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SYNTHESIS OF SILVER NANOPARTICLES FROM *PSEUDOMONAS PUTIDA* NCIM 2650 IN SILVER NITRATE SUPPLEMENTED GROWTH MEDIUM AND OPTIMIZATION USING RESPONSE SURFACE METHODOLOGY

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The environmental friendly process of silver nanoparticles synthesis using various biological systems was reported earlier. The bacterial strain was selected for study due to its ease of manipulation and maintenance. The present work focuses on the biosynthesis and optimization of silver nanoparticles using the bacterial strain *Pseudomonas putida* NCIM 2650. The bacterial strain, *Pseudomonas putida* is a gram negative facultative bacterium that has been certified as a biosafety host and also reported in bioremediation process. *Pseudomonas putida* NCIM 2650 was explored on silver nitrate (1mM) supplemented growth media and the silver nanoparticle synthesis was carried out. The extracellular nitrate reductase enzyme was assayed for the synthesis of silver nanoparticles; the activity of the enzyme was found to be 0.958 U/ml/min. The growth of an organism was monitored and transformation of silver nanoparticles was started from 22h to 48h. The biosynthesis of silver nanoparticles were optimized using Response Surface Methodology, the statistical tool which has the designs of experiments. The optimum synthesis was observed at 48 h, the optimum temperature was found to be at 37.5°C and pH of 6. The silver nanoparticles were characterized by UV spectroscopy, ICP-OES, Fourier transform infrared spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) confirmed the presence of silver nanoparticles crystals and the size of the silver nanoparticle by particle size analyze+r was found have uniform size of 70nm.

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Keywords: Biosynthesis, Silver nanoparticles, Transformation, Accumulation, Nitrate reductase assay

1. Introduction

The interdisciplinary science of nanobiotechnology is extensively used in all disciplines due to its environmental benign nature. Nanoparticles have unique physical and chemical properties and the nanoscale (1-100nm) particle implies its importance in various fields of medicine, pharmaceutical and environmental technologies[1]. Now a day biosynthesis of nanoparticles by exploring biological systems like bacteria, fungi, yeast, algae and plant has been intensively studied for its growing success[2].

The general methods of synthesizing nanoparticles by physical method, which yield low amounts of product and generates lots of heat and the chemical method of nanoparticles production causes toxic effects and produces hazardous by-products[3]. Apart from the above two methods the biological method of producing nanoparticles is said to be eco-friendly, economic and appreciable. Generally microorganisms have a tolerant capacity to heavy metals that induce the metal binding protein followed by their uptake into the cell [4,5,6]. The interaction and sequestration of metal ions by microbes have been exploited in various application fields of biomineralization, bioleaching and biocorrosion[7].

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Thus the microbial system is potential for the synthesis of nanoparticles. The synthesis of silver nanoparticles from different microorganisms like fungi [8,9,10,11,12,13], yeast [14], Dermatophytes [15], Plants [16,17,18,19,20] Bryophytes [21] and phytopathogen [22] has been employed. The bacterial strain *Pseudomonas putida* was explored in this study due to its ease of handling. In this study the attempts were made to optimize the synthesis of silver nanoparticle using Response surface methodology (RSM).

Response surface methodology (RSM) is a statistical and mathematical tool for analyzing and developing the optimization process. RSM is one of the design of experiments (DOE) method that used to compute the values for the unknown function approximately with fewer input values[23,24]. By using this statistical optimization tool, the independent variables and their influences were studied. According to the variable input, the experimental model will be generated by number of trial experiments. The conventional method of optimization is tedious and time consuming process. Where as in RSM reduces the experimental trials and has automated designing system according to the variable[25].

The present study reports about the biosynthesis of silver nanoparticles from *Pseudomonas putida* NCIM 2650 and characterized using simple analytical techniques of UV-visible spectrophotometer, SEM, FTIR, Particle size analyzer and XRD. The parameters for biosynthesis was optimized using statistical tool, RSM.

2. Experimental procedure:

2.1 Bacterial strain and growth conditions

The bacterial strain *Pseudomonas putida* NCIM 2650 was obtained from the National Collection of Industrial Microorganisms, Pune. The silver resistant *P.putida NCIM 2650 NCIM 2650* was grown on Luria Bertani (LB) agar substrate, containing 1mM silver nitrate at 37ºC for 24 h. The bacterial strain was routinely maintained on LB agar slants. A loop full of bacteria was taken from slant and inoculated in LB broth in sterilized condition for further studies. The culture flask was maintained for 24 h in an incubator at 37ºC. The turbid appearances of the culture flask have ensured the growth and taken for experimental study.

2.2 Determination of Bacterial Resistance Threshold of silver

The minimum tolerable level of silver ions was determined by exploring the bacterial strain on different concentrations of silver nitrate. The *P.putida NCIM 2650* was grown on LB agar plate supplemented with various concentrations of silver nitrate (1mM, 2mM and 3mM) and incubated at 37 ºC for 24 h.

2.3 Bacterial growth and transformation

The growth of *P.putida NCIM 2650* was periodically monitored spectrometrically at 600nm in LB medium for 24 h supplemented with silver nitrate (1mM) at 37ºC in a shaker (200rpm). The samples were withdrawn at different time interval and centrifuged and analyzed for growth at 600nm. The transformation rate was also monitored by withdrawn the sample and the absorbance was measured at 420nm.

2.4 Determination of metal accumulation

The silver accumulation potential of *P.putida NCIM 2650* was estimated using ICP-OES. The *P.putida NCIM 2650* was inoculated in LB broth containing 1mM silver nitrate and incubated for 24 h at 37ºC. An overnight culture sample was withdrawn and centrifuged at 10,000 rpm for 15 min at 4ºC. The biomass was harvested by draining the supernatant and the pellet was kept for overnight drying at 65 ºC. The dried cell mass was subjected to overnight acid digestion with 70% nitric acid. The digested samples were filtered using Whatman No.1 filter paper and the total volume of the sample was adjusted to 10ml and analyzed for silver accumulation by ICP-OES.

2.5 synthesis of silver nanoparticles

The extracellular synthesis of silver nanoparticles was carried out by growing *P.putida NCIM 2650* on LB broth containing 1mM silver nitrate for 48 h at 37^0C in a shaker at 200rpm. Periodical monitoring of the sample through UV-Visible spectrometrically was measured in the wavelength range 200-800nm. The change of culture color from brown to dark brown was the indication of synthesized silver nanoparticles.

2.6 Nitrate reductase assay

The activity of nitrate reductase was measured by putting the nitrate substrate along with enzyme and then measuring the nitrite formed after periodic incubation. The protocol uses the substrate as 25mM Phosphate buffer with 10mM potassium nitrate (KNO3) and 0.05mM Ethylene diamine tetraacetic acid having pH 7.3. For assaying the enzyme sample, the crude extract of supernatant solution of *P.putida NCIM 2650* culture was obtained by centrifugation. The supernatant obtained was taken as crude enzyme. The crude enzyme samples were taken in separate test tubes and the substrate was added to each test tube those were kept at varying incubation time of 0 min, 20 min, 40 min and 60 min. One of the enzyme sample test tubes immediately placed in a water bath for 5min after the addition of the substrate. Other sample test tubes were taken after incubation time and the sample aliquots were placed in a water bath for 5 min to denature the enzyme present in the sample. After cooling the samples to room temperature, the reduction reaction was terminated by adding 58mM Sulphanilamide solution in 3M Hydrochloric acid in each of the sample. N-(1-napthyl) Ethylenediamine Dihydrochloride solution (NEED) was then added and mixed by swirling. After 20 min of incubation, absorbance was measured at 540 nm using UV-Vis Spectroscopy. The control for the experiment was made for calibration to zero with phosphate buffer, 58mM sulphanilamide solution and NEED solution. The amount of nitrite found in the sample aliquots was compared with the Nitrite Standard Curve plotted using sodium nitrite. The enzyme activity was calculated based on the increased nitrite concentration of sample aliquots over time of incubation and expressed as U/ml/min.

2.7 Optimization of parameters for silver nanoparticles synthesis using RSM

The optimization tool of Response Surface Methodology (RSM) with Experiments of Design (DOE) was used to analyze the influence of various parameters like pH, temperature and reaction time. A Central Composite Design (CCD) was chosen to analyze the 3 factors with its low level and high level and have replicated at the center points and axial points. The CCD contains a total of 20 experimental trial runs that include 8 cube points, 6 central points and 6 axial points. After the experimental trial runs, the samples were measured spectrometrically at 420 nm for the synthesis of silver nanoparticles.

The predicted response value (Y) in each trial of the quadratic model was expressed as:

$$Y= \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1\beta_1 A^2 + \beta_2\beta_2 B^2 + \beta_3\beta_3 C^2 + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_2\beta_3 BC \quad \text{Eq. (A)}$$

Where Y was the measured response, β_0 – intercept, $\beta_1, \beta_2, \beta_3$ - linear coefficient, $\beta_1\beta_1, \beta_2\beta_2, \beta_3\beta_3$ – quadratic coefficient, $\beta_1\beta_2, \beta_1\beta_3, \beta_2\beta_3$ – Interactive Coefficient, ABC – independent variables.

2.8 Characterization of silver nanoparticles

The quantitative analyses of silver nanoparticle were performed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). The elemental concentration of the silver was obtained by repeating centrifugation of the supernatant containing silver nanoparticles stabilized in room temperature for two months. The pellet was digested using 70% nitric acid overnight. The digested sample was filtered and analyzed by ICP-OES.

The silver nanoparticles were characterized using SEM was done using Hitachi S-3400 N SEM machine. A thin film of the sample was prepared on a carbon coated copper grid by just dropping a very small amount of the sample and after sample coating, it was allowed to dry under a mercury lamp for 5 min and the analysis was performed.

The particle size was analyzed using particle size analyzer zetasizer ver. 6.20. A small drop of the sample was dispersed in a dispersant, water and analyzed under laser light beams in a disposable sizing cuvette.

Fourier Transform Infrared Spectroscopy (FTIR) of silver nanoparticles was carried out by putting a drop of sample and scanning the sample in the range of 450 – 4000 cm-1. FTIR graph gives the elemental composition.

X-Ray Diffraction (XRD) study was carried out using a dried sample of Silver nanoparticle. The supernatant was centrifuged at 5000 rpm for 10 min, the biomass obtained was discarded and the supernatant was added with acetone (1:4 ratio) and centrifuged at 5000 rpm for 30 min. The silver nanoparticle pellet was collected and dried for XRD analysis.

3. Results and Discussions

3.1 Determination of Bacterial tolerent concentration to silver

The minimum tolerant concentration of silver by *P.putida NCIM 2650* was determined by plating the organism with different concentration of silver nitrate (1mM, 2mM and 3mM). After incubation of 24 h at 37˚C, the growth at different concentration of silver nitrate was monitored. Among different concentration of plates the bacterial growth was noticed only on 1mM concentration and there was no growth observed in 2mM and 3mM concentration. The minimum threshold concentration of bacteria to silver nitrate was found to be 1mM and was taken for further study.

3.2 Bacterial growth curve and transformation to silver nanoparticles

The growth of *P.putida NCIM 2650* and transformation of silver nitrate to silver nanoparticles was monitored with a periodic time interval of 2h upto 24h. The growth of the organism showed the short lag phase of 5h, prolonged log phase of 12h and extended stationary phase from 18h was observed by taking optical density at 600nm. The transformation of silver nanoparticle was analyzed spectrometrically at 420nm and the transformation was noticed from 22h as shown in Figure 1.1.

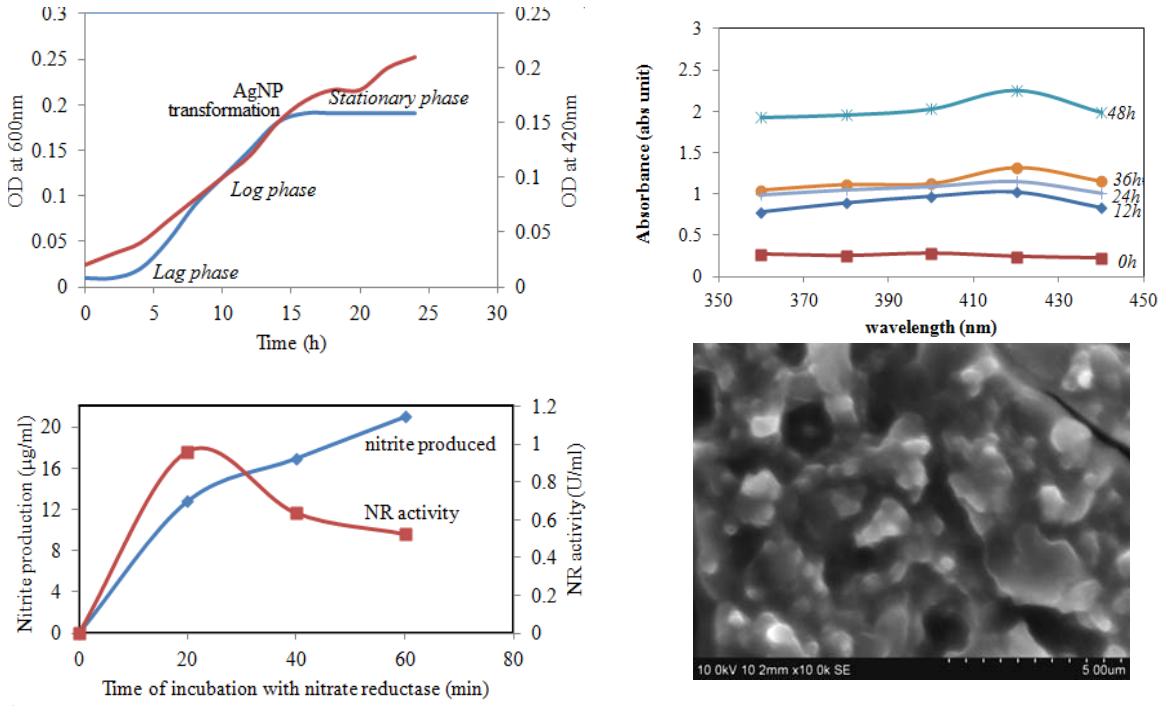

Fig. 1.1. Growth and Transformation of silver nitrate to silver nanoparticles, 1.2. Absorption spectrum of silver nanoparticles, 1.3 NR activity increased with nitrite production and decreased with increased of incubation time, 1.4. SEM Micrograph of Silver nanoparticles.

3.3 Estimation of silver accumulation

The accumulation of silver ions from the cell surface of *P.putida NCIM 2650* grown in the presence of silver nitrate in LB broth is determined by Perkin Elmer Optima 5300 DV ICP-OES, IIT, Chennai. The result corresponds to the concentration of silver ion in the acid digested biomass sample was 0.048mg/L at the element symbol wavelength of Ag328.068. This accumulated silver ions were further transformed to silver nanoparticles, mediated by the extracellular nitrate reductase enzyme.

3.4 Syntyesis of silver nanoparticles and UV-Visble spectrometric analysis

Synthesis of silver nanoparticles from the *P.putida NCIM 2650* broth supplemented with silver nitrate(1mm) was monitored from 0h to 48h at regular interval of 12h and observed under different wavelength of 360, 380, 400, 420 and 440 as shown in Figure 1.2. The color intensity of the broth was increased with time from yellow to dark brown color indicates the synthesized silver nanoparticles. After 24h of broth culture showed the peak which was prominent at 48h after that there was no noticeable increase of peak value. The maximum absorption peak was obtained at 48h under the wavelength of 420nm.

3.5 Estimation of Nitrate reductase activity

The nitrate reductase (NR) activity of culture supernatant measured about 0 in the boiled sample, at 20h incubation the activity was measured about 0.958 U/ml/min, 0.635 Uml/min of activity was measured at 40h incubation and 0.524 U/ml/min of NR activity was measured at 60h as shown in Figure 1.3. The reduction process of nitrate to nitrite was increased with the incubation time. The NR activity was higher at 20min incubation and 0.958 U/ml/min was taken as the enzyme activity of the supernatant. The decrease in NR activity with increasing incubation time is due to the depletion of the substrate. The nitrate to nitrite reduction was noticed from the colour change of purple due to the coupling of sulphanilamide with nitrite forming azo compound. In boiled enzyme sample there was no nitrite production and no colour change was noticed due to the denatured enzyme. The bioreduction process of silver nitrate into silver nanoparticle was mediated by the extracellular nitrate reductase enzyme of *P.putida NCIM 2650*.

3.6 Optimization of parameters for silver nanoparticles synthesis using RSM

Table-1 showed the Central Composite Design (CCD) with the response OD was measured at 420nm using UV-Visible Spectroscopy arranged in a run order of experiments. The maximum peak was measured at 420nm taken as the response of the reaction, The optimum temperature was found to be at 37.5˚C, pH 6 and incubation time was found to be 48 h. The temperature below and above the optimum temperature was not favoured the synthesis of silver nanoparticles. The more acidic and alkaline pH was denatured the enzyme activity showed reduced growth of silver nanoparticles. The predicted response value with respect to the optical density as an influence of pH(A), temperature (B) and time (C) was given below:

$$Y = -5.08068 + 0.579352\,A + 0.450435\,B - 0.233921C - 0.0496023A^2 - 0.00603927\,B^2 + 0.00334280\,C^2 + 0.000775000\,AB + 0.000286458\,AC + 9.16667E\text{-}05\,BC \qquad \text{Eq. (B)}$$

Table 1 CCD Experimental Design of trails with response

RunOrder	PtType	Factor A pH	Factor B Temperature(˚C)	Factor C Time(h)	OD 420nm	Predicted OD
1	0	6	37.5	36	1.36	1.27945
2	0	6	37.5	36	1.36	1.27945
3	-1	10	37.5	36	0.7	0.57945
4	0	6	37.5	36	1.36	1.27945
5	1	2	25	48	0.04	0.01580
6	-1	6	50	36	0.42	0.40382
7	1	10	25	48	0.02	0.15380
8	1	2	50	24	0.01	-0.18420

RunOrder	PtType	Factor A pH	Factor B Temperature(˚C)	Factor C Time(h)	OD 420nm	Predicted OD
9	-1	6	37.5	48	2.26	1.90382
10	-1	6	37.5	24	1.02	1.61782
11	1	2	25	24	0.01	-0.21520
12	1	2	50	48	0.02	0.10180
13	0	6	37.5	36	1.36	1.27945
14	1	10	25	24	0.01	-0.13220
15	0	6	37.5	36	1.36	1.27945
16	-1	6	25	36	0.01	0.26782
17	1	10	50	24	0.09	0.05380
18	0	6	37.5	36	1.36	1.27945
19	1	10	50	48	0.23	0.39480
20	-1	2	37.5	36	0.03	0.39182

The statistical significance of the experimental design was analyzed by the F-test for the analysis of variance (ANOVA) as shown in Table 2. The p- value given in the ANOVA table for linear and square values were < 0.005, indicates the model was highly significant and the p-values for interaction was > 0.005, negligible. The R-Square value of 0.905 for the synthesis of silver nanoparticles indicating the concurrence between the experimental and predicted values can be explained as 90.5% variability of the response. The graphs (Figure 1.1, Figure 2.2 and Figure 1.3) showed that the influence of parameters with the response of absorbance at 420nm. The Residual plot versus response of absorbance at 420nm showed the confidence level of above 95%.

Table 2 Analysis of Variance (ANOVA) for optimization of silver nanoparticles synthesis

Source	DF	Seq SS	Adj SS	Adj MS	F-value	P-value
Regression	9	8.59009	8.59009	0.95445	10.63	0.000
Linear	3	0.33909	3.89852	1.29951	14.48	0.001
Square	3	8.23596	8.23596	2.74532	30.59	0.000
Interaction	3	0.01504	0.01504	0.00501	0.06	0.982
Residual Error	10	0.89757	0.89757	0.08976		
Lack-of-Fit	5	0.89757	0.89757	0.17951	*	*
Pure Error	5	0.00000	0.00000	0.00000		
Total	19	9.48765				

3.7 Characterization of silver nanoparticles

The biosynthesized silver nanoparticles was quantitatively determined by Perkin Elmer Optima 5300 DV ICP-OES, IIT,Chennai. The result corresponds to the concentration of silver nanoparticles after acid digested sample of silver nanoparticle powder was 0.330mg/L at the elemental symbol wavelength of Ag328.068.

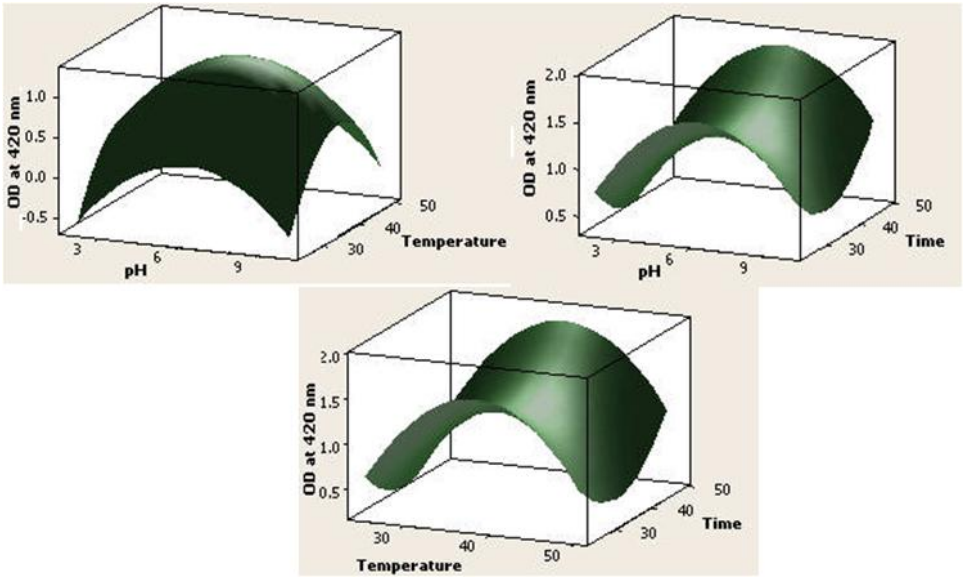

Fig. 2. 1. Surface Plot of OD at 420 nm vs Time, pH
2.2. Surface Plot of OD at 420 nm vs Temperature, pH
2.3. Surface Plot of OD at 420 nm vs Time, Temperature

The SEM micrograph recorded in Figure 1.4 of biosynthesized silver nanoparticles shows the aggregates of nanoparticles. It shows that the nanoparticles are well-dispersed on the surface and confirms the existence of small spherical uniformly distributed nanoparticles. The nanoparticles are agglomerated and the size observed varies from 50nm to 70nm.

The particle size was analyzed using particle size analyzer zetasizer ver. 6.20. A small drop of the sample was dispersed in dispersant, water and analyzed under laser light beams in a disposable sizing cuvette. The particle size was analyzed under the category of intensity of laser light on the sample particle. Laser diffraction revealed that the particles obtained are aggregated mixture with size ranging between nanometers and micrometers as shown in the Figure 3.1 and Figure 3.2. The average particle diameter was found to be 70nm.

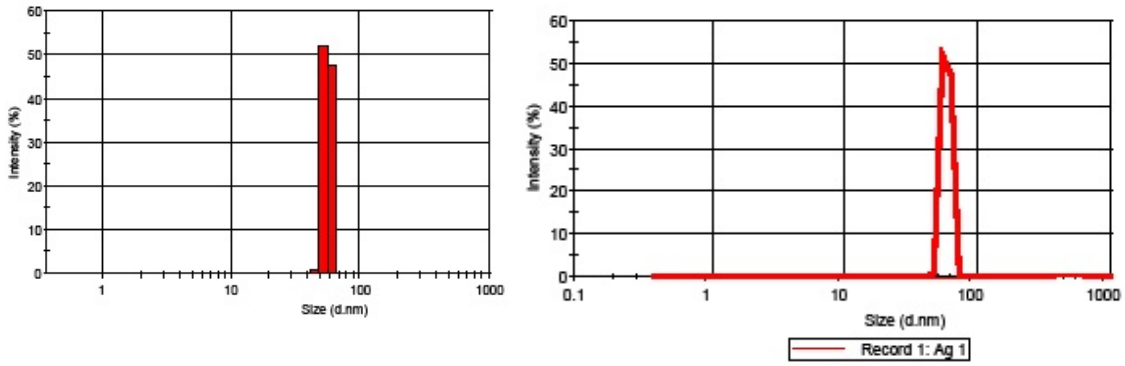

Fig. 3.1. Statistical graph of silver nanoparticles
3.2. Size distribution by intensity graph of silver nanoparticles

Fourier Transform Infrared Spectroscopy (FTIR) of silver nanoparticles was used to determine the interaction between the metallic silver nanoparticles to protein from the absorption of Infra Red (IR) radiation. The Figure 4 shows the FTIR spectra of silver nanoparticles with absorption peaks located in the region of 4000 cm^{-1} to 400 cm^{-1}. The absorption band seen at 3447.8 cm^{-1} corresponds to the stretching vibrations of primary amines, while their bending vibrations were seen at 1637.3 cm^{-1}. The two bands observed at 2370.3 cm^{-1}, 2079.0 cm^{-1} can be

assigned to the fingerprint of phenyl ring substitution overtones and the band observed at 696.5 cm−1 representing the bending vibrations of Alkynes. It is well known that proteins can able to bind with metals and metallic nanoparticles either through free amine groups or cysteine residues in the proteins. This is due to that one or more of these proteins may be enzymes(Nitrate reductase) that reduce silver nitrate ions and form the silver nanoparticle by reduction technique. The overall observations showed that the proteins or peptides might have formed a coating over the silver nanoparticles and stabilized the nanoparticles. Similar adsorption bands were reported in plant mediated *Dioscorea batatas* Rhizome [26].

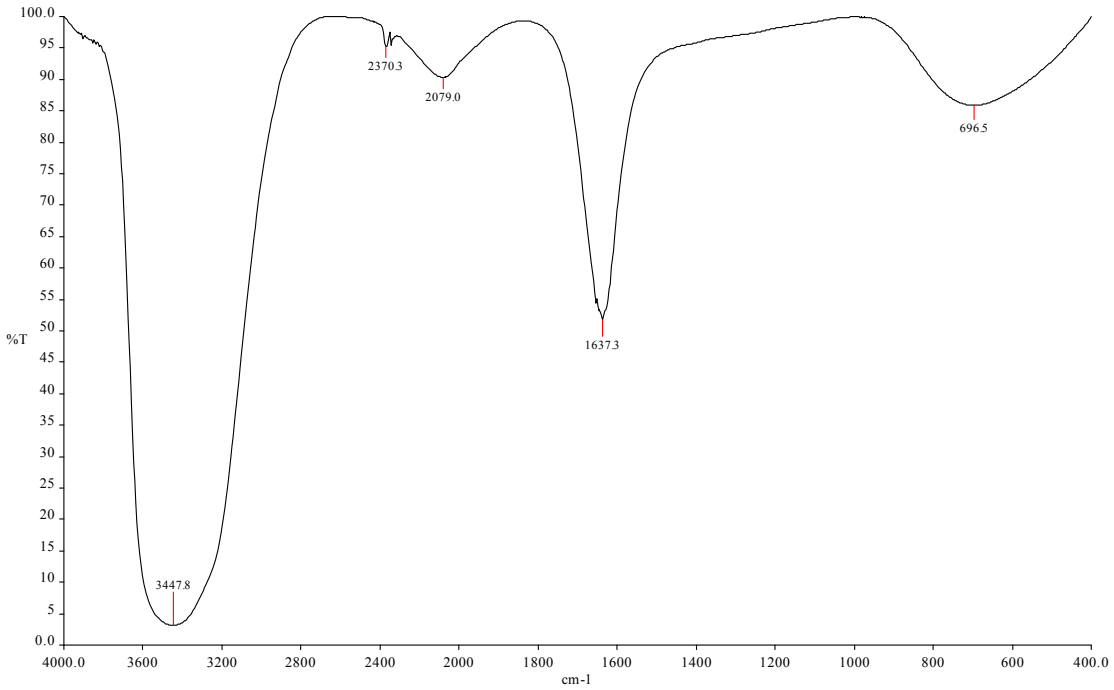

Fig. 4 FTIR spectra of silver nanoparticles.

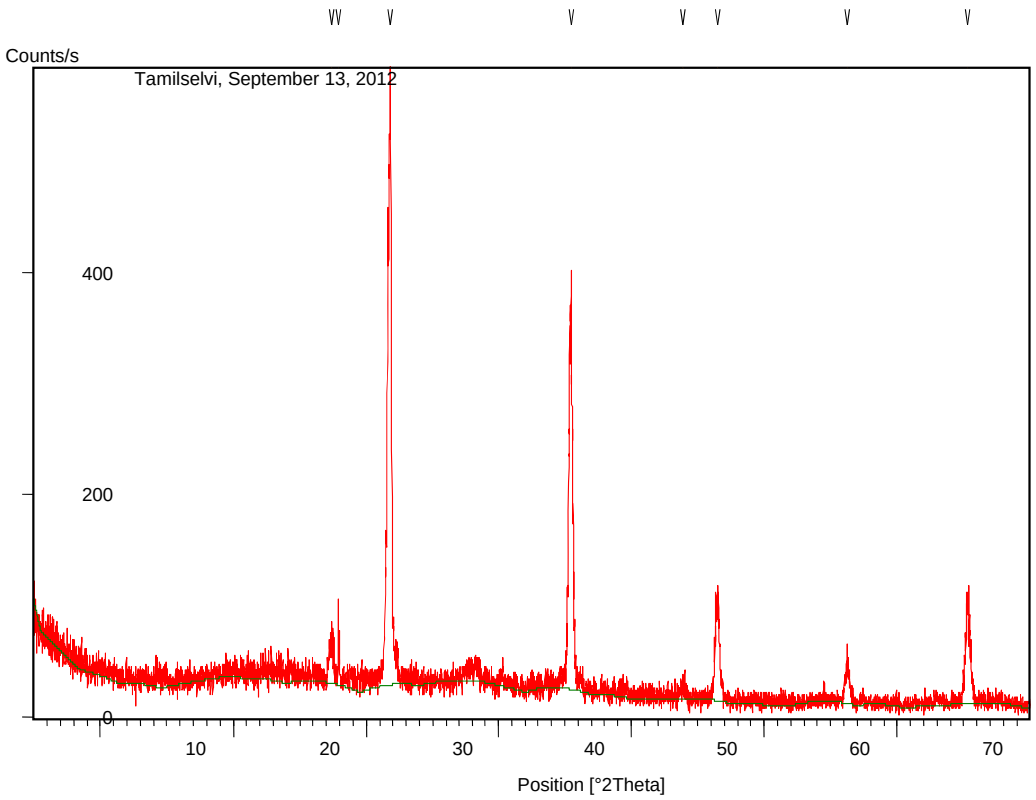

Fig. 5 XRD peaks of silver nanoparticles

In XRD pattern indexing is the process of determining the unit cell parameters from the peak positions. To index a powder diffraction pattern it is necessary to assign Miller indices, *hkl*, to each peak. A diffraction pattern cannot be analyzed until it has been indexed. The peak indices hkl can be measured using the Bragg's Law and the formula used for the calculation is

$$\sin^2\theta = (\lambda^2/4a^2)(h^2+ k^2+ l^2) \quad \text{Eq. (C.1)}$$

$$\text{Let } K= (\lambda 2/4a2) \text{ constant} \quad \text{Eq. (C.2)}$$

$$\sin 2\theta = K(h2+ k2+ l2) \quad \text{Eq. (C.3)}$$

The Figure 5 shows the XRD peaks of silver nanoparticles at 2θ positions of values 27.37°, 27.90°, 31.81°, 45.48°, 53.90°, 56.51°, 66.23° and 75.31° corresponds to the index of planes (111), (111), (200), (220), (311), (222), (400) and (420) planes of silver respectively . The XRD spectrum confirms the simple cubic crystalline structure of biosynthesized silver nanoparticles.
The Crystalline structure of the sample was calculated using Debye-Scherrer's Equation:

$$D= K\lambda / \beta.\cos\theta \quad \text{Eq. (D.1)}$$

$$\text{Where } \beta = \pi/ 180^* \text{ FWHM (Full Half Length Maximum)} \quad \text{Eq. (D.2)}$$

Where D is the mean diameter of particle in nm; B is the Line broadening at half the maximum intensity in radians; θ is the Bragg's angle. K is the Dimensional factor, constant as 0.89; λ is the X-ray Wavelength taken as 1.54°A; The calculated Kλ is 1.37; By substituting all the values to the Equation, the particle size of the sample was calculated and the average particle size was found to be 31.78nm.

4. Conclusion

The bacterial synthesis of silver nanoparticle using modified protocol of growth medium supplemented with silver nitrate(1mM) was observed to be rapid and the nanoparticle synthesis started from 22 h and completed at 48 h and analyzed by UV- Visible Spectroscopy. The bioreduction process of silver nanoparticle synthesis was mediated by the extracellular enzyme, nitrate reductase and its activity was measured spectrometrically as 0.958 U/ml/min, optimum at 20min. The parameters pH, temperature and time of incubation for synthesizing silver nanoparticles were optimized using RSM and the optimum results were shown as pH 6, temperature at 37.5ºC and the incubation time of 48 h. The RSM prediction data was found that the experimental design was significant and the R-square value was 90.5%. The characterization of silver nanoparticles by ICP-OES at Ag328.068 wavelength confirms the presence of silver and was found to be 0.330mg/l. The SEM micrograph shows the presence of silver nanoparticle crystals and its sizes were analyzed by particle size analyser zetasizer ver. 6.20 having an average size of 70nm.The FTIR and XRD studies also confirmed the presence of silver nanoparticles. The extracellular synthesis of nanoparticles is advantageous from the perspective of large scale production and downstream processing.

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