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An Overview on the Production of Microbial Copper Nanoparticles by Bacteria, Fungi and Algae

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Abstract- Bionanotechnology is an emerging field, which involves multidisciplinary areas such as engineering, chemistry, biology, among others. Bionanotechnology encompasses the production of organic and inorganic nanomaterials by living organisms such as vegetable, animal and microbial cells. In this sense, the microbial productions of metallic nanoparticles have drawn much attention mainly due to their alignment with the principles and concepts of green chemistry (no need for organic solvent). A wide diversity of biological organisms, such as bacteria, lichens, fungi, yeasts and algae, produce metallic nanoparticles. This mini-review specifically highlights the main keys to the production of copper nanoparticles by bacteria and fungi. In addition, this report indicates the lack of knowledge on the production of copper nanoparticles by algae, as well as the purification and application of metallic nanoparticles.

Keywords: *metallic nanoparticles, cooper nanoparticles, microorganisms, biotechnological processes.*

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An Overview on the Production of Microbial Copper Nanoparticles by Bacteria, Fungi and Algae

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Abstract- **Bionanotechnology is an emerging field, which involves multidisciplinary areas such as engineering, chemistry, biology, among others. Bionanotechnology encompasses the production of organic and inorganic nanomaterials by living organisms such as vegetable, animal and microbial cells. In this sense, the microbial productions of metallic nanoparticles have drawn much attention mainly due to their alignment with the principles and concepts of green chemistry (no need for organic solvent). A wide diversity of biological organisms, such as bacteria, lichens, fungi, yeasts and algae, produce metallic nanoparticles. This mini-review specifically highlights the main keys to the production of copper nanoparticles by bacteria and fungi. In addition, this report indicates the lack of knowledge on the production of copper nanoparticles by algae, as well as the purification and application of metallic nanoparticles.**

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I. Introduction

Nanotechnology is an emerging field. The application of nanomaterials is predicted to reach 58,000 tons by 2020 (Maynard et al., 2006). Nanoparticles are defined to range within 1-100 nm (diameter). The chemical composition, size and shape of nanoparticles have a significant effect on their properties (Singh et al., 2010; Gurav et al., 2014; Shobha et al., 2014).

One of the earliest studies on the production of metallic nanoparticles by microorganisms (bacteria) was reported by Temple and Le Roux, (1964). However, only in the 21st century has the production of metallic nanoparticles been more deeply investigated.

The electro-chemical method is the most feasible to produce copper nanoparticle (short period of time to synthesize large quantities of nanoparticles). In this sense, nChemi - a startup company located in São Carlos, Brazil - has been working on developing, customizing and fabricating metal oxide nanoparticles by the electro-chemical method (nChemi, 2017). However, the production of metallic nanoparticles by living organisms has competitive advantages over the electro-chemical method, such as being eco-friendly (green chemistry concept) (Shobha et al., 2014; Cuevas et al., 2015).

Among the metallic nanoparticles produced by living organisms, gold, silver and iron are the most well-known (investigated). The metallic nanoparticle producers (living cells) have unique characteristics such as magnetosomes (organelles) that store magnetic nanocrystals composed of greigite (Fe_3S_4) or magnetite (Fe_3O_4) (Singh, 2015). These metallic nanoparticles are produced by both intra and extracellular biocompounds. The wide range of molecules favors the reduction of metal ions - Brust–Schiffrin synthesis (bottom-up approach) (Singh et al., 2010; Usha et al., 2010; Le et al., 2013; Salvadori et al., 2014; Shobha et al., 2014; Singh et al., 2015).

Although researchers have focused mainly on silver, gold and iron nanoparticles, copper nanoparticles have drawn attention due to their unique properties such as electrical, magnetic, thermal, antimicrobial (*Escherichia coli*, *Bacillus subtilis*, *Vibria cholera*, *Pseudomonas aeruginosa*, *Syphillis typhus* and *Staphylococcus aureus*), optical and catalytic, which can be used in electronic devices (lithium batteries), magnetic phase transitions, gas sensors, industrial cooling and heating, mass transfer enhancement, energy storage devices, in the production of cosmetics and pharmaceuticals, etc (Varshney et al., 2010; Gurav et al. 2014; Shobha et al., 2014; Cuevas et al., 2015, Shankar et al., 2016).

Therefore, there is a trend towards nanotechnology; particularly that applying living cells (e.g., the production of copper nanoparticles) becomes increasingly important, due to its competitiveness, effectiveness and low operational cost (Salvadori et al., 2013).

II. Microbial Production of Copper Nanoparticles

The production of copper nanoparticles by microorganisms (e.g., bacteria, fungi and algae) is relatively a novel approach. There is a wide variation in the production of metallic nanoparticles by living cells (e.g., organelles and compounds responsible for production, shape and size of nanoparticles), which

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depends on the mechanisms of metal ions bioreduction (Singh, 2015).

a) Bacteria

In general, the production of metallic nanoparticles by bacteria takes advantages of shorter generation times, for instance *Escherichia coli* (bacteria) 18 minutes (Bremer, 1982), versus *Saccharomyces cerevisiae* (yeast) 100 minutes (Hartwell 1974) or *Chlorella vulgaris* (microalgae) 3.35 days (Andrade et al., 2014). Usually, the production of metallic nanoparticles by bacteria occurs during the stationary phase. In theory, when compared to the logarithmic phase, greater metabolic stress is observed during the stationary phase. Consequently, metabolites with greater capacity of chemically reducing other compounds are synthesized during the stationary phase. Thus, these metabolites are able to reduce metal ions, which lead to the production of metallic nanoparticles (Hasan et al., 2007; Shobha et al., 2014; Ammar, 2016).

Theoretically, the metallic nanoparticle production is a very general microbial detoxification mechanism (soluble metals → insoluble nanosized structures), since copper ions lead to change in the helical structure by cross-linking and, consequently, to many biochemical pathways (Abboud et al., 2014). A wide range of bacteria (Table 1) is able to reduce metal ions (metallic nanoparticle production) by their compounds such as proteins, polysaccharides and periplasmic proteins (Singh et al., 2010; Le et al., 2013; Shobha et al., 2014; Singh et al., 2015). For instance, Singh et al. (2010) described the production of nanoparticles from *Escherichia coli* proteins, in which *E. coli* was cultivated in citrate minimal medium. The biomass was recovered (centrifugation) and suspended in an aqueous 1 mM $CuSO_4$ solution. The secreted proteins were precipitate by trichloroacetic acid followed by dialysis (deionized water) for 24 hours. Then, the proteins were concentrated by membrane (molecular weight cut-off of 3 kDa) and their profile was studied by electrophoresis. In conclusion, the proteins with 22 kDa, 25 kDa, and 52 kDa were related to the production of copper oxide nanoparticles and acted on their stabilization (cooper oxide nanoparticle). Belchik et al. (2011) proved that the outer membrane c-type cytochromes of *Shewanella oneidensis* MR-1 played an important role in the reduction of Cr(VI). The authors evaluated the effects on Cr(VI) reduction by deleting the *mtrC* and/or *omcA* gene. When compared with non-engineered *Shewanella oneidensis* MR-1 (wild), the *mtrC* knockout led the rate of reduction of Cr(VI) to 43.5%, *omcA* by 53.4%, both *mtrC* and *omcA* genes by 68.9% of reduction. Then, the authors proved that purified MtrC and OmcA reduced Cr(VI).

Mat Zain et al. (2014) produced cooper nanoparticles by using ascorbic acid (reducing agent) in the presence of chitosan and microwave heating, in which 40 mL of copper nitrate solution (10, 30 or 50 mM) was mixed with 40 mL of chitosan solution (1, 2 or 3% w/v) and 4 mL of a 10% (w/v) ascorbic acid solution. Chitosan led to the higher stability of cooper nanoparticles and avoided agglomeration. The authors defined the synthesis of cooper nanoparticle as fast, inexpensive, environmentally friendly and high energy-efficient. In addition, the concentration of chitosan was positively correlated to cooper nanoparticle size.

Varshney et al. (2010) reported an easy, fast, and cost-effective production of copper nanoparticles by the non-pathogenic bacteria *Pseudomonas stutzeri*. The copper nanoparticles showed great stability. Thus, the metabolites from *Pseudomonas stutzeri* produced copper nanoparticles besides stabilizing them.

Therefore, many biocompounds are able to reduce metal ions, producing metal nanoparticles.

The initial concentration of cooper ions strongly affects the production of nanoparticles by living cells, for instance, Honary et al. (2012) tested three species of *Penicillium*: *P. aurantiogriseum*, *P. citrinum* and *P. waksmanii*, which were cultivated in a fluid zapex dox broth at 28 °C, 200 rpm for 10 days. Then, they were centrifuged and their supernatants were used for producing cooper nanoparticles, that is, the authors used the metabolites produced during the fermentation instead of the living cells (directly). In addition, the effects of cooper concentration (1, 3 and 5 mM of $CuSO_4$) and pH (5, 6, 7, 8 and 9) were investigated. The authors reported a direct correlation among pH, concentration of cooper, polydispersity index and particle size, that is, the 5 mM $CuSO_4$ (highest concentration) led to the largest copper nanoparticles (diameter), whereas pH 5 (the lowest pH), led to the production of smallest (diameter) copper nanoparticles. Moreover, the same trend (correlation among pH, concentration of cooper, polydispersity index and particle size) was observed among the three species.

Table 1: Production of copper nanoparticles by bacteria

Bacteria	Shape	Diameter*	Copper Source	Reference
Pseudomonas stutzeri	Spherical	8-15	$CuSO_4$	(Varshney et al., 2010)
Pseudomonas stutzeri	Cubic	50-150	$CuSO_4$	(Varshney et al., 2011)
Pseudomonas sp.	Cubic	84-130	Metallic copper	(Shobha et al., 2014)
Escherichia coli	quasi-spherical	10-40	$CuSO_4$	(Singh et al., 2010)
Streptomyces sp.	X	100-200	$CuSO_4$	(Usha et al., 2010)
Serratia sp.	Cubic	10-30	$CuSO_4$	(Shobha et al., 2014)

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Morganella morganii	Cubic	15-20	$CuSO_4$	(Shobha et al, 2014)
Serratia sp.	Spherical	10-30	$CuSO_4$	(Hasan et al., 2007)

* (nm)

b) Fungi

A wide range of the genera of fungi was already reported as metallic nanoparticle producers (my conanotechnology), such as *Penicillium aurantiogriseum*, *P. citrinum*, *P. waksmanii*, *Fusarium oxysporum*, etc (Table 2). Compared with the biotechnological processes that apply bacteria, algae, cyanobacteria and plants, fungi are more resistant to mutations and have the ability to synthesize silica nanoparticles. Nevertheless, there is no consensus on the biomechanism of the metallic nanoparticle production. In other words, there is no evidence that a specific type of protein, or carbohydrate, or lipid or any other molecule is the major responsible for the production of metallic nanoparticles (Singh, 2015).

In this sense, proteins appear to be fundamental to the production of copper nanoparticles, in which the amide groups lead to stability and to capping agents around copper nanoparticles (Shobha et al., 2014). On the other hand, studies have indicated that secreted enzymes by fungi act on the production of metallic nanoparticles (instead of only on the stability) (Cuevas et al., 2015). However, other compound types are also related to the production of metal (silver) nanoparticles such as anthraquinone pigments and their derivatives, which were produced by *Fusarium oxysporum* strains (Duran et al., 2005).

In a very specific study, Jain et al. (2010) detailed the profiles of the extracellular proteins (*Aspergillus flavus*) during the synthesis of silver nanoparticles. The authors investigated mainly two proteins, 32 kDa and 35 kDa, in which the 32 kDa protein acted as reductase (production of silver nanoparticles) and the 35 kDa protein enhanced the stability of the silver nanoparticles.

The oxidative stress is often related to a high concentration of metals (e.g. Ag, Fe, Cu, Co, Cd and Cu) (Jomova and Valko, 2011). Ramezani et al. (2010) highlighted the correlation between the production of glutathione (glutathione-like) and heavy metal stress (cadmium) in yeasts, in which metallic nanoparticles were produced. In theory, cells feel the decrease in glutathione/oxidized glutathione and then begin to synthesize more glutathione (injurious response). Thus, the glutathione antioxidant defense system is critical for the survival of the microbial cells.

In addition, other factors inherent in any biotechnological production seem to affect the production of metallic nanoparticles. For example, Salvadori et al. (2013) indicated the effect of pH on the production of metallic nanoparticles by *Hypocrea lixii*. On the one hand, at an acid pH (2-4), the membrane of microorganisms is positively charged with consequent reduction of metal biosorption. On the other hand, at pH 5, the cell membrane is negatively charged, which favors the biosorption of copper. Thus, the membrane is expected to be fundamental for the metallic nanoparticles, instead of cytoplasm (Salvadori et al., 2013).

An interesting approach was described by Ahmad et al. (2007) who produced the transparent p-type conducting oxide $CuAlO_2$ (bimetallic nanomaterial) by *Humicola* sp., exploiting the unique valence-controlled nanosynthesis capability of the *Humicola* sp. biosynthesis. Moreover, the material formed was free of any impurities (e.g CuO, Cu_2O or Al_2O_3).

Table 2: Production of copper nanoparticles by fungi

Fungi	Shape	Diameter*	Copper Source	Reference
Fusarium oxysporum	X	93-115	Metallic copper	(Majumder, 2012)
Pseudomonas sp	X	84-130	Metallic copper	(Majumder, 2012)
Hypocrea lixii	spherical	24.5	$CuCl_2$	(Salvadori et al., 2013)
Stereum hirsutum	spherical	5-20	$CuCl_2$	(Cuevas et al., 2015)
Rhodotorula mucilaginosa †	spherical	10.5	$CuCl_2$	(Salvadori et al., 2014)
Penicillium aurantiogriseum	spherical	89-250	$CuSO_4$	(Honary et al., 2012)
Penicillium citrinum	spherical	85-295	$CuSO_4$	(Honary et al., 2012)
Penicillium waksmanii	spherical	79-179	$CuSO_4$	(Honary et al., 2012)

* nm
† yeast

c) Algae

To the best of our knowledge, Abboud et al. (2014) were the first to report the bioproduction of copper oxide by algae microorganism (*Bifurcaria bifurcata*). The production of other metallic nanoparticles by algae was also reported (Table 3); for example, (i) iron nanoparticles by *Chlorella* sp. MM3 (Subramaniyam et al., 2016); (ii) gold nanoparticles by *Stoechospermum marginatum* (Rajathi et al., 2012), *Turbinaria conoides* (Vijayaraghavan et al., 2011), *Sargassum wightii*

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(Singaravelu et al., 2007), *Laminaria Japonica* (Ghodake and Lee, 2011) and *Kappaphycus alvarezii* (Rajasulochana et al., 2010); and (iii) silver nanoparticles by *Chlorococcum humicola* (Jena et al., 2013).

Table 3: Production of copper nanoparticles by algae

Algae	Shape	Diameter*	Copper Source	Reference
Bifurcaria bifurcata	spherical	5-45	$CuSO_4$	(Abboud et al., 2014) †
Other metallic nanoparticles				
Chlorella sp. MM3	spherical	5-50	$FeCl_3$	(Subramaniyam et al., 2016)
Stoechospermum marginatum	spherical	18.7-93.7	$HAuCl_4$	(Rajathi et al., 2016)
Sargassum wightii	spherical	8-12	$HAuCl_4$	(Singaravelu et al., 2007)
Turbinaria conoides	cubic	20-80	$HAuC_4$	(Vijayaraghavan et al., 2011)
Laminaria Japonica	cubic	15-20	$HAuCl_4$	(Ghodake and Lee, 2011)
Sargassum myriocystum	spherical	10-23	$HAuC_4$	(Dhas et al., 2012)
Kappaphycus alvarezii	spherical	10-40	$HAuC_4$	(Rajasulochana et al., 2010)
Chlorococcum humicola	spherical	2-16	$AgNO_3$	(Jena et al., 2013)

* (nm)

† small percentage of elongated particles

Therefore, the production of copper nanoparticles by algae should be investigated.

III. Recovery and Purification of Microbial Copper Nanoparticles

To the best of our knowledge, there is no report on the purification of copper nanoparticles and there is little information about the purification of metallic nanoparticles (biogenic production). However, Vijayaraghavan et al. (2011) cited that gold nanoparticles adhered to the surface of the biomaterial may be recovered by sonication. Thakkar et al. (2010) suspended the fungal mycelia in deionized water then filtered it (Whatman). Silver nitrate was added to the filtrated solution (metallic nanoparticle production). The metallic nanoparticle solution was dried under an infrared lamp. Singh, (2015) indicates that the procedures for recovering extracellularly synthesized nanoparticles are centrifugation or filtration. The nanoparticles should then be stored in the dark at low temperature. Yet, for the intracellular production, prior to the recovery of metallic nanoparticles, the microbial cells have to be lysed (lysis buffer, sonication and detergent solutions).

IV. Application of Microbial Copper Nanoparticles

a) Antimicrobial

In 2008, the United States Environmental Protection Agency approved copper as an antimicrobial agent, particularly against harmful bacteria (potentially deadly microbial infections). In this sense, attention has been drawn to the bactericidal effect of cooper nanoparticles (Theivasanthi et al., 2011).

The cooper nanoparticles produced by ascorbic acid, chitosan and microwave heating were slightly more effective (minimum inhibitory concentration) against *B. subtilis* instead of *E. coli* 0.313 and 0.469, respectively (Mat Zain et al., 2014).

Abboud et al. (2014) detailed the antibacterial properties of copper nanoparticles produced by algae extract against bacteria *Enterobacter aerogenes* and *Staphylococcus aureus* by using the agar disc diffusion method. Regarding cooper nanoparticles, the radial diameter of the inhibition against *E. aerogenes* and *S. aureus* were of 14 and 16 mm, respectively. Moreover, the algae extract did not show antibacterial activity. Theivasanthi et al. (2011) produced copper nanoparticles by dissolving $CuSO_4$ in distilled water and electrolyzing this solution. Then, the authors recovered the copper nanoparticles at the cathode and showed their antimicrobial properties against *Escherichia coli* and *Bacillus megaterium* by using the agar disc diffusion method, in which the diameter of inhibition against *E. coli* mm and *B. megaterium* were 15 mn and 5 mm, respectively. Merin et al. (2010) reported the antimicrobial activity of silver nanoparticles produced by *Chaetoceros calcitrans*, *Chaetoceros salina*, *Isochrysis galbana* and *Tetraselmis gracilis* (microalgae) against *Klebsiella* sp., *Proteus vulgaris*, *Pseudomonas aeruginosa* and against *Escherichia coli* by using the Muller Hinton agar disc diffusion method. The silver nanoparticles produced from *I. galbana* showed the highest zone of inhibition against *Klebsiella* sp. ($\approx$ 20 mm), whereas the silver nanoparticles produced by *C. salina* showed the highest zone of inhibition against *P. vulgaricus* and *P. aeruginosa*.

To the best of our knowledge, despite the broad potential application of copper nanoparticles (biogenic), only their antimicrobial properties were investigated.

V. Limitations on the Production of Metallic Nanoparticles by Living Cells

The two main limitations on the microbial production of metallic nanoparticles are to achieve the monodisperse size production and the lack of

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knowledge on the mechanism of the synthesis of metallic nanoparticles.

VI. Conclusion

Compared to copper, the biogenic recovery (production of metal nanoparticles) of other metals such as gold, silver and iron has been much deeply investigated. The biogenic production of copper nanoparticles by bacteria and fungi is relatively well known; on the other hand, the biogenic production of copper nanoparticles by algae is very rare. There is no consensus on which type of biomolecules (e.g. proteins, carbohydrates, lipids, etc) plays a major role in the production/stabilization of copper nanoparticles. To the best of our knowledge, there are no procedures concerning the purification of copper nanoparticles (biogenic). In addition, despite the many potential applications of copper nanoparticles (biogenic), only their antimicrobial properties were described.

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