

DOI : <http://doi.org/10.22438/jeb41/5/MRN-1395>

Investigating the applications of *Chlorella vulgaris* in agriculture and nanosilver production

M.F. Al dayel¹, N.A. El Semary^{*1,2}, K. Al Amer³ and K.M. Al Ali¹

¹Department of Biological Sciences, College of Science, King Faisal University, Al-Ahsa-31982, Kingdom of Saudi Arabia

²Department of Botany and Microbiology, Faculty of Science, Helwan University, Cairo-11795, Egypt

³Department of Chemistry, College of Science, King Faisal University, Al-Ahsa-31982, Saudi Arabia

*Corresponding Author Email : nelsemary@kfu.edu.sa

Paper received: 14.01.2020

Revised received: 03.03.2020

Accepted: 17.05.2020

Abstract

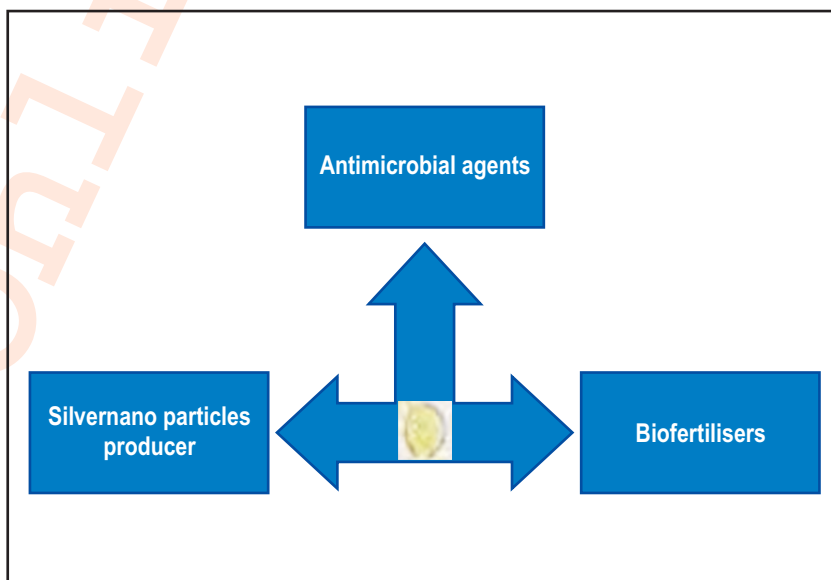
Aim: To investigate the dual beneficial effect of *C. vulgaris* as biofertilizer and antimicrobial agent in the form of nano particles.

Methodology: The antibacterial effect of aqueous extract was investigated by diffusion disk bioassay against five pathogenic bacterial strains. The plant growth promoting effect was investigated by performing germination tests on three types of seeds and measuring their morphometric growth parameters. The silver nanoparticles biosynthetic ability of alga was investigated by incubating with silver nitrate solution. The biogenic nanosilver was tested against multidrug-resistant *Staphylococcus aureus* (MRSA).

Results: The antibacterial effect of the extract, despite being derived from a miniscule biomass, was detected against all pathogenic strains. The inhibition zones ranged from 0.7 to 1 cm. In addition, the aqueous extract was effective bio-fertiliser that resulted in a significant increase in germination percentage and growth parameters of *Eruca sativa*, *Lepidium sativum* and *Vigna radiata* seedlings. Furthermore, the silver nanoparticles were observed to form both intra- and extracellularly. The UV-visible spectrum of nanoparticles showed a characteristic broad peak at 421-425 nm, while IR showed the presence of characteristic signal at 3297cm⁻¹. The nanosilver particles were effective antimicrobial agents against multi-drug resistant MRSA.

Interpretation: The results emphasize the multiple potential of *C. vulgaris*, which indicates its great potential if grown on a large scale for mass production. The present research also sheds some light on the potential of microflora inhabiting this rather underexplored part of the world.

Key words: Antibacterial, Bio-fertilizer, *Chlorella vulgaris*, Silver nanoparticle



How to cite : Al Dayel, M. F., N. A. El Semary, K. Al Amer and K.M. Al Ali: Investigating the applications of *Chlorella vulgaris* in agriculture and nanosilver production. *J. Environ. Biol.*, **41**, 1099-1104 (2020).

Introduction

Chlorella vulgaris is a green microalga that belongs to Order: Chlorellales, Family: Chlorellaceae (Bejerinick, 1890). It is widely distributed in both freshwater or marine ecosystem (Hodač et al., 2016). Cells of *Chlorella* species are small spherical or ellipsoidal whose chloroplast is cup-shaped lying in a parietal position. This alga is considered as a rich source of phytohormones (Tarakhovskaya et al., 2007) and essential nutrients. It is estimated to contain about 6% protein, 7.5% fat, and 18% carbohydrates. The protein of this alga contain essential amino acids, high percentage of beta-carotene and chlorophyll (Krienitz et al., 2018), vitamin A, thiamin (B1), riboflavin (B2), pyroxene (B6), niacin and cyanocobalamin (B12), C, E, K, minerals such as magnesium, potassium, phosphorus, copper, zinc, iodine, cobalt, calcium and iron and digestive enzymes (Sawant and Mane, 2018). All these attributes indicate high nutritional value of this alga. However, its use as a source of liquid bio-fertilizer (Ronga et al., 2019) with sparse reviews such as that of Priyadarshani and Rath (2012). (Faheed and Abd El Fattah, 2008; Bumandalai and Tserendulam. 2017). Interestingly, *Chlorella* extract has previously showed antibacterial activities (Acurio et al., 2018). Therefore, the beneficial role of aqueous extract of *C. vulgaris* both as biofertilizer and as antibacterial agent against *Bacillus* sp., *Streptococcus* sp., *Staphylococcus aureus* and *E. coli* was investigated. *Streptococcus* sp. bacterium is found in the digestive system of humans and animals and are widely spread in plants and dairy products. *Bacillus* sp. is a rod-shaped bacterium that plays an important role in the corruption of a number of foods and in food poisoning (Ouardien et al., 2018). *Escherichia coli* which lives mainly in the digestive system human and animal are also present in soil. Its presence in food is indicative of faecal contamination (Malar et al., 2018). Lastly, *Staphylococcus aureus* is a Gram-positive bacterium with several strains being multidrug-resistant. It is also the most common cause of food poisoning (Ondusko and Nolt, 2018). These pathogenic bacteria are of different characteristics and quite difficult to find one antimicrobial agent that has broad-spectrum activity against all in addition to having plant growth-promoting activities. This is a necessity nowadays as there is a surge for searching for eco-friendly fertilizers that replace harmful chemical fertilizers. In addition, if these biofertilisers have antimicrobial affect, they can reduce the use of harmful pesticides. Therefore, in order to address its protective ability against multidrug-resistant bacterial strain, the ability of nanosilver particles biosynthesis by this alga was investigated and the antibacterial action of biogenic nanosilver particles was tested against multidrug resistant *Staphylococcus aureus* (MRSA). *Chlorella vulgaris* has been reported to produce nanosilver (Soleimani and Habibi-Pirkoohi, 2017). Skulberg (2000) pointed out the need to exploit microalgae from rather underexplored areas for their applicable potential as they possess unique metabolites and bioactive compounds of wide array of uses and impact. Keeping in view the above, the present study was carried out to investigate the

application of *Chlorella vulgaris* in agriculture and nanoparticles biosynthesis.

Materials and Methods

Collection and identification of samples : The algal sample was collected in a sterile sampler from an irrigation canal supplied by groundwater in the east of Al-Ahsa Governorate. Light microscopic examination was performed to determine the types of algae present. Bold's medium was used to culture microscopic freshwater algae. After successive agar-streaking trials followed by purification, the green alga *Chlorella vulgaris* was identified as verified by microscopic examination.

Preparation of aqueous algal extract : The aqueous extract was prepared from 0.3 g fresh weight of *Chlorella vulgaris* biomass that was homogenized and extracted in 50 ml of sterile distilled water. To test the effect of aqueous *Chlorella vulgaris* extract on germination of seeds of different plants, ten seeds with replicates of *Vigna radiata*, *Eruca sativa* and *Lepidium sativum* were washed and sterilised using 6% (v/v) bleach for few minutes, then washed thoroughly with sterile water several times and soaked in the aqueous extract for a day. Then the seeds were planted on a sterile wet cotton wool and observed for their germination for a week. Germination percentage, radical and plumule length were recorded for the treatments and compared with control.

Antibacterial bioassay : The antibacterial potency of aqueous extract obtained from *Chlorella vulgaris*, was assessed against pathogenic bacterial strains namely, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus* sp. and *Bacillus* sp. that were freshly sub-cultured prior to assay. Sensitivity of pathogenic strains to the extract was assayed by modified Kirby Bauer Disk Diffusion Susceptibility method (Bauer et al., 1966). Sterilized-paper discs (6 mm in diameter) were saturated with 30 µl of extracts for antibacterial tests. Discs were dried and placed on the surface of inoculated nutrient agar medium with bacterial suspensions prepared in physiological saline (0.85 g NaCl 100 ml⁻¹ distilled water). Plates were kept for 2 hr at 4 °C to ensure diffusion of bioactive material, after that the plates were incubated at 37 °C. Control discs containing 30 µl of sterilized distilled water were left to dry, then used as negative control whereas positive control sets included discs of antibiotic Chloramphenicol. Plates were incubated for 24 hr and diameter of inhibition zones (mm) were measured in triplicates and the average standard deviation was recorded (El Semary, 2012). The antimicrobial bioassay was performed in a single experiment using nanosilver nanoparticles produced by *Chlorella vulgaris*, of low concentration 5 µg ml⁻¹, as antimicrobial agent against multidrug-resistant strain of Methicillin-resistant *staphylococcus aureus* (MRSA).

Biosynthesis of silver nanoparticles by *Chlorella* cells: A stock solution of silver nitrate (Sigma–Aldrich, USA) of 1M concentration was prepared and used in study. The solution was

Table 1 : Growth parameters (in percentage) of untreated (control) and treated seeds with aqueous extract of *Chlorella vulgaris*

Growth parameters	Seeds	Control	<i>Chlorella vulgaris</i> extract
Germination percentage	<i>Lepidium sativum</i>	100 ± 0	100 ± 0 ^a
	<i>Eruca sativa</i>	100 ± 0	100 ± 0 ^a
	<i>Vigna radiata</i>	100 ± 0	100 ± 0 ^a
Radicle length	<i>Lepidium sativum</i>	0.99 ± 0.3	1.31 ± 0.3 ^b
	<i>Eruca sativa</i>	1.03 ± 0.4	2.01 ± 0.1 ^b
	<i>Vigna radiata</i>	2.76 ± 0.3	3.73 ± 0.3 ^b
Plumule length	<i>Lepidium sativum</i>	0.67 ± 0.3	0.93 ± 0.2 ^b
	<i>Eruca sativa</i>	0.88 ± 0.3	1.31 ± 0.2 ^b
	<i>Vigna radiata</i>	0.65 ± 0.2	0.97 ± 0.3 ^b

^a Insignificant at $p < 0.05$; ^b Significant at $p < 0.05$ confidence

kept in dark bottle. *Chlorella vulgaris* biomass was collected by centrifugation (6000 rpm) and thoroughly washed with distilled water and centrifuged again. The biomass was incubated with silver nitrate solution for two months, both algal biomass and outer solution turned brown and were analyzed for silver nanoparticles production. The cells of *Chlorella vulgaris* were homogenized and the resultant homogenate was centrifuged for 30 min at 6000 rpm for complete precipitation of all cell debris. Supernatant was collected and filtrated using sterilized 0.2 µm millipore filter membrane. Filtrate was collected and used for the analysis of silver nanoparticles (AgNPs). Biogenic silver nanoparticles were analyzed and characterized by the following analyses.

UV-visible spectroscopic (UV-vis) analysis: The initial characterization was carried out by a UV-vis spectrophotometer (Genesys 10S UV-visible double beam spectrophotometer). The spectrum was recorded at room temperature using 200 µl test volumes with 1 cm path length quartz cuvette, in a wavelength range of 200 nm to 800 nm. Nanoparticle solution was diluted 5 times with deionized water to avoid errors due to high optical density of the solution.

Dynamic light scattering analysis: Dynamic light scattering (DLS) was studied with Dual Scattering Particle Size Analyzer (cilas, Nano DS). It was used to determine the average particle size and distribution of AgNPs. Each sample was interpreted in triplet at 25 °C with scattering angle of 135 °C. Deionized water was used as medium disperses.

Infrared spectrometer analysis of AgNPs : Characterization of AgNPs An Fourier-Transform infrared spectrometer (FT-IR, Agilent Cory 630, Agilent Technologies, USA) was used to analyze the chemical composition of nano particles.

Statistical analyses : Statistical significance of results was tested by Analysis of Variance (ANOVA) at $p < 0.05$. One way Anova test was performed using Minitab version 13.

Results and Discussion

Chlorella sp. is a widespread green alga found all over the world. Its wide presence in diverse habitat is an indicative of its

Table 2 : Antibacterial activity (zone of inhibition) of *Chlorella vulgaris* aqueous extract

Bacterial species	Inhibition zone
<i>Staphylococcus aureus</i>	0.8 ± 0.1
<i>Streptococcus</i> sp.	0.7 ± 0.1
<i>Bacillus</i> sp.	1.0 ± 0.2
Methicillin-resistant <i>staphylococcus aureus</i>	0.7 ± 0.1
<i>E.coli</i>	0.9 ± 0.1

adaptive metabolic capabilities (Hodač *et al.*, 2016). *Chlorella* sp. is rich in many bioactive compounds such as minerals, vitamins, growth factor and is used in healing wounds and as a food (Jensen, 1987). The use of *Chlorella* sp. for enhancing seed growth was reported by Faheed and Abd El Fattah (2008) who used *Chlorella* treatment on seeds of *Lactuca sativa* which significantly increased the seed growth. Addition of microalga to the culture medium or soil significantly increased the fresh and dry weight of seedlings. They attributed this enhancing effect to the presence of high amount of macronutrients, micronutrients and amino acids in the microalgal treatment. Al-Shakankery *et al.* (2014) showed that *Chlorella vulgaris* treatment significantly enhanced the growth of maize plants both quanti and qualitatively mainly due to the presence of many growth-promoting compounds. In the present study, *C. vulgaris* showed enhancing effect on different plant seeds. The germination rate of *Vigna radiata* seeds with *Chlorella vulgaris* aqueous extract was faster than that of control where the germination percent reached 90% on first day while that of control was only 70%. Both reached 100% germination percentage on the second day. The average length of radical and plumule were significantly greater than that of control (Table 1).

On the other hand, the germination rate of *Eruca sativa* seeds treated with *C. vulgaris* extract was equal to that of the control on all days. However, there was a different trend with regard to plumule and radical length. The highest radical length was obtained for the treatment with aqueous extract of *Chlorella*. Nonetheless, both radicle and plumule length were significantly greater than control.

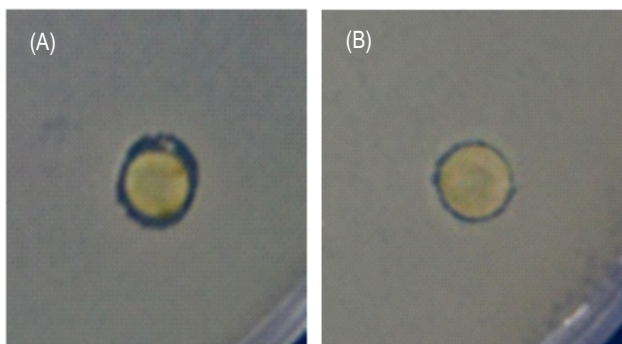


Fig. 1: Inhibition zone of aqueous extract of *Chlorella vulgaris* against (A) *Bacillus* sp. and (B) Methicillin-resistant staphylococcus aureus.

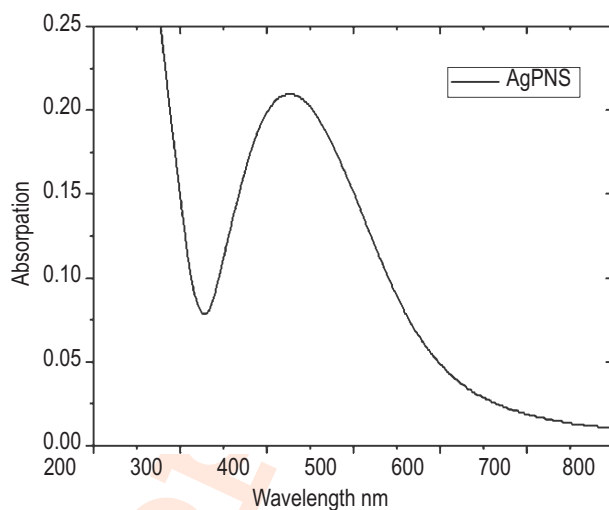


Fig. 2 : UV spectra for AgNPs.

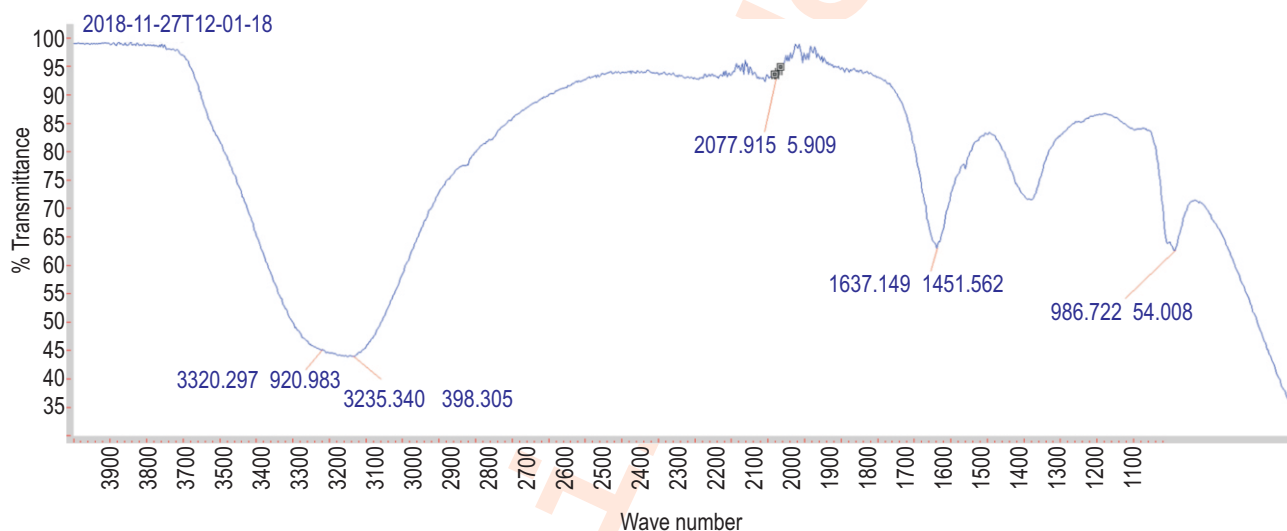


Fig. 3 : FTIR spectrum for nanosilver particles.

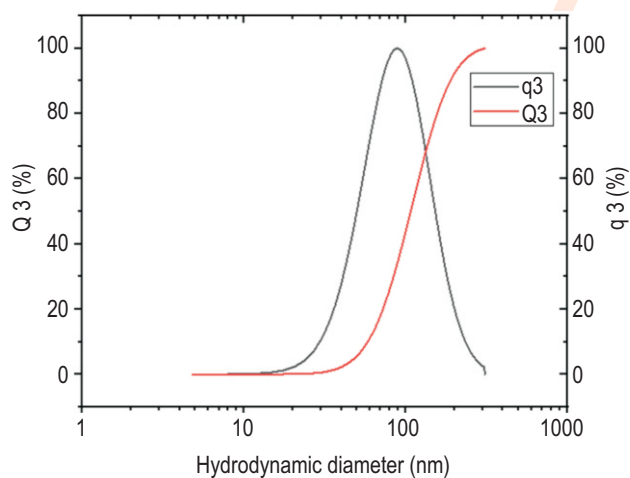


Fig. 4 : Distribution particle size by Dynamic light scattering for AgNPs.

Finally, the germination rate of *Lepidium sativum* seeds after treatment with extract was faster than control. The seeds showed 100% germination on the first day whereas the control showed only 90%. They both reached 100% on the second day (Table 1) Similar to the other two types of seedlings, the plumule and radicle length were significantly greater than control. Regarding the nature of plant growth promoting substances, Stirk *et al.* (2013 a, b) attributed this to the presence of several growth promoting hormones such as indole acetic acid, cytokinins, gibberellins and brassinosteroids. The latter accumulates in the nucleus and is responsible for significant induction of stem elongation. The fact that the aqueous extract of *C. vulgaris* had an enhancing effect on seeds reflect its agrobenevolent impact.

Regarding *Chlorella* extract as antimicrobial agent, the presence of several bioactive compounds such as flavanoids,

tannin, phenolic compounds, terpenes, glycosides, saponins has been reported (Shabudeen *et al.*, 2015). Acurio *et al.* (2016) reported the extraction of chlorellin as antimicrobial compound, which has antibacterial effect comparable to ampicillin and oxacillin. Moreover, Shabudeen *et al.* (2015) highlighted the importance of aqueous extract of *Chlorella vulgaris* rich in phenols antibacterial against different Gram positive and negative bacteria which is in complete accordance with our results. (Dantas *et al.*, 2015). Some of the phenols that *Chlorella* sp. contain include simple phenols, derivatives of benzoic acid and cinnamic acid, coumarins and flavonoids. In accordance with aforementioned reports, the aqueous extract derived from a very small biomass (0.3 g fresh weight) showed a broad-spectrum antibacterial effect against all tested bacterial pathogens, both Gram positive and negative (Table 2). The greatest inhibition zone was obtained for *Bacillus* sp. (Fig. 1A) whereas the smallest was that *Methicillin-resistant staphylococcus aureus* (Fig. 1B). However, low concentration silver of nanoparticles (5 $\mu\text{g ml}^{-1}$) showed antimicrobial agent against the multidrug resistant strain of *Methicillin-resistant staphylococcus aureus*. The inhibition zone was 1.2 cm.

Nano-silver particles were successfully synthesized both intra and extra-cellularly from green *Chlorella vulgaris* cells. The formation of AgNPs was confirmed by UV- vis absorbance measurement. UV-Vis spectra of AgNPs are presented (Fig. 2) in the range of 200–800nm. The colloidal solution of synthesized AgNPs showed a strong specific peak for synthesized AgNPs was obtained at 425 nm (Fig. 2). The presence of dark brown color confirmed the production of AgNPs, and the increased absorption refers to increased concentration. This is in accordance with the characteristic bands in the UV-Visible spectrometric analysis indicative of silver nanoparticles which showed plasmon resonance in the range of 410-450nm (Yasin *et al.*, 2016). In addition, FTIR confirmed their biosynthesis by *Chlorella*. The FTIR spectra of the AgNps. (Fig. 3) clearly showed that nanosilver particles were present at 3297cm^{-1} overlapping with OH signal (Verma *et al.*, 2015). At 1637 cm^{-1} there was a C-H stretching vibration of aromatic ring, and at 1040 cm^{-1} there was C-O-C stretching vibration of aromatic ether. The main components of *Chlorella vulgaris* is cellulose, which contain phenolic hydroxyl, benzene ring, double bond, - O -, and other groups. All these functional groups contain lone electron pairs. When nano-scale micro emulsion is formed in the aqueous phase, these functional groups were dispersed on the surface of emulsion, showing a strong adsorption capacity.

Dynamic light scattering technique was used to measure the light intensity reflected by particles in solution. DLS analysis (Fig. 4) showed a wide range of particle size distribution varying AgNPs from 55.9 to 106.9 nm with average value of 87.8 nm. The size of most particles was around 90 nm. The use of this technique is based on certain facts. The use of reflected light vary over time as particles change their position due to Brownian

motion. The rate of this process depends on the hydrodynamic diameter of particles. Therefore, by measuring the rate of change of reflected light, the size of suspended particles can be estimated. The majority of particles were 90 nm in size with some proportion being higher or lower than that. This size is quite favorable for different application of silver nanoparticles as it has a large surface area which means higher interaction surface than that of larger particles. In addition, the cost-effectiveness and pollution-free of biologically-synthesized nanoparticles make them more plausible than those synthesized using harmful and expensive chemical and physical synthesis methods (Mandal *et al.*, 2006). The proposed mechanism of nanosilver biosynthesis is that metals are trapped on the surface of microalgae via carboxylate group, the metal ions are reduced by reductase elemental form via the action of reductase enzymes (Kannan and Subbalaxmi, 2011). Finally, the use of produced biogenic nanosilver as antibacterial agent against multi-drug resistant strain MRSA is highly effective. It is in accordance with the reports of Marambio-Jones (2010). The reports emphasize the bacteriocidal effect of silver nanoparticles of three different sizes and its broad-spectrum activity against both Gram-positive and Gram-negative bacteria, including multi-resistant strains. This can be eco-friendly strategy for cost-effective bacterial control.

These promising results only indicate that this alga if grown on mass scale can be exploited in several ways. However, the cost of cultivation and maintenance of cultures on inexpensive growth media are issues of concern as the cost of microalga is high production (Mata *et al.*, 2010). Regard the use of wastewater or recycled water from hydroponic cultures such as that of tomatoes were proposed (Zhang *et al.*, 2017). This waste water is loaded with nutrients and represent cost-effective system for algal production and oxygen evolved in photosynthesis can be used in tomatoes respiration and growth which is a positive indication of success of hydroponic algal/tomato coproduction. Overall, this is the first report, to the best of our knowledge from Saudi Arabia that explores the multiple potentials of local green alga *Chlorella vulgaris* which showed promising potentials although the tested biomass was miniscule. This indicates great potential this alga, if used on mass scale. The study highlights the biotechnological potentials of microbial isolates from underexplored localities due to unique metabolism and activities.

Acknowledgment

The authors would like to express their deepest gratitude and acknowledge the financial support of Deanship of Scientific Research, King Faisal University, Al- Ahsa, Nasher track, grant number 186125.

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