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Isolation and Classification of Green Alga *Stigeoclonium attenuatum* and Evaluation of its Ability to Prepare Zinc Oxide Nanoflakes for Methylene Blue Photodegradation by Sunlight

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Abstract:

Algae have been used in different applications in various fields such as the pharmaceutical industry, environmental treatments, and biotechnology. Studies show that the preparation of nanoparticles by a green synthesis method is a promising solution to many medical and environmental issues. In the current study, the green alga *Stigeoclonium attenuatum* (Hazen) F.S. Collins 1909 was isolated and identified from the Al-Hillah River (Governorate of Babylon) in the middle of Iraq. The green synthesis by the aqueous extract of algae was used to prepare the nanoflakes of ZnO. Nanoflakes of ZnO are characterized by X-Ray diffraction (XRD) and scanning electron microscope (SEM) with flakes shape and dimensions ranging between 200-500 nm and thickness between 20-23 nm. And this study comprises a test of ZnO nanoflakes efficiency as a photocatalyst factor, thus experiments set of an aqueous solution of methylene blue with ZnO nanoflakes and exposed to sunlight have been conducted. The absorbance of methylene blue at 660 nm reduces over time and almost vanishes between 60-120 minutes. Consequently, it is obvious that in the presence of sunlight, pristine ZnO nanoflakes are photocatalytically active with a degradation efficiency of 97%. Furthermore, the antibacterial activity of ZnO nanoflakes that were prepared by aqueous extract of algae was evaluated against some resistant strains of bacteria *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Streptococcus sp.* and the antibacterial activity of NPs rises as concentration increases 50, 100, and 150 µg/ml.

Keywords: Active biochemical compounds, Antibacterial activity, Nanoparticles, Photocatalyst, *Stigeoclonium attenuatum*.

Introduction:

The green alga *S. attenuatum* (Phylum: Chlorophyta; Class: Chlorophyceae; Order: Chaetophorales; Family: Chaetophoraceae) was established by Collins, F.S. (1909)¹ Most of the algae within the green algae division (Chlorophyta) produce a wide range of active biochemical compounds such as fatty acids, Terpenes, Glycerides, Steroids, alkaloids, Cyclic peptides, pigments, vitamin C, iron nitric oxide, polyketides, polysaccharides hydrogen peroxide, phenolic compounds, chlorogenic acid, syringic acid, p-coumaric acid, myricetin, 3,4-dihydroxybenzoic

acid, vanillic acid, and 4-hydroxybenzoic acid and rutin²⁻⁵

Research has proved that the green synthesis of nanoparticles is a more effective, low cost and eco-friendly procedure, in addition to their potential to reduce the toxicity of NPs⁶⁻¹⁰. The physical and chemical properties of nanoparticles give them an active role in various medications such as cancer and antimicrobial and industrial products fields¹¹⁻¹³ Biologically, the antibacterial and antifungal effects of zinc oxide (ZnO) nanoparticles have been studied on some resistant strains of bacteria and fungi such as *Acinetobacter baumannii*^{14,15}. Nowadays, wastewater remediation is a big challenge to the

environment and there are physical, chemical, and biological technologies trying to solve this serious problem. The application of nanomaterials is a promising solution for environmental cleanup. Green synthesis is the preparation of nanoparticles by using a biological source. Green synthesis of nanoparticles by some algae has been investigated, such as preparing gold nanoparticles by *Chlorella Vulgaris* algae¹⁶. The special properties of zinc oxide nanoparticles such as conductivity, chemical stability, catalytic properties, antibacterial, antifungal, UV filtering properties, nontoxic nature, wide bandgap, and low-cost materials give it more activity against microbes and high effectiveness in environmental remediation applications like photocatalytic action¹⁷⁻¹⁹. The current study aims to isolate and purify *S. attenuatum* alga, study its ability to prepare ZnO nanoflakes as a green synthesis method, and use it in some biological and environmental experiments.

Materials and Methods:

Isolation and Purification of green alga *S. attenuatum*.

The liquid samples were brought from the Al-Hillah River (Governorate of Babylon) in the middle of Iraq, on the 24th of December 2021. Alga samples were examined under a microscope to identify and classify the alga^{1,20}. The alga was washed with distilled water many times to purify and eliminate the filaments of alga from the river's mud and attached bacteria and other algae, especially diatoms²¹.

Liquid extraction of Alga

For preparing an aqueous extract of alga, 25 gm of the dry weight of alga was harvested and dried in an oven at 60°C. The biomass was extracted by 500 ml of distilled water, with continuous mixing and heating by a magnetic stirrer at 60° - 70° for six hours²².

Preparing of ZnO nanoflakes:

The zinc oxide solution was prepared in distilled water, where 0.1 M of zinc sulfate in 100 mL deionized water and 50 ml of the algal extract were stirred for 30 min. The ZnO nanoflakes were precipitated by adding 0.1 M of sodium hydroxide slowly with monitoring the acidity value of the solution till reached pH =10. The precipitate was filtered and washed with water and ethanol. Finally, it dried at 110 °C for 8 hours. ZnO nanoflakes were characterized by X-Ray Diffraction (XRD) and scanning electron microscope instruments (SEM)²³

Photocatalysis activity of ZnO nanoflakes by sunlight:

The zinc oxide nanoflakes were tested for photocatalysis activity as follows: 100 mL

methylene blue dye 25 ppm was placed in a 400 ml beaker and ZnO nanoflakes powder was added to make slurry suspension and stirred for 30 min to reach adsorption equilibrium. The group was placed against sunlight at 11 a.m on August 20, 2021, where the sun was partially perpendicular, the weather temperature was 45 °C and the solution temperature was 34 °C. 2 ml of the slurry was withdrawn each 15 min, and the reaction lasted 120 min, then centrifuged at 10000 rpm for 5 min. Finally, the reaction absorbance was measured at 660 nm. In the same procedure, different weights of ZnO nanoflakes were used to evaluate the optimum weight of ZnO nanoflakes (0.01, 0.03, 0.05 and 0.1 gm per 100 ml). Also, different concentrations of the methylene blue at 25, 50, and 75 ppm were made to study the effect of concentration change on photocatalysis. Experiments by changing the initial pH of methylene blue solution were done in the range 6-10 by using sodium hydroxide or hydrochloric acid solution were used to study the effect of pH change on photocatalysis reaction and applied under the same conditions of sunlight, and the percentage degradation of methylene blue was calculated using Eq.1²⁴

$$\text{Degradation \%} = (A_0 - A_t / A_0) * 100 \dots\dots\dots 1$$

Where A_0 is the absorbance at the initial stage and A_t is the absorbance at a certain time. Also all kinetics were studied according to first-order reaction for all photocatalysis experiments.

Antibacterial activity of ZnO nanoflakes:

Several bacterial species *E.coli*, *K. pneumoniae*, and *S. aureus*, *Streptococcus Sp.* were examined for antibacterial activity of ZnO nanoflakes with concentrations 50, 100, and 150 µg/ml. The strains were inoculated with appropriate agar like Tryptone Soya Agar (supplied by Himedia, India) and incubated at 37 °C not less than 24 hours then stock cultures were yielded. These cultures, after inoculation, were maintained at 4°C. The working culture was transferred in a loopful into 5 ml of Brain Heart Infusion broth (supplied by Himedia, India) and incubated at 37 °C for 24 hours, yielding cultures that were ready for the well-diffusion method²⁵.

The well diffusion method for ZnO nanoflakes:

The goal of the agar well diffusion methods was a qualitative screening susceptibility of the ZnO nanoflakes against bacteria. Swabbing the working cultures on the agar surface was the first step and the concentration was around 109 CFU/mL then a sterile crock is used to engrave well about 9 mm in diameter. The ZnO nanoflakes suspensions were placed on the inoculated agar well. Each plate contains three wells and is filled

with different concentrations of ZnO nanoflakes about 50, 100, and 150 μg . All plates were incubated at 37 $^{\circ}\text{C}$ for 24 hours and the inhibition was estimated by measuring the inhibition zone in mm^2 .

Results and Discussion:

Description of alga *S. attenuatum*

The species of the current study have been recorded in the Iraq environment by Al-Kaisi *et*

*al.*²⁷. Thallus attached to submerged aquatic by rhizoid, bright green in color, elongate and upper branching mostly alternate Filaments, pseudo dichotomous. Some branches were short and spine-like or long with a sharp pointed cell or a series of cells forming a hyaline seta. Cells are cylindrical and the diameter on the main axis is 5 μ , while the length is 13 μ . The prostrate portion of the thallus is little-developed^{1, 20} (Fig. 1).

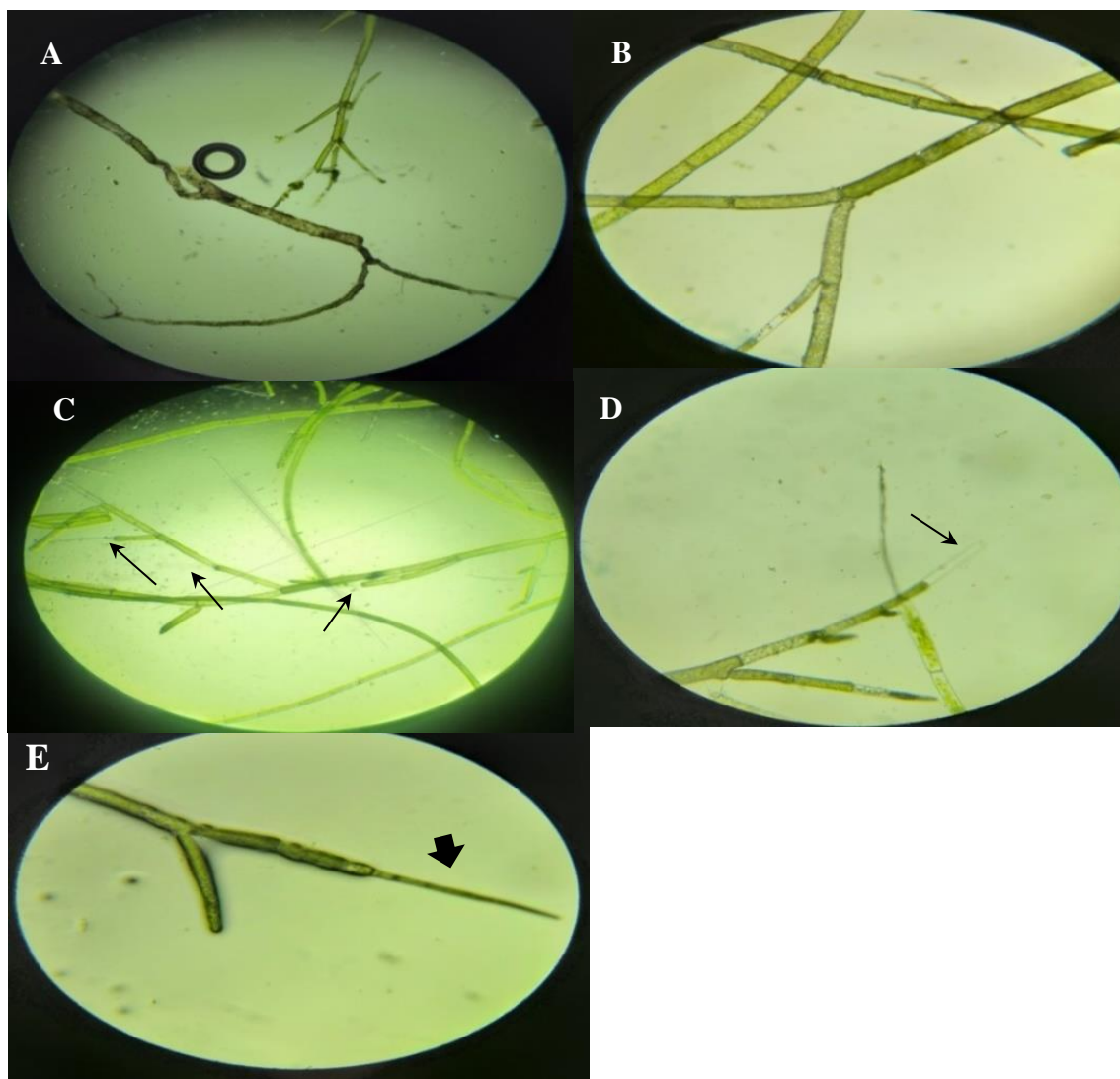


Figure 1. *S. attenuatum* under light microscope: 10X , 40X :

A: Prostrate portion with rhizoid; B: Below dichotomously branched; C-D: Alternate branching Show hyaline seta(Black arrow); E: Sharply pointed cell (Head black arrow).

The ZnO nanoflakes characterization:

In our results, the green synthesis is done by precipitation of ZnO as nanomaterials by green synthesis using *S. attenuatum*. The properties of this nanomaterial are dependent on the phytochemicals in the extract. The resulted powder tended to be white with a pale green color. The pattern of XRD diffraction results is in Fig.2. This

result revealed the hexagonal wurtzite of the examined ZnO nanoflakes. There were no peaks of any other phase in the nanoparticles, indicating great purity. According to the angle at 32.899 having the maximum intensity, grain size (D) was found to be equal to 15 nm, which is calculated by using the Debye–Scherrer Equation:

$$D = 0.9\lambda/\beta\text{Cos}\theta$$

The value 0.9 is constant, λ : is the wavelength of the X-ray, β : is the Full-Width Half Maximum, of Bragg's angle²⁸.

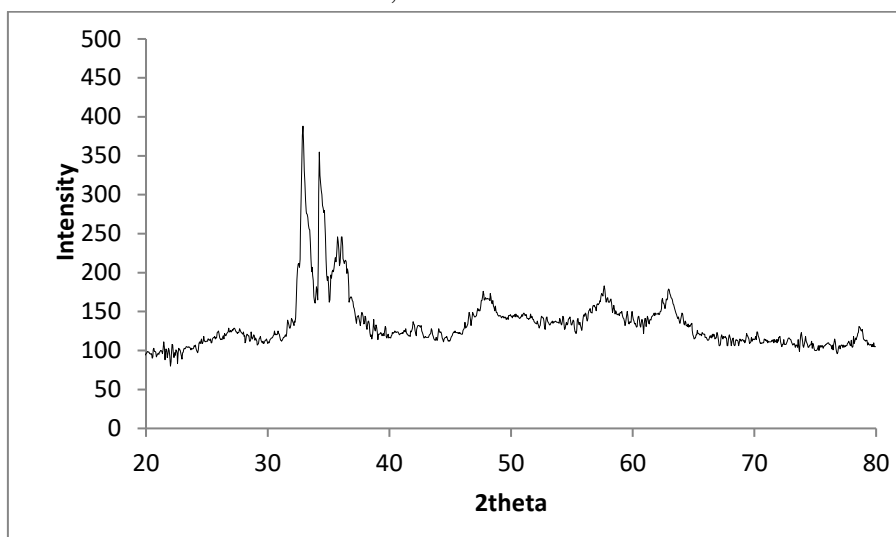


Figure 2. XRD patterned of zinc oxide nanoflakes synthesized by algae extract.

The ZnO nanoflake shape was characterized by SEM, where the image of different scales appears in Fig.3. On the scale of $1\mu\text{m}$, the shape of ZnO was like flakes aggregated in all directions and seemed like layers. These nanoflakes are different in size and the range was 200-500 nm, but the thickness

was 20-23 nm. This form is affected by the presence of phytochemicals that make uniform shapes. This method was a benefit to forming nanoflakes by the green method to precipitate ZnO in alkaline media, where the active ingredients in the extract work as a template to give nanoflakes (Fig.3)

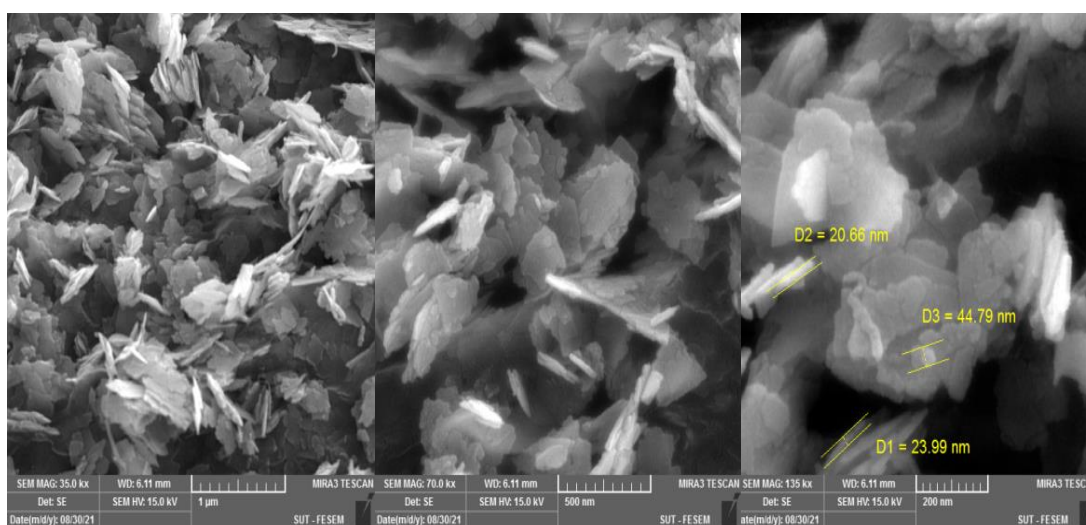


Figure 3. SEM image of zinc oxide nanoflakes in different scales.

Photocatalysis activity of ZnO nanoflakes by sunlight

The photocatalytic performance of methylene blue organic dye, a common contaminant in the textile sector that has a negative influence on the environment, was assessed²⁹. In our experiments, the exposure times to sunlight were studied. By utilizing ZnO nanoflakes as photocatalysts, the distinctive absorbance of methylene blue at around 660 nm reduces steadily over time and almost vanishes between 60-120

minutes. It is obvious that in the presence of sunlight, pristine ZnO nanoflakes are photocatalytically active, with a degradation efficiency of 97% (Fig.4).

Figure 4 depicts the degradation efficiency of the MB dye (25 mg/L) in the presence of varying doses of photocatalyst (0.01–0.1 g/dL), When the photocatalyst dose was changed from 0.01 to 0.1 g/dL, the photodegradation efficiency of MB was 58 to 97 percent, and the reaction time was 120 min. As the amount of photocatalyst increased, more

active sites were discovered on the photocatalyst surface, increasing radical production. As a result,

the higher dosage may boost the azo dye's degradation effectiveness^{30,31}.

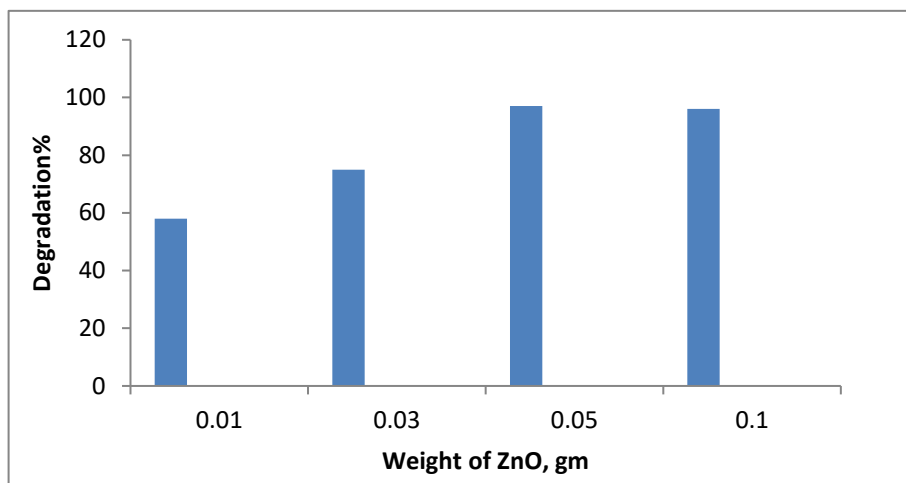


Figure 4. Effect of zinc oxide dose on photocatalytic degradation efficiency of 25 ppm methylene blue at pH=7

In Fig.5, the relation kinetics of ZnO nanoflakes dose to time of reaction, where the rate of pseudo-first reaction is increased at weight increased. The

apparent reaction constant (K_{app} , 1/min) is improved at a high loading of ZnO nanoflakes.

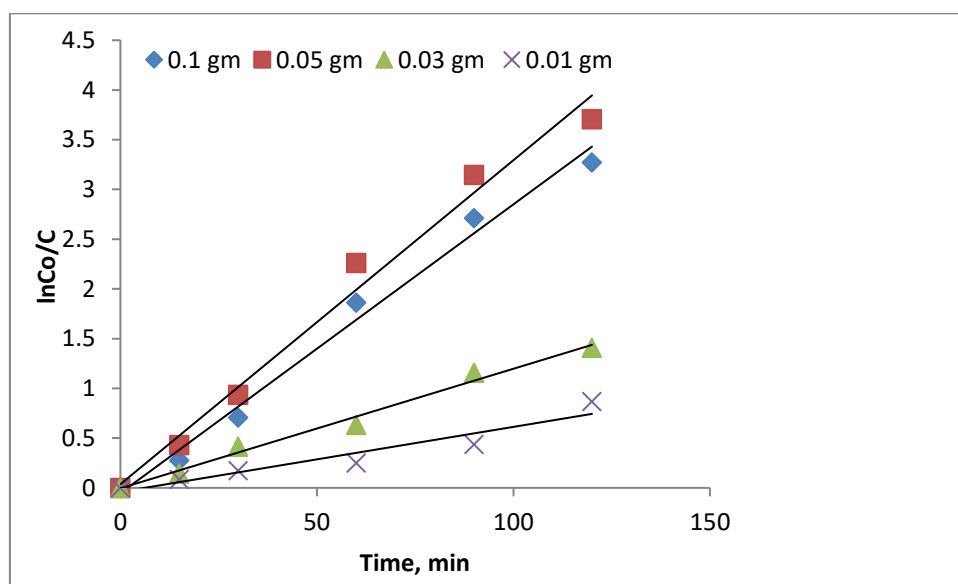


Figure 5. Pseudo– first- order kinetic relation zinc oxide dose on photocatalytic degradation of 25 ppm methylene blue at pH=7 and stacked figure is represent the kapp related to weight of catalyst.

The pseudo-first- order kinetics of the methylene blue degradation of ZnO photocatalysts are shown in Fig.6. Using the pseudo-first order model, the reaction constant (k) of methylene blue photodegradation by the ZnO nanoflakes was quantified according to first-order kinetic³².

$$\ln C_0/C_t = k \cdot t$$

The initial concentrations of MB (C_0) were adjusted from 25 to 75 mg/L and the concentrations (C_t) were monitored after an interval of time (t) to investigate the photodegradation activity.

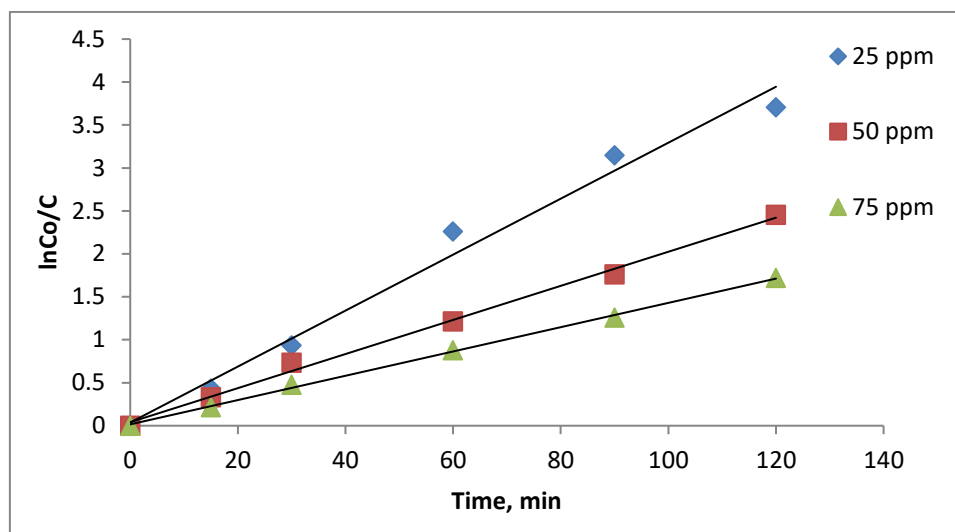


Figure 6. Kinetic relation of photocatalytic degradation in different concentration of methylene blue solution.

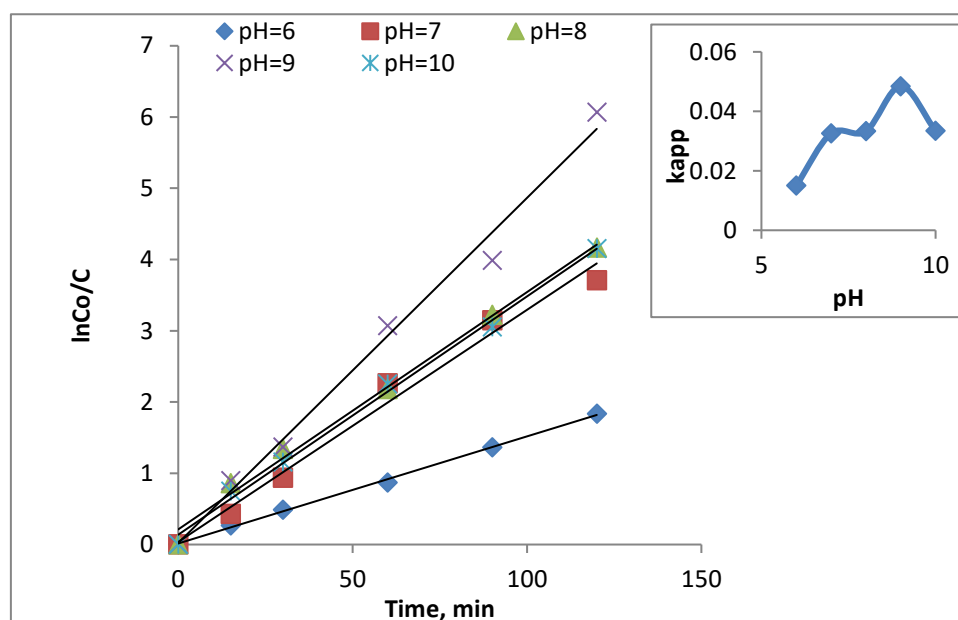


Figure 7. Pseudo-first-order kinetic relation of zinc oxide photocatalytic degradation of 25 ppm methylene blue at different pH and the stacked plot is relation of k_{app} with pH.

After 15 minutes of reaction time, the clearance rates of the dyes were compared. When all other circumstances are held constant, the clearance rates of methylene blue decrease as the starting concentration rises. As a result, the dye removal efficiency could be improved by a lower dye concentration. This effect can be understood and explained according to the effect of increasing concentration, the molecules were adsorbed more on the photocatalyst surface. Because the active site was filled with the dye molecules, where the competition appears more, leading to the reduction of O_2 and OH^- adsorption on the photocatalyst surface, where the production of radical species is reduced. Additionally, photons were prevented

before reaching the photocatalyst surface; hence a low number of photons supplied to the catalyst. As a result, at high starting dye concentrations, the clearance rate decreased^{18,33}. Typically, industrial effluent has a wide pH range. The photodegradation processes are influenced by the pH of the dye aqueous solution. The clearance efficiency of dye on ZnO at pH values of 6.0, 7.0, 8.0, 9.0, and 10.0 are shown in Fig.7. When the dye solution's starting pH is alkaline, the removal ratio is high, while at low pH values, on the other hand, the dye removal ratio is quite low. The dye's chemical features and the properties of surface-charge catalysts, both of which were electrostatically connected to the point of zero charges of the catalyst surface, may explain

this phenomenon. When the value of pH is lower than pH_{zpc} , the surface of ZnO becomes positively charged; whereas, when the pH is greater than pH_{zpc} , the surface becomes negatively charged. In these results, the degradation efficiency was dependent on pH_{zpc} , where the dye is more degradable at $pH=9$. As shown in Fig.7, the reaction rate (according to first-order kinetics) was increased at alkaline media ($pH=9$) and is low at acidic pH, which may be due to higher electrostatic attractive interaction between MB and ZnO nanoflakes with a higher pH value³⁴.

Antibacterial activity of ZnO nanoflakes

The ZnO nanoflakes produced by the algae extract were studied. The antibacterial activity of ZnO nanoflakes against *E.coli*, *S. aureus*, *K. pneumoniae*, and *Streptococcus sp.* was shown in Fig.8. According to the findings, the antibacterial activity of nanoflakes rises with concentration increases. This result was in line with recent research that found that the activity of ZnO

nanoparticles was dosage and morphology sensitive³⁵.

The formation of reactive oxygen species (ROS), the release of Zn (Zn^{2+}) ions inside the microorganisms, and the alteration in cell wall permeability are all factors that contribute to ZnO nanoflakes toxicity. Nanotoxicity is thought to be caused by the production of reactive oxygen species (ROS), which causes damage to biological components such as proteins, lipids, nucleic acids, phospholipids, and amino acids^{36,37}.

The dose of ZnO nanoflakes was studied by changing their concentration against bacterial species *E. coli*, *S. aureus*, *K. pneumoniae*, and *Streptococcus sp.* to validate the inhibition of each bacterium. ZnO nanoflakes doses were 50, 100, and 150 $\mu\text{g/ml}$ applied in each plate and we found the action is different as well as concentration is increased. Also, all bacterial species under study resist 50 $\mu\text{g/ml}$ of ZnO nanoflakes, but the action appeared at 100 and 150 $\mu\text{g/ml}$. This action undergoes a threshold of the concentration of ZnO nanoflakes, where each bacterium is affected at a level not less than 100 $\mu\text{g/ml}$ as in Table 1.

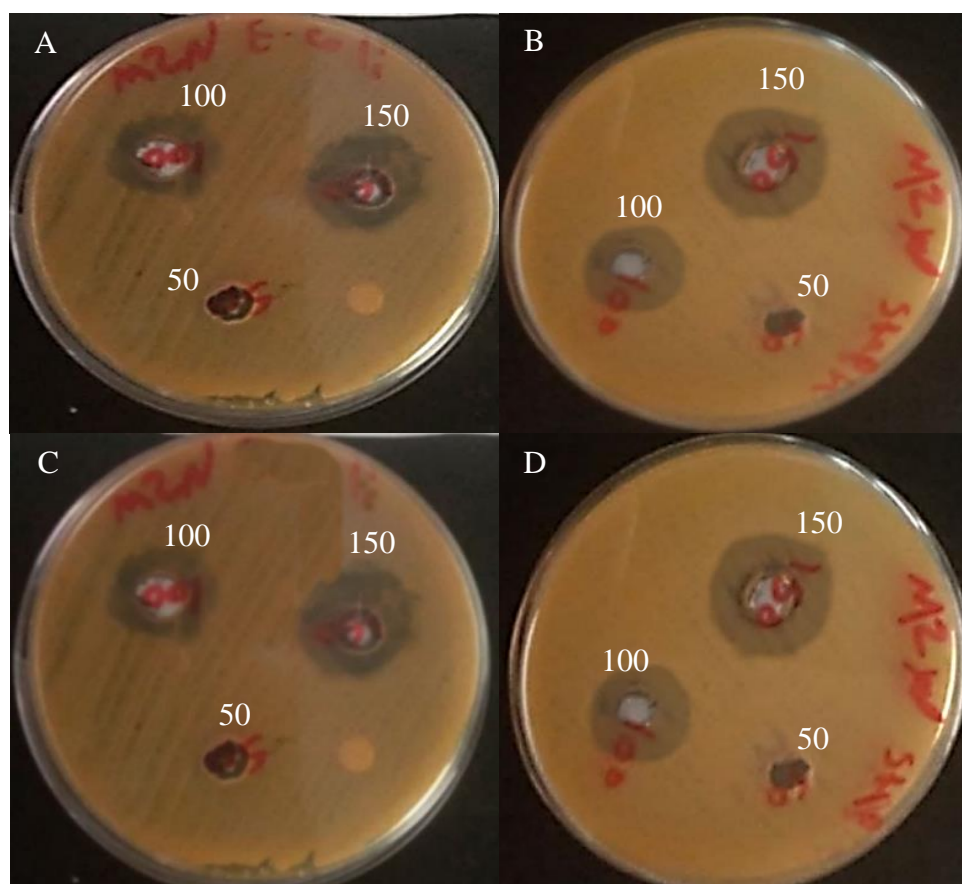


Figure 8. Antibacterial activity of ZnO nanoflakes against bacterial species *E. coli* (A), *S. aureus* (B), *K.pneumoniae* (C) and *Streptococcus sp.*(D)

Table 1. The inhibition zone of the effect of ZnO nanoflakes at different doses against *E. coli*, *S. aureus*, *K. pneumonia*, and *Streptococcus sp.*

Bacterial species	Inhibition zone (mm)		
	50	100	150
ZnO concentration, µg/ml			
<i>E. coli</i>	0	16	20
<i>S.aureus</i>	0	15	19
<i>K.pneumonia</i>	0	15	20
<i>Streptococcus sp.</i>	0	16	21

Conclusion:

In our research, we study the ability of *S. attenuatum* alga extract to be synthesized of nanoflakes without any spherical particles implying the effect of the phytochemical of alga for the first time. ZnO nanoflakes have been applied to photocatalysis using sunlight for the pigment of MB removal. The efficiency of photocatalytic degradation is affected by the dose of ZnO nanoflakes, the initial pH of MB solution, and the concentration. The nanoflake of ZnO showed antibacterial effectiveness against *E. coli*, *S. aureus*, *K. pneumonia*, and *Streptococcus sp*

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Besides, the Figures and images, which are not ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Basra.

Authors Contribution:

M A. A contributed to collecting the alga from Al-Hillah River (Governorate of Babylon) in the middle of Iraq, In addition to preparing an aqueous extract of alga, and the experiment of antibacterial activity of the ZnO nanoflakes.

A M O contributed to the ZnO nanoflakes preparation and Methylene Blue Photodegradation by Sunlight experiment and reading the results of nanoflakes chemical tests .

E Y.A. A-S contribute to confirming the final and accurate diagnosis of the alga, in addition to checking the working methods and results, bringing them out in their appropriate form, and writing and producing the research in its final form.

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عزل و تصنيف الطحلب الاخضر *Stigeoclonium attenuatum* و دراسة قدرته على تحضير اوكسيد الزنك النانوي zinc oxide nanoflakes و التفسير الضوئي لصبغة الميثيلين الازرق بواسطة ضوء الشمس

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الخلاصة:

ساهمت الطحالب في العديد من المجالات الحيوية كمجال الصناعات الدوائية و المعالجة البيئية و كذلك في مجال التقنيات الحيوية . كما يعد مجال تخليق المركبات النانوية مجال واعد وفي تطور سريع لتقديم مختلف الحلول لاهم المشاكل الحالية التي تواجه المجتمع الطبي و المشاكل البيئية. تضمنت الدراسة الحالية عزل و تصنيف الطحلب الاخضر *Stigeoclonium attenuatum* (Hazen) F.S. Collins من نهر الحلة (محافظة بابل) في وسط العراق. تم التخليق الحيوي لاوكسيد الزنك ZnO nanoflakes بواسطة المستخلص المائي للطحلب . تم تشخيص الجزيئات النانوية بواسطة (XRD) X-Ray diffraction و المجهر الالكتروني الماسح (SEM) بمختلفة الاحجام بمدى يتراوح بين 20-23 نانومتر. كما تضمنت تجارب الدراسة اختبار قدرة مسحوق اوكسيد الزنك النانوي كمحفز ضوئي لهذا اجريت مجموعة من التجارب على تحليل صبغة ازرق الميثيلين ضوئيا Photocatalyst و ذلك بتعريضها لأشعة الشمس ، و بطول موجي 660 نانومتر اظهر الصبغة تلاشي تدريجي الى ان وصلت الى نقطة الاختفاء التام عند حوالي 60-120 دقيقة ، و بهذا فقد اظهر مسحوق اوكسيد الزنك النانوي فعالية تحليلية ضوئية عالية في تحليل الصبغة بكفاءة 97% . كما تم اختبار اوكسيد الزنك النانوي ZnO nanoflakes المحضر كضاد حيوي تجاه بعض السلالات البكتيرية المقاومة وهي كل من *Escherichia coli* و *Klebsiella pneumoniae* و *aureus* *Streptococcus Sp.* و *Staphylococcus* ، وقد وجدت الدراسة ان فعالية المركب النانوي قد ازدادت بزيادة التراكيز وهي 50 و 100 و 150 مايكروغرام/ملييلتر.

الكلمات المفتاحية : المركبات البيوكيماوية الفعالة، فعالية المضاد الحيوي، الدقائق النانوية ، التحفيز الضوئي ، *Stigeoclonium attenuatum*.