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Study of Antimicrobial, Antioxidant and Photocatalytic Decolorization Activity of Bacteriologically Synthesized Zinc Oxide (ZnO) Nanoparticles

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ABSTRACT

Research on green synthesis of nanoparticles is gaining lot of importance owing to their stability, applicability and cost-effectiveness. This research aims to evaluate the properties of biologically synthesized Zinc nanoparticles using *Bacillus mycoides*. These nanoparticles were found to have significant antimicrobial property and the Minimum Inhibitory Concentration (MIC) against selected pathogenic strains of *Staphylococcus aureus, Streptococcus pyogenes* and *Klebsiella pneumoniae* was found to be 25µg/mL, 25µg/mL and 50µg/mL respectively. % Free radical scavenging activity of 100µg/ml of zinc nanoparticles assayed using DPPH was found to be 83.33%. Photocatalytic decolorization of 50ppm Methylene Blue (MB), Congo Red (CR) and Malachite Green (MG) was studied using free and immobilized nanoparticles by UV- visible spectroscopy. % Photocatalytic activity of free ZnO nanoparticles on decolorization of MB, CR and MG was obtained as 89.66%, 92.35% and 88.56% respectively. Percent decolorization of MB, CR and MG using sodium alginate encapsulated ZnO nanoparticles was found to be 84.37%, 86.93% and 79.45% respectively. Encapsulated ZnO nanoparticles could be reused for Photocatalytic decolorization of dyes as it gave significant decolorization of MB, CR and MG with 59.37%, 50.64% and 48.19% respectively till three cycles. Thus, nanoparticles can be utilized in medicinal field as well as textile effluent treatment plants.

***Key words*:** Zinc oxide, Antioxidant, of *Staphylococcus aureus, Streptococcus pyogenes*, *Klebsiella pneumoniae*

Nanoscience technology provides a wide range of applications in the fields like pharmaceuticals, medicines, biosensors, drug delivery, chemical industries, electronics, mechanics, space industries, energy science, environmental health, waste water treatments, biomedical science, plant tissue culture, health care, cosmetic products, etc. [1-2]. Its use has remarkable value in the treatment of various kinds of infections, cancer treatment, allergies, diabetes and inflammation. Over past few years**,** due to rising numbers of multidrug- resistant pathogens (MDRs), it has been very difficult for the clinical treatments particularly of epidermis and cutaneous infections. Owing to their unique chemical and physical properties along with high surface area to volume ratio, nanoparticles have come up as one of the novel solutions in combinations with existing antimicrobials [3]. The antibacterial properties of any nanoparticle mainly depend upon its morphology i.e., on their surface atomic arrangements [4]. Oxide nanoparticles of many metals such as silver, gold, iron, zinc, copper, magnesium, titanium, palladium, platinum etc., are widely used for waste water treatments and have a significant role in textile dye decolorization. Nanoparticles are used for waste water treatment, but can potentially cause risks to the environment. Thus, it becomes essential to encapsulate nanoparticles within a polymer matrix which withholds adding more pollution to water being treated. Both synthetic and natural polymers can be used for entrapment of nanoparticles [5]. Biopolymers such as alginate, starch, cellulose, chitosan etc. have been used for various applications along with treatment of waste water [6]. Sodium alginate is a natural polymer, non-toxic and biodegradable linear polysaccharide thus, can be used for encapsulation of nanoparticles, preventing pollution in environment [7-8].

Many metal oxides taken into consideration; Zinc oxide (ZnO) nanoparticles has received much greater attention. Being an n-type semiconductor and, wide band-gap and binding energy of 3.36 eV 60meV respectively, it has unique optical, electrical and chemical properties. It is non-toxic as well as less-expensive and is easily available [9-11]. It has wide range of applications in areas like piezoelectric sensors, magnetic materials, gas sensing, antimicrobial, photocatalytic

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decolorization, cosmetics, etc. [12]. Thus, utmost focus of the researchers is to figure out its potential applications [13].

Present study deals with evaluating the applicability of previously synthesized ZnO nanoparticles which were characterized by using UV- Visible double beam spectroscopy, XRD and confirmed using SEM. They were crystalline spherical structure of ZnO nanoparticles and had Z-average of 55nm [14]. ZnO nanoparticles were checked for their antimicrobial activity and antioxidant activity. Its MIC (Minimum Inhibitory Concentration) was also obtained. These nanoparticles were than immobilized using sodium alginate. The photocatalytic decolorization of three dyes i.e., Methylene blue, Congo red and Malachite green was studied using free and immobilized ZnO nanoparticles along with the sustainability of immobilized beads for photocatalytic decolorization.

MATERIALS AND METHODS

Antimicrobial activity and minimum inhibition concentration of ZnO nanoparticles

Antimicrobial activity of Zn NPs was determined using agar well diffusion method using Muller- Hinton agar. In order to study antimicrobial activity of ZnO Nanoparticles, clinical isolates namely; *Staphylococcus aureus*, *Streptococcus pyogenes* and *Klebsiella pneumoniae* were used. Each strain was inoculated separately into culture tube containing nutrient broth and were incubated overnight at 37^{o}C in incubator. 0.1mL of fresh bacterial culture (0.5 O.D. McFarland unit having approximately 1x 10^8 CFU/mL) was pipetted on Muller Hinton agar plate and swabbed using sterile cotton swab. Wells of 6mm diameter were made on Muller- Hinton agar plate using sterile cork borer [15]. Solution of 50 µg/mL concentration of ZnO nanoparticle was prepared and 30uL of solution was loaded in wells using micropipette. These plates were placed in refrigerator for 30 min for diffusion of solution and then were incubated at 37°C for 24 hours and zone of inhibition was measured latter.

Minimum Inhibition Concentration **(**MIC) is the lowest concentration of antimicrobial agent that inhibits the visible growth of any organism. MIC of ZnO nanoparticles for *Staphylococcus aureus, Streptococcus pyogenes* and *Klebsiella pneumoniae* was determined by agar well diffusion method (as mentioned before) using different concentrations of ZnO nanoparticles i.e.: 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL and 1.5 µg/mL (different concentrations was made by serial dilution of stock solution of ZnO nanoparticles) as performed by Zattindokht *et al*. [16]. To nullify effect of CTAB and Hexane on growth of bacteria, the control of 1mM zinc sulphateheptahydrate and mixture of 50uL of CTAB (2 x 10^{-4} M) + Hexane (35%) in the ratio 1:2 was used.

Antioxidant activity of ZnO nanoparticles

1,1-diphenyl-2-picryl-hydrazyl (DPPH) is a stable nitrogen-centered, lipophilic free radical which is broadly used for evaluating the antiradical activity of antioxidants within short time. Here DPPH acts as a free radical source and ZnO nanoparticles scavenge these free radicals. The DPPH is deep violet in color which gradually changes its color to pale yellow in the presence of ZnO nanoparticles [17]. The free radical scavenging activity of the ZnO nanoparticles was measured using DPPH. Ascorbic acid made of different concentrations same as that of ZnO nanoparticles was used as standard. Briefly, 1ml of different concentrations of ZnO nanoparticles (100µg/mL, 50µg/mL and 25µg/mL) were mixed individually with 1mL of 1mM DPPH and left to stand in the dark for 30min, absorbance was recorded at 517nm. 1mL of 1mM DPPH without ZnO was used as control [18]. The experiment was done in triplicate. The percentage scavenging was calculated using the following formula:

$$\text{DPPH Scavenging effect } (\%) = \frac{(\text{Acontrol} - \text{Asample})}{\text{Acontrol}} \times 100$$

Where, A control= absorbance of DPPH;
A sample= absorbance of the test solution after 30mins.

Immobilization of ZnO nanoparticles by sodium alginate beads

In a beaker, add 0.5gm of sodium alginate to 25 mL of distilled water with constant stirring using a glass rod. The solution was stirred for 10-20 minutes until a smooth viscous solution was obtained. It was then allowed to stand for 10-15 minute to remove any air bubble. To this 2mg/mL of zinc nanoparticles were added with gentle stirring. Later with the help of a syringe this mixture was added drop wise in 0.1 M chilled aqueous calcium hydroxide which was kept on a magnetic stirrer. After formation of sodium alginate beads, they were kept in chilled calcium chloride for 24 hours for hardening of beads. Next day the beads were washed two to three times with distilled water and were used for further application [19].

Photocatalytic decolorization of textile dyes by ZnO nanoparticles and encapsulated ZnO nanoparticles

Methylene Blue (MB) is also known as methylthioninium chloride with molecular formula $C_{16}H_{18}ClN_3S$ [20], Congo Red (CR) is a sodium salt of benzidinediazo-bis-1 napthylamine 4-sulfanic acid with molecular formula $C_{32}H_{22}N_6Na_2O_6S_2$ [21] and Malachite Green (MG) is an organic dye with molecular formula $C_{52}H_{54}N_4O_{12}$ [22]. Photocatalytic decolorization of 50ppm of individual dye was done using ZnO nanoparticles. Photocatalytic activity of zinc oxide nanoparticles on decolorization of Methylene blue, Congo Red and Malachite Green was investigated using 2mg/mL of zinc nanoparticles. The said amount of ZnO nanoparticles was added to 250mL Erlenmeyer flask containing 100ml of 50ppm dye solution and the reaction was allowed to happen in sunlight as a light source for 120 mins. with constant stirring on magnetic stirrer at 100rpm. Control was maintained, which contained 50ppm of pure dye solution (without zinc nanoparticles). 1 mL aliquots of sample were withdrawn and absorbance was checked at λ_{max} (Congo Red-498nm, Malachite Green-617nm and Methylene Blue-665nm) of each dye by using UV-Visible Spectrophotometer. To study dye decolorization by immobilized zinc nanoparticles, 5mg of the above encapsulated zinc nanoparticles beads were suspended in 100L of 50ppm dye solution and the reaction was allowed to proceed in normal sunlight for 120mins. with constant stirring on magnetic stirrer at low rpm (50rpm) to prevent external damage of beads.

To checked reusability of ZnO nanoparticle beads, the same beads were resuspended in fresh 100mL of 50 ppm of each dye solution and the reaction was allowed to take place in presence of sunlight for 120 mins with constant stirring on magnetic stirrer at 50rpm. To check the % decolorization of dye 1mL of aliquot was taken from the reaction flask and absorbance was recorded at the λ_{max} (Congo Red-498nm, Malachite Green-617nm and Methylene Blue-665nm) of each dye. The same experiment was performed thrice. Absorbance of all the experiments was checked using UV-Visible Spectrophotometer at λ_{max} of individual dye. % Decolorization was calculated using the formula given below:

$$\% \text{ Decolorization} = \frac{(\text{Acontrol} - \text{Asample})}{\text{Acontrol}} \times 100$$

Where $A_{control}$ = Dye solution
A_{sample} = Test solution containing dye and ZnO nanoparticles/ ZnO sodium alginate beads

UV-Visible spectrum of all the test solutions which include; dye solution with ZnO nanoparticles, dye solution with immobilized beads of ZnO nanoparticles for all three cycles to check reusability of beads along with that of pure dye solution was determined using double beam UV-Visible spectrophotometer within the range of 200-800nm after completion of the reaction after 120 mins.

RESULTS AND DISCUSSION

Antimicrobial activity and MIC of ZnO nanoparticles

The antimicrobial activity of ZnO nanoparticles was studies against selected microorganisms using agar-well diffusion method. The presence of inhibition zone clearly indicated the effect of ZnO nanoparticles over pathogenic strains. The diameter of zone of inhibition in mm around each well was determined with data analysis using ANOVA with alpha of 0.05 and mean ± SD was determined (Table 1). 50µg/mL of ZnO nanoparticle exhibited zone of inhibition of 22.5±0.2mm with MIC of 25µg/mL for *Staphylococcus aureus*, present study was found to be better when compared with the results of Zarrindokht *et al*. [16] as they obtained inhibition zone of 14mm with 78µg/mL of ZnO nanoparticle. Results for *Klebsiella pneumoniae* and *Streptococcus pyogenes* was also promising with 26.2±0.1 mm and 20.4±0.2mm zone of inhibition with MIC of 50 µg/mL and 25µg/mL respectively, which is much higher to that, observed by Syedahamed *et al*. [23] which is 9mm and 14mm zone of inhibition for *Klebsiella pneumoniae* and *Streptococcus pyogenes* respectively with 1000µg/mL of ZnO nanoparticles.

On basis of results obtained from the antimicrobial activity and MIC it can be stated that the Gram- negative bacteria is inhibited at higher concentration that that for Gram-positive bacteria. Similar results were reported by Zarrindokht *et al*. [16], having higher susceptibility of Gram- Positive organism for ZnO nanoparticles than that for Gram- negative bacteria.

Diameter of zone of inhibition for antimicrobial and MIC was measured and the values were subjected to one-way ANOVA test using Excel 2013(Windows10.) to determine the mean value and standard error for the data.

Table 1 Antimicrobial activity and MIC values of ZnO nanoparticles with $ZnSO_4$ and $CTAB^+$ Hexane as control

Organisms	Control*		ZnO nanoparticles 50µg/mL concentration (Zone of inhibition in mm)*
	CTAB+ Hexane	$ZnSO_4$	
Staphylococcus aureus (MIC-25µg/mL)	04.1±0.1	10.1±0.2	22.5±0.2
Klebshiella pneumonia (MIC-50 µg/mL)	08.2±0.2	15.5±0. 3	26.2±0.1
Streptococcus pyogenes (MIC-25 µg/mL)	05.4±0.2	08±0.2	20.4±0.2

*Data shown is mean ± SEM of triplicate experiment and are representative of two independent experiments, p-value<0.05

Antioxidant activity of ZnO nanoparticles

Percent Free radical scavenging activity of ZnO nanoparticles was determined using DPPH and compared with Ascorbic Acid which is a standard antioxidant. Percent scavenging activity for all concentrations of Ascorbic acid and ZnO nanoparticles is displayed in (Table 2).

Highest % radical scavenging activity was found to be 90.00% and 83.33% at 100µg/mL concentration of Ascorbic acid and ZnO nanoparticles respectively. Comparable % radical scavenging effect of ZnO nanoparticles was obtained by Tura *et al*. [24] which was 90% for the 100 µg/mL having average nanoparticle size of about 10nm. Soren *et al*. [25] also performed % free radical scavenging activity for 100 µg/mL of aqueous ZnO nanoparticles with activity of 93% which had particle size of 10-15nm. This indicated that the % radical scavenging activity depends upon the particle size of nanoparticles. Smaller particle size provides larger surface area and hence gives excellent activity [26].

Table 2 Antioxidant activity of ZnO nanoparticles and standard antioxidant ascorbic acid

Concentration of ZnO nanoparticles (µg/mL)	Percent free radical scavenging activity	
	Ascorbic acid	ZnO nanoparticles
100	90.00 ± 0.2%	83.33 ± 0.4%
50	83.33 ± 0.5%	76.66 ± 0.2%
25	80.56 ± 0.3%	73.33 ± 0.3%

Photocatalytic decolorization of textile dyes by zinc nanoparticles and encapsulated zinc nanoparticles

To determine the photocatalytic activity of ZnO nanoparticles on Methylene Blue, UV-Visible spectra of MB was studied (Fig 1a) which showed two maximum absorbance peaks, major at 655nm and minor at 343nm. % Decolorization of Methylene Blue by free ZnO nanoparticles of 2mg/mL was found to be 89.66% after 120 mins. which was better when compared with Isai *et al*. [27] who performed same experiment for 50mg/mL of ZnO nanoparticles which gave 86% decolorization within 180 mins. When the % decolorization of free ZnO nanoparticles (89.66%) was compared with that of encapsulated ZnO gave less % decolorization 84.37%. Reusability of these beads was performed which gave results as. 70.31% and 59.37% for cycle II and cycle III respectively (Fig 1b). Immobilization of ZnO on sodium alginate and Fe was also done by Isai *et al*. [27] which gave 92% decolorization. Here, efficiency of immobilized ZnO nanoparticles increased due to the use of Fe in combination with ZnO. UV-Visible spectra of Methylene Blue when compared with that of the decolorization experiments, the absorption peak of MB at 655nm declines. Same pattern of UV- Visible spectra was observed by Mardani *et al*. [28]. This proves the decolorization of Methylene Blue can be done using ZnO nanoparticles. In present study, the percent decolorization reduced by approximately 5 percent.

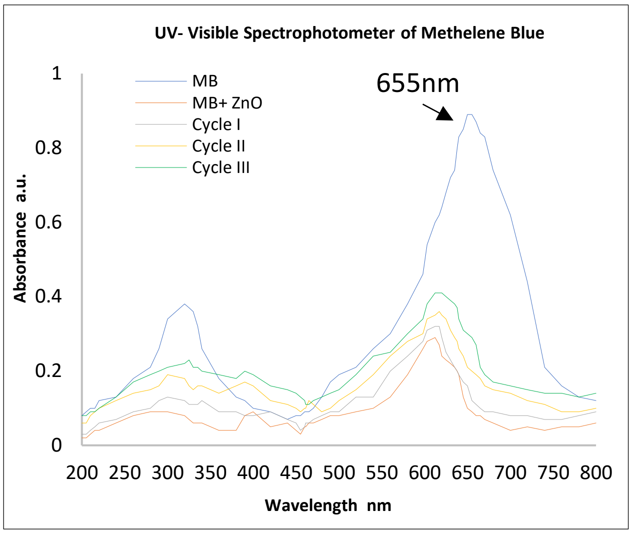

Fig 1a UV-Visible spectra of Methylene Blue, Methylene blue +ZnO nanoparticles (MB+ZnO), encapsulated ZnO with repeated test for reusability testing Cycle I, Cycle II and Cycle III (The figure represents the UV-visible spectra for Methylene Blue. The red line indicates identical absorption spectra for MB. The green, violet, yellow and blue lines indicate the absorption spectra for MB+ZnO, cycle I, cycle II and cycle III of MB decolorization)

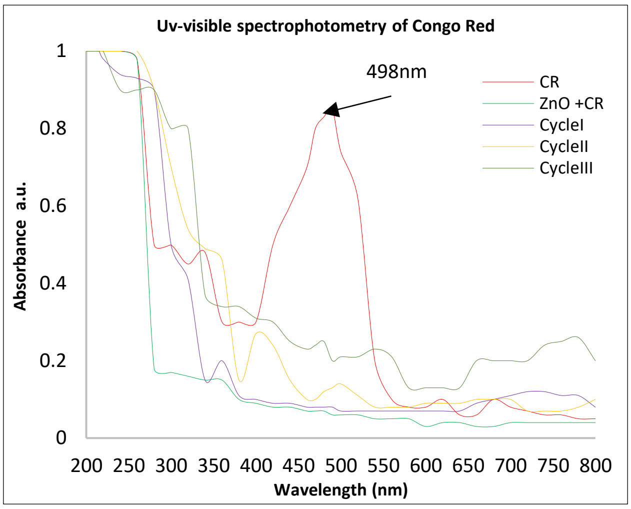

Fig 2a UV-Visible spectra of Congo Red, Congo Red +ZnO nanoparticles (CR+ZnO), encapsulated ZnO with repeated test for reusability testing Cycle I, Cycle II and Cycle III (The figure states the absorption spectra of Congo Red. Red color line depicts absorption spectra of CR. The absorption spectra for CR+ZnO, cycle I, cycle II and cycle III are shown by green, violet, yellow and blue lines respectively)

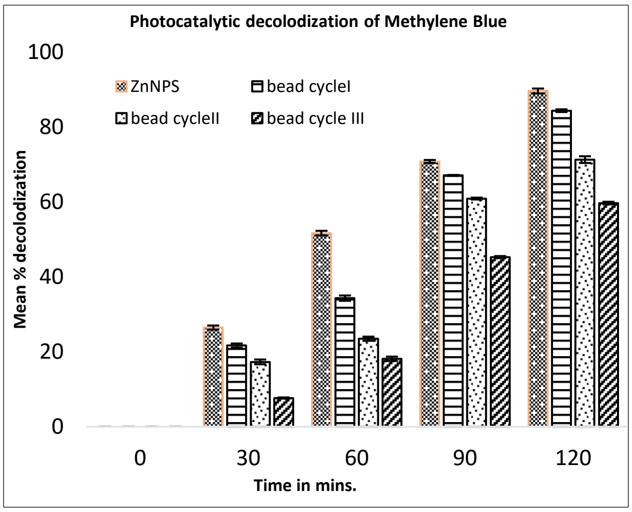

Fig 1b Photocatalytic decolorization of Methylene Blue (MB) by free and encapsulated ZnO nanoparticles

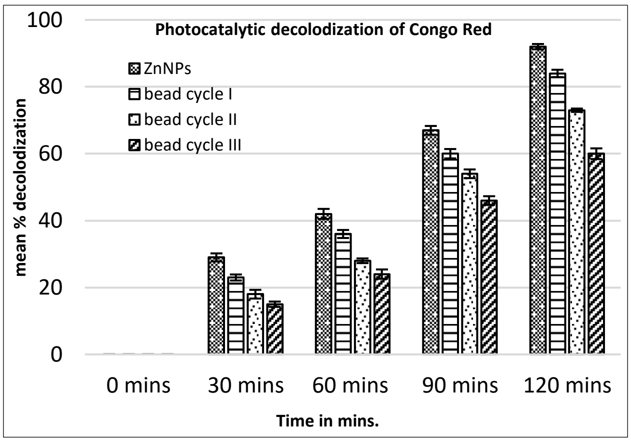

Fig 2b Photocatalytic decolorization of Congo red by free and encapsulated ZnO nanoparticles

Photocatalytic activity of ZnO nanoparticles on Congo Red was studied and monitored with the help of UV- Visible spectrophotometry. UV-Visible spectra of Congo Red (Fig 9) show two absorption peaks major at 498nm and minor at 340nm. % Decolorization of Congo Red by free ZnO nanoparticles was found to be 92.35% at 120 mins. Fowsiya *et al*. [29] got 97% decolorization of Congo Red with 1mg/ml of ZnO nanoparticles which is more when compared to present study because ZnO nanoparticles prepared by Fowsiya *et al*. [29] were synthesized using the microwave assisted extract of *C. edulis* fruits. Factors such as time, temperature, pH, method for synthesis of nanoparticles, concentration of dye, concentration of nanoparticles, etc., has a varying difference in results for % decolorization of dye [29]. 86.93% decolorization was obtained by encapsulated ZnO nanoparticles. When the beads were reused 73.29% and 60.54% of decolorization was obtained for II and II cycle respectively (Fig 6). Form the UV-Visible spectra for all the experiments (Fig 9), the absorption peak of Congo Red disappeared. This suggests that the dye might have been degraded along with the decolorization.

Effect of ZnO nanoparticles over the decolorization of Malachite green was commenced with study of UV-Visible spectra of Malachite green (Fig 10). Major absorption peak was observed at 617nm and minor peak was at 390nm. Babajani *et al*. [30] got major absorption peak at 620nm and two minor peaks at 410nm and 320nm. % Decolorization of Malachite Green by free ZnO nanoparticles was found to be 88.56% at 120mins. Babajani *et al*. [30] reported 92% decolorization of 15ppm of Malachite green using 50µg/ml of ZnO nanoparticles. % Dye decolorization using encapsulated ZnO nanoparticles was obtained to be 79.45% at 120mins. Sustainability of beads was checked which gave 62.20% and 48.19% decolorization for second cycle bead and third cycle respectively. The trend of % dye decolorization is shown in (Fig 7). Babajani *et al.* [30] reported 90% decolorization of 10ppm Malachite green with ZnO nanoparticles immobilized using Iridium (Ir). Here present study reported low % decolorization as the concentration of dye was 50ppm. Majority of studies reported that initial dosage concentration of dye and the dosage of catalyst i.e., ZnO nanoparticles has effect on efficiency % decolorization of dye [31-32]. The UV-Visible pattern for all % decolorization experiment of Malachite green is shown in figure 10. From the spectra it can be stated that the peak of maximum absorption for Malachite Green disappeared after using free and encapsulated

ZnO nanoparticles. Same pattern was observed by Babajani *et al*. [30] and Mohomed *et al*. [31] which reported the shift of maximum absorption peak of Malachite green. This suggests that there is degradation as well was decolorization of Malachite green. The detail studies of degradation will be done in future.

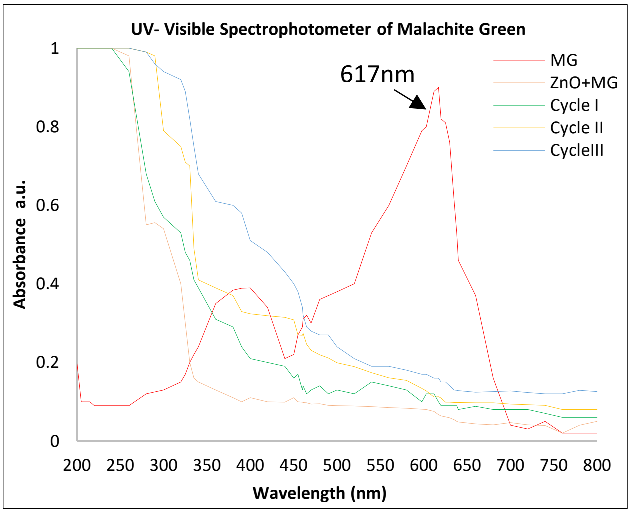

Fig 3a UV-Visible spectra of Malachite green (MG), Malachite green +ZnO nanoparticles (MG+ZnO), encapsulated ZnO with repeated test for reusability testing Cycle I, Cycle II and Cycle III (Above figure represents the trend of absorption spectra of MG,MG+ZnO, cycle I, cycleII and cycle III indicated by the lines in red, green, violet, yellow and blue color respectively)

The (Fig 1b, 2b, 3b) shows the records of percent photocatalytic decolorization of MB, CR and MG respectively, using free ZnO nanoparticles (first bar) and ZnO nanoparticles immobilized using sodium alginate. The result was recorded after every 30 mins. time interval until two hrs. The reusability of immobilized beads is studied and shown in the bars towards right which shows downline trend for photocatalytic decolorization of MB, CR and MG).

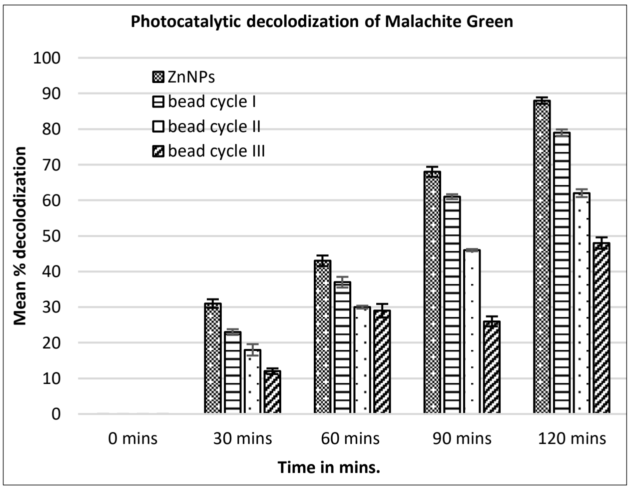

Fig 3b Photocatalytic decolorization of Malachite Green by free and encapsulated ZnO nanoparticles

CONCLUSION

Form the present study, it reveals that ZnO nanoparticles has significant antimicrobial activity for pathogenic strains of Gram positive as well as Gram negative bacteria. Bacterial synthesized ZnO nanoparticles also exhibits good antioxidant activity. Thus, ZnO nanoparticles can be used for making of personal care products like anti-ageing and skin care creams, antiseptic soaps and liquids. ZnO nanoparticles were also found to have a great ability for decolorization and degradation of Methylene Blue, Congo Red and Malachite Green. Thus, extracellularly synthesized ZnO nanoparticles using bacteria can be used for treatment of waste water from textile dye industries.

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