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Biosynthesis of Copper Nanoparticles and its Application in Vulindlela Wastewater Treatment Plant and Removal of Toxic Dye

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Chemical flocculants are known to pose environmental risks due to their toxicity, prompting interest in biodegradable and eco-friendly bioflocculants as alternatives. Despite their environmental benefits, the industrial adorption of bioflocculants is hindered by their lower efficiency and high production costs. Nanotechnology offers promising solutions for removing contaminant and pathogenic bacteria from potable water. The bioflocculant *Kytococcus sedentarius* was utilized to produce copper nanoparticles (CuNPs). Biosynthesized nanoparticles were characterized using UV-Vis spectroscope (UV-Vis), Fourier transform infrared (FT-IR) spectroscopy, scanning electron microscope (SEM), transmission electron microscope (TEM), X-ray diffractometer (XRD) and thermogravimetric analysis (TGA). CuNPs exhibited a wide pH stability with pH 7 having a highest flocculating activity of 98% with a low dosage size of 0.2 mg/mL. Cytotoxicity test results revealed that the nanoparticles are non-toxic at low concentrations up to 75 µL. Moreover, the synthesized nanoparticles have antimicrobial activity when tested. The biosynthesized CuNPs removed dyes effectively with the removal efficiency of ± 90% on all treated dyes. The CuNPs had a high biological oxygen demand (BOD), chemical oxygen demand (COD) removal efficiencies of 93% and 97%, respectively. Thus, the as-synthesized CuNPs have a potential to be applied in wastewater treatment to replace synthetic flocculants.

Keywords: Copper nanoparticles, Removal efficiency, Antimicrobial, Application, Flocculation.

INTRODUCTION

Water bodies are becoming contaminated by toxic chemicals and pathogenic microorganisms making it not suitable for human consumption [1]. It is therefore pertinent to find a means of purifying wastewater and larger polluted bodies. Every year, close to 1000 km^3 of wastewater is produced, 30% coming from municipal sources and 60% produced by different industries [2]. Rapid population growth, industrialization and agricultural activity have been responsible for the large amounts of waste produced as these resources demand clean water [3]. Natural organic matter in surface water, both dissolved and particulate, degrades water quality [4]. Wastewater must be treated in three phases namely; primary, secondary and tertiary to be deemed safe to drink [5]. Wastewater treatment methods such as flocculation, filtration, adsorption, sedimentation and ion-exchange [6] are reported in the literature. These processes have been used to remove pollutants, but their short outcomes have been noted. Hence, flocculation has been used extensively by wastewater treatment industries. The flocculation process in wastewater treatment occurs spontaneously when solids form large flocs that sediment faster. This technology is simple and not expensive, involving the aggregation and removal of solid particles, with the aid of flocculants [7]. Flocculants consist of two groups namely: natural and chemical (synthetic organic and inorganic) flocculants [8]. However, the short outcomes of chemical flocculants are expensive, non-biodegradable and their toxicity affects the environment and human health. Therefore, natural occurring flocculants which are eco-friendly, cost effective and innocuous to humans have been of interest. However, natural occurring flocculants have low flocculating efficiency, expensive production and low yield [6].

Nanotechnology is a science being exploited in wastewater treatment due to its effectiveness. Polysaccharides-based bioflocculants can be used to produce high-performance nanomaterials that are non-toxic and stable [9]. Chemical and physical techniques have experienced problems such as being hazardous as they use toxic chemicals and are costly [10]. Thus, the use of microorganisms or their products has been proposed as an eco-friendly alternative. The biological synthesis of metallic nanoparticles has been proposed to be eco-friendly, have low cost, require less time and have low energy consumption [11]. Copper nanoparticles exhibit antimicrobial activity, surface-area-to-volume ratio, magnetic and optical properties that are needed to reduce contaminants found in wastewater [12]. Nanotechnology has the ability to reshape the environment around us and its growth has led to fundamental changes in research. Thus, this study focused on the synthesis, characterization and application of copper nanoparticles in wastewater treatment. Their antimicrobial activity and cytotoxicity effects were also investigated.

EXPERIMENTAL

Biosynthesis of CuNPs using purified bioflocculant: CuNPs were synthesized using the green synthesis technique by Dlamini *et al.* [13]. In 200 mL of 3 mM $CuSO_4$ aqueous solution, bioflocculant (0.5 g) was added. To create a homogeneous solution, the reaction was agitated. For 24 h, the beaker was kept at room temperature. The colour change to a blue precipitate indicated that the CuNPs were successfully synthesized. A centrifuge was used to collect the produced precipitate (5000 rpm, at 4 ºC, 30 min) and dried to yield CuNPs.

Characterization: A scanning electron microscope (SEM) (JEOL USA, Inc., Massachusetts, USA), with an elemental analyzer was used to examine the surface structure of the bioflocculant. This method was followed to determine elemental analysis and particle images of the samples. The functional groups of the bioflocculant were analyzed using the Bruker Tensor 37 Fourier transform-infrared (FTIR) spectroscopy (Brucker, Gauteng, South Africa) at a range of 400-4000 cm^{-1} wavelength. The X-ray diffractometer (Brucker D8 Advance, Johannesburg, South Africa) was used to measure the crystallinity of the nanoparticles. The thermal stability of the samples was analyzed using the thermogravimetric analyzer (STA 449/C Jupiter, Netzsch, Germany) at a temperature ranging between 22-900 ºC in a nitrogen-inert environment. The UV-Vis spectrophotometric analyzer (Jasco V-730 Bio Spectrophotometer, JASCO Corporation, Japan) with a wavelength range of 200-700 nm was used to evaluate the absorption spectra of samples.

Determination of flocculation properties of CuNPs: The jar test was employed to determine the various parameters *viz.* dosage size, cations, pH, temperature and salinity. CuNPs solutions with a dosage range from 0.2 to 1.0 mg/mL were performed in autoclaved deionized water for each investigation [14]. Various cations such as lithium chloride, barium chloride, potassium chloride, magnesium chloride, aluminium chloride, ferric chloride and sodium chloride (1% w/v), were also evaluated. The pH was tested from 3-12 and the heat effect test ranged from 50-121 ºC. Lastly, different NaCl concentrations (5-30 g/L) were prepared to investigate the salinity effect on CuNPs flocculating activity [15]. Kaolin clay (4 g/L) was prepared to assess flocculating activity for each of the parameters, respectively. Thereafter, 2 mL of CuNPs mixture and 3 mL of 1% (w/v) $CaCl_2$ were mixed in the kaolin suspension in 250 mL flask, shaken and poured to graduated cylinders and left for 5 min. The solution was used to measure the flocculating activity (FA) and was calculated using the following formula:

$$\text{Flocculation activity (\%)} = \frac{A - B}{A} \times 100$$

The optical density of kaolin solution is represented by A and B is the optical density of sample measured at the wavelength of 550 nm [16].

Cytotoxicity effect: The cytotoxic effect of the CuNPs on human embryonic kidney (HEK 293) cells was performed using a tetrozolium-based columetric (MTT) assay. The cells were grown using 25 mL culture flasks, seeded into a 96-well plate at the density of 1×10^5 cells/mL and incubated at 37 ºC for 24 h. Growth medium (EMEM + 10% fetal bovine serum (FBS) + 1% antibiotics (penicillin-streptomycin) was replaced. Then, various concentrations of CuNPs (25-100 µg/µL) were poured into wells and incubated. The medium was then withdrawn and replaced with new serum-free media containing 10 µL MTT reagent (5 mg/mL in phosphate-buffered saline (PBS) as an indicator for cell survival. Medium containing MTT reagent was aspirated after incubation for 4 h. About 100 µL DMSO was added to solubilize formazan crystals. A microplate reader (Vacutec, Hamburg, Germany) measured the optical density at 570 nm, utilizing DMSO as the blank using the below formula:

$$\text{Cell viability (\%)} = \frac{D_0 - D_1}{D_0} \times 100$$

where D_0 is the optical density at 570 nm of untreated cells and D_1 is the optical density at 570 nm after treated with CuNPs [17].

Antimicrobial activity: Kamazeri *et al.* [18] method was used to assess the antimicrobial activity of the CuNPs and bioflocculant. The Gram-negative and Gram-positive bacteria selected were inoculated into the broth tubes and incubated overnight. About 1 mL of bacteria of choice was pipetted into 9 mL of nutrient broth in separate test tubes that were further incubated overnight at 37 ºC. The absorbance of the bacterial strains was read using the spectrophotometer at 600 nm. The turbidity of the suspension was diluted using nutrient broth to adjust the range of the absorbance between 0.08 to 0.132 which was comparable to McFarland turbidity standards of 0.5.

Minimum inhibitory concentration (MIC): About 50 µL of freshly prepared Muller-Hinton broth was added to each well of 96 micro-well plates. A 50 µL of CuNPs solution and 50 µL of bioflocculant were mixed on separate plates and poured into the first row. A mixture of 50 µL was removed from the first row wells, respectively to execute a three-fold serial dilution. The bacteria of choice (50 µL) were poured into respective wells. A 20 µg/mL of ciprofloxacin was used as a positive control. After incubation, 0.2 mg/mL (40 µL) of *p*-iodonitrotetrazodium violet (INT) solution was used as an indicator on each plate and incubated for 30 min at 37 ºC. A clear colour indicated antimicrobial activity while a red colour indicated no antimicrobial activity [19].

Minimum bactericidal concentration (MBC): A loopful of each culture medium from the wells with no bacterial growth was removed and streaked on Muller-Hinton Agar plates. About 12 h were spent incubating the plates at 37 ºC. The lowest concentration of CuNPs that killed the organisms was found to be the MBC [18].

Application of synthesized CuNPs in wastewater treatment: A 1 L sample of household wastewater was taken at the Vulindlela Water Treatment Plant. The wastewater was tested using the procedure provided by Li *et al*. [20]. To test for the flocculating activity of CuNPs on the sample, the following procedure was followed. A 2 mL of 0.2 mg/mL of CuNPs was mixed with 100 mL wastewater and poured in 250 mL flask. The solution was shaken at 200 rpm for 3 min and kept to settle for 10 min. The top phase was analyzed with the UV-visible spectrophotometer at an optical density of 680 nm. The removal efficiency (RE) of the CuNPs on biological oxygen demand (BOD), chemical oxygen demand (COD), phosphorus (P), total nitrogen (TN) was evaluated. The removal efficiency (RE) of the pollutants was calculated using the following equation:

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

where C_0 is the initial value of sample and C is the final value after treatment.

Removal of dyes: Experiments on dye were carried out in accordance to Gong *et al*. [21]. Dyes solutions (5 g/L) for carbol fuchsin, safranin, Congo red and methylene blue were prepared. The pH was adjusted to 7 on about 100 mL solution with 0.2 g/mL of CuNPs and shaken at 200 rpm for 3 min. After 10 min of sedimentation, the upper phase was collected for examination with a UV-visible spectrophotometer set to 550 nm. The following formula was used to calculate the removal efficiency:

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

where C_0 is the value before treatment and C is the value after the treatment.

Statistical, experimental and software analysis: All data was collected in triplicates and standard deviation values were calculated. GraphPad prism was used to perform a one-way analysis of variance (ANOVA) on data.

RESULTS AND DISCUSSION

UV-Visible spectra: UV-Vis spectra was recorded with an absorbance ranging from 200-1400 nm for both CuNPs (a) and the bioflocculant (b) with a peak maxima at 275 nm (Fig. 1). The maxima observed in the CuNPs in Fig. 1a gradually decreased with an increase in wavelength, whereas, the bioflocculant (Fig. 1b) was revealed to exhibit a weak peak. The confirmation of the biosynthesized CuNPs was affirmed by evaluating the absorption peak along the absorption band of the surface plasmon resonance (SPR) deducing nanoparticle's nano-size and shape. Initially, the bioflocculant solution exhibited a colour change to greenish blue due to the surface plasmon vibration owing to the formation of CuNPs. Metal nanoparticle conduction electrons interaction with photons was responsible for this colour change phenomenon [22]. The shift in the position of the SPR band depends primarily on the individual particle's properties mainly size, shape and capping agent [23].

SEM morphology: Fig. 2 depicts the surface structures of the CuNPs (a) and bioflocculant (b). The surface morphology of CuNPs revealed them to be amorphous in structure, whereas, the scanning electron micrograph exhibited the bioflocculant to have a distinct shape. The differences in their morphology signify the successful green synthesis of CuNPs. The biosynthesized CuNPs also revealed to be agglomerated due to the electric static force in the particles. The surface structure plays a critical role in determining their performance [24]. The remark-

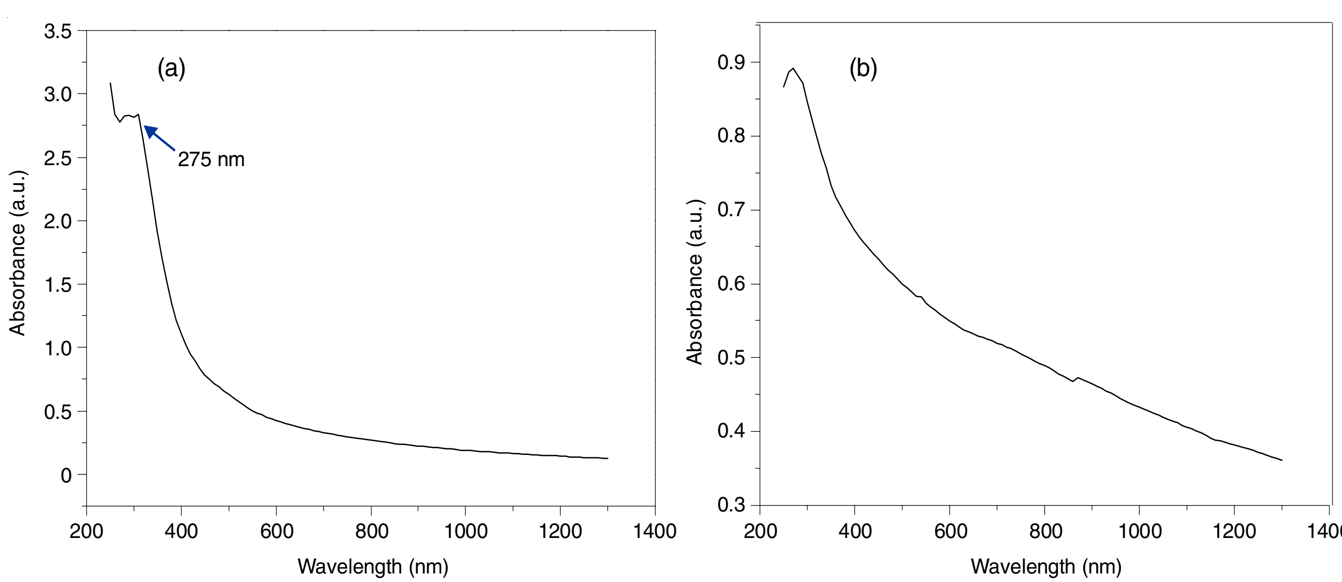

Fig. 1. UV-Vis spectra of the biosynthesized CuNPs (a) and bioflocculant (b)

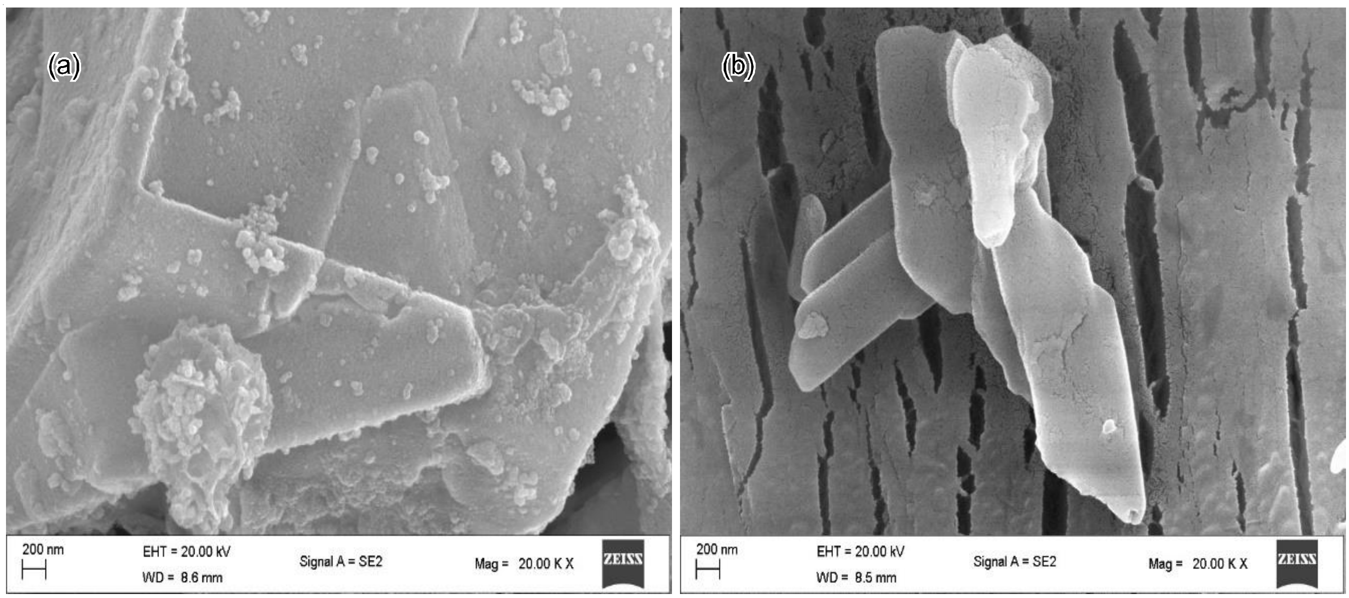

Fig. 2. SEM images of the biosynthesized CuNPs (a) and bioflocculant (b)

able flocculating activity exhibited by the CuNPs could be caused by these configurations.

TEM studies: The biosynthesized CuNPs are found be clustered due to the severe degree of agglomeration (Fig. 3a) whereas in Fig. 3b, the bioflocculant exhibited a uniform monodisperse spherical shape. Researchers have observed that globular nanomaterials are more powerful in terms of antibacterial activity due to their ability to simply perforate pathogen cell walls [11].

CuNPs and bioflocculant elemental composition: The elemental data in mass proportion (wt.%) of the bioflocculant and biosynthesized CuNPs is given in Table-1. The SEM-EDX results confirmed that the bioflocculant has elements namely;

TABLE-1
ENERGY-DISPERSIVE X-RAY ANALYSIS (EDX) OF BIOSYNTHESIZED CuNPs AND BIOFLOCCULANT

Elements	CuNPs (wt.%)	Bioflocculant (wt.%)
C	25.23	30.50
O	20.13	38.49
Mg	0.55	2.89
P	12.04	8.46
K	0.71	3.41
Ca	15.48	10.15
S	0.36	5.39
Al	1.60	0.67
Cu	23.37	–
Total	100	100

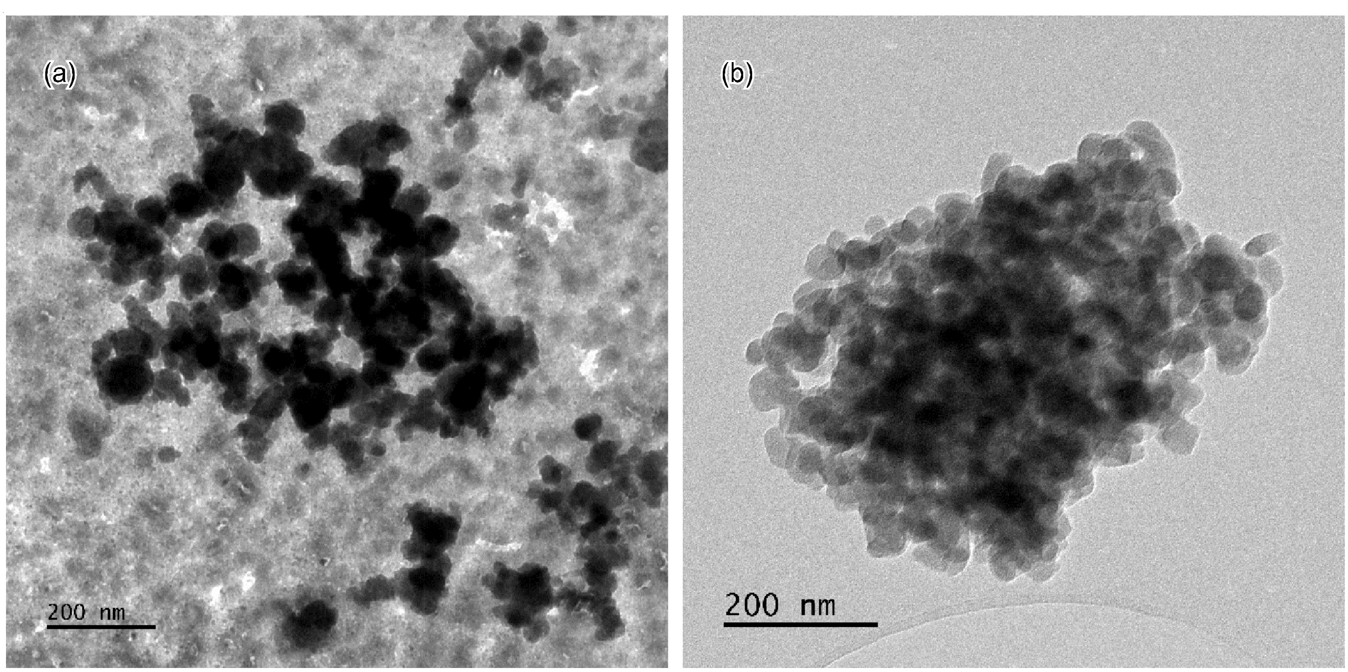

Fig. 3. As-synthesized TEM images of CuNPs (a) and bioflocculant (b)

C, O, Mg, Ca, P, Cl, K, S and Al. Oxygen had the highest weight (%) with 38.49%, followed by carbon (30.50 wt.%). CuNPs were found to contain a variety of components including C, O, Cu, P, Ca, Mg and Al. Carbon content was lowered to 25.23% from 30.50% achieved during the bioflocculant elemental composition examination. Furthermore, Cu (23.37%) was found in the biosynthesized CuNPs. This also confirmed the success of the CuNPs production (Table-1). Furthermore, the elemental composition may have been responsible for the shape and strong flocculating activity of the synthesized CuNPs.

FTIR studies: The FT-IR spectroscopy analysis of the bioflocculant and CuNPs. Bands observed at 3225 cm^{-1} (bioflocculant) and 3312 cm^{-1} (biosynthesized CuNPs) indicate the hydroxyl and amine (NH_2) groups (Fig. 4). Agunbiande [25] reported that the hydroxyl functional groups occurrence suggests the adsorption positions for suspended particles and one of the functional groups responsible for enhancing the flocculating activity. The bioflocculant's CH group stretching vibration was measured at 2993 and 2393 cm^{-1} and biosynthesized CuNPs at 2923 cm^{-1}. β-Glycoside bond presence in the sugar monomers is demons-trated by the tiny bands at 559 cm^{-1} (biosynthesized CuNPs) and 498 cm^{-1} (bioflocculant). Furthermore, the bands at 559 cm^{-1} is a typical Cu-O bonds in the synthesized CuNPs.

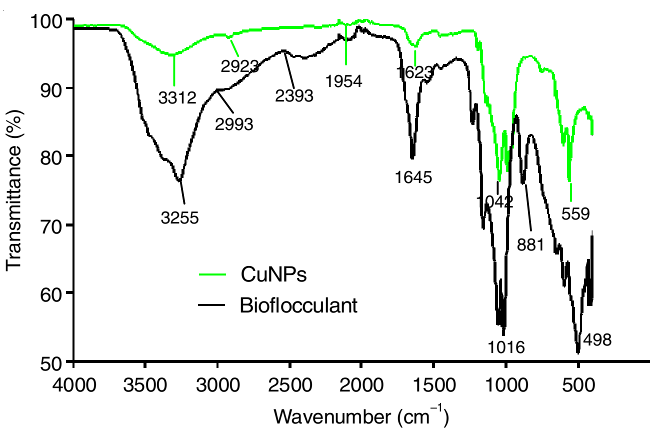

Fig. 4. FT-IR analysis of CuNPs and bioflocculant

Thermal studies: The thermogravimetric analysis of the CuNPs and bioflocculant exposed to high temperatures ranging from 100-900 ºC. The biosynthesized CuNPs retained 70% of its weight, whereas the bioflocculant retained only 50% (Fig. 5). Maliehe *et al.* [26] reported that the moisture loss may be caused by the presence of groups namely carboxyl and hydroxyl, which are responsible for attracting water to macromolecules. Thus, the biosynthesized CuNPs were concluded to be more thermal stable in comparison to the bioflocculant, proving that nanoparticles have enticing thermal properties that can withstand hush environmental conditions in various applications in industries.

XRD studies: Fig. 6 displays the X-ray diffraction of the CuNPs and bioflocculant. The biosynthesized CuNPs showed three dominant diffraction peaks at angle (2θ) between 20º and 70º, which are in line with the standard of CuNPs in good correlation with the JCPDS card no. 89-5899. The strong peaks observed represent the crystallinity and particle size. The cryst-

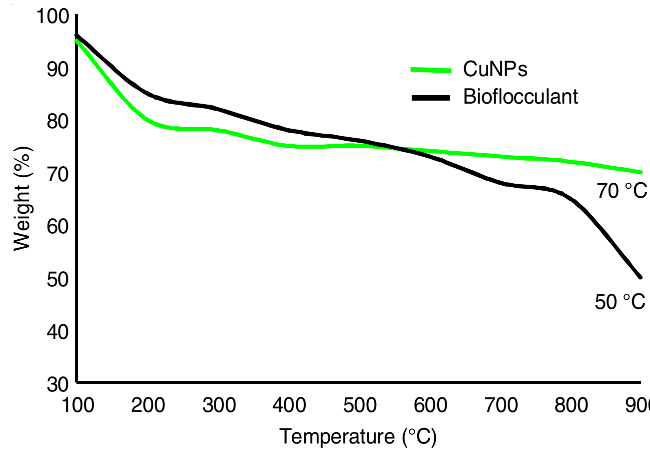

Fig. 5. Thermogravimetric analysis of CuNPs and bioflcoculant

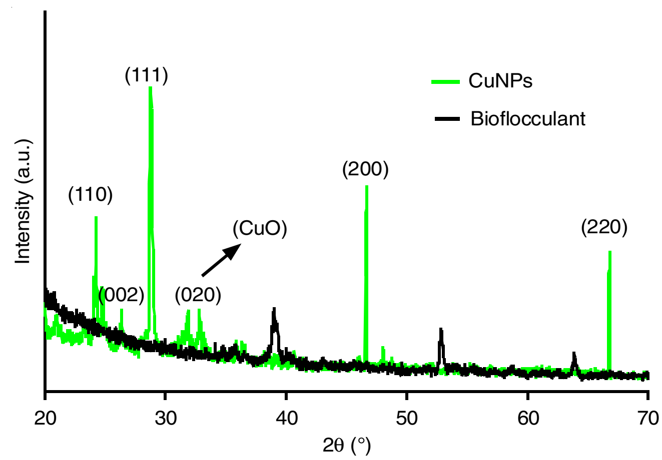

Fig. 6. XRD analysis of CuNPs and bioflocculant

allite size using the Debye-Scherrer equation was found to be 28.3 nm, which indicates a smaller biosynthesized CuNPs particle size. Smaller particle size contributes to contaminant removal in wastewater by increasing particle surface area [12]. This larger surface area allows for more particle-pollutant contact, which can lead to more effective pollutant removal [27].

Flocculation properties of biosynthesized CuNPs

Dosage size effect on flocculating activity: Table-2 represents the results obtained during the investigation of the dosage size of biosynthesized CuNPs. The optimum dosage size of biosynthesized CuNPs was 0.2 mg/mL as it gave 92% flocculating activity. Increasing the dosage size above 0.2 mg/mL resulted in a decrease in flocculating activity. The low flocculating activity of 70% at 1.0 mg/mL was caused by the flocculation deterioration phenomenon [28]. The phenomenon states that the decrease is caused by the enclosed colloidal particles in the concentrated flocculant triggering a colloidal protection function to occur reducing the flocculating activity. Furthermore, the reduction in flocculating activity with the increase in dosage size is also caused by the blockage of the binding sites of kaolin particles caused by excessive flocculant added. A similar study conducted by Dlamini *et al.* [13] reported that CuNPs had a significant flocculating activity of 96% at a modest dosage size of 0.2 mg/mL.

TABLE-2
EFFECT OF DOSAGE, CATIONS, TEMPERATURE, pH AND SALINITY ON FLOCCULATING ACTIVITY OF BIOSYNTHESIZED CuNPs

Dosage (mg/mL)	FA (%) ± SD	Cations	FA (%) ± SD	Temperature (ºC)	FA (%) ± SD	pH	FA (%) ± SD	NaCl (g/L)	FA (%) ± SD
0.2	92 ± 1.63a	Control	92 ± 4.32a	Unheated	98 ± 1.08a	3	45 ± 4.08a	5	98 ± 1.6a
0.4	88 ± 1.63a	K^+	92 ± 4.00a	50	93 ± 2.45a	4	60 ± 8.16b	10	98 ± 1.6a
0.6	82 ± 2.83b	Li^+	88 ± 8.04a	60	93 ± 2.08a	5	60 ± 4.08b	15	97 ± 0.82a
0.8	75 ± 4.08c	Na^+	87 ± 5.72a	70	92 ± 4.08a	6	83 ± 2.45c	20	96 ± 1.67a
1.0	70 ± 7.07c	Mn^{2+}	85 ± 4.08b	80	93 ± 1.97a	7	98 ± 1.63d	25	94 ± 1.45a
		Ba^{2+}	88 ± 8.04a	90	93 ± 2.23a	8	96 ± 1.83d	30	90 ± 4.08a
		Mg^{2+}	83 ± 2.45c	100	90 ± 1.63a	9	90 ± 6.34d	35	90 ± 4.08a
		Fe^{3+}	79 ± 0.81d	121	80 ± 13.5b	10	85 ± 4.00c		
						11	80 ± 3.56c		
						12	73 ± 2.45e		

*FA denotes flocculating activity while SD denotes standard deviation. Different letters (a, b, c, d and e) denotes statistical significance at ($p < 0.05$).

Influence of cations on CuNPs: The flocculating ability of biosynthesized CuNPs was evaluated using monovalent, divalent and trivalent metal ions. All cations promoted the flocculating activity of the CuNPs, with monovalent cations being the most effective followed by the divalent cations (Table-2). The CuNPs was found to be more effective when no cation was added as they resulted in an optimum flocculating activity of 92%, which was relatively equivalent to the effect of monovalent cation K^+. The K^+ was able to effectively stimulate the flocculating activity by neutralizing the zeta potential and stabilizing the functional group's negative charge stimulating the bridging mechanism [29]. However, the cation-independent CuNPs are advantageous as they can avoid secondary pollution and decrease the costs of adding metal cations to enhance flocculation making them commercially valuable [30]. The highest flocculating activity by CuNPs in the absence of cation was caused by the extensive surface area for adsorption causing negatively charged colloidal particles to serve as bridges with anionic polyelectrolytes to increase flocculating activity [31]. The same results were reported whereby CuNPs required no addition of cations and obtained a high flocculating activity of 96% [32]. Thus, the synthesized CuNPs in this study were used without the addition of cations in solutions.

Temperature influence on CuNPs: The synthesized CuNPs were evaluated by varying temperatures ranging from 50-121 ºC. The optimum flocculating efficiency of 98% was obtained with the control (unheated CuNPs) while the least activity was found when the nanoparticles were heated at 121 ºC. Although the highest activity was observed with the unheated CuNPs, they maintained a noteworthy flocculating activity (≥ 90%) even at high temperatures (≤ 100 ºC). This was indicative of the thermal stability of the formed CuNPs. The thermal stability of CuNPs was attributed to the presence of the hydroxyl functional group, which is responsible for the formation of hydrogen bonds [33]. The decrease in the flocculating activity at high temperatures (50-121 ºC) may be owed to the alteration of physiognomies of the synthesized CuNPs. The bioflocculant used might have lost some of its components, especially proteins, which are easily denatured at elevated temperatures, consequently losing their functionality and resulting in a decreased flocculating activity. Dlamini *et al*. [17] investigated the impact of heat on CuNPs and found them to be thermally stable, maintaining over 90% flocculating activity even at elevated temperatures.

pH effect on the flocculating activity of biosynthesized CuNPs: An evaluation of pH was conducted and the results are illustrated in Table-2. The optimum flocculating activity of 98% was shown at neutral conditions (pH 7). However, at the strongest acidic pH (pH 3) used, the lowest flocculating activity of 45% was observed. Moreover, a decrease in flocculating activity of CuNPs as the pH increased from neutral to alkaline conditions (pH > 7) was observed. Patil *et al.* [34] reported that the decrease in flocculating activity at low pH was caused by the adsorption of H^+ ions by the colloids in solution and CuNPs, consequently affecting the bridging mechanism. In addition, the decrease in the flocculating activity at alkaline conditions (pH > 7) was attributed to the interference of the hydroxide ions in the bond formation of CuNPs and kaolin solution [26].

NaCl concentration effect on the flocculating activity of biosynthesized CuNPs: The salt concentration effect on flocculation characteristics of CuNPs was investigated and the results are depicted in Table-2. CuNPs were not affected by the increase in sodium ion concentration as they maintained a high flocculating efficiency (>90%). The CuNPs obtained an optimal flocculating activity of 98% at 5 g/L. CuNPs stability under high salt concentrations is influenced mainly by the incorporation of bioflocculant from marine *K. sedentarius* into CuNPs. The isolated marine *K. sedentarius* grows best in a marine environment, which is mostly characterized by extreme variations in salt concentrations (33-37 g/L) of salt per kilogram or psu [35]. Thus, this automatically implied that its secondary metabolite (bioflocculant) can demonstrate NaCl tolerance. Therefore, CuNPs hold significant economic potential as they address the issues encountered by activated sludge methods, which often struggle to function effectively due to the elevated salt levels from seafood waste [36].

Cytotoxicity effect of biosynthesized CuNPs: Naz *et al.* [37] reported that the unique properties present in CuNPs allow them to be applied in broad applications and need to be evaluated for their long and short-term cytotoxic effect on human exposure before application. In this study, the cytotoxic effect of the biosynthesized CuNPs was studied against HEK 293 cells. The HEK 293 cell viability was lost in a dose-dependent manner. The higher the CuNPs concentration, the greater the cell death (Fig. 7). A high cell survival rate of 93% was obtained at 25 µg/µL and the lowest (58%) was at 100 µg/µL. An increase in concentration from 25-100 µg/µL caused a significant decrease in cell viability. However, a positive control (devoid of CuNPs) had 100% cell viability. Therefore, the results depicted that CuNPs have a margin of biosafety at low concentrations (≤ 25 µg/µL). The cytotoxicity of the CuNPs was perceived to be due to their shape and size. The results correlate with those of Ostaszewska *et al.* [38] who reported the bioassay of CuNPs synthesized from *Chlamydomonas reinhardti* CCC-125 to exhibit toxicity at higher concentrations above 25 mg/L.

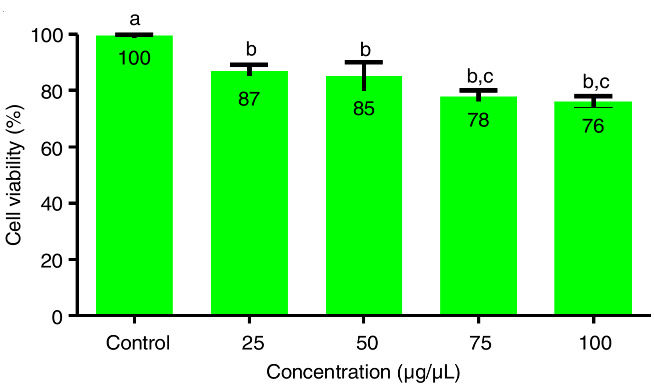

Fig. 7. *In vitro* cytotoxicity of biosynthesized CuNPs in HEK 293 cells, different letters (a, b and c) denotes statistical significance at ($p < 0.05$)

Antimicrobial activity effect of biosynthesized CuNPs: The biosynthesized CuNPs and ciprofloxacin were subjected to an antimicrobial activity test against Gram-positive and Gram-negative microorganisms. Table-3 shows the MIC and MBC results for both the Gram-negative organisms (*E. coli* and *P. flourescens)* and Gram-positive organisms (*B. cereus* and *S. aureus)*. The CuNPs exhibited to have significant antibacterial activity in both Gram-negative and Gram-positive bacteria tested. The inhibitory effect of 20 µL ciprofloxacin was investigated and showed minimal results as compared to the biosynthesized CuNPs that had a remarkable results throughout the investigation in all microorganisms. Even though the CuNPs were more effective in Gram-positive bacteria, they were also effective in Gram-negative bacterium (*P. flourescens*) with a low MIC of 5.25 mg/mL (Table-3). Contrary, ciprofloxacin had minimal effect as it couldn't block the microbial growth as the bacterium (*P. flourescens*) was able to grow in its presence. The high surface area to volume ratio of the small CuNPs caused them to be able to penetrate the Gram-negative cell and come into contact with the phospholipids of *P. flourescens* altering the morphology of the cell and inhibiting its growth. The results obtained suggested that the biosynthesized CuNPs has inhibitory properties that can be utilized in wastewater treatment to eliminate infectious microorganisms to reduce human health hazards especially those who are resistant to antibiotics. The high activity of CuNPs is caused by the penetration of metal nanoparticles ions in the cell inactivating enzymes, disrupting the helical structure of the DNA leading to cell death [39].

TABLE-3
MIC AND MBC IN mg/mL

Bacterial strains	CuNPs		Ciprofloxacin	
	MIC	MBC	MIC	MBC
E. coli	8.5	12.5	3.2	6.4
P. fluorescens	5.25	7.25	–	–
B. cereus	10.67	16	1.125	3.66
S. aureus	13.5	25	0.223	2.33

Flocculation efficiency of biosynthesized CuNPs, $FeCl_3$ and bioflocculant: Fig. 8 depicts the flocculation activity of CuNPs in comparison with conventional chemical flocculants ($FeCl_3$) and bioflocculant in various samples. Various wastewater samples were investigated; namely coal mine wastewater, domestic, industrial wastewater and kaolin clay solution. It has been reported that chemical flocculants have high flocculation efficiency but due to their negative impact on the environment bioflocculants have been seen as an alternative [8]. However, the bioflocculants having low flocculating efficiency and expensiveness have minimized their application. The dosage (0.2 mg/mL) of CuNPs was also compared with the same dosage for the traditional flocculant ($FeCl_3$) used in different wastewater samples and the optimum dosage of 0.8 mg/mL was used for the bioflocculant application. In this study, the biosynthesized CuNPs showed remarkable flocculation capability in all the tested wastewater as the flocculating activity of above 90% was obtained. The highest flocculating efficiency by CuNPs was obtained using kaolin clay suspension with 96% whereas 91%, 95% and 93% were obtained using domestic, coal mine and industrial wastewater, respectively. The $FeCl_3$ was more efficient than the bioflocculant as the flocculating activity above 75% was obtained in all tested wastewater, with kaolin clay suspension having 88% flocculating activity followed by the domestic wastewater with a flocculating activity of 80%.

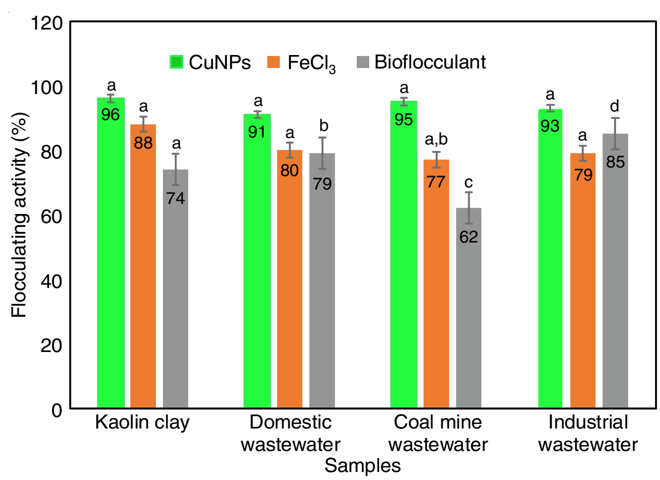

Fig. 8. Flocculation efficiency of biosynthesized CuNPs compared to iron(III) chloride and pure bioflocculant in different wastewater samples, the different letters (a, b, c and d) denotes statistical significance at ($p < 0.05$)

Influence of biosynthesized CuNPs on dye removal: The effect of CuNPs on dye removal from stains is depicted in Fig. 9. The as-synthesized CuNPs were revealed to have high removal efficiency above 85% for all the examined dyes with

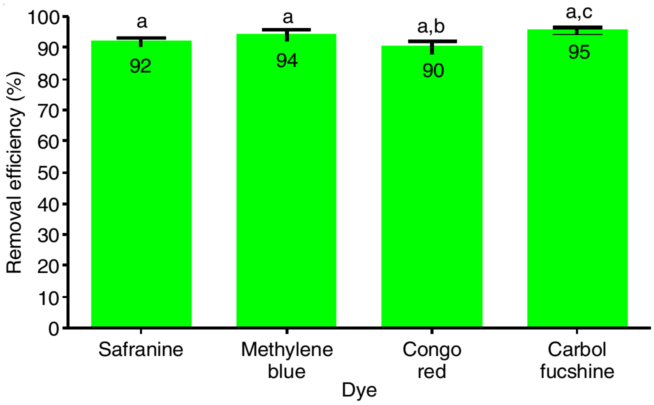

Fig. 9. Effect of biosynthesized CuNPs on staining dye removal, different letters (a, b and c) denotes statistical significance at ($p < 0.05$)

carbol fuchsine having the highest removal efficiency of 95% as the concentration of nanoparticles was constant at 0.2 mg/mL. The CuNPs exhibited functional groups in their molecular chain that could be capable of decolourizing dyes in wastewater treatment by interacting with the surface of the suspended particles to enhance their dye removal efficiency. The removal efficiency is caused by particle aggregation produced by bridging and charge neutralization [40]. Macczak *et al*. [41] observed that the application of low molecular weight polymeric compounds, like bioflocculants, to metal nanoparticles has effectively prevented aggregation, making them a successful agent for dye decolourization in wastewater treatment plants.

Removal efficiency of biosynthesized CuNPs in the treatment of Vulindlela wastewater: CuNPs ability to decrease suspended particles in wastewater was tested and the results are presented in Table-4. The CuNPs had remarkable removal efficiency on all tested parameters when compared to conventional chemical flocculants. The BOD and COD removal efficiency of CuNPs was 93% and 97%, respectively. However, $FeCl_3$ had a better removal efficiency of sulphates when compared to CuNPs and alum with 70% and 62%, respectively. The improved flocculation properties of CuNPs is owed by the presence of the hydroxyl, carboxyl groups and also the surface structure [42]. These groups are the preferred groups for adsorption-enhancing flocculating activity by bridging through suspended particles in wastewater [43]. Water intensively concentrated in BOD and COD causes an overgrowth of chemoorganotrophs, which consume oxygen [44]. Anoxic zones in water then form, causing macroscopic creatures to die and excessive concentrations of metals such as sulphur also lead to eutrophication [45]. Thus, the results displayed by CuNPs implied its potential to reduce high loads of chemoorganotrophs and eutrophication.

TABLE-4
REMOVAL EFFICIENCY OF BIOSYNTHESIZED CuNPs IN VULINDLELA WASTEWATER TREATMENT

Types of flocculants	BOD	COD	S
CuNPs	93^a	97^a	70^a
Alum	90^a	71^b	62^b
$FeCl_3$	93^a	74^b	75^c

*Different letters (a, b and c) denotes statistical significance at ($p < 0.05$).

Moreover, the results demonstrated the potential of CuNPs as an alternative material to chemical flocculants in the treatment of wastewater. The results are comparable to Sriprom *et al.* [46] who obtained 92% removal efficiency of COD in experimental wastewater in 120 min, making it significant for wastewater treatment.

Conclusion

The study aimed to biosynthesize CuNPs using the bioflocculant from *K. sedentarius* as the stabilizer and reducing agent and use them for pollutant removal from various wastewater. The UV-Vis, FT-IR, SEM, SEM-EDX and thermogravimetric spectra confirmed the successful biosynthesis of CuNPs. The CuNPs exhibited moderate flocculating activity at low dosage (0.2 mg/mL) and high thermal, pH and saline stabilities. Moreover, it demonstrated a margin of biosafety as it revealed a low cytotoxic effect on HEK 293 cells when used at low concentrations (≤ 25 µg/mL). The CuNPs demonstrated good removal efficiencies on BOD (93%) and COD (97%) during the treatment of the domestic wastewater from the Vulindlela wastewater plant. The biosynthesized CuNPs removed pollutants such as sulphur effectively with 70% removal efficiency and were capable of removing different dyes. These results confirm that the CuNPs can be used in wastewater treatment industries.

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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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