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Effect of Optimization Factors on the Production of *Bacillus subtilis* and *Escherichia coli* Synthesized Silver Nanoparticles

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Authors' contributions

This work was carried out in collaboration among all authors. All authors contributed to the design of the study. Author CCN performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed the literature searches. All authors managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

The recent discovery of silver nanoparticles and their production from *Bacillus subtilis* and *Escherichia coli* have enhanced optimization attempts. Extracellular biosynthesis of silver nanoparticles using the *Bacillus subtilis* and *Escherichia coli* cultured supernatants was done according to standard procedures. Optimization of the production of silver nanoparticles was done in a 3 X 3 (three factors) design involving temperature (25, 30 and 35 degrees), pH (6, 7 and 8), and time of incubation (24, 48 and 72 Hours) in a total of 15 non-randomized runs. The result showed a sharp decline in the synthesis of *B. subtilis* silver nanoparticles (BNP) 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. An increase in temperature from 25-30^{0}C resulted in a decrease in the production of BNP while the production of BNP increased over 30-35^{0}C. An initial lag in *Escherichia coli* synthesized silver nanoparticle (ENP) synthesis was observed with temperature variations. ENP synthesis maintained an exponential increase up to pH 7 but decreased with 7>pH≤8. The results showed

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that the increase in temperature resulted in a gradual decrease in production of ENP producing a negative slope. Therefore, the variations in optimization factors of silver nanoparticles produced from both *B. subtilis* and *E. coli* led to improved production.

Keywords: Silver nanoparticles; E. coli; B. subtilis; optimization.

1. INTRODUCTION

The term "nanoparticles" is used to describe a particle with size in the range of 1 nm 100 nm, at least in one of the three possible dimensions. In this size range, the physical, chemical and biological properties of the nanoparticles changes in fundamental ways from the properties of both individual atoms/molecules and of the corresponding bulk materials. Nanoparticles can be made of materials of diverse chemical nature, the most common being metals, metal oxides, silicates, non-oxide ceramics, polymers, organics, carbon and biomolecules. Nanoparticles exist in several different morphologies such as spheres, cylinders, platelets, tubes etc. Generally the nanoparticles are designed with surface modifications tailored to meet the needs of specific applications they are going to be used for. The unique properties of silver nanoparticles such as its electromagnetic characteristics, its shape and size, make them central to current research interests [1,2]. Not with standing that nanoparticles pass through several physical and chemical production stabilization processes [3], its properties support their incorporation as base materials for the production of electronic materials, antimicrobial agents, cryogenic superconductors, fibers and cosmetic products. Among these processes, the most popular include using inorganic and organic reducing agents, radiolysis, physicochemical and electrochemical techniques. The production of nanoparticles is generally referred to as green chemistry because of the environmental friendliness of its production techniques which yields silver nanoparticles with the desired size and morphology. In alternate to physicochemical methods of synthesizing nanoparticles, several biological methods have also been widely used [4]. This process mostly employs the use of vitamins, enzymes, polysaccharides, amino acids and proteins in a widely regarded environmentally friendly complex process. In addition, several reports have shown successful microbial synthesis of nanoparticles. *Pseudomonas stutzeri* AG259 strain isolated from silver mine, was the first reported bacteria with nanoparticle synthesizing capability [4]. With further studies involving other microbes with silver nanoparticle synthesizing potentials, their survival mechanisms have been described to be mainly by modifying metal solubility and toxicity by oxide reduction processes, metal precipitation, bioaccumulation, biosorption, or extracellular complexation [5]. Other factors leading to the improved biological synthesis of silver nanoparticles, have been considered. Hasnain et al. [6] reported that optimization techniques for silver nanoparticles mainly focus on the improvement of physicochemical properties, even with the use of biological method of production. It, therefore, becomes imperative to consider factors that can optimize the biosynthesis of silver nanoparticles. Thus, this study was carried out to investigate the effect of variations in optimization factors such as pH and temperature, on the productions of silver nanoparticles from *Bacillus subtilis* and *Escherichia coli*.

2. MATERIALS AND METHODS

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 Leuwenhoek'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 [7].

2.3 Extracellular Biosynthesis of Silver Nanoparticles using Culture Supernatant

Extracellular synthesis of silver nanoparticles was carried out as described by Shahverdi et al. [8] 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. 10 mm 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 400 nm.

2.4 Optimization Study

The Bohxbenken 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), pH (6, 7 and 8), and Time of incubation (24, 48 and 72 Hours). A total of non-randomized 15 runs were obtained as shown in the design table. (Table 1)

2.5 UV Analysis

Synthesized AgNPs were scanned by UV-Vis spectrophotometer at the wavelength of 190-800 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 surface modification.

3. 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 ^{0}C resulted in a decrease in the production of BNP while the production of BNP increased over 30-35 ^{0}C.

In Fig. 2 at constant time, an 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 ^{0}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 ^{0}C had an interaction that resulted to an overall decrease in BNP production.

The surface plots of the interactions among the optimization factors were presented in Figs. 3 – 5. At constant temperature, BNP synthesis increased with time and initial pH but declined at pH of 8 (Fig. 3). 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^{0}C. Also, at constant time, BNP synthesis was optimized at 35^{0}C while variations in pH showed increased BNP synthesis at elevated pH ≤8.

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^{0}C and a time of 72 hours. At these conditions the maximum yield that will be achieved will have a response of 1.1514.

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>pH≤8. The results showed that the increase in temperature resulted in a gradual decrease in the production of ENP as indicated in the negative slope.

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 the constant time showed dissimilar ENP response regardless of variations among three choice temperatures. This interaction produced a considerable increase in the production of ENP whereas temperature-temperature interaction resulted in a considerable decrease in the production of ENP.

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.

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.

Table 1. Design of experiment for nonrandomized 15 runs in box behnken design

Std order	Run order	Pt type	Blocks	pH	Temperature (°C)	Time (Hr)
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

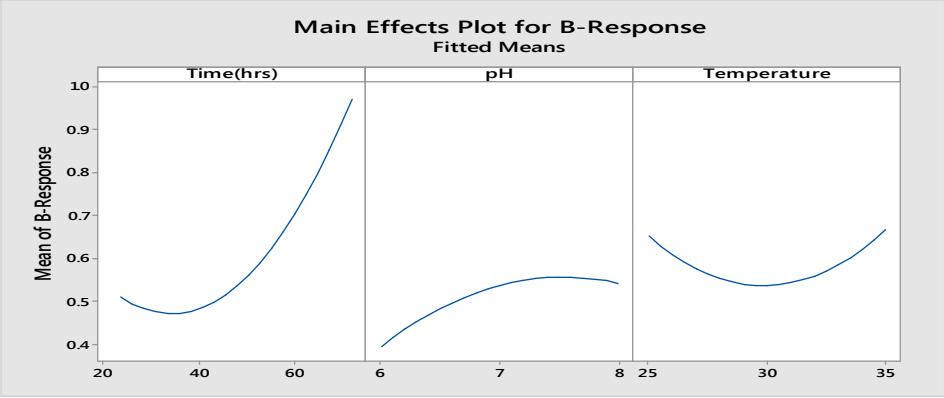

Fig. 1. Main effect plot for the production of BNP

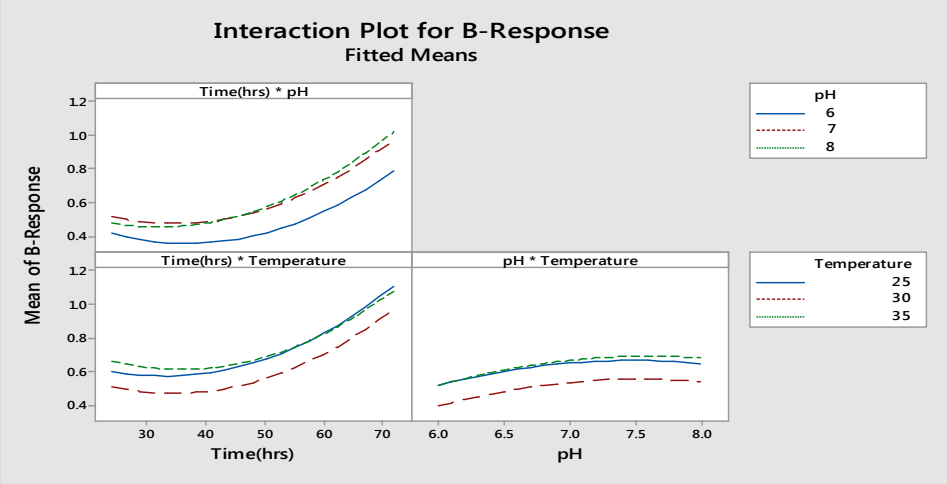

Fig. 2. Interaction plots for BNP production

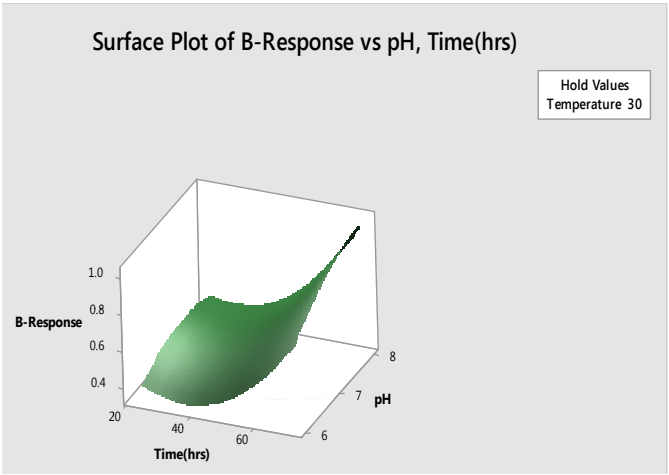

Fig. 3. Response surface plot of B-response vs pH, time (hrs)

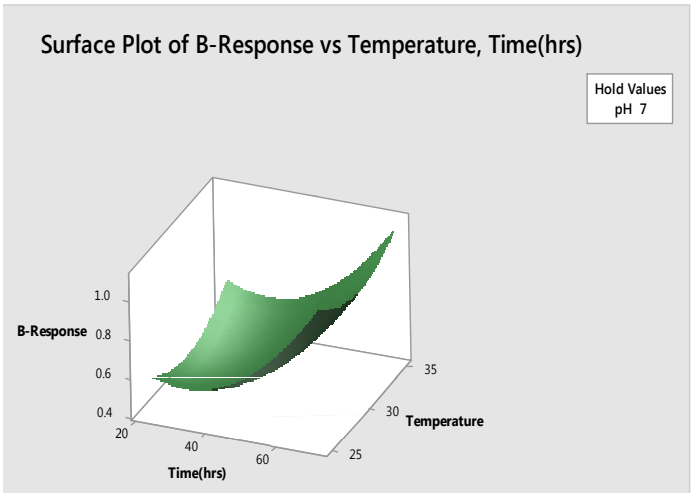

Fig. 4. Response surface plot of B-response vs. temperature, time(hrs)

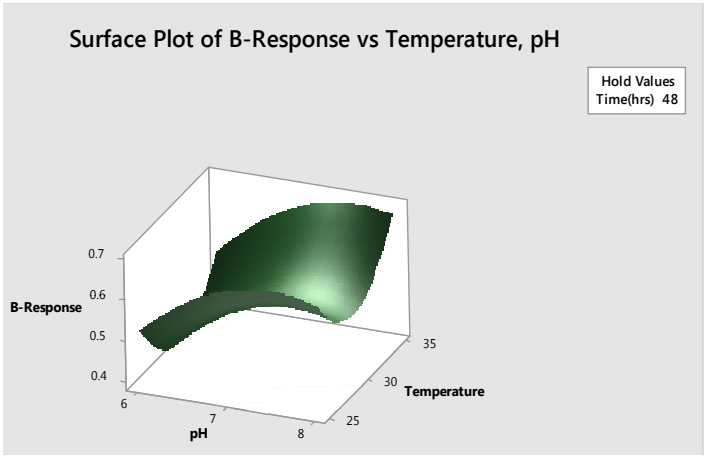

Fig. 5. Response surface plot of B-response vs temperature, pH

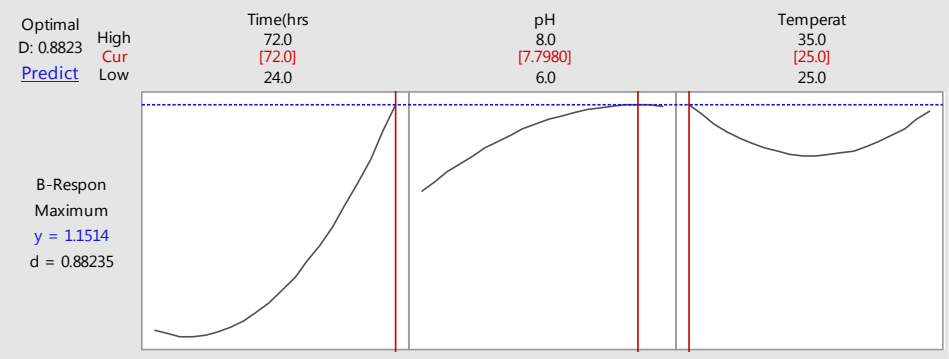

Fig. 6. Optimization plots of the production of BNP

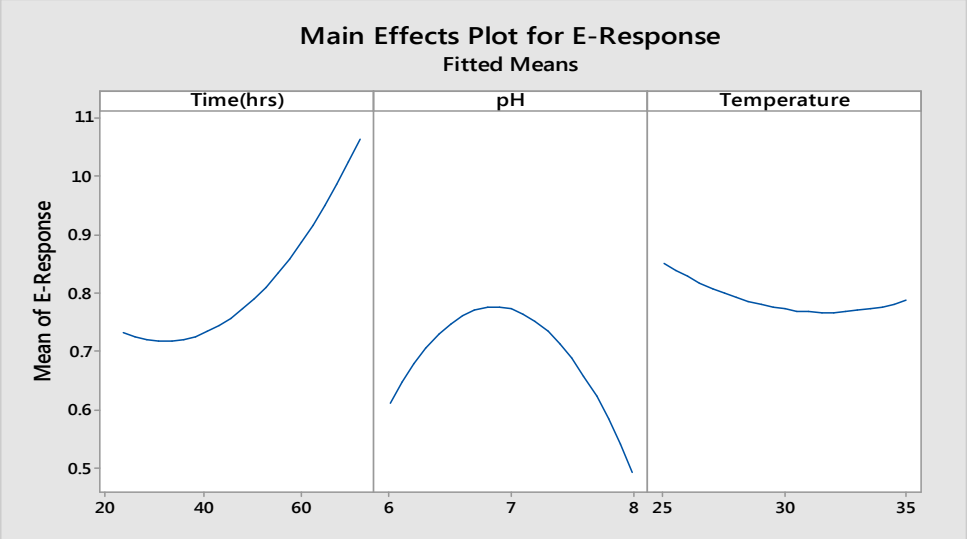

Fig. 7. Main effect plots for ENP production

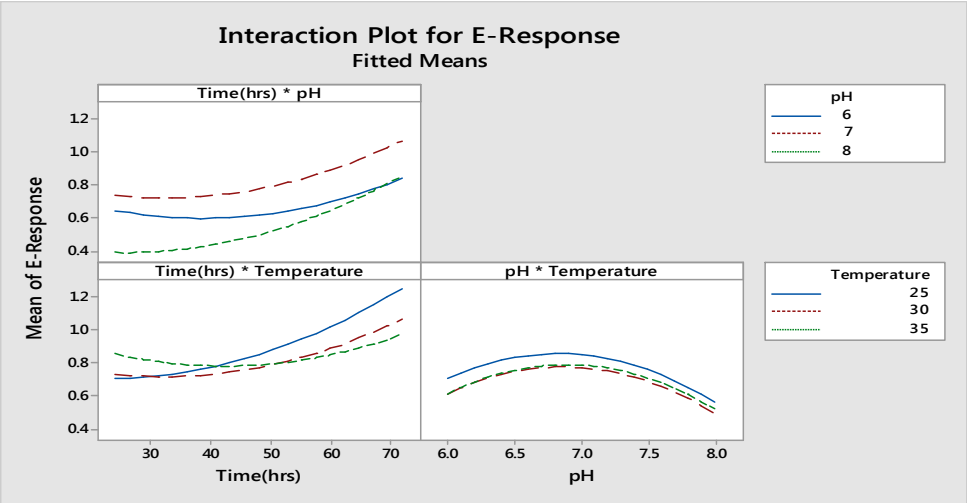

Fig. 8. Interaction plots for ENP production

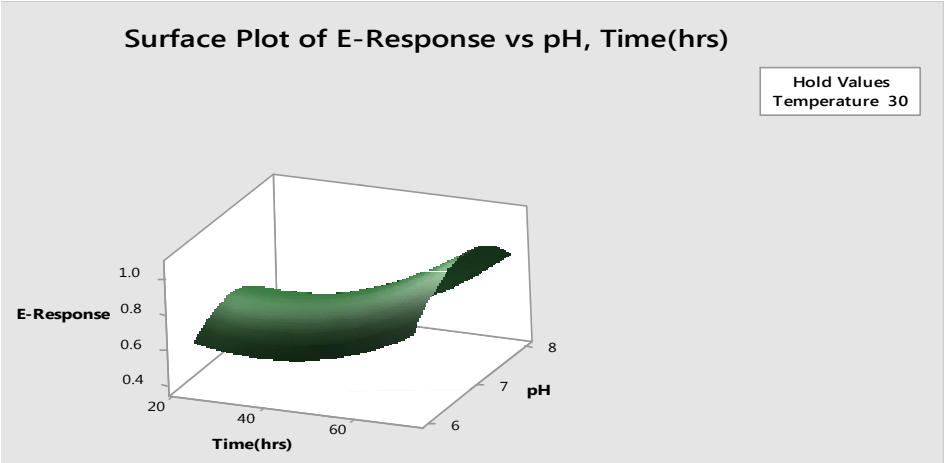

Fig. 9. Surface plot of E-response vs pH, time(hrs)

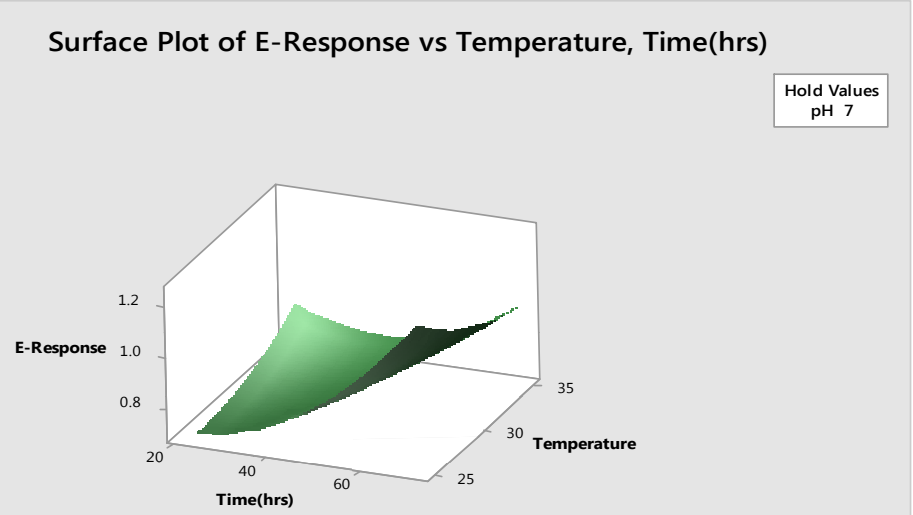

Fig. 10. Surface plot of E-response vs temperature, time(hrs)

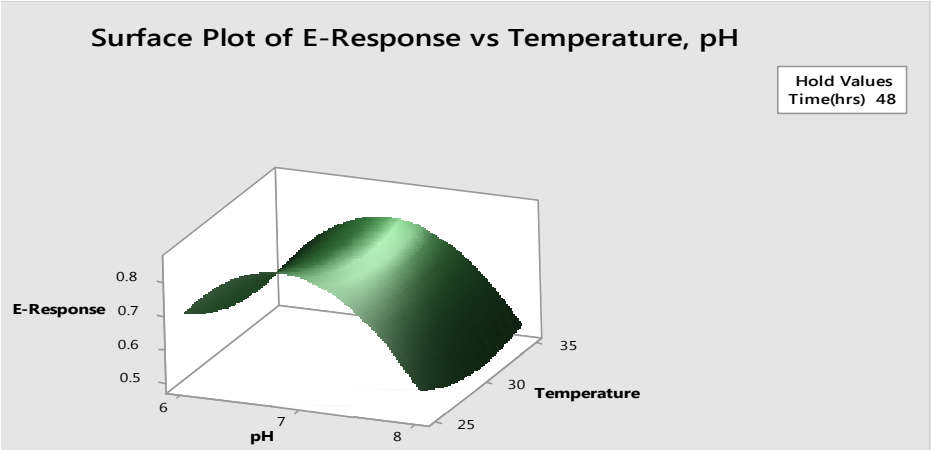

Fig. 11. Surface plot of E-response vs temperature, pH

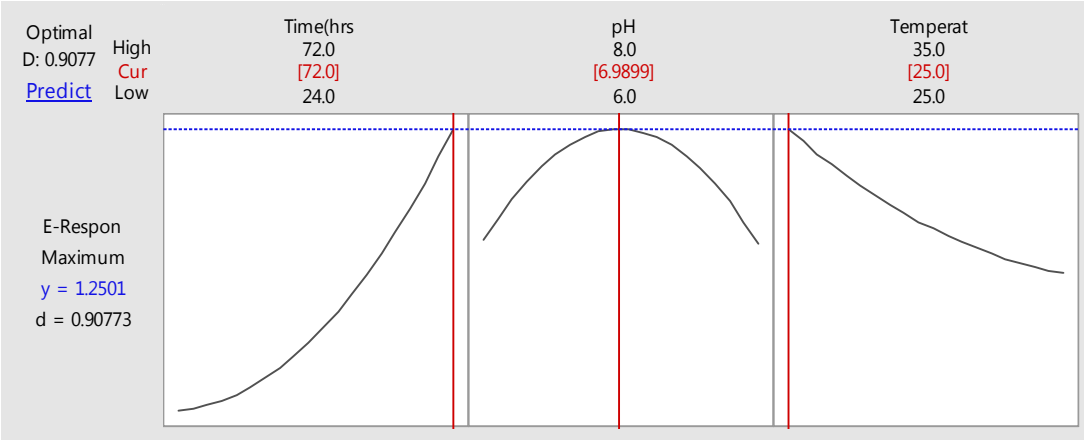

Fig. 12. Optimization plot for ENP production

Fig. 11 shows the response surface plot for the production of ENP at the 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 degrees.

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 ^{0}C and time of 72 hours. At these conditions, the *Escherichia coli* will have a yield of 1.2501.

4. DISCUSSION

This study seeks to identify optimal conditions that are required for the production of silver based nanoparticles from *Bacillus subtilis* and *Escherichia coli.* The study was designed to utilize only a very small concentration (10 mMol) of Ag nanoparticles. Particle size plays an influential role in the antibacterial properties of silver nanoparticles, with smaller particles exhibiting improved activities [9,10]. 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 [9].

The optimum conditions for the production of *Escherichia coli* (ENP) (pH 6.99, temperature 25 ^{0}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^{0}C 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 the conjugation of molecules of interest from the microbial extracts with those of silver nitrate, is time dependent. As a result, the longer the time of incubation, the more probable yield is obtained.

5. CONCLUSION

The variations in optimization factors of silver nanoparticles produced from both *B. subtilis* and *E. coli* led to improved production. The result showed that temperature, time, and pH play crucial roles in the synthesis of these bacteria-based nanoparticles. At very specific optimization condition, the synthesis of these nanoparticles attained either sharp increase of a sharp decline. The result showed that an increase in time and temperature improved the synthesis of *B. subtilis* nanoparticles whereas the increase in pH led to an initial decline. Nanoparticle production from *E. coli* was optimized mostly by increase in time, whereas both alterations in pH and temperature at some points led to production decline.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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