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Methylene Blue Dye Degradation using Mycosynthesized, Silver Metal Nanoparticles

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Abstract:- Growth and development of biologically synthesized metal nano particles have attracted significant attention in field of nanotechnology due to their potential use for human benefits. A natural, eco-friendly and green chemistry solution incorporating mycology, biotechnology and nanotechnology is the fungal synthesis of noble metal nanoparticles such as silver nanoparticles. There are several methods including physical, chemical and biological methods that can be used for the synthesis of silver nanoparticles. The aim of this research is to implement the green production of silver nanoparticles, i.e., the biological method using *penicillium rubens*aqueous fungal extract.

The aqueous fungal extract was added to silver nitrate solution where the color of the silver nitrate reaction medium was changed from Pale yellow to brown which indicates reduction of silver ions to silver nanoparticles.Synthesized silver nanoparticles are thus characterized by UV-Visible spectroscopy, which disclose a peak of 400-420 nm. The Fourier transform infrared spectrum was studied in order to distinguish the effective functional molecules responsible for the reduction and stabilization of fungal synthesized silver nanoparticles.

Changes in parameters such as pH, temperature and silver nitrate solution concentration were used to optimize the output of silver nanoparticles and to assess absorption in UV-Visible spectrophotometer. It is therefore concluded that the biosynthesis of silver nanoparticles using extracellular fungal filtrate was simple, eco-friendly and robust. Effective degradation of Methylene blue dye in 90 minutes of exposure time was observed, providing 97 percent efficiency.

Keywords:- *Green chemistry, Fungal-synthesis, Sustainable, Eco-friendly Silver nanoparticles, Penicillium rubens, Methylene Blue, Dye reduction.*

I. INTRODUCTION

Nanotechnology is an emerging field of science and technology due to its vast array of application towards human development. Due to their remarkable physical, chemical and optical properties, the use of noble metal nanoparticles has gained immense momentum for many decades. As an innovative stream from different means, a variety of noble metal nanoparticles such as gold **[1]**, platinum, titanium, silver, graphene **[2-3]** etc. have been formed. Amongst all, silver nanoparticle is repeatedly used in Food, pharmaceuticals, cosmetics, FMCG and other industrial sectors for its excellent behavioral properties. Peculiar application of silver nanoparticles includes Anti-bacterial (Consumer and Healthcare products etc.), Anti-cancer (Diagnostic and Drug delivery etc.), Anti-angiogenic (For tumors and blockade), Anti-inflammatory (For relieving stress and pain) and as Anti-oxidative compounds (Antioxidants and anti-corrosive agents etc.). In addition, they are used in wound healing as they are anti-inflammatory and anti-microbial agents **[4]**.

There are three methods to synthesis silver nanoparticles i.e., Physical method (spark discharge **[5]**, pyrolysis **[6]** etc.), Chemical method (cytochemical synthesis **[7]**, laser ablation **[8]** etc.) and biological method (plants extracts, microbes **[9-11]**, algae). These methods have been developed to synthesize noble metal nanoparticles such as silver nanoparticles of differing shape, size and solubility. Among these, as it is a green solution, environmentally friendly, inexpensive, non-toxic, compatible and quick **[12-15]**, we have preferred biological technique. Biological approaches, however, employs regulated scale size, shape, density, stability, etc. which are advantageous over other methods used. Also, compared to conventional synthetic techniques, biological system provides novel ideas for production of nano-materials **[16]**. It is also very preferable to use screened species such as fungi (*Penicillium rubens*) with high development potential and ease of control of reaction conditions. It is also very well-known that the characters of silver nanoparticles are greatly affected by its morphological, structural, physical and chemical properties Variety of physical and chemical methods including radiation assisted, thermal decomposition, laser ablation, sonochemical, photochemical and polyaniline synthesis have been reported to synthesize AgNPs.Therefore, both of these approaches require the use of hazardous elements that pose health and environmental risks. Biosynthesis of AgNPs using Fungal extracts does not involve the use of any toxic material and this approach is cost effective and very simple Process **[17]**.

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Fungal synthesis has many leads over bacterial synthesis in bulk development as they contain high amount of enzymes involved in silver nanoparticles **[18]**, and is easier to develop both in the laboratory and on an industrial scale. Fungal mediated silver nanoparticle synthesis is beneficial as it is eco favorable, improves environmental footprint, easy to handle, uncomplicated culture, can be genetically modified etc. Synthesis of noble metal nanoparticles has frequently been demonstrated by different species of *Penicillium*, Pseudomonas [**9]**, Lactobacillus**[10]**, Bacillus**[11].** In this research, we have used unique species i.e.,*Penicilliumrubens* for silver nanoparticles biosynthesis. *Penicillium rubens* of genus *penicillium produces* various acids and toxins like kojic acid, rubratoxin A, rubratoxin B and rubramin occurs in soybeans and grain corn. In addition, *Penicillium rubens*is also very nearly identical to *Penicillium chrysogenum*.

Fungal mediated silver nanoparticles synthesis is comparatively unique research stream among microorganisms. Some examples of fungal mediated intracellular synthesis of silver nanoparticles have been found in the fungus *Aspergillus flavus***[19]**.

Fungal mediated silver nanoparticle synthesis can be carried out by: (a) cell-free broth or (b) using dried or wet mycelia extract.In this study, we scrutinized the green synthesis of *Penicillium rubens*silver nanoparticles using cell-free broth. Characterization of silver nanoparticles were carried out by UV-Visible Spectroscopy and Fourier–transform infrared (FTIR) spectroscopy.

Only a few industries like textiles, cosmetics, paper, pharmacy etc. uses the organic dyes. Most of the industries currently using the synthetic dyes which are not only detrimental to human health but also to the environment **[20]**. The extracellular biosynthesized silver nanoparticles were subjected to dye reduction and Catalytic dye reduction activity was observed respectively.

II. MATERIALS AND METHODS

A. Materials

Fungal strain – Pure fungal strain was extracted by Jyoti Kedar it was sent to characterization and identification at NCMR Centre (National Center for Microbial Research), Pune, India and identification was carried out on ITS for 550 bp fungal sequence. Results were analyzed using NCBI (National Center for Biotechnology Information) database for identification of the fungal sample. After comparison of sequence, BLAST (Basic Local Alignment Search Tool) was performed for molecular identification. Silver Nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$) and Methylene Blue were obtained from Himedia, India. Chemicals used in this study were of experimental grade. All solution were prepared from double distilled water throughout the experiments.

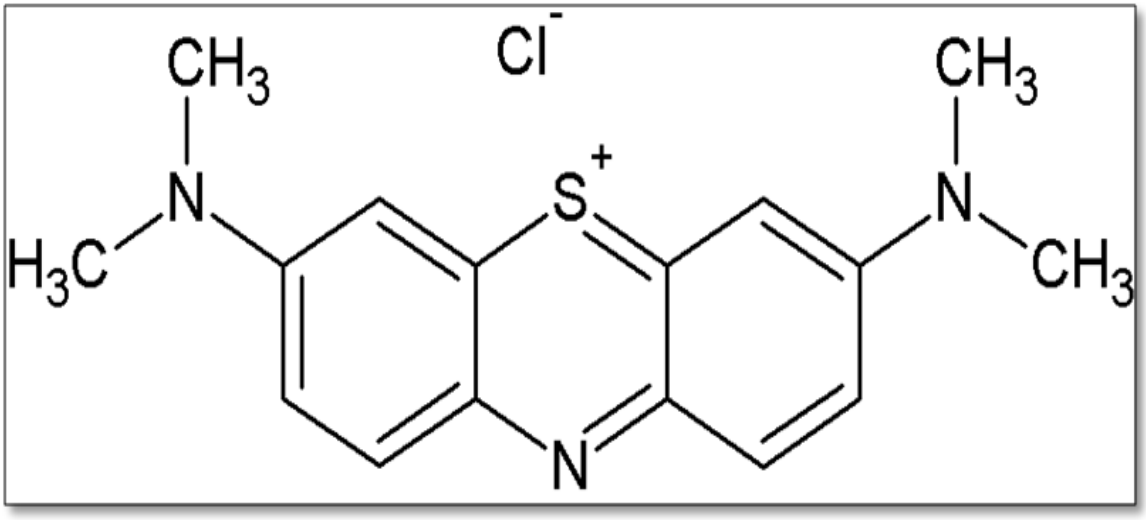

Fig. 1: Molecular structure of Methylene Blue

B. Methods:

a) Preparation of cell filtrate

Potato dextrose agar (PDA) was used as a medium for growing *Penicillium rubens* for 7 days at 28° C. The biomass was harvested by double filtration using Whatman filter paper, followed by washing with distilled water to remove any components of the media. It underwent two process-firstly the filtrate was boiled with distilled water and secondly it was incubated for 5 consecutive days on the rotary shaker.

b) Biosynthesis of silver nanoparticles by fungi *Penicillium rubens*

For Biosynthesis of silver nanoparticles, the cell filtrate was mixed with silver nitrate solution in 9:1 ratio. The prepared solution was kept for incubation at 35° C for 24 hrs. After incubation color change was observed from Pale yellow to dark brown color which detected the synthesis of silver nanoparticles. To prevent any photochemical reactions during the experiment, all the solutions were kept in the dark. The silver nanoparticles were purified by centrifugation at 10,000 rpm for 10 min and collected for further characterization as done **[21]**.

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c) Characterization of Silver Nanoparticles:
The reduction of silver nitrate to silver nanoparticles was confirmed by UV-Visible spectroscopy. The bio reduction of Ag+ in aqueous solution was monitored using spectrophotometer in range of 400 nm to 420 nm. The two significant aspects of pH and temperature were taken into account in the spectroscopy. FTIR spectroscopic studies were carried out to find possible bio-reducing agents present in the culture filtrate.

d) Optimization studies of silver nanoparticles production:
The environment in which an organism lives and that organism are constantly in interaction with one another. The growth and development of an organism are continually influenced by its surroundings. The environment that the organism is grown in has an impact on the production of enzymes by fungus. Therefore, optimization studies will increase product yield in addition to supporting fantastic growth.

a. Effect of pH:
The pH of the reaction was optimized by using various grades of pH, where the reaction pH was conditioned and maintained at 6, 7 and 8. The pH was adjusted by 0.1 N HCl and 0.1 N NaOH. The absorbance was measured by UV- Visible spectrophotometer respectively.

b. Effect of temperature:
Every reaction depends heavily on temperature. Temperatures of 28, 35, and 40 degrees Celsius were used for the optimization investigations, respectively. The sample underwent UV-Visible spectroscopy analysis, and the impact of temperature on nanoparticles was further investigated.

c. Effect of AgNO3 concentration:
Substrate concentration is one of the critical factors in the synthesis of nanoparticle. The concentration of silver nitrate was optimized using different concentration from range of 0.5 to 5 mM. The absorption of solution was measured by UV- Visible spectrophotometer.

d. Study of catalytic action of prepared silver nanoparticles against Methylene Blue:
Both a medicine and a stain, methylene blue is also known as methylthionine chloride **[22–24]**. It is mostly used to treat methemoglobinemia as a medicine. Prior to now, it was advised against using it to treat urinary tract infections and cyanide poisoning. Usually, an injection is delivered into a vein. Headache, nausea, dizziness, shortness of breath, and elevated blood pressure are typical adverse effects. Red blood cell degeneration, allergic responses, and serotonin syndrome are some other side effects. The use of methylene blue frequently results in the blue to green coloring of the faeces, urine, and perspiration.Metal nanoparticles have the potential to be used as catalysts in chemical reactions that otherwise wouldn't take place. NaBH4 evaluated the produced silver nanoparticles' catalytic activity using methylene blue (MB) as a substrate, a harmful dye. In a typical test, 3 mL of freshly made 1 mM NaBH4 solution and 10 mL of a 10 mM stock solution of MB was combined. There were three different samples made. In two samples, a previously created mixture of MB and silver nanoparticles was further enhanced by the addition of 1.5 and 3.0 mL of manufactured colloidal silver nanoparticles. Additionally, a sample without any silver nanoparticles was created. By adding ddH2O, the final reaction mixture volume in all three samples was changed to 16 mL. Samples containing silver nanoparticles gradually turned from deep blue to brown over time. A UV-Vis spectrophotometer was used to measure the absorbance at 665 nm in order to track the reduction of MB at regular intervals of time (10 min).

Percentage of dye degradation was estimated by the following formula:

$$\% \text{ Decolorization} = \frac{100 \times (C_0 - C)}{C_0}$$

Where C0 is initial concentration of dye solution and Cis concentration of dye solution after catalytic degradation.

III. RESULT AND DISCUSSION

A. Identification of fungi

a) Phenotypic characterization:
Identification and Characterization of collected fungal strain was performed at NCMR by analyzing obtained results and species was identified as *Penicillium rubens* with 99% similarity.

PRN	Strain No.	Closed neighbor	Access ion No.	% Similarity
A-NOV_17_160	MG	*Penicilliumrubens*CBS 129667	NR_111815.1	99%

Table 1: ITS 18S RNA sequencing

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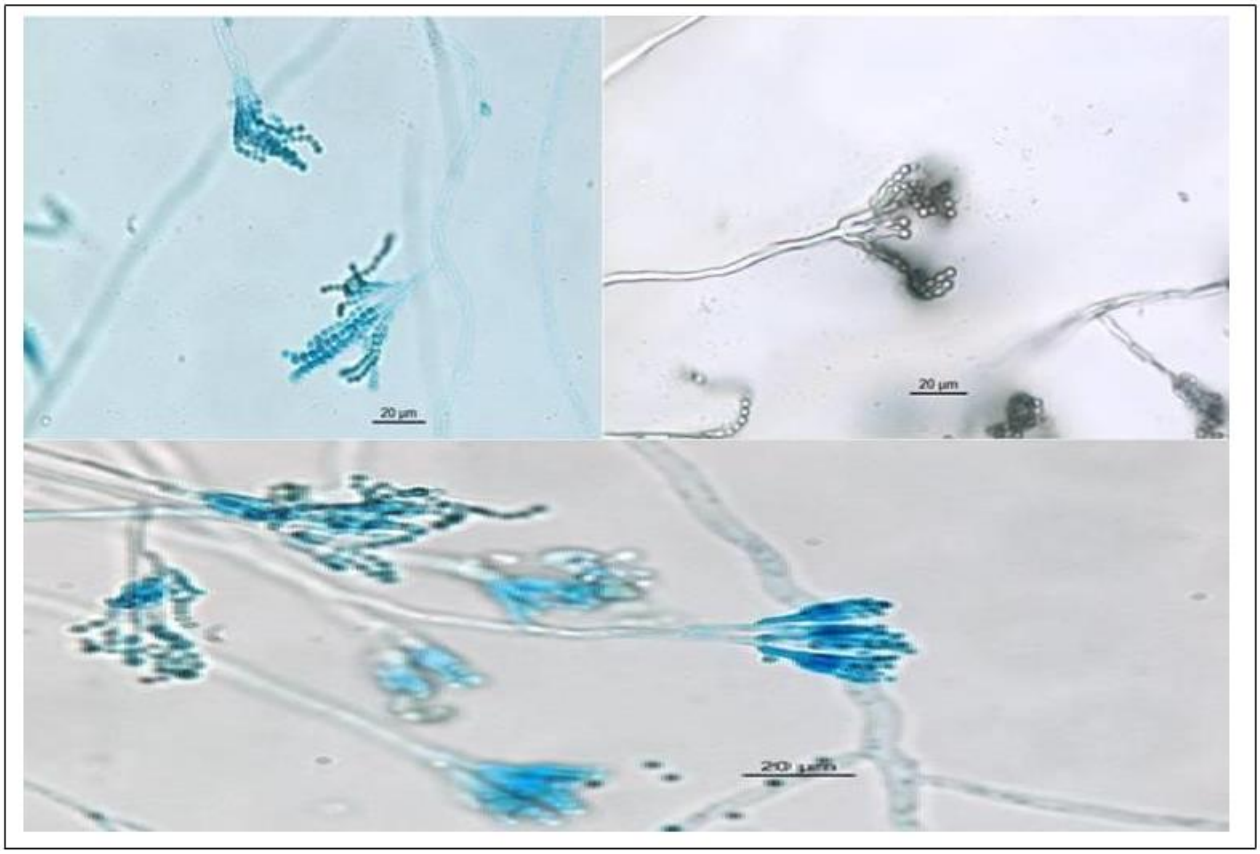

Photos 1: Microscopic structure of *Penicillium rubens*

Microscopic Observation were performed to study phenotypic expression. Branched, septate, smooth, hyaline hyphae was observed with width 37.66 x 5.82 µm. Conidiophore is un-branched and cylindrical. Size of Metulla is 8.67 x 3.06 µm and Phialides is 7.43 x 2.09. Conidia is single chains, brown, and smooth, oval with size 1.56 µm.

b) Synthesis and Characterization:

In this experimental study, with a reduction of aqueous Ag+, silver nanoparticles were synthesized, by mixing cell filtrate with silver nitrate solution in 9:1 ratio. After an incubation, color change was observed from pale yellow to dark brown color in 24 hrs., indicating formation of silver nanoparticles. No tangible aggregate was observed, which indicate that the particle was well dispersed in the solution. Also, the color intensity of mixture of $AgNO_3$ and cell filtrate was suspended for 2 hrs. periodic monitoring of reaction mixture were recorded at regular time interval by UV visible spectroscopy.

The UV spectra of mixture of cell filtrate of $AgNO_3$ shown a maximum absorption at 411 nm, which indicates the presence of silver nanoparticles. The silver nanoparticles formed were highly stabled for about 160 hours. Similar results were obtained using Aspergillus *flavus* **[25]**. Moreover, **Afreen et al [26]** reported peak at 422 nm with *Rhizopus stolonifera*. The result were also consistent since they obtained similar absorption spectrum of AGgNPs produced by *penicillium* with a maximum peak between 420-450 nm **[27]**. FTIR analysis measurement accounts for identification of specific functional group which are responsible for synthesis and stabilization of silver nanoparticles. This FTIR spectrum revealed that, the synthesis of silver nanoparticles showed us a strong absorption peak at 3292.45 cm^{-1} which indicates the presence of carboxylic group. Similarly, the broad absorption found was observed at 3425 and 2927 cm^{-1} due to O-H stretching and H bounded alcohol and phenol group. A weak bond was observed at 1635 cm^{-1} corresponding to N-H bending primary amino. A small peak was formed at 773 cm^{-1} due to occurrence of alkalide halides. This analysis showed the functional biomolecule are hydroxyl, carboxyl, phenol and amine groups that strongly supported the formation of silver nanoparticles by fungi *Penicillium rubens*to stabilize the silver nanoparticle.

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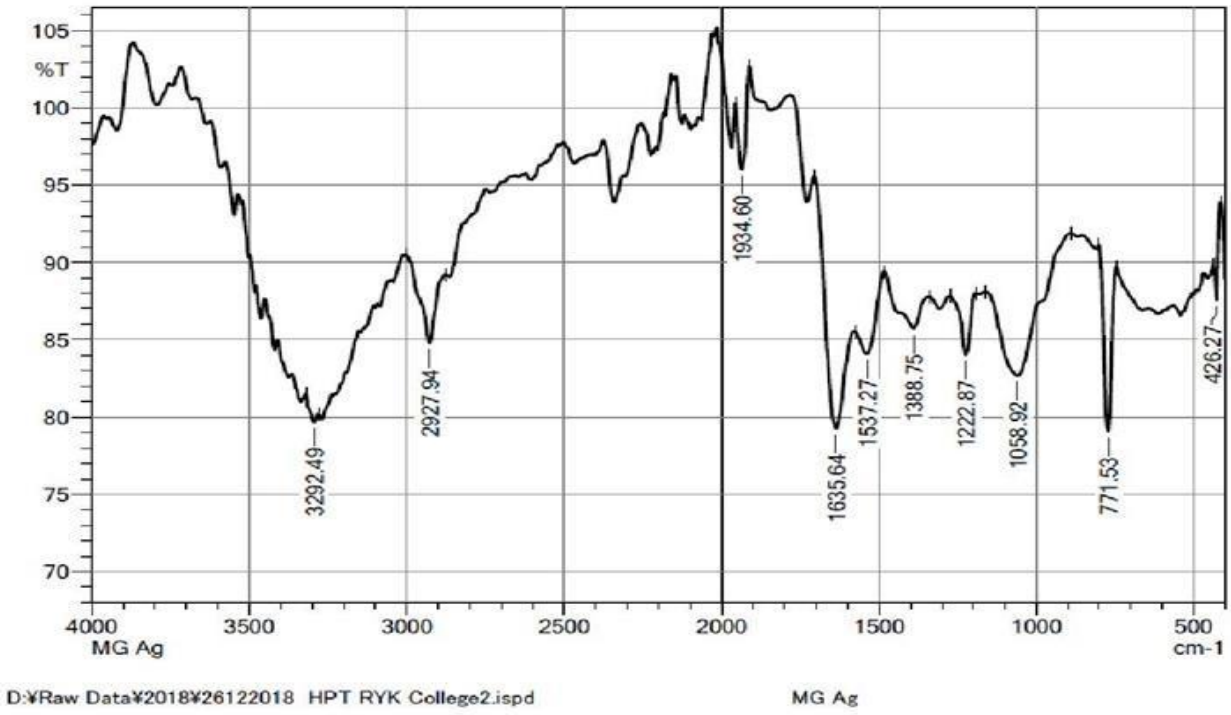

Fig. 2:FTIR analysis of silver nanoparticles

	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	426.27	87.54	4.16	437.84	412.77	237.808	33.676
2	771.53	79.06	11.34	800.46	744.52	842.012	306.135
3	1058.92	82.67	6.82	1159.22	891.11	3538.572	848.445
4	1222.87	84.02	3.91	1274.95	1193.94	1108.270	128.740
5	1388.75	85.72	2.61	1483.26	1340.53	1847.572	219.133
6	1537.27	84.08	3.11	1577.77	1483.26	1342.458	157.746
7	1635.64	79.26	10.81	1705.07	1577.77	1902.476	696.679
8	1934.60	96.01	5.42	1955.82	1911.46	74.822	140.532
9	2927.94	84.76	4.96	3001.24	2877.79	1504.786	251.245
10	3292.49	79.67	0.91	3319.49	3280.92	761.368	22.005

Table 1: FTIR analysis of silver nanoparticles

c) Optimization study:

The growth and metabolism of fungi is intensely altered by environmental conditions, similarly culture condition and other parameter also greatly influence the productivity and growth of fungi respectively. Various parameter like concentration, pH and temperature can directly affect the rate of which the silver nanoparticles are synthesized.

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Photos 2: Formation of silver nanoparticles was detected by color change from Pale yellow to dark brown.

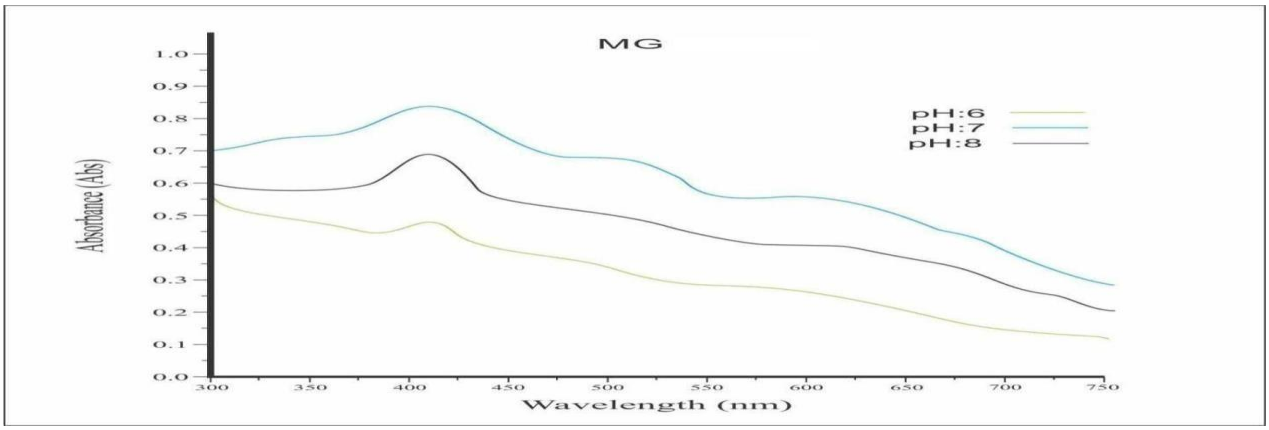
A. Effect of $AgNO_3$ concentration:

Fungal filtrate was used to study the production of silver nanoparticles at various concentrations of silver nitrate solution (0.5 mM to 5 mM). The maximal absorbance of 411 nm in the UV-visible spectrum allowed for the detection of the optimum substrate concentrate as 1 mM. Our findings are consistent with those of **Banu et al. 2011 [28]**, who also discovered that *Rhizopus stolonifera* produced silver nanoparticles at an optimal concentration of 1 mM AgNO3.

B. Effect of pH:

To monitor certain features of the nanoparticles, modifying the pH range is preferable. The pH that is required for silver nanoparticles biosynthesis has a strong influence on growth and the production of enzymes. To research the effect of pH on the development of silver nanoparticles from fungi *Penicillium rubens,* a different pH ranging from 6 to 8 with a difference of 1 was used. At acidic ranges, small and broad peaks were observed whereas at neutral or slightly alkaline pH, studies have reported successful formation of narrower and sharper peaks indicating successful biosynthesis of silver nanoparticles. From the results obtained, the graph indicates maximum growth at the pH 7 at 410nm which indicates the presence of synthesized nanoparticles. Low pH or acidic ranges cause the protein structure to be compromised, the protein to become inactive, and as a result, nanoparticles agglomerate.

The AgNP that is produced is stable at pH 7, but not at acidic pH, it can be inferred. Our findings are consistent with those of **Banu et al. 2011 [29]** and **Jain et al. 2001 [30],** who used Aspergillus flavus at pH 7.0 and found a maximum absorbance peak at 422 nm.



Graph 1: Optimization of condition at different pH

C. Effect of temperature:

In all reactions, temperature plays an important role. Temperature optimization experiments were carried out at temperatures of 28 ℃, 35 ℃ and 40 ℃ respectively. UV-Visible spectroscopy was used to analyze the sample, and further effects of temperature on nanoparticles were studied. The temperature used in the synthesis of fungal mediated silver nanoparticles can affect parameters such as the synthesis, speed, size and stability of the nanoparticles. Earlier studies shows that synthesis rate increases as the temperature increases and the maximum rate of synthesis was observed at 35 ℃ and 40 ℃ and is considered to be the ideal temperature. While the majority of studies have recorded faster rates of synthesis at higher temperatures, the quality of nanoparticles should be taken into account. The temperature can affect the size and stability of the nanoparticle, in addition to affecting the synthesis rate. Comparing with the ideal conditions of temperature we have obtained optimum temperature of our research to be 35° C. At low temperature, Broad peaks are observed

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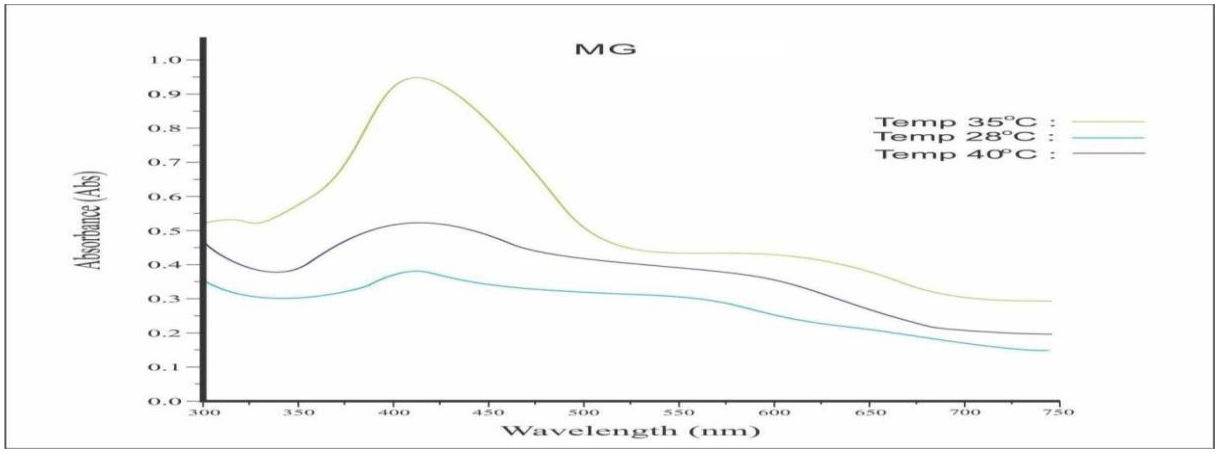
whereas at high temperature narrow and sharp peaks exist. When compared with other temperature range detected by UV spectrum, the maximum absorption spectra were observed at 425 nm which indicated the production of silver nanoparticles. The peak was somewhat symmetric at 35° C. On the other side, at greater temperature like 40^0 C, the enzyme activity gets lowered, hence the synthesis of silver nanoparticles is affected and peak formed is unsymmetrical.



Graph 2: Optimization of conditions at various temperature.

d) *Mechanism of dye reduction:*

Bond dissociation energy (BDE) is a crucial factor in the breaking and creation of new bonds during chemical reactions. Methylene blue dye serves as an electron acceptor while NaBH4 serves as an electron donor throughout the process [31]. When silver nanoparticle is added, the BDE is reduced and the electron transfer is made more effective. Silver nanoparticle decomposition efficiency was calculated to be 97.7% in 90 minutes. This quickens the rate at which NaBH4 reduces the methylene blue dye. Additionally, a progressive decline in the dye's absorbance value can be used to determine dye degradation. Therefore, the silver nanoparticles made from the fungus Penicillium rubens showed promising results in methylene blue dye removal.

Silver nanoparticles were added to the reaction mixture as a potential intermediary between the BH4- ions and the methylene blue dye. It first reduced the BDE and improved the efficiency of the electron transport between them. As a result, the rate at which MB was reduced by NaBH4 increased when silver nanoparticles were present.

Various application such as antimicrobial activity for multidrug resistant microorganisms as well as reduction of harmful industrial dyes, artificial food coloring, toxic food dyes are noted.

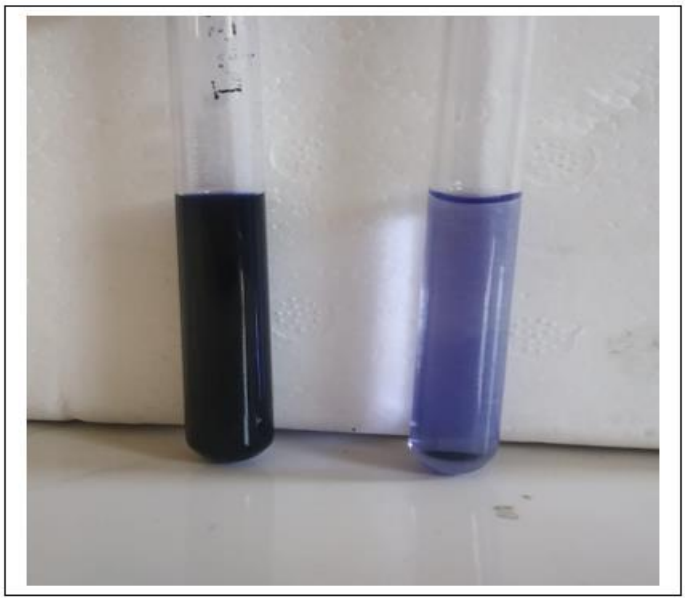
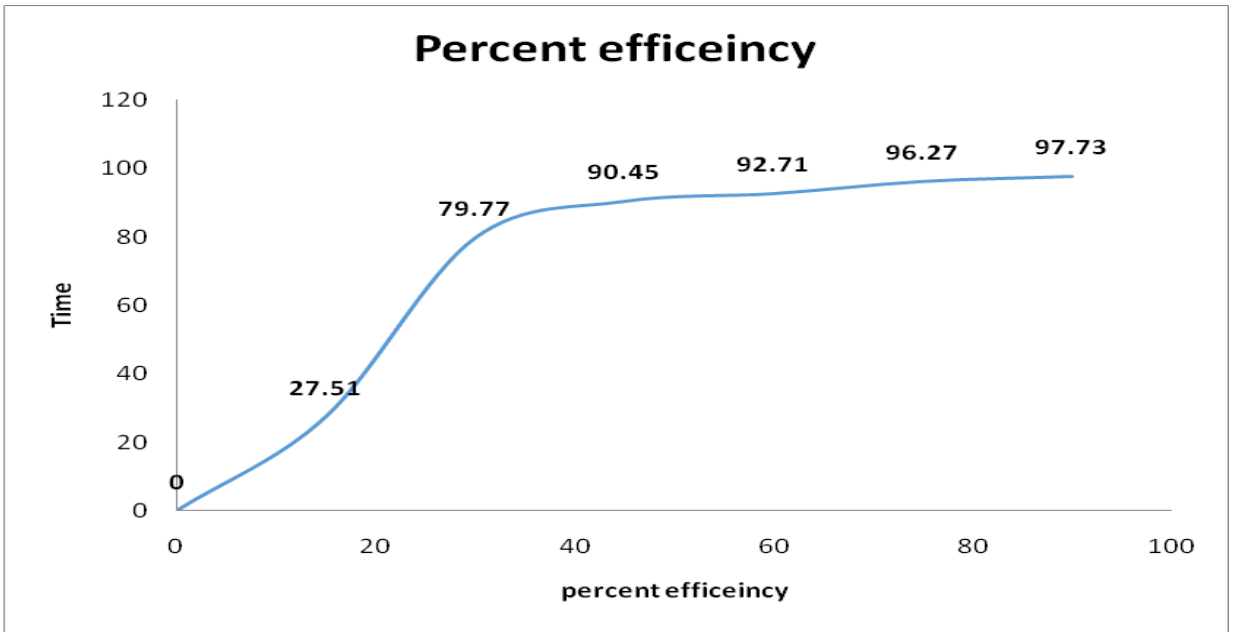


Photo 3: Catalytic dye reduction indicating color change from deep blue to brown

Time (Min)	Absorbance et. at 665 nm	Percent efficiency
0	0.618	0
15	0.448	27.508
30	0.125	79.77
45	0.059	90.45
60	0.045	92.71
75	0.023	96.27
90	0.014	97.73

Table 3: Percent dye reduction at 665 nm



Graph 3: Absorbance of methylene blue reduced by silver nanoparticles at 665 nm.

IV. CONCLUSION

The results of this work confirm the synthesis of silver nanoparticles. The synthesized nanoparticles were characterized by optimization through UV-Visible spectroscopy and FTIR. The synthesis of fungus *Penicillium rubens* may therefore serve as a simple, cheap and ecofriendly approach. Hence, silver nanoparticles prepared by cost effective reduction method.

DECLARATIONS

- **Availability of data and materials**
  All data generated or analyzed during this study are included in this article and in the Supporting Information.

- **Competing interests**
  The authors declare that there is no conflict of interest regarding publication of this study.

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- **Authors Contribution**
  J.A.K; Systemic Methodology and Investigation, A.K, N.P; Analysis and Interpretation, P.T; writing- original draft preparation, S.B; data curation and visualization. All authors read and approved the final manuscript.

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