

Optimization of Microbial Synthesized Silver Nanoparticles and its Activities on Selected Bacterial Isolates

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Abstract

The antimicrobial properties of bacterial synthesized silver nanoparticles of *Bacillus subtilis* and *Escherichia coli* origin were tested against five isolates namely: *Pseudomonas* sp, *Bacillus* sp, *Salomonella* sp, *Shigella* sp and *Escherichia coli*. The silver nanoparticles were synthesized from 10 Mm of AgNO₃ and the bacterial culture supernatant. Optimum physiological conditions of bacterial nanoparticles' synthesis were determined using the Box behnken design with three factors and three levels which include pH (6, 7, 8), time (24, 48, 72hrs) and temperature (25, 30, 32°C). The different significance of the physiological factors was determined. The optimal conditions for the synthesis of silver nanoparticles were determined as pH 6.9, Temp. 25°C and Time 72 hrs for *Escherichia colinanoparticles*(ENP) and pH 7.79, Temp. 25°C and Time of 72 hrs for *Bacillus subtilis* nanoparticles (BNP). The antimicrobial activity of the microbial synthesized silver nanoparticles was determined using the kill kinetics and the Kirby bauer well-in-agar diffusion method. ENP had better activity than BNP on *Shigellasp*, while the reverse was the case when tested against *Salmonella* sp. Time kill kinetics shows that BNP and ENP inhibited the growth of *Salmonellasp*, *Pseudomonas* sp, *Bacillus* sp, *Shigella* sp and *E. coli* at 12 hrs and 20 hrs, 28 hrs and 32 hrs, 36 hrs and 32 hrs, 24 hrs and 16 hrs and 32 hrs and 16 hrs respectively. All nanoparticles recorded lower activity than the control drug, Ciprofloxacin.

Keywords: Nanoparticles, Antimicrobial activity, Kill kinetics and Isolates.

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1.0 INTRODUCTION

Massive industrialization and urbanization have predisposed the environment to extensive decay. This has primarily led to the release of harmful gases and chemicals thereby necessitating proactive measures of environmental restoration. It was to this effect that the advent of nanoparticles came to existence. The suitability of nanoparticles for most bioprocesses relates to their unique properties. Biomolecules are subjected to controlled assembly to enhance their Eco-friendliness during their use of metal nanoparticles synthesis (HareKrishna *et al.*, 2009). The production of semiconductor and metal nanoparticles features widely in several research areas with their own attendant advantages. The peculiarity of nanoparticles is relative to distinct size, shape, morphology and particle distribution.

Nanoparticles refers to discrete particles in size of 1nm – 100nm in at least, one possible dimension out of the three. In such discrete sizes, the biological and physicochemical properties of the nanoparticles are quite different from the constituent atoms or the bulk.

Nanoparticles can be synthesized from a wide range of materials of varying chemical states where the most used are carbon, silicates, polymers and silicates. Based on morphology, nanoparticles can be classified as spherical, cylindrical, or conical (Atul *et al.*, 2010).

In addition, nanoparticles have large atomic surface area as well as high surface area. Furthermore, due to the peculiarities regarding the physicochemical properties of nanoparticles, especially it's magnetic, optical, catalytic, antibacterial (Catauro *et al.*, 2005) and optical properties, their new synthetic approaches has become very topical. Nano crystalline silver particles has been applied in areas ultra-sensitive detectors especially in biomolecular studies, therapeutics diagnosis and Microelectronics (Duran *et al.*, 2005). These nanoparticles can either be synthesized by biological method of synthesis involving capping agents to carry out bulk synthesis of nanoparticles that occurs over a very short period of time. However, these chemicals are extremely toxic and less eco-friendly. Thus, the need for more eco-friendly innocuous synthetic approaches for nanoparticles had led to the discovery of biological

approaches for the synthesis of nanoparticles referred to as green technology (Grima *et al.*, 2011). Thus, recent studies on nanoparticles have been centered on the use of biological approaches involving plants and microorganisms for both intracellular and extracellular nanoparticles synthesis (Mukherjee *et al.*, 2001; Grima *et al.*, 2011).

The quest for newer forms of antibiotics is unending as a result of the ever-increasing antibiotic resistance pattern observed in microorganisms. Indeed, some microorganisms are resistant to two or more antibiotics. On the other hand, nanotechnology has come to solve so many life problems and has currently found applications in the fight for antibiotic resistance. Silver nanoparticles are a group of antimicrobial agents that have been synthesized chemically and biologically. Among others, biological sources (plants, animals and microorganisms) are preferred over chemically synthesized particles due to its low human toxicity, ease of raw materials and eco-friendliness. Microbial synthesis of Silver Nanoparticles are preferred over Plant and animal sources because of the absence of food chain competition, vast abundance, rapid growth rate and ease of cultivation. Microbial products (antagonistic) in combination with silver nitrate can be a lifeline and a broad-spectrum antimicrobial. Despite this fact, optimization studies for the production of these important metabolites are limiting.

The aim of this project is the optimization of microbial synthesized silver-nanoparticles and its activities on selected clinical isolates.

2.0 MATERIALS AND METHOD

2.1 Collection of Isolates

The test organisms were obtained from the Department of Microbiology, Federal University of

Technology Owerri and Verified at the Anthony van Leeuwenhoek's Research Centre in Nekede, Imo state. The viability and identities of the isolates were confirmed using routine laboratory methods before being adopted for the research

2.2 Identification of the test organisms

The biochemical screening and identification of the isolates were carried out according to microbiological guidelines and standards (Cheesbrough, 2009; Buchanan and Gibbons, 2000).

2.3 Extracellular biosynthesis of silver nanoparticles using culture supernatant

Extracellular synthesis of silver nanoparticles were carried out as described by Shahverdian *et al.*, (2007) using *Bacillus subtilis* and *Escherichia coli*. The isolated colonies were sub-cultured in nutrient broth and incubated for 24 h at 37°C. The broth was centrifuged at 8000 rpm for 10 min to collect the culture supernatant. 10mM silver nitrate solution was prepared in double distilled water. 200 mL of aqueous solution of 1mM silver nitrate was treated with 100 mL of culture supernatant in a 500 mL Erlenmeyer flask. The whole sample kept in the shaker at 150 rpm and maintained in dark condition. The reduction of silver nitrate was monitored by visible color change of the solution at 400nm.

2.4 Optimization study

The Box-Behnken design was adopted for the optimization of the production of silver nanoparticles in a 3 X 3 design, that is, three factors in three levels using Minitab (Design of Experiment). Factors include Temperature (25, 30 and 35 degrees Celsius), pH(6, 7 and 8), and Time of incubation (24, 48 and 72 Hours). A total of non-randomized 15 runs was obtained as shown in the design table (Table 1).

Table 1: Design of experiment for Non randomized 15 runs in Box behnken design

Std Order	Run Order	Pt Type	Blocks	pH	Temperature	Time
1	1	2	1	6	25	48
2	2	2	1	8	25	48
3	3	2	1	6	35	48
4	4	2	1	8	35	48
5	5	2	1	6	30	72
6	6	2	1	8	30	72
7	7	2	1	6	30	48
8	8	2	1	8	30	48
9	9	2	1	7	25	72
10	10	2	1	7	35	72
11	11	2	1	7	25	72
12	12	2	1	7	35	72
13	13	0	1	7	30	48
14	14	0	1	7	30	48
15	15	0	1	7	30	48

2.5 UV-Vis Spectroscopy

Synthesized AgNPs were scanned by UV-Vis spectrophotometer at the wavelength of 200-800 nm on Labman UV-Vis spectrophotometer. It is basically done for monitoring the AgNPs as UV-Vis spectroscopy is used for the characterization of colloidal particles. Noble metal particles possess strong surface plasmon resonance (SPR) absorption in the visible region and are highly sensitive to the surface modification.

2.6 Antimicrobial susceptibility assay using Kirby Bauer Techniques

This was done in line with the protocol described by Cheesbrough (2009) and Mohamed *et al.* (2010). In vitro antimicrobial activity was screened by using Mueller Hinton Agar (MHA) obtained from Himedia (Mumbai). The MHA plates were prepared by pouring 15 ml of molten media into sterile petri dishes. The plates were allowed to solidify for 5 min and 0.1 % inoculum suspension was swabbed uniformly and allowed to dry for 5 min. A sterile cork borer was used to create uniform wells on the surface of the inoculated plates with a diameter of 6mm. Concentrations of the antimicrobial agents, *Pseudomonas* sp, *Bacillus* sp, *Salmonella* sp, *Shigella* sp and *E.coli*, were added into the different wells. The plates were kept for incubation at 35-37°C for 16–18 hours. Negative control was prepared using respective solvent. Ciprofloxacin (5µg/disc) was used as positive control. At the end of incubation, zones formed around the disc were measured with transparent ruler in millimeter.

2.7 Antimicrobial activities of AgNPs using Spectrophotometer

An overnight broth culture of the test organism was maintained in Nutrient broth and standardized to 0.5

Macfarland standard. Freshly prepared nutrient media were inoculated with 0.1 ml of the Test isolate each. 1ml of the AgNPs was added to the test set up and 1ml of sterile water was added as control set up. A third set up contained only the nutrient broth and the Medium but no isolate. The experiment was done in triplicates. The test tubes were incubated at 37°C for 18 hours and readings was obtained at 2 hours intervals using a UV-Vis Spectrophotometer at 600nm. The growth curve of the isolates was obtained and the specific growth rate of each isolate was determined.

2.8 Statistical Analysis

Results obtained from this work was subjected to statistical Analysis at $P<0.05$ using the MINITAB 17 Software to obtain response surface plots, Statistical Significance of Factors as well as interval plots of differences and deviations across the Mean.

3.0 RESULTS AND DISCUSSION

3.1 Results

The plot showing the main effects of the synthesis of *B. subtilis* silver nanoparticles (BNP) was presented in Fig. 1 while Fig. 2 shows the interactions of the optimization factors. The considered optimization factors were time, pH, and temperature. The synthesis of BNP showed a sharp decline within the first 40 hours but attained steady optimization between 40 – 60 mins. An exponential increase in BNP synthesis was observed between pH 6 – 7 with a slight decline observed between pH 7 – 8. Increase in temperature from 25-30°C resulted in a decrease in the production of BNP while the production of BNP increased over 30-35°C.

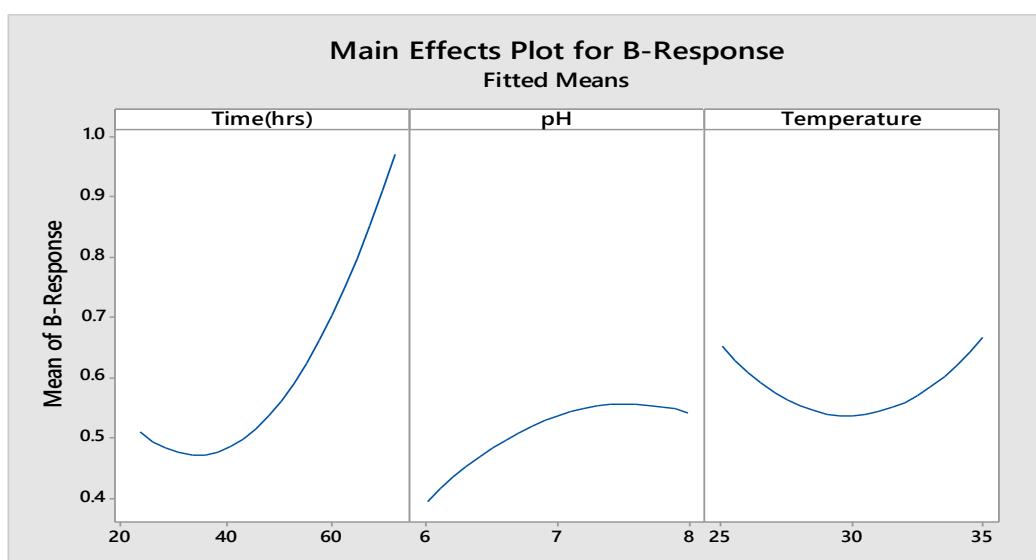


Figure 1: Main effect plot for the production of BNP

Fig. 2 shows the interactions of the optimization factors. At constant time, increase in pH from 7 to 8 produced similitude of BNP. At pH 6 no interaction with

the activity of the other pH ranges was observed. Between 40 - 50°C the BNP at pH 7 and 8 synchronized. At a temperature of 30 degrees, the production of BNP

had no interaction with other effects at other temperatures. At constant pH, that is pH*Temperature Interaction, the temperatures of 25 and 35°C had an

interaction which resulted to an overall decrease in BNP production.

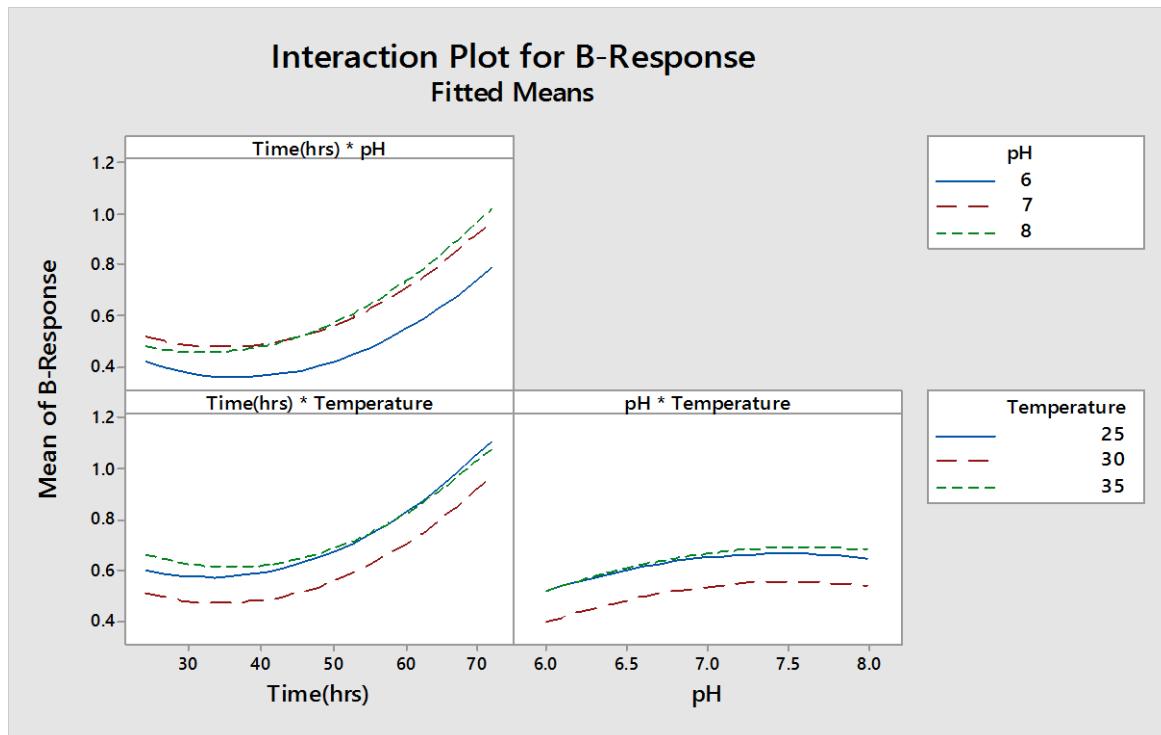


Figure 2: Interaction plots for BNP production

The surface plots of the interactions among the optimization factors were presented in Fig. 3-5. At

constant temperature, BNP synthesis increased with time and initial pH but declined at pH of 8 (Fig. 3).

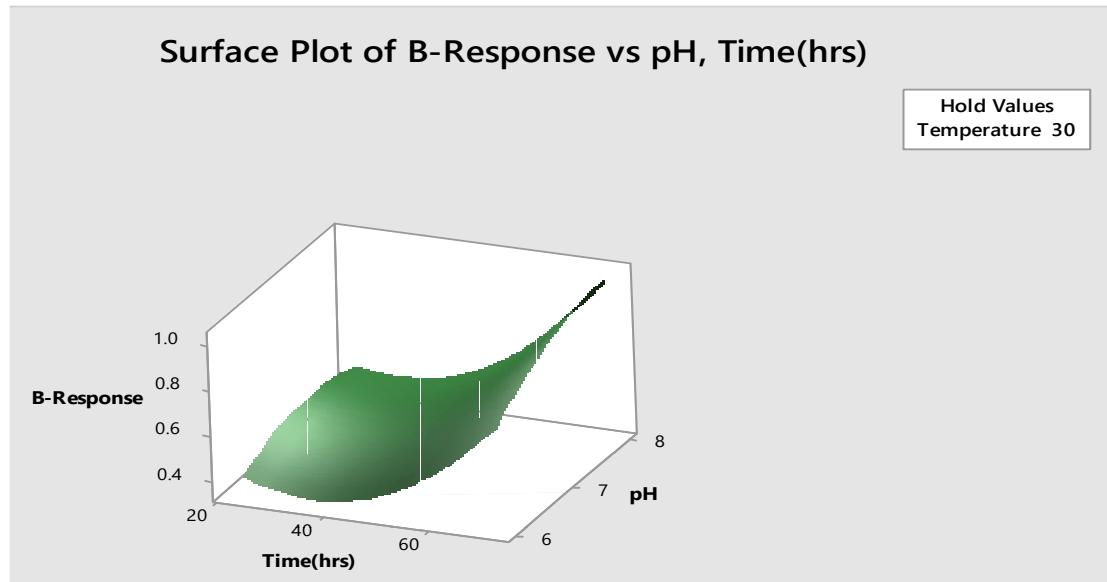


Figure 3: Response Surface Plot of B-Response Vs pH, Time (hrs)

The result in Fig. 4 shows that at constant pH, BNP synthesis increased with time but showed a decline

with increased temperature but attained optimal synthesis at 35°C.

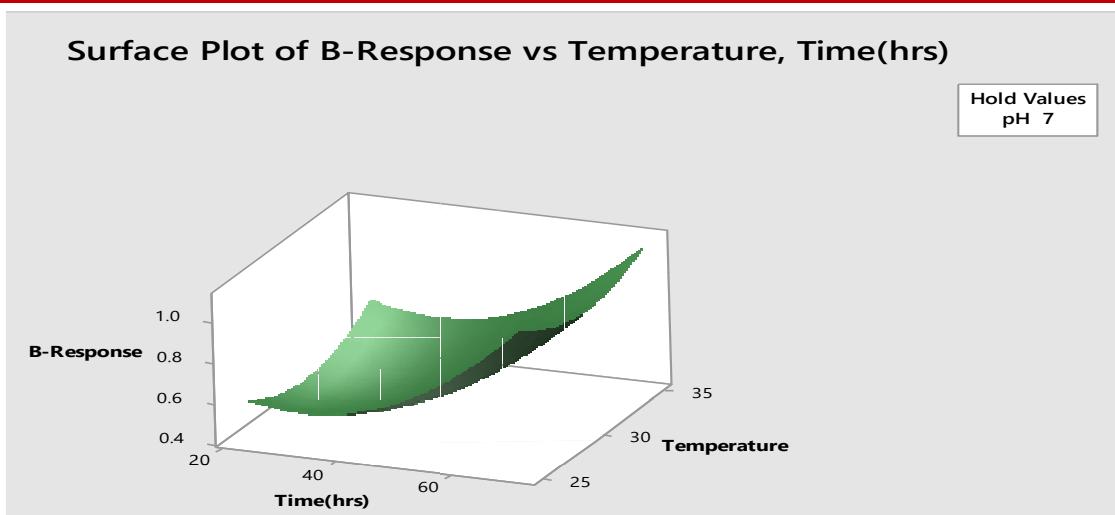


Figure 4: Response Surface Plot of B-Response vs. Temperature, Time(hrs)

Also, at constant time, BNP synthesis was optimized at 35°C while variations in pH showed increased BNP synthesis at elevated pH ≤ 8 (Fig. 5).

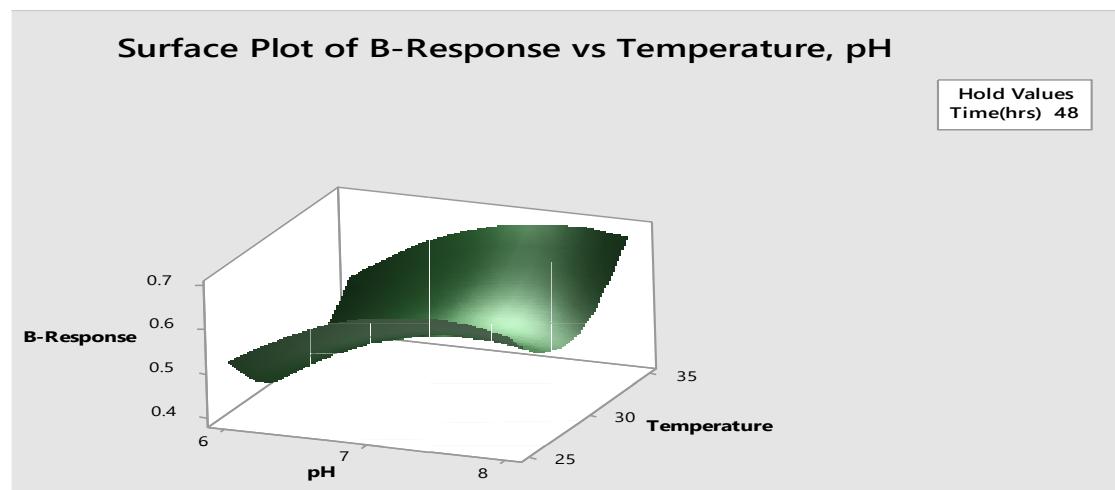


Figure 5: Response Surface Plot of B-Response vs Temperature, pH

Fig. 6 shows the optimization plots for the production of BNP. The results show that the optimum conditions for the production of BNP are pH 7.8,

temperature of 25°C and a time of 72 hours. At these conditions the maximum yield that will be achieved will have a response of 1.1514.

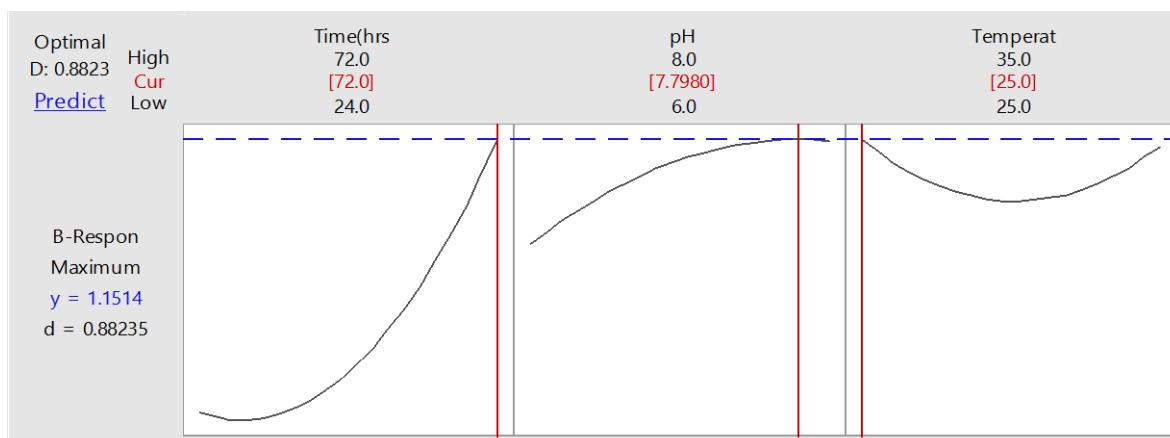


Figure 6: Optimization Plots of the production of BNP

Fig. 7 shows the main effects plots for the production of *Escherichia coli* synthesized silver nanoparticle (ENP). The result shows an initial lag in ENP synthesis with temperature variations. ENP

synthesis maintained an exponential increase up to pH 7 but decreased with $7 > \text{pH} \leq 8$. The results showed that the increase in temperature resulted in a gradual decrease in production of ENP as indicated in the negative slope.

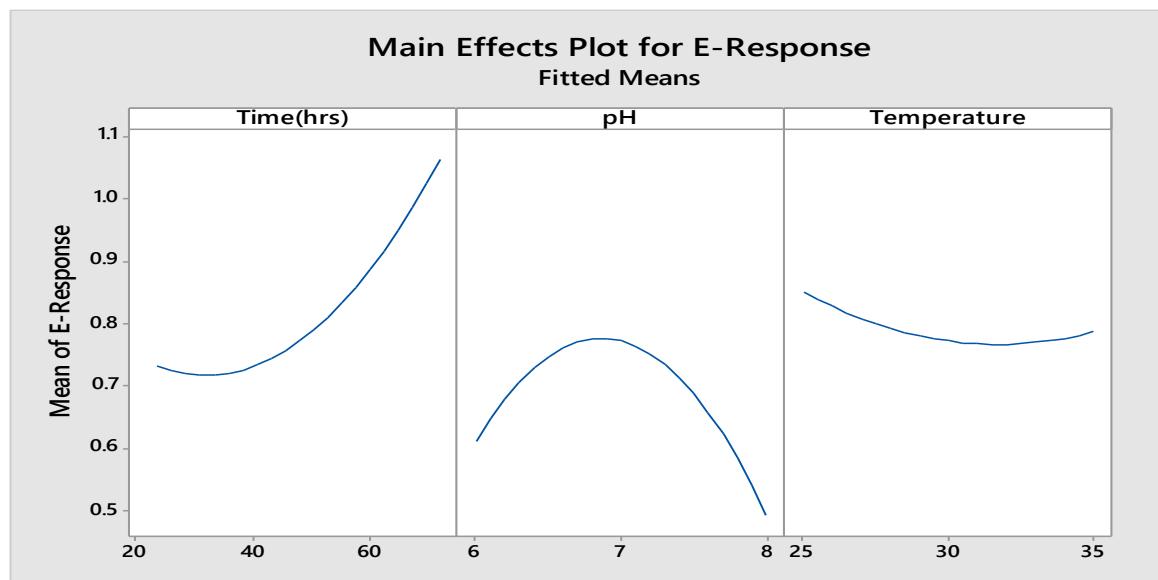


Figure 7: Main effect plots for ENP production

In Fig. 8, the results of the interaction plots of the production of ENP shows that at constant time, a similitude of ENP synthesis was achieved at pH 6 and 8. On the other hand, temperature variability at constant time showed dissimilar ENP response regardless of

variations among three choice temperatures. These interactions produced considerable increase in the production of ENP whereas temperature-temperature interaction resulted in a considerable decrease in the production of ENP.

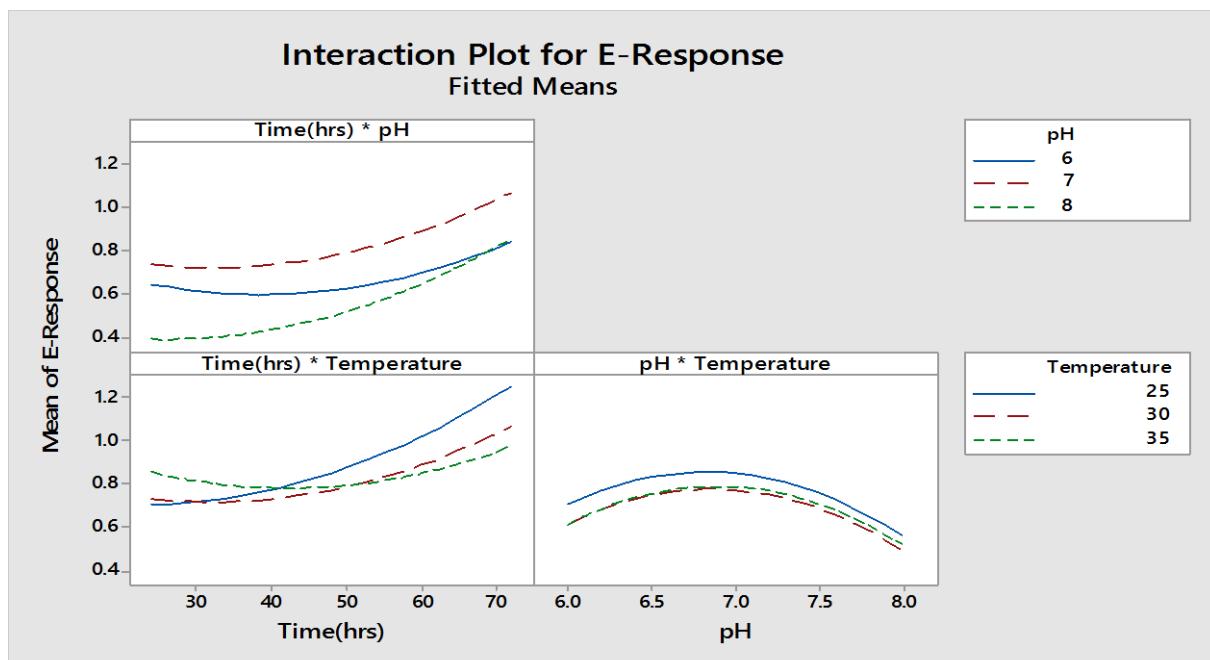


Figure 8: Interaction plots for ENP production

Fig. 9 shows the response surface plot of the production of ENP at constant holding Temperature. A considerable increase in the production of ENP as the

time increased was observed. The pH initially increased the production of the ENP followed by a decrease in production as pH approached 8.

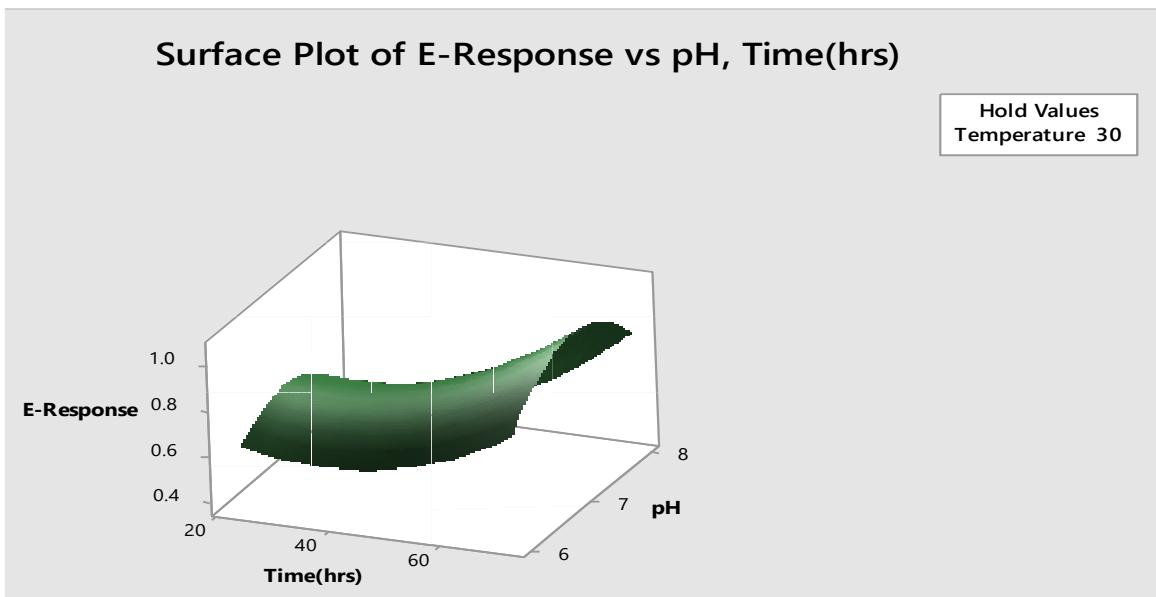
**Figure 9: Surface Plot of E-Response vs pH, Time(hrs)**

Fig. 10 shows the response surface plot for the production of ENP at constant holding pH. A considerable increase in production of the ENP as the

time increased was observed with a slight negative slope found for the production of ENP as temperature increased.

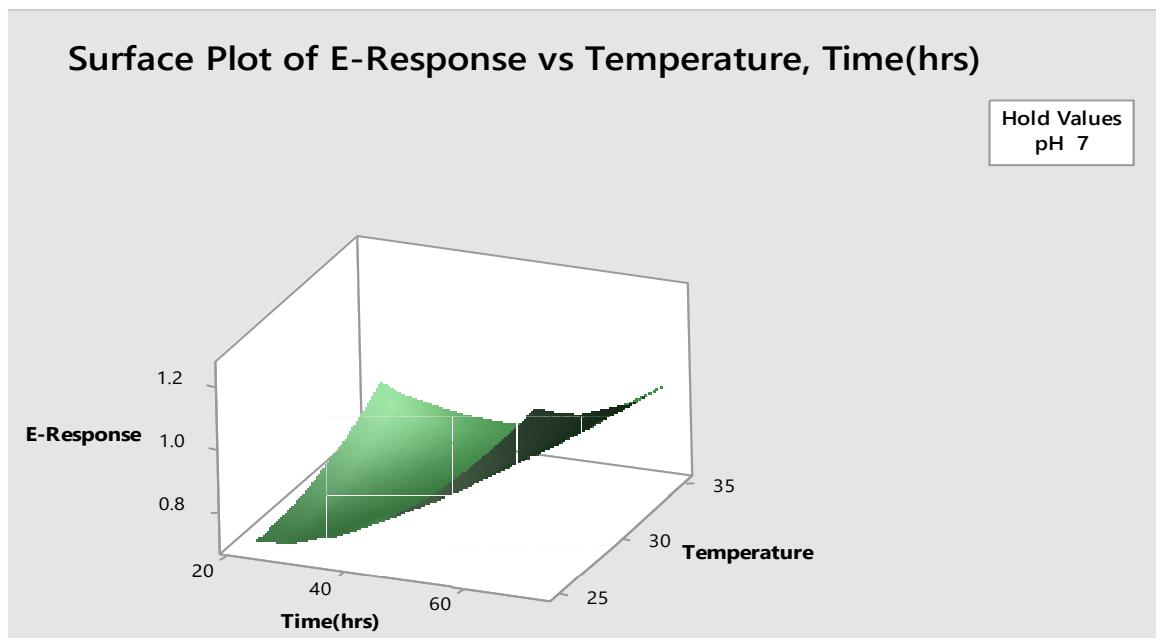
**Figure 10: Surface Plot of E-Response vs Temperature, Time(hrs)**

Fig. 11 shows the response surface plot for the production of ENP at holding time value. The production of the ENP increased initially with increased pH which later decreased as the pH approached 8. Temperature

variations produced a slight decline in ENP production followed by a slight increase as the temperature approached 35°C.

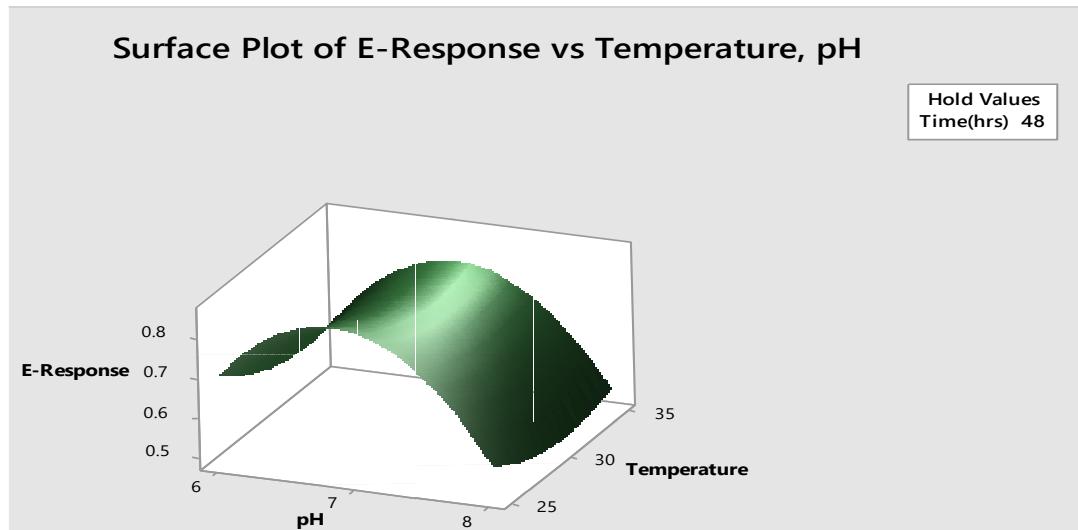


Figure 11: Surface Plot of E-Response vs Temperature, pH

Fig. 12 shows the optimization plots for the production of ENP. The result shows that the optimal conditions for the production of ENP were pH 6.99,

Temperature 25°C and time of 72 hours. At these conditions, the *Escherichia coli* will have a yield of 1.2501.

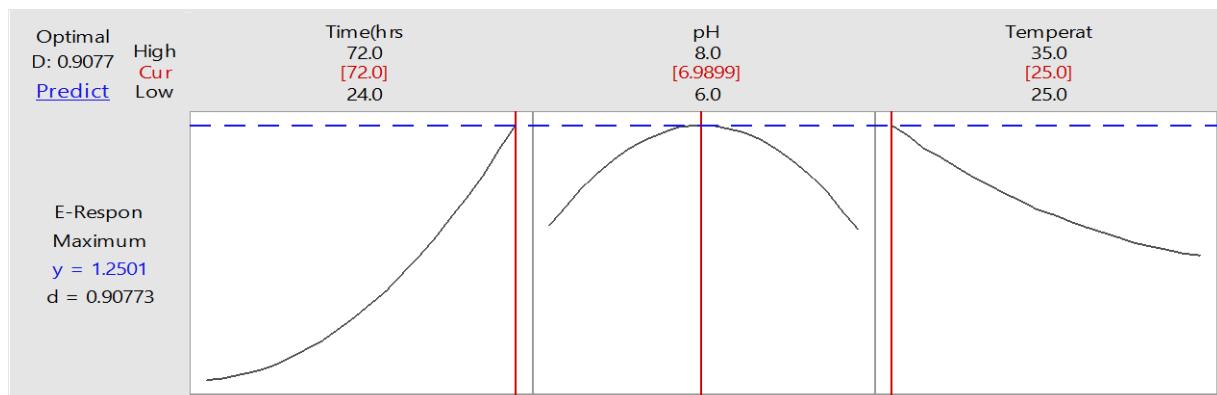
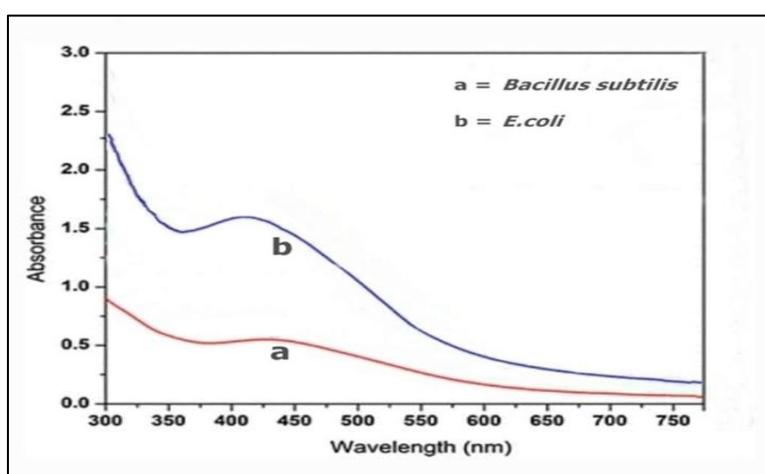


Figure 12: Optimization plot for ENP production

3.1.1 UV-Vis Spectroscopy

Fig. 13 shows that the strong UV-Visible spectra of silver nanoparticles was a broad peak and

existing between 400nm and 450nm. It centred at 445nm for *B. subtilis* and at 440nm for *E. coli*.

Fig. 13: UV-Vis Spectra of the produced AgNPs by *B. Subtilis* and *E. coli*.

3.1.2 Antimicrobial activity of ENP and BNP

Fig. 14 shows the antimicrobial property of ENP and BNP using the Kirby Bauer method. The Nanoparticles were tested against *Pseudomonas* sp, *Salmonella* sp, *Bacillus* sp, *Shigella* sp and *Escherichia coli*. BNP had the highest activity against *Salmonella* sp with a diameter of zone of inhibition of

14mm and the lowest activity against *Bacillus* sp with a diameter of zone of inhibition of 8mm. On the other hand, ENP had the highest activity against *Shigella* sp with a diameter of zone of inhibition of 14mm and the lowest activity against *E. coli* with a diameter of zone of inhibition of 7mm. Both ENP and BNP had lower activity than the control drug, Ciprofloxacin.

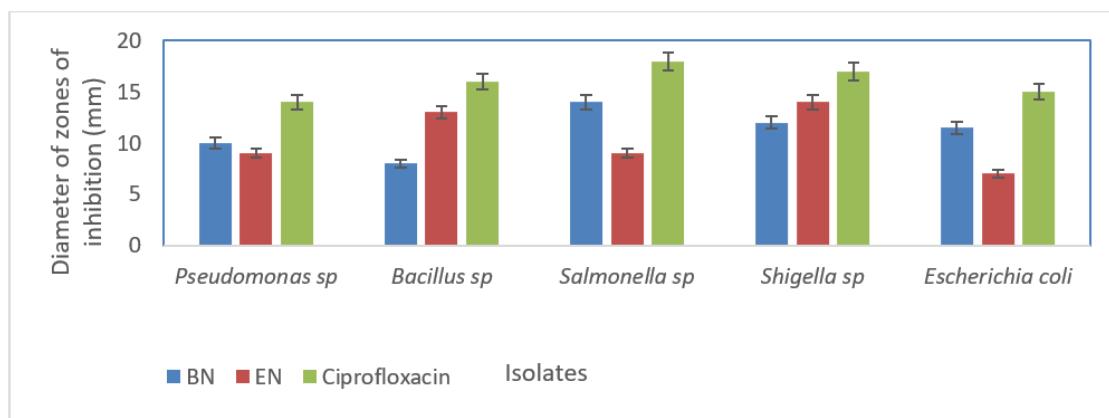


Figure 14: Antimicrobial activity of ENP and BNP using Kirby Bauer Method

BN= *Bacillus subtilis* synthesized Nanoparticle

EN= *Escherichia coli* synthesized Nanoparticle

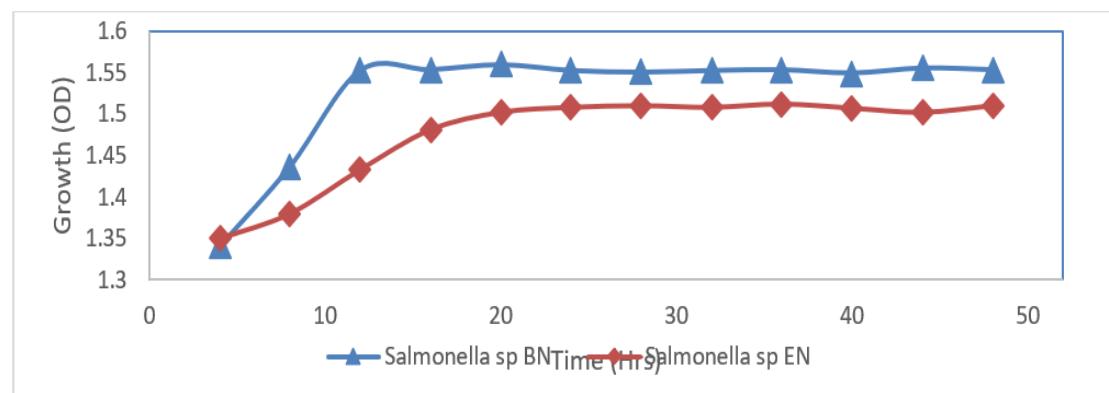


Figure 15: Time kill kinetics for the inhibition of *Salmonella* sp by BNP and ENP

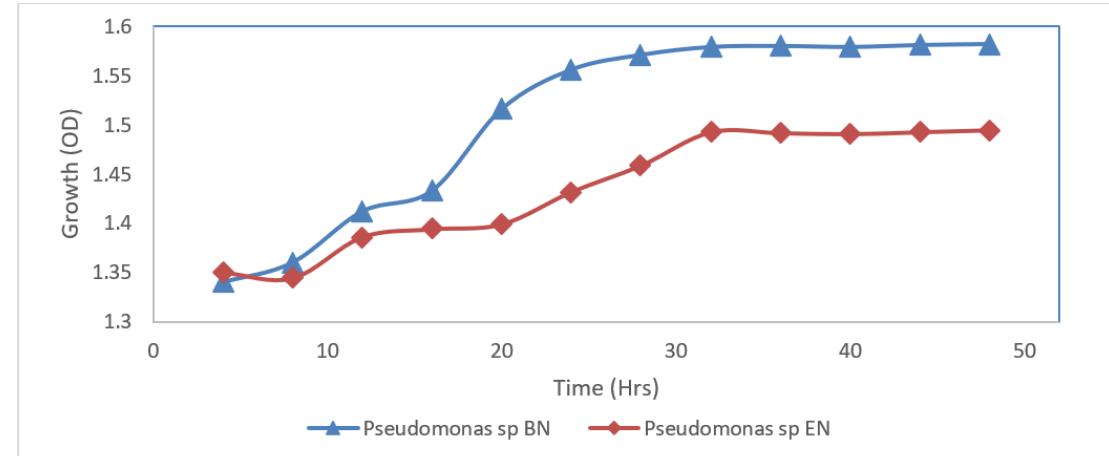
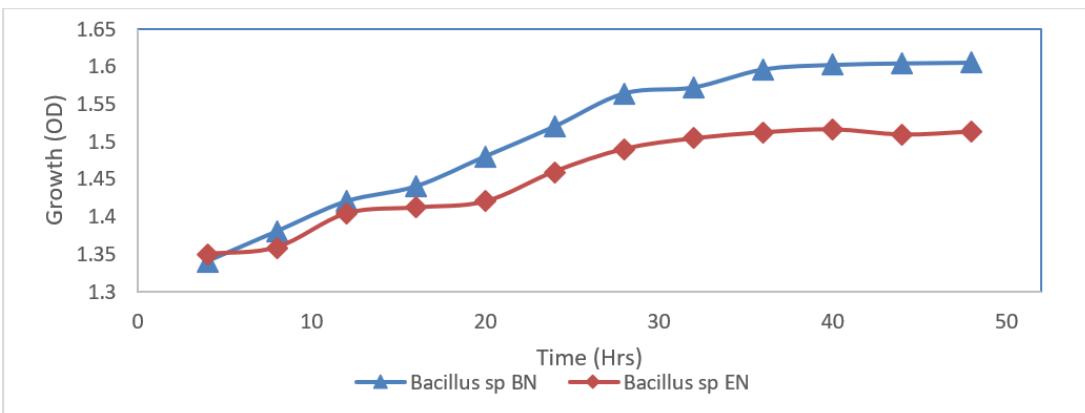
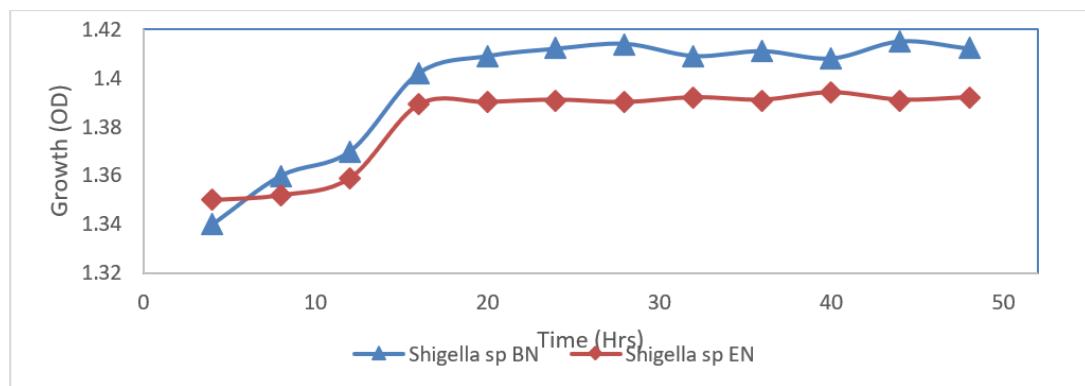
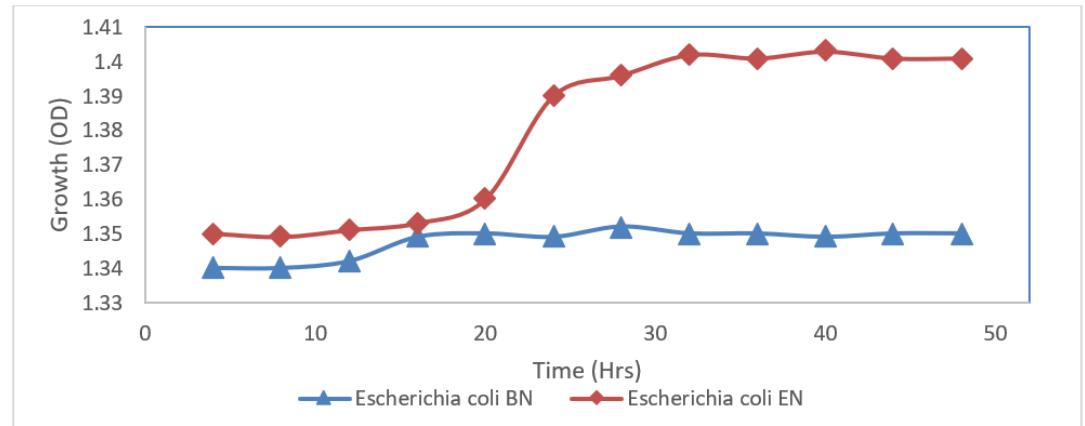


Figure 16: Time kill kinetics for the inhibition of *Pseudomonas* sp by BNP and ENP

Figure 17: Time kill kinetics for the inhibition of *Bacillus* sp by BNP and ENPFigure 18: Time kill kinetics for the inhibition of *Shigella* sp by BNP and ENPFigure 19: Time kill kinetics for the inhibition of *E. coli* by BNP and ENP

4.2 DISCUSSION

This research seeks to identify optimal conditions that are required for the production of silver-based nanoparticles from *Bacillus subtilis* and *Escherichia coli*. The Ag Nanoparticles were produced from 10mM concentration which is quite small as required. Particle size plays an influential role in the antibacterial properties of silver nanoparticles, with smaller particles exhibiting improved activities (Burt *et al.*, 2004; Pal *et al.*, 2007). However, it must be noted that the smaller nanoparticles have a tendency to agglomerate in a media with high electrolyte content

resulting in a loss of antibacterial effectiveness (Burt *et al.*, 2004).

The optimum conditions for the production of ENP (pH 6.99, Temperature 25°C and Time of 72 hours) had a predicted yield which was higher than that produced by *B. subtilis* (BNP) at its predicted optimum conditions (pH 7.8, Temperature of 25 degree Celsius and a time of 72 hours). The experiment also demonstrated that among other parameters measured, Time was a factor of great significance. This is because it takes a lot of time for the molecules of the Microbial extracts to conjugate with the molecules of Silver

Nitrate. As a result, the longer the time of incubation, the more probable yield is obtained.

The antimicrobial activities of ENP and BNP demonstrates that the nanoparticles had antimicrobial properties. The *E. coli* silver nanoparticles has been reported to have antibacterial activity against *Bacillus subtilis*, *Klebsiella pneumonia*, *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. (Ramya and Silvia., 2012). On the other hand, Marambio-Jones (2010) has proposed that the biologically synthesized nanoparticles from *Bacillus subtilis* have a good antibacterial activity against various pathogens organisms such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella typhi*, *Klebsiella pneumonia* and *Vibrio cholera* using agar well diffusion method. In his research, the synthesized silver nanoparticles showed highest antibacterial activity against all the pathogens except *Vibro cholera*.

Nanoparticles show antimicrobial activity against a range of bacteria. Among the various nanoparticles, silver nanoparticles exhibit broad inhibitory behavior towards nearly 650 species of microbes, and more importantly against antibiotic resistant bacterial strains (Jeonget *et al.*, 2005; Raiet *et al.*, 2012; Marambio-Jones, 2010). In one of the findings, it was shown that silver nanoparticles showed superior antibacterial activity against *E. coli* and *S. aureus* when compared to gold nanoparticles (Amin *et al.*, 2009). Gram positive bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*) and Gram-negative bacteria (*Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Proteus vulgaris*) has already been tested and NPs show great activity against them. The silver nanoparticles showed high activity against *Salmonella typhi* followed by *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *E. coli* and least activity was against *Klebsiella pneumonia* (Kim *et al.*, 2007).

It has been reported that medically, small concentration of AgNPs is harmless for human cell but deadly for majority of viruses and bacteria. AgNPs reduce toxicity of cell without affecting the antibacterial efficacy (Karimzadeh and Mansour, 2010). Nanoparticles show high antibacterial activity because of their finely honed surface and they are small enough to penetrate through the membrane of cell to disturb the intracellular processes. AgNPs show higher antibacterial effect because of their formation of free radicals on its surface (Kim *et al.*, 2007). The Mechanisms of these antimicrobial activities has been suggested by Zewde. (2016) ranging from (a) formation of pits in cell wall, (b) disruption of cell membrane via free radical formation by nanoparticles and inhibition of respiratory enzymes by free oxygen species produced by silver nanoparticles and silver ion, and (c) binding of silver nanoparticles with soft basic residues within the cell, e.g., DNA. Depending upon their size, shape, and composition, they are capable

of penetrating the cell membrane and influencing the intracellular processes.

However, BNP had an average higher activity than the ENP as evaluated using the Tukey's test. This could be because most of the isolates tested were gram negative bacteria and had similar receptors on the bacterial cell wall or cell membrane. On the other hand, BNP and ENP had reduced activity against *Bacillus subtilis* and *E. coli* respectively. The reasons for this activity is unknown. The activity of silver nanoparticles are broad spectrum.

5.0 CONCLUSION AND RECOMMENDATION

5.1. Conclusion

Biological synthesis of nanoparticles is a novel and effective approach of nanoparticles synthesis. It that, the microorganisms or plant biomass involve as a reducing agent which are environmentally benign and non-toxic and has significant advantages over other processes since it takes place at relatively ambient temperature and pressure.

Biological synthesis of nanoparticles is thus effective and economic approaches which even control the size and shape of the nanoparticles (Narayanan and Sakthivel, 2010).

It is important to quantify specific microbial metabolites following an initial separation using HPLC and characterization using GC-MS so that the activity of microbial metabolites forming Silver Based Nanoparticles can be attributed to specific Metabolites. Also, Surface engineering and modification of the silver-based Nanoparticle can be adopted for improved antimicrobial activity.

5.2. Recommendation

Other bacterial isolates can also be used for the production of Silver Nanoparticles under optimized conditions. This will create a wider range of newer antimicrobials since microorganisms are of vast abundance.

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Conflict of Interest: The authors declared that there is no conflict of interest regarding the publication of this manuscript.

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