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Biosynthesis of Biologically Active Silver Nano Particles Using Natural Red Pigment Under Optimized Conditions

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Abstract

The microbial production of natural red pigment was investigated using *Penicillium purpurgenium* .Optimization of pigment production was carried out under different growth conditions involving different carbon source, fermentation time, pH value as well as addition of some additives. The study aims to use the obtained pigment as reducing agent for synthesis of silver nano particles. The results revealed that the maximum production of pigment (295 mg/L) was obtained by using sucrose conc. (20g/L) , production time 8 days, pH 6 at 30C accompanied by the addition of $Mg^{+2}$and Mn^{+2} salts. The separated pigment was used for synthesis of nano-silver particles (AgNPs) , characterization of the obtained nano-particles was carried out and determination of their Antimicrobial activity. TEM images showed that the generated nanoparticles were spherical and, hexagonal shaped with particle size (4-70)nm.

Key words: Biosynthesis , nono-particles, natural pigment ,characterization.

Introduction

Many natural pigments in addition to their function as coloring agents are known as interesting bioactive compounds with potential health benefits. These compounds have a wide range of applications in cosmetics, medicine agrochemicals,, pharmacology and food additives[1].

The disadvantages of synthetic food colorants which destroy the nutrients in food, cause many dangers to consumers that led to some health problems, most notably types of cancer, Sudden mood swings, hyperactivity in children, DNA damage or genotoxicity, attention-deficit disorder and detrimental effects on environment.

Numerous microbial bioactive pigments have been discovered and many of them show antioxidant, anti-inflammatory, and/or antimicrobial properties.

In comparison to plant and animal sources, microbial pigment produced by fermentation technology is more dynamic and economic, resulting in biodegradable compounds that may have wide industrial applications as colorants[2]. Although microbial pigments are not widespread in colorant formulations, they represent an important alternative that has the long-term ability to compete with synthetic dyes [3]. The successful application of microbial pigments relies on high production yields, reasonable production costs, regulatory approval, pigment characterization, and stability to environmental factors such as temperature and light [4].

Green synthesis of AgNPs by microorganisms is attractive because it can take advantage of their metabolism. Fungi are interesting for this purpose because they have a high growth rate in a great variety of substrates and, considering their eukaryotic metabolism, have a higher chance of being similar to human conditions. Fungi have proven to be effective in the production of silver nanoparticles, owing to their versatile polyphenols and pigments [5]. Research focusing on these bioactive compounds from fungal sources is therefore essential to enhance the stability and size control of metal nanoparticles.

A number of microorganisms have been reported by many authors as producer of large quantities of colorants including *Paecilomyces, Monascus, Cordyceps, Serratia, Streptomyces, Penicillium atrovenetum* and *Penicillium herquei* [6].

Nanotechnology is widely used in nanoparticle research, including the production of silver nanoparticles. Silver nanoparticles are widely utilized across a range of applications due to their distinctive physicochemical and antimicrobial characteristics [7]. They exhibit promising potential for therapeutic purposes and can be incorporated into various health-related products, household items, and industrial uses [8].

The present study aims for the optimization of natural pigment production using *Penicillium purpurogenum* and its application as a reducing agent for the production of silver nanoparticles to be used in some biological activities.

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2-Materials and Methods

2.1-Microorganism and maintenance

The culture of *Penicillium purpurogenum* was purchased from Assiut University Mycology Centre (AUMC), it was maintained on Czapeck ،s medium that contained the following ingredients (g/l): Glucose 30, yeast extract 2, peptone 10, $NaNO_3$ 3, KCl 0.5, $MgSO_4$ 0.5 and Agar 25incubated for 7 days at 28°C and stored at 4°C.[9].

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2.2-Production medium

250 ml Erlenmeyer flasks containing 50 ml of modified Czapeck،s medium containing the following nutrients (g/l) Sucrose 20, $NaNO_3$ 3.0, KCl 0.5, $MgSO_4.7H_2O$ 0.5, KH_2PO_4 1.0), $FeSO4.7H_2O$ (0.1).).The culture was shake at 200 rpm and 30°C for 7 days [9].

2.3-Pigments Extraction

Cultures obtained from Czapeck،s production media were filtered through filter paper (No. 1; Whatman), the biomass was dried at 50ºC for 48 h till constant weight. Red pigment was extracted with ethanol and the extract was concentrated till dryness , absorbance was read at 500 nm by a UV-visible spectrophotometer.

2.4-Synthesis of Silver Nanoparticles (AgNPs) using pigment

The pigment that was extracted served as the basis for the synthesis of silver nanoparticles (AgNPs). A reaction mixture consists of 5 mL was prepared by incorporating 0.5 g/L of the pigment along with 5ml of 2 mM of silver nitrate (AgNO3). The reaction mixture obtained was underwent gentle vortexing to ensure thorough mixing and was subsequently incubated at 28 °C for a duration of 48 h. Following this incubation period, the formation of nanoparticles was confirmed through a visual observation of color change and analysis via UV-Visible spectroscopy (V-550 spectrophotometer, JASCO, Hachioji, Tokyo, Japan). A control was established using a silver nitrate solution devoid of pigment, alongside a pigment solution at a concentration of 0.5 g/L that did not contain the precursor salt[6].

2.5-The characterization of silver nanoparticles (AgNPs)

An aqueous solution of AgNPs was examined using a UV-Vis spectrophotometer (Model UV-1600, China), covering a wavelength range of 300 to 800 nm. To identify the functional groups present in the mixture of MPs and biosynthesized AgNPs, Fourier-transform infrared spectroscopy (FT-IR) was employed (Model IS50, USA). A silicon wafer was utilized to drop-cast the AgNPs solution, and the resulting X-ray diffraction (XRD) pattern was acquired using a Bruker D8 advance powder X-ray diffract meter with Cu-Kα radiation[6]. The dimensions and shape of the produced silver nanoparticles were analyzed the JEOL JEM-100 CX model of Transmission Electron Microscopy (TEM). For the TEM analysis, silver nanoparticles were applied onto carbon-coated TEM grids through a drop-coating technique. After allowing the film on the grids to dry, any excess solution was carefully eliminated with blotting paper.

2.6-Antimicrobial activity of AgNPs.

Antibacterial activity of the obtained Ag NP was assessed against the Gram-negative bacteria (*Escherichia coli* NRRL B210 , *Pseudomonas aeruginosa* ATCC278531) and gram-positive bacteria (*Bacillus subtilis* NRRL-B543) by well diffusion agar method. The optical density of the inoculum was adjusted at 0.125 for freshly grown bacteria, wells were made to add Ag NP (1, 2, 3, 4 mg/ml) suspended in 1 mL (DMSO) , and ceftriaxone was used as an antimicrobial reference for bacteria[10]. The plates were incubated for 24 h at 37°C. Antimicrobial activities were evaluated by measuring the inhibition zone diameter (cm).

2.7-Minimum inhibitory concentration (MIC)

Ag NP was prepared in various concentrations (10, 20, 30, and 40 mg/ml) and applied to a series of test tubes that contained 0.1 ml of gram-positive *Bacillus subtilis* NRRL-B543a and gram-negative *Escherichia coli* NRRL B210 and *Pseudomonas aeruginosa* ATCC278531. The culture allowed to grow at 35°C for 24h., bacteria alone with the nutrient broth was kept as control and they were examined for inhibition studies. MIC was the lowest concentration of the nanoparticle that did not permit any visible growth of bacteria. CFU was calculated by subculturing the above (MIC) serial dilutions after 24 h in nutrient agar Petri plates using 0.01ml loop and incubated at 35°C for 24h [11].

3-Results and discussion

3.1-Effect of different carbon source on the production of red pigment

In the present experiment the effect of different carbon sources (mannitol, sucrose, Glucose starch . maltose , galactose and fructose) on the production of the red pigment was tested. The result presented in figure (1) showed that the maximum production(198mg/l) was obtained by sucrose .Consequently, a remarkable output of natural pigment was obtained (176mg/l) by using glucose as carbon source .The lowest pigment output (100mg/l) was obtained by using starch as carbon source. In agreement with our results of priority of using sucrose as carbon source [6].On the other hand. manitol was the best carbon source for pigment production[9].The lowest yield (90.9mg/l)was obtained by using starch.

3.2-Effect of different sucrose concentration

In the present study the effect of different sucrose conc. (5, 10, 15, 20, 25 and 30g/l) was tested. The results presented in figure (2) showed that the production of natural pigment was increase by the increasing sucrose concentration and reached the maximum (260mg/l) at 20g/l. The higher concentrations (25 and 30 g/l) produce reduced levels(210 and 220mg/l) respectively. However, Sharad *et al* .(2019) revealed that the best of pigment was obtained by using 50g/l sucrose . On the other hand. Eman et al., [8] showed that the maxiumum pigment production was obtained by using 25g/l manintol

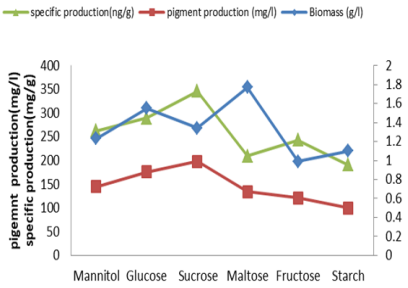


Fig.(1) Effect of different carbon source on the pigment prodction

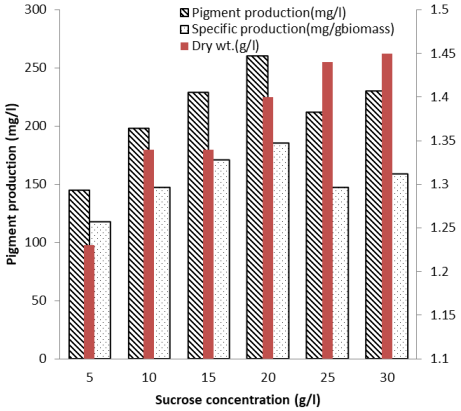


Fig.ure 2: Effect of different sucrose concentration on the production of pigment

3.3-Effect of different fermentation time on the production of pigment

The effect of different fermentation time (1,2,3,4,5,6,7,8,9and 10day).The results presented in figure (3) showed that the maximum yield (295mg/l) was obtained at 8 days .The fermentation time before 8day((1.2.3.4.5.6and 7days) produced low yields(100,121, 122, 162, 193, 260 and 277 mg/l) respectively. The fermentation time above 8 days (9.and 10 days) showed reduced levels of pigment (210 and 195 mg/l).Sharad [6] indicated that the maximum pigment production was obtained after 10 days of growth under dark conditions.

3.4- pH value

The effect of different pH value fermentation medium (4,5,6,7,8,9,and 10).The results presented in figure (4) and figure 4 showed that the maximum yield (244g/l) was obtained at pH 6 .The upper pH value (7,8, and 9) produced low yields(176,134,and121mg/l) respectively. On other hand, the lowest yield (111mg/l) was obtained at pH 10 . Because of enzymes are proteins, their molecular structure is determined by interactions between the charges of the amino acids that compose the protein chains. These interactions take the form of hydrogen bonds, which are altered by pH. These positive charges impact the charges of the amino acids inside the protein, making the enzyme more or less active depending on the enzyme's optimum pH[6,9].

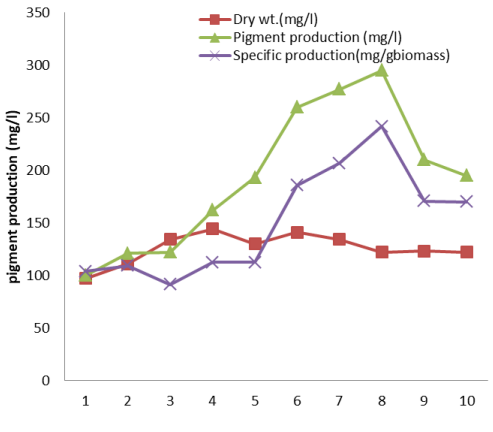


Figure 3: Effect of different fermentation time on the production of pigment

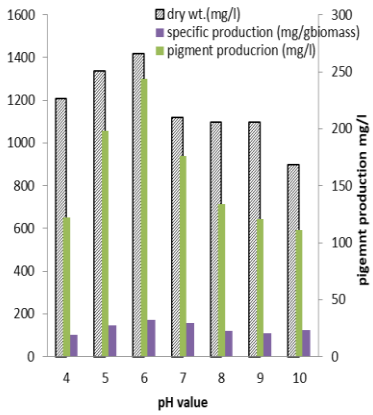


Figure 4 : Effect of different pH value of the fermentaton medium on the production of pigemnt

3.5-Effect of different metal salts on the production

In the present experiment the effect of different in organic salts on the production were tested. The results in table (5) revealed that the addition of magnesium sulphate showed enhancing effect (210mg/l) as well as manga nous sulphate 224mg/l).The results presented in table 1 revealed that the pigment production affected by the addition of some metal salts. The addition of calcium chloride ,magnesium sulphate and manganous sulphate showed stimulating effect (198 , 210 and 224mg/l) respectively. On the other hand, zinc sulphate and ferrous sulphate showed inhibitory effects[12].

Table 1: Effect of different metal salts on the production

Time (day)	Dry wt. (g/l)	Pigment production mg/ml	Specific production (mg/g biomass)
$MgSO_4$	1,15	210	182.60
$ZnSO_4$	1.21	139	114.87
KCl	1.34	198	147.76
$(NH4)_2SO4$	1.22	177	145.08
NaCl	1.21	189	156.19
$CaCl_2$	1.34	198	147.76
MnSO4	1.44	224	155.55
CuSoO4	1.33	188	141.35
FeSO4	1.23	167	135.77

Preparation of silver nano particles using separated pigment

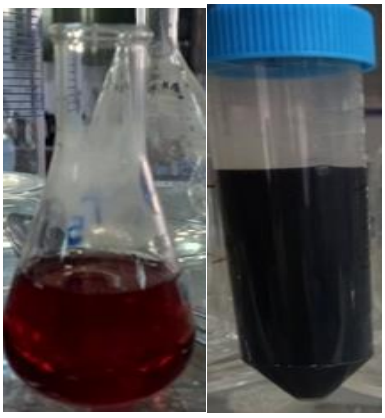
In this experiment, we presented a straightforward one-step, bottom-up approach for the synthesis of silver nanoparticle (Ag Np) utilizing silver nitrate salts and fungal red pigment derived from *Penicillium purpurgenium* culture. Fungal pigments act as reducing agents in the production of silver nanoparticles. They promote the conversion of silver ions (Ag^+) into metallic silver (Ag^0) via a range of biochemical mechanisms, leading to the formation of silver nanoparticles [13].

The initial observation figure 5 of the reduction of silver nitrate to AgNPs utilizing the pigment was conducted visually, followed by a spectrophotometric analysis. The solution's color progressively transitioned from red to dark brown, indicating the reduction of silver nitrate..

3.6-Characterization of the synthesized silver nanoparticles (AgNPs).

3.6.1-UV-Visible Spectrophotometer

UV-Vis spectroscopy serves as a crucial method for the preliminary validation of the biosynthesis of metal nanoparticles, as each type of metal nanoparticle exhibits a distinct surface plasmon resonance (SPR) characteristic **(***Hafeez et al.*, 2020). The absorption peaks of silver nanoparticles (AgNPs) are observed within the wavelength range of 400 to 500 nanometers [14].The UV-visible spectrum of the solution displayed a prominent peak around 410 nm, which is characteristic of the surface plasmon resonance (SPR) associated with silver nanoparticles (AgNPs), as illustrated in Figure 6. This results agree with Sharad[6].



A B

Figure 5: A: fungal pigment, B: silver nanoparticles formation

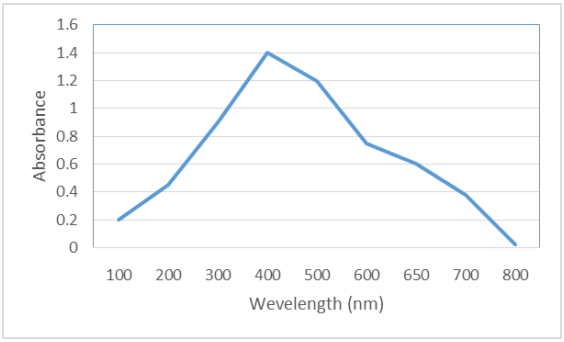


Figure 6 : UV-Vis spectrum for AgNPs

3.6.2-TEM analysis

The identification of nanomaterial structures is a crucial initial step in comprehending and controlling various properties. Transmission Electron Microscopy (TEM) analysis stands out as one of the most effective methods for obtaining direct images of nanostructures at atomic resolution. TEM analysis (fig 7) revealed a distribution of particles with spherical and hexagonal shapes with size from 4 to 70 nm [15].

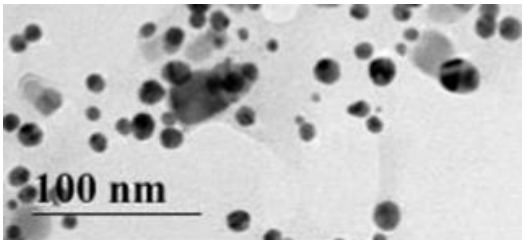


Figure 7 :TEM analysis

3.6-3-FTIR Analysis of Ag NP

FTIR serves to verify the presence of functional groups. In this investigation, FTIR was employed to analyze the silver nanoparticles aiming to identify the functional groups present in the pigment, which may have significantly contributed to the reduction and stabilization of AgNPs [6]**.** In figure 8 The bands 3325.764cm^{-1} and 1634.30 cm^{-1} ware assigned to N-H (amine group) and C=C (alkene) stretching vibration respectively which indicates presence of proteins, Band at 2066.73 cm^{-1} in the spectra corresponds to C≡C (alkyne)[16].The peak observed at 633 cm−1 was attributed to the –O–H bond formed between the oxygen atom of Ag2O and the hydrogen atom of the phenolic compound present on the surface of the nanoparticles, signifying the formation of silver nanoparticles (AgNPs) [17].

3.6.4-XRD of Ag NP

IN figure 9 the XRD of the AgNPs , The 2θ peaks observed at 27.5, 44.2708 , 64.3125 , and 77.222 align with (111), (200), (220), and (311) respectively indicates the silver nanoparticles are fcc and crystalline in nature (JCPDS file no. 84-0713 and 04-0783[16].

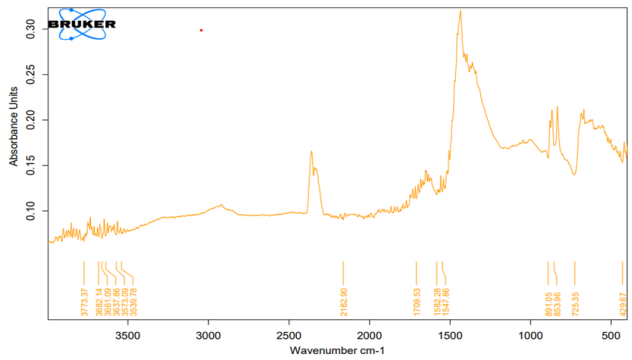


Figure 8 : FTIR of Ag NP

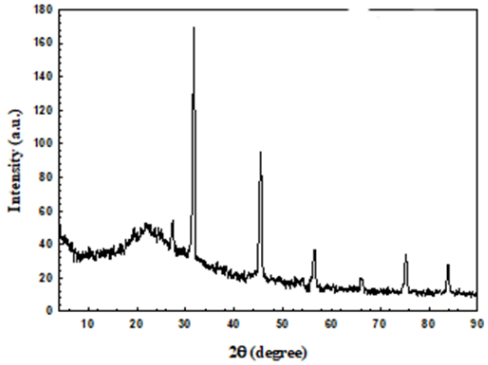
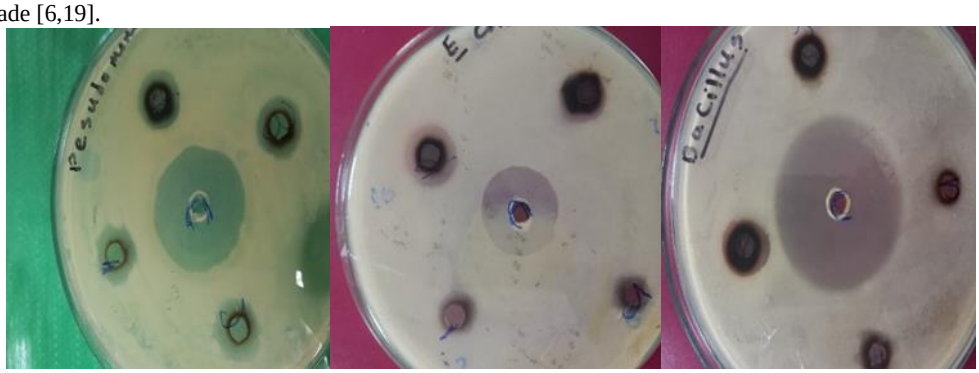


Figure 9 : XRD of Ag NP.

3.7-Antibacterial activity

In this study, the AgNPs demonstrated significant antibacterial efficacy against the Gram-negative bacteria (*Escherichia coli* NRRL B210 , *Pseudomonas aeruginosa* ATCC278531) and gram-positive bacteria (*Bacillus subtilis* NRRL-B543). The results presented in figure 10 showed that the inhibition zone were found to be 20 mm and 19 mm at 4mg/ml for *E.Coli* NRRL B210 and *P. aeruginosa* ATCC278531 ,respectively. *Bacillus subtilis* NRRL-B543 showed inhibition zone 12 mm at 4 mg /ml . From this results Ag NP more effective on Gram-negative bacteria than gram positive this results agree with **Slavin** [18] . Gram-negative bacteria are more susceptible to AgNPs because they have negatively charged lipopolysaccharides on their surface, which increase their interaction with the positive silver ions and ultimately cause the cell wall to degrade [6,19].



A B C

Figure 10: Antimicrobial activity of the obtained pigment nanoform against three bacterial pathogens (A : *P. aeruginosa* ATCC278531, *B. E.Coli* NRRL B210 and C: *Bacillus subtilis* NRRL-B543).,

The least inhibitory concentration (MIC) of any antibiotic is the lowest concentration required to prevent the observable growth of a bacterium in culture. Figure 11 findings demonstrated that Ag NP exhibited antimicrobial activity against every examined bacteria, with a minimum inhibitory concentration (MIC) ranging from 10 to 40 mg/ml. The MIC values of AgNPs against gram-negative bacteria (*Escherichia coli* NRRL B210, *Pseudomonas aeruginosa* ATCC278531) of 10 mg/mL. While gram-positive bacteria (*Bacillus subtilis* NRRL-B543) showed the MIC value of 30 mg/mL. Gram-negative bacteria are more susceptible to AgNPs because they have negatively charged lipopolysaccharides on their surface, which increase their interaction with the positive silver ions and ultimately cause the cell wall to degrade [20,21].

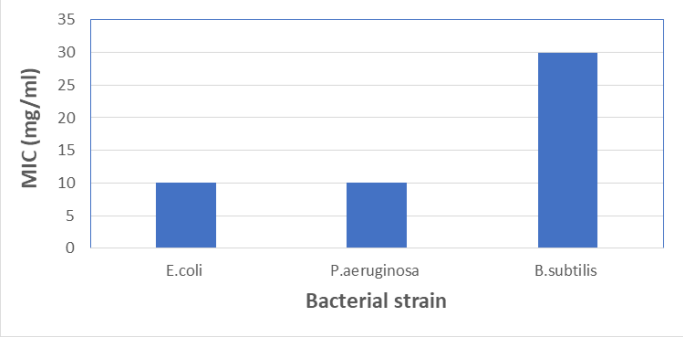


Figure 11: MIC of Ag NP.

4-Conclusion

In the last decades the sustainable application of natural pigment in many pharmaceutical. Medicinal, food industries were increased. The using of natural pigments has the priority synthetic dyes .In the present study the maximum natural red dye production was obtained by using *P. purpurgenium* at sucrose 20g/l , as a carbon source ,pH 6 fermentation time 8days as well as the presence of some inorganic salts specially Mn. Mg < K and Ca ions . the separated pigment was used to synthesized the silver nanoparticles .Tem analysis of the of the nanoparticles formed revealed that it has a size range from (4.70 nm) , and UV analysis range 400-500nm . The synthesized sliver anno partice showed antimicrobial activity against both g^{+ve} and g^{-ve} bacteria.

Conflicts of interest

"There are no conflicts to declare".

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