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Characterization of Gold Nanoparticle (NP-AU) Biosynthesis with Bioreductor *Chaetoceros calcitrans* as a Disease Inhibitory Power of AHPND in Vannamei Shrimp (*Litopenaeus vannamei*)

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ABSTRACT

Indonesia is the third largest global shrimp exporter after Ecuador and India. Shrimp production increased by 21% from 1.21 million tons to 1.48 million tons in 2021-2022, but decreased to 1.097 million tons in 2023. This is thought to be due to AHPND (*Acute Hepatopancreatic Necrosis Disease*). Our research aims to determine the biofabrication, characterization and effectiveness of gold nanoparticle biofabrication mediated by *Chaetoceros calcitrans* as an antibacterial against AHPND in white shrimp. The method used is experimental. The findings were the antibacterial potential of gold nanoparticle biofabrication mediated by *C. calcitrans* as an AHPND drug in vaname shrimp. Based on the research results, biofabrication was successfully formed with UV-vis characterization at a wavelength of 533 nm, in the FTIR test the AU^{3+} group was successfully reduced by the bioactive compound *C. calcitrans*. The results of the SEM and TEM tests showed that nanoparticles were dominated by a spherical shape, the particle size according to the PSA test was 8.25 nm and the XRD test answered that Np-Au crystallization had formed with the appearance of a high intensity peak.

Keyword: Biosynthesis, Gold Nanoparticle, *Chaetoceros calcitrans,* AHPND, Vannamei shrimp.

INTRODUCTION

Indonesia is the third largest global shrimp exporter after Ecuador and India. Shrimp production increased by 21% from 1.21 million tons to 1.48 million tons in 2021-2022, but decreased to 1.097 million tons in 2023 (KKP, 2023). Vanname shrimp farming is developed in several technologies and systems, including biofloc systems (Abidin, 2022). However, one of the obstacles that farmers still face is disease. *Acute Hepatopancreatic Necrosis Disease* (AHPND) is one of the causes of the decline in production, because it results in 100% death of whiteleg shrimp. As many as 35% of farmers are only able to complete one cultivation cycle with shrimp survival of 50% of the population (Anissa *et al.*, 2024). Therefore, innovation in handling AHPND is needed to prevent greater losses. Given that whiteleg shrimp is a leading export commodity whose productivity is targeted at 2 million tons in 2024 (Arasa *et al.*, 2024).

AHPND is caused by *V. parahaemolyticus* bacteria which have a unique virulent plasmid. The plasmid contains the Pir toxin gene. Pir toxin consists of ToxA and ToxB encoded by the Pir-A and Pir-B genes. This toxin forms a heterodimeric complex that binds to receptors on the shrimp hepatopancreas, causing acute necrosis (Nainggolan *et al.*, 2020). Shrimp infected with AHPND show symptoms like lethargy, anorexia, slow growth, empty

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digestive tract, and pale to white hepatopancreas (Suryana *et al.*, 2023). *V. parahaemolyticus* infects shrimp for 30 days after the spread of the fry.

AHPND in whiteleg shrimp is treated with siphoning and antibiotics. Siphoning is not effective in reducing ammonia levels in the water, so that shrimp experience stress and shrimp growth is inhibited (Parikan and Rahim, 2021). Meanwhile, high doses of antibiotics cause bacterial resistance and leave residues. Rezaee *et al.* (2020) showed that bacteriophages can inhibit *V. parahaemolyticus* bacteria, but also cause resistance to pathogenic bacteria. Sun *et al.*, (2023) only reported that gold nanoparticles can diagnose *V. parahaemolyticus* in whiteleg shrimp, but not as antimicrobials.

Gold nanoparticles are a type of metal nanoparticles used as drug carriers and stabilizers. Metal nanoparticles have a unique non-specific toxicity mechanism, causing bacteria to be non-resistant (Hassan *et al.*, 2023). However, its toxicity is high in target organisms and the environment. The biosynthesis method of metal nanoparticles with herbal plants, phytoplankton, and polychaeta can reduce its toxicity. Secondary metabolites in natural materials such as: amino acids, citric acid, terpenoids, polyphenols, and polyols can function as reducing agents as well as capping agents (stabilizers) for nanoparticle metal ions (Pal *et al.*, 2019; Wang *et al.*, 2019). This method is superior because it is environmentally friendly, energy efficient (room temperature), and minimal use of chemicals. Muniz *et al.* (2020) reported that the results of silver nanoparticle biosynthesis can inhibit AHPND disease. However, the toxicity of silver nanoparticles is higher than gold nanoparticles (Talarska *et al.*, 2021). Hassan *et al.* (2023) further researched gold nanoparticles with *M. morbidi* (polychaeta) as an antibacterial for AHPND. The bioactive content of phenols, esters, and fatty acids in *M. morbidi* can reduce toxicity and stabilize Au^{3+} metal ions into Au^{0} nanoparticles. However, the extraction of active ingredients in *M. morbidi* is considered suboptimal due to the less polar and organic solvents. Secondary metabolites are polar and easier to extract with organic solvents, such as ethanol (Arioen and Indriyani, 2022). *M. morbidi* is very difficult to obtain, because it is located on the coast of Malaysia. Therefore, it is necessary to have an alternative *M. morbidi* as a reducer and stabilizer of gold nanoparticles that is easily obtained, abundant and extracted with appropriate solvents to obtain more secondary metabolites.

C. calcitrans is a cosmopolitan of the Bacillariophyceae type and is widely found on the coast of Pamekasan, East Java, up to 700–9100 ind/L in July-August (Indriyawati *et al.*, 2023). *C. calcitrans* contains many secondary metabolites: terpenoids, flavonoids, tannins, and steroids that can inhibit pathogenic bacteria (Okunowo, 2019). Soto-rodriguez *et al.* (2021) reported that hydrophilic compounds of *C. calcitrans* cells have antibiotic activity (Vp M0904) that can trigger AHPND in shrimp. However, its antibiotic activity is still low so it needs to be optimized. Optimal polychaeta extraction is carried out using methanol (Shantar *et al.*, 2021). However, methanol is toxic so that similar alternatives such as ethanol are needed which are safer. Therefore, secondary metabolites of *C. calcitrans* are very potential as reducing agents and stabilizers (capping agents) of gold nanoparticles through biosynthesis methods to produce effective AHPND antibacterials at low doses, do not cause bacterial resistance and residues so that they can be used in the long

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term.

RESEARCH METHODOLOGY

Type of Research

This type of research is experimental, namely a research method used to find the effect of certain treatments carried out directly by observing health protocols.

Time and Place of Research Implementation

The research was carried out for four months (May-August) in the integrated Labs one and five FIKKIA, FST Analytical Chemistry Lab, LIHTR Engineering, FTMM Nanotechnology Engineering Research Center Lab, Mpl-3 Lab, Faculty of Pharmacy, UNAIR Institute Tropical Disease Freeze Dry Lab and TEM Service Unit, FMIPA UGM.

Research Tools and Materials

Tools used in this research: hot plate, magnetic stirrer, autoclave, micropipette, incubator, vortex, rotary evaporator, petri dish, test tube, test tube rack, refrigerator, centrifuge, centrifuge tube, dropper, volume pipette, bulb, ose, drigalski, tweezers, analytical balance, 100 ml erlenmeyer flask, laminar air flow, bunsen, stirrer, incubator shaker, oven, cuvette, measuring flask, measuring cylinder, petri dish, 250 ml beaker, UV – Vis spectrophotometer (Shimadzu UV-1800), PSA (Biobase BK-802N), SEM (Thermo Fisher Scientific Phenom P-Series), TEM (Jeol JEM-1400), FTIR (Shimadzu, IRTracer-100) and XRD (Rigaku, Japan).

Materials used in research namely: *C. calcitrans*, 96% ethanol, $HAuCl_4.3H_2O$ 1 mM, *V. parahaemolyticus* isolate as much as 108 CFU/ml (0.5 McFarland standard), TCBS (*Thiosulfate Citrate Bile Salt Sucrose*) media, PCA (*Plate Count Agar)*, MHA (*Mueller Hinton Agar*), NB (*Nutrient Broth*), distilled water, disc paper, microtips, tissue, and 70% alcohol.

Research Variables

Independent Variable: Concentration of *C. calcitrans*-mediated Np-Au solution (70%; 75%; 80% (v/v)). Control Variables: *C. calcitrans* extract, $HAuCl_4.3H_2O$ solution, temperature, pH and biosynthesis time of *C. calcitrans*-mediated Np-Au, and density of *V. parahaemolyticus* strain bacteria. Dependent Variable: Antibacterial activity of *C. calcitrans*-mediated Np-Au.

Research Procedures

Extraction of C. calcitrans

Extraction of *C. calcitrans* was adopted from Maftuch *et al.* (2018). *C. calcitrans* was obtained from BPBAP (*Center for Brackish Water Cultivation*) Jepara. *C. calcitrans* powder was soaked in 96% ethanol (1:6). Macerated for 1x24 hours, filtered, and concentrated using a vacuum rotary evaporator at 60ºC until it became a paste.

Biofabrication of Gold Nanoparticles (Np-Au) Mediated by C. calcitrans

Biofabrication of Np-Au which started by taking 20 ml of 1 mM make sure solution plus 5 ml of crude extract of *C. calcitrans,* adjusted pH = 7.8. The mixture was stirred for 1 hour until the color changed to ruby red or purple, incubated for 24 hours and the color change was observed periodically. The obtained Gold Nanoparticles (Np-Au) were characterized by UV-Vis, FTIR, SEM, TEM, PSA, and XRD.

Characterization Techniques and Data Analysis

UV-Vis Analysis

UV-vis characterization to determine the SPR value and maximum wavelength of the stable Gold Nanoparticles (Np-Au) absorption graph (Au^0).

PSA (Particle Size Analyzer) Analysis

PSA characterization to determine the size

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and distribution of gold nanoparticles (Np-Au). PSA characterization is measured based on the hydrodynamic diameter (Dh) and polydispersity index (PDI) which indicates the particle size distribution.

RESULT AND DISCUSSION

Results of *C. calcitrans*-Mediated Np-Au Biofabrication

The initial indicator of the success of Gold Nanoparticle (Np-Au) bio-fabrication is marked by a change in the color of the solution. The color change of the gold solution from pale yellow (Figure 1a) to ruby red after the addition of *C. calcitrans* extract and 24-hour incubation.

The ruby red color indicates that stable Np-Au has been formed. This is in line with the research of Hassan *et al.* (2023), that the color change from yellow to dark red to blue or purple indicates the formation of gold nanoparticles. This phenomenon is caused by the reduction of Au^{3+} ions to Au^{0} by secondary metabolite compounds of *C. calcitrans* extract such as: phenols, amino acids, terpenoids, and flavonoids (Okunowa., 2019). Reduction occurs due to the interaction of electrostatic forces of hydroxyl and carbonyl groups in negatively charged secondary metabolites with the positive charge of the Au^{3+} gold solution.

UV-Vis Analysis

The ruby red color of Np-Au was characterized quantitatively by UV-vis to determine the maximum Surface Plasmon Resonance (SPR) (λmax nm). The excitation of SPR vibrations in the Np-Au solution causes the color change. The typical SPR of gold nanoparticles is at 520 nm - 560 nm (Ee Pei *et al.*, 2020). Based on Figure 1, stable Np-Au was successfully biofabricated with the help of the capping agent *C. calcitrans,* showing the emergence of a single maximum absorption at λ = 533 nm. The SPR of Np-Au which is in the range of 530-555 nm is possible to be spherical (Osibe and Aoyagi, 2019). The difference in the size of gold nanoparticles also affects their absorbance. The absorbance value of gold nanoparticles measuring 10-100 nm has an SPR range of 517-575 nm (Manzila *et al.*, 2020). However, the shape and size of the particles will be confirmed more clearly by SEM characterization.

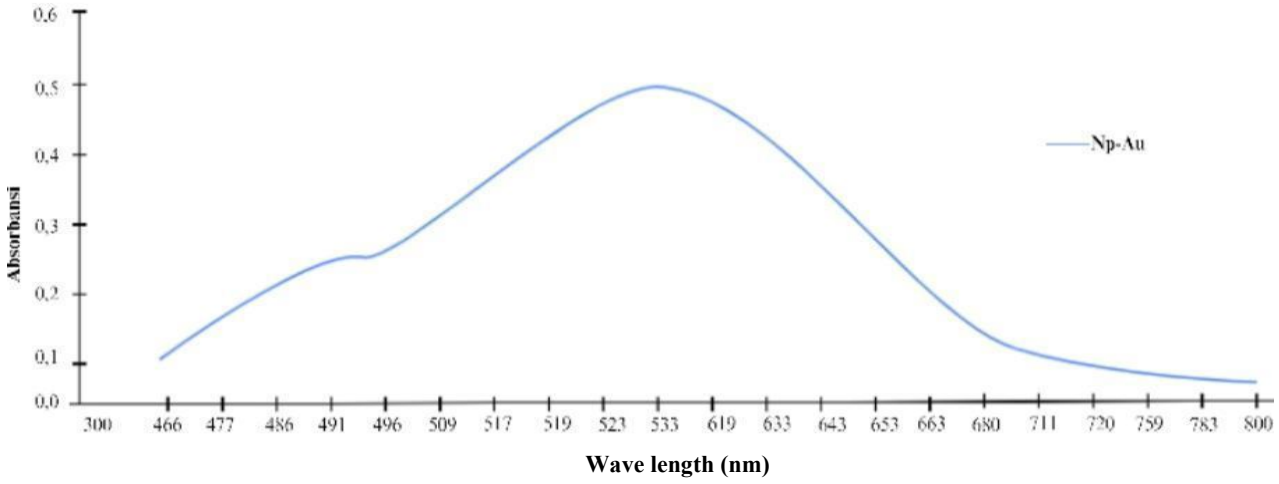

Figure 1. Results of UV-Vis Np-Au

FTIR Analysis

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Functional groups of *C. calcitrans* extract such as: amines, carbohydrates, proteins, and amino acids that can act as capping agents, reducers, and stabilizers of gold nanoparticles. Based on the FTIR results (Figure 2), there is a broad peak with high intensity at 3473 cm-1 indicating the stretching vibration of -OH phenolic *C. calcitrans* extract (Fouda *et al.*, 2022). Absorption at 2366.66 - 2335.80 cm-1 indicates stretching vibration of C≡C bonds (alkyne) (Geetha *et al.*, 2013) 2073 cm/cm broad absorption with low intensity is the stretching vibration of the HC≡N group (Ee Pei *et al.*, 2020). Reduction in the biofabrication of Gold Nanoparticles (Np-Au) by *C. calcitrans* was successfully carried out, as indicated by the presence of absorption at wave number 1639 cm^{-1} from the symmetric stretching vibration of C=O of flavonoid functional groups or carboxylate ions, and 1361 cm-1 indicating the stretching vibration of C-N aliphatic amines both of which originate from the polypeptide amino acid residue of the Np-Au ligand which functions as a caping agent (stabilizer) of Np-Au (Suryakala *et al.*, 2022), and 601 cm-1 indicating Au-O. These characteristics indicate that the typical functional groups of *C. calcitrans* are around Au^0 on Gold Nanoparticles (Np-Au) after biofabrication occurs.

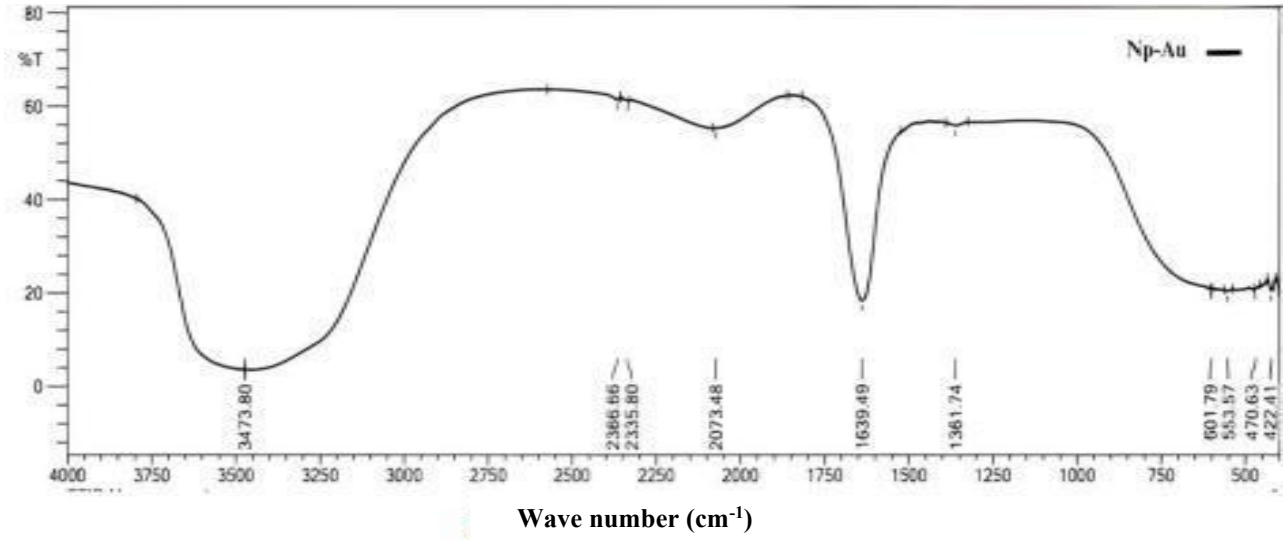

Figure 2. Results of FTIR

SEM Analysis

The morphology of biofabricated Np-Au is predominantly spherical (Figure 3a). These results are consistent with the biosynthesis of gold nanoparticles using natural materials and polychaeta (Suryakala *et al.*, 2022; Hassan *et al.*, 2023). In addition to being spherical, there is also an irregular morphology at higher magnification (Figure 3b).

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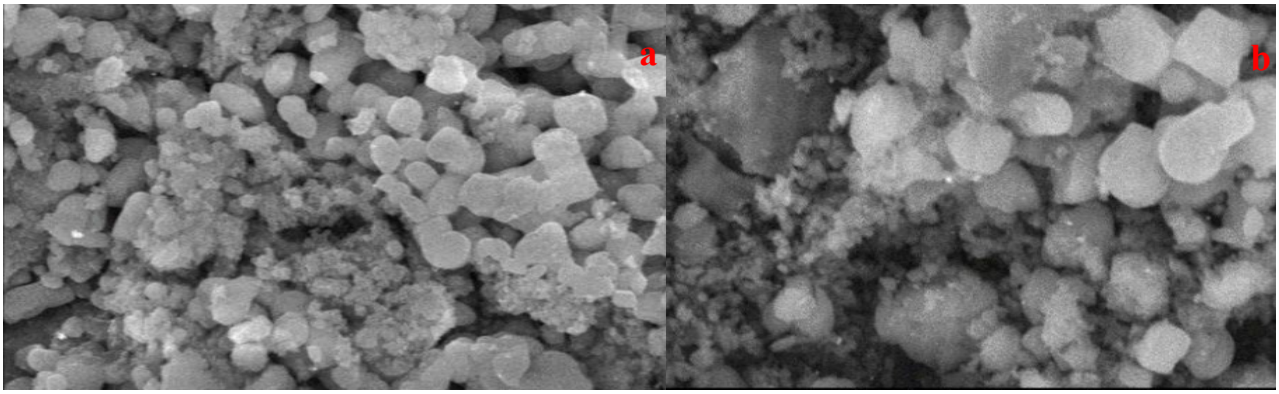

Figure 3. a) SEM 25,000x magnification, and b) SEM 30,000x magnification.

TEM Analysis

The morphology of Np-Au biosynthesis results in TEM is in line with SEM, which is dominated by a round shape (Figure 4a). Irregular trigonal and tetragonal shapes were also confirmed at magnifications of 20,000 and 80,000 times (Figures 4b and 4c). Meanwhile, the size of the Np-Au particles produced also varies, namely: 50, 100, and 200 nm. The size of Np-Au is influenced by the pH of Np-Au biosynthesis (pH 7.8). The lower the pH, the larger the particle size because the hydroxyl group on the capping agent is protonated, reducing its reactivity (Annur, 2018). Therefore, the distribution of Np-Au particle sizes varies.

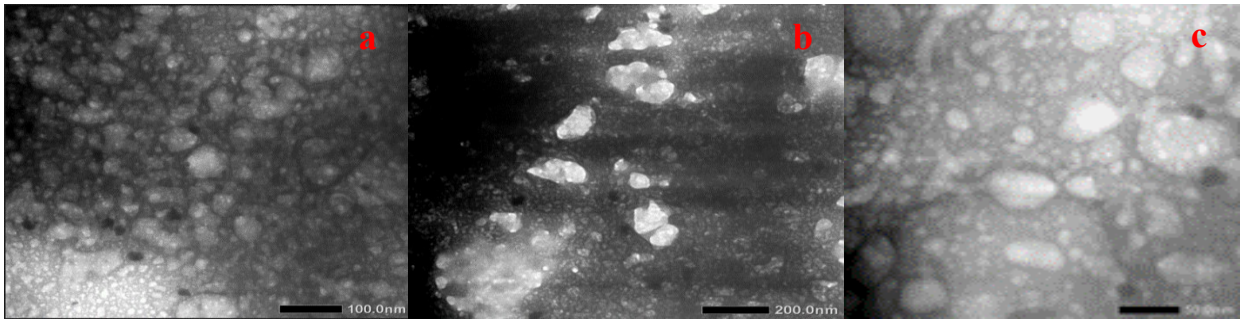

Figure 4. TEM Results of Np-Au Magnification a) 40,000x, b) 20,000x, c) 80,000x

PSA Analysis

Based on the results of the PSA analysis (Figure 5), it shows that the average particle size of Gold Nanoparticles (Np-Au) biosynthesized with *C. calcitrans* is 8.25 nm, in line with the statement of Osibe and Aoyagi (2019) regarding the particle size of gold nanoparticles ranging from 2-20 nm. This value is smaller than the TEM results. This is possible because TEM can show the morphology and diameter of gold nanoparticles with high accuracy compared to PSA which is only on certain sample samples. However, these results are in accordance with the morphology of Np-Au which tends to be round if the particle size is <20 nm (Osibe and Aoyagi, 2019). Thus, the particle size of the Np-Au formed varies, namely: 8.25, 50, 100, and 200 nm. The PDI (Polydispersity Index) of Np-Au was obtained at 0.12, indicating that the particle size was distributed polydisperse and homogeneous. Because the PDI value is in the appropriate range, which is <0.500 (Ee Pei *et al.*, 2020). This statement strengthens the previous TEM results which showed a variety of Np-Au morphologies, namely round, trigonal and irregular tetragonal.

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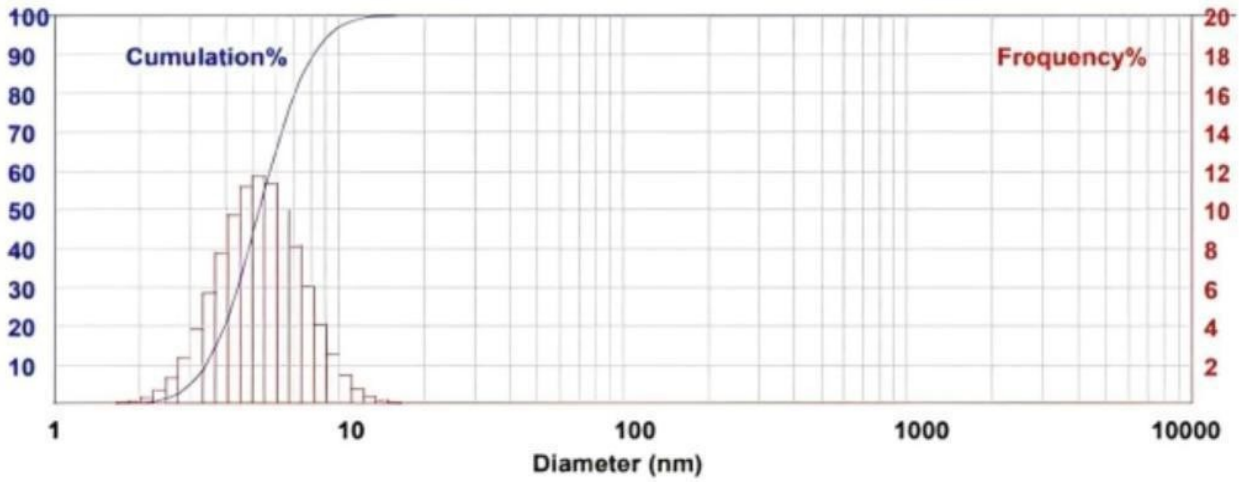

Figure 5. PSA Results of Np-Au

XRD Analysis

Based on the diffractogram (Figure 6), there are four characteristic diffraction peaks of Np-Au 2θ at 31.7, 45.4, 66.2, 75.2, and 83.9, which correspond to the Bragg diffraction standards at (111), (200), (220), and (311) respectively (Hassan *et al.*, 2023). There is a shift in the 2θ peak of Np-Au from the gold nanoparticle standard according to JCPDS 04-0784. This peak indicates the purity of the gold nanoparticles formed, so the shift can be caused by several factors, namely: 1) the content of abundant secondary metabolite compounds of *C. calcitrans* bound to the surface of Np-Au, 2) oxidation of hydroxyl groups in the capping agent, and 3) changes in the fcc (face centered cubic) crystal lattice of gold nanoparticles (Suryakala *et al.*, 2022).

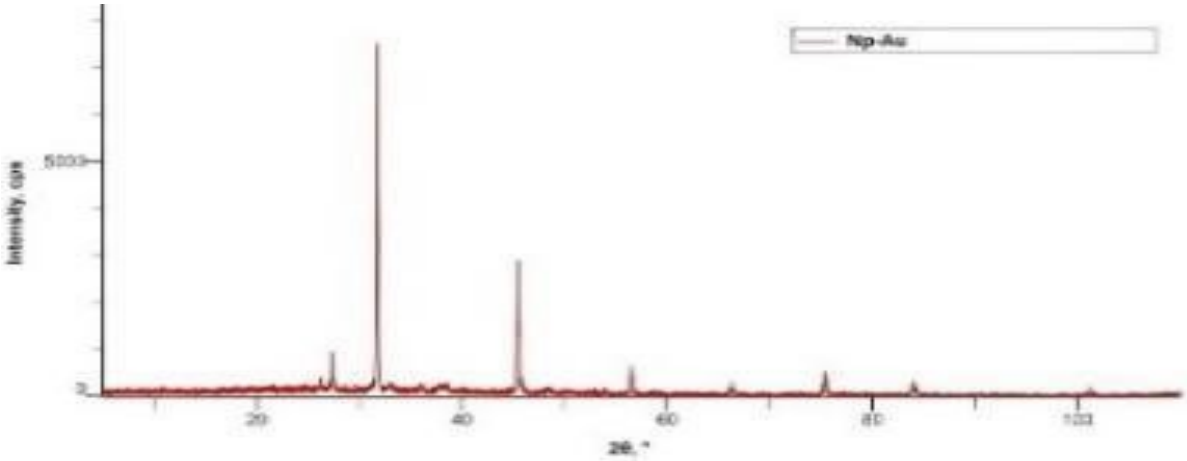

Figure 6. XRD Results of Np-Au

CONCLUSION

Based on the research results, biofabrication was successfully formed with UV-vis characterization at a wavelength of 533 nm, in the FTIR test the AU^{3+} group was successfully reduced by the bioactive compound *C. calcitrans*. The results of the SEM and TEM tests showed that nanoparticles were dominated by a spherical shape, the particle size according to the PSA test was 8. 25 nm and the XRD test answered that Np-Au crystallization had formed with the appearance of a high intensity peak.

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AUTHORS' CONTRIBUTIONS

The contributions of each author are as follows, ARP; collect data and design research. DD and SA; realizing research. DPH; perform data analysis and create graphs. MICG; write scientific articles. All authors discussed the results and contributed to the end.

CONFLICTS OF INTEREST

All authors declare that they have no conflicts of interest.

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