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Biosynthesis of Chitosan and Chitosan AgNps of *Penaeus monodon* and their potential antimicrobial activity.

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ABSTRACT

The noval discovery of biopolymer with antibacterial properties gains importance, hence the present study deals with the synthesis of chitosan from biodegradable shell waste of *Penaeus monodon* through several process like demineralization, deproteinization and deacetylation. The Chitosan AgNps were synthesized and characterized by UV, FTIR and XRD. The Chitosan AgNps of *Penaeus monodon* were subjected to antibacterial activity against six bacterial strains namely, *Bacillus subtilis , Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus mutans* respectively. Among all tested bacteria, *Escherichia coli* showed maximum zone of inhibition at 300 µg/ml concentration and *Klebsiella pneumonia* showed minimum zone of inhibition at 300 µg/ml concentration

INTRODUCTION

Shrimp wastes are generally treated as trash and for the disposal of wastes either extra money is spent or additional manpower is needed. In the form of head, shell, leg, appendages and tail, around 40-50% of the shrimp is wasted and according to an estimate, every year 30,000 tons of shrimp wastes are dumped by shrimp processing industries (Nowsad, 2005). Large quantities of shell wastage generated by the shrimp aquaculture industry is considered one of the most important environmental challenges due to their accumulation in the environment and theirs low rate of degradation in the long term, which can lead to significant environmental impacts and increase the ecological footprint produced by the shrimp aquaculture industry. Dumping and burning shrimp shells are the most common non-eco-friendly methods of disposal (Mathew *et al.*, 2022).

Chitin is a natural polymer found in the skeleton of crabs, shrimps, lobsters and also in exoskeleton of corals and jelly fishes (Shahidin and Abuzaytoun, 2005). Chitosan is made of ß 1, 4-linked glucosamine, and it is in deacetylated form of chitin obtained from fungi, shrimps, crab, etc. Chitosan has been used as a biomaterial, pharmaceutical, drug delivery, medical, textile, agricultural, preservative, wastewater purification, plant pesticide, and dressing material for wounds. Chitosan is known to have antibacterial activity, thus used in making gloves, wound bandages and textiles, etc. (Fernandes *et al.*, 2008). The only difference between chitosan and cellulose is the amine (-NH2) group in the position C-2 of chitosan instead of the hydroxyl (-OH) group found in cellulose (Hudson and Smith, 1998).

Nanoparticles are becoming increasingly important in many areas, including catalysis, biological applications and information storage. Their unique size-dependent properties make these materials superior. Recently the attention has been focussed around the metallic nanoparticles such as silver gold and zinc oxide nanoparticles (Kavitha *et al.*, 2017) etc. Silver nanoparticles (AgNPs) have emerged as an arch product owing to broad antibacterial activity and low toxicity towards mammalian cells (Hien *et al.*, 2015). Silver nanoparticles (AgNPs) have attracted much research interest due to its advantageous applications in biomedical, drug delivery, food industries, agriculture, textile industries and water treatment (Venugopal *et al.*, 2017).

Nontoxic and antibacterial highlights of chitosan make it usable for some, regions identified with human wellbeing. Chitosan is utilized as metal nanoparticle-chitosan material in biomedical applications in view of its points of interest of biodegradability, antibacterial properties, and superb chelating operator. Both of Ag and chitosan are antibacterial specialists, so chitosan Ag nanoparticle composite material has a more antibacterial impact (Rabea *et al.*, 2003).

The present study deals with the synthesis of chitosan and chitosan AgNps from shell waste of *Penaeus monodon*. The obtained chitosan AgNps of *Penaeus monodon* were characterized by UV, FTIR and XRD and also we demonstrate its anti-bacterial activity against *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtilis* and *Streptococcus mutans* respectively. The most important novelty of this work lies in the use of chitosan derived from a cheap waste source.

2. Materials and Methods

2.1. Collection of shrimp shell and preparation of Chitosan

Shrimp shells were collected from fish industry and collected shell wastes were identified as *Penaeus monodon*. Shrimp shells were scraped free of loose tissue, washed with cold water and dried in oven at 60 °C. Dried shells were grind in mixer, sieved to particle size 500 µm followed by 250 µm and were stored in air tight containers at ambient temperature until further processed. No *et* al. (1989) modified method was used for preparation of chitosan which included demineralization, deproteinization, and deacetylation processes. Initially, the shrimp shells powder was demineralized with 1N HCl, with solid to solvent ratio of 1:15 (w/v) with constant stirring for 30 min at ambient temperature followed by vacuum filtration. The residue was washed for 30 min with tap water and then oven dried for overnight. Further, demineralized powder was deproteinized with 3.5% NaOH solution in the ratio 1:10 (w/v) for 2 h, at 65ºC with constant stirring. The mixture was vacuum filtered and the residue was washed as above and oven dried for 2 h. Further, removal of acetyl groups from chitin was achieved by refluxing for 12 h at 110°C using 50% sodium hydroxide with solid to solvent ratio 1:15 (w/v). The resulting chitosan (C3) obtained was washed and neutralized with tap water followed by rinsing with hot distilled water at 90ºC, filtered and oven dried at 60ºC for 24 h and stored in airtight containers till further use.

2.2 Biosynthesis of Chitosan AgNps of *Penaeus monodon*

Chitosan synthesized from *Penaeus monodon* was taken to synthesis chitosan silver nanoparticles. A chitosan solution was made by dissolving 0.2 g of chitosan in 1% acetic acid followed by stirring for 30 min. This was then filtered to obtain clear solution. About 0.1 M of 3 mL of freshly prepared $AgNO_3$ and then 100 µL of 1 M NaOH was added to 1% of acetic acid containing chitosan solution. The solution was mixed under stirring for 10 h at 90 °C. The color of the solution was changed from colorless to light yellow and finally to yellowish brown. This indicates the formation of Ag NPs. The solution was centrifuged at 6000 rpm for 10 min. The supernatant was then discarded. The resulting residual powders were washed with 30 mL of double-distilled water (ddH_2O) to remove the alkaline solution. The washing process with ddH_2O was carried out twice. The resulting powders were then dried at 40°C for 1 h to obtain chitosan silver nanoparticles (Senthilkumar et. al., 2019).

2.3 Characterization of chitosan AgNps of *Penaeus monodon*

i) UV-Vis Spectral Analysis

The UV–Vis spectral was analyzed for chitosan AgNps of *Penaeus monodon* a pinch of each samples were diluted with one milliliter of water and loaded those cuvettes in the Shimadzu, UV 2500 (China) instrument, the scan rate was set from 200 to 750 nm. The absorption of the samples were observed using a UV-Vis spectrophotometer (Priya *et al.,* 2020).

ii) Fourier Transforms Infrared spectra analysis

Ultraviolet (UV) spectrums were recorded on Shimadzu UV-170 spectrophotometer. The extracted chitosan was analyzed to elucidate the possible functional groups present in it. One milligram of the sample was dissolved in 10 ml of water and the spectra were recorded at 200–400nm range. The infrared spectra were recorded on a Shimadzu IR-470 Model. The spectra were scanned in the 400 to 4000 cm-1 range. The spectrum was obtained using potassium bromide pellet technique. Potassium bromide was dried under vacuum at 100°C for 48 h and 100mg of KBr with 1mg of the sample was taken to prepare a KBr pellet. The spectrum was plotted as intensity versus wave number (Ashokkumar and Ramaswamy, 2014).

iii) X-ray diffraction (XRD) analysis

X-ray diffractograms on powder samples were obtained using a Bruker's X-ray Diffraction (USA) with Cu tube radiation (k = 1.54184 Å), a graphite monochromator and Lynxeye detector at 30 kV, and a current of 10 mA. The diffractometer was controlled and operated by a PC with the DIFFRAC.SUITE™ Software package. Measurements were taken over an angular range of $0.99° \leq 2\theta \leq 89.99°$ with a scanning step of 0.05 and a fixed counting time of 10 s. Divergence, scattered, and receiving radiation slits were 1°, 1°, and 0.2 mm, respectively (Badawy *et al.*, 2018).

2.4. Antimicrobial activity of chitosan AgNps of *Penaeus monodon:*

The test microorganisms used for antibacterial analysis are *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtilis* and *Streptococcus mutans* was purchased from Microbial Type Culture Collection and Gene Bank (*MTCC*) Chandigarh. The bacterial strains were maintained on Nutrient Agar (NA). Pure culture from the plate were inoculated into Nutrient Agar plate and sub cultured at 37ºC for 24 h. Inoculum was prepared by aseptically adding the fresh culture into 2 ml of sterile 0.145 mol/L saline tube and the cell density was adjusted to 0.5 McFarland turbidity standard to yield a bacterial suspension of 1.5×108 cfu/ml. Standardized inoculum used for antimicrobial test. The medium was prepared by dissolving 38 g of Mueller-Hinton Agar Medium (Hi Media) in 1000 ml of distilled water. The dissolved medium was autoclaved at 15 Lbs pressure at 121^{0}C for 15 min (pH 7.3). The autoclaved medium was cooled, mixed well and poured in to Petri plates (25 ml/plate). The plates were swabbed with Pathogenic bacterial culture viz *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtilis* and *Streptococcus mutans.* The standard drug Ciprofloxacin 5 mcg concentration disc was used for positive control depends on the sensitivity of bacterial culture and empty sterile disc was used for negative control. The plates were kept for incubation at 37°C for 24 hours. At the end of incubation, inhibition zones were examined around the disc and measured with transparent ruler in millimetres. The size of the zone of inhibition (including disc) was measured in millimetres. The absence of zone inhibition was interpreted as the absence of activity (Mathabe *et al.*, 2006; Assam *et al.*, 2010)

3. Results and Discussion

3.1. UV-Visible Spectroscopy of Chitosan AgNps of *Penaeus monodon*

UV–visible spectroscopy was used to characterize the biosynthesized Chitosan AgNps of *Penaeus monodon.* AgNPs were successfully synthesized when the color of the solution changed from colorless to light yellow to yellowish-brown as the synthesis progressed. The $AgNO_3$ surface plasmon resonance's (SPR) excitation is what was responsible for the color change that happened gradually. The UV-Visible spectral absorption pattern between 200 and 900nm was observed. In the analysis of the UV-visible spectrum, a noteworthy absorbance peak with a wavelength falls in the range of 419 nm was observed. This distinct peak was observed in the spectrum of AgNPs that were synthesized using chemically extracted chitosan obtained from *Penaeus monodon* The UV–vis spectrum, as depicted in Figure. 1 clearly exhibits this characteristic absorbance feature, indicating the presence of AgNPs with specific optical properties.

3.2. FTIR Spectroscopy of Chitosan AgNps of *Penaeus monodon*

Figure. 2 shows the absorption efficiency of chitosan AgNps of *Penaeus monodon.* Our present study confirms the IR spectral data for the Chitosan AgNps of *Penaeus monodon* by indicating that the absorption of wavelengths at 3415.90 cm^{-1} is the N–H stretching of the amino group. The absorption at the wavelength of 2924.50 cm^{-1} is the C–H stretching thought to come from lipids. The absorption at the wavelength of 1638.07 cm^{-1} and 1618.43 cm^{-1} is the C–N bond of the amide compound. The absorption at wavelengths of 1481.09 cm^{-1} and 1423.45 cm^{-1} is from the (–CH2) group. The absorption at a wavelength of 1279.26 cm^{-1} is the C–O bond from the carboxyl group. The absorption at a wavelength of 616.23 cm^{-1} and 457.79 cm^{-1} belonged to the C-Br stretch of alkyl halides. The presence of N–H and C–N absorption seems to be due to the interaction between the amino groups and the metallic surface of silver nanoparticles (AgNPs), where the amino groups act as capping

agents for the stabilization of silver nanoparticles (Mukherjee *et al.*, 2001; Ali *et al.*, 2011).

3.3. X-Ray Diffraction of Chitosan AgNps of *Penaeus monodon:*

The analysis of the crystalline size and structure of the Ag NPs was carried out by XRD. The XRD pattern of Chitosan AgNps of *Penaeus monodon* was shown in Figure. 3. The XRD exhibited intense peaks in the whole spectrum of 2θ value ranging from 20° to 80°. The XRD analysis showed that the numbers of Bragg reflections with 2θ values of 37.9, 44.2, 64.3, 77.3 corresponding to (111), (200), (220) and (311) respectively indicated that the Ag NPs were spherical structured and crystalline in nature (JCPDS card no 01-076-1393). The sharp peak indicated some bioorganic compounds/proteins in the NPs during synthesis. (Shankar *et al.*, 2004). The obtained XRD pattern clearly illustrated that the silver ions have been reduced to AgO_2 by the stabilization of chitosan under reaction condition. The pattern showed the reflection planes that confirmed the presence of face-centered cubic form of metallic silver and no peaks of other impurity crystalline phases were observed. (Bogle *et al.*, 2006; Zamiri *et al.*, 2011).

3.4. Antimicrobial activity of chitosan AgNps of *Penaeus monodon*

Our present study revealed that chitosan AgNps of *Penaeus monodon* of different concentrations have excellent enhancement of antibacterial activity against six strains namely *Bacillus subtilis* , *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus mutans*. The results are tabulated and represented in table. 1and plate.1.

The antibacterial activity of chitosan AgNps of *Penaeus monodon* at 100 µg/ml concentration, the maximum zone of inhibition was recorded in *S. aureus* and S. mutans at 10 mm, 9 mm zone of inhibition was observed in *E. coli, P. aeruginosa* and *B. subtilis* followed by 8 mm in *K. pneumoniae*. In 200 µg/ml concentration, the maximum zone of inhibition was recorded in *E. coli* (15 mm) and minimum zone of inhibition was recorded in *K. pneumoniae* (9 mm) followed by 11 mm in *P. aeruginosa, S. aureus, B. subtilis* and *S. mutans*. At 300 µg/ml concentration, the maximum zone of inhibition was found against *E. coli* (19 mm) and least zone of inhibition was observed in *K. pneumoniae* (10 mm). *P. aeruginosa, S. aureus, B. subtilis* and *S. mutans* shows 12 mm zone of inhibition.

Chitosan extracted from prawn shell showed antibacterial activity against *Salmonella typhi, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. The effect of antibacterial activity of Chitosan, at a concentration of 1000µg/ml, it showed the maximum zone of inhibition against *Bacillus subtilis* (24mm). The least zone of inhibition of 8mm was observed for *Escherichia coli*. Zone of inhibition of *Salmonella typhi, Staphylococcus aureus* and *Pseudomonas aeruginosa* were 12mm, 10mm and 18mm respectivelyAt 750µg/ml concentration, 16mm zone of inhibition was found against *Bacillus subtilis* and *Pseudomonas aeruginosa,* 10mm zone of inhibition was observed in *S. typhi* while 8mm and 7mm were observed against *S. aureus, E. coli* respectively. At 500µg/ml concentration, the maximum zone of inhibition was seen against *P. aeruginosa* at 16mm, *S. typhi* and *B. subtilis* showed 8mm while *E. coli* and *S. aureus* showed 6mm (Prabha and Sivakumar, 2017).

The chitosan preparations of different concentrations have excellent enhancement of antibacterial activity against four strains namely *E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Staphylococcus aureus*. Similarly, microorganisms' growth is inhibited by 1 % chitosan and 0.0075%. Chitosan of 70 µg and 80 µg concentrations were shown to be a potent antibacterial agent against selected bacterial pathogens. This might be the polycationic nature of chitosan, which can easily bind to a negatively charged bacterial cell wall and creates impact (Abirami *et al.*, 2021). The bacterial inhibition experiments involving Cs-AgNPs indicated high antibacterial activities against *B. subtilis, K. pneumoniae*, and *S. typhi* . At 90ppm concentration of AgNPs inhibited the growth of *B. subtilis* (inhibition zone 26.76mm), *K. pneumoniae* (inhibition zone 22.66mm), and *S. typhi* (inhibition zone 24.33mm). It is also noted that at the concentration of 30ppm the zone of inhibition exceeded 13mm, and at the concentration of 60ppm the zone of inhibition exceeded 18mm for all the three bacteria tested, thereby showing high antibacterial activity (Alshehri *et al.*, 2020).

CONCLUSION

The present study deals with the synthesis of chitosan and Chitosan AgNps from shell waste of *Penaeus monodon*. The extracted Chitosan AgNps were characterized by UV, FTIR and XRD and was subjected to antibacterial activity. The Chitosan AgNps of *Penaeus monodon* were tested against six bacterial strains. The result of the present study revealed that among six bacterial strains, the maximum activity was recorded against *Escherichia coli* (19mm) at 300 µg/ml concentrationfollowed by 12mm zone of inhibition in *Bacillus subtilis* , *Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus mutans* respectively . We conclude that chitosan AgNps of *Penaeus monodon* can be used as an antimicrobial agents for many different pathogens and would replace the existing inadequate and cost effective antibiotics.

Figure.1. UV pattern of Chitosan AgNps of *Penaeus monodon*

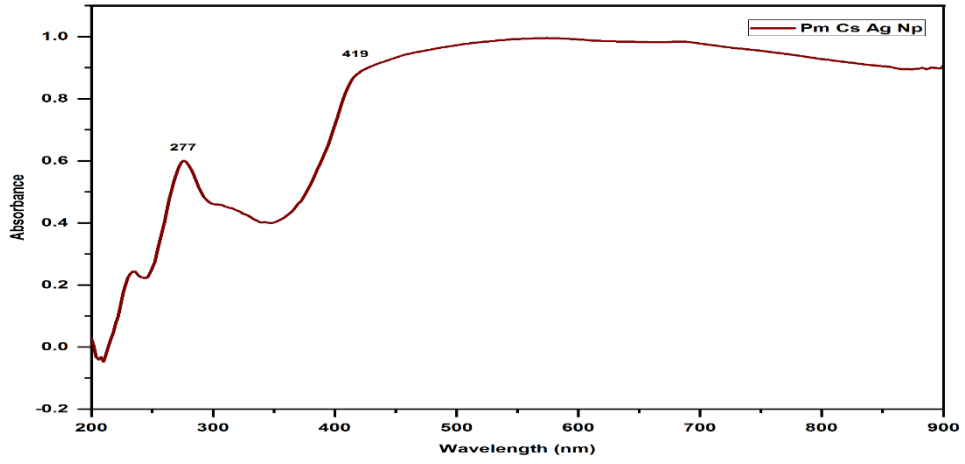

Figure.2. FTIR spectral analysis of Chitosan AgNps of *Penaeus monodon*

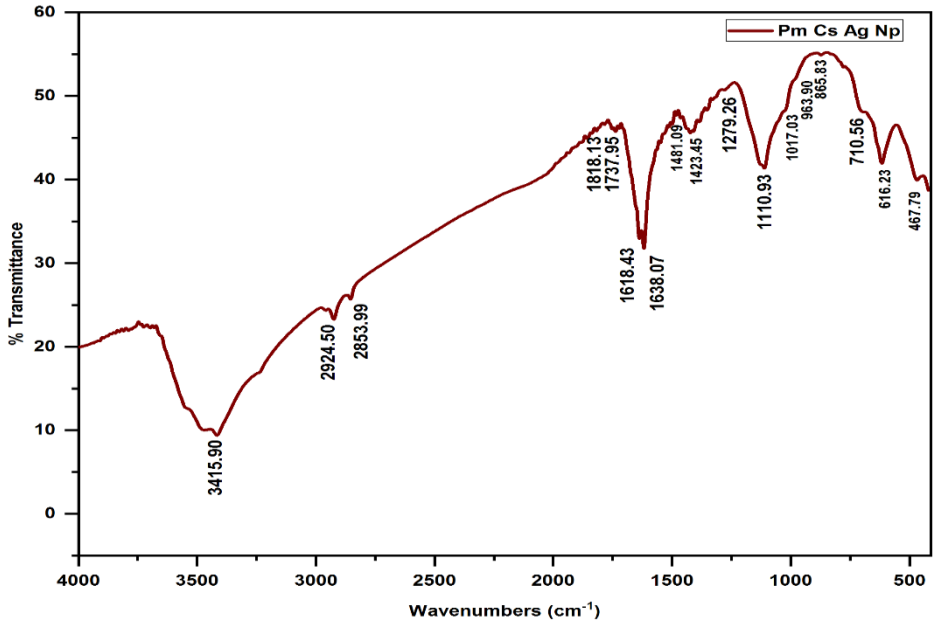

Figure.3. XRD pattern of Chitosan AgNps of *Penaeus monodon*

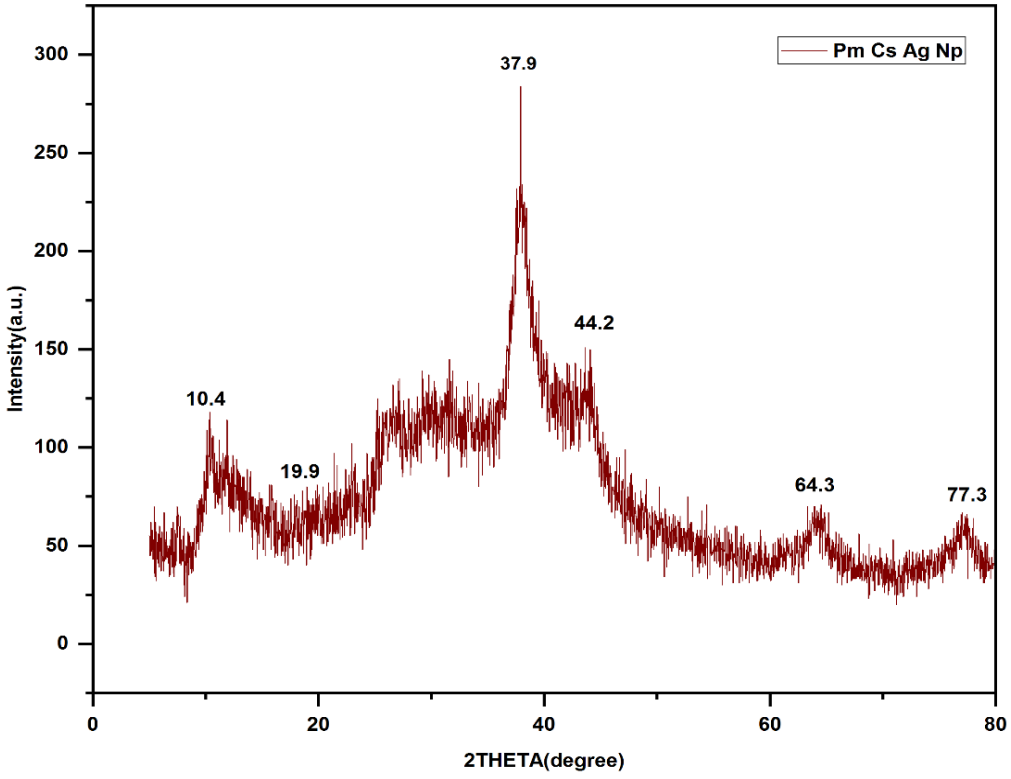

Table:1 Antibacterial activity of Chitosan AgNps of *Penaeus monodon* against tested pathogens.

Test Pathogens	Zone of Inhibition (mm)			
	100 (µg/ml)	200 (µg/ml)	300 (µg/ml)	+ve control Ciprofloxacin
Bacillus subtilis	9 mm	11 mm	12 mm	31 mm
Escherichia coli	9 mm	15 mm	19 mm	30 mm
Klebsiella pneumoniae	8 mm	9 mm	10 mm	26 mm

Pseudomonas aeruginosa	9 mm	11 mm	12 mm	27 mm
Staphylococcus aureus	10 mm	11 mm	12 mm	32 mm
Streptococcus mutans	10 mm	11 mm	12 mm	30 mm

Plate:1 Antibacterial effect of Chitosan AgNps of *Penaeus monodon* against tested pathogens

Conflict of interest

The authors declare that they have no conflict of interest regarding this investigation

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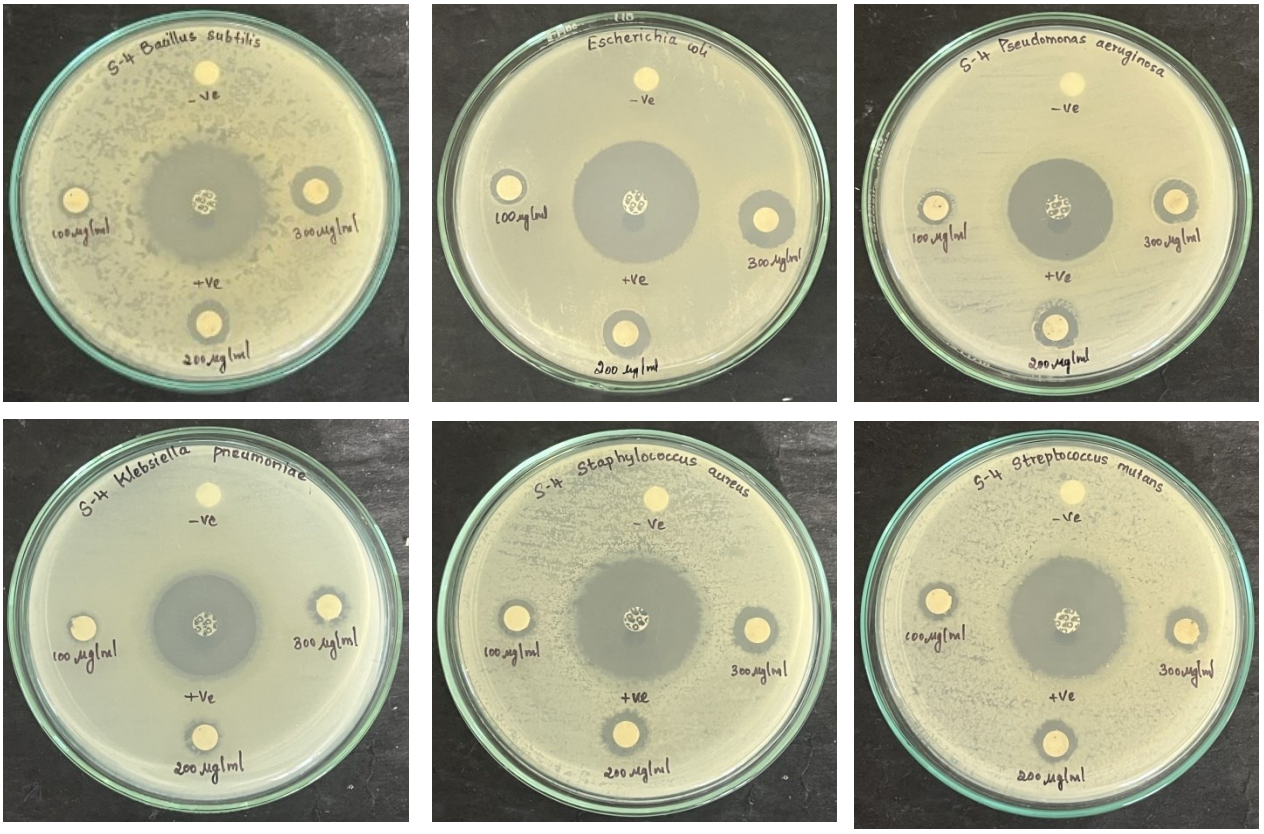

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