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Green synthesis of Silver nanoparticles and their Activity against Bacterial Biofilms

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Abstract

Emergence of new resistant strains of microorganisms to current antibiotics has become a major issue in public health; therefore, it is imperative to develop new bactericides. This study, thus, aimed at synthesizing silver nanoparticle (AgNPs) and determining their antibacterial activities on bacterial biofilms. Sachet water and well water samples were collected from sachet water industries and two hand-dung wells around Gbonagun piggery house, respectively from where isolation and identification of microorganisms was carried out using standard techniques. Identified bacterial isolates were screened for biofilm formation using tube test as well as Congo red method and the fungal isolate was used to synthesize AgNPs. The formation of silver nanoparticles was confirmed by the physical change of colour and absorption peak between 250-800 nm was evaluated using UV-Vis spectroscopy. Further, the synthesized AgNPs were characterized by Fourier transform infrared spectroscopy. Antibacterial activities of the silver nanoparticles on the biofilms formers was investigated using agar well diffusion method and the potential to prevent the growth of biofilms was evaluated. Predominant bacterium and fungi were *Bacillus subtilis* and *Aspergillus* species. Biofilm formers as revealed by the tube test and **C**ongo red method are: *B. subtilis*, *Enterobacter cloacae, Proteus mirabilis, Klebsiella pneumoniae, Streptococcus pyogenes* and *Staphylococcus aureus.* The color of the synthesized silver nanoparticles by *A. niger* was yellowish brown and control showed no colour change. The UV-visible spectra of the nanoparticles exhibited an absorbance peak at approximately 425 nm. The FTIR analysis revealed the presence of coordinating NH_2, OH, amides I and II among others, ligating functional groups in the biomass of *A. niger* responsible for efficient capping and stabilizing of the AgNPs. Synthesized AgNPs prevented the growth of biofilm formers by exhibiting highest zone of inhibition of 18.5 mm. The ability of the synthesized silver nanoparticles to inhibit growth of bacterial biofilms by *Bacillus subtilis* presents the mould (*A. niger*) as a vital bioresource in synthetizing AgNPs with good bactericidal activities.

1. Introduction

Microorganisms naturally or artificially adhere themselves to either living or non-living surfaces to form complicated matrix known as biofilms. Biofilms are responsible for causing a wide range of chronic diseases and infections have really become difficult to treat with efficacy due to the emergence of antibiotic resistance [1]. Development of drug resistance micro-organisms against antibiotics suggests

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for new drug or material to combat infectious microorganisms [2, 3]. In the last decades, the treatment of microbial infections have become more complex and undesirable due to the resistance mechanisms of these microbial isolates that have resulted in serious debilitating and even life-threatening infections, thus, the need for the search of novel antibacterial agents [4, 5].

Nanotechnology has drawn in a great attraction over the last few years due to its positive impact on many scientific fields such as pharmaceutical industries, electronics, energy, medicine and space industries [6]. Nanoscience have become relevant due to their size congenial with cells (10-100 nm), viruses (20-450 nm), proteins (5-50 nm) and genes (2 nm wide by 10- 100 nm long). They are also mobile within the body without hindrance of normal body functions [7]. Several microflorae can synthesize nanoparticles through their extracellular and intra levels [8]. Similarly, Delbarre *et al*. [9] and Iravani *et al*. [10] submitted that enzymes, vitamins and purified polysaccharides from plants, animals and microorganisms could be used as reducing and stabilizing agents for the synthesis of nanoparticles.

Metallic nanoparticles have gained most outstanding attention in biomedical field for the treatment of various acute infectious diseases. Amidst all the potential metallic nanoparticles, silver nanoparticles (AgNPs) are the main and essential candidates of choice for resolving various medical problems due to their chemical biocompatibility, inertness, oxidation resistance and broad spectrum of antimicrobial activity against diverse range of microbial infections [11]. Salem *et al*. [12] and Gahlawat *et al*. [13] have reported that silver nanoparticles have emerged as potential antimicrobial agent to treat infectious diseases and eradicate multidrug resistance issue. Moreover, silver ions cause damages to enzymes, proteins, microbial cell wall including the DNA and silver nanoparticles have the efficacy to hinder directly the microbial growth by intracycling with the cell wall and steadily destroying the metabolic responses [14].

At present, the biological approach to the production of nanoparticles is gaining more attention over the physicochemical methods, which have numerous demerits because biological approach have distinctive attributes for the synthesis of nanomaterial with clear and distinct features [15]. Certain microorganisms such as fungal isolates have become the best suited choices for the nanotechnologist as a result of the wide variety of merits they offer over bacteria, yeast, actinomycetes, plants and other physico-chemical properties [16].

Vahabi *et al*. [17] reported that some characteristics for chosen fungi to synthesize nanoparticles include: simple nutrient requirements, secretion of large amount of proteins, increase wall-binding capability and an intracellular metal uptake capacity, ease of handling, provision of relatively quick and sterile metallic nanoparticles. However, there is paucity of information on the antibacterial activities of silver nanoparticles on bacterial biofilm formers from sachet water samples and also due to huge developing applications of silver nanoparticles in distinct fields, there is an urgent need to identify new sources of microbial nanoparticles producers. The present study therefore, focuses on the biosynthesis of AgNPs and its effect on bacterial biofilm formers.

2. Materials and Methods

2.1 Collection of samples

Sachet water samples were purchased from three different water industries in Abeokuta, Ogun State and the sachet water samples were stored at different time intervals. Also, one liter of water samples was collected from two hand-dug wells around a piggery-house.

2.2 Microbial analysis

Sachet water samples were inoculated into sterile prepared plates of plate count agar, nu-

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trient agar, eosin methylene blue agar, *salmonella shigella* agar and macconkey agar. Plates were incubated at 37°C for 24 h and 48 h, respectively, after which colonies were taken and re-purified. Well water samples were serially diluted up to the fifth factor and aliquot was inoculated into Sabouraud dextrose agar plates and was incubated for five days [18].

2.3 Identification of isolates

Bacterial isolates were purified and identified based on their microscopic, physiological and biochemical characteristics. Fungal isolates were identified based on microscopic and macroscopic characteristics [19].

2.4 Screening of Isolates for Biofilm formation

Bacterial isolates were screened for the ability to produce biofilms using the tube and congo red agar screening methods.

2.5 Tube method

A loopful of the positive biofilm formers from the complimentary screening test was inoculated into tryptic broth and incubated at 37 °C for 72 h. After incubation, the broth was discarded and the test tubes were washed with prepared buffer and stained with safranin. The tubes were air-dried and observed for the occurrence of a visible film along the walls of the test tube which indicates the presence of biofilm [20].

2.6 Congo red agar method

Congo Red Agar (CRA) was prepared according to Thilakayathy *et al*. [21] and composed of 37g/L of Brain heart infusion (BHI) broth, 10g/L agar base, 50 g/L sucrose, 1 L water and 0.8 g/L Congo Red indicator. The Congo red indicator was prepared as concentrated liquid part of other media constituents, and was autoclaved at 121 °C for 15 min and then added into the cooled agar. The pure isolate was inoculated on the sterile congo red agar plates, and were incubated at 37 °C for 24 h. Positive result were indicated by black colored colonies which indicate biofilm producers while negative results were indicated by pink colored colonies which indicate non-biofilm producers.

2.7 Fungal biomass preparation for AgNPs biosynthesis

Biomass of *A. niger* was prepared by introducing the spores into Erlenmeyer flasks containing 100 ml of sterile Potato dextrose broth, placed in an orbital shaker, incubated at 25°C and agitated at 100 rpm for 72 h. The biomass was harvested and then centrifuged at 3000 rpm for 40 min. The filtrates obtained were discarded and the biomass (residue) was washed twice with sterile distilled water [22].

2.7 Synthesis of AgNPs

Silver nitrate was prepared by dissolving 0.17g of silver nitrate into 1000 ml of distilled water. Biomass (10g) was inoculated into conical flasks containing 100 ml of distilled water and placed in the orbital shaker again at 100 rpm for 72 h. The mixture of the biomass and the distilled water was then centrifuged again after which the resulting cell filtrate (50 ml) was mixed with 50ml of 1 mM silver nitrate solution. The flask was then finally agitated in the orbital shaker for 72 h. A conical flask containing only the fungal biomass was also placed in an orbital shaker to serve as a control sample [22].

2.8 Characterization of AgNPs

2.8.1 Visual observation

This was done using the development of color change as compared to the control.

2.8.2 UV-Vis spectroscopic analysis

The reduction of pure silver ions synthesized by fungal culture was monitored by measuring the UV- Vis spectrum of the reaction mixture in the range of 250–800 nm.

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2.8.3 Fourier-Transform Infrared spectroscopy

The filtrate was freeze-dried and diluted with potassium bromide in the ratio of 1:100. The FTIR spectrum of samples was recorded on a FTIR instrument (model, maker and country) with diffuse reflectance mode. All measurements were carried out in the range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1} [23].

2.8.4 Antibacterial activity of the synthesized AgNPs

Biosynthesized silver nanoparticles produced by *A. niger* were assayed for antimicrobial activity as suggested by Gudikandula *et al*. [14] using various bacterial biofilm formers by the agar well-diffusion method. Bacterial biofilms formers (100 µl) in nutrient broth was used to prepare bacterial lawns (1×10^5 CFU/ml). Agar wells of 8 mm diameter were prepared with the help of a sterilized stainless steel cork borer. The wells were loaded with 100 µl of AgNPs and 100 µl of culture broth from A. niger as control. The plates were incubated at 37 °C for 24 h and then were examined for the presence of zones of inhibition. The diameter of zones of inhibition was measured and the mean value for each organism was recorded and expressed in millimeter unit.

3. Results and Discussion

3.1 Enumeration of microbial isolates

The physical and microbiological states of packaged water are very important aspect that should be observed by all the packaging companies. Bacteriological analyses results (Table 1) showed that all the brands of sachet water had growth after 24 h of incubation. At day 1, sample A water gave the least microbial count of 2.0 CFU/ml while sample O water had the highest growth of 4.0 CFU/ml. At week 6 of sample F water recorded the highest microbial count of 11 CFU/ml. Total fungal counts obtained from hand-dung well samples are as presented in Table 2. Highest and lowest fungal counts recorded were 7 CFU/ml (Well 1) and 5 CFU /ml (Well 2). The result of total heterotrophic bacteria count obtained in this study disagrees with the findings of Duwiejuah *et al*. [24] where higher value of 73 CFU/ml were obtained.

Bacterial growth was detected in all the brands of sachet water and as the number of weeks increases, the bacterial count increased and also gradually decreased numerically throughout the study period. It was observed that after day 1, there was an increase in the microbial count but the bacterial counts greatly reduced at week 4 for sample F and sample A. This observation is in line with Akpen *et al*. [25] that reported that indicator organism's loose viability in fresh water environment with time. Studies conducted in the United Arab Emirates and United States of America demonstrated that organisms multiply more easily between 25 and 37°C [26]. In the same vein, Akinde *et al*. [27] reported proliferation of bacteria in sachet water when stored beyond 4 weeks.

Table 1: Total bacterial counts in the sachet water samples at different time intervals

Water samples	BACTERIAL ISOLATES IN CFU/ML						
	Day 1	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Sample F	3±1.0	6.5±2.0	10±1.5	4±2.0	2±0.0	8±1.0	11±2.5
Sample O	4.0±1.0	9±1.5	8±2.0	5±1.0	8±1.2	5±2.0	3±1.0
Sample A	2.0±0.0	9±1.0	2±0.0	6±1.0	3±1.0	7±1.3	6±2.0

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A gradual increase in total aerobic heterotrophic bacterial counts followed by decease in counts up to the end of the experiment, is a growth pattern typical of microorganisms growing in a closed system and the total aerobic heterotrophic bacterial count method determines the general microbiological quality of treated drinking water [27]. The fungal population recorded disagrees with the previous studies of Umar et al [28], that showed total heterotrophic fungal count of 1.1×10^4 to 8.0×10^4 CFU/ml. Fungi are able to colonize, multiply and survive in diversified habitat that makes them ubiquitous and cosmopolitan.

Table 2: Total bacterial counts of the water samples at different time intervals

Water samples	Fungal counts (cfu/ml)
Well 1	7±2.0
Well 2	5±1.0

3.2 Identification of microbial isolates

The presence or absence of these microbial isolates in treated water determines the quality of water and whether water treatment and disinfection was adequate [29]. Microbial growth in water may be unnoticed even in transparent packaged water and the presence of some of these microorganisms may pose a potential risk to consumers. The non-compliance of the water processors with NAFDAC regulation could pose serious threats to public health [30]. World Health Organization (WHO) has reported that the occurrence of pathogens or indicator organisms in water sources mainly depends on the intrinsic physical and chemical characteristics of the catchment area, the magnitude and range of the human activities and animal sources that release pathogens to the environment [31].

Different Gram positive and Gram negative bacterial isolates were recovered from the sachet water samples. *Bacillus subtilis* was the most predominant isolate (27%) followed by *Klebsiella pneumoniae* (21%) while *Streptococcus pyogenes* had the least occurrence of 3%. Figure 1 shows the

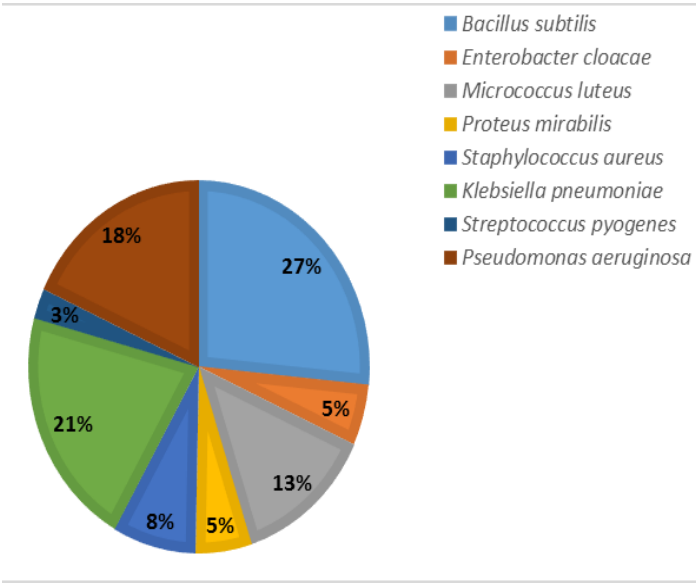

Fig. 1: Percentage occurrences of the identified bacterial isolates

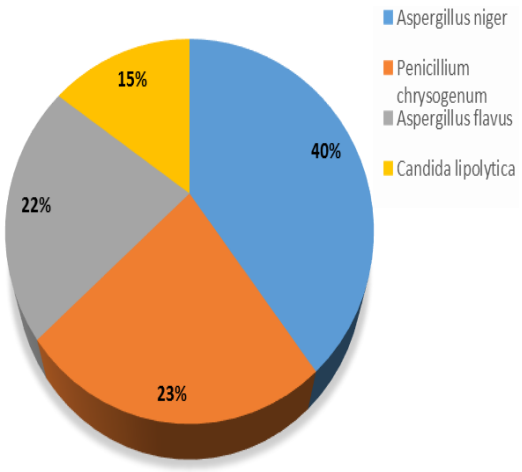

Fig. 2: Percentage occurrences of the identified fungal isolates

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percentage occurrences of the identified bacterial isolates. No faecal coliform (*Escherichia coli*) was detected in the sachet water samples but different *Enterobacteriaceae* were identified. Figure 2 depicts the distribution of different fungal isolates in the hand-dug well around the poultry house. *Candida* sp had the least occurrences, while *Aspergillus niger* was the dominant mould followed by *Penicillium chrysogenum*. Geographic location, climatic conditions, microhabitat, substrate type, distribution of fauna and flora are the significant factors contributing to fungal distribution and diversity [32]. Similar fungal isolates identified in this study were reported in the works of Akinnibosun and Ayejuyoni [33], Arroyol *et al*. [34] and Carregaro *et al*. [35]. It is known that *Aspergillus* spp. and *Penicillium* spp. have greater adaptation to aquatic environments, and that they commonly have the ability to survive in treated water [36]. This was seen in the present study, in which both genera had high survival rates. Bacteria isolated in this study such as: *Klebsiella* spp., *Pseudomonas* spp., *Bacillus* spp., *Proteus* spp., *Staphylococcus* spp., *Enterobacter* spp, *Streptococcus* spp and *Micrococcus* spp had also been reported by several researchers [37, 38, 39, 40].

Table 3: Tube method screening for biofilm formers

S/N	Bacterial isolates	Result
1	*Proteus mirabilis*	Positive
2	*Micrococcus luteus*	Positive
3	*Streptococcus pyogenes*	Positive
4	*Enterobacter cloacae*	Positive
5	*Staphylococcus aureus*	Positive
6	*Klebsiella pneumoniae*	Positive
7	*Bacillus subtilis*	Positive
8	*Streptococcus pyogenes*	Positive
9	*Klebsiella pneumoniae*	Positive
10	*Bacillus subtilis*	Positive
11	*Klebsiella pneumoniae*	Positive
12	*Enterobacter cloacae*	Positive

Table 4: Congo red agar screening assay for biofilm formers

S/N	Bacterial isolates	Diameter of zone
1	*Staphylococcus aureus*	6.7±1.7
2	*Streptococcus pyogenes*	10.5±0.4
3	*Bacillus subtilis*	2.1±0.3
4	*Klebsiella pneumoniae*	8.1±0.7
5	*Klebsiella pneumoniae*	5.3±1.1
6	*Proteus mirabilis*	9.4±0.7
7	*Enterobacter cloacae*	9.5±1.9

The bacteria characterized and identified from the sachet water samples were found mostly to be opportunistic pathogens and are usually isolated from unhygienic environments or materials. These microorganisms are versatile in their nutrient requirements, can survive with limited nutrient availability, are mostly indigenous to aquatic environments and their presence suggests problems with the processing of water which could be due to ineffective treatment [29]. The organisms isolated are not pathogenic in healthy individual, but some have been discovered to be of health concern in immune-compromised individuals [41].

Contamination of sachet water may result from the raw source of water, treatment employed and handling during production [25]; short distance of sanitary facilities from wells, shallow depth of walls, irregular changing of water filters and unhygienic environment [42]. The findings suggest the need for the government and other stakeholders to intensify surveillance activities and enforce strict hygienic measures in this rapidly expanding industry to improve water quality. These organisms are reasonably sensitive to disinfectants and their entry into distribution systems can be prevented by adequate treatment [29].

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3.3 Screening of bacterial isolates for biofilm formers

Biofilms are distinct microbial matrices that depend on a solid surface and extracellular polymeric substances for survival [43]. In this study, Congo red agar and tube method were used as screening methods to demonstrate varying capacity of biofilm formation. It was observed that *B. subtilis*, *Enterobacter cloacae, Proteus mirabilis, Klebsiella pneumoniae, Streptococcus pyogenes* and *Staphylococcus aureus* displayed a strong tendency to form biofilm by the two screening methods. On the other hand, *Micrococcus luteus* and some strains of *B. subtilis*, *Enterobacter cloacae, Klebsiella pneumoniae* and *Streptococcus pyogenes* were unable to form biofilm by the Congo red agar method. The tube method (Table 3) depicted that out of 38 bacterial isolates recovered from the sachet water samples, only 12 isolates were biofilm formers while 7 isolates showed as biofilm-producing organism using the Congo red agar method (Table 4). *Streptococcus pyogenes* gave the highest diameter zone while *Bacillus subtilis* showed the lowest zone of diameter. Different results were obtained from the Congo red agar and tube method. This is however in agreement with the previous studies [44] concluded that tube method should not be recommended as general screening test to identify biofilm producing isolates. Moreover, Panda *et al*. [45] documented similar disparities in results, in which out of 300 bacterial isolates, tube method showed that 118 isolates (39.3%) were positive while 33 isolates (11%) only showed positive result by the Congo red agar test. The reason for the disparities in the two methods could be because of the difficulty in discriminating between the weak and biofilm negative isolates due to the

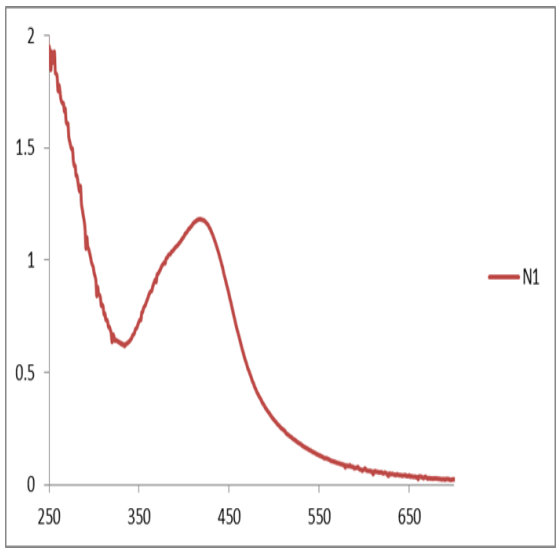

Fig. 3: UV-vis spectra of biosynthesized AgNPs of *A. niger*

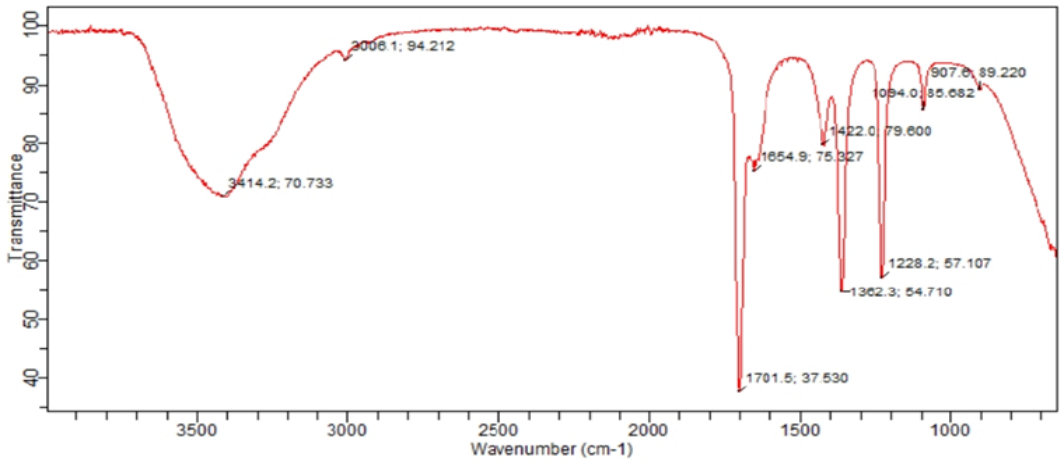

Fig. 4: Fourier Transform Infrared Spectroscopy (FTIR) FTIR spectrum of silver nanoparticles synthesized by *A. niger*

that varia-

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bility in observed results using the tube method. It is also likely that Congo red agar showed only strong biofilm producers compared to tube method which might assigned positive result to both strong and weak biofilm formers [46].

3.4 Characterization of the synthesized AgNPs

3.4.1 Visual observation

The color of the fungal extract was light yellow before addition of $AgNO_3$ but changed to dirty brown or yellowish brown after treatment with $AgNO_3$ while the control (only $AgNO_3$) showed no colour change. This indicates the bio-reduction of Ag^+ to Ag^0 to form AgNPs. Gudikandula *et al*. [14] reported that the reduction of silver nitrate into silver nanoparticles is clearly visible when the sample solutions change its color from colorless to brown and also reported that the brown color of the solution was due to the excitation of surface plasmon vibrations in the AgNPs.

3.4.2 UV-Vis spectroscopy

Synthesis of AgNPs exhibited strong absorption in the visible range due to the local surface plasmon resonance. The UV-visible spectra of the nanoparticles exhibited an absorbance peak at approximately 425 nm (Figure 3). Similar UV absorption band result was observed in the report of Khan *et al.* [47] and Saravanana *et al*. [16]. This absorbance peak can be attributed to the surface plasmon resonance band occurring due to collective oscillation of free electrons in metal nanoparticles in resonance with the light wave. No absorption band was observed in the control. Earlier reports of Kanmani and Lim [48], also showed a strong surface plasmon resonance band at 400–550 nm, indicating the formation of AgNPs.

3.4.3 Fourier Transform-Infrared Spectroscopy (FTIR)

Conversion of silver ions to silver nanoparticles is due to the secretion of nitrate reductase enzyme by the microbes [49]. FTIR spectroscopy gives information about the capping and interactions of protein with silver ions which is responsible for stability of silver nanoparticles. The functional groups in the FTIR analysis: N-H Primary and Secondary amines and amides (stretch), C-O Alcohols, S=O Sulfones, and C-H Aromatics, C-H Alkanes and C=O Carboxylic acid (Figure 4) indicated that the biomolecules present in *A*.

Table 3: Antibacterial activity of the synthesized nanoparticles by *A. niger*

Isolates	Zone of inhibition (mm)	Culture broth
Proteus mirabilis	4.0 ± 1.0	-
Micrococcus luteus	10.6± 2.5	-
Streptococcus pyogenes	8.7 ± 1.9	-
Enterobacter cloacae	9.3± 1.4	-
Staphylococcus aureus	7.3± 3.2	-
Klebsiella pneumoniae	10.0± 2.0	-
Bacillus subtilis	18.5± 1.8	-
Streptococcus pyogenes	5.2± 2.1	-
Klebsiella pneumoniae	8.6± 2.8	-
Bacillus subtilis	12.5± 1.2	-
Klebsiella pneumoniae	4.6± 2.5	-
Enterobacter cloacae	7.4 ± 3.1	-

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niger biomass was responsible for the reduction of silver ions to AgNPs.

3.4.4 Antibacterial activity of the synthesized silver nanoparticles

The antibacterial activity of silver nanoparticles against different pathogens had been previously reported [50]. In bacteria, the key component for bacterium-host cell interactions and biofilm structures are the exopolysaccharides and cell surface hydrophobicity. Nanoparticles directly disseminate through the exopolysaccharide layer and exert their antimicrobial role [51]. Table 5 shows the antibacterial activity of the biosynthesized AgNPs. The AgNPs inhibited the growth of all the biofilm formers. Highest and lowest inhibition zone of inhibition was 18.5 mm and 4 mm by *Bacillus subtilis* and *Klebsiella pneumoniae,* respectively.

The ability of the silver nanoparticles to inhibit all tested organisms suggests that the nanoparticles contained potential antibacterial agents against infections. More also, various studies have proposed that silver nanoparticles attach to the surface of the cell membrane to hinder the permeability task of the cell [52]. Gudikandula *et al*. [14] documented that the bacterial membrane contains sulfur containing proteins and the silver nanoparticles interrelate with these proteins in the cells, as well as with the phosphorus containing compounds like DNA to eventually attack the respiratory chain and cell division leading to the microbe cell death.

The potential of the silver nanoparticles to inhibit the growth of the biofilm formers could be that the nanoparticles release silver ions in the bacterial cells which led to their bactericidal activity [53]. The growth of both the weak and strong biofilm formers were inhibited by the biosynthesized nanoparticles. The antibacterial activity reported in this study agrees with the work of Metuku *et al*. [54] that reported highest zone of inhibition of 19 mm against *Bacillus subtilis.* The differences in the inhibition zone may be due to the difference in the susceptibility of tested biofilm formers, their morphology, physiology, metabolism and interaction with the positively charged silver nanoparticles [55]. Also, the difference in the inhibition zone could be due to the different biofilm formers resistance and varying degree of cell membrane depth penetration of silver nanoparticles [56].

4. Conclusion

The present study revealed the presence of bacteria in stored sachet water samples over time and the potential of certain bacteria to produce biofilms. Fungi are generally regarded as safe and efficient as compared to bacteria for industrial applications and this prompted the choice of chosen *A. niger* to produce silver nanoparticles. *A. niger* isolated from hand-dug well was used to synthesize silver nanoparticles through simple techniques and the antibacterial activities of the synthesized silver nanoparticles revealed varying capability of the silver nanoparticles to remove biofilms. Further studies on other fungal isolates with desirable characteristics as presented in this study and molecular mechanism leading to the formation of silver nanoparticles should be investigated. It is also recommended that there should be regular monitoring, inspection and sanctions by regulatory bodies to enforce existing water regulations.

Conflict of interest

The authors declare that they have no conflict of interest.

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