



Biosynthesis of Copper nanoparticles using bacterial supernatant optimized with certain agro-industrial byproducts

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Received: 7 December, 2019; Accepted: 21 December, 2019; Published online: 28 December, 2019

Abstract

Biosynthesis of green nanomaterials using microorganisms is considered clean, eco-friendly and viable, instead of the physical or chemical methods. This study aimed in the biosynthesis of copper nanoparticles (CuNPs) exploiting Egyptian local bacterial isolates. Sixteen copper-resistant isolates out of 160 bacterial isolates; were captured from various plant rhizospheres including; chamomile, hibiscus, neem, iris, and pea, beside samples collected from the Sharm El-Sheikh seawater. Among the 16 copper-resistant isolates, one promising isolate I108 was chosen which synthesized CuNPs of diameter about 87.1 nm, showed UV absorbance of 0.54 at 580 nm, with a concentration of 12.21 mg/l. This isolate was characterized by phenotypic and genotypic features. Based on 16S rRNA gene analysis and compared with the sequences presented in NCBI GenBank, the phylogeny positions assessment confirmed that it belonged to Genus *Pseudomonas*, and was closely related to *Pseudomonas silesiensis* strain A3 (98% similarity). For the bacterial synthesis of CuNPs, optimization of the *P. silesiensis* strain A3 cell-free supernatant was carried out using seven agro-industrial residues, added to the basal medium as different carbon sources. Results showed that 2% blackstrap sugar cane molasses was the most efficient carbon source for CuNPs biosynthesis, when incubated for at 30°C for 24 h using shaking speed of 120 rpm. The biosynthesized CuNPs has a size of 66.12 nm at a concentration of 19.2 mg/l, and maximum surface plasmon peak (SPR) of 0.85.

Keywords: Copper nanoparticles, *Pseudomonas silesiensis*, Agro-industrial by-products, Dynamic light scattering, Surface plasmon resonance

1. Introduction

Nanoparticles (NPs) gained a lot of attention from scientists owing to their size, which occur between the

size of their original materials and their structures at the nano size. In a previous study, Soheyla *et al.*,

(2012) reported that most of the physicochemical properties of the materials are size-dependent, so unlike the bulk materials (usually larger than 1,000 nm in size) NPs despite their minute structures have novel characteristics rather than their metal ions. Nanoparticles synthesis can be undergone using specific protocols such as; chemical, physical or biological (Subbaiya and Selvam, 2015). Despite the extensive use of the physical and chemical procedures in the synthesis of nanoparticles, they still have high cost and require utilizing dangerous chemicals. Therefore, there is an increasing concern to create easy, cost-effective and applicable techniques. According to Cuenya, (2010), novel cost-effective, eco-friendly procedures and large-scale production possibilities are being developed called the green synthesis. Din *et al.*, (2017) added that green synthesis is considered as a novel procedure for synthesizing NPs using living organisms including; plants, algae, fungi, and bacteria. By circumventing extreme pH and temperature ranges, biochemical synthesis has different benefits over chemical processes. Recently, Mangesh *et al.*, (2018) stated that large energy requirements are met by the specific oxidation reduction enzymes and protein molecules for metal reduction, these proteins however possess catalytic and metal-binding properties. This is in addition to their hydrophobic lipid molecules which play a promising role as capping agents, and serve to stabilize the NP through preventing their oxidation. Din *et al.*, (2017) reported that microbial methods have achieved perspectives through their strain selection, optimization of cultivation conditions including; nutritional factors, pH, temperature, incubation time, metal ion concentration, and volume of the biological material to be used in large-scale and commercial applications.

Recently, the bacterial synthesis of NP has attracted researcher's attention for its massive benefits such as; ease of culturing the bacteria, production of extracellular NPs, and its simple experimental conditions as pH, temperature and short generation time (Jang *et al.*, 2015). Copper is the most commonly

used metal around the world, as it is a primary micronutrient and is necessary for health of all living organisms. Moreover, it plays an important role as a coenzyme for many enzymes and metallo-enzymes needed by the plant cells\ organisms to perform multiple metabolic operations. Biosynthesis of CuNPs is a novel research field because of copper availability, inexpensive (compared to other metal ions such as gold, platinum and silver), and can be easily obtained. Besides, Din *et al.*, (2017) explored that CuNPs has low toxic impact compared with the AgNPs. Previously, Prabhu *et al.*, (2015) documented that CuNPs will have promising massive industrial, medicinal applications and future use in nano-devices.

According to Varshney *et al.*, (2011), many bacterial strains including; *Serratia* sp., *P. stutzeri*, *P. fluorescens*, *Morganella morganii*, *Thermoanaerobacter* sp and *Bacillus subtilis*, have been used for the biosynthesis of CuNPs. As illustrated by Ali *et al.*, (2011), components in the culture medium including carbon and nitrogen sources are crucial factors that affect the microbial cultivation, to obtain cell-free extract that plays a major role in the biosynthesis of NPs.

Ghosh *et al.*, (2017) stated that for the great benefits of the agro-industrial wastes as cheap, available, and renewable sources of carbon or nitrogen, they are used for the production of valuable NP in low-cost medium. The aims of the current work were to biosynthesize CuNPs through a local bacterial strain exploiting agro-industrial wastes to reduce the product costs, and as a trial to overcome the environmental pollution problems arising from the accumulation of the agricultural wastes.

2. Material and methods

2.1. Collection of samples

Different samples of Sharm El-Sheikh seawater, and eight rhizosphere soils from different fields of El-Qalyubia and Sharqia governorates, Egypt, planted with a basil (*Ocimum basilicum*), chamomile (*Matricaria chamomilla*), Egyptian clover (*Trifolium*

alexandrinum), hibiscus (*Hibiscus rosa-sinensis*), iris (*Iris versicolor*), pea (*Pisum sativum*) and thyme (*Thymus vulgaris*) were collected. The soil samples were collected from a depth of 5 to 30 cm after disposing 5 cm from the ground surface. These samples were collected in sterile plastic bottles and bags; respectively, and kept in ice boxes throughout their transport to the laboratory. In the laboratory, all these samples were preserved at 4°C until use.

2.2. Culture media used

Glucose agar and broth media were used for preservation of the bacterial cultures, and were prepared according to DIFCO. (1984). They consist of; Glucose, 10 g; Peptone, 5g; Beef extract, 3 g; Agar, 15 g\ liter, and the pH was adjusted to 7. These media were supplemented with 1 mM of CuSO₄.5H₂O for the biosynthesis and production of CuNPs. The King's B medium (King *et al.*, 1954) was used for the phenotypic identification of *Pseudomonas* spp. It was composed of Proteose peptone, 20 g; K₂HPO₄, 1.5 g; MgSO₄.7H₂O, 1.5 g; Glycerol, 10 ml; Agar, 15 g\ l, and then the pH was adjusted to 7.2.

2.3. Isolation of the copper resistant bacteria

For the isolation of copper-resistant bacteria, the enrichment culture technique was followed according to the serial dilution plate procedure using Glucose agar medium supplemented with 1 mM CuSO₄.5H₂O solution. Approximately, 10 ml of seawater sample and 10 g of a soil sample were suspended individually in 90 ml sterilized water, mixed well for 10 min. to obtain the initial dilution (10⁻¹), and then ten-folds dilutions were prepared till the dilution of 10⁻⁷ using sterilized water. These suspensions were spread on Glucose agar medium supplemented with CuSO₄, and then incubated. After 48 h of incubation, the development of the brown colonies on the surface of culture plates indicated the isolation of the copper resistant bacteria, according to Subbaiya and Selvam, (2015). For confirming that the produced brown color is not revealed from bacterial physiological pigmentation behavior, the colonies were re-streaked

onto the control Glucose agar plates without CuSO₄.5H₂O (Oremland *et al.*, 2004). Pure cultures of the recovered isolates were maintained on slants of Glucose medium at 4°C till further use.

2.4. Biosynthesis of the CuNPs

The CuNPs has been synthesized following the procedure described by Jafari *et al.* (2010). Flasks containing 50 ml of Glucose broth medium were inoculated individually with 0.1 ml inoculum (5.77×10⁵ cells/ ml) of each of the recovered bacterial isolates, and then placed on a shaking incubator (Shin Saeng) 150 rpm at 30°C. After 12 h of incubation, 50 ml sterilized 1 mM CuSO₄.5H₂O solution was added under aseptic conditions, then re-incubated (150 rpm) at 30°C for 24-48 h. The biosynthesis of CuNPs was indicated by changing the color of the medium from pale yellow or green to deep brown. After that, the promising broth culture was centrifuged at 10000 rpm for 10 min. using a SIGMA 2-16 P centrifuge. The bacterial pellets were collected to investigate the intracellular synthesis of the CuNPs, whereas the cell-free extract was collected separately to investigate the extracellular synthesis of the CuNPs.

2.4.1. Investigation of the intracellular synthesis of CuNPs

To investigate the intracellular biosynthesis of the NPs within the bacterial cells, the separated bacterial cells were pre-washed 3 times by suspending in 0.9% NaCl solution equal to the volume of the growth medium, and then centrifuged at 8000 rpm for 10 min. After that, the pellets were suspended in a volume of 0.5 M sucrose solution equal to the volume of the growth medium, incubated in a rotary shaker 120 rpm for 20 min. at 25°C, centrifuged at 8000 rpm for 10 min., and then re-suspended again in 0.5 M sucrose solution equal to the volume of the growth medium to get rid of any pollutants. The final washing step was conducted by re-suspending the pellets in a complete saline solution equal to the volume of the growth medium composed of (g/l): 17.5, NaCl; 0.74, KCl; 12.3, MgSO₄.7H₂O; 0.15, Tris HCl and adjusted pH to

7.5, and then centrifuged at 8000 rpm for 10 min. After the washings have completed, the pellets were collected in a flask containing dist. deionized water and disrupted by ultrasonication (Biologics 300 V/T ultrasonic) at 120 Watts, 40 Hz for 10 min. The resulting suspension after sonication was centrifuged at 12000 rpm for 30 min. to get rid of the remaining's of the bacterial cells. Finally, the resultant extraction supernatant was used wisely for CuNPs investigation according to the modified procedure of Laddaga and MacLeod, (1982); Oremland *et al.*, (2004).

2.4.2. Investigation of the extracellular synthesis of CuNPs

For investigating the extracellular biosynthesis of the CuNPs, about 10 ml of the collected cell free extract was re-centrifuged at 15000 rpm for 30 min., in order to remove the non-protein components, and then the supernatant was collected according to Dhoble and Kulkarni, (2015). This supernatant was filtered to separate the NPs and then washed 4 times (3 times with de-ionized water, and the last time with ethanol), to remove the remaining CuSO₄ and the other impurities. The separated precipitate (CuNPs) was dried in air for 24 h, and then stored at room temperature till being used for characterization. A control treatment composed of the Glucose broth medium supplemented with 1 mM CuSO₄ but without bacterial inoculation was used as recommended by Sayedeh-Fatemeh *et al.*, (2014).

2.5. Identification of the potent CuNPs synthesizing bacterial isolate

According to Niall and Paul, (2009), Bergey's Manual of Systematic Bacteriology 2nd ed was applied to identify the potent bacterial isolate following its morphological and physiological characterization. These findings were confirmed by the 16S rRNA sequencing in reference to Sarker *et al.* (2012). The partial 16S rDNA gene sequence was aligned, analyzed in comparison with several nucleotide information, using the BLAST application provided in the NCBI GenBank. For discovering the closest

homologous bacterial members, data was retrieved through constructing a phylogenetic tree using the Neighbour-joining method.

2.6. Influence of medium components and temperature on the synthesis of CuNPs by *P. silesiensis*

In this experiment, the Glucose broth medium and 1 mM CuSO₄ were used independently and\ or mixed to study their effects on CuNPs synthesis by the *P. silesiensis* isolate after incubation for 48 h at 30°C on the rotary shaker (150 rpm). After incubation, the broth culture was centrifuged for 20 min. at 10000 rpm, and then the supernatant was collected. The formation of CuNPs was detected using the UV-vis spectral analysis (UNICO UV-2100) at 400-700 nm, which has been suggested by Shantkriti and Rani, (2014). Existence of protein molecules that reduced the CuSO₄ was studied by the denaturation treatment as described by Shantkriti and Rani, (2014). The cell-free supernatant sample was heated at 100°C using a water bath for 30 min., and then Cu NPs formation was detected as described before.

2.7. Time course of the CuNPs biosynthesis

To trace out the site and the time needed for CuNPs synthesis by *P. silesinsis* strain A3, a time course was performed by taking 2 ml aliquots of the culture supernatant at different incubation times (every 6 h for 48 h) under aseptic conditions, centrifuged, and then analyzed for CuNPs production by UV-vis analysis at 400-700 nm according to Shantkriti and Rani, (2014).

2.8. Preparation of the agro-industrial residues

The utilized solid agro-industrial residues such as; olive cake, broken rice, whey of Arish cheese, glucose syrup, sugar beet waste, and sugar cane molasses, were washed with cold water then warm water, and then dried overnight at 50°C to constant weights. The dried substrates were ground and sieved to remove the coarse particles and kept for further studies (Singh and Rani, 2014). For blackstrap sugar cane molasses, water

was added in the ratio of 1:1 for dilution, acidified to pH 4, heated at 100°C for 1 h in a water bath, and then kept overnight to precipitate the undesirable metal salts in reference to the method of Pyke, (1958). The pH of whey of Arish cheese was adjusted to pH 4.5 then heated at 121°C for 15 min. to denature the protein, and then centrifuged at 10000 rpm for 15 min. at 4°C; finally the precipitates were discarded (Guerra and Pastrana, 2002).

2.9. Supplementation of the broth culture with the agro-industrial residues affecting CuNPs synthesis

To select the most appropriate carbon source, the basic carbon source for the synthesis medium (glucose) was replaced with a similar concentration of each of the tested carbon sources (i.e. sugar cane molasses, sugar cane bagasse, glucose syrup, olive cake, broken rice, sugar beet waste, and Arish cheese whey). Various concentrations ranging between 0.5% and 4.0% of the tested carbon sources were added individually to the basal medium. The total carbon content of the sugar cane molasses, glucose syrup, olive cake, broken rice, sugar beet waste, sugar cane bagasse and Arish cheese whey were; 48.3, 40.0, 54.9, 81.2, 50.0, 46.9 and 4.8%; respectively, according to reports of the Central Laboratory, Horticulture Research Institute, Agriculture Research Center, Giza, Egypt.

2.10. Bio-reduction of divalent (Cu^{+2}) to monovalent (Cu^{+1}) by copper reductase

Monovalent Cu^{+1} was estimated using 1 mM Neocuproine (NC) (2, 9-dimethyl-1,10-phenanthroline) reagent which has a high affinity for binding with Cu^{+1} (Smith and McCurdy, 1952; Andreatza *et al.*, 2011). The Cu reductase assay was carried out as follow: prepare 1 mM stock solution of Cu^{+2} by dissolving 0.249 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1 l dist. water, 1 mM stock solution of Neocuproine hemihydrate (NC) by dissolving 0.0108 g in 100 ml ethanol, 0.1 M phosphate buffer pH 7.0. Add 1 ml of 1 mM Cu^{+2} in 3 ml of 0.1 M phosphate buffer (pH 7.0), and then incubate at 30°C for 5 min. After

incubation, add 1 ml of the bacterial cell-free supernatant (enzyme), mix and incubate at 30°C for 5 min. After that, add 2 ml of 1 mM NC solution and incubate at 30°C for 30 min. The reduction of Cu^{+2} was observed through the formation of orange-yellow [Cu^{+1} -NC] complex (which stable for at least 2 h). Maximum absorbance of this solution was measured spectrophotometrically at 448 nm against a [Cu^{+2} - NC] blue-green blank solution, which normally does not show absorbance in this range of the spectrum. The concentration of the reduced Cu was calculated from a standard curve of Cu^{+1} prepared in the same medium using CuCl_2 .

2.11. Characterization of the biosynthesized CuNPs

The preliminary observation for CuNPs formation was detected by visual color change. Primary investigation for CuNPs formation was detected through spectral analysis of the culture supernatant using UV-vis spectroscopy at wavelength of 400-700 nm (Prema, 2010). According to Kohler and Fritzsche, (2004), the particle size determination and zeta potential for CuNPs suspension were investigated using Dynamic light scattering (DLS), and Zetasizer (Malvern Zetasizer ZS90, UK) instrument; respectively. High-resolution Transmission electron microscopy (HR-TEM) (model JEOL JEM-2100) was used to study the shape of the CuNPs, using an amorphous carbon-coated copper grid loaded with an aliquot of CuNPs suspension, dried and then analyzed at 200 kV voltage in reference to Subbaiya and Selvam, (2015). The complementary investigations including concentration estimation was carried out using; the Atomic absorption spectrophotometer (Perkin Elmer A Analyst 100), the crystalline structure of the CuNPs powder was determined by X-ray diffraction (XRD) (Shimadzu XRD-7000) (Neelakandeswari *et al.*, 2014), whereas the Fourier-transform infrared spectroscopy (FTIR) (400-FTIR) was used to identify the functional groups in the agro-industrial responsible for reducing the Cu such as; amine, carbonyl, and hydroxyl groups according to Subbaiya and Selvam, (2015).

2.12. Statistical analysis

Data were analyzed statistically by SPSS Statistics (version 25) software according to Duncan's, (1955) at 5% level.

3. Results and Discussion

3.1. Isolation and screening for CuNPs synthesizing bacteria

Data represented in Fig. (1) indicate that 100 isolates are obtained from the plant rhizospheres (62%), whereas 60 isolates are recovered from the seawater (38 %) samples. Basil, Chamomile, Egyptian clover, Hibiscus, Iris, Neem, Pea, and Thyme isolation

samples gave; 6, 20, 6, 15, 12, 10, 22, and 9 isolates which represent; 4, 12, 4, 9, 7, 6, 14 and 6 %, respectively. These isolates were preliminarily screened for CuNPs synthesizing on Glucose agar medium supported with 1 mM CuSO₄ solution. Out of 160, sixteen bacterial isolates exhibited growth on the plates containing Cu, which demonstrates their resistance capacity (tolerance) to Cu. The 16 isolates were collected from seawater were designed symbols as; S54 and S57 isolates, and plant rhizospheres samples of chamomile; C74, 80, 83, 84, 86 and 87 isolates; Hibiscus; H96 isolate; Iris; I108, I109, I10 and I14 isolates, Neem; N125 isolate, and Pea; P146 and 149 isolates.

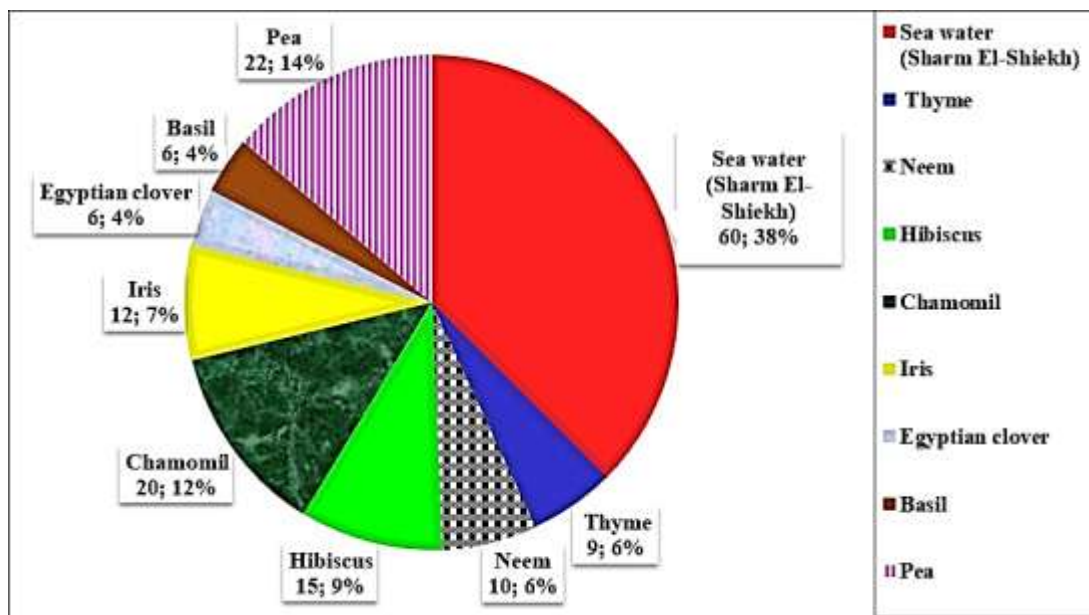


Fig. 1: The number and percentage (%) distribution for the isolates obtained from different cultivated sea water and soil rhizosphere samples.

3.2. Biosynthesis of CuNPs

The inocula of mid-logarithmic growth of the 16 selected isolates were inoculated into Glucose broth medium supplemented with 1 mM CuSO₄ solution, and then incubated at 30°C on a rotating shaker (150 rpm), for 48 h. Through visual observation, the mixture's reaction colour are turned from pale green to deep green or brown Fig. (2a). Color formation in the mixture's reaction indicates excitation of the surface plasmon resonance in the metal NP (Shahverdi *et al.*, 2007). Current results indicate that the maximum surface plasmon peak (SPR) for the mixture's reaction appeared at λ max 580 nm (data not shown). According to Ramyadevi *et al.*, (2012), CuNPs exhibits SPR at wavelength ranged from 556 to 590 nm. Results represented in Fig. (2b-d) show various characteristics including; particle size of the Cu NPs, maximum absorbance SPR at 580 nm, and Cu NPs concentration (mg\ l). Results impacted that among the 16 resistant isolates, 3 isolates designed as; I108, I114, and S57 showed a denser color change in the reaction mixture with high significant ($p < 0.05$), SPR of (0.54, 0.45 and 0.40), and small particle size of (87.1, 189.1 and 203.6 nm), respectively. Isolate (I108) expressed the highest production capacity of CuNPs, characterized by having the smaller particle size and maximal SPR, thus it is selected as a promising isolate for subsequent investigations. This result may be related to the higher reduction rate of Cu ions, or more stability of the synthesized CuNPs, as recorded previously by Tiwari *et al.*, (2016).

3.3. Identification of the promising CuNPs synthesizing isolate

3.3.1. Phenotypic characteristics

The most promising isolate I108 was identified according to culture growth pattern, cell morphology, and biochemical properties based on phenotypic characteristics according to Bergey's Manual of Systematic Bacteriology (Niall and Paul, 2009). Colonies of the isolate I108 grown on the Glucose agar

medium are round, smooth surface and edges, with milky-yellow color. The isolate is Gram-negative, small, short rods, flagellated, and is non-spore forming bacterium. Moreover, the physiological properties of this isolate indicated that it is aerobic, capable to grow in 0- 4% NaCl, at pH range between 6 to 9 (optimum at 7), and at a temperature range of 4- 37°C (optimum at 30°C). The isolate gives positive results for casein hydrolysis, catalase, and nitrate reduction; whereas negative results are obtained for gelatin hydrolysis, starch hydrolysis, citrate utilization, Voges-Proskauer, and fluorescent pigment production when cultivated on King's B medium.

3.3.2. Genotypic characteristics

Results of the 16S rRNA gene sequence analysis of isolate I108 shows that it belong to Genus *Pseudomonas*, closely related to *P. silesiensis* strain A3 (98% similarity) after analysis and comparison with nucleotide sequences of NCBI. The amplified DNA produced a single band of 1300 bp, as shown in Fig. 3(a- b).

3.4. Mechanism of CuNPs biosynthesis by *P. silesiensis* strain A3

To confirm whether the biosynthesis of CuNPs was extracellular or intracellular, the cell-free extract and cell pellets of *P. silesiensis* strain A3 were investigated according to color change and particle size. Results showed that when 1 mM CuSO₄ solution was added to the cell free extract of *P. silesiensis* strain A3 and incubated for 48 h, the color of the reaction mixture changed from pale blue to deep green or brown, whereas the particles size is 87.1 nm, as presented in Fig. (2a-b). On the other hand, the addition of CuSO₄ solution to the cell pellet does not reveal any change in the color, and no particles are detected (data not shown). Accordingly, these results confirm that the biosynthesis of CuNPs is in an extracellular manner.

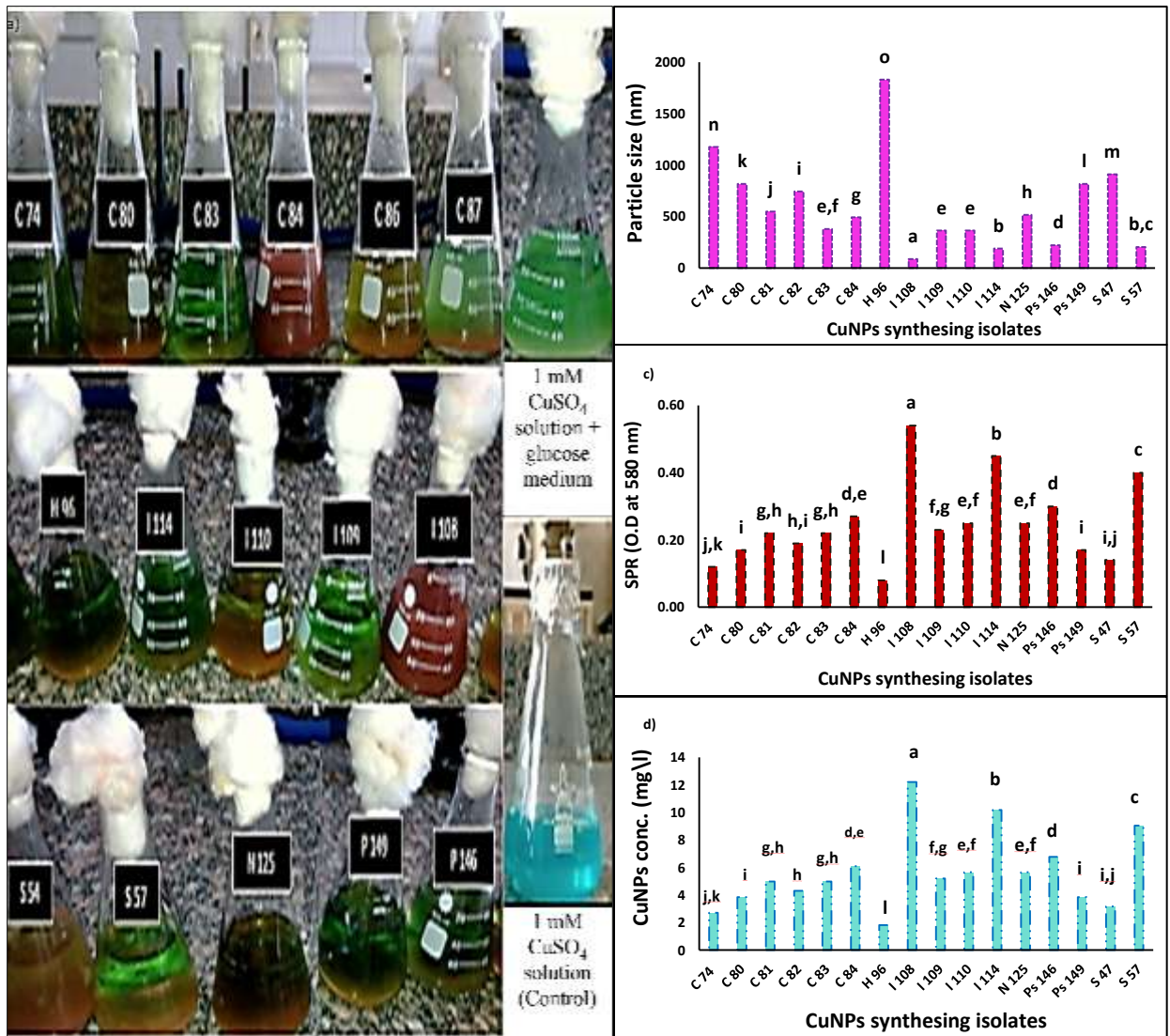


Fig. 2: (a): Change mixture's reaction color, (b): particle size; (c): SPR; (d): concentration (mg/l), of the CuNPs synthesized using the cell-free extract of the potent isolates on Glucose broth medium after incubation at 30°C for 48 h. SPR= surface plasmon resonance, C=Chamomile, H= Hibiscus, I=Iris, N= Neem, Ps=Pea, and S=Sea water. Values in the same column followed by the same letter do not represent significant difference from each other, according to Duncan's, (1955) at a 5% level.

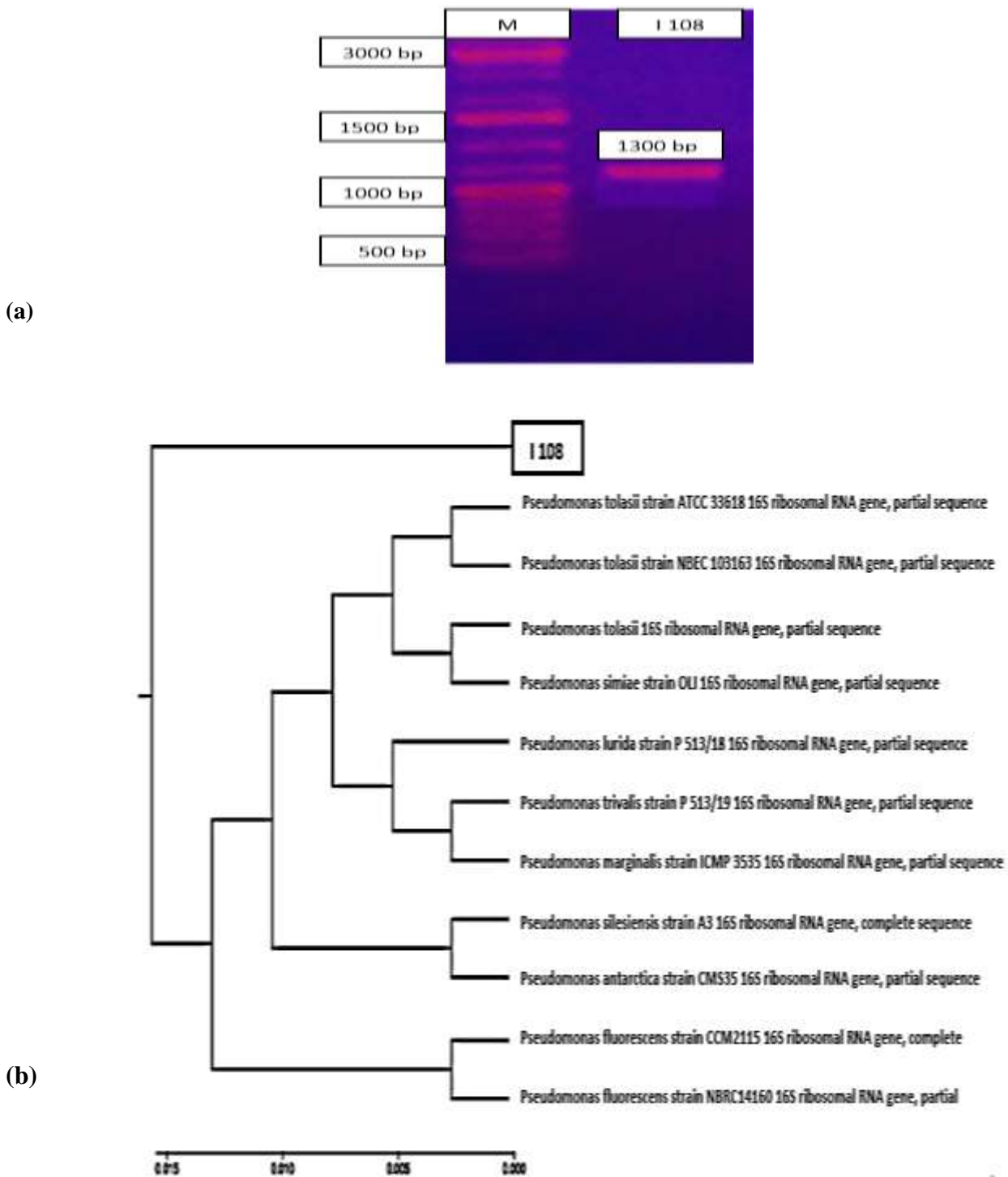


Fig. 3: Phylogeny tree of the genus *Pseudomonas* based on comparative analysis of 16s rRNA sequences. a) PCR products for 16S DNA of the isolate I108; M: marker. b) Obtained neighbor-joining tree from BLAST search based on 16S rRNA sequences of the genus *Pseudomonas* showing the position of I108 isolate compared with the related strains.

3.5. Effect of growth medium components on CuNPs biosynthesis

This investigation attempted to identify interaction between bacterial cell-free extract and media components during the synthesis of CuNPs by *P. silesinsis* strain A3. Results in shown Fig. 4 (a) revealed that the un-inoculated control Glucose broth medium showed a maximum absorbance peak at 680 nm, while the 1 mM CuSO₄ solution showed a peak at 480 nm. On the other hand, the maximum peak is observed at 660 nm upon addition of 1 mM CuSO₄ solution to the un-inoculated medium, which is not a characteristic of CuNPs formation. On the contrary, when the cell-free extract was added to 1 mM CuSO₄, a peak is observed at 580 nm indicating the formation of CuNPs. These results may be attributed to factors in the cell-free extract that converted and stabilized the NP during its biosynthetic process as stated by Din *et al.*, (2017). Previously, Shivaji *et al.* (2011) suspected that there was an interaction between cell-free supernatant and the media components during the synthesis of AgNPs by Psychrophilic bacteria. To get rid of this suspicion, the intensity of SPR peak observed in the specific wavelength is the most powerful indication for the bacterial culture supernatant effect compared to the medium components. On the other hand, when the reaction mixture (cell-free extract + 1 mM CuSO₄) was treated with heat at 100°C for 15 min., there was no absorption at 560-580 nm compared with the non-heat treatment. These observations confirmed that NP synthesis is referred to the bio-reduction of Cu ions throughout heat-labile protein biomolecules released by the bacteria into cell-free supernatant in accordance with Shantkriti and Rani, (2014).

3.6. Time course of CuNPs biosynthesis

Results presented in Fig. (5) clearly show the maximum absorption of *P. silesinsis* strain A3 reaction mixture recorded at λ max 580 nm during the 6 to 48 h incubation periods. At zero time of incubation, the

maximum absorption is registered at λ 680 nm, which is associated with the CuSO₄ solution-Glucose medium mixture. The SPR peaks increased gradually on increasing the incubation time to 24 h and are stable up to 48 h later. After 24 to 48 h of incubation, the highest SPR peaks are recorded in the ranges from 0.64 to 0.66. This indicates the good dispersion and non-agglomeration of the CuNPs, owing to the capping agents that exist in the cell-free extract. These results are in agreement with those obtained by Tiwari *et al.*, (2016), that CuNPs synthesis was taking place within 36 h of incubation by *Bacillus cereus* SWSD1 and after that period the particles size did not increase due to its non-agglomeration. Currently, the SPR are 0.36 and 0.37, after 24 and 48 h of incubation, respectively.

3.7. Effects of different agro-industrial wastes on the production of CuNPs by *P. silesiensis*

To investigate the impacts of different agro-industrial residues as carbon sources on CuNPs production by *P. silesiensis* strain A3, about seven sources were used (blackstrap sugar cane molasses, glucose syrup, olive cake, broken rice, sugar beet waste, sugar cane bagasse, and Arish cheese whey) instead of glucose (control 1%) in the basal medium. Data illustrated by Fig. (6) indicate that the maximum SPR at 580 nm, minimum particle size, CuNPs concentration and Cu reductase activity being 0.75, 80.5 nm, 16.5 mg/l, 23.2 mg/l; respectively, which are recorded on using blackstrap sugarcane molasses as the sole carbon source. This is followed by glucose and glucose syrup with SPR of 0.55 and 0.58, diameter size of 87.1 and 90.0 nm, Cu reductase activity of 22.7 and 18.7 mg/l, and CuNPs concentration of 12.44 and 13.12 mg/l, respectively. On the other hand, the lowest SPR, CuNPs concentration, Cu reductase activity, and the highest particle size values are recorded on using broken rice with 0.33, 7.5 mg/l, 8.9 mg/l, and 135.2 nm, respectively. These results indicate that utilization of blackstrap sugarcane molasses increases the SPR by 36.4%, CuNPs

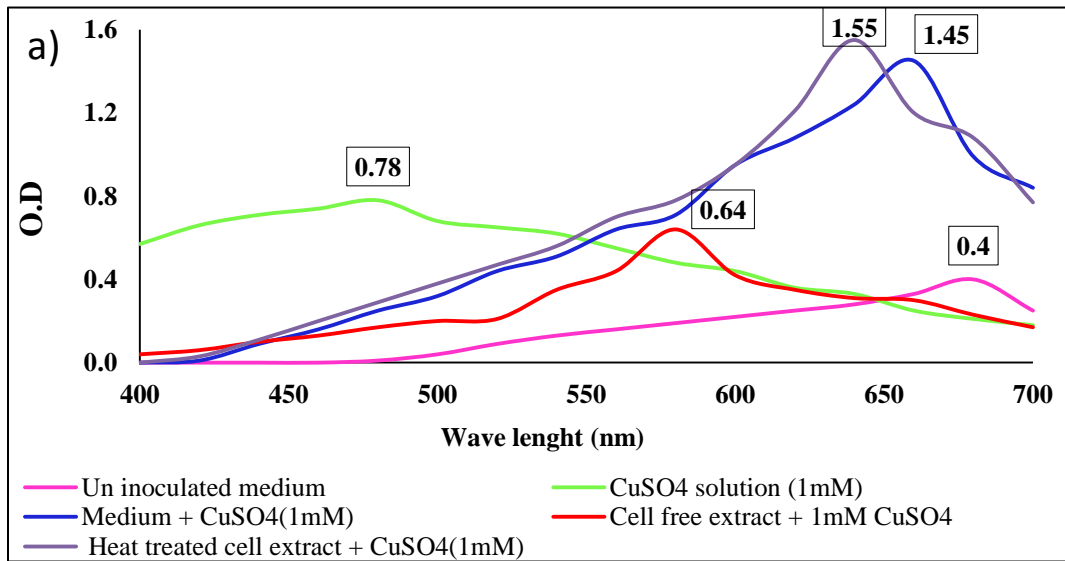


Fig. 4: Effect of media components on the UV–vis absorbance of the cell free supernatant during CuNPs synthesis by *P. silesinsis* strain A3 cell free extract.

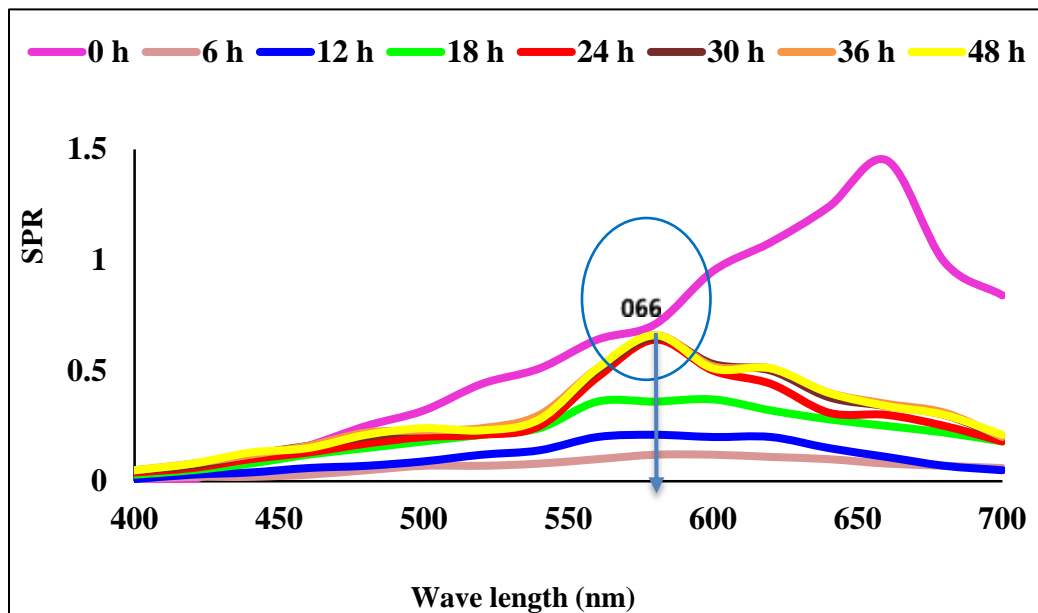


Fig. 5: Time course of CuNPs SPR synthesized by *P. silesinsis* strain A3 cell free extract.

concentration by 25.0% and Cu reductase activity by 33.0%. However, the reduction of particle size by 7.6% is recorded compared with glucose (control). These results may be attributed to blackstrap molasses as being rich in vitamins, sugars and growth factors essential for microbial growth and enzymes production. These results coincided with those obtained by Anna *et al.*, (2015). According to Satyanarayana *et al.*, (2004), the significant reduction in SPR, CuNPs concentration and Cu reductase activity on using olive cake, broken rice, sugar beet waste, and sugar cane bagasse may be referred to the thickness of the growth medium structure which contribute to the decrease in culture aeration, which is essential for the bacteria growth and Cu reductase activity. These results are in accordance with Anna *et al.*, (2015), who reported that cell-free extract of *B. subtilis* T-1 cultivated on brewery liquor and molasses, were the most suitable sources for AgNPs biosynthesis. Similarly, Sherien *et al.*, (2017) several utilized agricultural wastes such as; carrot peel, potato peel, apple peel and olive cake waste for the biosynthesis of ZnNP by *Chaetomium globosum*, and found that olive cake waste was the most promising carbon source.

3.8. Effect of different concentrations of blackstrap sugar cane molasses on the production of CuNPs by *P. silesiensis*

About six concentrations of blackstrap sugar cane molasses ranging from 0.5 and 4% were utilized for CuNPs synthesis by the potent bacterial strain. Data presented in Fig. (7) clearly show that the lowest diameter size of 66.12 nm is recorded after 24 h of incubation using cell-free extract obtained from broth culture containing 2% blackstrap sugar cane molasses. This lead to the increase in SPR by 13.3%, NPs concentration by 16.4%, enzyme activity by 16.5%, and lead to reduction of particle size about 17.9%, compared with 1.0% blackstrap sugar cane molasses in the control. Whereas, the highest diameter of the CuNPs of 119.7 nm is recorded on using the cell-free

extract obtained from broth culture supplemented with 0.5% blackstrap sugar cane molasses after 24 h of incubation.

3.9. Characterization of the biosynthesized CuNPs

3.9.1. HR-TEM investigation

From determining the shape of the CuNPs synthesized by *P. silesiensis* strain A3, HR-TEM was used. Fig. 8(a) demonstrates that these particles have spherical aggregates. Similarly, Shantkriti and Rani, (2014) indicated that CuNPs synthesized by *P. fluorescens* were spherical and/or hexagonal in shape.

3.9.2. XRD analysis

An increase in CuNPs crystallinity level is presented with diffraction angles of 43.44°, 43.58° and 50.58°, respectively. The face-centered cubic (FCC) characteristic of the copper lines are plotted at 264, 253 and 55; respectively, as clear in Fig. (8b). Also, there are some extra peaks at 32.53°, 53.35°, 58.22°, 61.58°, 66.12°, 68.06°, and 75.18°, which could be attributed to the existence of copper oxides using manual instrument (JCPDS card No: 80–1917 and 78–2076). In agreement with the previous observations of Tiwari *et al.*, (2016), peaks are recorded at 45.55°, 48.84°, and 56.97°, corresponding to 111, 200 and 220 planes of the copper, and corresponding to the face-centered cubic (FCC) of this CuNPs. Moreover, extra peaks at 31.39°, 35.83°, 39.17°, 65°, and 72.2° are detected, due to the presence of copper oxides.

3.9.3. FTIR analysis

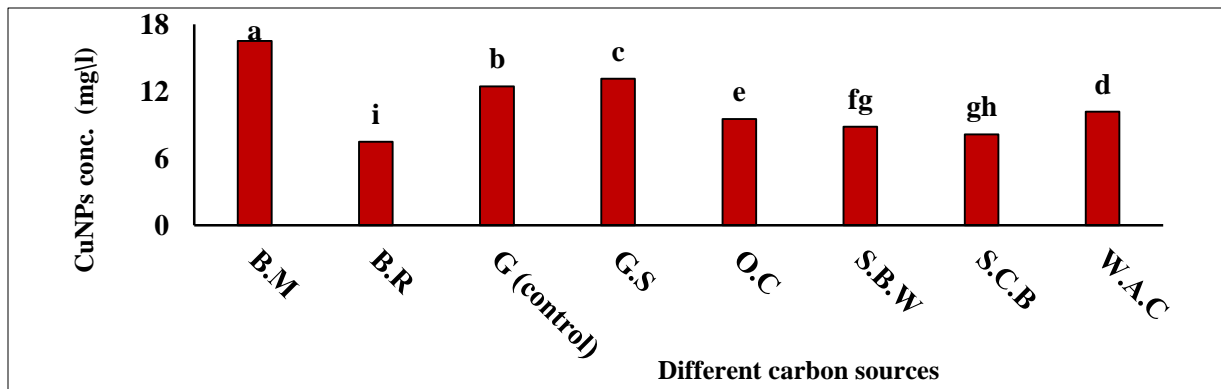
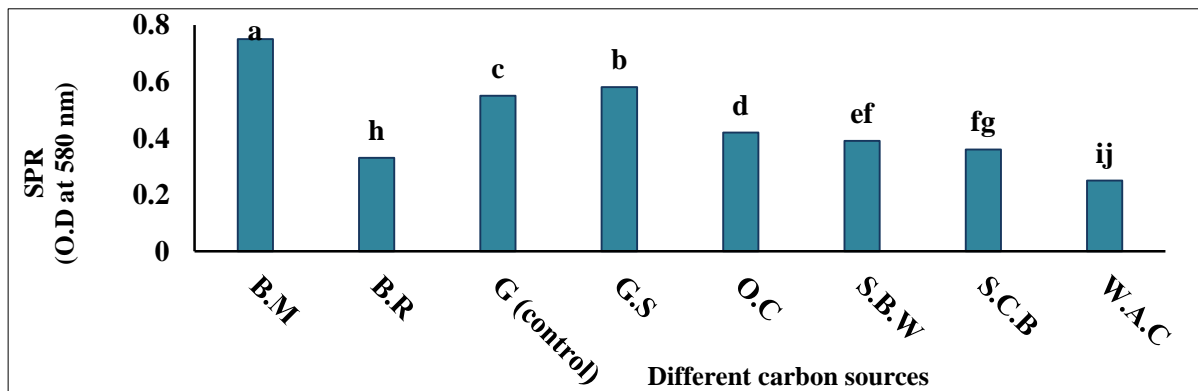
The observed spectra of the FTIR analysis show molecular relationships between CuNPs and the media components. Results presented in Fig. (8c) illustrate the specific amide peak and amine stretching and bending indicating the presence of proteins. It is observed that characteristic peaks are assigned at 1080, 1384, 1620 and 3433 cm⁻¹ correspond to -C-O- (str), C-H aromatic (str), C=C (str) and N-H (str), respectively. In accordance with Tiwari *et al.*, (2016),

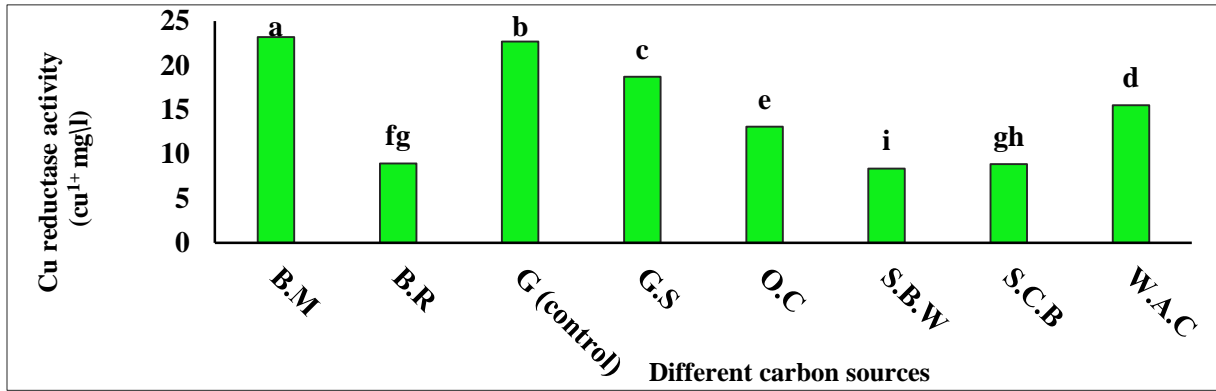
the FTIR spectra analysis of CuNPs showed specific peaks of amide and amine groups confirming that the CuNPs are coated by proteins. On the other hand, Jang *et al.*, (2015) reported that the proteins that coated CuNPs are known to reduce their oxidation and increase their long-term stability.

3.9.4. Zeta potential analysis

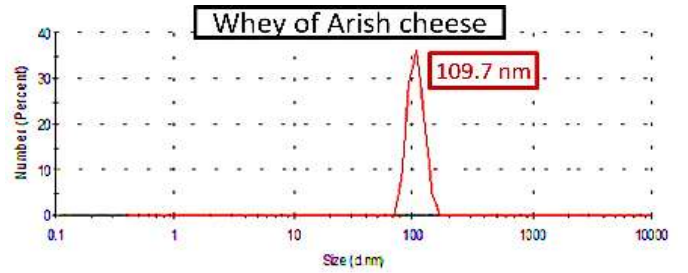
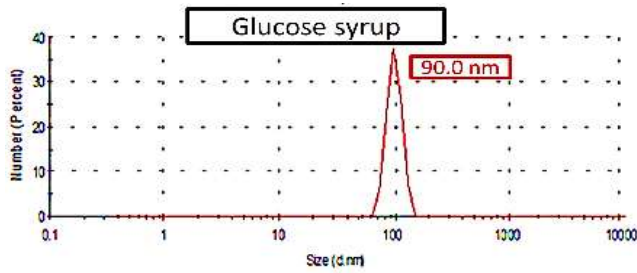
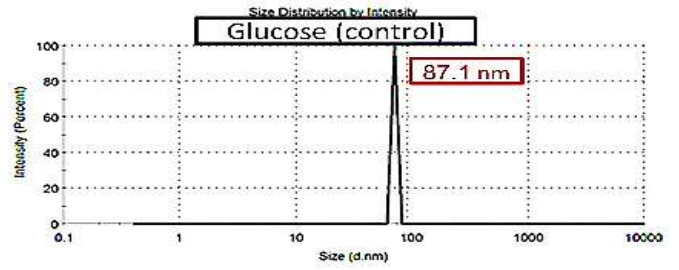
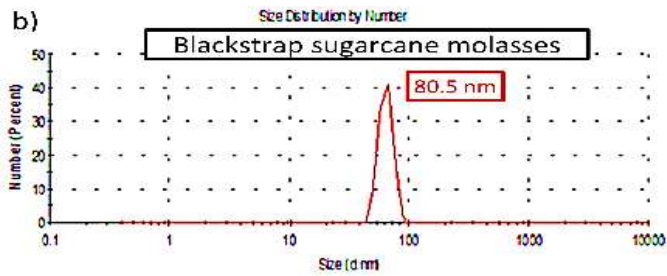
The zeta potential investigation of the CuNPs is observed at -14.6 mV that is attributable to the non-

ionic nature of the cell-free extract capping molecules. Due to the negative charge zeta potential shown in Fig 8(d), it induces the repulsion between the NPs and prevents the aggregation, thus gave the long-time stability owing to the electrostatic stabilization. Similarly, Tiwari *et al.*, (2016) reported that the negative zeta potential of CuNPs is such a unique property that could be referred to the protein coating of the NPs.





(a)



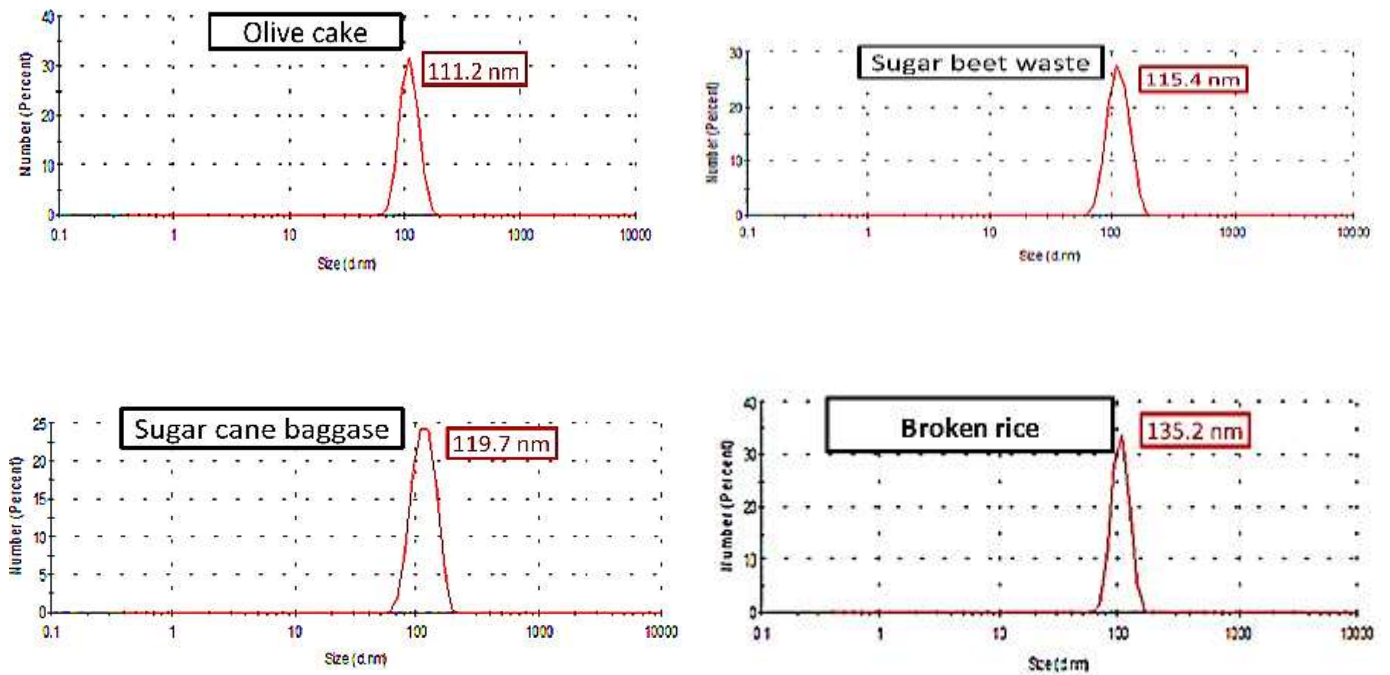
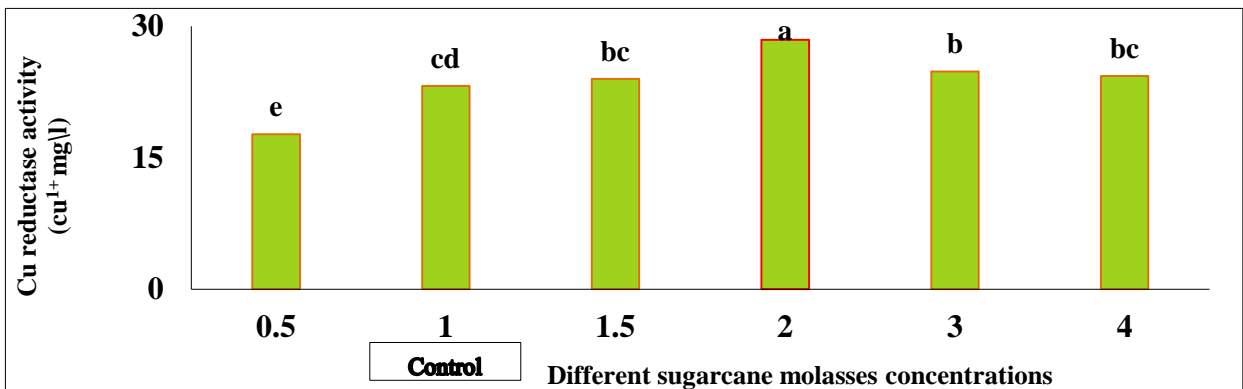
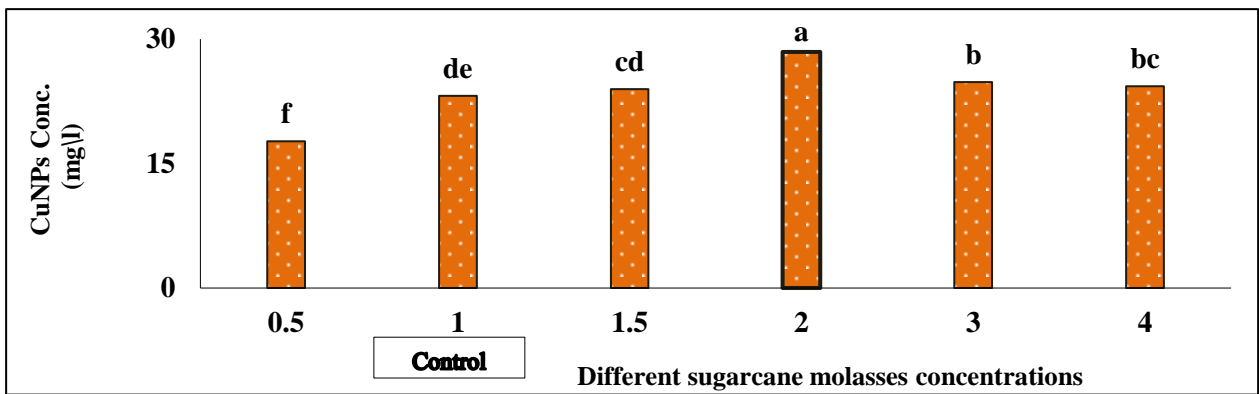
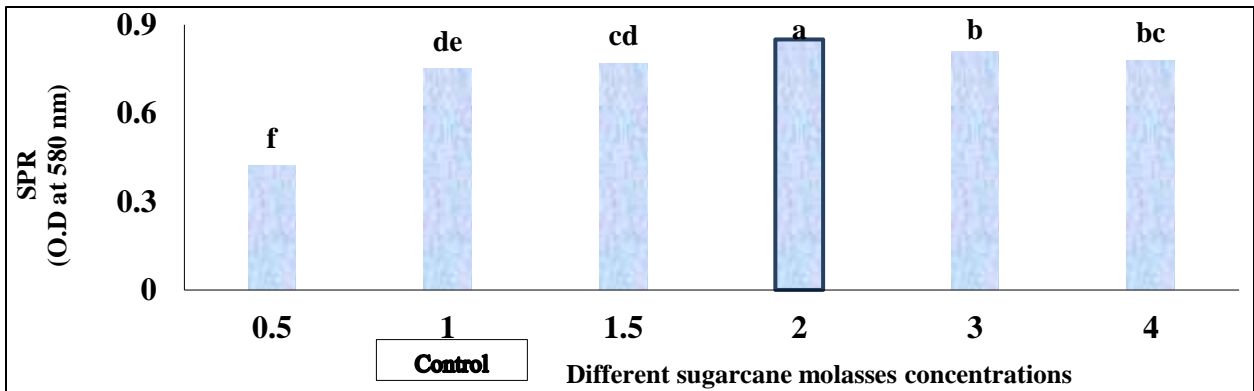


Fig. 6: Effect of different agro-industrial wastes on CuNPs synthesis by *P. silesiensis* strain A3 at 30°C during 24 h of shaking incubation. a) CuNPs parameters produced by cell free extract of *P. silesiensis*. b) NP size determination using DLS characterization. Where; B.M= Blackstrap sugarcane molasses, G.S = Glucose syrup, G=Glucose, O.C=Olive cake, B.R= Broken rice, S.B.W= Sugar beet waste, S.C.B= Sugarcane bagasse and W.A.C= Whey of Arish cheese. Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's, (1955) at 5% level.



(a)

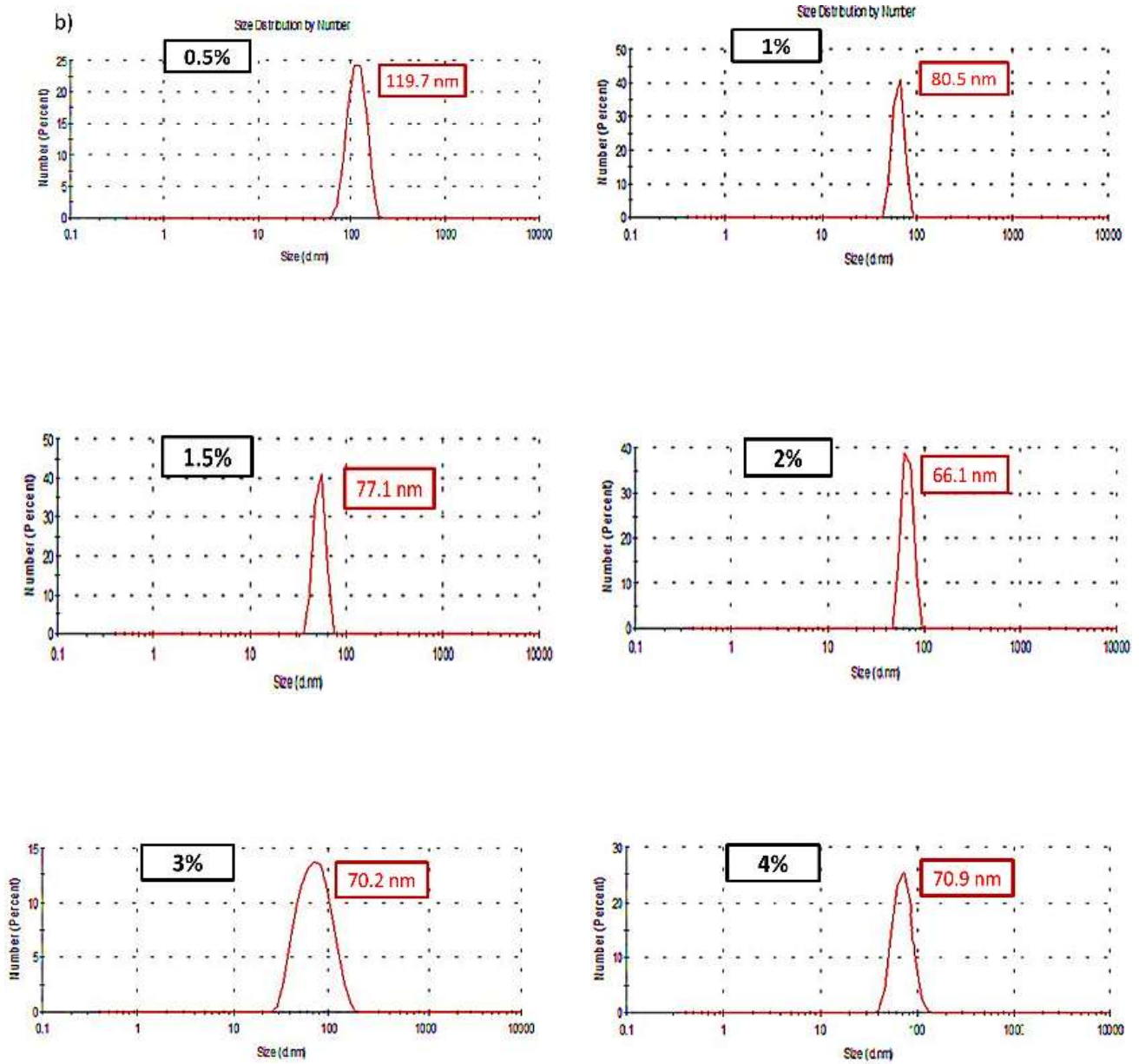


Fig. 7: Effect of different concentrations of sugarcane molasses waste on CuNPs produced by *P. silesiensis* strain A3 at 30°C after 24 h of shaking incubation at 150 rpm. a) CuNPs parameters produced by cell free extract of *P. silesiensis*. b) Particles size using DLS characterization. Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's, (1955) at 5% level.

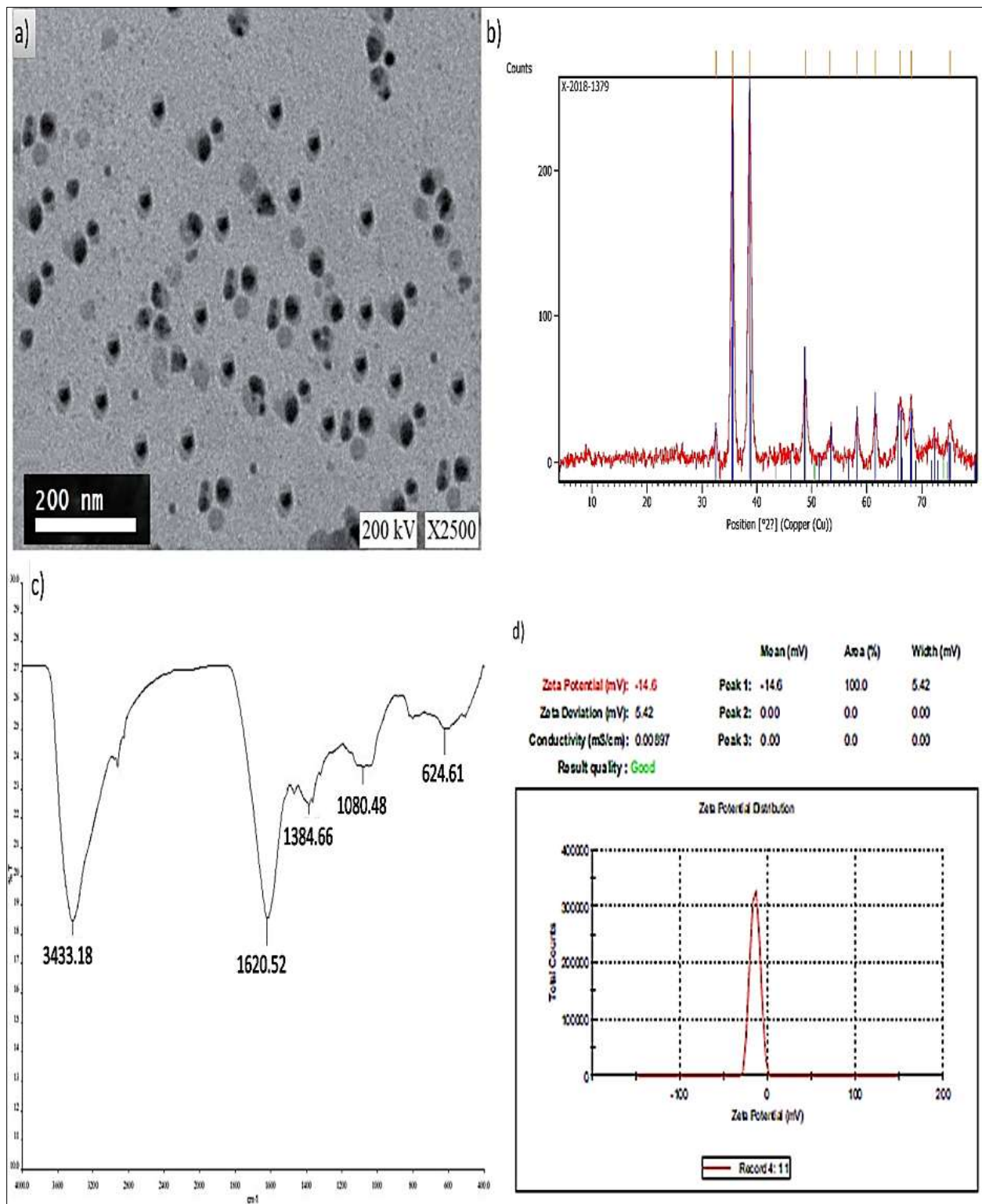


Fig. 8: Characterization of the CuNPs using (a) HR-TEM, (b) XRD, (c) FTIR and (d) Zeta potential

Conclusion

Of the 106 isolates recovered from different Egyptian ecosystems (soil and seawater), only single copper resistant isolate I108 is chosen and identified as *P. silesiensis* strain A3 based on the phenotypic and genotypic properties. The CuNPs has a size of 87.1 nm and SPR of 0.54 at 580 nm are synthesized after 24 h incubation of the *P. silesiensis* cell-free extract with 1 mM CuSO₄ at 30°C, using shaking incubation at 150 rpm. The use of inexpensive substrates is an economic process for biosynthesis of the CuNPs. *P. silesiensis* strain A3 I108 free-cell extract is capable of synthesizing CuNPs in the growth medium supplemented with 2.0% of blackstrap sugar cane molasses, which increases the biosynthesis process by about 35.0 % SPR, particles concentration by 35.0%, copper reductase activity by 20.0%, and reduces the particle size by about 32.0%, compared to that the basal medium containing glucose. It could be summarized that utilization of the agro-industrial by-products as eco-friendly supplements for the microbial biosynthesis of NPs has a great positive impact on the production process, as these NPs will be applied in the future as antitumor, antimicrobial agents and in many industrial applications.

Acknowledgment

Authors would like to thank the Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt, for supporting out the current experiments.

Conflict of interest

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

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