Biocatalysis and Agricultural Biotechnology 18 (2019) 101085

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Stimulating effect of nanoparticles and salts on thermo and halo-tolerant cell-bonded laccase synthesis in *Acinetobacter* sp. UIETPU

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ARTICLE INFO

Keywords:
Acinetobacter sp UIETPU
CuO
Nanoparticles
Halophilic
Thermo-tolerant
Laccase

ABSTRACT

Laccase synthesizing *Acinetobacter* sp. UIETPU was isolated from the rhizosphere of the paddy plant. Enzyme was firmly cell attached and no extracellular laccase activity was detected up to 96h, 37°C, and 150 rpm. Nanoparticles (NPs) of Cu and CuO (100 μM) increased the cell-bonded laccase synthesis by 2.1 and 2.3 fold respectively, but potent laccase inducer $CuSO_4$ enhanced the enzyme production by 1.5 fold at the same concentration. There was no adverse effect of NaCl (0.5–5.0%) on laccase synthesis when added to the production media. Enzyme oxidizes the syringaldazine and 2, 6-dimethoxyphenol (specific substrates of laccase) and inhibited by the dithiothreitol (DTT), sodium azide and cystein by 98, 65 and 52% respectively. Cell-bonded laccase showed the 100% activity up to 2h and $t_{\frac{1}{2}}$ of the bonded enzyme was 4h at 80°C. Field emission scanning electron microscopy (FESEM) revealed that there was no lysis of *Acinetobacter* sp. UIETPU cells in the presence of CuO NPs.

1. Introduction

Production and purification of enzymes before their applications at industrial scale is difficult and time consuming task, moreover it's too cost intensive and needed prerequisite technical skills. As an effect of these entire difficulties, enzyme based technologies are less popular in textile and clothing sector, cosmetics production and paper processing units (Singh et al. 2007, 2008; Sharma et al., 2015). Utilization of the microbial enzymes should be easier, eco-friendly and cost effective. Biocatalysts if strongly adhered to the cell components, and not released to the reaction mixture even by change of environmental conditions (pH, salinity and temperature) then no requirement of their extensive down streaming processing. On the other hand enzymes will be remained in their natural environment and usually act as more stable than the free soluble enzymes (Švitel et al., 2007). Bacteria from the genus *Arthrobacter*, *Alcaligenes, Burkholderia, Pseudomonas* and *Staphylococcus* have been characterized as the whole cell biocatalysts for the different industrial purposes (de Carvalho, 2017). But there is no report available on the bacterial laccases, where enzyme attached to the prokaryotic cells and showed “whole cell” bonded biocatalytic potential in non growth conditions (without nutrients). Many species of *Acetobacter and Gluconobacter* were studied as the whole cell (non viable) source of oxidative enzymes for the fabrication of biosensors and applied for the detection of mono and poly-alcohols, multiple aldoses and ketoses. In the case of heat killed yeasts, *Pichia pastoris* and *Saccharomyces cerevisiae,* possessed β-fructosidase (BfrA) in their periplasmic membranes was evaluated frequently for the purpose of inversion of sucrose to fructose and glucose (Martínez et al., 2014). Furthermore search for thermo and halo-tolerant bacterial laccases have been continuing process for the several years (Miyazaki, 2005; Singh et al., 2011). Halophilic and thermo-tolerant bio-catalysts reduced the cost of bio-processes, because sterile conditions are not much required and moreover easy product recovery is possible due to less viscosity of the reaction mixture. Only few bacterial laccases has been characterized according to the stipulation of industries, otherwise most of them are like fungal enzymes, works at low pH and temperature (Singh et al. 2011, 2015). In present study, the cell-bonded laccase of *Acinetobacter* sp. UIETPU was characterized in the presence of harsh (thermo, alkaline and salty environment) conditions. The stimulatory effect of Cu and CuO NPs was also determined on *Acinetobacter* sp. UIETPU cells for the increased enzyme synthesis. This work is the first report on bacterial laccase where no need of its purification (since the laccase was bonded to the cell) before industrial applications that represents the unique characteristic of a biocatalyst.

2. Materials and methods

The CuO nanopowder (average particle size (APS): 40 nm and

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https://doi.org/10.1016/j.bcab.2019.101085
Received 23 December 2018; Received in revised form 27 February 2019; Accepted 7 March 2019
Available online 11 March 2019
1878-8181/

specific surface area (SSA): $80 m^2 g^{-1}$) and Cu nanopowder (APS: 50 nm, SSA: $12 m^2 g^{-1}$) were obtained from the SRL, India. The syringaldazine, ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)), 2,6-Dimethoxy phenol, pyrogallol and guaiacol were purchased from the Sigma-Aldrich. The media components like yeast extract, agar and tryptone were purchased from the Himedia, India.

2.1. Isolation and screening of the laccase producing bacteria

The M162 media (Degryse et al., 1978) was utilized in the present study for isolation and screening of bacteria. The environmental samples (soil from rhizosphere, effluent from pulp and paper industries) were diluted appropriately and spread plated on M162 agar plates containing 5.0 mM guaiacol. The plates were incubated for 96 h at 37 °C (Kaur et al., 2016).

2.2. Enzyme production

All experiments for the evaluation of laccase synthesis by newly isolated bacteria were performed in 0.2% tryptone and yeast extract (TY), pH of the media was adjusted to 7.2 with 0.1N NaOH after the process of autoclave. One ml of inoculum (overnight culture) was used to inoculate the 20 ml of TY media, incubated at 37 °C and 150 rpm for 96 h. The pelleted cells were obtained after the centrifugation at 10,000 × *g*, 4 °C for 10 min. Thereafter cells were washed twice and resuspended in 20 ml of 0.1M phosphate buffer (pH 7.2). The newly isolated pure culture of bacteria was maintained as a suspension in 20% glycerol at −70 °C and was routinely cultured.

2.3. Enzyme assay

The cell-bonded laccase activity was measured by monitoring the oxidation of 5.0 mM guaiacol at 465 nm, incubation time was 20 min at 80 °C and pH 8.0 (100 mM phosphate buffer). The reaction mixture contains 1.0 ml bacterial cells (~0.4, OD), 2.0 ml buffer and 1.0 ml substrate. One unit of enzyme activity was defined as the amount of enzyme that increased the absorbancy by 0.001 absorbancy units per min (Bains et al., 2003). Same procedure was followed with syringaldazine (525 nm), catechol (450 nm), pyrogallol (450 nm), ABTS (420 nm) and 2, 6-dimethoxy phenol (468 nm). Turbidity of the cells was observed by spectrophotometer at 600 nm.

2.4. Dynamic light scattering (DLS)

The particle size of CuO and Cu NPs was measured by using the light scattering technique by using Zeta Sizer Nano (Malvern Instruments, UK). The instrument used 633 nm wavelength to measure the size distribution of dissolved or suspended particles. This instrument is capable of determining the particle size distribution in solution, from 0.3 nm to 10 μm. Dispersion efficiency of engineered nanomaterials (ENMs) under each set of dispersion conditions was determined in triplicate by taking intensity weighted particle size measurements using DLS. The measurements are reported as the z-average hydrodynamic diameter (dh, z ave.) and the particle size polydispersity index (PDI), which is a measure of the broadness of the particle size distribution. The PDI scale ranges from 0 to 1, with 0 being monodisperse and 1 being polydisperse. The measurements were performed within 5 min after sonication, and before and after each measurement, the electrophoretic cell was washed with distilled and deionized water to prevent cross contamination. The temperature of the laboratory was kept at 22 ± 2.5 °C during all experiments.

2.5. Field emission scanning electron microscopy

FESEM study of *Acinetobacter* sp. UIETPU cells was performed by using the FESEM (Hitachi SU8010) at CIL, Panjab University. This microscopy provides useful information about the shape, size, and production of extracellular polymeric substances produced by microbes.

2.6. Effect of NPs (Cu and CuO), $CuSO_4$ and NaCl on laccase synthesis

To study the effect of NPs Cu (100–500 μM) and CuO (100–1000 μM), both were added separately at different concentrations after the autoclavization of TY media. $CuSO_4$ (100 and 200 μM) and NaCl (0.5–5.0%) were added before autoclaving of the media.

2.7. Optimum temperature and pH of the cell-bonded laccase

To evaluate the optimum working temperature of enzyme, the laccase activity was performed at 5.0–90 °C. The optimum pH of the enzyme was determined at pH 3–10 using guaiacol as a substrate in the following buffers: 0.1M sodium acetate buffer (pH 3.0–5.0), 0.1M phosphate buffer (pH 5.5–8.0), 0.1M Tris-HCl buffer (pH 7.0–9.0) and Glycine-NaOH buffer (pH 9.0–11).

2.8. Thermo stability of cell-bonded laccase

The temperature stability of cell-bonded laccase was determined by pre incubating the bacterial cells (OD, ~1.0) in 0.1M sodium phosphate buffer (pH8.0) at 80 °C for different time intervals (0.0–5.5h).

2.9. Substrate specificity and effect of laccase inhibitors

The substrate specificity of bacterial laccase was evaluated with a range of substrates that included non-phenolic compounds, substituted phenols and aromatic amines. All the substrates were studied at 1.0 mM and the oxidation of each of the substrate was measured at their specific λ_{max}. Five potential laccase inhibitors (DTT, Ethylenediaminetetraacetic acid (EDTA), NaN_3, cystein and $FeSO_4$) 1.0 mM were evaluated for their inhibitory effects on cell-bonded enzyme. The cells were incubated with various inhibitors for 20 min at optimum temperature (80 °C) and the laccase activity was measured with 5 mM guaiacol.

2.10. Statistical analysis of data

All the experiments were performed in triplicate and results presented were the mean of three experiments. The difference in the values within the triplicate samples never exceeds 10%. The significance difference (p-value) of each group was calculated by the analysis of variance (ANNOVA).

3. Results and discussion

Cell-bonded laccase synthesized by the *Acinetobacter* sp. UIETPU was identified as the true laccase, because of its ability to oxidize the syringaldazine to dark purple color reaction of quinones. This substrate is non-autooxidative compound and not reacted with the tyrosinase, lignin peroxidase, or with hydrogen peroxidase, and has been considered to be uniquely as the substrate of laccase only (Harkin et al., 1974; Sanchez-Amat and Solano, 1997; Singh et al., 2007).

3.1. Isolation and identification of the organism

Soil samples were obtained from the rhizosphere of paddy plant (Paddy field near to the city, Sahibjada Ajit Singh Nagar (Mohali), India). Effluent from the pulp and paper industry was collected from the paper industry situated in Rupnagar, Punjab, India. Around 50 different isolates (Gram positive and negative bacteria) were isolated from these samples. Five isolates were screened as the laccase producing organisms, among them four were Gram positive bacteria (isolated from the

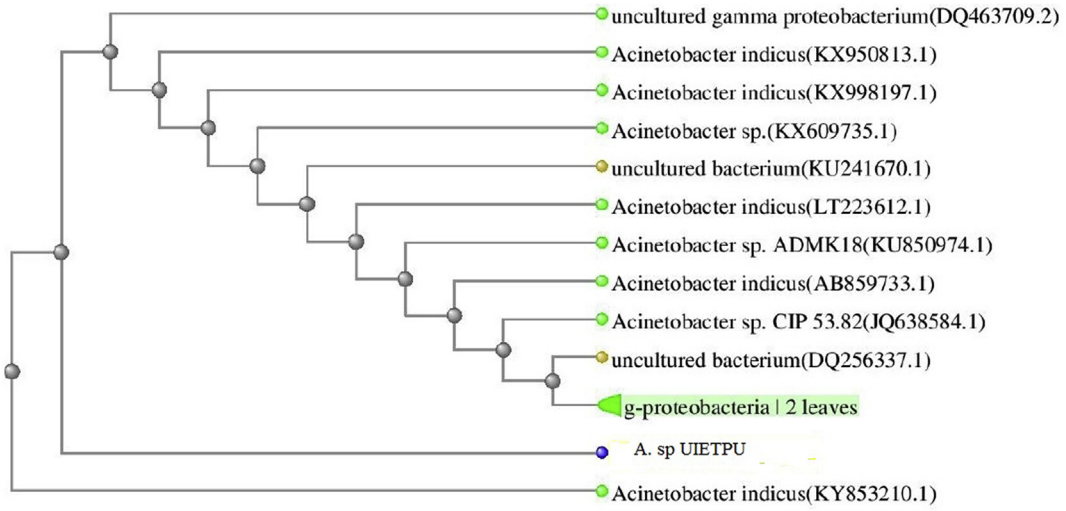

Fig. 1. Phylogenetic tree (constructed by the neighbor joining (NJ) method).

effluent) and *Acinetobacter* sp. UIETPU was isolated from the rhizosphere. During the comparison of these five bacteria, *Acinetobacter* sp. UIETPU showed the intense reddish-brown colonies on M162 agar plate contained 5.0 mM guaiacol. *Acinetobacter* sp. UIETPU was proved as the best isolate due to its ability to work in the harsh environmental conditions. Presumptive identification of the organism was performed at the genus level, peculiar characteristics of the genus *Acinetobacter* (oxidase-negative, catalase-positive, motility negative and coccobacillary morphology after Gram staining) were found in the *Acinetobacter* sp. UIETPU. The 16S rRNA sequencing was performed outside of our laboratory by Eurofins Analytical Services, Pvt. Ltd., Bangalore, India. 16S rDNA sequence of the organism was deposited to the Gene Bank under the accession number of MF682322, phylogenetic tree was also constructed (Fig. 1). *Acinetobacter* is ubiquitous in the nature, taxonomy of this genus has undergone extensive revision during the last two decades, at least 31 named and unnamed species have already described (Dijkshoorn et al., 2007). Maximum growth of *Acinetobacter* sp. UIETPU was observed after 24h and later it was slightly declined up to the 48h in TY media. Cell associated laccase synthesis was observed maximum after the 72h, at 37 °C, 150 rpm (data not shown). The initial pH of the media was adjusted to 7.2 before the inoculation but final pH of the culture broth was 8.6 after 72h of incubation. Singh et al. (2007) observed the pH of production media played the significant role in extracellular production of laccase by γ-proteobacterium JB. The organism produced maximum laccase (~20 nkat), when pH shifted from 7.0 to 8.0 in unbuffered production media.

3.2. Location of the cell-bonded laccase in Acinetobacter sp. UIETPU cells

The exact location of laccase in *Acinetobacter* sp. UIETPU cells was not understood clearly, but it was an apparent manifestation, enzyme attached to the cell wall or embedded firmly elsewhere in the bacterial cell. Before the each experiment prokaryotic cells were washed twicely with phosphate buffer (pH 8.0) and no laccase activity was determined in the supernatant. Only one report of the attached laccase is available from yeast *Cryptococcus neoformans* where laccase acted as the major virulence factor to oxidize the brain catecholamines. Monoclonal antibodies against the *C. neoformans* laccase were generated and exploited to reveal the localization of the laccase in cell wall of the representative serotype A (H99) and D (B-3501) strains by electron microscopy. Laccase was found tightly associated to the cell wall and enzyme was readily accessible for the interactions with host immune cells. Before this observation laccase was assumed as the industrial enzyme only and never considered as the sheer pathogenesis component of any laccase producing microbe (Zhu et al., 2001). Virulent species of *Acinetobacter* are known for their less permeability of cell wall and as a major rational behind their resistance to high molecular weight antibiotics (Zahn et al., 2016). Interestingly in the present study, potent laccase substrates used have less molecular weight and may easily permeable to the cell wall of *Acinetobacter* sp. UIETPU cells. Many studies have shown that it is possible to improve the substrate transfer across the cell wall and membranes by increasing their permeabilization level by chemical (detergents and solvents) or physical (temperature shock) methods (de Carvalho, 2017).

3.3. Induction/stimulation of laccase synthesis in Acinetobacter sp. UIETPU by Cu, CuO NPs and $CuSO_4$

Metallic NPs used in the present study were not stabilized with any protective coating or surfactants. When NPs were added to the production media, aggregation of the particles was observed. In case of Cu NPs, the aggregates were of the average diameter ~571 nm, while the average size of CuO particles was ~385 nm. Among the different concentrations of Cu NPs evaluated, only 100 μM of Cu increased the enzyme synthesis by 2.1 fold, rest of the other concentrations reduced the synthesis of enzyme (Fig. 2). In the presence of CuO (100 μM), synthesis of cell-bonded laccase was increased up to 2.3 fold. Cell growth was also enhanced at each evaluated concentration (100–1000 μM) of CuO (Fig. 3). On the other hand $CuSO_4$ (100 μM) increased the laccase synthesis by 1.5 fold, but showed significant reduction in enzyme production and growth of the organism at higher

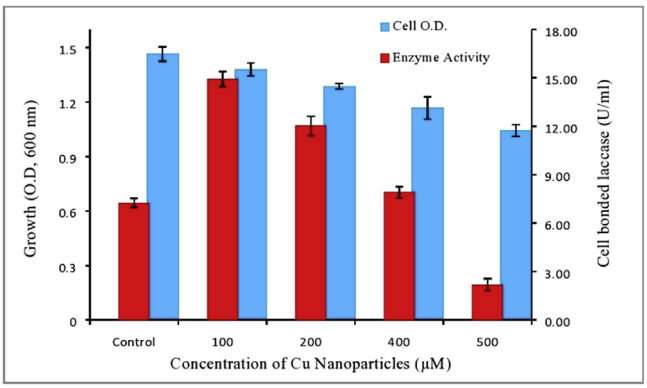

Fig. 2. Impact of Cu NPs on laccase synthesis by *Acinetobacter* sp. UIETPU, (p-value < 0.05).

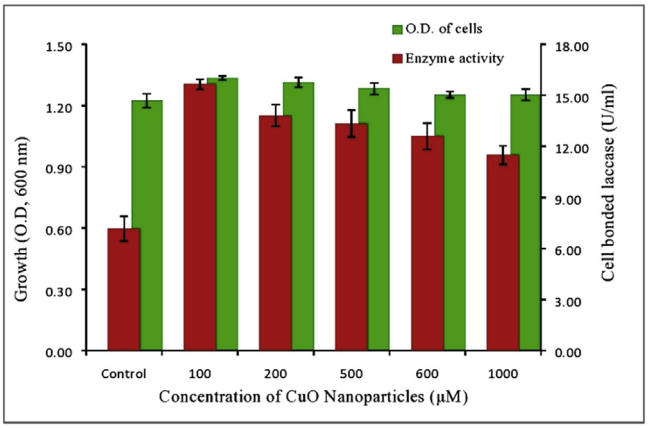

Fig. 3. Impact of CuO NPs on laccase synthesis by *Acinetobacter* sp. UIETPU, (p-value < 0.05).

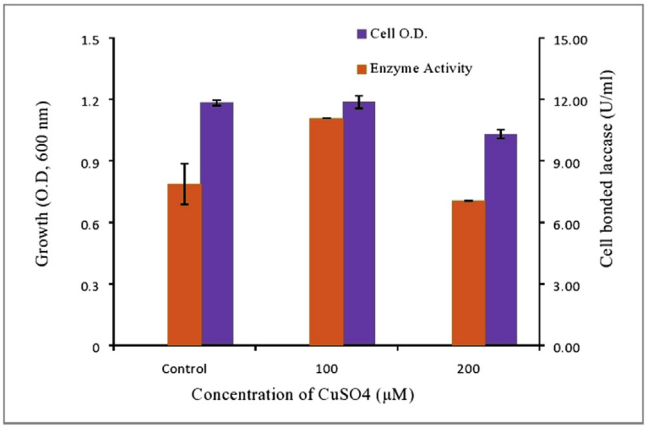

Fig. 4. Impact of $CuSO_4$ on laccase synthesis by *Acinetobacter* sp. UIETPU, (p-value > 0.05).

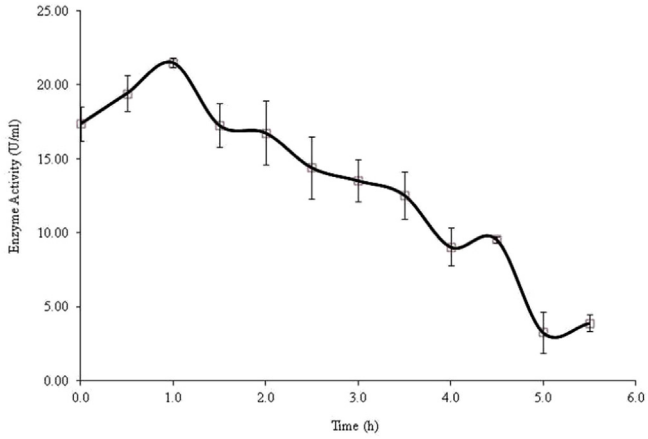

Fig. 5. Thermo stability of the cell-bonded laccase at optimum temperature 80 °C.

concentration of $CuSO_4$ (Fig. 4). Physiological responses and tolerance mechanism of *Acinetobacter* sp. UIETPU bacterial cells against the CuO and Cu NPs was not elucidated well in the present study. But increased in cell-bonded laccase synthesis seems the probable metal detoxification mechanism of bacteria against the CuO and Cu particles. Interestingly in case of several species of *Acinetobacter*, quorum sensing (QS) phenomena is also regulating a diversified network of signaling cascades which helps the organism to resist infinite hostile conditions that are yet to be unveiled. QS Network in *Acinetobacter* is mediated by the acyl homoserine lactones (AHL) (Dijkshoorn et al., 2007). The best-studied bacterial laccase is CotA, the endospore coat component of *Bacillus subtilis*, the CotA participates in the biosynthesis of brown spore pigment, which is thought to be a melanin-like product and responsible for the protection of spore coat against UV light and hydrogen peroxide (Dalfard et al., 2006). Laccase synthesized in γ-proteobacterium JB was increased to several fold in the presence of xenobiotics and mutagenic dyes due to unknown reasons (Singh et al., 2009). The brown pigmented spores of *Bacillus* sp. HR03 contained laccase, showed remarkable resistance against H_2O_2, UVA and UVC rays (Mohammadian et al., 2010). Laccases have undefined role in the bacterial physiology and yet not understood evidently (Singh et al., 2011). Su et al. (2015) investigated the negative influence of CuO NPs on bacterial denitrification process by playing the significant alteration to the expression of key proteins. Bao et al. (2015) reported *Saccharomyces cerevisiae* protected themselves against the CuO NPs due to the presence of specific genes that were responsible to decrease the permeability of cell to NPs. In comparison to the wild-type strain of *S. cerevisiae*, copper was readily taken up by the mutant strains when cell permeability genes were knocked out and the mutants with deletions of genes regulated under oxidative stress (OS) were likely producing more reactive oxygen species (ROS). In case of $CuSO_4$ the growth and laccase synthesis by *Acinetobacter* sp. UIETPU were inhibited at 200 µM, it shows copper sulphate is more toxic than Cu and CuO NPs. Copper (Cu) has a dual role in the biological systems of living organisms. It acts as an essential trace element by working as a cofactor for the several biocatalysts, including superoxide dismutase (providing protection against the free radicals), cytochrome c oxidase (mitochondrial electron transport chain) and tyrosinase (pigmentation). At the same time, copper is toxic to microorganisms and may lead to the death of microorganisms within minutes of their exposure to metal (Krumova et al., 2012). Copper is extremely toxic to the bacteria, even slightly above the concentration normally present in the regions from where they isolated (Wilson and Ray, 1956). In present study we are not clear about the level of toxicity that created by $CuSO_4$, but the aggregation of NPs may decrease their toxic effect on the cell growth of *Acinetobacter* sp. UIETPU. The effect of NPs and $CuSO_4$ was observed only on the synthesis of enzyme but not on activity. The enzyme activity of cell-bonded laccase was checked in the presence of NPs and $CuSO_4$ separately, but there was no change observed in the activity of cell-bonded enzyme when compared to the control experiments (laccase activity without NPs and $CuSO_4$).

3.4. Effect of NaCl on laccase synthesis by Acinetobacter sp. UIETPU

Synthesis of the cell-bonded laccase was increased ~1.6 fold in the presence of 0.5% NaCl, there was no adverse effect of NaCl up to 5.0% on the enzyme production and no elution of the laccase was detected in the production broth (data not shown). *Acinetobacter* sp. UIETPU may consider as the moderate halophilic bacteria according to the classification of Kushner and Kamekura (1988), because *Acinetobacter* sp. UIETPU bacteria can bare easily 3.0–5.0% NaCl in production broth. In the recent years halotolerant microorganisms have been explored for their biotechnological potential in different fields like bioremediation processes of textile dyes, effluent treatment of pulp and paper industries (Singh et al., 2015).

3.5. Substrate specificity and effect of inhibitors on the cell bonded bacterial laccase

Bacterial laccase oxidize the syringaldazine, guaiacol, dimethoxyphenol, ABTS and catechol successfully. Only pyrogallol was not oxidized by the *Acinetobacter* sp. UIETPU bacterial laccase. Enzyme was inhibited fully by the DTT but less (38 & 40%) inhibition was observed with EDTA and $FeSO_4$. Laccase from γ-proteobacterium JB oxidized the different substrates like L-methyl DOPA, catechol, pyrogallol, *p*-

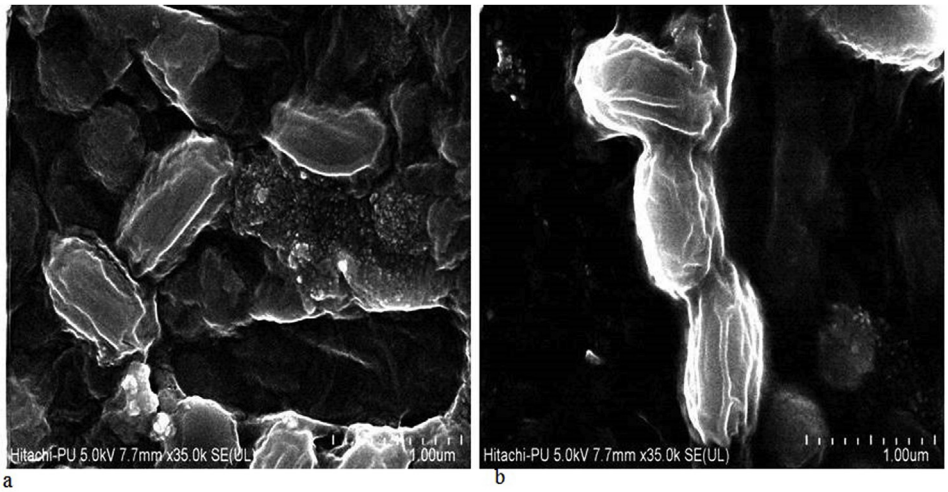

Fig. 6. Effect of CuO on the morphology of *Acinetobacter* sp. UIETPU cells, (a) FESEM image of cells in absence of CuO NPs, (b) FESEM image of cells in presence of 100 μM, CuO NPs after, 72h

phenylenediamine, syringaldazine and guaiacol. The same enzyme was also inhibited by the sodium azide, sodium diethlydithiocarbamate, sodium thioglycollate and cysteine-hydrochloride (Singh et al., 2007). Fungal laccases, also showed the inhibition from sodium azide, cysteine-hydrochloride and EDTA (Patel et al., 2014).

3.6. Thermo stability and half life of the enzyme

Bacterial laccase showed the optimum working temperature between 70 to 85 °C. Enzyme was 100% active up to 2h and $t_{\frac{1}{2}}$ of the cell-bonded laccase was 4h at 80°C (Fig. 5). Thermostability of the biocatalysts is very influential factor before their selection in biotechnological applications at industrial scale (Miyazaki, 2005; Singh et al., 2018). Laccases are considered as the moderately thermostable and several attempts have been initiated for the isolation of highly thermostable laccases. Kiiskinen et al. (2004) isolated the novel laccases from wood rotting fungi, most of them had $t_{\frac{1}{2}}$ of 3–6 h **at** 60°C but pH optima of these laccases were 2.0–4.0. Laccase from the spores of *Bacillus vallismortis* retained more than 50% activity after 10 h at 70 °C and demonstrated broad pH stability in both acidic and alkaline conditions (Zhang et al., 2013). Optimum pH of the cell-bonded laccase of *Acinetobacter* sp. UIETPU was 8.0 (data not shown).

3.7. FESEM study of Acinetobacter sp. UIETPU bacterial cells

Antibacterial mechanisms of NPs are poorly understood, but it is widely accepted that prokaryotes have a difficulty to develop a resistance against the NPs (Wang et al., 2017). But in the present case, FESEM study of the *Acinetobacter* sp. UIETPU cells showed no lyses effect of 100 μM of CuO NPs (Fig. 6). Why the cells of *Acinetobacter* sp. UIETPU showed the resistance to CuO NPs was not clear. Maybe the cell-bonded laccase acts as a protective protein against the CuO NPs, but without the detailed study it's too early to hypothesize. Indeed, there was no lytic effect of CuO on bacterial cells even at 1 mM (Fig. 3); growth was more than control experiment (absence of NPs) at each concentration of CuO. The NPs of CuO showed the toxic effect on bacteria by damaging to their cell membrane by formation of cavities. These toxic effects may probably be due to the ions interaction, the oxide-reduction reactions and the generation of reactive species (Concha-Guerrero et al., 2014).

4. Conclusions

The current study illustrates CuO and Cu NPs have more potential than $CuSO_4$ for increasing the cell-bonded laccase synthesis in newly isolated bacterium *Acinetobacter* sp. UIETPU. Moreover, laccase showed the potential to work under the harsh environmental conditions (high temperature, alkaline pH and moderate saline), this performance makes it befitting for the industrial use. Enzyme bonded to the *Acinetobacter* sp. UIETPU cells; it's a great mileage from the industrial point of view, because there is no need of purification of laccase before its use.

Acknowledgments:

Authors are thankful to the Science and Engineering Research Board (SERB)/Department of Science & Technology (DST) (SB/FT/LS-315/2012), Delhi, India, for providing the research funding under Fast Track Young Scientist Program.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101085.

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