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# Characterization of *Arthrospira platensis* cultured in wastewater of Clarias catfish farming media: DNA barcode, helical form, growth, and phycocyanin

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**Abstract.** *Wijayanti M, Syafudin M, Yulisman, Nurianti Y, Hidayani A, Gofar N. 2020. Characterization of* Arthrospira platensis *cultured in wastewater of* Clarias *catfish farming media: DNA barcode, helical form, growth, and phycocyanin. Biodiversitas 21: 5872-5883. Arthrospira* production technology in catfish waste media can be an alternative to reduce environmental pollution. However, some environmental factors such as nutrition, light, and water content can influence characterization of *Arthrospira* at the genetic and physiologic level. *Arthrospira platensis* is one of the phycocyanin-producing *cyanobacteria* and can be cultured using catfish culture wastewater. Water quality especially pH and salinity can effect of growth rate and residue of phycocyanin from *Arthrospira platensis*. This study aimed to identify the species and morphological forms of *Arthrospira* cultured using technical fertilizer and waste media, as well as to know the phylogenetic trees between species in this study and the GeneBank based on the 16S rRNA gene, and determine the optimum of pH and salinity required in the medium of catfish culture wastewater to phycocyanin maximum production of *Arthrospira*. The optimation of pH and salinity method used Completely Randomized Design (CRD) factorial with 2 factors consisting of the first factor with 3 treatments and the second factor with 4 treatments and 3 replications. The first factor was pH of culture medium i.e. pH 6.5 ± 0.2, pH 8.5 ± 0.2 and pH 10.5 ± 0.2. The second factor was salinity of culture medium, that was salinity 0 ppt (parts per thousand/‰), 10 ppt, 20 ppt, and 30 ppt. Parameters observed in *Arthrospira* include density, growth rate, rendement of phycocyanin, and decreased total nitrogen and phosphate content in culture media. The results showed that morphology *Arthrospira* cultured on technical fertilizer media (AF) had a longer and helix filament compared to *Arthrospira* cultured on waste media (AW) which showed several linear and shorter filaments. Both samples have a genetic distance of 0.068 (6.8%). Phylogenetic trees indicated that AF had a close relationship with *Arthrospira platensis* petH from Japan (bootstrap value 95%). While AW formed a separate sub-cluster of AF isolates and *Arthrospira platensis* petH from Japan (bootstrap value of 85%). The best treatment in this study was P2S3 (pH 8.5 ± 0.2 with salinity 20 ppt), which produced 0.867 grams maximum density, growth rate of 22.026 %.day$^{-1}$ and 11.347 mg.g$^{1}$ rendement of phycocyanin.

**Keywords:** 16S rRNA, *Arthrospira,* catfish culture wastewater, DNA barcode, pH, phycocyanin, phylogenetic analysis, salinity, *Spirulina*

## INTRODUCTION

*Arthrospira* is a genus of cyanobacterial microalgae, commonly known under the taxonomically incorrect brand name '*Spirulina*' (Papapanagiotou and Gkelis 2019). The cyanobacterial genus *Arthrospira* Stizenberger ex Gomont 1892 contains at present 23 species, along with 12 infraspecific taxa (Guiry and Guiry 2010). They have variety characteristics of molecular, morphology and physiology that based on polyphasic approach. Various phenotypes are adaptable to various specialized ecosystems. The combination of different methods should be based on molecular sequencing as the basic approach, to which must be added other criteria (morphological, ecological) if they are available and which are distinct and recognizable in cyanobacterial populations. A polyphasic approach to include all the criteria obtained from morphological, biochemical, molecular studies, and phylogenetic to understand cyanobacterial classification as like as *Arthrospira* classification (Komárek 2018).

Recent studies have shown that *Arthrospira* can be used for treating wastewater, including effluent from fish culture, because the biomass can metabolize the nutrients and remove the pollutant from aquaculture effluent efficiently (Zhang et al. 2019). Industrial and processing wastes and by-products for culturing *Spirulina* (*Arthrospira*) are also being considered as alternative culture media, as like as aquaculture wastewater (Wijayanti et al. 2018; Widyantoro et al. 2018; Ragaza et al. 2020). Aquaculture could apply an integrated strategy of simultaneously treating aquaculture effluent while producing the biomass to supplement fish diets. The nutrient composition in their biomass depends on their environmental factor for growing biomass. Their character could be different from the various media for growth.

Basically, *Arthrospira*'s morphology is characterized by trichomes that circular regularly (helical). However, abnormal morphology can also occur in *Arthrospira* as a circular shape that is irregular even linear. In some cultivation conditions, linear filaments can spontaneously return to the helix. However, there are significant

differences in morphology, ultrastructure, physiology, biochemistry, and genetic characteristics between the original filament and the linear filament but not the difference between the origin and the returned filament. Linearization in *Arthrospira* is a variation on the genetic level that can be caused by several environmental factors such as nutrition, light, and content of water media for growth (Wang and Zhao 2005). According to Liu et al. (2016), DNA barcoding has developed as a reliable technology for identifying species based on variations in the sequence of standard DNA regions. This method is used successfully in a variety of biological applications including finding cryptic species, detecting invasive species, and identifying plants. DNA barcoding is a simple short genome sequence amplified via PCR using appropriate primers (Adamowicz 2015). DNA barcoding using the 16S rRNA gene has been widely used to determine bacterial DNA characterization. Therefore, identification of *Arthrospira* using the 16S rRNA gene needs to be done to get the characterization of *Arthrospira* that is cultured on technical fertilizer and waste media and determine the phylogenetic tree structure that has been recorded in GeneBank.

Culture of *Arthrospira* (*Spirulina*) in *Clarias* pond farming wasted water could have specific characterization for optimal pH value and salinity. Their adaptation to grow in organic wastewater makes change in bioactive and important compound production. Their biomass has a nutritional value of 55-70% protein, 6-10% lipid 20% carbohydrate, besides being rich in minerals, vitamins, and pigments (Borowitzka et al. 2016; Vernes et al. 2015). Some color pigments that can be produced such as phycocyanin (blue pigment), allophycocyanin (blue-green), and phycoerythrin (red pigment) (Sharma and Tiwari 2011; Vernes et al. 2015). Phycocyanin is pigment in *Arthrospira* which has functions as an antioxidant (Pirenantyo and Limantara 2008), a source of food coloring, cosmetics, pharmaceuticals, and drugs (Tang et al. 2020; Tiwari and Tiwari 2020), anti-inflammatory, antioxidative and anticancer (Liu et al. 2013). One of the factors that influence phycocyanin levels is biomass (Taufiqurrahmi et al. 2017). The pH and salinity of culture media can affect the biomass of *Arthrospira* (Ciferri 1983; Marek et al. 1987; Planes et al. 2002). Ismaiel et al. (2016) showed that the diversity of the chemical composition of biomass is influenced by the pH of the growth media. Value of pH and environmental factors, especially salinity, influence the productivity of cell biomass, photosynthesis, shape, and flow of cellular metabolic activities that affect the dynamics of cell composition (Hu 2004). The optimal pH value for growth of *Arthrospira* sp is 7-10.5 (Hariyati 2008), and salinity from 15-30‰ (Thajuddin and Subramanian 2005). The salinity and pH value of *Arthrospira* culture media have been known to affect the morphology of the filament.

The aims of this study are characterizing morphological forms and DNA barcode based on the 16S rRNA gene of *Arthrospira* (*Spirulina*) cultured in fertilizer and waste water effluent of *Clarias* pond farming media, and determining optimal pH value and salinity of culture media for growth and phycocyanin production, especially in *Clarias* pond farming waste water media and morphological changes of their filament.

## MATERIALS AND METHODS

### *Arthrospira* cultured in agar media

Bacto agar was weighed as much as 2 g dissolved in 100 mL of water. The water used was swamp water and catfish culture waste that has been filtered and sterilized using an autoclave. Sterilized swamp water was then added with 0.02 gram $MgSO_4$ fertilizer; $CaCl_2$ 0.004 gram; EDTA 0.008 gram; urea 0.03 gram; ZA (Sulphate of Ammonia) 0.132 grams; 0.4 gram baking soda; AB solution 1 mL mix A solution (Calcium Nitrate 64.26%, Potassium Nitrate 33.66%, Fe EDTA 2.08%) 2 grams / 10 mL and B solution (Potassium dihydro phosphate 25.83%, Ammonium sulfate 9.41%, Potassium sulfate 2.78%, Magnesium sulfate 60.91%, Cupric sulfate 0.03%, Zinc sulfate 0.12%, Boric acid 0.31%, Manganese sulfate 0.62%, Ammonium heptamolybdate 0.01%) 2 grams/10 mL water and TSP (Triple Super Phosphate) 0.05 grams were then homogeneous using magnetic stirrers. Next, wasted water was sterilized by an autoclave then cooled. Bactoagar was added to the technical fertilizer and waste solution to be homogenized using a magnetic stirrer and then boiled using a hot plate until all the ingredients dissolve and then autoclave again. The agar media was made with a pH of 7 and a salinity of 10 ppt or 10 $g.L^{-1}$ (Hidayani et al. 2019).

*Arthrospira* cultivated in liquid media was taken 100 $\mu$L using micropipette and spread to the surface of a petri dish containing bactoagar media by using a sterilized spreader rod. Petri dishes were wrapped in wrapping plastic and then given a lamp lighting (light intensity 2000-4000 lux) with a dark: light ratio = 0: 24 hours. *Arthrospira* was observed every day until it grows blue-green. After growing, *Arthrospira* was re-cultured in agar media by the 4 quadrant scratch method. The cultures were used as isolate samples for determining DNA barcodes. The biomass of *Arthrospira* was isolated from commercial *Spirulina* TopSpira East Jakarta, Indonesia.

The commercial *Arthrospira* was cultured in technical fertilizer media directly, and we used the media based on our previous study about fertilizer media on laboratory scale (Laboratorium of Aquaculture, Faculty of Agriculture, Sriwijaya University). The result showed that this fertilizer media can substitute Zarrouk Medium (ZM) for growing *Spirulina* biomass in cheaper medium than ZM (Wijayanti et al. 2018).

### DNA extraction

DNA extraction was carried out according to procedures in which there was a Presto TM Mini gDNA Bacteria Kit (Geneaid Biotech Ltd.). DNA extraction consisted of several stages: sample preparation, lysis, purification, and precipitation or washing. The sample used was 0.15 grams of wet weight for one extraction (Geneaid manual).

**DNA amplification**

The process of DNA amplification using the PCR (Polymerase Chain Reaction) method was performed using 2 $\mu$L forward primers 63f (5'-CAGGCC TAA CAC ATG CAA GTC-3 ') and reverse primer 1387r (5'-GGG CGG WGT GTA CAA GGC-3 ') (Marchesi et al. 1998). The total composition of the PCR mixture was 50 $\mu$L which consisted of 25 $\mu$L Go Taq Green, 13 $\mu$L NFW (Nuclease Free Water) and 8 $\mu$L *Arthrospira* DNA extraction template. DNA amplification was carried out in stages: the initiation cycle at 95°C for 5 minutes, followed by 30 denaturation cycles at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, then the extension stage at 72 °C for 1 minute, and the final stage 72 °C for 7 minutes (Lee et al. 2003).

**Electrophoresis**

Electrophoresis was carried out using 1% agarose gel at 75 V for 35 minutes. Agarose that has been electrophoretic was immersed with a mixture of 10 $\mu$L diamond dye solution and 100 mL TAE 1x buffer solution for 30 minutes without exposure to light. The results were visualized through gel documentation by observing DNA migration using a transilluminator UV.

**Gene sequencing**

*Arthrospira* DNA samples that were successfully amplified using PCR were then sequenced in the fragments of 16S rRNA gene. The amplified products were sequenced through the services of the Macrogen Institute in Jakarta. The DNA sequences obtained in the form of fasta format were aligned using MEGA 6.0 software and then uploaded through the Basic Local Alignment Search Tool (BLAST) program. BLAST was a program to search for and analyze the homology of an organism's sequence, on the ncbi.nlm.nih.gov website so that its homology can be identified with another genus *Arthrospira* 16S rRNA gene sequences registered in the GenBank database. The genetic distance and phylogenetic trees between genera were constructed using the Neighbor-Joining (NJ) method. The phylogenetic tree was constructed through the Mega 6.0 software application using the Neighbor-Joining (NJ) method of the Maximum Composite Likelihood model and Substitutions to include d: Transitions + Transversions with 1000x bootstrap. Meanwhile, *Arthrospira* morphological form analysis was presented in the form of images and discussed descriptively by referring to the appropriate literature.

**Optimization of pH and salinity for growing *Arthrospira* in Catfish farming wasted water**

The experimental design for optimizing pH and salinity media for growing *Arthrospira* in Catfish farming wasted water was a Factorial Completely Randomized Design (CRD) consisting of the first factor with 3 treatments and the second factor with 4 treatments and 3 replications. The first factor was the difference of pH in culture media, including P1: culture media pH 6.5 ± 0.2, P2: culture media pH 8.5 ± 0.2, and P3: culture media pH 10.5 ± 0.2. The second factor was the difference of salinity in culture media i.e. S1: salinity of 0 ‰, S2: salinity of 10 ‰, S3: salinity of 20 ‰, and S4: salinity of 30 ‰.

**Culture preparation**

The equipment used in this study was sterilized using 70% alcohol to minimize the contaminants that inhibit the productivity of *Arthrospira*. The containers used plastic bottles with capacity of 5 L volume of 36 units. The plastic bottle was sterilized using a potassium permanganate solution (2 mg. $L^{-1}$). Catfish culture wastewater obtained from catfish farming ponds measuring 2 m x 1 m x 1 m, and high water media was 20 cm (Figure 1). The density used in the pond was 330 fish.400 $L^{-1}$ with 150 grams $fish^{-1}$, maintained for 2 months by providing artificial feed (protein 31%-33%), twice per day at satiation. Catfish culture wastewater was previously sterilized by boiling in an autoclave and then cooled, while the steril wastewater was treated with salinity. In treatments S1, S2, S3, and S4 were added with salt until salinity was obtained according to the treatment. The wastewater media had a pH of 7.3, therefore there was an addition of HCl 1 N of 0.75 mL $L^{-1}$ in P1 treatment to reach a pH of 6.5. Meanwhile,. in treatments P2 and P3, to get a pH of 8.5 and pH 10.5 there was an addition of NaOH 8 N as much as 0.07 mL $L^{-1}$ and 0.45 mL $L^{-1}$.

***Arthrospira* cultivation**

*Arthrospira* previously used was cultured in catfish culture wastewater for culture stock with a density of 2 g $L^{-1}$. The stock was taken as much as 400 mL in 3600 mL of catfish culture wastewater in accordance with treatment. Aeration was used for agitation, the lighting using 36 watt TL lamps for 24 hours $day^{-1}$ during maintenance (Figure 2). Harvest of biomass. The components of wastewater were total phosphorus 2,6 mg.$L^{-1}$, total nitrogen 1,9 mg.$L^{-1}$, total organic carbon 11.4 mg.$L^{-1}$. Harvest of the biomass was after exponential phase by filtering. The biomass was dried using an oven for 14 hours at 40°C (Hidayani et al. 2019). The dry biomass was used for the phycocyanin extraction process.

**Phycocyanin extraction**

The dry biomass was 0.04 g added by 1 mL of phosphate buffer pH 7, then homogenized and frozen in the freezer for 24 hours at a temperature of -4 °C. After 24 hours from the freezer, thawing process for 15 minutes. Samples were centrifuged for 30 minutes at 3000 rpm. After that, the sediment and the supernatant were separated. The resulting supernatant was phycocyanin which be analyzed using the Bennett and Bogorad method (1973).

**The density of *Arthrospira* biomass**

Biomass density measurements were performed at each treatment and 3 replications every day at the same hour. The density of biomass was 1 mL of sample in each treatment with 3 replications. The 1 mL of sample into aluminum bowl. The sample and the aluminum bowl were weighed, then dried in the oven for 14 hours at 40 °C. The sample of water that had dried was weighed again. The dry biomass weight of *Arthrospira* biomass was converted to g $L^{-1}$.

**Figure 1.** Catfish farming pond

**Figure 2.** *Arthrospira* cultivation

The growth rate of *Arthrospira* can be calculated using the following formula according to Vonshak (1997):

$$\mu = \frac{\ln Nt - \ln No}{t} \times 100\%$$

Where:
$\mu$ = daily growth rate (% days$^{-1}$)
t = time (days) from $N_0$ to $N_t$
$N_0$ = initial density (g L$^{-1}$)
$N_t$ = density at the time t (g L$^{-1}$)

Measurement of total nitrogen and phosphate content in culture media was carried out at the beginning and the first day after the peak phase of each treatment.

The measurement of phycocyanin refers to Bennett and Bogorad (1973). The absorbed supernatant was measured using a spectrophotometer at wavelengths of 615 nm and 652 nm.

$$\text{C-phycocyanin (mg.mL}^{-1}) = \frac{(OD\ 615) - 0.474\ (OD\ 652)}{5.34}$$

$$\text{Rendement of phycocyanin (mg g}^{-1}) = \frac{\text{C-phycocyanin x V}}{DB}$$

Rendement percentage of phycocyanin (%) = Rendement of phycocyanin (mg g$^{-1}$) x 100%

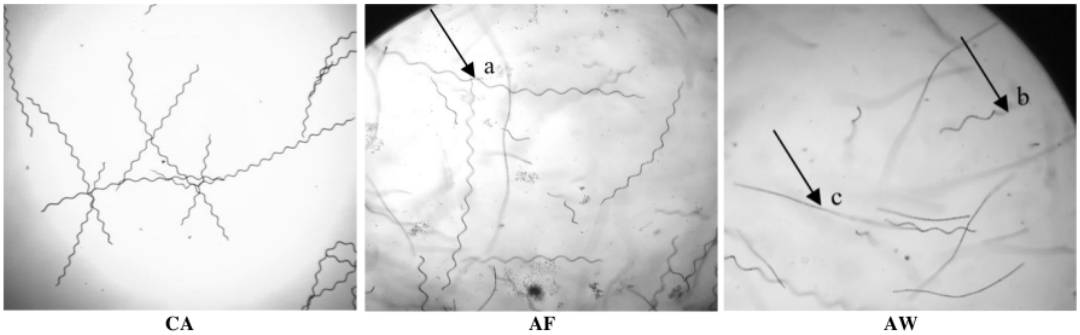
Where:
C-phycocyanin = C-phycocyanin consentration (mg. mL$^{-1}$)
V = Solvent Volume (mL)
DB = Dry Biomass (0.04 g)
0.474 and 5.34 = coefficient of extinction (Bennett and Bogorad 1973)

The results were submitted to simple analysis of variance tests (ANOVA) ($p < 0.05$) and in the case of significant differences, the means were compared by the Least Significant Differences test ($p<0.05$).

## RESULTS AND DISCUSSION

### Morphology of *Arthrospira*

*Arthrospira* was cultivated using two different fertilizer media namely technical fertilizer and waste media. The morphology of commercial *Arthrospira* before fertilizer treatment was presented in Figure 3.




**CA** **AF** **AW**

**Figure 3.** Morphological identification results of *Arthrospira* isolate. (CA =Commercial *Arthrospira*; AF= commercial *Arthrospira* cultured with technical fertilizer media; AW= commercial *Arthrospira* cultured with waste media) 40x magnification. Note: a = helical form AF, b = helical form AW, c = straight form AW

**Tabel. 1.** The results of the BLASTn analysis of *Arthrospira* samples cultivated in technical fertilizer and waste medium with data in GenBank

| Description | Identity (%) | Access code | Sample origin |
|---|---|---|---|
| *Arthrospira* (fertilizer media) (AF) | | | |
| *Arthrospira platensis* petH | 100 | AB113346 | Japan |
| *Spirulina platensis CCC 478* | 90,48 | JX014313.1 | India |
| *Spirulina platensis cyaG* | 94,4 | D49531.1 | Japan |
| *Arthrospira platensis PCC 7345* | 90,12 | JN831264.1 | USA |
| *Arthrospira maxima* EEW2 | 74,4 | HQ008225 | Australia |
| *Arthrospira* (waste media) (AW) | | | |
| *Arthrospira platensis* petH | 94,3 | D49531.1 | Japan |
| *Arthrospira platensis DKCAS2* | 81,4 | MG912588.1 | India |
| *Spirulina platensis CCC 478* | 74,4 | JX014313.1 | India |
| *Arthrospira maxima* str. Lefevre 1963/M-132-1 | 73,3 | FJ798612 | Venezuella |
| *Arthrospira maxima* EEW2 | 72,2 | HQ008225 | Australia |

The results of the identification of isolates showed that the isolate had a twisted filament shape resembling a spiral (helical). Based on Davis's identification book (1955), it is known that the isolate used in the study was *Spirulina (Arthrospira) platensis*. *Arthrospira* is cyanobacteria belonging to the order Oscillatoriales which has a filament (trichome) that resembles a spiral (helical) but does not have heterocyst cells (Sze 1998). Heterocyst cells are special thick-walled cells that play a role in nitrogen fixation from the air (Issa et al. 2014). In this study, *Arthrospira* cultured in different media had several linear/straight morphologies.

Based on Figure 2, *Arthrospira* which was cultured on technical fertilizer media has a longer and spiraling morphological form compared to another cultured on waste media. Their filaments have more linear morphological form, some spirals but not too long. According to Astiani et al. (2016), *Arthrospira* growth is influenced by nutritional and environmental factors. Wang and Zhao (2005) explained that linearization that occurs in *Arthrospira* is a variation on the genetic level that can be caused by environmental factors such as lack of nutrition and high light intensity. In this study, isolates were cultured with the same light intensity of 2000-3000 lux with a light-dark ratio of 0: 24 hours. Linear filaments in AW have a lower metabolic rate compared to helical filaments. This is one of the adaptive mechanisms for *Arthrospira* to survive some environmental conditions that are not appropriate.

Yadav et al. (2020) showed that helical and linear morphotypes of *Arthrospira* sp. display genomic differences. Vonshak (2000) showed that polyphasic in *Arthrospira* morphotypes can be caused by: growing in agar/solid media, light stress-photoinhibition, irradiation, and temperature, effect of physical and chemical conditions. *Arthrospira* is prokaryotic organism, so it is easy to mutate and change sequences in their genome.

**Phylogenetic tree**

The results of the 16S rRNA encoding gene sequences from AF and AW isolates were traced to other *Arthrospira* isolates present in GenBank through the BLAST program. The results of the BLASTn analysis of *Arthrospira* samples cultivated in technical fertilizer and waste media with data in GenBank are presented in Table 1. Table 1 results of the BLAST analysis show the closeness between AF and AW isolates with other isolates in GenBank. It shows that *Arthrospira* technical fertilizer isolates and *Arthrospira* waste isolates have the closest homology to *Arthrospira platensis* petH species from Japan with percentage values respectively 100% and 94.3%.

Genetic distance was used to see kinship relationships from *Arthrospira* both AF and AW samples with sequential data from GenBank. AF isolates indicated a genetic distance of 0.068 with AW isolates. AW and AF isolates showed the lowest genetic distance respectively 0.089 and 0.060 with *Arthrospira platensis* petH from Japan. Analysis based on genetic distance showed that both isolates belonged to the same species namely *Spirulina platensis*, however, the genetic distance was 0.068 (6.8%) meaning that there are intraspecies variations in the sample caused by mutations.

Phylogenetic tree *Arthrospira* isolates from technical fertilizer and waste media were presented in Figure 4. The phylogenetic tree is a two-dimensional graph showing relationships between organisms or population classifications based on their evolutionary history. The result of phylogenetic tree construction showed that both samples formed branches with a cluster. Phylogenetic tree from AF and AW isolate sequences formed cluster was separate with several other *Arthrospira* species from GenBank data.

The AF isolates had a close relationship with *Arthrospira platensis* petH species from Japan with a bootstrap value of 95%. Hadiati (2003) states that bootstrap analysis is performed to determine the level of confidence in grouping. Bootstrap value is considered high because according to Hall (2001), a clade can be trusted with a bootstrap value of 90%. In addition, Hillis and Bull (1993) state that bootstrap analysis with values of 70% or higher indicates a reliable grouping. The AW isolates formed a separate branch of AF isolates and *Arthrospira platensis* petH species. Genetically, they had diverse, and adapted to

environmental conditions. The AW isolate indicated different strains from AF isolate groups. Ballot et al. (2004) stated that *Spirulina* from the same species and cultured under different conditions can form a separate subcluster with a bootstrap value of 79%. Zhao et al. (2006) identified and analyzed the number of restriction-modification genes in the cyanobacterial genome, seeing that more restriction-modification genes were found in cyanobacterial filaments (*Anabaena, Spirulina,* and *Nostoc*) than dispenses (*Synechocystis, Synechococcus* and *Prochlorococcus*) this was due to the organism adapting to various environmental conditions, or the many variations in sources of nutrients that cause mutations.

**Density and Growth Rate of *Arthrospira* cultured in wastewater of catfish farming**

The biomass of *Arthrospira* displayed mechanism of adaptation in culture media. The wastewater media could make different characteristic of growth as like as filament. The maximum densities of *Arthrospira* cultured in wasted water were achieved on a different day. The daily density of *Arthrospira* during culture can be seen in Figure 5.

The graph presented in Figure 5, it shows that in the culture period from day 1 to day 2, *Arthrospira* in each treatment-experienced slow growth, because the cells were still adapting to their new environment. The exponential phase for the treatment of P2S2, P2S3, and P3S3 lasts from day 1 to day 8 of the culture period. The treatment of P2S1, P2S4, P3S1 and P3S2 takes place from 2nd until 8th day of the culture period. While the treatment of P1S1, P1S2, P1S3, P1S4, and P3S4 lasted from 3rd - 8th day of the culture period. The decreasing *Arthrospira* density for the treatment occurred from day 9 to day 11 of the culture. Lesmana et al. (2019) explained that the adaptation phase lasts from day 0 to day 1, while the exponential phase occurs from day 1 to day 7, and experiences a stationary phase from day 7 to day 9 then enters the death phase after the 7th day and 9th day. The decrease of density could because of reducing the nutrients in the culture media. Soni et al. (2019), the concentration of nutrients in the media decreased after reaching the peak period. This is due to the higher density of *Arthrospira* in the culture media.

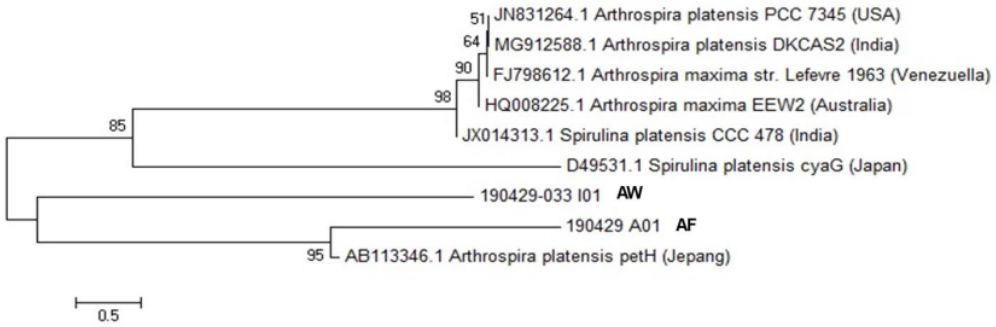



**Figure 4.** Phylogenetic analysis with 1000 bootstrap AW (*Arthrospira* cultured in wastewater media) and AF (*Arthrospira* cultured in fertilizer media)

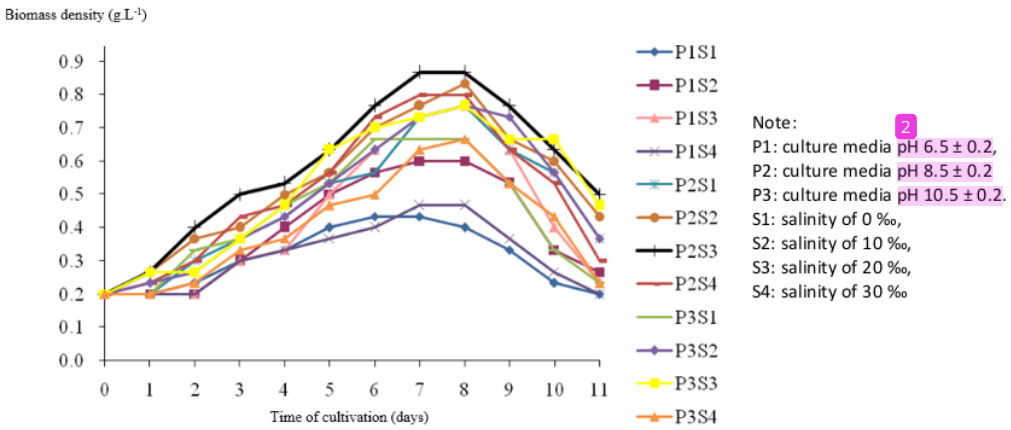


**Figure 5.** Cell density (dry weight with a moisture content of 1.2%) of *Arthrospira* in catfish rearing pond wastewater

The maximum density of *Arthrospira* could be achieved on different days, between 5 - 8 days after culturing. The mean of maximum density could be 0.433-0.867 g $L^{-1}$ of dry biomass cultured in catfish rearing wastewater. The maximum cell density of *A. platensis* which cultured in nile fish rearing wastewater, resulted in the production of 0.22 g $L^{-1}$ of dry biomass and maximum productivity of 0.03 g $L^{-1}$ $day^{-1}$ (Nogueira et al. 2018). The catfish rearing pond wastewater has high potential as cultivation media for *Arthrospira* production.

The analysis of variance showed that differences in pH, salinity, and interaction between factors (pH and salinity) significantly affect the maximum density and growth rate of *Arthrospira platensis*. The results of the $LSD_{0.05}$ maximum density test and growth rate sequentially were presented in Tables 1 and 2. $LSD_{0.05}$ test results on the main factors of differences in pH, density, and growth rate of *Arthrospira platensis* in the P2 treatment (pH 8.5 ± 0.2) were significantly higher than those in the P1 (pH 6.5 ± 0.2) and P3 treatments (pH 10.5 ± 0.2). According to Ismaiel (2016), the highest biomass of *Arthrospira platensis* is produced in media with a pH of 8.5-9.5. Although *Arthrospira platensis* can tolerate a wide pH range, a pH range farther from its optimal pH can reduce its growth rate. A low growth rate will also cause low biomass production.

Furthermore, the different salinity treatment factors showed that the maximum density and growth rate in treatment S3 (salinity of 20 ‰) were significantly higher compared to S1 (salinity of 0 ‰), S2 (salinity of 10 ‰) and S4 (salinity of 30 ‰) treatments. The S1 and S4 treatments were not significantly different and were the treatments that produced the lowest density compared to other treatments. Tables 1 and 2 showed that the highest density and daily growth rate was found in the S3 (salinity of 20 ‰) treatment. This is supported by the results of Kouhgardi et al. (2015), that *Arthrospira platensis* cultured on Conway media was able to produce the highest density of 912.07 $mL^{-1}$ cells at a salinity of 20 ‰. While the density and growth rate between S1 and S4 treatments showed no significant difference. This is because the salinity of 0-30 ‰ is still within the range of salinity that can be tolerated by *Arthrospira*. Ughy et al. (2015) said that *Arthrospira platensis* is one of the species of *Cyanobacteria* that can grow in an euryhaline environment.

The interaction between pH and salinity factors showed that the density and growth rate in treatment P2S3 (pH 8.5 ± 0.2 and salinity of 20 ‰) were significantly higher compared to other treatments. At maximum density, treatment P2S3 was not significantly different from treatments such as P1S3, P2S1, P2S2, P2S4, and P3S3. While in the growth rate, treatment P2S3 was not significantly different from P1S3, P2S2, P2S4, P3S2 and treatments P3S3. Tables 1 and 2 show that treatment P2 (pH 8.5 ± 0.2) is more dominant causing higher density and growth rate of *Arthrospira platensis* despite being in different salinity ranges. As for the treatments P1 (pH 6.5 ± 0.2) and P3 (pH 10.5 ± 0.2) provide the highest density when combined by the treatment S3 (salinity 20 ppt). The treatment of P1, P2, and P3 (pH 6.5 ± 0.2, 8.5 ± 0.2, and 10, 5 ± 0.2) and S1, S2, S3 and S4 treatments (salinity of 0 ‰, 10 ‰, 20 ‰, and 30 ‰) still support the growth of *Arthrospira platensis* with the best treatment found in the combined treatment P2S3 (pH 8.5 ± 0.2 with salinity of 20 ‰ ).

**Table 1.** Maximum density of *Arthrospira platensis* (g $L^{-1}$)

| Single INFLUENCE of pH (P) | Single influence of salinity (S) ($LSD_{0.05}$ = 0.107) | | | | Main influence of pH (P) ($LSD_{0.05}$=0.053) |
|---|---|---|---|---|---|
| | S1 (0 ‰) | S2 (10 ‰) | S3 (20 ‰) | S4 (30 ‰) | |
| P1 (pH 6.5) | 0.433 $^{a}$ | 0.633 $^{b}$ | 0.767 $^{cdef}$ | 0.467 $^{a}$ | 0.575 $^{a}$ |
| P2 (pH 8.5) | 0.767 $^{cdef}$ | 0.833 $^{ef}$ | 0.867 $^{f}$ | 0.800 $^{def}$ | 0.817 $^{c}$ |
| P3 (pH 10.5) | 0.667 $^{bc}$ | 0.733 $^{bcde}$ | 0.767 $^{cdef}$ | 0.700 $^{bcd}$ | 0.717 $^{b}$ |
| Main influence of salinity (S) ($LSD_{0.05}$=0.062) | 0.622 $^{a}$ | 0.733 $^{b}$ | 0.800 $^{c}$ | 0.656 $^{a}$ | |

**Table 2.** The growth rate of *Arthrospira platensis* cultured in pH and salinity treatment (% $day^{-1}$)

| Single influence of pH (P) | Single influence of salinity (S) ($LSD_{0.05}$ = 3.224) | | | | Main influence of pH (P) ($LSD_{0.05}$=1.612) |
|---|---|---|---|---|---|
| | S1 (0 ‰) | S2 (10 ‰) | S3 (20 ‰) | S4 (30 ‰) | |
| P1 (pH 6.5) | 14.333 $^{a}$ | 18.659 $^{cd}$ | 19.192 $^{cde}$ | 13.348 $^{a}$ | 16.383 $^{a}$ |
| P2 (pH 8.5) | 18.343 $^{bcd}$ | 20.570 $^{de}$ | 22.026 $^{e}$ | 20.723 $^{de}$ | 20.416 $^{b}$ |
| P3 (pH 10.5) | 20.023 $^{de}$ | 19.527 $^{cde}$ | 20.623 $^{de}$ | 16.417 $^{abc}$ | 19.147 $^{b}$ |
| Main influence of salinity (S) ($LSD_{0.05}$=1.861) | 17.566 $^{a}$ | 19.585 $^{b}$ | 20.614 $^{b}$ | 16.829 $^{a}$ | |

Note: ‰ means part per thousand (ppt), it is not percent (%). Salinity of freshwater (0 ppt), brackish water (5-20 ppt), and sea water (more than 25 ppt). The salinity used in this study still supports the growth of *Arthrospira* (Vonshak 1997).

Mismatch of pH will cause lysis and can change the shape of pigment growth (Hariyati 2008). The process of photosynthesis affects pH value. In daylight, aquatic plants release carbon dioxide from water for use in photosynthesis. The release of carbon dioxide by plants occurs through respiration. When carbon dioxide is released, carbonate builds up and hydrolyzed so that the pH of the water will increase (Boyd 1990). Prasadi (2018) showed that growth of *Arthrospira* could be inhibited if it was in the pH range above 10.5 or less than 7. Salinity is one of the factors that can influence osmotic pressure for *Arthrospira* as like others of microalgae. Pisal and Lele (2005) microalgae can experience cell shrinkage in conditions outside the cell salinity higher than inside the cell (hypertonic), and vice versa under conditions of low salinity outside the cell (hypotonic) cell swelling will occur due to water molecules outside moves into the cell. This condition affects the process of photosynthesis, and makes microalgae to produce secondary metabolites in the form of β-carotene to sustain life against changes in salinity in culture media. While in isotonic conditions, cell fluid is isotonic to its external media which causes low active ion transport and osmoefector exchange, making the Na-K-ATPase enzyme activity at a maximum level and more energy will be utilized for growth (Rahmawati et al. 2012 ). The optimal combination of pH and salinity causes the growth of *Arthrospira* to be maximal. The optimal salinity range for *Arthrospira* is between 15-20 ‰, from the related research showed that the results of *Arthrospira* culture with 20 ‰ of media salinity, pH 7.5-8.5 using fertilizer media (0.010 g $L^{-1}$ TSP, 0.030 g $L^{-1}$ Urea, and 0.030 g $L^{-1}$ ZA) and a culture period of 9 days produced a dry weight of *Arthrospira* of 0.0375 g $L^{-1}$ (Prasadi 2018).

**Rendement of phycocyanin**

The rendement of *Arthrospira* was presented in Table 3. The pH condition of maintenance media can be affect protein content in *Arthrospira* cells. The results of LSD $_{0.05}$ on the main factor of pH showed that the rendement of phycocyanin *Arthrospira* in treatment P2 was significantly higher compared to other treatments. Taufiqurrahmi et al. (2017), the amount of *Arthrospira* biomass influences the high content of phycocyanin. Table1 showed that the highest of *Arthrospira* biomass was found in treatment P2. It showed that the highest *Arthrospira* biomass produced the highest rendement of phycocyanin (Table 3). The culture medium of *Arthrospira* pH of 8.5 produced the highest C-phycocyanin content (Ismaiel et al. 2016). Rahmawati et al. (2017) said that the higher C-phycocyanin followed the higher of rendement of phycocyanin.

LSD $_{0.05}$ showed that the main factor of salinity showed the rendement of phycocyanin in treatment S3 (salinity of 20 ‰) was significantly higher than other treatments. It is thought that the difference in salinity treatment has an impact on the external osmotic pressure of *Arthrospira* cells which results in changes in cell composition especially phycocyanin. Sodium will flow into the cell and cause the release of phycobilin (phycoerythrin, phycocyanin and allophycocyanin) from PS II (Photosystem II) and stop the electrons transporting to PS I (Photosystem I) followed by activation of the protective mechanism. *Arthrospira* will produce carbohydrates to balance intracellular osmotic pressure and require more energy to remove sodium ions from cells. In this case it will produce ammonium assimilation causes inhibition of protein synthesis (Zhou et al. 2017).

The environment includes the availability of nutrients, pH, salinity, light and temperature can affect the growth and accumulation of biopigments from microalgae (Sharma and Tiwari 2014). The condition of culture media is able to influence the growth phase of *Arthrospira*, causing changes in the composition and proportion of phycobilin (phycoerythrin, phycocyanin and allophycocyanin) (Simeunovic et al. 2013). The results of the LSD $_{0.05}$ was showed that the rendement of phycocyanin *Arthrospira* on the interaction between factors in treatment P2S3 was significantly higher than in other treatments, but it was not significantly different from treatment P2S2. This showed the density and growth rate of *Arthrospira*. The increasing salinity will cause maintenance media to be hypertonic towards cells and result in poor absorption of nutrients by cells. These cells could reduce protein and increase in carbohydrates from *Arthrospira* cells (Ravelonandro et al.,2011).

Production of phycocyanin was able to reach 12.4 % - 17.6% of biomass dry weight of *Arthrospira* cultured in Zarrouk Medium (ZM) (Prates et al. 2018; Garcia-Lopez et al. 2020). There are several factors that affect the rendement of phycocyanin include temperature, extraction time, mixing rate, biomass, type of solvent, and the ratio of biomass to the solvent (Taufiqurrahmi et al. 2016). The content of phycocyanin in cyanobacteria increases when grown in low light intensity. Phycocyanin is a pigment associated with protein, polar and water-soluble. The protein content of microalgae is influenced by environmental conditions such as temperature, age of culture, light intensity, pH, salinity, and nutrient limits (especially nitrogen). Nitrogen is an essential element needed for the synthesis of accessory pigments and chlorophyll. When microalgae are growing fast, it requires large amounts of nitrogen and could consume phycocyanin as an alternative source of nitrogen for the production of biomass (Hsieh-Lo et al. 2019). It must be optimized for biomass production and phycocyanin content. The higher concentration of phycocyanin will be followed by the rendement of phycocyanin. In this study, the phycocyanin content is lower than previous study, because of different media for culturing *Arthrospira*. The nutrient from ZM (pro analysis substances) is more complete for growing and forming phycocyanin than wastewater catfish pond media, especially the trace mineral in ZM.

**Reduction of total nitrogen and phosphate content**

The percentage reduction of total nitrogen in the culture media was presented in Table 4.

**Table 3.** Phycocyanin (%) yield in *Arthrospira* dry biomass at 8 days after inoculation

| Single Influence of pH (P) | Single influence of salinity (S) ($LSD_{0.05}$ = 0.194) | | | | Main influence of pH (P) ($LSD_{0.05}$=0.096) |
|---|---|---|---|---|---|
| | S1 | S2 | S3 | S4 | |
| P1 | 7.881 [a] | 8.783 [c] | 9.441 [d] | 8.387 [b] | 8.623 [a] |
| P2 | 9.657 [e] | 10.906 [h] | 11.347 [i] | 10.423 [g] | 10.583 [c] |
| P3 | 8.970 [c] | 9.408 [d] | 10.134 [f] | 9.262 [d] | 9.444 [b] |
| Main influence of salinity (S) ($LSD_{0.05}$=0.111) | 8.836 [a] | 9.699 [c] | 10.307 [d] | 9.357 [b] | |

**Table 4.** Reduction of total nitrogen content in *Arthrospira* culture medium (%)

| Single Influence of pH (P) | Single influence of salinity (S) ($LSD_{0.05}$ = 1.290) | | | | Main influence of pH (P) ($LSD_{0.05}$=0.645) |
|---|---|---|---|---|---|
| | S1 | S2 | S3 | S4 | |
| P1 | 80.990 [a] | 82.250 [ab] | 83.767 [cde] | 81.897 [ab] | 82.226 [a] |
| P2 | 83.880 [de] | 84.377 [ef] | 85.420 [f] | 84.857 [ef] | 84.633 [c] |
| P3 | 82.940[bcd] | 81.143 [a] | 84.950 [ef] | 84.813 [ef] | 83.462 [b] |
| Main influence of Salinity (S) ($LSD_{0.05}$=0.745) | 82.590 [a] | 83.856 [b] | 84.712 [c] | 82.603 [a] | |

The mechanism for removing nitrogen in water is determined by several factors, including bacterial activity (Gersberg et al. 1986), uptake by plants (Breen 1990), and evaporation (Sanchez-Monedero et al. 2001). In Cyanobacteria, nitrogen is a macronutrient that plays an important role in the formation of biochemical compounds such as nucleic acids (DNA, RNA), amino acids (protein), and pigments (chlorophyll and phycocyanin) (Markou et al. 2014). The results of the $LSD_{0.05}$ on the main pH factor showed that the reduction in total nitrogen content in *Arthrospira* culture media in treatment P2 was significantly higher than in treatments P1 and P3. This is due to the higher density of *Arthrospira* in treatment P2 compared to other treatments, so that the utilization of nitrogen by *Arthrospira* in culture media is greater than in others. Markou et al. (2014) showed that the higher density of *Arthrospira* could be higher the absorption of nutrients including nitrogen.

The value of reducing total nitrogen content in S3 treatment was significantly higher than for other treatments. This showed that treatment S3 caused *Arthrospira* to absorb nitrogen higher than other treatments in line with the high density and rendement of phycocyanin *Arthrospira* obtained in this study. Jabeen and Ahmad (2011) showed that salinity in culture media influences nitrogen absorption and protein biosynthesis. Reduction of total nitrogen content in *Arthrospira* culture media due to interactions between pH and salinity factors showed that treatment P2S3 was significantly higher than other treatments. With the optimal conditions (pH and salinity), *Arthrospira* is able to make maximum use of nitrogen. This can be seen from the highest density, growth rate and rendement of phycocyanin found in treatment P2S3. The amount of nitrate and phosphate decreases with increasing growth of *Chlorella vulgaris* and *Arthrospira platensis* (Sayadi et al. 2016). This is because algae have the ability to absorb nutrients such as nitrogen and phosphate are use to carry out photosynthesis and protein production. Reduction of phosphorus content in waters is influenced by the process of absorption, complexation, deposition and assimilation (between microbes and plant biomass) (Tanner et al. 1999).

The results of $LSD_{0.05}$ on the main factor of pH showed that each treatment did not significantly affect the reduction of phosphate content in *Arthrospira* culture medium. Plants can only absorb phosphorus in the form of $H_2PO_4$ and $HPO_4^{-2}$ free orthophosphate ions (Becquer et al. 2014). The orthophosphate content decreases with increasing media pH. Cerozi and Fitzsimmons (2016) showed the orthophosphate content increases in the pH range from 5.5 to 8.5 and decreases when pH 10. The fall in the orthophosphate content at pH 10 is due to an increase in calcium phosphate formation. The value of reducing the phosphate content in *Arthrospira* culture medium was presented in Table 5.

The salinity factor, administration of different salinity in each treatment has no significant effect on reducing the phosphate content. Bassin et al. (2011) explained the reduction of phosphorus will be inhibited when a combination of $Cl^-$ and nitrite and $Cl^-$concentration more than 2.5 g $L^{-1}$. The interaction effect of differences in pH and salinity, each treatment had no significant effect on reducing phosphate content. Hong et al. (1995) showed that the utilization of Dissolved Organic Phosphorus (DOP) can be through active uptake into cells or by extracellular mineralization by phosphatase enzymes. However, most DOP compounds cannot be assimilated directly with microalgae because they have been mineralized. Markou *et al*. (2014) stated that phosphorus is a macronutrient that plays an important role in the preparation of nucleic acids (RNA and DNA), phospholipids and energy-carrying molecules (ATP). The phosphorus content in plants is lower than Ca, N, and K (Sasaqi et al. 2018). Although the analysis of variance shows that the results are not significantly different, but the highest phosphate reduction still existed in treatment P2S3.

**Table 5.** Reduction of phosphate content in *Arthrospira* culture medium (%)

| Single influence of pH (P) | Single influence of salinity (S) | | | | Main influence of pH (P) |
|---|---|---|---|---|---|
| | S1 | S2 | S3 | S4 | |
| P1 | 70.500 | 71.667 | 73.000 | 72.333 | 71.875 |
| P2 | 74.667 | 74.000 | 74.667 | 72.667 | 74.000 |
| P3 | 70.333 | 70.333 | 73.667 | 72.667 | 71.750 |
| Main influence of salinity (S) | 71.833 | 72.000 | 73.778 | 72.556 | |

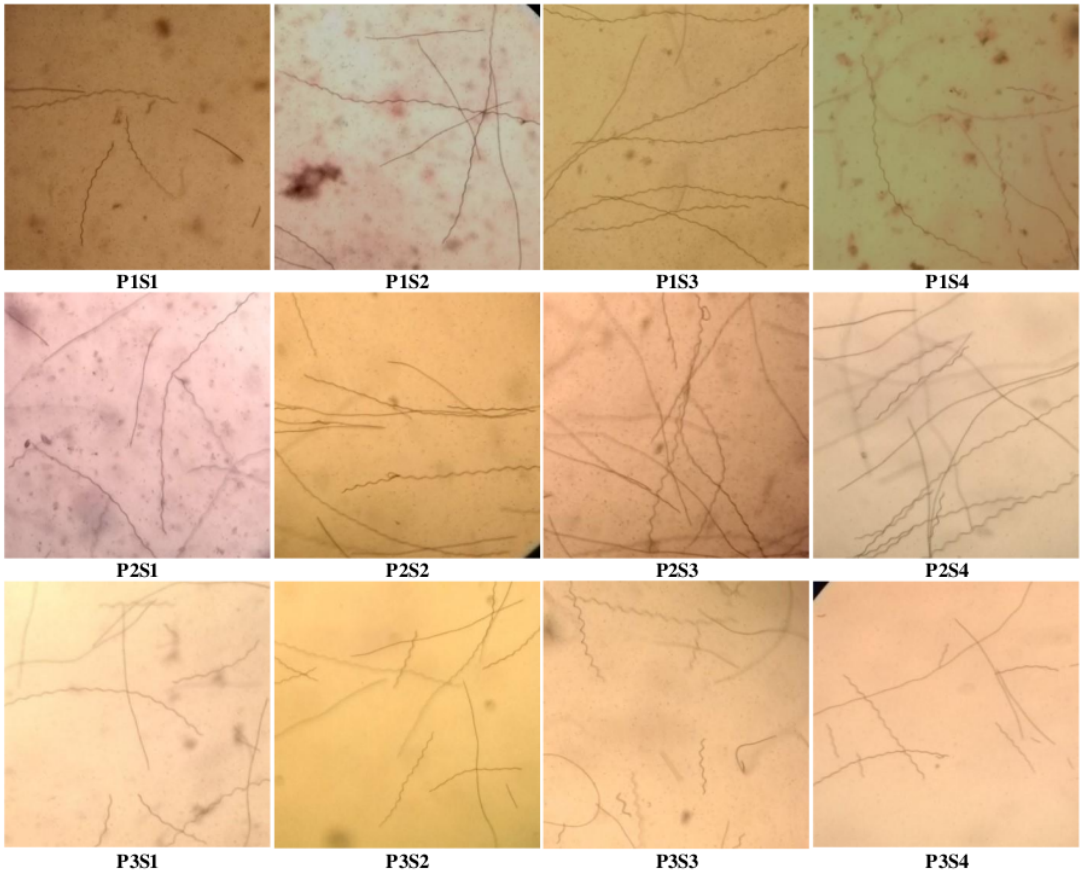



**Figure 6.** Morphology of *Arthrospira* in catfish wastewater culture media at several treatments of pH and salinity

**Morphology of *Arthrospira* in various pH and salinity media**

Morphology of *Arthrospira* was affected by increasing or decreasing physical or chemical factors in their culture media. Salinity and acidity value combination at this study didn't change the filament of *Arthrospira* significantly as their filament of *Arthrospira* under microscope with 100x magnification (Figure 6). The morphological forms of *Arthrospira* were not different in all pH and salinity treatments. The wastewater catfish pond media could be affected in the linearizing filament. This study indicated that salinity and acidity of culture media didn't effect various forms of filament, either straight and helical.

The straight filaments were observed for *Arthrospira* strains during two years of cultivation, and their presence in *Arthrospira* sp. Nigrita C1 cultures was constant. The various morphological plasticity, greatly influenced by the growth stage and exogenous parameters, such as temperature and light intensity, was observed for *Arthrospira* strains (Papapanagiotou and Gkelis 2019). There are indications that adaptability to change in environmental conditions is relatively rapid and also

subsequent changes at the genetic level can be realized quickly. This means that we can easily find different genotypes in various stable, ecologically different habitats. Morphologically similar strains were cultured for a long time under uniform and stable conditions (Komarek 2016). But the morphological changes could not be effected by acidity and salinity of culture media.

In conclusion, *Arthrospira* that is cultured on waste media (observed in liquid culture) indicated some short and linear filaments. Identified *Arthrospira* had a genetic distance of 6.8% between AF and AW isolates. AF isolates had a close relationship with *Arthrospira platensis* petH species originating from Japan (bootstrap value of 95%) while AW isolates form phylogenetic branches which are separated from AF isolates and *Arthrospira platensis* petH species originating from Japan (bootstrap value 85%). The catfish culture wastewater media at different pH and salinity affects the density, growth rate, and rendement of phycocyanin *Arthrospira platensis*. The highest density, growth rate and rendement of phycocyanin were in P2S3 treatment (pH 8.5 ± 0.2 and salinity of 21 ‰) which produced a maximum density of 0.867 g $L^{-1}$, growth rate of 22.026% $day^{-1}$ and the rendement of phycocyanin of 11.334 %.

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