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Research Article

Bacteriophage and Bacterial Lysate Guarantee an Additional Source of Proteins for the *Arthrospira fusiformis*

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Abstract

Arthrospira fusiformis is a cyanobacteria could resist both of the salinity and the alkalinity. It is showing an increasing interest while it is safe as a food and contain valuable active compounds particularly their billins. Their billins is proposed to have antisickling and antiviruses agents. In this study bacteriophage coexisted with the *Arthrospira fusiformis* might has a critical role in their survival in a closed cultivation system for more than ten years. The existence of the bacteriophage in the *Arthrospira platensis* ecosystem was proved either by normal plate cultivation or by using the electron microscope. Bacteriophage, bacterial cells, the lysis bacterial cells, and the *Arthrospira fusiformis* exopolysaccharide guarantee the survival of the *Arthrospira fusiformis* for more than ten years in a closed cultivation system. The idea and the explanations included in this study might open a new possibility for increasing the cultivation biomass in the open ponds. We proposed both of the bacteriophage and the alkalinity as agents could lysis bacterial cells and supply the *Arthrospira fusiformis* with additional source of nutrients.

Keywords: *Arthrospira fusiformis*; Closed cultivation; Bacteriophage; Exopolysacharide

Introduction

Arthrospira is utilized as food additive and as an animal feed [1]. It is rich in vitamins, minerals, proteins and fatty acids [2-4]. It is able to activate the immune response [5,6] and producing anticarcinogenic [7,8]. It has an antiviral polysaccharides [7,9] inhibiting the infection for herpes simplex virus type 1 and type 2, pseudorabies virus, human cytomegalovirus [10] as well as measles virus, mumps virus, influenza A virus [11] and HIV-1 [11,12], producing allophycocyanin, which inhibits enterovirus-71 [13]. *Arthrospira* was also found to excrete exo-polysaccharides (Spirulan-like substances) with antiviral effect [14-16]. The highest effective antiviral compound purified from *Arthrospira* was found to be Ca-Spirulan, a sulfated polysaccharide attached to Calcium ions, which is extracted after 1 hour of boiling the cyanobacterium [10,11]. In general *Arthrospira fusiformis* is used as it is (without any treatment) from unknown time by different trips of the Africans particularly in Chad Lack. Species of *Arthrospira* were found in a variety of environments including soil, sand, marshes, brackish water, seawater, and freshwater [17,18]. Rich (1931) has reported it as a dominant phytoplankton in a number of lakes in the Rift Valley of East Africa [19]. 113 years after its first microscopic identification, *Arthrospira* was reintroduced to the world by Dangeard (1940) from a sample collected by Mr. Creach (a pharmacist) from a local market in Chad [17,20]. The alkaline lakes enable the growth of one of the few nontoxic cyanobacterial species, *Arthrospira fusiformis* [17]. Kebeda (1997) reported that in Ethiopia, farmers and herdsmen living in areas close to the soda lakes make their cattle drink *Arthrospira* water about once a month and believe that it has therapeutic effects and compensates for some lack in dietary food [21]. The invention of the microscope enabled Turpin in 1827 to identify and describe *Arthrospira* as spiral cyanobacteria [22]. *Arthrospira* contains high levels of proteins (50-70%), lipids (7-16%), vitamins, and omega-3 fatty acid [23-25]. For economic production of *Arthrospira*, it is usually cultivated in open ponds, so the absorbed solar energy is used to fix inorganic carbon. *Arthrospira* is produced in quantities exceeding 3000 tons/year of dry material [26]. *Arthrospira fusiformis* strain from Chad prove to have unique tolerance to resist complete dryness and dehydration by producing exopolysaccharide biofilm [27-29]. Recently, Amara et al. (2014) introduced a protocol for producing bacterial ghosts using critical chemical compounds in their minimum inhibition concentrations and in their minimum growth concentrations. The proposed compounds were SDS, NaOH, $CaCO_2$ and H_2O_2 [30-32]. Additional chemical compounds were used and proposed. $NaHCO_3$ was used based on the study conducted by Amara and Steinbüchel (2013) [33]. $NaHCO_3$ is one of the basic component in the Zarrouck medium [34]. The water evaporation in the open lack increase both of the alkalinity and the salinity and cause lysis for the biota existed in the ecosystem except some microbial strains including the *Arthrospira fusiformis* as proved by Amara and Steinbüchel (2013) [34]. The $NaHCO_3$ which used in 16.80 g/l do the same in addition to the other medium components. Additionally, the existence of both of the bacteria and the bacteriophage is another opportunity to both of degrading the dead cells and supplying additional nutrient from the lysis bacterial cells. The existence of the phages and bacteriophages in the ecosystem play a critical role in microbial lysis. Phages which have *E lysis* genes induce microbial cell lysis [35-38]. The evacuated microbes release their cytoplasm content in the surrounding environment [35,39,40].

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Figure 1: Five liter flask with a ten year age *Arthrospira fusiformis* culture.

This study propose strong evidence for *in vivo* lysis of both of algae, bacteria and other microbes during the growth over seasons due to the increase in alkalinity and salinity as well as the existence of the bacteriophage as an additional factor.

Materials and Methods

Chemical

All chemicals used were analytical grade and obtained from Sigma-Aldrich and Roth.

Cyanobacteria strain

The extremoalkalophilic cyanobacteria strains used in this study were obtained from strain isolated from Chad. The strain was identified using a light microscope as *Arthrospira fusiformis*. The strain was identified using the molecular biology tools by Sharaf et al. (2010) as *Arthrospira fusiformis* by sequencing and analysis of the PC-IGS regions in the gene of phycocyanin [26].

Zarrouck medium

$NaHCO_3$ 16.80 g; $NaNO_3$ 2.50 g; K_2SO_4 1.00 g; NaCl 1.00 g; K_2HPO_4 0.50 g; $MgSO_4.7H_2O$ 0.20 g; Disodium EDTA.$2H_2O$ 0.08 g; $CaCl_2.2H_2O$ 0.04 g; $FeSO_4.2H_2O$ 0.01 g; Trace Metal Mix A 1.00 ml; Trace Metal Mix B 1.00 ml; pH 9.0 ± 0.2 at 25˚C [34].

Components were added to distilled/deionized water and brought to volume 1 l and autoclaved for 15 min at 15 psi pressure 121˚C.

Trace Metal Mix A

H_3BO_3 2.86 g; $MnCl_2.4H_2O$ 1.81 g; $ZnSO_4.7H_2O$ 0.222 g; $CuSO_4.5H_2O$ 0.079 g; MoO_3 0.015 g; Components were added to distilled/deionized water and brought to volume 1 l and autoclaved for 15 min at 15 psi pressure 121˚C [34].

Trace Metal Mix A

NH_4NO_3 22.96 g; KCr $(SO_4)_2.12H_2O$ 192.00 g; $NiSO_4.6\ H_2O$ 44.80 g; $Na_2WO_4.2H_2O$ 61.10 g; $Co(NO_3)_2.6H_2O$ 43.98 g; Components were added to distilled/deionized water and brought to volume 1 l and autoclaved for 15 min at 15 psi pressure 121˚C [34].

Cultivation Conditions

One liter of sterile Zarroul medium in 5 liter sterile flask was used to growing *A. fusiformis* under static condition and in lab., exposed to sunlight as well as the room normal light. The flask left for ten years. Water is added only if the culture is nearly to be out of their water content.

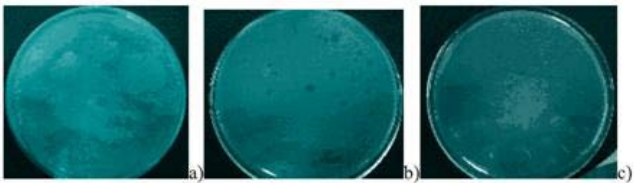

Figure 2: Clear zoon(s) (2a,b,c) due to bacteriophage infections.

Sample preparation for electron microscopy

In this study the bacteriophage activity was examined using electron microscope which was used to scan the surface of the dried bacterial growth media show phage infection. The part where the bacteriophage cause clear zone due to their killing to the bacteria was taken carefully after complete dry of the agar.

The whole agar layer was separated from the surface of the glass Petri dish and the clear zoon was cut-off. The cut part then put on the surface of the light microscope slide. 20µL of distilled water was put on the surface of the glass slide and the cut part of the agar then put just on the water surface and fixed gently. The sample then left until complete drying. The dry and fixed part of the agar surface then coated with approximately 15 nm gold (SPI-Module™ sputter Coater).

Scanning of the agar surface

The golden coated sample then subjected to be scanned by analytical scanning electron microscope (Jeal JSM-6360LA) with secondary element at 20 KV acceleration voltages at room temperature. The digital image were adjusted and saved.

Bacterial cell surface scanning and counting the phage number

The infected cells image under the scanning electron microscope was enlarged to a propitiate size and the number of phages infected a single cells from one side was calculated manually.

Qualitative determination of the exopolysaccharide

The exopolysaccharide which performed either in the biofilm or distributed in the cultivation medium was isolated simply by using 95% cold ethanol (-80˚C), where 10 ml sample was taken and filtered using tissue to remove the cyanobacteria biomass. After that, 750µl of the filtrate was taken and 1 ml of the cold ethanol was loaded over it in Eppendorf tube and the sample then centrifuged at 13000 rpm. Then the supernatant was discarded and the rest of the sample was washed with 75% cold ethanol and centrifuged again at 13000 rpm. The obtained exopolysaccharide dried and then preserved in -20˚C for further investigations.

Results and Discussion

The observation and the analysis of the nature phenomena have learned the human a lot. The understand for the biological ecosystem emerge new idea, technology and opportunity. Usually, the native biological ecosystem succeeded in issues did not solved by scientists,

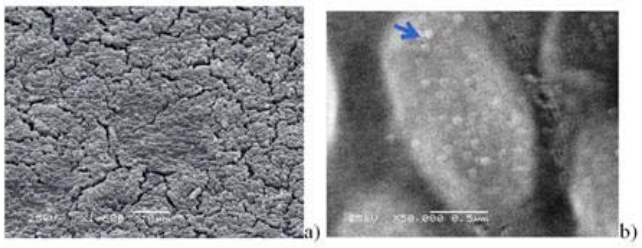

Figure 3: a) Agar surface show clear zone due to bacteriophage infection; b) Bacterial cells show bacteriophage growth on their surface.

that because of the power given to it, its variation, resources and due to the adaptations of the creatures over generations. The symbiotic activity between microbes could exceed the domain to include various and unparallel creatures. In this study various factors play roles in the surviving of the *Arthrospira fusiformis* in a single 5 liter flask for more than ten years as in Figure 1. At the first we could not explain such phenomena correctly, but only proposed it to the tolerance of the *Arthrospira* strain to the harsh cultivation conditions. In fact after conducting research for a period longer than twelve years in various subjects, the image become more clear. Conducting research not only on *Arthrospira fusiformis*, bacteriophages, salinity, and biopolymers but also on the lysis of the microbes including both of the eukaryotic and the prokaryotic cells enable such understand. The presence of the bacteriophage in the growth medium of the *Arthrospira fusiformis* was observed on the cultivation plats as in Figures 2 (a, b and c). The plates which contain clear zones due to phage infections were kept in 37˚C till complete drying. The clear zoon was cut-out and fixed on microscopic slide and prepared using standard criteria and tested using the electron microscope. The plates which show clear zones contain no bacterial growth as in Figure 3 (a) using magnification 1500X. The enlarging of the image enable counting the number of infected phage per single cells. About 47 bacteriophages were counted as in Figure 3(b) using magnification 50000X.

Recently Amara and Steinbüchel (2013) proved that salinity is a critical factor in eradicating microbes other than *Arthrospira fusiformis*. Additionally, alkaline chemical compounds such as $CaCO_3$ and $NaHCO_3$ are used for preparing microbial ghosts and proved to have efficient effect on introducing pores in both of the prokaryotic and the eukaryotic [30,31,40-44].

The conditions which enable such survive over ten years could be summarized in the following points:

1. *Arthrospira fusiformis* is able to produce exopolysaccharide.

2. The exopolysaccharide which produced by the *Arthrospira fusiformis* (data not shown) not protect it only but protect other microbes as proved by Amara (2011) [45].

3. The seasonal change as well as the evaporation of the water content increase the salinity and the alkalinity which induce lysis for the dead cells and for intolerant microbes. That cause some sort of water purity and provided additional source of nutrient for the *Arthrospira fusiformis*.

4. Bacteriophages existed in the ecosystem induce bacterial lysis and reduce the microbial load as well as the cytoplasm of the evacuated cells and supply the *Arthrospira fusiformis* with additional source of nutrients.

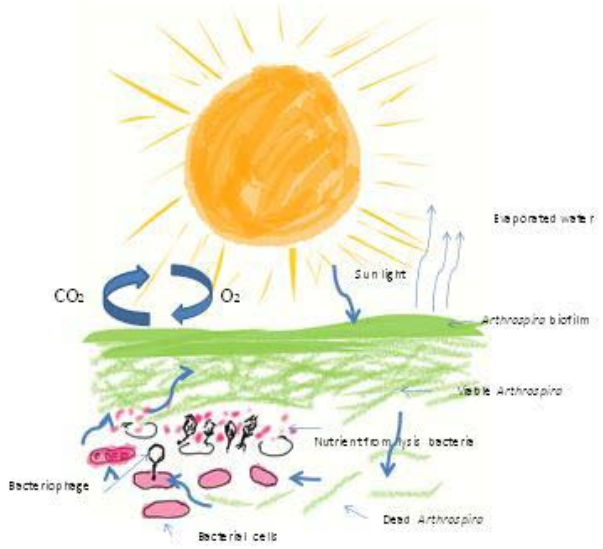

Figure 4: Model diagram show the proposed symbiotic relationship between the bacteria, *Arthrospira fusiformis* and the bacteriophage.

5. Apparently, each of the bacteriophage, the bacteria and the *Arthrospira fusiformis* are adapt themselves in a balanced live which did not lead to eradicate any of them. The dead *Arthrospira fusiformis* used as a food by the bacteria and the bacteria used by the bacteriophages which lysis them and the evacuated cells release again the nutrients in the medium which enrich the growth of the *Arthrospira fusiformis*.

Conclusion

In conclusion the presence of the bacteriophage in the cultivation medium grantee the lysis of the bacterial cells not protected by the *A. fusiformis* exopolysaccharide. And the bacterial cells or their enzymes are responsible for degrading the dead cells of *A. fusiformis*. The reducing of the water content is responsible for increasing the salinity and the alkalinity ad cause also lysis for the bacterial cells. The bacterial cell remove the waste from the medium and the bacteriophage reduce their number and release nutrient to the *A. fusiformis*. Only water is needed to be added particularly in rising the temperature in summer (nearly 1 liter for two time/year). *A. fusiformis* biomass as well as their exopolysaccharide were stay nearly stable during the ten year cultivation and the *A. fusiformis* regenerate their biomass and their biofilm. Microbes could collaborate to safe themselves and could sense the changes in the surrounding environment and reduce their number based on the nutrients around.

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