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Green Synthesis of Copper Nanoparticles using a Bioflocculant Extracted from *Pseudomonas yamanorum* and their Characterization

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This study used a bioflocculant produced by *Pseudomonas yamanorum* as a reducing and stabilizing agent in the green synthesis of copper nanoparticles (CuNPs). Several techniques like UV-Visible absorption, Fourier transform infrared (FT-IR), X-ray diffraction (XRD), scanning electron microscopy (SEM), energy dispersive X-ray (EDX), thermogravimetric analysis (TGA) and transmission electron microscopy (TEM) were used to characterize the bioflocculant and biosynthesized CuNPs. Elemental analysis showed the existence of various elements, including copper (10.30 wt.%) in the CuNPs. FT-IR analysis showed carboxyl, hydroxyl and amide groups being present in the samples. The samples appeared almost spherical and agglomerated through SEM and TEM analysis, with sizes between 40 and 50 nm. The UV-visible spectra showed absorption peaks at 280 nm (bioflocculant) and 580 nm (CuNPs). After exposure to a high temperature of 900 ºC, the samples retained more than 60% of their weight, indicating good thermal stability.

Keywords: Bioflocculant, Biosynthesis, Copper nanoparticles, *Pseudomonas yamanorum*.

INTRODUCTION

A field in science called nanotechnology was introduced in the 20th century and is concerned with manipulating atoms and molecules between 1-100 nanometers (nm) in size [1]. Nanotechnology was introduced to synthesize nanoparticles with the potential to benefit humans and have a variety of shapes, sizes, chemical compositions and controlled dispersion [2]. The chemical and physical properties that nanoparticles possess are a result of their increased surface area and nanoscale size [3]. Various methods to synthesize nanoparticles such as biological, chemical and physical methods have been reported. However, synthesizing nanoparticles using physical methods is known to be time-consuming and energy-intensive [4]. The chemical methods use toxic chemicals to synthesize nanoparticles, making the produced nanoparticles unsuitable for cosmetic, medical and food applications [5]. Therefore, to mitigate such problems, the creation of low-cost, non-toxic and eco-friendly processes for synthesizing nanoparticles is necessary [6].

Copper nanoparticles have been gaining much attention over the years, among other metal nanoparticles under study [7,8]. CuNPs are inexpensive compared to various noble metals like platinum, gold and silver. In addition, CuNPs possess significant properties that include biocidal, magnetic, catalytic, optical, heat transfer and high surface area-to-volume ratio properties [9,10]. The synthesis of CuNPs can be achieved using different techniques including chemical reduction [11], vapour deposition [12], thermal decomposition [13], electrochemical reduction [14] and radiolysis [15]. Although CuNPs can be synthesized using the aforementioned techniques, there are still some limitations, such as the need for pressurized conditions, high temperatures and hazardous chemicals during the copper reduction process [16,17].

The green synthesis method is advantageous compared to these other methods in that it is simple, inexpensive and doesn't use toxic substances [18]. The harmlessness, biodegradability and minimal secondary pollution of microbial flocculants such as bioflocculants have drawn a great deal of interest [19]. Microbial flocculants in the wastewater treatment industry are used to remove suspended particles, at which point colloids emerge from suspension as flocs or flakes [20]. The bioflocculant is utilized in the chemical reduction process to get around the toxicity issues associated with reducing agents and to synthesize CuNPs in an eco-friendly way [21]. In this study, a safe, affordable and environmentally friendly method was used to synthesize CuNPs whereby a bioflocculant was used as a reducing and protective agent. Bioflocculants have been implicated in the synthesis of various nanoparticles [22-25].

The activated sludge was used to isolate *Pseudomonas yamanorum* MG269627.1 as a promising bioflocculant producer that belongs to the family *Pseudomonadaceae* at a treatment plant located at KwaDlangezwa, on the north-east coast of KwaZulu-Natal, Republic of South Africa. *P. yamanorum* is aerobic, straight rod-shaped and non-spore-forming and contains cells that are Gram-negative [26]. The bacterium is motile using two flagella and most commonly found in moist areas, soil and water [26]. It is one of the least studied species in the genus *Pseudomonas* but has been reported in industrial applications [27-29]. Bioflocculants produced by different *Pseudomonas* species have been documented [30-32], but studies on *P. yamanorum* as a bioflocculant producer have not been reported. Therefore, this study aimed to biosynthesize and characterize copper nanoparticles (CuNPs) using the bioflocculant from *P. yamanorum* MG269627.1. Confirmation of the formation of CuNPs was done by evaluating the acquired morphology, functional groups, elemental composition, thermal stability, UV-Visible adsorption and crystallinity using SEM, TEM, FT-IR, SEM-EDX, TGA, UV-visible spectroscopy and XRD analysis.

EXPERIMENTAL

Medium for bioflocculant production: A fermentation medium containing 20 g of fructose, 5 g of K_2HPO_4, 2 g of KH_2PO_4, 0.5 g of yeast extract, 0.2 g of $(NH)_2SO_4$, 0.2 g of $MgSO_4$ and 0.1 g of NaCl was prepared in 1 L of filtered activated sludge wastewater at an adjusted optimum pH of 7 and sterilized for 15 min at 121 ºC. The medium was left to cool down followed by inoculation with a fresh culture broth (1 mL) (v/v) of the previously isolated *P. yamanorum* MG269627.1 and placed in a shaking incubator at an optimum speed of 160 rpm for 72 h at 30 ºC [33].

Extraction of bioflocculant and its purification: The production medium (1 L) after being inoculated with the bacterial suspension (2% v/v) was incubated at an optimum temperature of 30 ºC. Following 72 h of incubation, the culture broth was exposed to centrifugation for 15 min at 8000 rpm with the temperature adjusted to 4 ºC. The supernatant was collected and added to 1 L of distilled water (dH_2O) and put through centrifugation again using previous conditions. The supernatant was recollected and added to 2 L of cold ethanol and mixed well before being placed at 4 ºC for 12 h. A precipitate formed after 12 h, from which the crude bioflocculant was obtained and vacuum dried. To purify the obtained crude bioflocculant, a solution (1% w/v) was prepared by dissolving the crude bioflocculant into 100 mL of dH_2O, followed by the addition of a chloroform-butanol mixture in a 5:2 ratio (v/v) and thoroughly mixing the solution. The purified bioflocculant powder was collected after the mixture had been left in a separating funnel at room temperature for 12 h, separated and vacuum dried [34].

Biosynthesis of CuNPs: In distilled water (200 mL), 3 mM $CuSO_4$ and 0.5 g of freshly prepared bioflocculant were added and mixed. To allow the solution to precipitate, it was stored at room temperature overnight while covered with a foil. After storage, the biosynthesized CuNPs were obtained through centrifugation for 5 min at 8000 rpm and vacuum dried [35].

Characterization: The samples were investigated for UV-visible spectra using a Varian Cary 50 UV-Vis spectrophotometer (California, USA). The Bruker Tensor 27 FT-IR spectrophotometer (Gauteng, South Africa) with a resolution of 4 cm^{-1} was used to acquire the FT-IR spectrum of the samples over a wavenumber ranging from 400 to 4000 cm^{-1}. The Bruker D8 ADVANCE powder X-ray diffractometer (Johannesburg, South Africa) was employed to examine the XRD patterns of the samples. The X-ray generator operated at 40 kV, current at 40 mA and copper monochromatized Cu-$K\alpha_1$ radiation (λ = 1.5406 Å). Diffraction intensities were recorded from 20º to 80º at scanning steps of 0.03º in 2θ scanning mode and at a scanning rate of 4º/min. Carl Zeiss FE-SEM Sigma VP-03-67 (Oberkochen, Germany) was utilized to analyze the surface morphology properties of the samples at operating voltages of 20 kV. The elemental analysis of the bioflocculant and CuNPs was performed using an Oxford Instruments X-MaxN 50 model 54-XMX1003 EDX analyzer (Oberkochen, Germany). On the slide coated with silicon, 5 mg of samples were each placed and fixed with a spinner coater for 60 s at 1000 rpm before SEM analysis. The JEOL 1010 transmission electron microscope (Massachusetts, USA) was employed for TEM images of the bioflocculant and biosynthesized CuNPs operating at an accelerating voltage of 100 kV. Sample preparation was done by placing a diluted drop of the suspension that had been diluted using a micropipette in toluene on carbon-coated copper grids (150 mesh) and leaving the sample to utterly dry. A Mega-View III camera was used to capture the images. Thermogravimetric analysis of the samples (10 mg) was conducted using a Perkin-Elmer Thermal Analysis Pyris 6 TG analyzer (Massachusetts, USA) at 10 ºC/min, over a temperature between 40-900 ºC and using nitrogen gas at a constant rate of 40 cc/min to obtain a pyrolysis profile.

RESULTS AND DISCUSSION

The purified bioflocculant from *P. yamanorum* as a capping agent was used in the biosynthesis of CuNPs and the observation of colour change of the experimental sample in the presence of aqueous $CuSO_4$ signified the synthesis of the CuNPs and confirmed using a UV-visible spectrophotometer. The sample solution turned from white to blue, which was due to the formation of the chemical reaction whereby $CuSO_4$ was reduced in the presence of bioflocculant, yielding CuNPs [36]. Thus, the surface plasmon vibration being excited was regarded as the basis for the formation of blue colour [37]. The experimental control showed no colour change. The solution changing colour during the biosynthesis of CuNPs is an indication of CuNPs formation as reported [38].

UV-Visible spectral studies: The UV-Vis absorption spectrum of the bioflocculant recorded an intense peak at 280 nm, while an absorption peak at 580 nm was recorded in the CuNPs, which could be due to the formation of CuNPs (Fig. 1). Often, in the range between 550-650 nm, a surface plasmon resonance peak is exhibited in the CuNPs [39-41]. The nanoparticles properties, including capping agent, size, shape and nature of the surrounding medium all affect the wavelength of oscillation [42]. However, according to Mie's theory, particle size has no effect on the spectra of particles with radii between 2 and 10 nm, whereas a broadening and longer wavelength absorption peak is observed in particles larger than 10 nm [43]. The results are in agreement with *Aloe vera* extract mediated biosynthesis of CuNPs, with an absorption peak at 578 nm [44]. The bioflocculant produced by *Enterobacter aerogenes* also observed a peak at 280 nm [45].

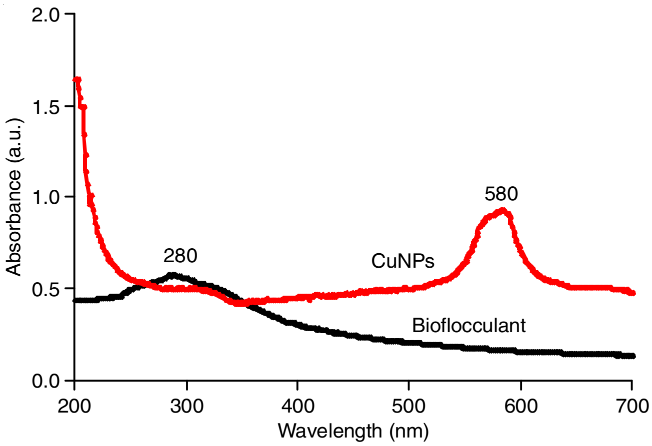


Fig. 1. UV-Visible spectrometric analysis of the (a) bioflocculant and (b) biosynthesized CuNPs

FT-IR spectral studies: The IR spectra of the bioflocculant and biosynthesized CuNPs is displayed in Fig. 2, which exhibits the existence of different functional groups. The absorption peaks at 3262 and 3269 cm^{-1} in the bioflocculant and CuNPs, respectively, correspond to O-H stretching and stretching vibrations of N-H in the amide group [46,47]. Furthermore, the absorption bands at 1643 cm^{-1} (bioflocculant) and 1650 cm^{-1} (CuNPs) could be attributable to the C=O stretching vibration in the amide group or NH_2 bending [48]. The C-N bending vibrations correspond to the peak at 1161 cm^{-1} in the bioflocculant [49]. The C-O-C group in the bioflocculant and CuNPs is represented by the peaks in the region of 1025 cm^{-1} [50]. The absorption bands between 1200 and 800 cm^{-1} in both samples are linked to the polysaccharide bands and suggest the presence of alcohol and ether -C-O- bonding [50]. The small absorption peak at 1244 cm^{-1} (CuNPs) signifies sugar existence shown by C-O bonds [51]. The formation of CuNPs is confirmed by the absorption peak at 528 cm^{-1}, which correlate with the Cu-O bond [52]. In addition, furan saccharides are shown by the peak at 882 cm^{-1} in bioflocculant [53]. The existence of carboxyl, hydroxyl and amide groups was validated in the FT-IR spectra, which was compatible with most results on bioflocculants and CuNPs produced through green synthesis [54,55]. However, the FT-IR spectrum of bioflocculant extracted from *Bacillus velezensis* displayed the presence of aliphatic bonds, carboxylates and methoxyl groups [56].

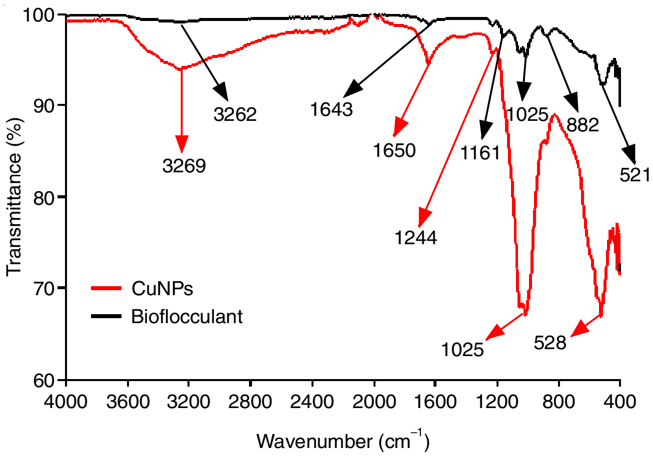


Fig. 2. FT-IR analysis of the bioflocculant and biosynthesized CuNPs

XRD studies: The bioflocculant and biosynthesized CuNPs were subjected to XRD analysis (Fig. 3). The bioflocculant displayed distinct diffraction peaks at 2θ with peaks observed between 10 and 45° angles (2θ). The distinct diffraction peaks were observed at 11.52°, 13.76°, 15.97°, 23.79°, 25.53°, 26.66°, 27.86°, 29.25°, 31.87°, 40.41°, 41.48° and 43.07° in the bioflocculant (Fig. 3). The existence of sharp peaks in the XRD patterns is assigned to the crystallinity of sample, while the broadening of the peaks is related to the size of the particles. The smaller the particle, the broader the peaks, therefore, the bioflocculant and CuNPs were crystalline in nature as they contained sharp peaks and contained bigger particles as there were no broad bands. The CuNPs had distinct diffraction peaks at 43.78°, 50.66° and 75.84°. These peaks corresponded to the (111), (200) and (220), respectively, of the face-centered cubic copper lines (JCPDS card No. 04-0836). Additional peaks at 29.31° and 35.71° are associated with the (110) and (020) planes,

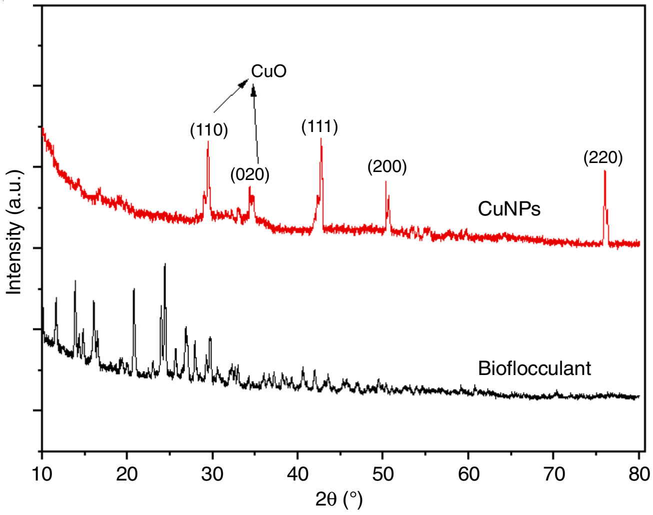


Fig. 3. XRD patterns of the bioflocculant and synthesized CuNPs

which are consistent with CuO [17]. Employing the Debye-Scherrer equation:

$$D = \frac{k\lambda}{\beta\cos\theta}$$

Here, K (0.94) is the Scherrer's constant; D is the CuNPs crystalline size; λ is the X-ray source wavelength (0.154 nm); β is the full width at half maximum and θ is the Bragg angle. Through green synthesis, the bioflocculant-passivated CuNPs crystals had an average size of 42 nm.

SEM studies: The surface morphology of the bioflocculant (Fig. 4a) and CuNPs (Fig. 4b) at a magnification scale of 5 µm was determined using the scanning electron microscope. The SEM micrograph of the bioflocculant appeared flake-shaped, almost spherical and agglomerated, while the CuNPs were predominantly spherical. The majority of nanoparticles acquire a spherical morphology, accompanied by agglomeration in small aggregates [57].

EDX analysis: Elemental analysis was done to evaluate the composition of elements in pure bioflocculant and biosynthesized CuNPs. The results display that the bioflocculant (Fig. 5a) has an elemental composition of O (49.88 wt.%), P (20.82 wt.%), Ca (10.40 wt.%), Mg (9.31 wt.%), C (4.68 wt.%), K (2.43 wt.%), Cl (1.72 wt.%), S (0.67 wt.%), Al (0.11 wt.%) and Na (0.08 wt.%). Oxygen had the highest presence in bioflocculant, while sodium had the lowest percentage weight. Carbon and oxygen elements being present in the structure indicate carbohydrates are present as the main component. The CuNPs constitute elements such as O (42.71 wt.%), C (22.62 wt.%), Cu (10.30 wt.%), P (10.96 wt.%), Ca (7.39 wt.%), Mg (4.08 wt.%), Na (0.84 wt.%), S (0.74 wt.%), Al (0.32 wt.%) and Cl (0.04 wt.%) (Fig. 5b). Oxygen was the highest element in the synthesized CuNPs with 42.71 wt.%. Cu was the 4th highest element present with 10.30 wt.%, which confirms that CuNPs were able to be synthesized using a bioflocculant. The existence of elements including Al, C, Ca, Cl, Mg, Na, P and S was due to the interaction with the bioflocculant during the formation of CuNPs. The presence of these various elements may have contributed to the stability and flexibility of the bioflocculant and biosynthesized CuNPs [58].

TEM studies: The shape and size of the bioflocculant and biosynthesized CuNPs were studied using TEM technique. The majority of the bioflocculant particles were spherically shaped and agglomerated (Fig. 6a). The CuNPs were also predominantly close to spherical-shaped structures (Fig. 6b), having particle sizes between 40 and 50 nm, which corresponds with 42 nm particle size calculated from the XRD pattern. The nanoparticles shape and size is determined by the kind of reducing and stabilizing agents utilized [23]. The study results are in close conformity with the CuNPs synthesized using *Streptomyces griseus,* which had sizes ranging from 30 to 50 nm and were predominantly spherical and aggregated [59].

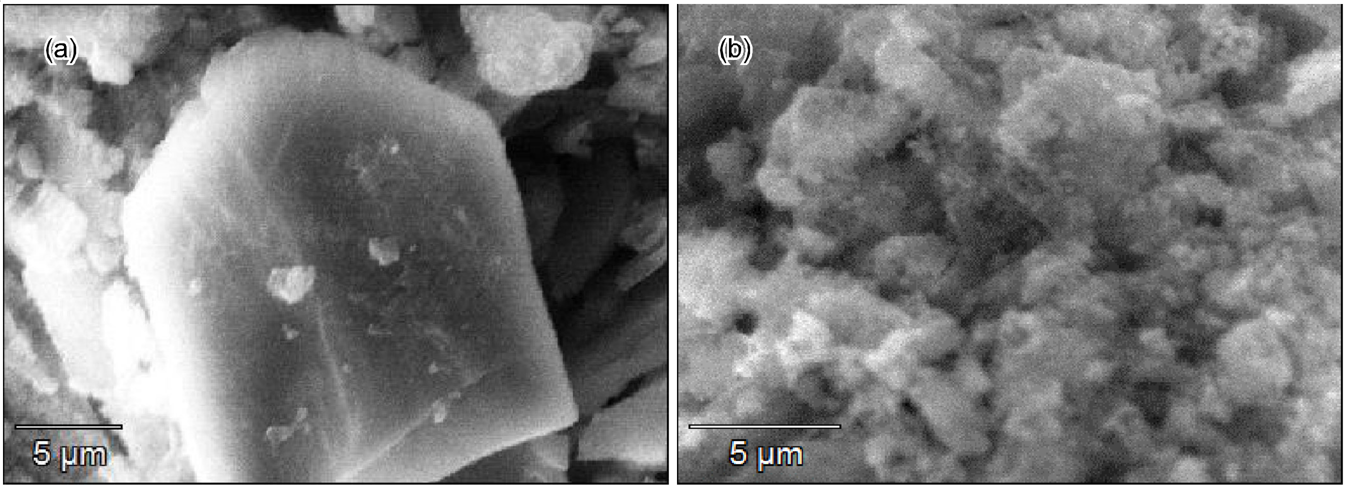
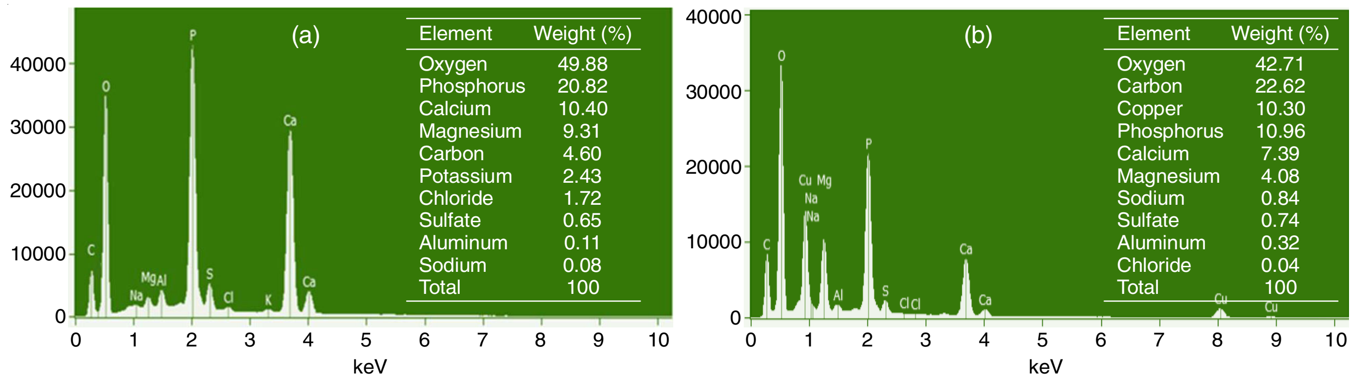


Fig. 4. SEM images of (a) bioflocculant and (b) biosynthesized CuNPs at 5 µm scale



(a)

Element	Weight (%)
Oxygen	49.88
Phosphorus	20.82
Calcium	10.40
Magnesium	9.31
Carbon	4.60
Potassium	2.43
Chloride	1.72
Sulfate	0.65
Aluminum	0.11
Sodium	0.08
Total	100

(b)

Element	Weight (%)
Oxygen	42.71
Carbon	22.62
Copper	10.30
Phosphorus	10.96
Calcium	7.39
Magnesium	4.08
Sodium	0.84
Sulfate	0.74
Aluminum	0.32
Chloride	0.04
Total	100

Fig. 5. EDX spectra of (a) bioflocculant and (b) CuNPs

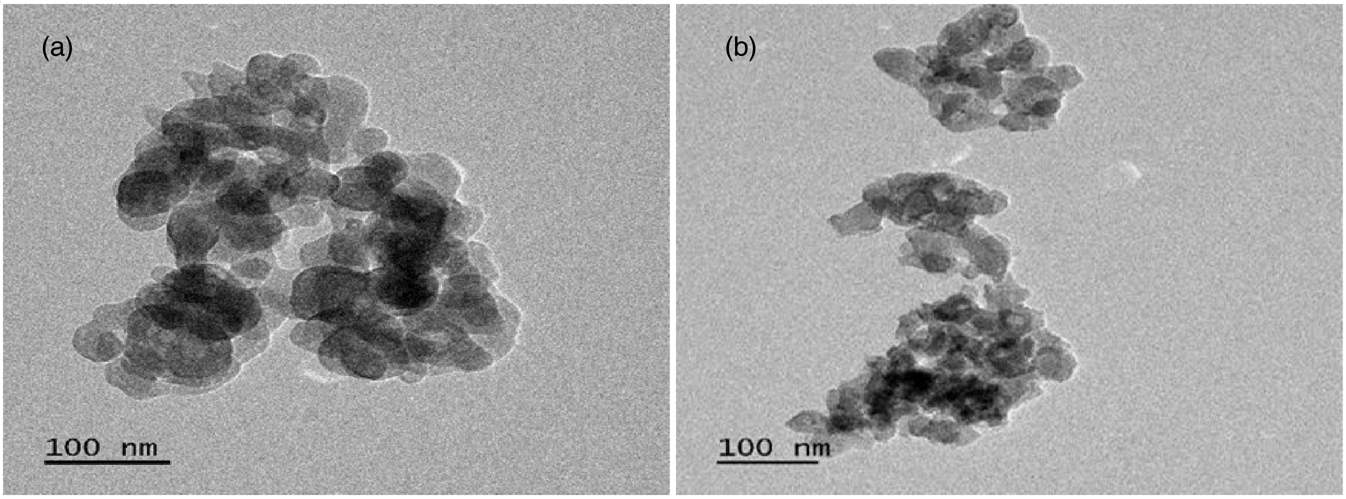


Fig. 6. TEM micrographs of the (a) bioflocculant and (b) CuNPs

Thermal studies: To reveal the behaviour of samples under temperature, the bioflocculant and CuNPs were subjected to thermogravimetric analysis. This reveals the material's pyrolytic properties when exposed to high temperatures. The pyrolysis properties of the bioflocculant are shown in Fig. 7. From 40 to 200 ºC, about 19% weight loss was recorded in the bioflocculant. Moisture content loss may have caused the initial weight loss [60]. When the temperature was raised further to about 400 ºC, the weight loss increased to 30%, indicating the second phase of bioflocculant degradation and thus affecting the bioflocculant stability. Due to the continuous thermal decomposition of the residual components, a gradual decrease in mass weight was observed, with 38% total weight loss. The gradual decrease might have resulted from the release of volatile hydrocarbons in the bioflocculant polysaccharides from heat decomposition [53]. After being exposed to a high temperature of 900 ºC, the bioflocculant produced by *P. yamanorum* retained more than 60% of its weight, indicating good stability. The weight above 60% was maintained by bioflocculant, signifying the bioflocculant to be thermostable. The initial weight loss of 18% by CuNPs was observed at 190 ºC, which could be caused by the evaporation of water in the nanoparticles [61]. The weight loss accumulated as the temperature was raised, which could be a result of the degradation of the bioflocculant that capped the CuNPs, leading to weight loss [62]. The ability of the as-synthesized CuNPs to retain a weight greater than 70% even at 900 ºC indicates that the CuNPs are thermostable. These findings are relatable to those documented by Prabhu *et al*. [61], where the initial weight loss of CuNPs synthesized using *Garcinia mangostana* extract occurred at a temperature below 200 ºC with an initial weight loss of about 1.309% and a total of 3.328% weight loss, indicating good thermal stability.

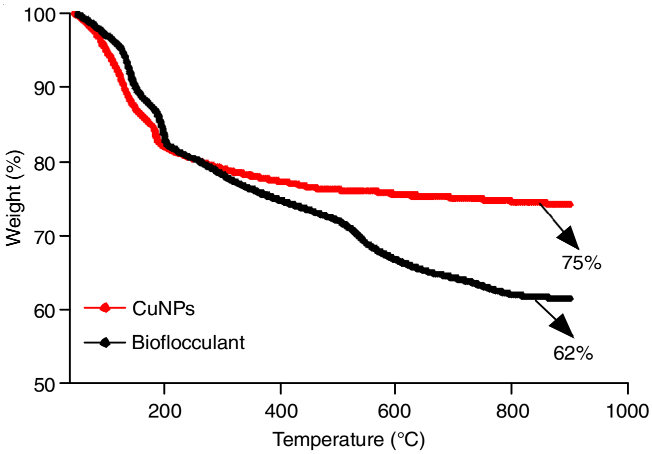


Fig. 7. Thermogravimetric analysis of the bioflocculant and CuNPs

Conclusion

The biosynthesis of metal nanoparticles is receiving a lot of attention as a developing area of interest in nanotechnology. Thus, using a bioflocculant from *Pseudomonas yamanorum* as a reducing and stabilizing agent, CuNPs were synthesized with $CuSO_4$ used as a precursor in this study. The flocculants consisted of Al, C, Ca, Cl, Mg, Na, O, P and S. The elemental analysis of the CuNPs revealed a significant amount of copper (10.30 wt.%), which was undetectable from the bioflocculant used to synthesized CuNPs. The bioflocculant was less heat stable than the produced CuNPs, according to the thermogravimetric measurements, which showed that they kept roughly 75% and 62% of their weight after exposure to 900 ºC, respectively. The bioflocculant and CuNPs had similar functional groups (carboxyl, hydroxyl and amide) with different intensities with CuNPs containing a Cu-O bond in the molecular chain. The bioflocculant and CuNPs were aggregated, almost spherically shaped and had particle sizes between 40 and 50 nm, as observed through SEM and TEM and confirmed through XRD analysis. The UV-Vis spectrometric analysis showed peaks at 280 nm (bioflocculant) and 580 nm (CuNPs). It can therefore be deduced from these findings that green synthesis can be effectively used for cost-effective, quick and eco-friendly copper nanoparticle synthesis, which can then be used in waste-water treatment.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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