Effect of pH on phytochemical and antibacterial activities of *Spirulina platensis*

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Abstract

This study presents the effect of pH changes on productivity of *Spirulina platensis*. The highest content of its dry weight (0.0853 g/20 ml), chlorophyll *a* (2.8 µg/ml), carotenoids (2.6 µg/ml), protein and carbohydrates are recorded at pH 10 (425 mg/g and 97 mg/g) respectively. As its phenolic content was maximized with acetone extract (0.52 mg/g) while flavonoids were detected with ethanol extract (7.6 mg/g). Antimicrobial activity of *S. platensis* was prepared in 70% (methanol, ethanol and acetone) at different pH values, and tested against some pathogenic bacterial. Its highest antibacterial activities were reported with 70% acetone extract. The maximum inhibition of *Staphylococcus aureus* NCTC-7447 was observed in acetone extract at pH 8.0 and 10, whiles it was recorded at pH 8.0 for *E. coli* NCTC-10418.

**Key words:** *Spirulina*, pH, antibacterial.

INTRODUCTION

The problem of microbial resistance to dings is growing and an outlook for the use of antimicrobial drugs in the future is still uncertain. The majority of clinically used antimicrobial drugs have drawbacks like toxicity, lack of efficacy, inhibiting cost and their frequent use leading to the emergence of resistant strains. Thus, there is an urgent need to develop alternative biodegradable agents, which should be free from side effects. Natural compounds are biodegradable and so more environmentally...
acceptable (Pradhan et al., 2012). Cyanobacterial metabolites showed an interesting and exciting range of biological activities ranging from antimicrobial, anticancer, antiviral, immunosuppressant, insecticidal, anti-inflammatory to proteinase-inhibiting activities which are striking targets of biomedical research (Shweta Yadav et al., 2011, Sachin Chauhan et al., 2014 and Pandey 2015). *Spirulina* is a cyanobacterium which acquired the ability for photosynthesis before any other organism and is considered to be the ancestor from which the higher plants evolved (Desai and Sivakami, 2004). *Spirulina platensis* is one of the important micro-alga showing antimicrobial activity against many pathogenic bacteria and fungi (Kumar et al., 2013). It is contains many functional bioactive ingredients with antioxidant and anti-inflammatory activities, including phenolic phytochemicals (Jensen et al., 2015). *Spirulina* refers to various species of blue-green algae found naturally in lakes and grown commercially. It is grows in fresh, salt and marine, as well as in brackish water. It grows best in a high alkaline environment of pH 10-12 (Capelli and Cysewski, 2010). Previous studies investigated the effect of pH on *Spirulina* growth, pigment production, and protein content (Pandey and Tiwari, 2010). It is rich in important nutrients, such as proteins (50-70 %), vitamins, minerals, carbohydrates and essential fatty acids such as gamma linolenic acid (Vonshak et al., 1988).

The present study aims to optimize the growth conditions in respect to pH value of *Spirulina platensis* for the highest biomass production and its antibacterial activities.

**MATERIALS AND METHODS**

**Microorganism cultivation and growth conditions**

*Spirulina platensis* was obtained from National Institute of Oceanography & Fisheries, hydrobiology Lab. It was cultivated axenically as batch culture using Zarrouk medium described by (Zarrouk 1966). It was grown in Erlenmeyer flasks (1000 ml) each flask containing 700 ml of Zarrouk’s medium, adjusted at different pH values (8.0, 9.0, 10.0, 11.0 and 12.0). Each flask was inoculated with 70 ml of a pre-culture of the experimental organism. These flasks were placed on shelves illuminated by fluorescent lamps at light intensity of 26 µE/m²/s and 30±1°C. At the end of the experimental period, the growth and production of pigments were determined.

**Growth evaluation**

Cyanobacterial growth was monitored using optical density of the culture according to Fatma et al. (1994) at 750 nm (OD750) and by determination of cyanobacterial cellular dry weight (CDW). Biomass productivity was calculated according to APHA (2005).
Estimation of pigment (chlorophyll \textit{a} and carotenoids)

A known volume of \textit{S. platensis} culture was centrifuged at 6000 rpm for 10 min. The supernatant was decanted and an equal volume of methanol added to the pellet, then it was incubated in a water bath at 55°C for 15 min and centrifuged. Absorbance of the extract (A) was measured against blank of free methanol at 650, 665 and 452 nm. Chlorophyll \textit{a} and carotenoids were estimated as \( \mu g \text{ ml}^{-1} \) of culture suspension using the following equation (MacKinney 1941):

\[
\text{Chlorophyll a (} \mu g \text{ ml}^{-1} \text{)} = 10.3 E665 - (0.918 \times E650) \text{ and Carotenoids (} \mu g \text{ ml}^{-1} \text{)} = 4.2 E452 - (0.0246 \text{ chl a}).
\]

Estimation of total carbohydrates

Total carbohydrates were quantitatively determined by phenol sulphuric acid method described by Kochert (1978) using glucose as a standard reference.

Estimation of total soluble proteins

After pigment extraction, residual cells were extracted using 1 N NaOH in a boiling water bath for 2 h, as described by Payne and Stewart (1988). Protein concentration, as mg ml\(^{-1}\), was determined according to Lowry et al., (1951), using bovine serum albumin as a standard reference.

Estimation of total phenolic compounds

Total phenolics were estimated quantitatively according to Jindal and Singh (1975), using pyrogallol as a standard reference.

Estimation of total flavonoid compound

Aluminum chloride colorimetric method, as modified by Chang et al (2002) was used to estimate flavonoids content. Different concentrations of quercetin were used to make the calibration curve.

Preparation of \textit{Spirulina platensis} extracts:

About 0.2 gm of alga was soaked in 70% Methanol (5 ml) for 24h and centrifugation the residues were repeated soaked in 70% Methanol (5ml) for 24h and centrifugation then by 70% Acetone and 70% Ethanol. The filtrate was taken and concentrated in vacuum until drying. Extracts were stored in airtight glass bottles in refrigerator until use. For testing antimicrobial activity the extracts were mixed with 5 ml of the same solvent used for each extract.
Microorganisms tested
Reference bacteria required for present studies were obtained from Microbial Physiology-Botany and Microbiology department, Faculty of science, Al-Azhar University. They included five bacterial strains, *Pseudomonas aeruginosa* NCIB-9016 and *Escherichia coli* NCTC-10418 (gram-negative bacteria), *Bacillus subtilis* NCTC-10400, *Bacillus diminuta* ATCC-19146 and *Staphylococcus aureus* NCTC-7447 (gram-positive bacteria). In addition, four pathogenic bacterial strains were obtained from faculty of Medicine, Menoufia University, *Acinetobacter* sp, *Klebsiella pneumonia*, *Salmonella typhi* (gram-negative bacteria) and *Staphylococcus aureus* (as gram-positive bacteria). All of the cultures were maintained in nutrient agar slants.

Preparation of bacterial suspension:
The bacteria used were obtained from slants which were less than 30 days old. Loop full samples were taken from the slants which were grown in sterile 50 ml nutrient broth medium, autoclaved at 121°C under 1.5 atmospheres pressure for 15 min. Then left grow for 16 h at 37°C in a shaking incubator agitating, at 140 rpm (Mtolera and Semesi, 1996). All bacterial suspensions of tested microorganism were adjusted to $10^8$ CFU.

Antimicrobial activity test
Paper disc diffusion method (Pelczar et al., 1993) was used for testing antimicrobial activities of *S. platensis*. The media was poured into sterile petri-dishes (9.0cm diameter) and allowed to set. The paper disc diffusion method was employed for antimicrobial susceptibility. Whatman filter paper discs (No. 1, Diameter 6mm) saturated with about 50 μl of the different extracts, was placed on culture medium seeded with the test organism. Disc fed with corresponding solvent alone served as the control. These Agar plates were incubated at 37±2°C for bacteria. After 24 h, inhibition zone around the disc was measured in mm. the experiment was carried out three times and the mean values were presented.

RESULTS
1-Effect of initial pH on growth parameters and pigment production of *S. platensis*
Effect of initial pH values on growth of *S. platensis* was recorded as OD 750 for 30 days incubation, with 3 days intervals (figure 1). Generally, the obtained results revealed that decreasing and increasing of pH values showed significant changes in growth pattern or biomass productivity.
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![Effect of pH Values](image)

**Figure 1:** Effect of pH values on growth of *Spirulina platensis* even 30 days of incubation

It is evident from the data presented in table (1) that the maximum dry weight (0.0853 g/20 ml) of *S. platensis*, proteins (425 mg/g) and carbohydrates (97 mg/g) were recorded at pH 10. As the maximum pigments content (table 1), chl *a* (2.8 µg/ml) and carotenoids (2.6 µg/ml) were reproduced at pH 10.

The total phenolics and flavonoids of *S. platensis*, with different solvent extracts 70 % (acetone, methanol and ethanol), were presented in table (2). The highest phenolic content was found in acetone extract (0.52mg/g) while the highest flavonoid content was reported with ethanol extract (7.6 mg/g), at pH 10.0.

Significant variations in the growth, proteins, carbohydrates, pigments contents (chl *a* and carotenoids), flavonoid and phenol content were attained at different pH values.

2- Effect of different initial pH values on production of antibacterial substances from *S. platensis* extracted with different solvent

The experiments were adopted in order to determine the effect of initial pH values on production of antibacterial substance of *S. platensis*. It was cultivated in favorable conditions, as mentioned in material and methods, but the inoculums was exposed to different pH values and the same extraction method were tested against different bacterial strains.

The acetone extract showed antibacterial effect on wide range of bacteria and recorded highest zone of inhibition against *Staphylococcus aureus* NCTC-7447 (20.00 mm), at pH 8.0, 10.0 and *E. coli* NCTC-10418 and *S. aureus* at pH 8.0.
At the same methanol extract showed maximum inhibition of 20.0 mm with *S. aureus* NCTC-7447, while it was reported 17.00 mm with ethanol extract at pH 8.0, as shown in figure (2, 3, 4).

**Table 1.** Effect of initial pH values on the growth, proteins and carbohydrates of *Spirulina platensis*.

<table>
<thead>
<tr>
<th>pH Values</th>
<th>D. Wt. g/20ml</th>
<th>Proteins mg/g d.wt</th>
<th>Carbohydrates mg/g d.wt</th>
<th>Chlorophyll a µg/ml</th>
<th>Carotenoids µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>0.0562±0.00025d</td>
<td>363±6c</td>
<td>71±0.2d</td>
<td>1.4±0.025c</td>
<td>1.78±0.015d</td>
</tr>
<tr>
<td>9.0</td>
<td>0.0642±0.0002b</td>
<td>373±4bc</td>
<td>81±0.1b</td>
<td>1.8±0.014b</td>
<td>1.99±0.008b</td>
</tr>
<tr>
<td>10.0</td>
<td>0.0853±0.00035a</td>
<td>425±3a</td>
<td>97±0.8a</td>
<td>2.8±0.051a</td>
<td>2.60±0.013a</td>
</tr>
<tr>
<td>11.0</td>
<td>0.0581±0.00025c</td>
<td>377±6b</td>
<td>73±0.5c</td>
<td>0.8±0.013d</td>
<td>1.84±0.008c</td>
</tr>
<tr>
<td>12.0</td>
<td>0.0363±0.00035c</td>
<td>166±4d</td>
<td>59±0.8e</td>
<td>0.3±0.016e</td>
<td>1.14±0.012e</td>
</tr>
</tbody>
</table>

Each value is the mean of three readings ± standard deviation. Values with the same small letter in the same column showed insignificant difference (at p≤0.05).

**Table 2.** Effect of initial pH values on the phenol and flavonoid content of *Spirulina platensis*.

<table>
<thead>
<tr>
<th>pH Values</th>
<th>Phenol</th>
<th>Flavonoid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetone mg/g d.wt</td>
<td>Methanol mg/g d.wt</td>
</tr>
<tr>
<td>8.0</td>
<td>0.221±0.0006c</td>
<td>0.186±0.0007b</td>
</tr>
<tr>
<td>9.0</td>
<td>0.180±0.0006d</td>
<td>0.158±0.0006c</td>
</tr>
<tr>
<td>10.0</td>
<td>0.524±0.0004a</td>
<td>0.189±0.0006a</td>
</tr>
<tr>
<td>11.0</td>
<td>0.263±0.0006b</td>
<td>0.121±0.0004d</td>
</tr>
<tr>
<td>12.0</td>
<td>0.120±0.0004d</td>
<td>0.047±0.0002a</td>
</tr>
</tbody>
</table>

Each value is the mean of three readings ± standard deviation. Values with the same small letter in the same column showed insignificant difference (at p≤0.05).
**Figure 2:** Effect of pH values on antibacterial activity of acetone extraction of *S. platensis*

**Figure 3:** Effect of pH values on antibacterial activity of methanol extraction of *S. platensis*
Figure 4: Effect of pH values on antibacterial activity of ethanol extraction of *S. platensis*.

Values with the same small letter in the same row showed insignificant difference (at p≤0.05). Error bars indicate SD

**DISCUSSION**

Growth of *S. platensis* started to decline after 21 days of incubation. This could be attributed to increase of death over the growth. Most of the previous works measured biomass of *S. platensis* after 15 and 25 days and none of them used extra time to follow biomass depletion (Murugan and Radham, 2010 & Kumer et al, 2011). Abo-Shady et al., (1992) found that the optimum culture conditions for the formation of highest biomass, protein and phycocyanin by *S. platensis* were achieved by inoculating cultures for 20 days. Delrue et al., (2017) found that the optimum culture conditions were even observed after 21 days of batch cultivation.

In the present study, biomass production of *S. platensis* has been evaluated under different pH levels. Its maximum growth rate was introduced at pH 10.0. This can be attributed to the optimal capacities of both photosynthesis and respiration which are associated with its enzymatic activities within the associated pH. (Kaushik and Shanna 1997). The given results was more or less similar with that reported by Abd El-Baky et al., (2003) who stated that *S. platensis* and *S. maxima* were grown best, for biosynthesis and production of some important chemicals, such as carotenoids and tocopherols, at pH 10.5. Whereas Ismaiel et al. (2016) recorded the highest biomass yield of *Spirulina* sp. was recorded at pH 9.0.

Also the present finding was parallel with Soundarandian and Vasanthi (2008), they were reported that the maximum growth of *S. platensis* was observed at pH 10.0, and could be attributed to optimal activity of all the enzymes needed for photosynthesis and respiration at such pH. Other with Pandey et al., (2010) was
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maintained that the various strains of Arthrospira (Spirulina) showed maximum growth and pigment biosynthesis at alkaline pH. The higher biomass production recorded in Zarrouk’s medium was most likely due to its high alkalinity.

On this respect, Abo-Shady et al., (1992) obtained the highest values of biomass, protein and phycocyanin of S. platensis, in Zarrouk's medium, at pH 8.3. While Rafiqui et al., (2005), recorded the maximal protein contents of S. platensis and S. fusiformis at pH 9.0 and 10.0, respectively. In the previous studies, it was reported that optimization of growth of Spirulina platensis was observed at pH 10.0 (Thirumala 2012)

The present studies introduced that total phenolic compounds and flavonoids of S. platensis with the different used solvents (70% Acetone, 70% Methanol and 70% Ethanol). The highest contents was represented with Acetone extracts. That was agree with Elbagowry (2014),They stated that the stronger inhibitory effect on some tested microorganisms induced by 70% acetone, followed by 70% methanol and finally 70% ethanol. As Salamatullah (2014) was tested total phenolic and flavonoid with different solvent extracts (Acetone, Methanol and Ethanol). He found that the highest phenolic was determined with Acetone extraction while flavonoid was found in methanol. On the other side, Indumathi (2016) had shown acetone, ethanol and diethyl ether extracts of S. platensis and other microorganisms, acetone extracts show least or no sensitiveness to most bacteria except Enterobacter sp. and K. Pnuemoniae.

Chakraborty et al. (2015) tested the antibacterial efficacy of Spirulina platensis extract against few Gram positive and Gram negative bacteria. Maximum zone of inhibitions were maximally shown by extract from water followed by methanol, acetone and ethanol when tested against S. aureus, S. epidermidis, K. pneumonia, P. aeruginosa and E. coli by well diffusion assay. Elshouny et al. (2017) was stated the effect of the various extracts of Spirulina platensis, Chlorella vulgaris, Saragassum wightii and Saragassum latifolium using different solvents (methanol, ethanol, ethyl acetate and chloroform) as antimicrobial agents against five bacterial pathogens; S. aureus, E. coli, P. aeruginosa, Salmonella sp, Shigella sp, and indicated that among the various extracts used, methanol extracts of tested cyanobacteria land algal species appeared to be the most effective ones showing maximum antibacterial activity against the selected bacterial pathogens. Spirulina platensis appeared to be the most effective against all the tested pathogens.

In this present study, acetone extract gave the highest zone of inhibition of 20 mm of Staphylococcus aureus. This the result don’t agree with Chakraborty et al., (2014), had shown Spirulina platensis extracts were prepared in water, methanol, ethanol and acetone with different concentration and tested against Staphylococcus aureus. Water extract gave the highest zone of inhibition of 19 mm in diameter followed by methanol extract which gave zone of inhibition of about 16 mm in diameter, ethanol extract exhibited zone of inhibition of 13 mm in diameter and acetone extract provided the lowest zone of inhibition of 9 mm.
CONCLUSIONS
Optimal growth rates of *Spirulina platensis*, cultivated in Zarrouk’s media, were obtained at pH 10.0 that was associated with biosynthesis and production of carotenoid, protein and carbohydrates. Acetone extract showed its highest phenolic and flavonoids content. It has antibacterial activity against some pathogenic bacteria.

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