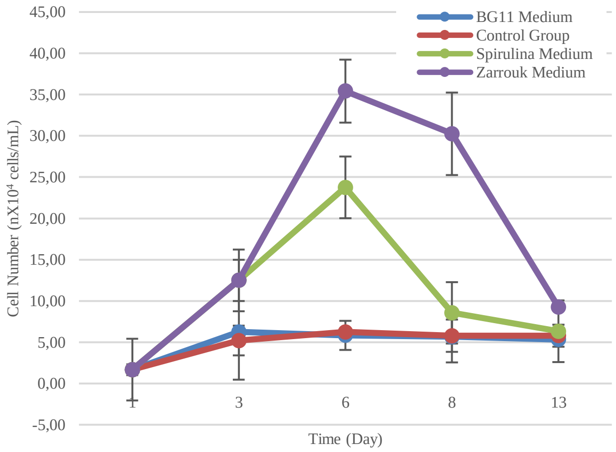

Figure 5. The number of *Arthrospira platensis* cells cultured with different nutrient media and geothermal water.

DISCUSSION

Various photosynthetic microorganisms were present in the emergency pond; however, our studies resulted in the isolation of algae exhibiting different morphology of *A. platensis* in the Maspo Energy geothermal power plants emergency pond. The isolated species was shown to have similar green-blue, coiled filaments and to be morphologically compatible with the genus *Arthrospira* (Morais & Costa, 2008). *A. platensis*, a filamentous cyanobacterium, is widely used in many countries as health food due to its protein content and biochemical substances for immune system. It is known that the environmental conditions, especially culture temperature and light intensity greatly influence the composition and physiological state of phytoplankton (Reynolds, 1984) protein and amino acid metabolism in the cells (Becker, 1994; Borowitzka & Borowitzka, 1988).

Using native species that naturally adapt better to local conditions not only provides the potential to increase productivity and a more favorable cost-benefit ratio, but also minimizes the risk of environmental impact (Morais & Costa, 2008). It is envisaged that microalgae can be used to make the wastewater of such enterprises useful. However, the isolation and cultivation of commercially important microalgae in the facility's own waters is of great importance. It has been observed that the species that adapt to the region simultaneously exhibit superior growth and reproduction, which reveals their importance. There is no prior evidence that this species has been isolated from geothermal energy wastewater. The presence of this species in the waters considered as waste and its ability to thrive are important indicators for the potential use of these waters.

Abundant in terms of proteins and bioactive pigments, microalgae offer a promising potential as a source of new food and agricultural products due to their various applications (Lafarga, 2019; Refaay et al., 2021). Due to these characteristics, the species formed in thermal energy wastewater have been evaluated for fertilizer and agricultural industry and the potential of organic fertilizer in the agricultural field has been revealed. In the experiments conducted on apple trees, it has been recorded that the yellowing of the leaves on the trees has been eliminated. In addition, seasonal temperature increase stress was reduced on the ages and an increase in sprouting and fruit quality was recorded.

When evaluated before and after algae production, it was found that the increase in the resistance level of water and the decrease in conductivity measurements usually decreased the electrical conductivity of water or the water contained less ionic substances. It has been analyzed that when the water resistance increases, the ions or dissolved minerals in the water decrease, or the water is purified. This may indicate that the water may have been purified or purified more, or that the ionic substances in its content have decreased. However, the increase in the resistance level alone may not be enough to make a full assessment of the water quality. The complete water content and chemical composition should be evaluated to understand whether it is suitable for algae cultivation. In order to better understand the reasons for the increase in the resistance level, other properties of water and analyses should also be taken into account.

Such a decrease in Ca hardness usually indicates a large decrease in calcium in the water content. The fact that geothermal water showed such a significant decrease in Ca hardness only when growing spirulina-type algae may indicate that it may have used calcium for algae growth. A decrease in mg hardness indicates a decrease in the magnesium level in the water. Usually, the decrease in Mg hardness can be associated with the magnesium consumption of algae in the process of algae cultivation. This dramatic decrease in the sulfate level indicates that the sulfate in the water has almost completely disappeared. In the geothermal water supply, it is common for sulfates to be found naturally, and normally a high amount of sulfates can be found in the water content. However, such a significant decrease in water in a process where only *A. platensis* is produced may indicate that the algae may have used sulfate during the growth process. Algae use various minerals to grow and maintain their metabolism. In this case, it can be thought that the algae may have continued their growth processes by consuming the sulfate in the water. A decrease in the phosphate level indicates that the amount of phosphate in the water is decreasing. Such a decrease in the process of algae cultivation signals that they may have exhausted the phosphate necessary for the algae to grow. Phosphates are an essential nutrient for the growth and cell division of algae. Algae reproduce by using phosphates in their growth processes. Therefore, this decrease in phosphate level can be associated with the phosphate consumption of algae during algae cultivation. Chen and Pan, (2002) concluded that living *A. platensis* cells have a high tolerance to lead (concentration below 50 mg L^{-1}) and are good adsorbing agents.

The growth performance of *A. platensis* isolated from geothermal inert water in different nutrient media was investigated. Optical density and the number of cells are the main indicators for the growth of algae. It has been observed that Zarrouk medium and Spirulina medium are more effective in this type of algae compared to other nutrient media, both in terms of optical density and cell number. It can be thought that these environments better provide the nutrients necessary for algae's photosynthesis and cell growth.

A. platensis consists of 55-70% protein, 15-25% carbohydrates, 5-6% total lipids, 6-13% nuclear acids (DNA and RNA), 2.2-4.8% minerals, 25% total polysaccharides carbohydrates and 1.5-2% of the total lipid content Polyunsaturated Fatty Acids (PUFAs) (Hosseini et al., 2013; Wan et al., 2016). *A. platensis* contains high levels of protein (50-70%) that is associated with health food, pharmaceuticals and nutraceuticals (Cohen et al., 1987) Low temperatures cause a decrease in *growth* and the protein content of *Spirulina* (Tomaselli et al., 1988). In this study, as a result of the production of *A. platensis* species in the emergency pond water of Maspo Energy geothermal power plants, the production of species containing a maximum of about 43% protein was realized. While the protein value is expected to be 60-65% under normal conditions, it is assumed that temperature, production conditions and drying temperature will be effective. For instance, negative effect of increased phosphate concentration on protein content of *A. platensis* was stated (Markou et al., 2012). Also, another study conducted with Zarrouk medium shows that the initial culture pH of *A. platensis* affected the protein content directly (Pandey et al., 2010). Colla et al., (2007) stated that *A. platensis* culture at 35 °C with 1.875 g L^{-1} sodium nitrate concentration provided 21% more protein content than at 30 °C with 1.250 g L^{-1} sodium nitrate concentration. In line with the previous literature data, no definitive conclusion can be drawn about the suitability of the *A. platensis* strain obtained in this study regarding protein production. It is thought that future studies conducted with this strain under different culture conditions will be helpful.

Compliance with Ethical Standards

Conflict of Interest : The authors declare that there is no conflict of interest.

Ethical Approval: For this type of study, formal consent is not required.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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