**Green Synthesis of Silver Nanoparticles obtained from *Lysinib*acillus Sphaericus C3-41, Its Chemical Compositions and Biotechnological Utilization in the Treatment of Biofilm**

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**ABSTRACT**

Green synthesis of nanoparticles has become a new catching area of nanoscience and nanotechnology and its utilization in the different fields of life is increasing everyday. It involve development of clean, low-cost, bio-compatible, non-toxic and eco-friendly methods for nanoparticles synthesised from microbes as compared to conventional method like physical and chemical which are often toxic. In the present study, variety of nanoparticles with well-defined chemical compositions have been synthesized and characterized from *Lysinib*acillus sphaericus C3-41 using the Ultraviolet-visible spectroscopy and Fourier transform infrared (FTIR) spectroscopy. The potentials of the synthesized nanoparticles were then tested on the treatment of wastewater biofilms. The biosynthesis nanoparticles obtained from the cell free extract showed variations in the UV-Vis spectra and the FTIR absorption spectrum showed distinct peaks at range of 3444.98, and 873.78 cm -1 for *L. sphaericus-*AgNPs. The peak bands were an indicative that the particles are composed of carbohydrates and proteins. Also, wastewater microbial biofilm are not functioning in the presence of Ag-NPs. The significant change in the viability of the biofilm bacteria implies that there was reduction in the relative abundance of microbial mass of wastewater biofilms significantly. It can therefore be reported that the study represents the first reference on the application of biosynthesized AgNPs which is capable of potentiating antimicrobial activities against microbial biofilms and there is great possibility that the organism has future prospects which is needed in order to allow them to be used in a wider range of industries.

**INTRODUCTION**

In the recent years, the topic of nanoparticles has received particular interest in a wide range of fields. The term “nano” comes from the Greek word “nanos” meaning short which means a measurement on the scale of one-billionth (109) of a metre in size (Narayanan and Sakthivel, 2010; Thakkar *et al*., 2010). ‘Nanoparticles’ may be defined as particulate dispersions of solid particles with at least one dimension at a size range of 10-1000 nm (Thakkar *et al*., 2010). One of the most important feature of nanoparticles is their surface area to volume aspect ratio, allowing them to interact with other particles easily (Narayanan and Sakthivel, 2010; Thakkar *et al*., 2010). In order for microorganism to survive in the environments containing high levels of metals, organisms have adapted by evolving mechanisms to cope with them. These mechanisms may involve altering the chemical nature of the toxic metal so that it no longer causes toxicity, resulting in the formation of *nano*particles of the metal concerned (Mohanraj and Chen, 2006). Thus *nano*particle formation is the “by-product” of a resistance mechanism against a specific metal, and this can be used as an alternative way of producing them. *Nano*particles have unique thermal, optical, physical, chemical, magnetic and electrical properties compared to their bulk material counterparts (Durán *et al.,* 2007; Husseiny *et al*., 2007). These features can be exploited for next generation biosensors, electronics, catalysts and antimicrobials (Durán *et al.,* 2007). Metallic *nano*particles are one important and widely studied group of materials, showing great diversity and many different uses.

There are important links between the way *nano*particles are synthesised and their potential uses. Silver *nano*particles (AgNPs) have been shown in numerous studies to display antibacterial properties (Durán *et al.,* 2007; Guzmán *et al.,* 2009; Krishnaraj *et al*., 2010; Guzman *et al*., 2012). For instance, *nano*particles such as silver and gold have been shown to be effective in inhibiting growth of both Gram-positive and Gram-negative bacteria (Guzmán *et al*., 2009; Lima *et al*., 2013). With the rise in antibiotic resistance in recent years and the development of fewer new antibiotics, research has begun to focus on these antibacterial *nano*particles as potential new medical tools.

Silver *nano*particles have also been used as optical sensors for the formation of small molecule adsorbates (McFarland and Van Duyne, 2003), whereas catalysts based on Pt *nano*particles have been shown to exhibit high activity for the electrooxidation of formic acid (Waszczuk *et al*., 2002). The most common methods for preparing all of these *nano*particles are wet-chemical techniques, which are generally low-cost and high-volume. However, the need for toxic solvents and the contamination from chemicals used in *nano*particle production limit their potential use in biomedical applications (LiX *et al*., 2011). Therefore a “green”, non-toxic way of synthesising metallic *nano*particles is needed in order to allow them to be used in a wider range of industries. This could potentially be achieved by using biological methods.

**METHODOLOGY**

**2.1 Preparation of extract for Synthesis of nanoparticles**

**2.1.1 *Lysinibacillus sphaericus* C3-41 extract**

*Lysinibacillus sphaericus* C3-41strain was inoculated into peptone water (15g of peptone in 1000 ml of distilled water), sterilized at 1210C for 15 min and incubated for 24h at 370C. After the incubation period, the culture broth was centrifuged at 4000 rpm; 250C for 30 min (Kumar *et al*., 2014). The supernatant obtained from the broth was decanted, allowed to cool and stored at 4 0C until further use.

**2.2 Synthesis of Silver nanoparticles (AgNPs)**

The cell-free supernatant of *Lysinibacillus sphaericus*C3-41 was used to synthesize both AgNPs and MgNPs as described by Lateef *et al.* (2015a). About One mille (1 mL) of each of the extracts was added to the Mc Cartney bottle each containing 40 mL of 1mM silver nitrate (AgNO3) solution for the reduction of silver and magnesium ions respectively. The reaction was carried out for 15min under bright condition for photoactivation. The formation of AgNPs and MgNPs was monitored through visual observation of change in colour (Lateef *et al*., 2015a; Shahverdi *et al.,* 2007).

**2.3 Characterisation of AgNPs nanoparticles**

The AgNPssynthesised by each of the supernatants were characterized using the UV-visisble spectroscopy and Fourier transform infrared (FTIR) spectroscopy (Jain *et al*., 2012).

**2.3.1 UV-visible spectroscopy**

When the detection of biosynthesized AgNPs by colour change was observed, the particles were then subjected to optical measurement and these were carried by using UV-visible spectrophotometer (Cecil, USA) which determines the optical properties of a solution. Light was passed through the samples and the amount of absorbed light was measured. The absorbance peaks of the biosynthesized AgNPs was by scanning the UV-visble between 200 and 800 nm (Jain *et al*., 2012).

**2.3.2 Fourier Transform Infrared Spectroscopy (FTIR)**

Biological molecules responsible for capping as well as stabilization of the AgNPs were studied using FTIR spectrophotometry. Fourier Transform Infrared (FTIR) spectroscopy analysis was carried out using IR Affinity-IS Spectroscopy (Shimadzu, UK) on the powder sample according to Bhat *et al*. (2011). Spectrums were taken in the range of 500 to 4000 cm-1. The data of FTIR revealed information about functional groups that are present. The AgNPs and MgNPs solutions were then centrifuged at 1000 rpm for 20 min. The pellets recovered were then dried at room temperature, and the powder obtained was used for FTIR measurement using KBr pellets.

**2.4 Effect of varying volume of (AgNO3) solution on the formation of AgNPs**

Varying concentrations of (AgNO3) solution in the formation of AgNPs by *L. sphaericus* C3-41 were tested to know the best volume. About 1 ml of *L. sphaericus* C3-41extract was added to various volumes of mM (AgNO3); 20 – 80 ml. The best volume where there was peak absorbance was obtained using UV-vis spectrophotometer at specific wavelength and this was chosen for further analysis.

**2.5 Wastewater biofilm sample**

Wastewater biofilms were extracted from the wastewater sample of Falegan Restaurant, Ado-Ekiti and taken to the laboratory. The biofilm thickness was 0.5mm. The biofilms were prepared by cutting out a section (0.5 cm x 0.5 cm attached to the substratum) of the biofilm and the substratum just before each experiment. The prepared samples were stored in a Petridish on ice during transport and then within 30 min of arrival at the laboratory were analysed (Zhiya *et al*., 2015).

**2.6 Silver nanoparticle (AgNPs) treatment on wastewater**

The effect of AgNPs was tested on wastewater biofilm. For each experiment, replicate biofilms were placed in 5mL of water and 50µg/mL AgNPs suspension and these were then incubated with shaking (100 rpm) for 24 h in the dark at room temperature (27±20C). For each enumeration and cell counting, biofilm was scraped off from the substratum after the 24 h incubation. The experiment was carried out in duplicates, such that one plate contains only biofilm while the other contains biofilm and AgNPs suspension (Zhiya *et al*., 2015). Bacterial enumeration was done using Heterotrophic Plate Count (HPC) by the modified method of Liu *et al*. (2007). A series of 10-fold dilutions were performed and 10µL of each dilution was plated on R2A agar in the duplicate sample. Plates are incubated at room temperature (300C) for 24h. Microbial load was performed with a lower detection limit of 102 CFU/mL. The result was then converted into CFU/cm2 based on the area of each biofilm sample. T-tests were performed to examine the statistical significance of the results and corresponding p-values (*p* > 0.05) were calculated using a type 3 two tailed test (unequal SD).

**3.0 RESULTS AND DISCUSSION**

Cell free supernatant of *Lysinibacillus sphaericus* mediated the synthesis of AgNPs within 8mins of reaction in the vessel at the temperature of 30oC and pH 8 resulting into the characteristic ‘dark brown’ colour which further changed with time and then stabilized within 11min when exposed to sunlight. The colour change of the reaction between *Lysinibacillus sphaericus*/AgNO3 from light brown to dark brown indicated that there was reduction in the ions of the nanomaterials. The variable colours of AgNPs colloidal solution might be due to the compositions of bio-reductant responsible for the synthesis of the nanoparticles. Interestingly, this reduction from Ag+NO3- to Ag0NO30 simply explained that the nanoparticles (AgNPs) synthesized was completely safe and ecological friendly. Several authors have reported variations in this colour change of AgNPs. For instance, it was reported by Kalimuthu (2008), Minaeian (2008), Sadowski (2008) and Gurunathan (2009b) that the biomass of *Bacillus cereus* obtained after centrifugation when challenged with silver nitrate solution resulted into formation of nanoparticles whose changed from pale white to brown indicating a reduction reaction. The characteristic brown color of AgNPs arose from the excitation of the surface plasmon vibrations in the silver metal nanoparticles (Minaeian 2008; Sadowski, 2008). Lateef *et al*. (2015a) also reported the formation of dark brown AgNPs solution using the crude extracellular keratinase of the strain of *B. safensis* LAU 13 while Jeevan *et al*. (2012) reported the formation of yellowish brown AgNPs using the culture supernatant of *Pseudomonas aeruginosa.*

The Ultraviolet-Visible spectra obtained from the cell free extract treated with 1mM each of silver nitrate (AgNO3) solution showed variations in spectra of biosynthesizedAgNPs. The absorbance peaks were monitored within the range of 200-900nm. The UV-Vis spectra of the biosynthesized AgNPs had wide absorbance peaks of wavelengths within the range of 241 and 268 nm (Fig. 1). The spectrum showed a strong surface plasmon absorption bandwidth and a long tailing from 435 to 900 nm. A long tailing on the large-wavelength which may be due to small amount of particle aggregation while the broad band indicated the presence of spherical or roughly spherical AgNPs that remained the same throughout the reaction period, suggesting that the particles are polydispersed in the aqueous solution (Minaeian, 2008). However, this report disagrees with the work of Saifuddin (2009) and Swetha and Valli (2012) who did not show any evidence for aggregation in the dispersion of the particles. The reason for aggregation in this work might be due to the fact that at this peak (241 and 268 nm), there was concentration of salt solution which shortens the scattering of the nanoparticles.

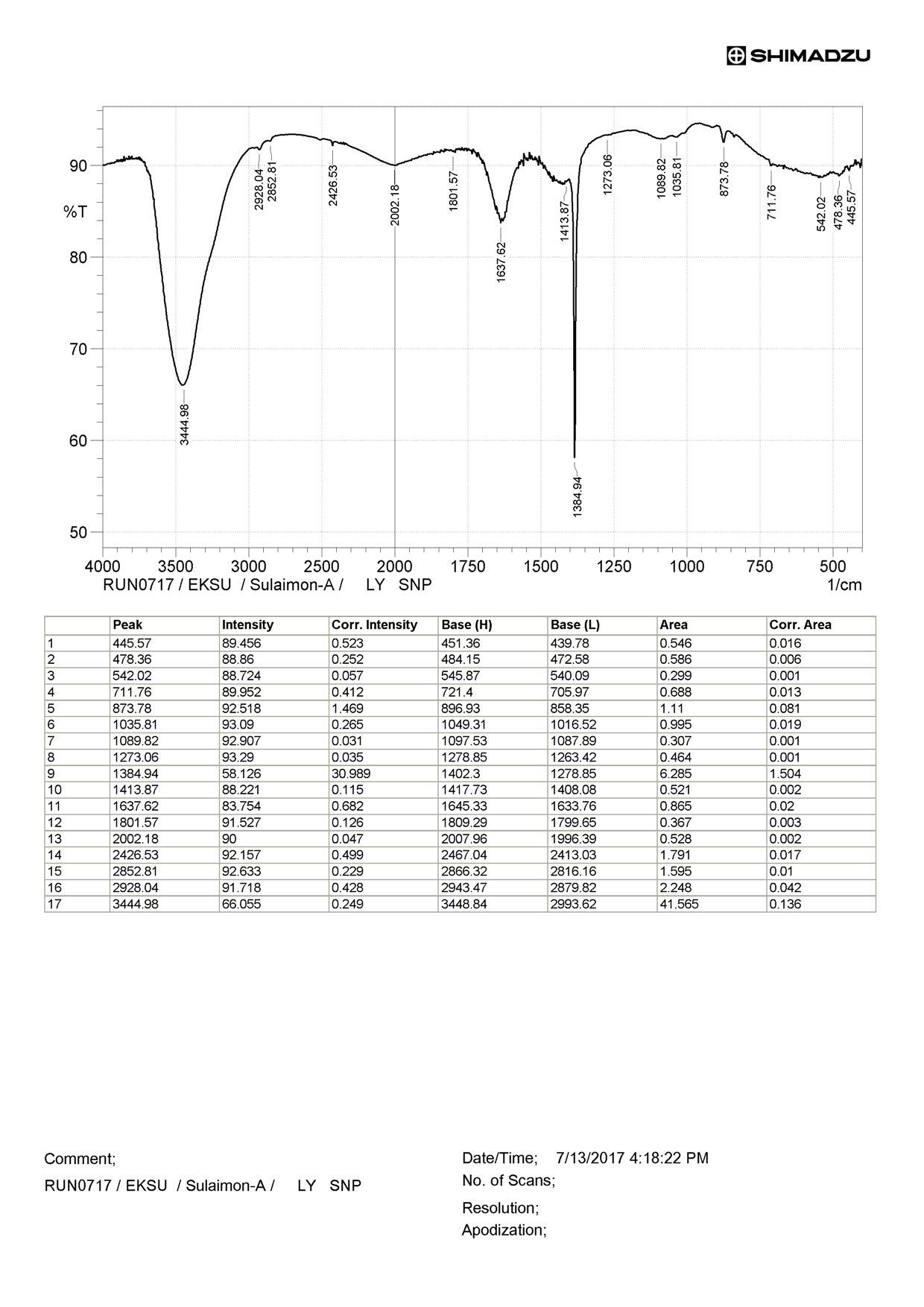
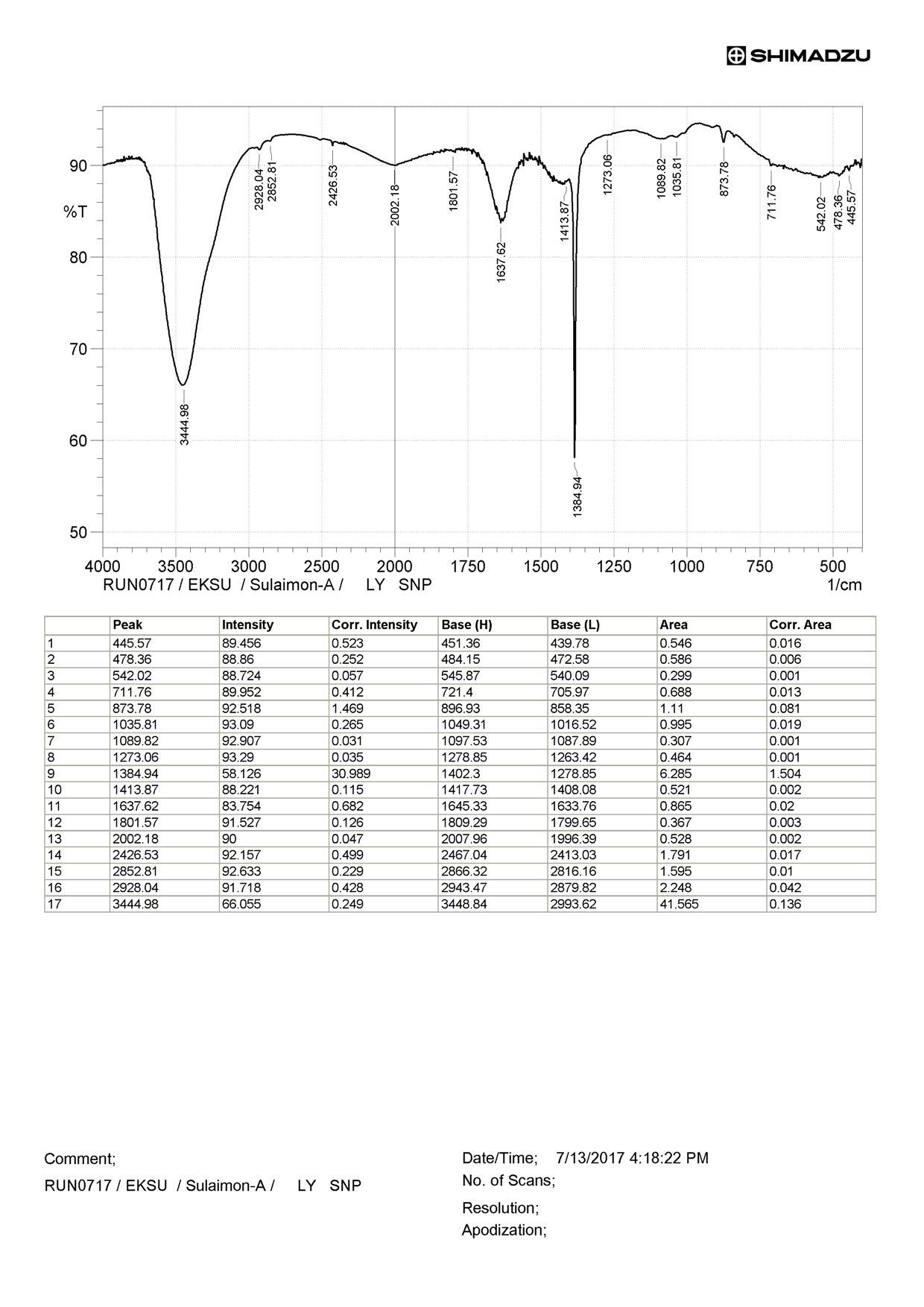
FTIR spectroscopy revealed a very strong broad absorption band width in the range of 873 cm -1 - 3444.98 cm -1 with a maximum at 3444.98 cm-1 and this wide range represents –OH (hydroxyl), -CH (alkane), and –NH (amine) stretching vibrations. The distinct peaks of the FTIR absorption spectrum were found at 3444.98, 2928.04, 2852.81, 2002.18, 1637.62, 1384.94, and 873.78 cm -1 for *L. sphaericus-*AgNPs. The peak bands at 1637.62 and 873.78 cm -1 observed from AgNPs are typical of -N-H bond of amines (1°, 2° amines) (Fig. 2). This has been noticed to be a characteristic of carbon-containing compounds with amino groups bonds of protein confirming that there were lots of proteins containing substance in the sample (Ismail *et al*., 2013). Also, there were about four peak bands ( 1147.68, 1109.11, and 630.74 cm-1) with AgNPs that are also recognized as amine I and amine II which arose due to carbonyl stress and -N-H stretch vibrations in the amide linkages of the protein correspondingly. A relatively sharp and narrow, weak-to-moderate absorption, normally centered around 1635 to 1650 cm -1 (1637.62 cm -1) was indicative of olefinic unsaturation which signifies CO-NH stretching vibration, suggest the presence of carbonyl functionality present in the carboxylate or amide moieties of protein and peptide amines and also an indicative of olefinic unsaturation (John, 2000). The presence of amine groups and the stability of the AgNPs may be discussed by the proteins that may be involved in their synthesis. This indicates that proteins were the capping and stabilization biomolecules in the synthesis of AgNPs which agrees with Shankar *et al*. (2014) and Roozbahani *et al* (2014). The proteins functions as capping agents so also as carbonyl group from the amino acid residues showed stronger ability to bind to metals (Sathyavati, 2010). It has already been reported that the biological molecules (proteins) perform dual functions of formation and stabilization of silver nanoparticles in the aqueous medium (Mallikarjuna, 2011). This report also conform with the work of Saimmai *et al.* (2011) and Jain *et al*. (2012) who reported that sharp peak observed at 1635 to 1650 cm -1 related to the presence of protein and peptide amines. Frequency lowering, accompanied by intensification of the band, is characteristic of conjugation with another double bond structure, such as few aliphatic and aromatic ring (John, 2000). Amine (N-H) bending of primary amines amide I bonds of proteins may arise due to carboxyl stretch and N–H deformation vibrations (Sathyavati, 2010). The FTIR peaks obtained from this work were thesame with those commonly found in the ‘IR spectra’ of the biosurfactant produced by several *Bacillus* species in other researches (Das *et al*., 2008; Oliveira *et al*., 2013).

Functioning groups of alcohol (O-H) stretch and alcoholic related substance such as phenol was also present with peak bands of 3444.98 cm-1 for AgNPs. The peak bands with 2928.04 and 2852.81 cm-1 were also an indicative of the presence of C-H stretch of alkanes for AgNPs and –C≡≡C– of alkynes with a peak band of 983.73 cm-1 for MgNPs. The peak bands at 1413.87 and 873.78 cm -1 observed from AgNPs are also typical of C–H wag (–CH2X) bond of alkyl halides. The weak band of 1400.37 cm-1 falls in the absorption range of 1370-1470 cm-1 resulting from deformation and bending vibrations of alkene and alkane–C-CH2 and –C-CH3 groups (alkyl groups) in aliphatic chains. This observation conformed to Ismail *et al*. (2013) who reported that the weak band at 1403.7 cm-1 is in the absorption range 1370-1470 cm-1. Also, the strong sharp band observed at 1109.2 cm-1 in this work showed the presence of polysaccharide or polysaccharide-like substances as reported by Aparna *et al*. (2012). IR absorption found at 868 cm-1 was due to out of plane C–H bending, a characteristic of sugar and aromatic compounds (Das *et al*., 2008). The adsorption peak of CH (858–934 cm-1) suggested that the polysaccharides are composed of sugar derivatives (Zheng *et al*., 2012). The absorption peak observed at 530.76 cm-1 was also known to be characteristic of sugar derivatives. Therefore, it can be inferred that the macromolecules produced by *Lysinibacillus sphaericus* C3-41 was glycolipopeptide in nature which could be used for the building up and assembly of macromolecules into cellular structures (Zheng *et al*., 2012). The presence of hydrocarbons, halides, alcohol, amines, amides and amino groups showed that the nanoparticles consisted of different functioning groups. Hydroxy or amino groups mainly dominated these regions, and their presence give rise to characteristic band profiles which showed that the particles are composed of carbohydrates and proteins.

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Fig. 1: The UV-Vis absorption spectrum of freshly prepared ‘AgNPs’ using *Lysinibacillus*

*sphaericus* extract



td

Transmittance (%)

**Wave number (cm-1)**

Fig. 2: The FTIR spectrum of the *Lysinibacillus sphaericus* mediated AgNPs

**Effect of varied concentrations of AgNO3 solution on the formation of ‘AgNPs’**

The highest peak of AgNO3 solution (50 mM) for the formation of AgNPs was observed in the dilution ratio of 1:50 (AgNO3; H2O, v/v) with maximum absorbance of 1.68 at 520nm. The synthesis of the large numbers of AgNPs at this dilution showed that the functional group (glycosidic and carbonyl group) present in the bioreactant reacted well with the silver ions present (Fig. 3).

The observation of large synthesis of AgNPs at the dilution ratio of 1:50 (AgNO3; H2O, v/v) was as a result of those functional groups such as (glycosidic and carbonyl group) present in the bioreactant which reacted well with silver ions at this dilution ratio. John (2000) observed that at relatively low-dilution around 1:30 to 1:50 confirms the terminal glycosidic vibration. Metal carbonyls have unique bonding, where the bond order between the carbon and oxygen is between two and three, and where the additional electrons are provided by bonding from the accompanying transition metal atom. The multiple bonded CO group provides an extremely intense absorption and formation of nanoparticles and also the actual position of the bond(s) and the complexity of the bonds being dependent on the structure of the compound John (2000).

Fig. 3: The effect of increasing concentration of AgNO3(aq) solution on the absorbance of *L.*

*sphaericus* C3-41 mediated AgNPs at the wavelength of 520 nm

**Antimicrobial activities of biosynthesized AgNPs on biofilm**

*Lysinibacillus* sp. cell free extract-mediated AgNPs exhibited noticeable antimicrobial efficacy against the wastewater biofilms. After 24h, the mean value of the heterotrophic plate count (HPC) in the wastewater biofilm with no Ag-NP treatment was 2.03 × 108 CFU/cm2 and the HPC in the wastewater biofilm with introduction of Ag-NP treatment was 1.41 × 108 CFU/cm2 respectively (Table 4.9). About 46.4 % reduction was achieved with the incorporation of *Lysinibacillus* sp. mediated AgNPs. There was significant change in the viability of heterotrophic bacteria (*p* > 0.05) with the concentration of (50mM) Ag-NPs applied.

Wastewater microbial biofilm are not functioning in the presence of Ag-NPs. The significant change in the viability of the biofilm bacteria implies that there was reduction in the relative abundance of microbial mass of wastewater biofilms significantly after 24 h when *Lysinibacillus*-mediated silver nanoparticle was used. This is not found to be consistent with previous research ([Davies, 2003](file:///C:\Users\ILEGRAMMS%2082\Downloads\water%20treatment_files\aids%2022_files\NANODIS%20MAIN.htm#B13); [Liu *et al*., 2007](file:///C:\Users\ILEGRAMMS%2082\Downloads\water%20treatment_files\aids%2022_files\NANODIS%20MAIN.htm#B31); [Sheng and Liu, 2011](file:///C:\Users\ILEGRAMMS%2082\Downloads\water%20treatment_files\aids%2022_files\NANODIS%20MAIN.htm#B47)) where that there was no significant change at the categories (different species population) (*r* > 0.99) of silver nanoparticle on biofilms. The variation might be due to the relative thickness of the biofilm (0.5mm) compared to 1.5mm used in the previous research and significant level adopted in this work. However, the inhibition or reduction in the microbial population was always compensated for by a decreased in population of probable pathogenic residual strains. It was clear that silver nanoparticle might have affected the functional gene in the biofilms microbes which led to redundancies in functions and populations ([Liu *et al*., 2007](file:///C:\Users\ILEGRAMMS%2082\Downloads\water%20treatment_files\aids%2022_files\NANODIS%20MAIN.htm#B31)). It was observed that necessary reduction was required to ensure the stability of an ecosystem under disturbance. This is to say that these species are functionally redundant and thus, this could be substituted with minimal impact on the total function of the ecosystems ([Rosenfeld, 2002](file:///C:\Users\ILEGRAMMS%2082\Downloads\water%20treatment_files\aids%2022_files\NANODIS%20MAIN.htm#B45); [Briones and Raskin, 2003](file:///C:\Users\ILEGRAMMS%2082\Downloads\water%20treatment_files\aids%2022_files\NANODIS%20MAIN.htm#B6); [Siripong and Rittmann, 2007](file:///C:\Users\ILEGRAMMS%2082\Downloads\water%20treatment_files\aids%2022_files\NANODIS%20MAIN.htm#B48)).

**Table 1: Bacteria viability in wastewater biofilms under *Lysinibacillus* sp. mediated**

**‘Silver nanoparticle’ (Ag-NP) treatment**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Experimental set up Ag-NP (50µg/ml) Mean± SD ρ value (tcal)** | | | | |
| (CFU)/cm2 | | | | |
| X Y XY/2 | | | | |
| A | 2.15 x 10 8 | 2.03 x 10 8 | 2.09 ±0.042 | 7.46 |
| B | 0.91 x 10 8 | 1.32 x 10 8 | 1.12±0.145 |  |

A: Wastewater biofilms without Ag-NPs,

B: Wastewater biofilms with Ag-NPs,

Percentage reduction: A-B/A x 100%,

X: HPC value of first treatment,

Y: Duplicate treatment of HPC value.

ρ value: (ρ > 0.05, t0.95 = 6.314); when tcal > ttab: significant,tcal ˂ ttab: significant

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