Impact of Copper and Its Nanoparticles on Growth, Ultrastructure, and Laccase Production of *Aspergillus niger* using Corn Cobs Wastes

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The influence of copper and its nanoparticles was studied relative to growth and ultrastructure of *Aspergillus niger*. Laccase production by *A. niger* using corn cobs as substrate at different concentrations of $CuSO_4$ and copper nanoparticles (CuNPs) is reported. Fungus growth was induced at 100 ppm of CuNPs and $CuSO_4$, while at 300 ppm, the growth inhibition was 65.6% and 86.9%, respectively. Fungus sporulation was reduced to 30.4% and 47.6% at 300 ppm of CuNPs and $CuSO_4$, respectively, compared to the control (100%). Transmission electron microscopy revealed that $CuSO_4$ and CuNPs treatments encouraged the deformed appearance of the fungus at 200 ppm and 300 ppm, particularly CuNPs. The CuNPs and $CuSO_4$ induced laccase production at 1.67 U/mL and 1.51 U/mL at optimum concentrations 0.15 mM and 0.25 mM, respectively. The optimum concentrations of CuNPs and $CuSO_4$ led to reduced incubation periods of 12 d and 14 d, respectively, required to produce the highest amount of laccase (1.66 U/mL and 1.53 U/mL), while without treatments, the incubation period increased to 16 d required for the highest amount of laccase production (1.36 U/mL). Induction of laccase production at acidic pH and at 30 °C was recorded with the addition of $CuSO_4$ and CuNPs, while its effects were slight at pH above 6.

Keywords: Copper nanoparticles; Ultrastructure; Laccase; A. niger; Corn cobs

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INTRODUCTION

Several studies have shown the biocidal activity of copper with broad-spectrum effectiveness against bacteria and fungi. Metal nanoparticles (NPs) are mainly used as fungicidal agents against phyto- and human-pathogens (Abdel Ghany 2013; Abdel Ghany *et al.* 2013, 2018a,b; Ganash *et al.* 2018). Among the metal NPs, copper nanoparticles (CuNPs) have been used increasingly in numerous applications in the current decade due to their cost-effectiveness; however, their impact on agricultural soil microorganisms has been poorly investigated (Rajput *et al.* 2019). Copper at the nanoscale demonstrates major antimicrobial activity (Gamalero *et al*. 2009; Raffi *et al*. 2010; Durán and Seabra 2012; Jia *et al*. 2012). Banik and Pérez-de-Luque (2017) recommend that CuNPs be applied as an alternative to chemical fungicides or non-nanoform copper, or at minimum scale used as an additive for increasing and enhancing the impact of commercial products of fungicides. Therefore, the antifungal properties of CuNPs alone or with other chemical fungicides have

been studied (Banik and Pérez-de-Luque 2017; Chalandar *et al.* 2017). Moreover, the antibacterial activity of CuNPs was also investigated against Gram –ve and Gram +ve bacteria (Gopalakrishnan *et al*. 2012; Amatya and Joshi 2020).

Incorporation of 50 mg/L CuNPs with copper oxychloride (non-nano copper) enhances the inhibition of *Phytophthora cinnamomi* growth compared to the inhibition at copper oxychloride application alone. Banik and Pérez-de-Luque (2017) recorded synergistic action of CuNPs with copper oxychloride against growth development and sporulation of *Alternaria alternata*. Recently, results of Sarkar *et al.* (2020) detected highest activity of defense enzymes and total phenolic compounds in the roots of *Lens culinaris* treated by 0.025 mgmL−1 CuNPs; therefore, CuNPs may be used as a potent plant defense booster. An increase in lignification of soybean root cells was recorded after CuNPs treatment (Nair and Chung 2014).

Microbial cell walls have a greater capability to accumulate metals from the surrounding environment. *Penicillium ochrochloron* accumulates Cu^{2+} from the environment in cell walls (Fukami *et al*. 1983). High levels of potentially toxic elements create side effects on microbial growth. Besides the toxicity, the potentially toxic elements can cause changes in or block enzyme action, inhibit proteins, carbohydrates, lipids, and nucleic acids synthesis, as well as induce disturbance of internal organelles in the cell (Denkhaus and Salnikow 2002). Changes in fatty acids of cell membrane as well as lowered polyunsaturated fatty acid content also have been reported (García *et al*. 2005). Hefnawy *et al*. (2009) investigated the rate of fungal sporulation under effects of copper in growth medium, where perithecia and spores numbers were markedly reduced with injure of seta, conidiophores, and phialides. Stohs and Bagchi (1995) declared that the elevated levels of Cu (II) and Zn cause a fast decline in membrane integrity, which is generally manifested by leakage of mobile cellular solutes and cell death.

The relationship between laccases and copper is due to structural properties, where several studies have identified laccases as glycosylated polyphenol oxidases that include four copper ions for each molecule. Fernandes *et al.* (2008) report that laccases are broadly spread among microorganisms as well as plants. The importance of laccases for various applications includes degradation of a large range of synthetic dyes, delignification of lignocellulosics, detoxification of waste, conversion of textile dye, food technological applications, and biosensor as well as analytical uses (Mayer and Staples 2002; Patel *et al*. 2019). Laccases catalyze oxidation of organic compounds, such as aromatic amines, polyphenols, methoxyphenols, and ascorbate (Yaropolov *et al.* 1994; Leonowicz *et al.* 2001).

Several species of rot fungi, such as *Trametes versicolor*, *T. villosa*, *Pleurotus ostreatus*, *Ganoderma lucidum*, *Panus tigrinus*, and *Agaricus bisporus* have been reported as sources for laccases (Ko *et al.* 2001). However, other fungi such as *Aspergillus spp.* have been reported to produce laccase, including *A. nidulans* (Aramayo and Timberlake 1993; Ko *et al.* 2001), *A. niger* (Abu and Ado 2004; Vandana *et al.* 2014), *A. flavus* (Kumar *et al.* 2016; Ghosh and Ghosh 2017), and *Aspergillus sp.* (Bhamare *et al.* 2018). Also, *Penicillium chrysogenum* (Senthivelan *et al.* 2019) and *Alternaria solani* (Abdel Ghany and Bakri 2019) were recorded as a producer of cellulases as well as laccase.

According to Revankar and Lele (2006), numerous conditions such as constituents of media, carbon and nitrogen sources, carbon-to-nitrogen ratio, pH, temperature, and aeration rate play a critical role in fungal laccases secretion. Bhamare *et al.* (2018) reported the vital role of toxic elements ions, mainly Cu^{2+}, for induction or inhibition of laccases production by fungi. Many studies report that laccases are cupro-proteins; therefore, the

addition of copper salts as a growth medium constituent at appropriate conditions lead to improved enzyme production (Fonseca *et al.* 2010; Nakade *et al.* 2013; Gomaa and Momtaz 2015). Enhancement of laccase secretion from *Fusarium solani*, *Pleurotus ostreatus*, and *Agaricus bisporus* have been observed by Kumar *et al.* (2007, 2011) at 1 mM of copper sulfate. In another study, Mann *et al.* (2015) reported that the medium growth supplemented by 0.4 mM and 0.75 mM copper increased laccase secretion in *Ganoderma lucidum* and *Cerrena consors*, respectively. Bhamare *et al.* (2018) attributed the role of copper sulfate in enhancement of laccase production to the fact that copper acts as a strong inducer of laccase in fungi. An earlier study reported that the promoting role of copper is due to regulation of laccase gene transcription (Collins and Dobson 1997). Currently, minimization of the potentially toxic elements using its nano-forms are required for reduce its biohazards. Therefore the objective of this research was to investigate the influence of copper and its NPs on growth and ultrastructure of *A. niger*, as well as study its role on laccase production by *A. niger* using corn cobs.

EXPERIMENTAL

Materials and Methods

Fungal isolation and identification

Fifteen samples of corn cobs that showed fungal infection were collected from storage in Monufia Governorate (Lat. 30° 31′ 12″ N, Long. 30° 59′ 24″ E), Egypt. The infected samples were kept in sterile plastic bags and transferred to a microbiology lab for fungal isolation. Parts of infected corn cobs ranged from 3 mm to 5 mm in diameter were placed on the surface of potato dextrose agar (PDA) medium, then incubated at 30 °C (similar to temperature of storage place) for 7 d, and the developed fungal colonies were purified and sub-cultured. The colonies that appeared on all samples were similar in all morphological and microscopical characterizations. According to available criteria by Raper and Fennell (1973) and Samson *et al.* (1981), the fungal isolate was identified with regards to texture and color of fungal colony, reverse color, pigmentation, and colony diameter measured after 7 d of inoculation on different media, including PDA and Czapek's dox agar (CDA) media, where the diameter (μm) of conidiophores, conidial heads, hyphae, conidiospores, and vesicles as well as phialide length, were measured.

Copper nanoparticles

By chemical reduction method of $CuCl_2.2H_2O$; CuNPs were obtained from Sigma-Aldrich (St. Louis, MO, USA) and used in the current study. Their properties included nano-powder composition, 40 nm to 60 nm particle size, and assay with more than 99.5% trace metals basis according to producer pamphlet.

Antifungal assay of $CuSO_4$ and CuNPs using well diffusion and poisonous food methods

The well diffusion method was applied to determine the antifungal activity $CuSO_4$ and CuNPs against *A. niger*. Wells by sterile cork borer (5 mm) were made on CDA surface and seeded by 0.1 mL of fungus spores suspension. The $CuSO_4$ and CuNPs at different concentrations (100, 200, 300, 400, and 500 ppm) were added separately in each well. The plates were incubated at 30 °C for 10 d; then the inhibition zone was observed and recorded (Roy *et al.* 2013). The poisonous food technique was also applied to determine the antifungal activity of $CuSO_4$ and CuNPs against *A. niger* (Singh *et al*. 2010), and studying

the morphological and sporogenesis of the fungus. For studying the morphological properties, a transmission electron microscopy investigation of *A. niger* under different separately concentrations of $CuSO_4$ and CuNPs (100, 200 ppm and 300 ppm) was done. With using 5 mm of cork borer, colony margin of the growing isolated fungus (5 d old) was cut, followed by inoculation at the middle of the Petri dish containing growth medium CDA supplemented with different concentrations of $CuSO_4$ and CuNPs, and then incubated for 7 d at 30 °C. Fungus sporulation was recorded at different concentrations of $CuSO_4$ and CuNPs using hemocytometer slide, regarding the control as 100%. Moreover, the inhibition of growth (%) was estimated based on Kumar *et al*. (2007) using the following equation,

$$\text{Growth inhibition (\%)} = \left(\frac{\text{Growth without treatment} - \text{Growth with treatment}}{\text{Growth without treatment}}\right) \text{x}100 \quad (1)$$

where medium without any treatment was used as control.

Morphological and transmission electron microscopy investigation of A. niger

The PDA medium containing different concentrations of $CuSO_4$ and CuNPs as mentioned in poisoned food method was autoclaved and poured, and then *A. niger* spores were seeded. Under aseptic conditions the sterilized cover slips were dipped obliquely in the seeded agar layer along the line where the medium meets the upper surface of the cover slip; then they were incubated for 7 d at 30 °C. Using a tiny drop of Canada balsam (Carl Roth, Karlsruhe, Germany), the cover slips were fixed on glass slides and examined under a microscope. For electron microscopy studies, hyphal tips were cut from the margin of growing colony and fixed with 5% gluteraldehyde as a primary fixative for 24 h. The specimens were washed three times with phosphate buffer (pH 7.2), followed by buffer removal, then covered for 2 h by osmium tetraoxide (1%), followed by removal of the osmium tetraoxide. The specimens were dehydrated by passage through a sequence of ethanol levels, which ranged from 50% to 96%, followed by alcohol removal and substitution by propylene oxide for 1 h. The specimens were placed in propylene oxide and Epon 812 resin (2:1) (Carl Roth, Karlsruhe, Germany), then placed in a pure resin overnight, and then placed in an oven for 48 h at 60 °C. Small blocks were sectioned (50 nm) *via* ultramicrotomy, stained using uranyl acetate-lead citrate 500A (Agar Scientific, London, UK), and then examined using the transmission electron microscope (C JEOL Jem-1200 EX II. Acc. Voltage 120 KV. MAG-medium, JEOL, Tokyo, Japan) in the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

Induction of laccase production by A. niger

Five g of non-fungal infected ground corn cobs (size less than 2 mm) were moistened with 20 mL of water as moistening agent, placed in a 250 mL conical flask, sterilized at 121 °C for 30 min, then inoculated by 5 discs (5 mm) of growing colony, each disc containing 5 x 10^6 spores of *A. niger*, and incubated at 30 °C for 12 d. Under aseptic conditions, different concentrations of $CuSO_4$ and CuNPs (0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, and 0.50 mM) were added at the second day of incubation period as inducer for laccase production. The control was without any concentrations of $CuSO_4$ or CuNPs. Distilled water (50 mL) was added after incubation period to each flask and incubated for 60min on shaking incubator (150 rpm). Then, the metabolized substrate of each flask was filtered through muslin cloth, and the filtrates were centrifuged at 80,000 rpm (35,000 RCF) for 10 min by cooling centrifuge (Minispin; Eppendorf AG, Hamburg,

Germany). The enzyme in the supernatants was assayed as described by Garzillo *et al*. (2001).

Laccase assay

The supernatant (100 µL) was added to the reaction mixture of 1 mL containing 2 mM of 22,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate (ABTS) (Sigma Aldrich, St. Louis, MO, USA), in citrate-phosphate buffer (pH 5.0). The enzymatic activity was estimated in IU by monitoring the absorbance change at 420 nm, ϵ=36 $mM^{-1}cm^{-1}$ by spectrophotometer (Model 6300, EU, JENWAY, Stone, UK) at 30 °C.

Effect of temperature, pH, and incubation period on enzyme production

In order to study the optimal period of incubation for maximum laccase production, the production medium was adjusted at pH 6 and inoculated with spore suspension of *A. niger* (5 x 10^6 spores/mL), then incubated at 30 °C for different incubation periods from 2 d to18 d. The metabolized medium was withdrawn at different periods for measuring enzyme activity. The prepared productive medium was adjusted at p

H 6 and inoculated by 5 discs (5 mm) of growing colony and incubated at different temperatures *viz.* 10 °C, 20 °C, 30 °C, 40 °C, and 50 °C for 12 d. The effect of pH on laccase production was carried out by adjusting the growth media at different initial pH values *viz.* 3, 4, 5, 6, 7, 8, and 9, then inoculated and incubated at 30 °C for 12 d. At all conditions the growth medium was supplemented with the optimum concentrations of CuNPs and $CuSO_4$ at the second day of incubation period under aseptic conditions. The enzyme was estimated as mentioned earlier.

Statistical analysis

The mean ± SD (standard deviation) was calculated using three independent replicates of each treatment. The SPSS ver. 22.0 computer software was applied for statistical analyses of data.

RESULTS AND DISCUSSION

One fungus that was isolated from all collected corn cobs samples showed black fungal infection (Fig. 1), and the isolate fungus was identified as *A. niger* according to the mentioned criteria in material and methods. Therefore, corn cobs wastes were used as a low-cost substrate by using $CuSO_4$ and CuNPs as inducers of laccase production by *A. niger*. The inhibitor effect of copper ions and its NPs has already been established in various reports. For this reason, in the current study different concentrations of $CuSO_4$ and CuNPs were tested against *A. niger* growth as well as their ultrastructure to determine the noninhibitor concentration for laccase production.

The well diffusion agar technique revealed the antifungal activity of copper ion represented by $CuSO_4$ and its NPs on *A. niger* growth. However, CuNPs showed stronger antifungal activity than $CuSO_4$ (Fig. 2) at the same used concentrations (100 ppm to 500 ppm). Surprisingly, the resistance to low concentrations of $CuSO_4$ (100 to 400 ppm) appeared after 8 d of incubation period, where the inhibition zone was covered with new growth of *A. niger*, while the inhibition zone with CuNPs was still clear until 8 d after inoculation (Fig. 2). The lowest concentration of CuNPs (100ppm) showed no antifungal activity. The present results agreed with the obtained results of Banik and Pérez-de-Luque

(2017), who reported that *Trichoderma harzianum* growth was completely inhibited at 1000 ppm of CuNPs but not inhibited at low concentrations.

Through the poisonous food technique and measuring the mycelial radial growth, *A. niger* growth was promoted at low concentrations (100 ppm), but the growth was inhibited at 200 and 300 ppm of $CuSO_4$ and CuNPs, where the growth inhibition was 58.1% and 86.9% using CuNPs and 30.3% and 65.6% using $CuSO_4$, respectively. At the same time, sporulation decreased at 300 ppm of CuNPs and $CuSO_4$, reaching 30.4% and 47.6%, respectively, compared to control at 100% (Table 1). Decreasing of *Alternaria alternata* and *Botrytis cinerea* sporulation was observed as a result of CuNPs treatment at 15mg L^{-1} (Sahar 2014). Not only did *A. niger* tolerate the low concentration of copper ion and its NPs, but other fungi were reported. Hefnawy *et al*. (2009) established that *Chaetomium globosum* and *Stachybotrys chartarum* were able to grow at 800 mg/L, while they failed to grow at 1000 mg/L of copper. In a prior study (Viet *et al.* 2016), *Fusarium* sp. growth was inhibited at concentrations more than 200 ppm of CuNPs, where its inhibition reached to 94.0% at 450 ppm after 9d of incubation period. Recently Banik and Pérez-de-Luque (2017) observed that the growth of *Alternaria alternata*, *Botrytis fabae*, *Fusarium oxysporum* f. sp. *ciceris*, and *F. oxysporum* f. sp. *melonis* was promoted at low concentrations (100 ppm) of CuNPs. Fungal growth enhancement at low concentrations of $CuSO_4$ and CuNPs might be due to biological activity of copper ion as a cofactor of enzymes. Similar results have been reported for antifungal activity of CuNPs (Pariona *et al.* 2019), where *Neofusicoccum* sp. exhibited great tolerance to CuNPs at low concentrations, but its growth was inhibited at concentration greater than 500 ppm.

The morphological changes of *A. niger* at control and CuNPs treatments are reported in Table 2 and shown in Fig. 3. Microscopic examination showed normal structural characteristics of *A. niger* cultivated in medium without treatments. In contrast, $CuSO_4$ and CuNPs treatments promoted deformation of fungus. Conidial heads, vesicles, conidiospores, and hyphae diameters, as well as phialides length, were reduced at high concentrations (200 ppm and 300 ppm) of $CuSO_4$ and CuNPs (Tables 2 and 3). The CuNPs were more effective than $CuSO_4$ with respect to morphological changes of *A. niger*.

Transmission electron microscope (TEM) revealed deformation of *A. niger* mycelia at high concentrations (200 ppm and 300 ppm) of $CuSO_4$ and CuNPs (Fig. 4). In contrast, at control and 100 ppm of $CuSO_4$ and CuNPs, no changes appeared in the cell wall and cell membrane, with clearing nucleus inside the cell. The cytoplasmic membrane was collapsed outside the cell wall at 200 ppm of CuNPs. Furthermore, numerous granules were aggregated inside the cytoplasm at 300 ppm of CuNPs, and these might be due to accumulation of copper within fungal cell.

Little is known regarding the impact of CuNPs on ultrastructure of fungi, because most of scientific papers have focused on the growth inhibition only. Using TEM, Sahar (2014) revealed collapse of *Alternaria alternata* and *Botrytis cinerea* hyphae treated CuNPs, beside hyphae damage and precipitations of NPs on walls of cells.

Many large vacuoles appeared as a response to high concentrations of $CuSO_4$ (200 ppm and 300 ppm) and CuNPs (300 ppm). Under stress conditions of potentially toxic elements, Gamalero *et al*. (2009) reported that the potentially toxic elements were accumulated in *Glomus intraradices* cell wall and vacuoles, but slight changes in the potentially toxic elements levels were detected in the cytoplasm. Recently, Pariona *et al.* (2019) observed strong morphological changes in the mycelium and cell membranes of *Fusarium oxysporum*, *F. solani*, and *Neofusicoccum* sp. exposed to CuNPs. Salvadori *et al.* (2013) investigated the dead biomass of *Hypocrea lixii* treated by CuNPs. They found

NPs inside cell wall but not in cytoplasm and cytoplasmic membrane.

Fig. 1. Corn cobs samples as a source of *A. niger* isolate

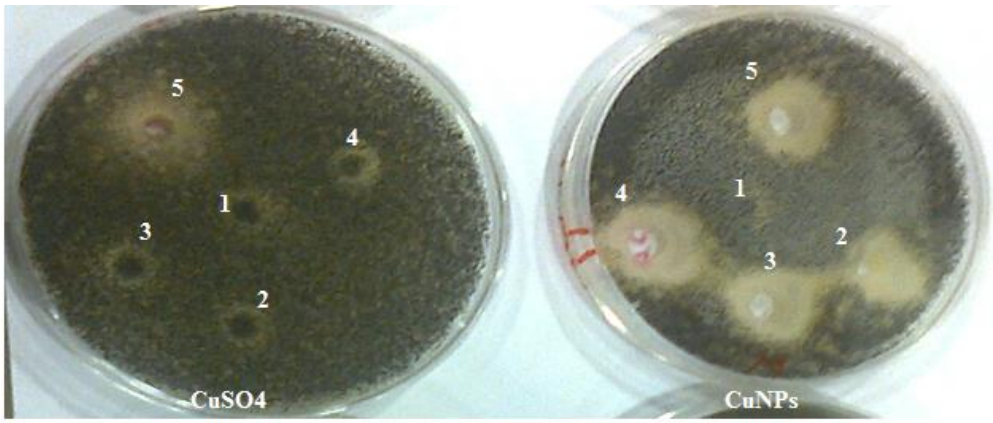

Fig. 2. Impact of different concentrations of $CuSO_4$ and CuNPs on *A. niger* growth: (1) 100 ppm, (2) 200 ppm, (3) 300 ppm, (4) 400 ppm, (5) 500 ppm

Table 1. Growth of *A. niger* at Different Concentrations of CuNPs

Concentration (ppm)	CuNPs			$CuSO_4$		
	Growth (cm)	Inhibition (%)	Sporulation (%)	Growth (cm)	Inhibition (%)	Sporulation (%)
Control	6.10±0.03	0.00	100.0	6.10±0.03	0.00	100.0
100	6.25±0.04	0.00	100.0	6.30±0.02	0.00	100.0
200	2.55±0.05	58.10	68.25	4.25±0.03	30.32	76.68
300	0.80±0.05	86.89	30.40	2.10±0.06	65.57	47.55

±, Standard deviation

Table 2. Morphological Characterization of *A. niger* at Different Concentrations of CuNPs

Concentration (ppm)	Conidial Head Diameter (µm)	Vesicle Diameter (µm)	Phialide Length (µm)	Spore Diameter (µm)	Hypha Diameter(µm)
Control	270.25±8.81	178.26±5.56	50.07±1.22	12.08±0.54	38.50±1.65
100	273.50±15.20	177.23±5.63	48.55±3.42	11.87±1.01	36.57±1.78
200	166.10±6.06	83.35±2.72	34.78±2.90	9.11±0.52	38.45±1.39
300	105.22±2.33	56.34±1.36	25.15±0.43	8.85±0.33	22.76±1.11

±, Standard deviation

Table 3. Morphological Characterization of *A. niger* at Different Concentrations of $CuSO_4$

Concentration (µm/L)	Conidial Head Diameter (µm)	Vesicle Diameter (µm)	Phialide Length (µm)	Spore Diameter (µm)	Hypha Diameter (µm)
Control	270.25±8.81	178.26±5.56	50.07±1.22	12.08±0.54	38.50±1.65
100	275.42±15.20	179.20±5.63	51.55±3.42	11.79±1.01	35.50±1.78
200	200.15±6.06	100.35±2.72	45.70±2.90	10.15±0.52	39.20±1.39
300	175.52±2.33	78.57±1.36	35.17±0.43	10.56±0.33	33.06±1.11

±, Standard deviation

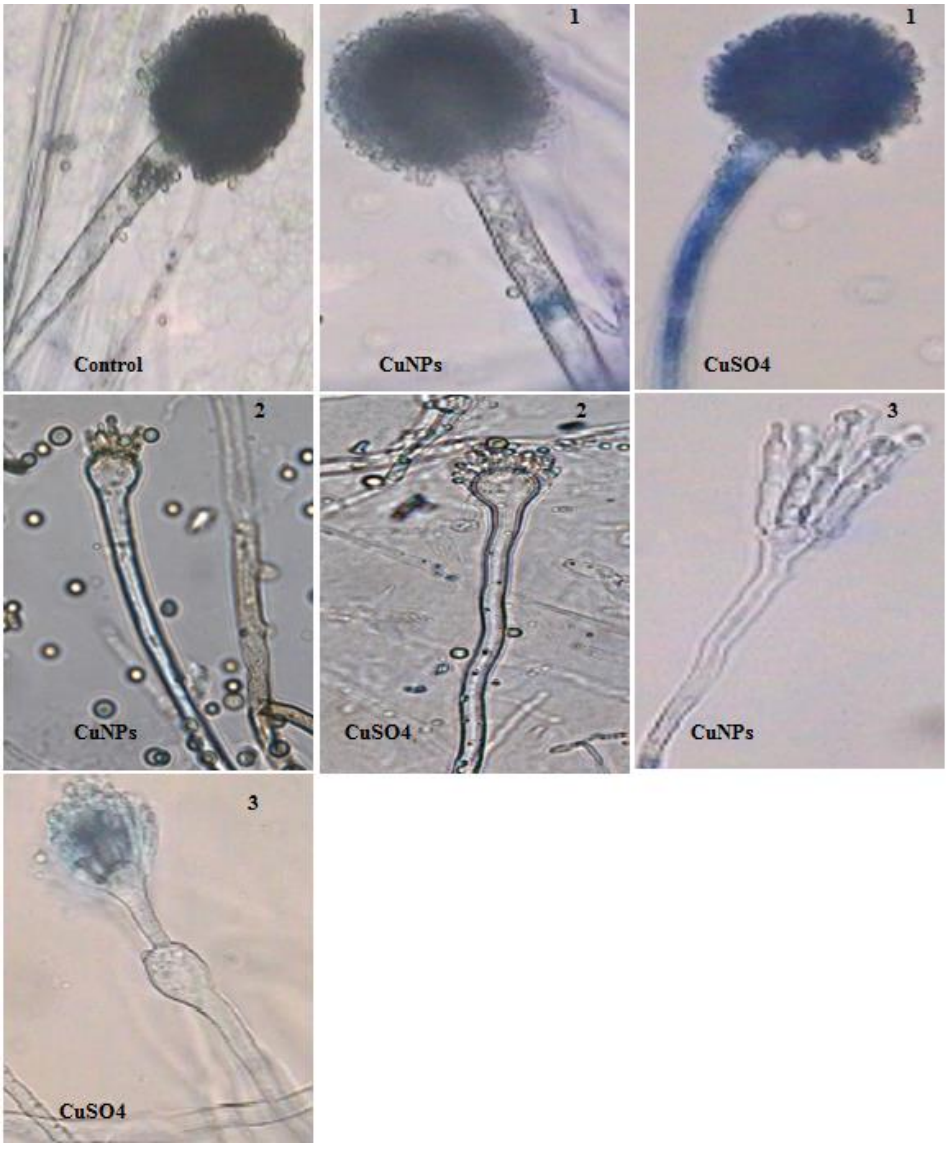

Fig. 3. Morphological changes of *A. niger* at different concentrations of CuNPs and $CuSO_4$: (1) 100ppm, (2) 200ppm, (3) 300ppm. Mag=400x

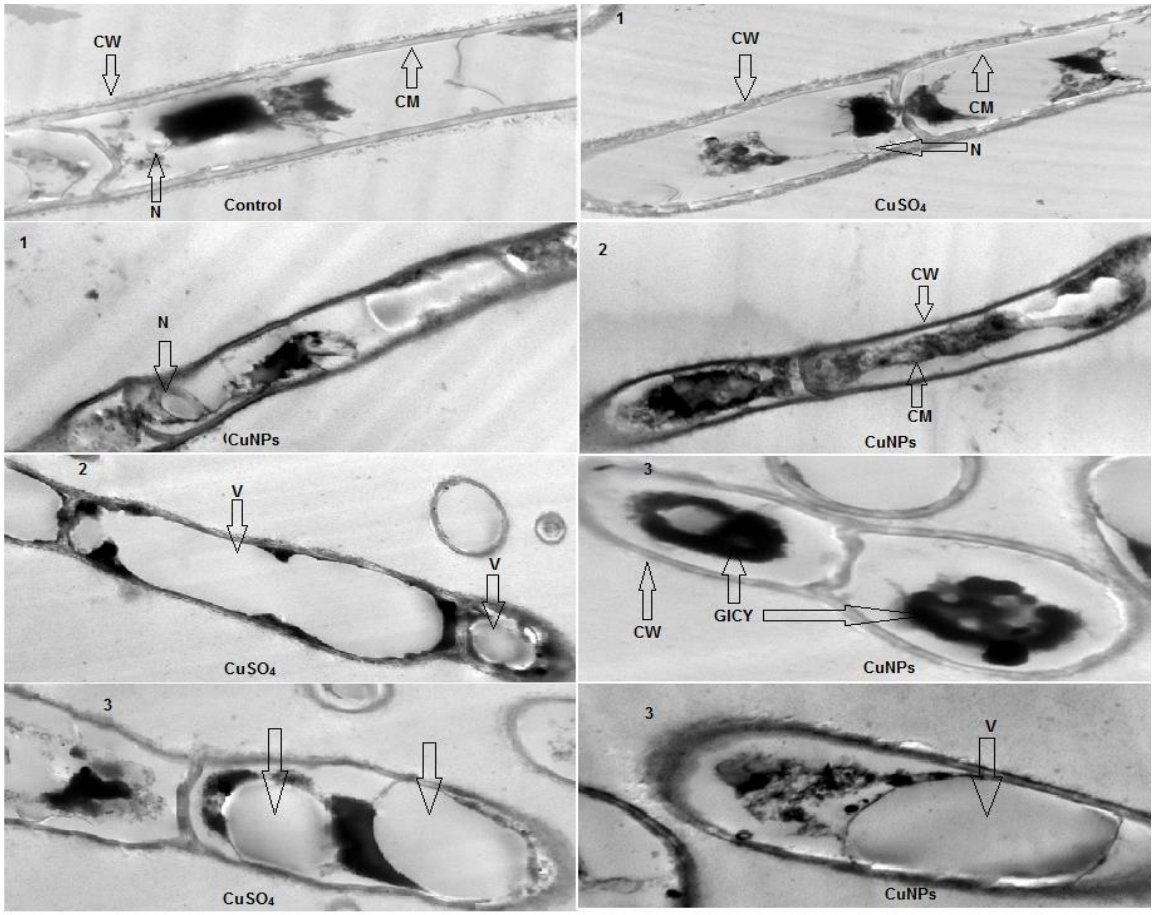

Fig. 4. TEM of *A. niger* mycelia at different concentrations of CuNPs and $CuSO_4$: (1) 100 ppm, (2) 200 ppm, (3) 300 ppm; N: nucleus, CW: cell wall, CM: cytoplasmic membrane, V: vacules, GICY: granules inside cytoplasm. TEM mag=6000X

Metallic ions play an essential role in regulation of laccases secretion by fungi (Piscitelli *et al.* 2011), particularly copper (Palmieri *et al.* 2000; Vrsanska *et al.* 2016). However, there are very few studies on the impact of metal NPs on production of laccase (Maurya *et al.* 2017). Therefore, the present study focused on the effect of CuNPs on laccase production compared to its non-nanoparticle form.

In the current results, CuNPs and $CuSO_4$ at concentrations of 0.15 and 0.25 mM, respectively, induced maximum amounts of laccase at 1.67 and 1.51 U/mL, respectively. At concentrations greater than those, enzyme production decreased (Fig. 5). However, a previous study demonstrated that an increase in copper concentration causes a raise in laccases production in *Trametes versicolor* (Collins and Dobson 1997). Increased laccase production by *T. versicolor* was observed with increasing $CuSO_4$, but over concentrations of 80 mM, the activity decreased (Lorenzo *et al.* 2005). In another study, 25 μM of $CuSO_4$ was shown to inhibit the activity of laccases in *F. oxysporum*. f. sp. *lycopersici* (Hernández-Monjaraz *et al.* 2018).

A similar situation to the current results has been observed in numerous studies. Saparrat (2004) has observed that the highest activity of *Grammotheles subargentea* laccase occurs at level range of 0.6 mM to 1.2 mM $CuSO_4$, whereas at higher levels of the metal (1.5 mM and 1.8 mM), the activity decreases. Copper acts as a cofactor in the catalytic center of laccase, but at high concentrations it might induce oxidative stress and

result in damage to proteins, and therefore decreases in laccase production at high concentrations of CuNPs and $CuSO_4$ were observed. Four copper atoms in the catalytic center of laccases were detected (Pannu and Kapoor 2014), which had various characteristics, and therefore copper at low concentrations could stimulate laccase production. Fillat *et al.* (2016) used $CuSO_4$ as an inducer to increase laccase production up to 85%. In contrast, Bhamare *et al.* (2018) discovered that 0.025 mg/L $CuSO_4$ was the best concentration for laccase production *via Aspergillus* sp. The CuNPs were more efficient than $CuSO_4$ at lower concentrations as an inducer for laccase production by *A. niger*, while at higher concentrations CuNPs were also more an inhibitor to enzyme activity (Fig.5). From this point of view, CuNPs in general were potent inhibitors of fungal growth as well as enzymatic reactions. CuNPs had nano-size to large surface area which caused the highest adsorptions of enzymatic protein on the surface of NPs and therefore could amplify activity of enzymes (Galhaup and Haltrich 2001). Recently, Singh *et al.* (2018) observed that microparticles enhanced production of *Aspergillus oryzae* enzymes up to ten-fold.

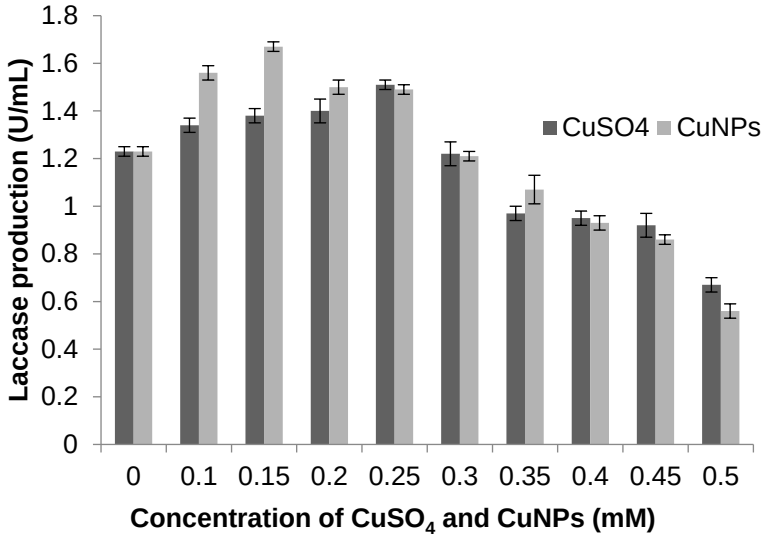

Fig. 5. Effect of different concentrations of $CuSO_4$ and CuNPs (mM) on laccase production by *A. niger*

Further studying was achieved for optimization of laccase production. An increase in incubation time resulted in increased laccase secretion from *A. niger* cultivated in medium without $CuSO_4$ and CuNPs up to 14 d, while addition of optimum concentrations of CuNPs and $CuSO_4$ (0.15 mM and 0.25 mM, respectively) reduced the appropriate time to 12 d using CuNPs and to 14 d using $CuSO_4$ for highest level of laccase (1.66 U/mL and 1.53U/mL, respectively) compared with untreated (1.36 U/mL at 16 d) (Fig. 6). These validated that production of laccase was dependent on fungal growth and the presence of metallic ions. Singh *et al.* (2018) recorded that the highest production of amylase, cellulose, phytase, and xylanase by *Aspergillus oryzae* was on the fourth day of fermentation, but addition of microparticle to medium growth resulted in production of enzymes at the third day. At the second day, the enzyme was not detected, but at the fourth day it appeared in low quantity; hence the laccase production began at the third day of incubation period (Fig. 6). The induction impact of $CuSO_4$ and CuNPs on laccase production was observed at the starting process of production. Optimum incubation period for laccase production might

differ according to fungal species, substrate type, presence of inducers, and environmental conditions. Earlier studies reported maximum laccase production by *Ganoderma lucidum* at 14 d (Songulashvili *et al.* 2007), by *Aspergillus fumigatus* at 6 d using banana peel (Vivekanand *et al.* 2011), by *Trametes hirsuta* at 20 d (Bakkiyaraj *et al.* 2013) using wheat bran, by *A. niger* at 5 d (Priyam *et al.* 2014), by *A. flavus* at 7 d using various agro-wastes, including heat bran and apple peel (Ghosh and Ghosh 2017), and by *Aspergillus* sp. at 9 d (Bhamare *et al.* 2018).

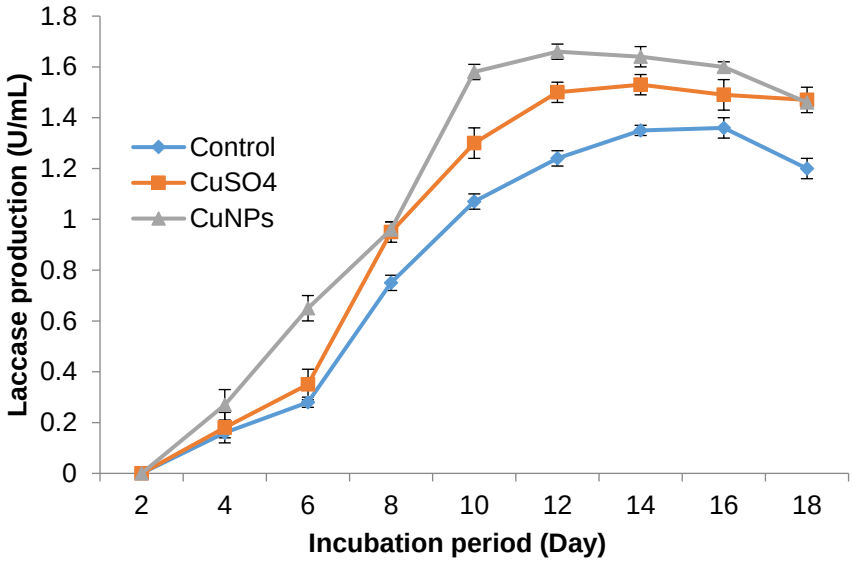

Fig. 6. Impact of different incubation periods on laccase production by *A. niger* at optimum concentrations of $CuSO_4$ and CuNPs (mM)

Laccase productivity varied with the different pH of growth medium, but optimum production was mainly obtained at pH 5 (Fig. 7). Acidic pH was more favorable than alkaline for enzyme production. Therefore, productivity at pH 3 and pH 4 were better than productivity at pH 8 and pH 9. A similar behavior has been observed in numerous studies (Ravikumar *et al.* 2012; Patel and Gupte 2016), with the highest enzyme production at pH 5.0. Laccase production by *A. niger* was reported at pH 5 (Priyam *et al.* 2014). When the copper level was less than optimal, laccase production improved with the increase in copper supplementation, but when the copper concentration was above the optimal concentration, the laccase production was induced with a reduction in copper supplementation (Tavares *et al.* 2005; Zhao *et al.* 2017). For this reason, the laccase production was studied at different pH ranging from 3 to 9 and temperature ranged from 10 °C to 50 °C (Figs. 7 and 8). Addition of $CuSO_4$ and CuNPs induced laccase production at acidic conditions, while its effects were negligible at pH above 6. At pH 9, laccase productivity in medium growth without $CuSO_4$ and CuNPs was higher than medium supported by them. In the present result, maximal laccase production was obtained at 30 °C, and further increases in temperature showed decreased enzyme production (Fig. 8).

The current results were similar to the obtained results of Priyam *et al.* (2014), who noted that the optimum temperature was 30 °C for laccase production by *A. niger*. Laccase was not detected at low temperatures such as 10 °C, and this might be attributed to the failure of the fungus to grow at these temperatures. The current results agreed with the results obtained by Ghosh and Ghosh (2017), who revealed that the optimal values of

laccase production by *A. flavus* were at 25 °C and pH 4. Recently, Senthivelan *et al.* (2019) recorded that maximum laccase activity from *Penicillium chrysogenum* was at pH 5.5 using 1.3 g/L of yeast extract. Addition of copper in the NPs form or non-nanoform supported enzyme production at different temperatures, particularly at optimum temperature 30 °C. Unsuitable pH or temperature might restrict fungal growth and inhibit metabolic rate and therefore restrict fungus activity as well as laccase production. From the current results, CuNPs played a greater role for laccase induction than $CuSO_4$, and this might be due to unique properties of NPs related to their size. In contrast, Bhamare *et al.* (2018) revealed that 34 °C and pH 6.0 were optimal for laccase production by *Aspergillus* sp. Additionally, Abd El Monssef *et al.* (2016) reported that the maximum yield of laccase enzyme by *Trichoderma harzianum* was at pH 5 and 35 °C after 6 d.

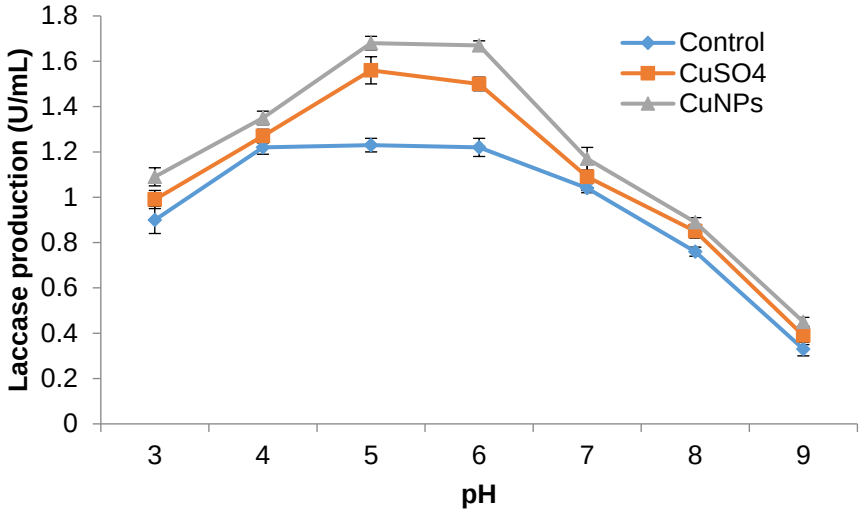

Fig. 7. Impact of different pH on laccase production by *A. niger* at optimum concentrations of $CuSO_4$ and CuNPs (mM)

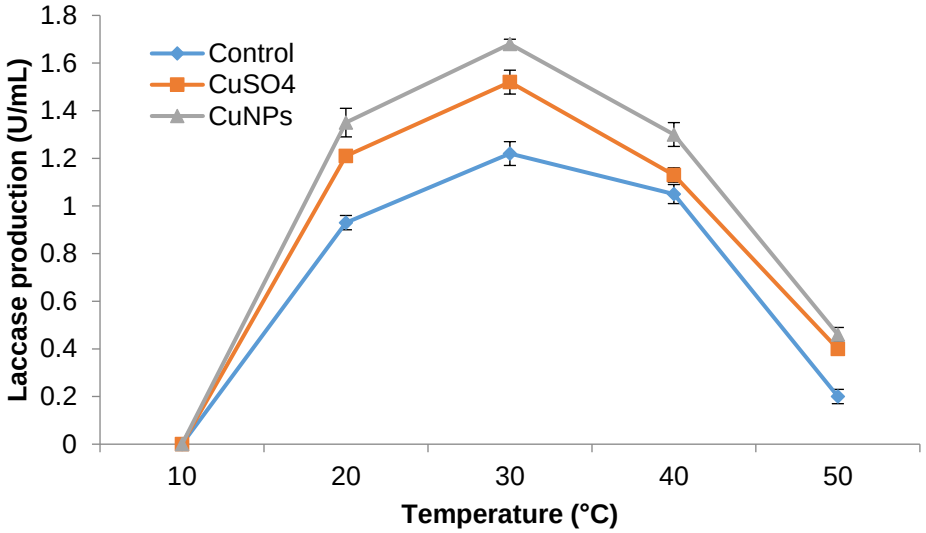

Fig. 8. Impact of different temperatures on laccase production by *A. niger* at optimum concentrations of $CuSO_4$ and CuNPs (mM)

CONCLUSIONS

1. The results showed the fungistatic effects of $CuSO_4$ and CuNPs on *A. niger* contaminated corn cobs and their grains.
2. CuNPs resulted in higher yields of laccase, but their higher concentrations negatively affected the production of laccase by *A. niger*.
3. Production of laccase by *A. niger* was controlled *via* numerous cultural conditions such as pH, temperature, incubation periods, and composition of culture medium.
4. The addition of $CuSO_4$ and CuNPs minimized the incubation periods required for laccase production by *A. niger*.

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