Universidade do Minho
Escola de Engenharia

Juliana de Castro Carvalho

Mechanistic aspects of extracellular silver nanoparticles synthesis by filamentous fungi

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UMinho | 2014

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Dissertação de Mestrado
Mestrado Integrado em Engenharia Biomédica
– Ramo Engenharia Clínica

Trabalho efetuado sob a orientação do
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e da
Doutora Nicolina Marques Dias

julho de 2014

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Título dissertação

Mechanistic aspects of extracellular silver nanoparticles synthesis by filamentous fungi

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Ano de conclusão: 2014

Designação do Mestrado:

Mestrado Integrado em Engenharia Biomédica – Ramo Engenharia Clínica

Universidade do Minho, ____/____/_____

Assinatura: _____

Agradecimentos

O espaço limitado desta secção de agradecimentos, seguramente, não me permite agradecer, como devia, a todas as pessoas que, ao longo do meu Mestrado Integrado em Engenharia Biomédica me ajudaram, directa ou indirectamente, a cumprir os meus objectivos e a realizar esta etapa da minha formação académica.

Desta forma, agradeço à Micoteca da Universidade do Minho (MUM) pela oportunidade de realizar a dissertação no seu laboratório. Estou extremamente grata aos meus orientadores Doutora Nicolina Dias e ao Professor Doutor Nelson Lima pelo conhecimento, disponibilidade, estímulo, apoio constante e paciência que sempre dispensaram. Aos investigadores da MUM, Doutor André Antunes, Doutora Célia Soares, Doutora Lúcia Simões e Doutora Marta Simões um muito obrigado pela disponibilidade, companheirismo e atenção prestada ao longo do projecto. Foi um enorme privilégio ter feito parte deste grupo.

Agradeço aos meus amigos que me acompanharam desde sempre, Ana, Catarina, Carina, Liliana, Regina e Patrícia pela amizade, pelos bons momentos, no qual foram e sempre serão muito importantes para mim. À Liliana e à Raquel pela boa disposição e bons momentos compartilhados ao longo destes dois anos.

À minha Família, aos meus pais, Manuel e Armandina, ao meu irmão, José Pedro, à minha tia Eva, aos meus padrinhos, Joaquim e Eduarda, e aos meus primos, Tiago, Diogo e Maria Carolina, um enorme obrigada por acreditarem sempre em mim, pela boa disposição, compreensão e por todos os ensinamentos de vida. A eles dedico todo este trabalho.

O meu profundo e sentido agradecimento a todas as pessoas que contribuíram, de uma forma directa ou indirecta, para a concretização desta dissertação, estimulando-me intelectualmente e emocionalmente.

Resumo

A produção biológica de nanopartículas metálicas foi estudada por muitos investigadores, devido a obtenção de pequenas partículas estabilizadas por proteínas. No entanto, o mecanismo envolvido nessa produção, ainda não foi esclarecido, embora existam diversos mecanismos hipotéticos propostos na literatura.

As estipes dos fungos *Aspergillus ibericus* MUM 03.49, MUM 03.50 e MUM 03.51; *Penicillium chrysogenum* MUM 03.18, MUM 97.43 e MUM 92.11; e *Neurospora crassa* MUM 11.01 e MUM 92.08 foram testados como produtores de nanopartículas de prata (AgNPs) biogénicas. Através da análise das características físico-químicas de AgNPs produzidos por cada um dos filtrados de células fúngicas, a melhor estirpe produtora de cada espécie fúngica foi seleccionada. Deste modo, as estipes seleccionadas *P. chrysogenum* MUM 92.11 e *N. crassa* MUM 92.08 foram usadas para estudos posteriores da mutagénese e da modulação de mecanismos bioquímicos da produção de AgNPs.

Os mutantes defeituosos no gene estrutural do nitrato reductase *niaD*⁻ foram facilmente recuperados a partir do fungo filamentoso *Penicillium chrysogenum* MUM 92.11 a partir da sua resistência ao clorato. Apesar das várias tentativas realizadas para a estirpe *N. crassa* MUM 92.08, a mutagénese não foi bem-sucedida e consequentemente não houve nenhum crescimento de mutantes. O filtrado do tipo selvagem e o mutante foram ambos capazes de produzir nanopartículas de prata após a adição de nitrato de prata no filtrado. Por fim, para verificar se cada composto do filtrado identificado como influência fundamental na produção de nanopartículas foi desenvolvido um ensaio *in-vitro*. A síntese de AgNPs não ocorreu nos ensaios sem filtrado celular, no entanto, ocorreu nos ensaios com filtrado celular, mesmo após a desnaturação térmica das proteínas, indicando que os aminoácidos podem estar envolvidos na produção de nanopartículas de prata. Outras biomoléculas presentes no filtrado podem também desempenhar um papel-chave no mecanismo de biorredução dos iões de prata, em filtrados fúngicos, que exemplifica o entendimento do mecanismo de produção de AgNPs biogénicas fúngicas.

Abstract

Biological production of metal nanoparticles has been studied by many researchers due to the convenience of the method that produces small particles stabilized by protein. However, the mechanism involved in this production has not yet been elucidated although hypothetical mechanisms have been proposed in the literature.

The fungal strains *Aspergillus ibericus* MUM 03.49, MUM 03.50 and MUM 03.51; *Penicillium chrysogenum* MUM 03.18, MUM 97.43 and MUM 92.11; and *Neurospora crassa* MUM 11.01 and MUM 92.08 were tested as producers of biogenic silver nanoparticles (AgNPs). Through the characterisation of the physiochemical features of the AgNPs produced by each fungal cell filtrate the best producer strain from each fungal species was selected. Therefore, selected strains *P. chrysogenum* MUM 92.11 and the *N. crassa* MUM 92.08 were used to further studies of mutagenesis and modulation of biochemical mechanisms for the production of AgNPs.

Defective mutants on the nitrate reductase structural gene *niaD*$^{-}$ were recovered easily from the filamentous fungi *Penicillium chrysogenum* MUM 92.11 by their resistance to chlorate medium. Although several attempts performed for the strain *N. crassa* MUM 92.08 the mutagenesis was not successful and none of the mutants grew. Wildtype and the mutant filtrate were both able to produce silver nanoparticles after the addition of silver nitrate in the filtrate. Finally, to verify if each filtrate compound identified as key-compound influence the production of nanoparticles an *in-vitro* assay was developed. The synthesis of AgNPs did not occur without cell filtrate, however it did occur in cell filtrate even after the heat denaturation of proteins, indicating that amino acids could be involved in the production of silver nanoparticles. Other biomolecules present in the filtrate are also believed to play a key-role in the bioreductive mechanism of silver ions in fungal filtrates, which exemplify understanding the production mechanism of fungal biogenic AgNPs.

Abbreviations

Ag^{+}	Silver ion
Ag^{0}	Reduced silver
AgNP	Silver nanoparticles
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
ddH_2O	Double-distilled water
Da	Dalton
EtOH	Ethanol
FAD	Flavin adenine dinucleotide
MUM	Micoteca da Universidade do Minho
NADPH	Nicotinamide adenine dinucleotide phosphate
NR	Nitrate reductase
SDS	Sodium dodecyl sulfate
TEMED	N,N,N',N'-Tetramethyl ethylenediamine
Tris	Tris(hidroximetil)aminometane
Tween 80	Polyoxyethylene (80) sorbitan monooleate
UV	Ultraviolet radiations
WT	Wildtype

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General introduction, aims and outline

1. General introduction, aims and outline

1.1. General introduction

The synthesis of nanoparticles is currently an area of intense scientific interest, because of its physicochemical characteristics that are not found in conventional materials (Krolikowska et al. 2003; Albrecht et al. 2006). Besides the various physical and chemical methods employed for the synthesis of metal nanoparticles, biological methods have better advantageous (Shankar et al. 2004; Panacek et al. 2006).

Many reviews have been published related to the biological synthesis of silver nanoparticles by filamentous fungi and their applications in several areas (Mandal et al. 2006; Chen & Schluesener 2008; Marcato & Durán 2008; Durán et al. 2008, 2009, 2010). However, the mechanism involved in this production has not yet been elucidated although hypothetical mechanisms have been proposed by several authors. (Durán et al. 2005; Rai et al. 2009).

The hypothetic model proposed by Durán et al. (2005) suggests that the silver nanoparticles are synthetized with the presence of the NR enzyme which is present in the nitrogen cycle (Kumar et al. 2007; Kalimuth et al. 2008). Besides the presence of NR enzyme, the fungus secrete the cofactor NADH and NADH-dependent enzymes that might be responsible for the bioreduction of Ag^{+} to Ag^{0} and mediates the extracellular reaction process (Kalimuth et al. 2008; Li et al. 2011).

Filamentous fungi nitrogen metabolism is a process controlled by highly complex regulatory proteins, which ensures greater efficiency in the utilization of available nitrogen sources. For a better comprehension of the nitrate assimilation in filamentous fungi it is essential to understand the nitrogen metabolism, as well as the mechanisms of the control of metabolic regulation (Msore-Landecker 1990).

1.2. Aims

The aims of this dissertation were to optimize the biosynthesis process and characterisation of biogenic AgNPs produced by selected filamentous fungi following with the modulation of the biochemical mechanism of silver reduction for biosynthesis of silver nanoparticles by NR enzyme.

1.3. Outline

The theme and relevance of the dissertation are initially described. On the chapter I is selected the best fungal strain from non-pathogenic and non-producers of mycotoxins filamentous fungi and which have the ability to synthesize specific metallic nanoparticles. The chapter II defines the role of NR on the reduction mechanism of biosynthesis of silver nanoparticles and confirmed by the use of defective mutants on the NR structural gene *nia*D^{-}. The chapter I and II are divided in introduction, methods, results, discussion and conclusions. Finally, general conclusion and futures perspectives of the dissertation are described.

Chapter I:

Fungal screening for optimization of extracellular Bio-AgNPs synthesis.

1. Introduction

1.1. Nanoparticles

Particles are small size objects classified according to their diameter. Particles with a size between 1 and 100 nm are known as nanoparticles. They have been found to have a diverse range of applications and are currently used in a variety of fields such as nanotechnology, biology, chemistry, physics, botanic, zoology, chemistry, earth sciences, medicine, pharmacology, mycology, microbiology, pathology and biotechnology (Bhushan 2010).

One of the first references to nanotechnology was made by Richard Feynman (Feynman 1959), where he affirmed that one day it would be possible to manipulate objects with atomic dimensions and make structures with a greatly reduced size. In the late 1960s, the first nanoparticles for drug delivery purposes and for vaccines were developed.

In 2010, the ISO/TS 80004 defined the term of nanotechnology as: "The definition of scientific knowledge to control and utilize matter at the nanoscale, where size-related properties and phenomena can emerge" (Luther and Zwech 2013). It is important to refer that the ISO/TS 80004, from the International Organization for Standardization, provides terms, definitions and applications in the field of nanotechnologies. These series of standards were originally elaborated in 1985 by Eric Dexter and are still a current reference for health, safety and environment concerns. In 2011, nanoscale was defined as a size range with approximately 1 to 100 nm (Hatto 2011).

The characteristics of the nanomaterials are dependent upon a number of properties, including particles size and size distribution, solubility and state of aggregation, elemental composition, mass and concentration, shape and crystal structure, surface area, charge, chemistry and the presence of impurities (Castro-Longoria et al. 2011; Tiede et al. 2009).

Among nanomaterials, metallic nanoparticles have been attracting attention mainly due to their unique physicochemical properties, like: conductivity, reactivity, resistance, and durability. Therefore these are particularly interesting for their many potential applications in innumerable scientific fields, namely on the development of nanocomputers, biomolecular detection, catalysis, optical devices, and biomedicine (Ghorbani et al. 2011). In this field, metallic nanoparticles are mostly promising due to their antibacterial properties improved by their large surface area to

volume ratio, which is of the interest to researchers because of the increasing microbial resistance to antibiotics, and the development of multiresistant strains.

1.2. Silver nanoparticles

Silver is a well-known antimicrobial agent widely used on infectious diseases (Marshall and Schneider 1977; Drake and Hazelwood 2005). The use of silver and copper ions has also been recommended as superior disinfectants for wastewater generated from hospitals containing infectious microorganisms (Lin et al. 1996). On the other hand, silver ions have the disadvantage of forming complexes and when are used to treated ions may adversely affect human health (Lee et al. 2009).

Among metallic nanoparticles, silver nanoparticles (AgNPs) are recognized potent and broad-spectrum antimicrobial agents being one of the most widely used engineered nanoparticles in consumer products (Shrivastava et al. 2007; Wijnhoven et al. 2009). The AgNPs have been widely investigated for their ability to produced composites further used as disinfecting filter and coating materials. Beyond that, they are used as antimicrobial agents in most of the public places such as elevators and railway stations; in surgically implanted catheters in order to reduce the infections, burn wounds, dental work. Antifungal, anti-inflammatory, anti-angiogenic and anti-permeability activities have been claimed by several authors (Wiederrech 2010).

Silver nanoparticles lead to a rise of number of particles per unit area and, consequently, a maximized antibacterial effect was described (Yeo et al. 2003; Morones et al. 2005).

The volume, V, of spherical nanoparticles is described by:

$$V = (4/3)\pi r^3 \quad [1.1]$$

And, the area (A) is defined by:

$$A = 4\pi r^2 \quad [1.2]$$

The r is the particle radius.

Therefore, the ratio between the area and the volume is given by the follow equation:

$$\left({}^{A}/_{V}\right) = {}^{3}/_{r} \qquad [1.3]$$

From the equation [1.3] it is possible to extrapolate that the ratio between the area and the volume is inverse to the particle radius. In this way, to improve the proprieties of the nanomaterial and consequently its antimicrobial activity, the size of the particles has to decrease as the area of activated spots increase. In others words, small particles exhibit higher antimicrobial activity than big particles (Panacek et al. 2006; Durán et al. 2010). This outcome can be explained by the fact that high penetration of particle is due to the reduced particle size (White 2001; Durán et al. 2010). Recent publications suggest that silver nanoparticles are shape-dependent on the interaction with the bacterial cells. Pal et al. (2007) and Sharma et al. (2009) confirmed that the triangular form of the silver ions displayed the strongest biocidal action against *E. coli* when compared with spherical and rod-shaped nanoparticles. Moreover, the high affinity of silver to sulfur or phosphorus is the key element for the antibacterial effect (Yeo et al. 2003; Morones et al. 2005).

1.3. Non-biological vs. biological methods to synthesize AgNPs

The AgNPs can be synthesized by physical/chemical (non-biological) and biological methods. Non-biological methods include irradiation, electrolysis, hydrothermal method, pyrolysis, physical vapor condensation (PVC), arc-discharge methods, chemical reduction and precipitation, and chemical vapor deposition that involve the use of toxic organic solvents and strong reducing chemical agents (for example: N,N-dimethylformamide and sodium borohydride) (Ghorbani et al. 2011). In general, nanoparticles synthesis mediated by non-biological methods requires high temperature and high pressure, consuming energy and producing toxic wastes that are environmentally hazardous, so research efforts have been undertaken for greener production alternatives. Biological methods use microorganisms such as: bacteria (Nair and Pradeep 2002; Lengke and Southam 2006; Husseiny et al. 2007; Shahverdi et al. 2007), filamentous fungi (Ahmad, Mukherjee, et al. 2003; Parikh et al. 2008; Durán et al. 2005; Durán et al. 2010; Anilkumar et al. 2007; Gajbhiye et al. 2009; Govender et al. 2009), actinomycetes (Ahmad et al. 2003; Ahmad et al. 2003) and yeasts (Kowshik et al. 2003); plant extracts (Shankar et al. 2003; Huang et al. 2007; Song & Kim 2009; Bar et al. 2009; Jha et al. 2009) and also peptides (Naik

et al. 2002; Tomczak et al. 2007) to synthetize nanoparticles by biological pathways. Many studies have reported that the biological methods represent an inexpensive, less labor-intensive and eco-friendly method, that is causing an emerging focus on the nanotechnology and biotechnology fields (Kowshik et al. 2003; Kalishwaralal et al. 2008). Notwithstanding the production of biosynthesized silver nanomaterial, biological method allows for the production of different types of other metal nanoparticles such as: copper (Ito et al. 2007), zinc (Bai et al. 2006), titanium (Bansal et al. 2005; Prasad et al. 2007), cadmium (Kowshik et al. 2002), magnesium (Gu et al. 2003), zirconia (Bansal et al. 2004), silver and gold (Nair and Pradeep 2002), platinum (Lengke et al. 2006), and palladium (Yong et al. 2002).

Biological methods allow manipulating the properties of metallic nanoparticles by achieving control over the physicochemical parameters that determines their size and shape. Silver nanoparticles can be induced to have different forms such as: spherical, cubic, wire, and triangular shapes (Chen et al. 2010). Rai and Durán (2011) described that form depends on the cultural conditions: culture medium, quantity of biomass, filtrate volume, salt concentration; and physical conditions: pH, temperature and light intensity that affect the maximum yield, presence of light, rate of synthesis and size of nanoparticles.

Furthermore the microbial cultures are easy to handle and also the downstream processing of biomass is much easier as compared to the non-biological methods (Ingle et al. 2008).

1.3.1. Biosynthesis of silver nanoparticles

Biosynthesis of AgNPs mediated by microorganisms occurs within their cell walls (Ahmad et al. 2002) resulting in the production of extracellular or intracellular nanoparticles (Ahmad et al. 2003; Mukherjee et al. 2008; Shaligram et al. 2009; Ingle et al. 2008; Rai et al. 2008). Despite being observed both in prokaryotes and eukaryotes not all organisms are competent for the synthesis of silver nanoparticles.

Besides that, the interaction of AgNPs with biomolecules released by microorganism metabolism, namely proteins will influence surface chemistry of nanoparticles and modify their electronic charge and agglomeration state leading to the improvement of their biological activity

(Brett 2006; Liau et al. 1997; Gade et al. 2008; Bhainsa and D'Souza 2006a). Also, the presence of proteins in the AgNPs solution has the function of stabilizing the nanoparticles preventing aggregation (Murdock et al. 2008; Greulich et al. 2009). The aggregation of the metal particles resulting in the geometric shapes observed can be originated by the location of the cysteine/histidine residues.

Some of the organisms used in the biosynthesis of AgNPs are summarized in the next paragraph.

Bacteria

Bacterial-mediated biosynthesis has received the most attention in the area of metal nanoparticle biosynthesis (Das and Marsili 2007). First evidence of synthesis of silver nanoparticles was with the microorganism *Pseudomonas stutzeri* AG259, obtained in 1984 (Nair and Pradeep 2002; Bhainsa and D'Souza 2006).

It is known that many bacteria are unaffected by silver while most metals are toxic for the majority of microorganisms. Such bacteria have the advantage of being resistant and, frequently accumulate nanoparticles in intracellular spaces. This was observed by e.g. Parikh et al. (2008) in *Morganella* sp., which reduced metals as a mechanism to decrease toxicity (Hennebel et al. 2009).

The use of bacteria for the synthesis of nanoparticles regularly involves the intracellular synthesis method. In this process, the bacterial cell filtrate is treated with metal salt solution and retained in a shaker in dark at ambient temperature and pressure conditions (Nair and Pradeep 2002; Ahmad et al. 2003).

Nair and Pradeep (2002) demonstrated that the initial step of synthesis of nanoparticles in *Lactobacillus* sp. consists in the nucleation of clusters of metal ions, and consequent electrostatic interaction between these clusters and the bacterial cell. Parikh et al. (2008) used *Morganella* sp. and Saifuddin et al. (2009) used *Bacillus subtilis* to demonstrate that in the presence of silver ions, the supernatant solution produced extracellular silver nanoparticles.

Parikh et al. (2008) demonstrated that the reductase together with electron shuttling compounds (Newman and Kolter 2000) and other peptides/proteins are responsible for the reduction of silver ions and the subsequent formation of silver nanoparticles in a similar way as in fungi (Durán et al. 2005).

The advantages of using bacteria for the production of silver nanoparticles include their ease of handling and their capability to be manipulated and enhanced by genetic techniques (Parikh et al. 2008).

Fungi

In Fungi, the reduction of silver ion to silver nanoparticles is associated with a NADH-dependent reductase enzyme produced as secondary metabolite. This was determined in a preliminary protein assay of silver nanoparticle formation by *Fusarium oxysporum,* Ahmad et al. (2003), and further confirmed by Durán et al. (2005) and Ingle et al. (2008).

Another study using *Fusarium moniliforme* showed no production of intra- or extracellular silver nanoparticles, suggesting that the reductase enzyme is not present in all fungi. However, a later study with this species detected nitrate reductase (NR) but pointed to the absence of naphthoquinone. These findings are evidence that not only the reductase enzyme is necessary but also the electron shuttle is needed for the ion metal reduction (Durán et al. 2005). Another study conducted by Kumar et al. (2007) confirmed the participation of all these molecules in the formation mechanism of metal nanoparticles, i.e. in the absence of one of these compounds: enzyme, quinone, naphthoquinones or NADPH, silver nanoparticles were not produced (Kumar et al. 2007; Durán et al. 2011). More details about fungal-mediated biosynthesis of AgNPs will be described in section 1.4.

Yeasts

Of all the eukaryotes, yeast species are unquestionably the most studied and applied in bioprocesses, which qualify them as attractive microorganisms for the synthesis of nanoparticles.

Nanoparticle biosynthesis by yeasts includes two steps: the synthesis of nanoparticles, where the metal salt solution is added to the yeast culture and incubated in the dark for 24 h;

and the recovery of the synthesized nanoparticles, where the cells are separated from the medium by centrifugation and the cell-free extract is used for recovery (Kowshik et al. 2003).

The production of nanoparticles by yeasts is usually intracellular, but a few exceptions are present. Kowshik et al. (2003) reported that the silver tolerant yeast strain MKY3 reduces silver ions to metallic silver resulting in extracellular nanoparticles. This study suggested that the result from the excretion of intracellular nanoparticles as a response to silver stress.

Plants

Plants have been extensively researched regarding the biosynthesis of nanoparticles. The exact mechanism for the plant-mediated synthesis of nanoparticles is still unclear, but several possible mechanisms have been proposed (Ingle et al. 2008; Rai et al. 2008; Thakkar et al. 2010).

In 2007, Li et al. proposed recognition-reduction-limited nucleation besides a growth model to explain the silver nanoparticles production. In this hypothetical mechanism, the recognition phase of the silver ions was trapped on the surface of proteins present in the *Capsicum annum* extract through electrostatic interaction. They hypothetically said that the proteins probably reduce the silver ions, resulting in the nucleation of silver. Then, the proteins and biomolecules present in the reaction mixture lead to isotropic growth of silver nuclei stabilizing the silver nanoparticles.

Furthermore, in 2008, another method of production was employed where the plant product from *Cinnamomum camphora* leaf was used to synthesize silver nanoparticles in a continuous flow tubular microreactor (Huang et al. 2007).

It is known that for the extracellular synthesis of silver using plants, the biomolecules act as reducing agents and the heterocyclic compounds act as capping agents for the nanoparticles. The literature describes that the reduction of silver ions and stabilization of the nanoparticles were respectively made by polyol components and the water-soluble heterocyclic components. This study concluded that hydroxyls in the terpenoids present in the leaf extract (citronellol and geraniol) are oxidized to carbonyl groups and hence act as a reducing agent for silver ions (Shankar et al. 2003; Safaepour et al. 2009). This supported the study made by Shankar (2003)

through the directly used geraniol extract for the reduction of silver ions and found that geraniol possesses the ability to synthesize silver nanoparticles by reducing silver ions.

Peptides

In 2002, Naik et al. demonstrated the biosynthesis of silver nanoparticles through the use of peptides with silver-binding capability. The authors proposed that the addition of the peptide to a silver ion solution and interaction with preformed nanoclusters or nuclei of silver metal. This interaction generates a chemically reducing environment around the cluster resulting in the reduction of silver ions at the peptide-metal interface. Arginine, cysteine, lysine, and methionine are the amino acid residues that can be used for this production. Advantages of this method include the easy separation from water, and the possibility to produce Au core–Ag (when using tyrosine) (Naik et al. 2002; Durán et al. 2005).

We can conclude that the mutual intermediates in the mechanisms of bacteria, fungi, yeast and plants are NR and an electron shuttle (quinones or naphthoquinones). Peptides also appear to have a reductase-like activity due to their conformation.

When comparing fungi with bacteria, the fungi have been known to secrete much higher amounts of bioactive substances, which make them more suitable for large-scale production and the extracellular biosynthesis by fungi could make downstream processing much easier (Sagar and Ashok 2012).

1.4. Fungal-mediated biosynthesis of AgNPs

The biosynthesis of silver nanoparticles by fungi is a relatively recent research area. Filamentous fungi have emerged up as very good candidates for environmental and friendly synthesis of metal nanoparticles. The use of fungi is potentially interesting since they exhibit metal ions resistance, bioaccumulation (Mandal et al. 2006) and have the ability to secrete large amount of proteins which are desirable features shared by those microorganisms (Vahabi et al. 2011). Moreover, because their biomass is easy to handle, they need simple nutrient possess high wall-binding capacity (Dias et al. 2002; Sanghi and Verma 2009) which made the scale-up synthesis of nanoparticles to a larger scale easier (Durán et al. 2011)

Fungi have the capacity to synthetize biogenic, geometric metal particles, in the nanometer range through a bioreductive process, when exposed to metal chloride or nitrate solutions.

The biosynthesis of AgNPs can be performed by intra or extracellular method according to the location where NPs are formed. Nevertheless the formation of NPs in the solution by reduction of metal ions outside the fungal biomass is advantageous from the practical point of view. Screening studies has been done using different species of fungi for extracellular biosynthesis of metal NPs (Table 1). Nevertheless some of those studies used pathogenic strains without regard on the potential presence of mycotoxins in the AgNPs solution (Mandal et al. 2006; Li et al. 2011).

According to Ahmad et al. (2003) the first report on the extracellular synthesis of silver nanoparticles with eukaryotic fungi used the species *Fusarium oxysporum.* In this study they point out that even though silver nanoparticles have been synthesized using prokaryotes such as bacteria, and eukaryotes such as fungi the nanoparticles grow intracellularly. They also showed that one of the proteins was an NADH dependent reductase that has the responsibility for the reduction of Ag^{+} ions and the subsequent formation of silver nanoparticles.

Also, Mukherjee et al. (2001) demonstrated the bioreduction of aqueous Ag^{+} ions with the *Verticillium* sp. and verified that the reduction of the metal ions occurs on the surface of the mycelia leading to the formation of silver nanoparticles.

Furthermore, the preliminary study of Bhainsa and D'Souza (2006), used the filamentous fungi *Aspergillus fumigatus* to reduce silver ions extracellularly proving that this fungus is a good candidate for rapid biosynthesis of silver nanoparticles.

Table 1 – List of fungi that have been used in the production of extracellular AgNPs and their respective size (Adaptation of Rai and Durán 2011).

Organism	Size range (nm)	Author (publication year)
Aspergillus clavatus	10–25	Verma et al. (2010)
Aspergillus flavus	8.92 ± 1.61	Vigneshwaran et al. (2007)
Aspergillus fumigatus	5–25	Bhainsa & D'Souza (2006)
Aspergillus sp.	20	Gade et al. (2008)
Cladosporium cladosporioides	10–100	Balaji et al. (2009)
Fusarium acuminatum	5–40	Ingle et al. (2008)
Fusarium oxysporum	5–50	Ahmad et al. (2003)
Fusarium semitectum	10–60	Basavaraja et al. (2008)
Fusarium solani	5–35	Gade et al. (2009)
Penicillium brevicompactum WA	23–105	Shaligram et al. (2009)
Penicillium fellutanum	1–100	Kathiresan et al. (2009)
Phanerochaete chrysosporium	100	Vigneshwaran et al. (2006)
Trichoderma asperellum	13–18	Mukherjee et al. (2008)
Trichoderma viride	5–40	Fayaz et al. (2010)
Verticillium sp.	25 ± 12	Mukherjee et al. (2001)

1.5. Mechanisms and metabolism of nanoparticles biosynthesis

The synthesis of metal nanoparticles by different microbial species has been reported, but the exact mechanism of nanoparticle biosynthesis is still not well understood. The analysis and identification of active species in the nucleation and growth of metal nanoparticles is complex, mainly due to the interaction process along with metabolic complexity of microorganisms (Das and Marsili 2007).

It is not clear why not all organisms are capable to synthetize silver nanoparticles. Recently, Parikh et al. (2011) described the improvement in the biological synthesis and showed that the shape of silver nanoparticles can be altered from nanospheres to nanoprisms by controlling the growth kinetics of a silver resistant bacteria *Morganella psychrotolerans.*

The mechanism of nanoparticles by intra- and extracellular synthesis is different in numerous biological agents. Extracellular production of nanoparticles is stabilized by proteins and they have the ability to reduce agents secreted by the fungus itself (Durán et al. 2005). Relatively to the intracellular synthesis of nanoparticles, it is known that the cell wall of microorganisms has a major role on their synthesis and also needs a special ion transportation system into the microbial cell (Mann 2001). Also, the fact of the cell wall being negatively charged can interact electrostatically with the positively charged metal ions. Inside of the cell wall, the enzymes reduce the metal ions to nanoparticles and make the smaller sized nanoparticles diffuse through the cell wall.

The metabolic activity of microorganisms can lead to an extracellular precipitation of nanoparticles where the fungi are considered to be extremely good candidates for this processes. The extracellular synthesis of silver nanoparticles by fungi was described in *Colletotrichum* sp. (Mandal et al. 2006) and *Aspergillus fumigatus* (Bhanska et al. 2006 cited in Sadowski et al. 2008). Mukherjee et al. (2001) proposed that the synthesis of silver nanoparticles using *Vericillum* has two-steps. The first involves trapping silver ions at the surface of the fungal cells; and the second, occurs through the reduction of silver ions by the enzymes present in the cell (Mukherjee et al. 2001).

The NR-mediated synthesis is normally related to biosynthesis of nanoparticles using microbes with the extracellular mechanism. The bioreduction of metal ions and synthesis of nanoparticles is carried out by the NR produced by the fungi (Durán et al. 2005; Kumar et al. 2007; He et al. 2007; Gade et al. 2008; Ingle et al. 2008).

The reduction of Ag^{+} with combinations of biomolecules such as enzymes/proteins, amino acids, polysaccharides and vitamins is environmentally benign, yet chemically complex. The biosorption and bioreduction process for the fabrication of nanoparticles depends on the

source extract that contains proteins, in the microorganisms (Sanghi and Verma 2009) or carboxylic groups, amino groups, proteins and carbohydrates in the plants (Huang et al. 2007).

The role of the biomolecules in the biosynthesis of silver nanoparticles is not yet very clear. It is known that the nanoparticles formed on the surface of the mycelia. The silver ions are first trapped on the surface of the fungal cells via electrostatic interaction among the ions and negatively charged cell wall from the carboxylate groups in the enzymes. The enzymes reduced the metal ions to form silver nuclei, which subsequently grow through further reduction and accumulation (Bansal et al. 2004; Sneha et al. 2010). In 2005, Durán and his work group have proposed a hypothetic model that supports the experimental data for the bioreduction mechanism of the synthesis of extracellular silver and gold nanoparticles formation by the fungus *Fusarium oxysporum*. This hypothetic model proposes that the silver nanoparticles are synthetized with the presence of the "NR" enzyme which is present in the nitrogen cycle (Kumar et al. 2007; Kalimuth et al. 2008).

Also, Durán et al. (2005) have described the reduction of silver ions with gold nanoparticles as a possible mechanism under the name of electron shuttle enzymatic metal reduction process (Figure 1).

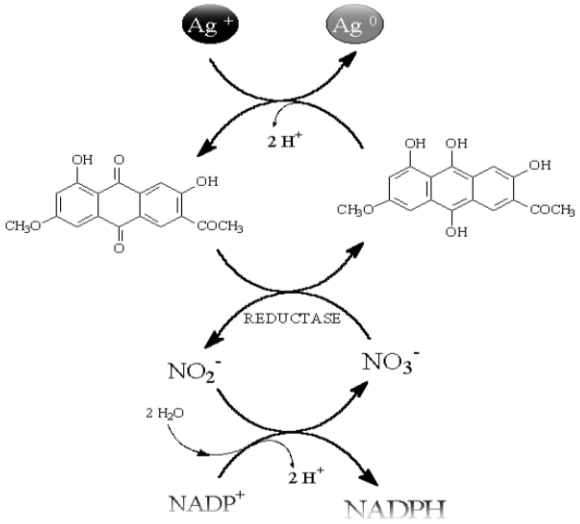


Figure 1 – Hypothetical diagram of the synthesis of silver nanoparticles by the electron shuttle enzymatic metal reduction process, through the NR dependent of NADPH. (Adaptation of Durán et al. 2005)

In this mechanism, the fungus secrete the cofactor NADH and NADH-dependent enzymes, especially NR, that might be responsible for the bioreduction of Ag^{+} to Ag^{0} and the subsequent extracellular formation of silver nanoparticles (Kalimuth et al. 2008). Beyond that,

Kumar et al. (2007) have shown evidences of the used of NR for synthesis of silver nanoparticle by *Fusarium oxysporum*. The mixture that contained NR enzyme, silver nitrate and NADPH demonstrated that, gradually, the reaction mixture turned brown which proves the presence of silver nanoparticles. This became the first evidence of the involvement of NR in the synthesis of silver nanoparticles because the metal nanoparticles usually show a strong plasmon resonance extinction bands in the visible spectrum as a consequence of the deep colours suggestive of molecular dyes (Fedlheim and Fos 2002). Besides that, Li et al. (2011) demonstrated that *Aspergillus terreus* at room temperature synthesized spherical silver nanoparticles polydispersed, with a size range from 1 to 20 nm and found that the reduced NADH is an important reducing agent that mediates the extracellular reaction process.

It is well known that enzymes play an important role in metal transformations, but, their role in the nanoparticle formation is a relatively new and still a developing field of study.

2. Materials and methods

2.1. Fungi

The current study investigated the biosynthesis of AgNPs by several fungal strains obtained from Micoteca da Universidade do Minho. We focused on two mesophilic species: *Aspergillus ibericus* and *Penicillium chrysogenum;* and a thermophilic one: *Neurospora crassa* (Table 2).

Table 2 – Fugal strains used to study the biosynthesis of AgNPs.

Species	Strain
Aspergillus ibericus	MUM 03.49
	MUM 03.50
	MUM 03.51
Penicillium chrysogenum	MUM 92.11
	MUM 03.18
	MUM 97.43
Neurospora crassa	MUM 11.01
	MUM 92.08

2.2. Culture and storage conditions

The strains were maintained in PDA (Oxoid, UK) plates at 25 °C for mesophilic or 37 °C for thermophilic strains for 5 to 7 days. Then, the fungi were cut in small agar blocks and set in MGYP (0.3 % malt extract (Oxoid, UK); 1.0 % glucose (Fisher Chemical, USA); 0.3 % yeast extract (Oxoid, UK); 0.5 % peptone (Oxoid, UK)) liquid medium for 72 h at 30 °C (mesophilic strain) or 37 °C (thermophilic strain).

2.3. Biosynthesis of AgNPs

For the biosynthesis of AgNPs the methodologies presented by Ahmad et al. (2003) and Durán et al. (2005) were taken into consideration. However, some modifications were put in place, as follow. Fungal cells were grown in MGYP liquid medium, and incubated at 30 °C (mesophilic) and 37 °C (thermophilic) with 150 rpm for 72 h. The fungal biomass was then harvested followed by extensive washing with Milli-Q deionized water. In a glass flask 10 g (wet

weight of biomass) were added to 100 mL of Milli-Q deionized water and incubated with shaking for 72 h (30 and 37 °C for mesophilic and thermophilic strains, respectively at 150 rpm). The fungal biomass in the aqueous suspension was then filtered through a Whatman grade 1 filter paper (Whatman, UK) and the fungal filtrate was finally obtained.

Biosynthesis of Bio-AgNPs was achieved by adding 1 mM silver nitrate ($AgNO_3$ – Sigma-Aldrich, Germany) to 100 mL of the fungal filtrate, which was incubated in the dark at 30 °C and 150 rpm for up to 96 h. Control flask, without the $AgNO_3$, was incubated at same conditions. Aliquots of the reaction solution were taken at 96 h of incubation for characterisation of Bio-AgNPs by UV-Vis spectroscopy analysis and Dynamic Light Scattering (DLS) and Zeta-potential. A volume of 25 mL was stored at 4 °C for ICP-OES analysis. The remaining filtrate was freeze-dried and the samples were ready for further analysis by Scanning Electron Microscopy (SEM/EDS).

2.4. Physicochemical characterisation of the Bio-AgNPs

2.4.1. Ultraviolet-visible spectroscopy

The reduction of Ag^+ ions was analysed by UV-Vis spectrophotometry with a specific surface plasmon absorption band between 380 to 420 nm after 96 h of incubation. This specific surface plasmon absorption band indicates the presence of spherical nanoparticles. Thereby, the density was measured with disposable cuvettes of low volume with 1 mL of extract. It was prepared three measurements by spectrophotometer V-560 (Jasco, Japan) with a wavelength between 300 and 800 nm.

2.4.2. Dynamic Light Scattering (DLS)

The size and the size distribution of AgNPs dispersed in the filtrate were analysed by the Dynamic Light Scattering (DLS) and measured by Malvern Instruments Zatasizer 1000 (Malvern, UK) equipment at 25 °C. In this method, it was used disposable cuvettes of low volume with 1 mL of filtrate. It was prepared three measurements after 96 h of incubation.

2.4.3. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

To determinate the silver concentration in the filtrate Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) equipment was used. One milliliter of filtrate with a Sterile Syringe Filter with 0.2 µm cellulose acetate membrane (VWR, North America) before the

analysis to remove any organic material existent. The filtrate was diluted up to 5 mL with 2 % (v/v) HNO_3. The used instrument was an ICP-OES OPTIMA 8000 from PERKINELMER, with an absorbance of 338,289 nm. The analysis was performed in triplicate.

2.4.4. Zeta-Potential analysis

The Zeta-Potential analysis was used to determinate the charge of the solution that cover the silver nanoparticles. This analysis was prepared with 1 mL of filtrate with a clear disposable zeta cell after 96 h of incubation. The measurement was made with the Malvern Instruments Zatasizer 1000 (Malvern, UK) equipment at 25 °C.

2.4.5. Scanning Electron Microscopy (SEM)

Silver nanoparticles topographic analyses were performed in an ultra-high resolution Field Emission Gun Scanning Electron Microscopy (FEG-SEM), NOVA 200 Nano SEM, FEI Company. The freeze-dried samples were analysed with a Backscattering Electron Detector (BESD) at an acceleration voltage of 10 kV.

2.4.6. Energy-dispersive X-ray spectroscopy (EDS)

EDS is an analytical technique used for the elemental analysis of a sample. It depends on the interaction of some source of excitation and a sample. This involves a qualitative analysis that identified the lines in the spectrum and quantitative analysis, which define the concentrations of the elements present. These quantitative analyses involve measuring the line intensities for each element in the sample and for the same elements in calibration standards of known composition.

Chemical analyses of samples were performed with EDS technique, using an EDAX Si(Li) detector with an acceleration voltage of 15 kV and were analysed with an Ultra-high resolution Field Emission Gun Scanning Electron Microscopy (FEG-SEM), NOVA 200 Nano SEM, FEI Company.

3. Results

This chapter is focused on the optimization of biosynthesis process and characterisation of biogenic AgNPs produced by selected filamentous fungi. It has the purpose to determinate the best strain of the best fungus from non-pathogenic and non-producers of mycotoxins filamentous fungi and which have the ability to synthesize specific metallic nanoparticles.

3.1. Biosynthesize of AgNPs

For the study of the biosynthesis of AgNPs the filamentous fungi species of *Aspergillus ibericus, Penicillium chrysogenum* and *Neurospora crassa* were used. The production of AgNPs was monitored through the change of colour of the filtrate treated with 1 mM of $AgNO_3$. A brownish colour of the filtrate was observed after 96 h of incubation for *A. ibericus* (Figure 2), *P. chrysogenum* (Figure 3) and for *N. crassa* strains (Figure 4). A negative control was prepared for each strain without $AgNO_3$.

Different results were found from different species and also within each species. The strain *A. ibericus* MUM 03.51 produced the most intense brownish filtrate comparing with its negative control and when compared to other *A. ibericus* strains. All the strains of *P. chrysogenum* produced brownish filtrate comparing to their negative control. From all the fungi *N. crassa* MUM 92.08 produced the most brownish filtrate although *N. crassa* MUM 11.01 did not shown any colour change when compared with the negative control.

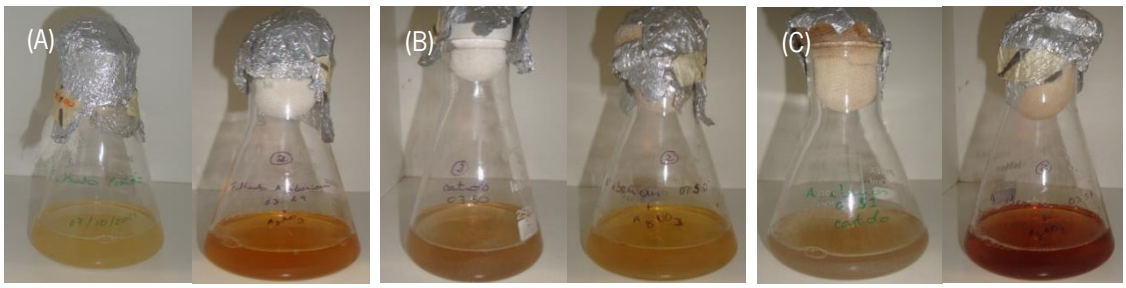


Figure 2 – Cellular filtrate of *Aspergillus ibericus* MUM 03.49 (A), MUM 03.50 (B) and MUM 03.51 (C), treated with 1 mM of $AgNO_3$ after 96 h of incubation in the dark. Corresponding negative controls are shown on the left of each image.

Figure 3 – Cellular filtrate of *Penicillium chrysogenum MUM* 97.43 (A), MUM 92.11 (B) and MUM 03.18 (C), treated with 1 mM of $AgNO_3$ after 96 h of incubation in the dark. Corresponding negative controls are shown on the left of each image.

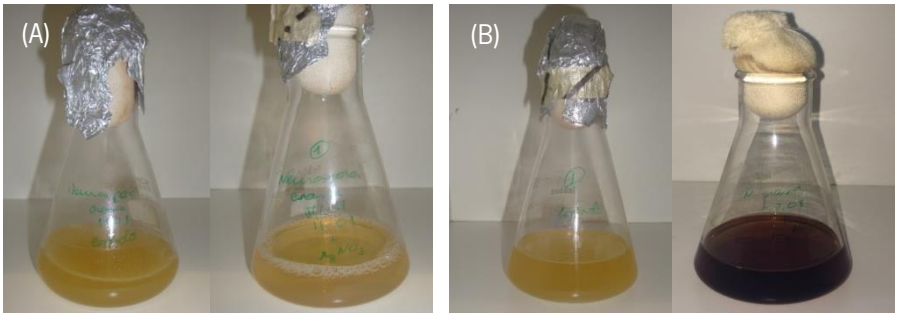


Figure 4 – Cellular filtrate of *Neurospora crassa* MUM 11.01 (A) and MUM 92.08 (B), treated with 1 mM of $AgNO_3$ after 96 h of incubation in the dark. Corresponding negative controls are shown on the left of each image.

The production of AgNPs was monitored by spectrophotometric analysis and a UV-Vis spectrum was obtained through the excitation of Ag^0 surface plasmon vibrations at wavelengths ranging from 380 to 420 nm.

Each species exhibited different absorbance peaks comparing with other species. The strains *A. ibericus* MUM 03.49, *P. chrysogenum* MUM 92.11 and *N. crassa* MUM 92.08 (Figure 8) showed the highest absorbance peak for each species. Differences on peak absorbance were also observed within the same species suggesting intraspecific variability. The strain *A. ibericus* MUM 03.51 (Figure 5) and the strain *N. crassa* MUM 11.01 (Figure 7) did not show any absorbance peak on the UV-Vis spectra.

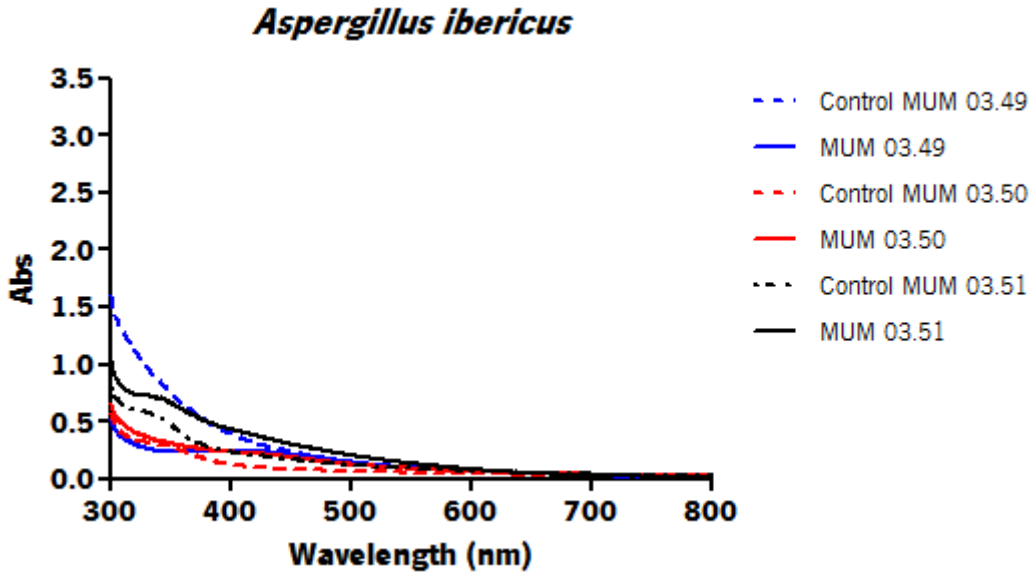


Figure 5 – UV-visible spectrum of *Aspergillus ibericus* filtrate with 1 mM of $AgNO_3$ aqueous solution with and its respective strains after 96 h of incubation.

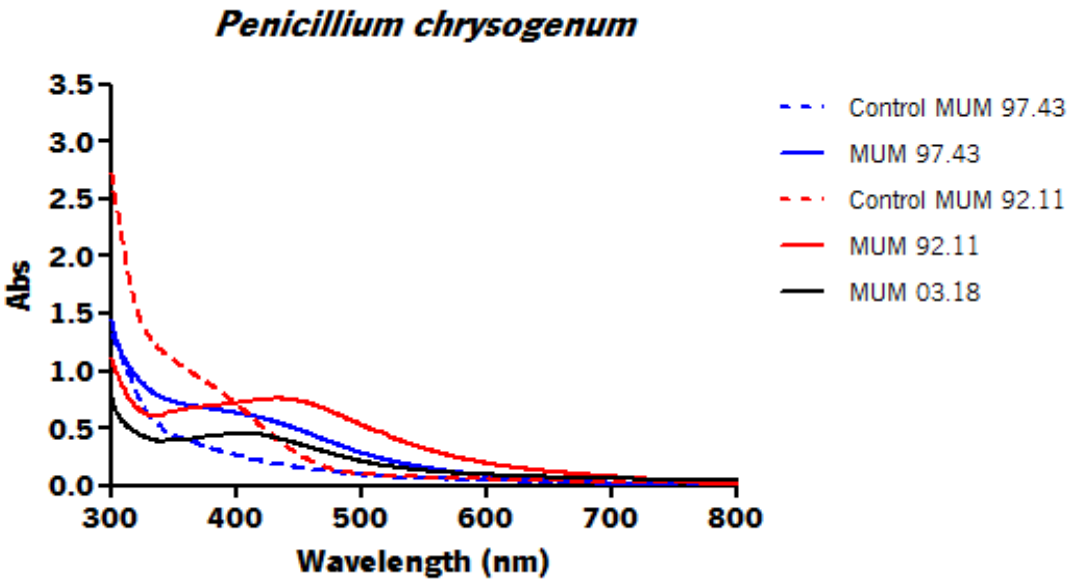


Figure 6 – UV-visible spectrum of *Penicillium chrysogenum* filtrate with 1 mM of $AgNO_3$ aqueous solution with and its respective strains after 96 h of incubation.

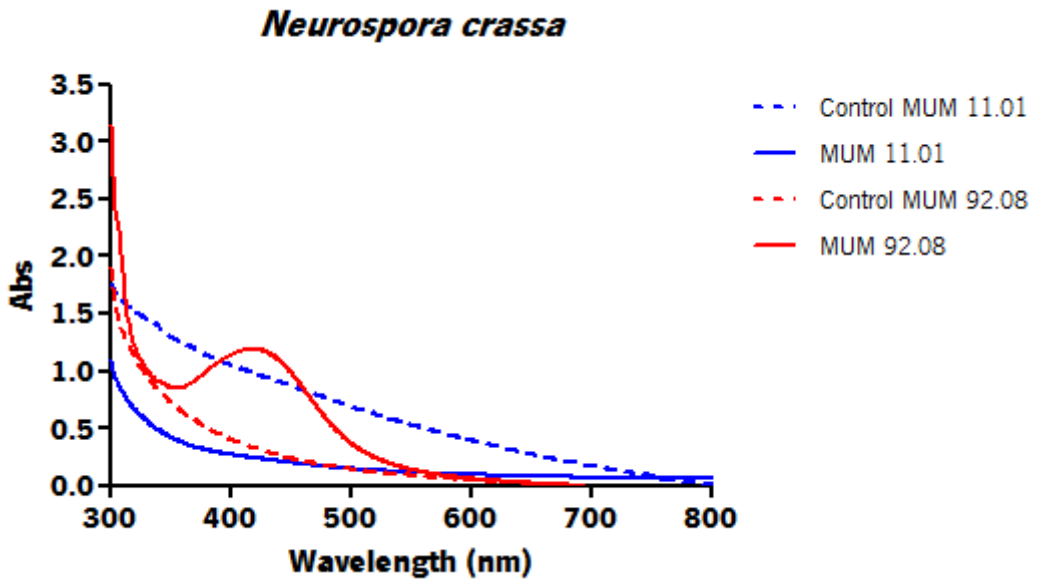


Figure 7 – UV-visible spectrum *Neurospora crassa* filtrate with 1 mM of $AgNO_3$ aqueous solution with and its respective strains after 96 h of incubation.

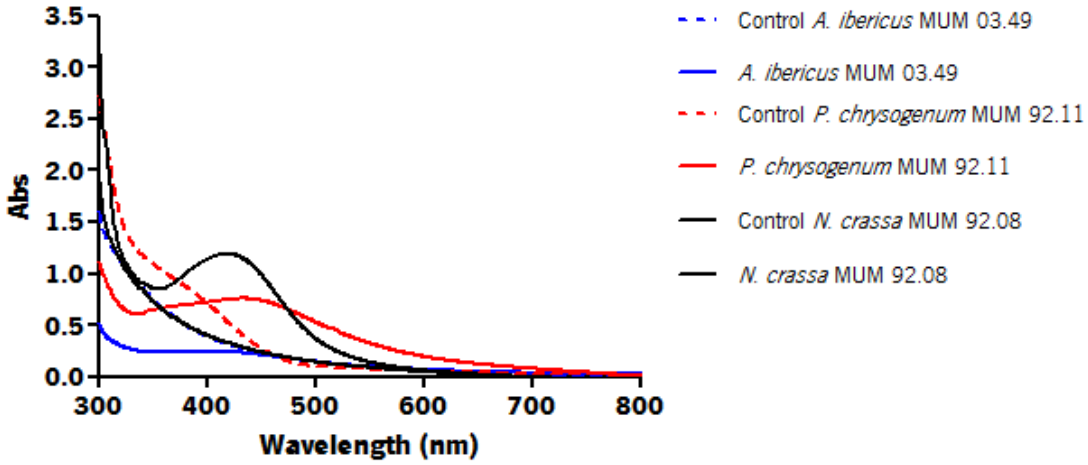


Figure 8 – UV-visible spectrum *Aspergillus ibericus* MUM 03.49, *Penicillium chrysogenum* MUM 92.11 and *Neurospora crassa* MUM 92.08 filtrates with 1 mM of $AgNO_3$ aqueous solution with and its respective strains after 96 h of incubation.

The size distribution of AgNPs produced for each fungal strain (Figures 9 - 16) was determined by Dynamic Light Scattering (DLS). Table 3 represents the average size (nm) and polydispersity of AgNPs. In terms of these physicochemical features different size distribution curves, average size and polydispersity values were obtained for AgNPs produced by each species and within each species. For *A. ibericus* species the strain *A. ibericus* MUM 03.49

produced AgNPs with a unimodal size distribution, showing the lowest average size, as well as the lowest polydispersity value. The AgNPs produced by *P. chrysogenum* MUM 97.43 exhibited the lowest average size, however AgNPs produced by *P. chrysogenum* MUM 92.11 exhibited the best size distribution and the lowest polydispersity values. Overall the strain *N. crassa* MUM 92.08 produced AgNPs with unimodal size distribution the lowest average size, and the lowest polydispersity.

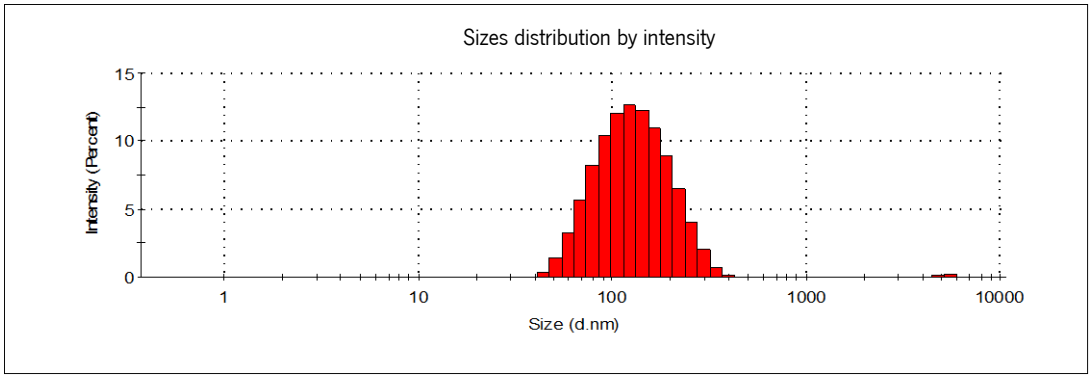


Figure 9 – AgNPs sizes distribution by intensity of *Aspergillus ibericus* MUM 03.49.

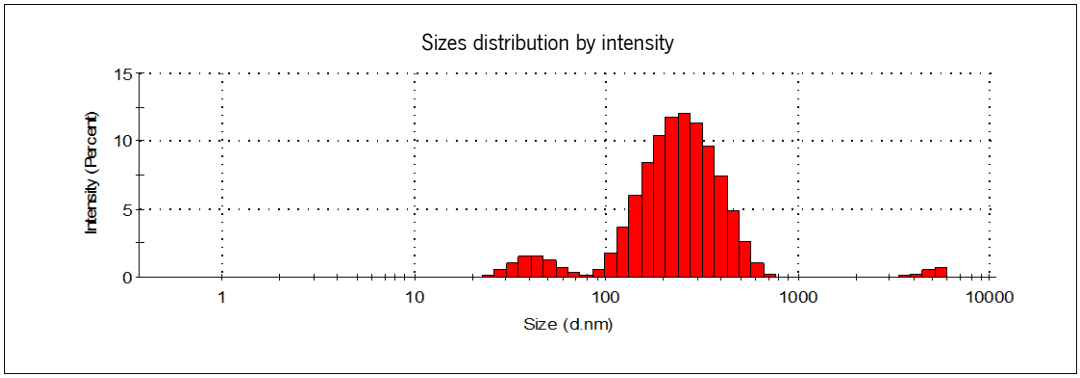


Figure 10 – AgNPs sizes distribution by intensity of *Aspergillus ibericus* MUM 03.50.

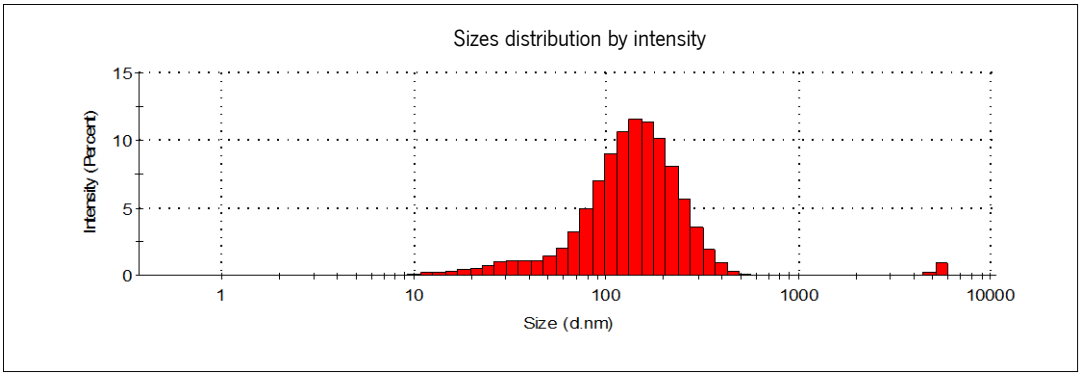


Figure 11 – AgNPs sizes distribution by intensity of *Aspergillus ibericus* MUM 03.51.

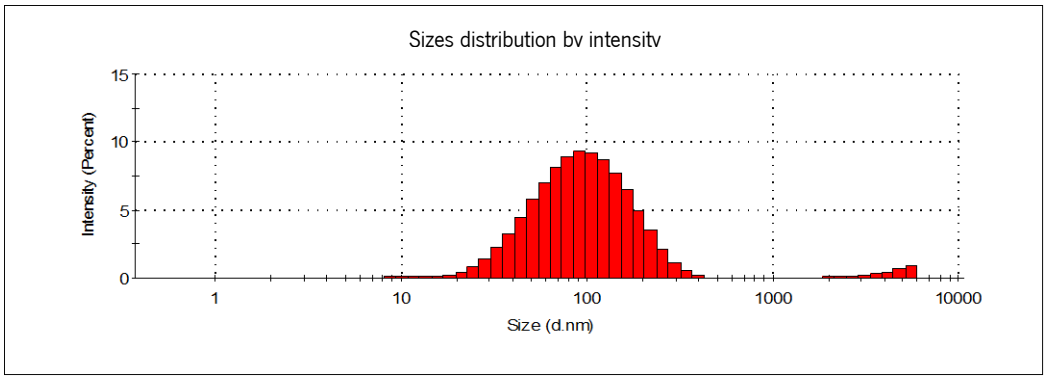


Figure 12 – AgNPs sizes distribution by intensity of *Penicillium chrysogenum* MUM 92.11.

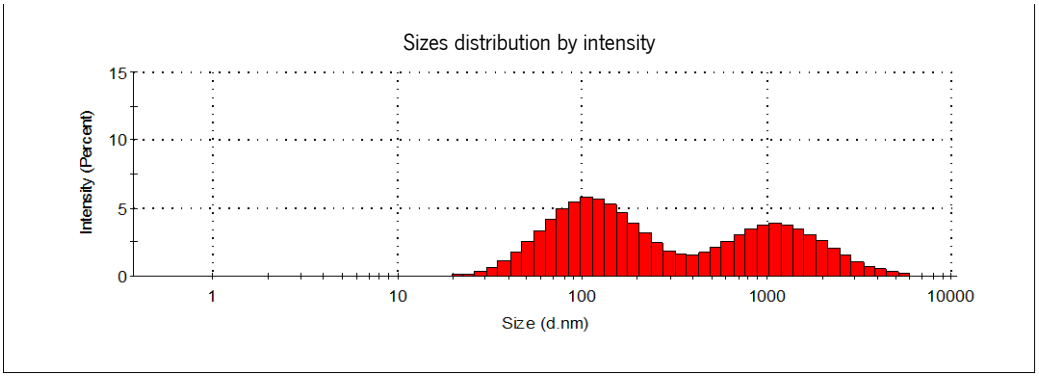


Figure 13 – AgNPs sizes distribution by intensity of *Penicillium chrysogenum* MUM 97.43.

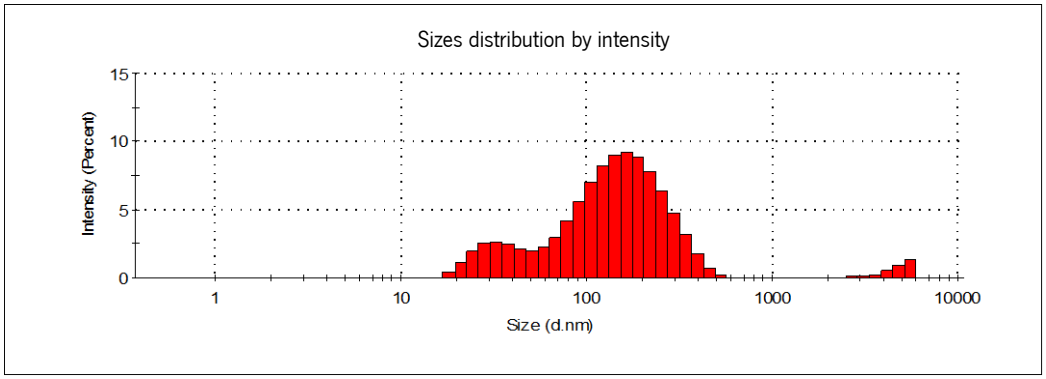


Figure 14 – AgNPs sizes distribution by intensity of *Penicillium chrysogenum* MUM 03.18.

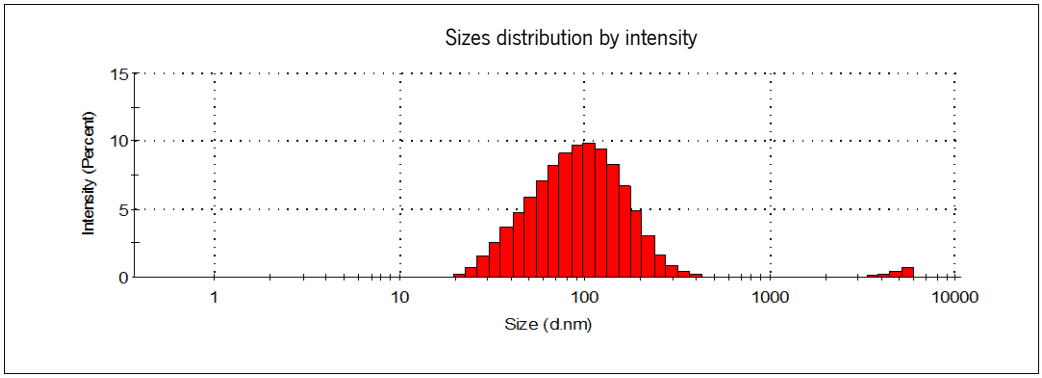


Figure 15 – AgNPs sizes distribution by intensity of *Neurospora crassa* MUM 11.01.

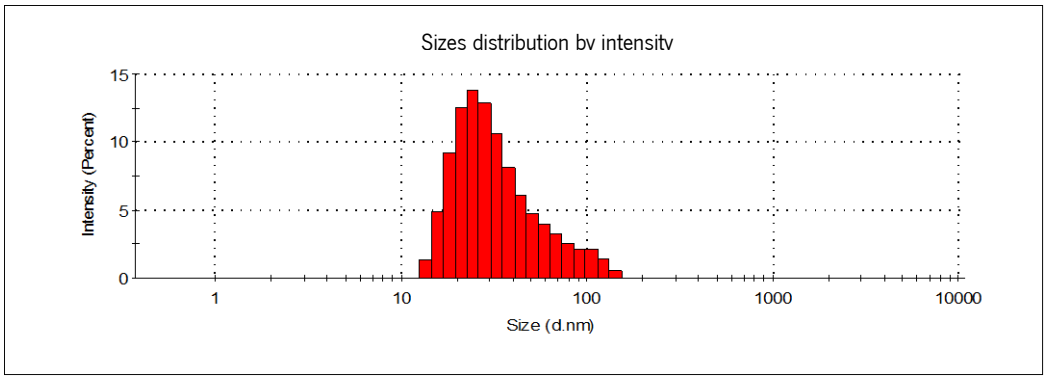


Figure 16 – AgNPs sizes distribution by intensity of *Neurospora crassa* MUM 92.08.

Table 3 – Average of size (nm) and polydispersity of each fungus and respective strains analyse by DLS.

Fungus	Average size (nm)	Polydispersity
A. ibericus MUM 03.49	122.4	0.179
A. ibericus MUM 03.50	255	0.337
A. ibericus MUM 03.51	141.8	0.390
P. chrysogenum MUM 92.11	141.8	0.242
P. chrysogenum MUM 97.43	105.7	0.672
P. chrysogenum MUM 03.18	164.2	0.435
N. crassa MUM 11.01	105.7	0.325
N. crassa MUM 92.08	24.36	0.269

Inductively coupled plasma optical emission spectrometry (ICP-OES) method was used to determinate the silver concentration. Table 4 represents the silver concentration (10^3 mg/mL) in the filtrate after 96 h of incubation with 1 mM $AgNO_3$ of all fungal strains. Theoretical value is the calculated concentration of silver in 1 mM $AgNO_3$ and real value was obtained by measuring the concentration of 1 mM $AgNO_3$ in distilled water by ICP-OES. A decrease of silver concentration in the filtrate was observed for all the strains.

Table 4 – Concentration (10^3 mg/mL) of silver of each fungus and respective strains filtrate analyse by ICP-OES.

Fungus	Concentration (10^3 mg/mL)
A. ibericus MUM 03.49	65.578
A. ibericus MUM 03.50	57.225
A. ibericus MUM 03.51	31.335
P. chrysogenum MUM 92.11	43.496
P. chrysogenum MUM 97.43	65.732
P. chrysogenum MUM 03.18	64.079
N. crassa MUM 11.01	22.142
N. crassa MUM 92.08	51.203
Real Value	107.870
Theoretical Value	111. 590

From the previous results the strains *A. ibericus* MUM 03.49, *P. chrysogenum* MUM 92.11, *N. crassa* MUM 92.08 were found the best candidates to producer of AgNPs from each species. Further analyses as zeta-potential, Energy-dispersive X-ray spectroscopy (EDS) and Scanning Electron Microscopy (SEM) were conducted on these strains.

The Zeta-potential analysis (Table 5) clearly shown that the surface charge of the produced AgNPs for *P. chrysogenum* MUM 92.11 and *N. crassa* MUM 92.08 was highly negative indicating that the AgNPs surface has an anionic charge.

Table 5 – Solution charge (mV) of previously selected strains of each fungus analyse by Zeta-Potential.

Fungus	Charge (mV)
A. ibericus MUM 03.49	- 9.7
P. chrysogenum MUM 92.11	- 25.1
N. crassa MUM 92.08	- 22.2

Real charge (mV) of ddH_2O with $AgNO_3$ = -1.1

The morphology and size range and the elemental composition of AgNPs in the freeze-dried filtrate of each selected fungi were analysed by SEM and EDS, respectively. A representative micrograph of the AgNPs was obtained by the SEM microscopy (Figure 17). It was found that the AgNPs exhibited variable shapes but most of them were spherical. Besides a size range of 40.9 to 204.0 nm for AgNPs produced by *A. ibericus* MUM 03.49 (Figure 17 (A)); a size range of 40.9

to 52.5 nm for AgNPs produced by *P. chrysogenum* MUM 92.11(Figure 17 (B)) and a size range of 43.7 to 72.9 nm for AgNPs produced by *N. crassa* MUM 92.08 (Figure 17 (C)) was observed. Most of the AgNPs were found as aggregates.

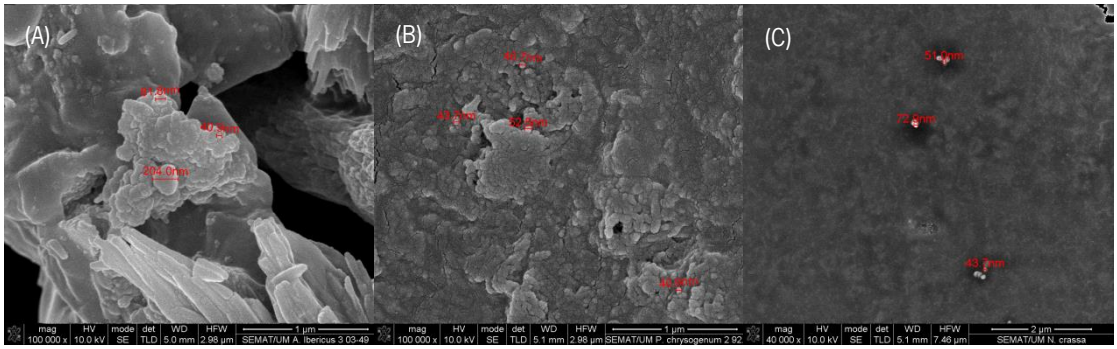


Figure 17 – SEM images of AgNPs synthetized by: *Aspergillus ibericus* MUM 03.49 (A), *Penicillium chrysogenum* MUM 92.11 (B) and *Neurospora crassa* MUM 92.08 (C).

Elemental composition of the AgNPs in the freeze-dried filtrate of selected fungal strain was determined by EDS (Figure 18). It can be observed that the *P. chrysogenum* MUM 92.11 shown the highest signal on the silver region (proximally at 3 KeV) comparing with *A. ibericus* MUM 03.49 and *N. crassa* MUM 92.08. Other elements such as C, N, O, P, S, Cl and K were also found in the freeze-dried filtrate of all the selected strains. In addition the *A. ibericus* MUM 03.49 and the *N. crassa* MUM 92.08 filtrate showed the presence of Na and *P. chrysogenum* MUM 92.11 filtrate shown the presence of Mg.

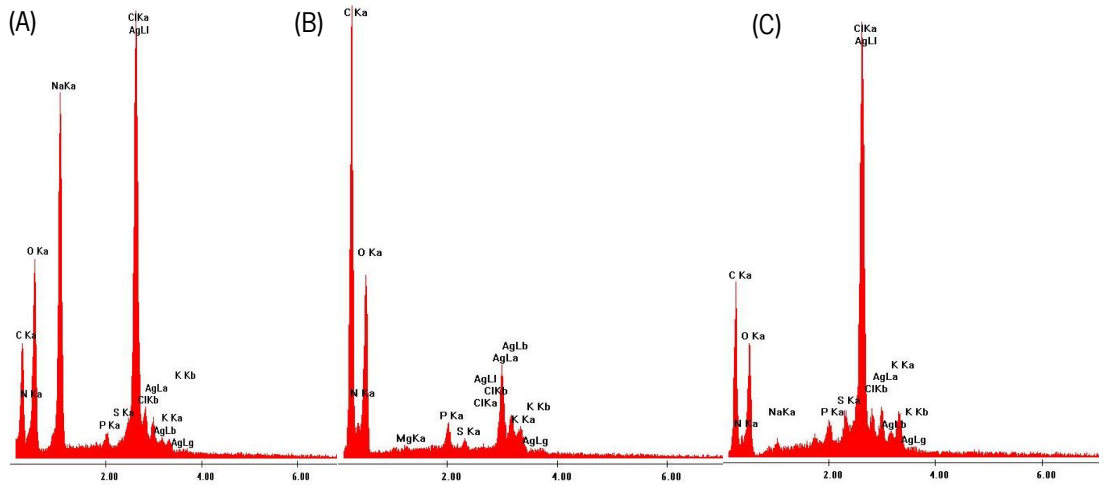


Figure 18 – EDS spectrum of the fungi filtrate after 96 h of incubation: (A) *A. ibericus* MUM 03.49, (B) *P. chrysogenum* MUM 92.11 and (C) *N. crassa* MUM 92.08.

4. Discussion and conclusion

The potential to manipulate fungus to synthetize nanoparticles involves several key parameters (temperature, agitation, light, control growth and other cellular activities) to achieve an optimized production of nanoparticles. The aim of this study consisted in selecting the best producer of stable biosynthesised AgNPs through the analysis of physicochemical features nanoparticles of selected fungi species.

Therefore, the reduction of Ag^{+} ions was monitored by the change of colour of the filtrate from yellow to brown after 96 h of incubation, and the presence of an absorbance peak on UV-Vis spectroscopy analysis at wavelengths ranging from 380 to 420 nm indicated the production of spherical AgNPs. According to previous published data, the changing in the reaction solution colour is due to the excitation of surface plasmon vibrations in the AgNPs (Mulvaney 1996; Elechiguerra et al. 2005). Furthermore, the absorbance band between 380 and 420 nm indicates the formation of spherical or roughly spherical AgNPs (Hasell et al. 2007; Pal et al. 2007). In our study, the fungal strains *A. ibericus* MUM 03.49, *P. chrysogenum* MUM 92.11 and *N. crassa* MUM 92.08 showed the presence of surface plasmon vibrations between 380 and 420 nm suggesting that these strains are the best candidates to produce spherical AgNPs.

Differences of size in AgNPs when comparing the DLS results with the SEM were found. These differences were above two fold which leads to conclude that AgNPs size is overestimated by DLS. In fact, DLS technique measures the hydrodynamic diameter of the particle. It gives the information of the inorganic core along with any coating material and the solvation layer attached to the particle. It means that nanomaterial sizing determined by techniques that use wet dispersion are commonly overestimated (De Palma et al. 2007; Dhawan and Sharmam 2010). In contrast, SEM analysis uses dehydrated samples, where only the inorganic core and the coating material are take into account. In addition, due to poor contrast the measurement of the coating layer can sometimes be underestimated (Gaumet et al. 2008). In addition, the surface plasmon resonance plasmon at 420 nm is characteristic of AgNPs between 20 and 50 nm (Durán et al. 2005).

Zeta-potential analysis showed that the surface charge of the formed nanoparticles was highly negative. This result suggested that the nanoparticle surface has an anionic charge, as

stated by Kumar and Mamidyala (2011). It has long been recognized that the zeta potential is a very good index of the magnitude of the interaction between colloidal particles and its measurements are commonly used to assess the stability of colloidal systems (Malvern 2009). In our study, all selected strains had negative surface charge which has been referred to play a role as a cellular signature to study the surface interaction of AgNPs (Kumar and Mamidyala 2011).

The SEM micrographs show the high density of nanostructures, confirming the presence of AgNPs in the selected fungi. The EDS analysis allows confirms the reduction of Ag^{+} ion to elemental silver (Ag^{0}). The absorption peak on SEM-EDS was observed at approximately 3 keV, which is characteristic of silver nanocrystals (Kumar and Mamidyala 2011; Jain et al. 2011). The presence of other peaks besides silver products as C, N, O, S, Cl and K in the EDS spectra indicated the heterogeneity of the reaction solution. The peaks found for C, N, O and S indicated the presence of fungal proteins as a capping material on the surface of AgNPs (Jain et al. 2011; Durán et al. 2010). Besides that, thick cap surrounding the nanoparticles could also be observed. This is probably due to the biomolecules released from the fungus acting as stabilizing agents of the nanoparticles (Durán et al. 2005; Gade et al. 2008). In our study, the strain *P. chrysogenum* MUM 92.11 produced roughly spherical AgNPs with a range size of 40.9 to 52.5 nm but most of them were aggregated. The range of sizes of AgNPs produced by *N. crassa* MUM 92.08 was 43.7 to 72.9 nm and the nanoparticles were found less aggregated.

From the previous results, the best fungal strains for the production of AgNPs were *P. chrysogenum* MUM 92.11 and *N. crassa* MUM 92.08. Both strains were used in furthers studies of mutagenesis and modulation of biochemical mechanisms for the production of AgNPs.

Chapter II:

Mechanism approach of biosynthesis of AgNPs.

1. Introduction

1.1. Nitrogen requirement in filamentous fungi

Nitrogen is a major element found in many of the simple compounds and in nearly all of the complex macromolecules of living cells. It is essential to all organisms, to synthesize amino acids and, consequently, proteins. Fungi can use inorganic nitrogen in the form of nitrates, nitrites, ammonia or organic nitrogen to produce amino acids. It is known that fungi are different in their ability to use nitrogen compounds for growth, and also, that they may have a requirement for nitrogen in a specific form (Msore-Landecker 1990).

The principal process of conversion from inorganic nitrogen into organic compounds is done by nitrate assimilation. This conversion occurs in a diversity of microorganism such as: bacteria (Stouthamer 1967; Piéchaud et al. 1967), yeast, fungi (Lewis & Fincham 1970; Cove 1979), algae (Nichols & Syrett 1978; Huskey et al. 1979) and plants (Birkett & Rowlands 1981; Martins et al. 2009).

Extensive studies of nitrogen metabolism and its control have been carried out in several fungi, such as: *Neurospora crassa*, *Aspergillus nidulans*, and *Saccharomyces cerevisiae*. Although certain compounds, like ammonia, glutamate and glutamine, are preferred over others as nitrogen sources, these fungi are able to use many diverse secondary sources, including nitrate and nitrite, purines, proteins, amino acids, acetamide and acrylamide. These secondary nitrogen sources require the synthesis of catabolic enzymes or an activation of previously remaining enzymes (Msore-Landecker 1990).

Synthesis of nitrogen-regulated enzymes involves two conditions: an excitation of nitrogen catabolic repression in required and, secondly, a specific induction by subtract or in the presence of an intermediate pathway. Several works have been developed to study the metabolic pathway of nitrate assimilation of filamentous fungi. Until now, the *Ascomycetes* (Strauss et al. 1998) and the *Aspergillus* (Unkles et al. 1989; Daboussi et al. 1989; Ventura & Ramón 1991) have been the most studied fungi for that purposes.

The regulation of nitrogen metabolism on fungi is derived from genetic analysis of structural genes mutant (Msore-Landecker 1990). However, the numerous fungi that are unable to utilize nitrates require a more reduced form of nitrogen, probably because they are unable to reduce the nitrate ion (Msore-Landecker 1990).

1.2. Nitrate assimilation mechanism and NR regulation

The nitrate ion must be reduced to the oxidation level of ammonia (NH_4^+), before the nitrogen can be assimilated into organic compounds (Figure 19).

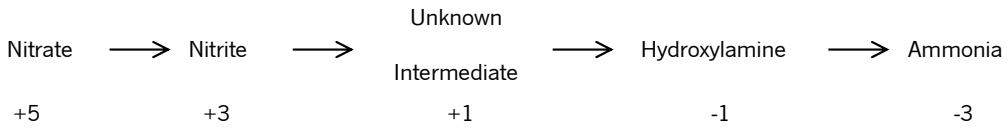


Figure 19 - Stepwise reduction of nitrate ion into ammonia and respectively oxidation states (adapted from Msore-Landecker 1990).

The nitrate is carried intracellularly and mediated by the nitrate permease, and after its assimilation, occurs a sequential reduction of nitrate to nitrite and nitrite to ammonium, catalyzed through the nitrate to nitrite enzymes. In fungi, the nitrogen anabolic metabolism starts on the ammonium. Its incorporation in organic molecules can be performed by two systems: glutamate dehydrogenase (GDH) or glutamine synthase (GS) (Pereira et al. 2003).

The catalytic steps and genes involved in many fungi are shown in figure 20.

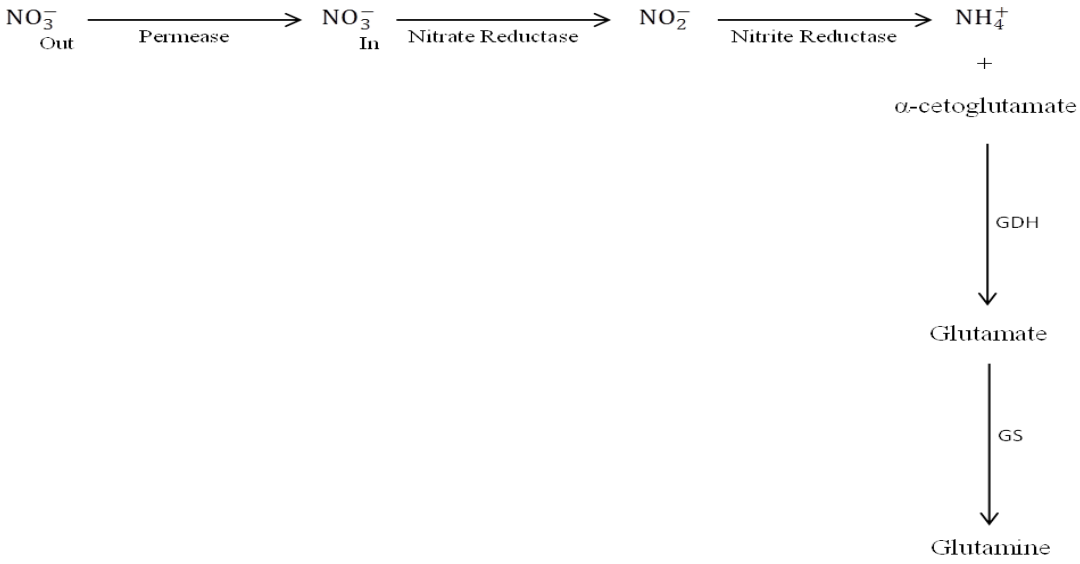


Figure 20 – Nitrate assimilation on filamentous fungi (adapted from Msore-Landecker 1990)

For a better comprehension of the nitrate assimilation in filamentous fungi it is essential to understand the nitrogen metabolism, as well as the mechanisms of the control of metabolic regulation (Figure 21) (Msore-Landecker 1990). NR catalyzes the pyridine nucleotide-dependent reduction of inorganic nitrate to nitrite. The NR enzyme is a homodimer composed with two

identical 100 kDa subunits, containing one equivalent of flavin adenine dinucleotide (FAD), heme-Fe, and Mo-molybdopterin (Mo-MPT) (Campbell 1999; Capela 2013; Haas et al. 1996). Two types of NR are known. One is an "assimilatory" enzyme that is a soluble protein complex found in many aerobic microorganisms and higher plants where it functions; and, together with nitrite reductase form NH_3 for the biosynthesis of nitrogenous compounds. The other, a "dissimilatory" (or "respiratory") enzyme, that is a membrane bound protein complex found in microorganisms that use nitrate as a terminal electron acceptor under anaerobic conditions (Cove 1979).

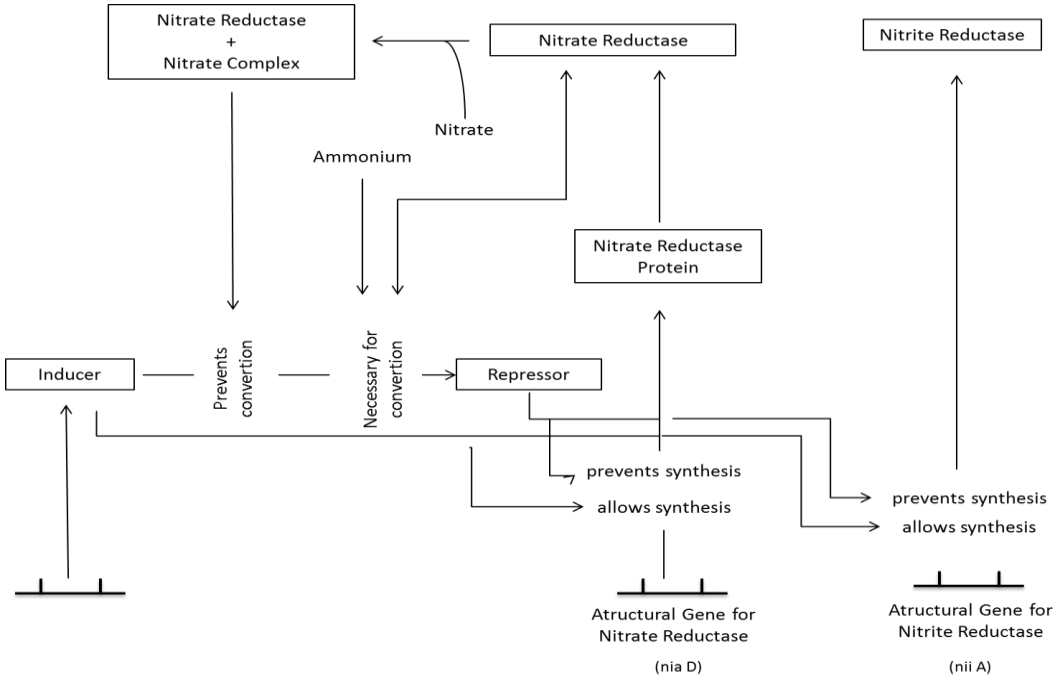


Figure 21 - Illustrative model of a possible mechanism of nitrate and nitrite production controls in *Aspergillus nidulans* (adapted from Moore-Landecker, 1990).

NR, from algae and higher plants, prefers NADH as reducer, while the fungal enzyme prefers NADPH. Besides that, there is another major difference between the fungal and green plant enzymes. Plant enzyme is maximally activated in the absence of added flavin coenzyme. Purified fungal enzyme, however, has an absolute requirement for added flavin coenzyme and it is known that FAD is far more active than FMN (Cove 1979).

The genetic mechanism involved in the nitrate assimilation in *Aspergillus nidulans* has been the most extensively studied among filamentous fungi. This fungus utilizes enzymatic equipment for the reduction of nitrate, which is induced by the presence of nitrate and nitrite, and repressed by the presence of ammonia (Cove 1976).

1.3. Mutagenesis

Spontaneous and induced mutations are a source of genetic variation that is used on the industrial microbiology. Here, important microorganisms, e.g. *Penicillium chrysogenum* and *Aspergillus oryzae*, with sexual reproduction are used. This is the reason why the meiotic variation is replaced by mutagenesis programs with the chance of increasing the productivity of microorganisms (Lima 1998). Induced spontaneous mutations are a genetic variability source with big impact on the industrial microbiology. The initial strains selection depends on the spontaneous variability founded inside of the natural population of microorganisms (Lima 1993).

Physical and chemical mutagenesis allows obtaining two types of mutations: bigger and smaller mutations. Bigger mutations are related to mutant selection with a genetic character by a marked alteration with practical interest. For example, the unpigmented *Penicillium chrysogenum* has the ability to produce penicillin and create genetic marks on strains, like, auxotroph - resistance to antibiotics and heavy metals. On the other hand, smaller mutations affect the quantity of the product synthetized, distinguishing phenotypically the mutant from the parental strains. They show subtle alterations on a particular genetic character leading to the improvement of 5 to 10 % on the desire product formation (Elander et al. 1987).

Mutant strains deficient in nitrate assimilation have been isolated on the bases of chlorate toxicity through spontaneous and induced mutagenesis (Kanan & Al-Najjar 2010). Chlorate probably resulted from its activity as a non-metabolizable analogue of nitrate. The resistance of chlorate by positive selection is due to the resemblance between the nitrate and the chlorate, which favors the mutant growth relatively to the wildtype. Besides that, the chlorate enters on the cell with a permease of the nitrate and is reduced to chlorite by the action of the NR. It is known that the chlorite stimulates the oxidative stress of the fungi cells, and thereby, becoming toxic and, consequently, provoke the cell death (Ingram et al. 2003). Therefore, the cell that has the functional enzyme NR dies and the ones who don't have the non-functional forms live, because it is not capable of reducing the chlorate into chlorite (Pereira et al. 2003).

Mutations in at least ten genes of *A. nidulans* can produce a chlorate-resistant phenotype where seven of those genes are involved in the production of an active NR (Cove 1976). One of that genes is the structural gene for NR (*nia*D), other (*nir*A) is believed to encode the regulatory element essential for the synthesis of nitrate and nitrite reductase, and the

remaining five genes (*cnx*A, B, C, E, F, G, H) are involved in the synthesis of a cofactor shared with the xanthine dehydrogenase (Pateman et al. 1964; Patemand and Cove 1967; Cove 1970; Scazzocchio 1974; Cove 1976).

The *nia*D gene is the structural gene for NR and its mutation, in at least 16 genes, can prevent or reduce the use of nitrate as nitrogen source. The mutations in only one of the *nia*D genes can prevent the NR activity. The *nia*D mutants were the first to be isolated because they have the inability to use nitrate as a nitrogen source, while they are able to use nitrite and ammonium (Cove 1979). At least 28 different *nia*D mutants isolated in this manner have been examined for nitrate-reductase activity, and it was found that they have enzyme levels appreciably lower than those in wildtype strains (Cove 1979).

Defective mutants in the structural gene *nia*D, for the NR apoprotein but not for the nitrite reductase (*nii*A), can be obtained on the basis of their resistance to chlorate (Cove 1976). In addition, the mutations resulting of chlorate resistance can occur in a series of genes, such as the transport gene *crn*A and genes *cnx*A required for the biosynthesis of a molybdenum co-factor which is necessary for NR as well as xanthine dehydrogenase activity. Chlorate resistant mutants can also be obtained as a result of mutation within *nir*A and *are*A, positive-acting control genes for the system. Fortuitously, *nia*D mutants can be differentiated from *crn*A, *cnx*A-J, *nir*A, and *are*A mutants on the basis of simple growth tests (Unkles et al. 1989).

It is known that at least five mutations can make a chlorate resistant phenotype:

a) Gene mutation of the nitrate permease (*crn*A), which incapacitates the cell of absorbing the extracellular chlorate.
b) Mutation on the specific regulator gene of the nitrate assimilation (*nir*A), that impossibilities the cell to express the NR enzyme.
c) Gene mutation of the general regulator of the nitrogen assimilation (*are*A), which makes possible the expression of the NR enzyme.
d) Mutation on a gene that is involved on the biosynthesis of the molybdenum cofactor (*cnx*A-J) that, if it is not present, makes the NR enzyme inefficient and others enzymes dependents of that cofactor.
e) Mutation of the nitrate NR itself (*nia*D), preventing the synthesis of that enzyme. (Cove 1976; Cove 1979).

In this way, the chlorate resistant colonies are needed to make a mutant with a phenotypic breakdown through the growth medium test with different nitrogen sources: nitrate, nitrite, hypoxanthine, glutamate and ammonia. Positive growth in different sources of nitrogen is related with different mutations described on the table 6.

Table 6 – Mutants growth tests resistant's to chlorate with different sources of nitrogen (Adaptation of Unkles et al. 1989).

Mutation	Name	Growth Tests					
		Chlorate	Nitrate	Nitrite	Ammonia	Hypoxanthine	Glutamate
-	WT	-	+	+	+	+	+
NR	*niaD*⁻	+	-	+	+	+	+
Specific Regulator	*nirA*⁻	+	-	-	+	+	+
Molybdenum cofactor	*cnxA-J*⁻	+	-	-	+	-	+
General regulator	*areA*	+	-	-	+	-	-
Permease	*crnA*⁻	+	+	+	+	+	+

(+) positive for growth; (-) negative for growth

The hypoxanthine growth test requires the NR enzymes that are dependent of the molybdenum cofactor and NAD(P)H. The cofactor absence makes the use of adenine, hypoxanthine and xanthine as the only sources of nitrogen, impossible to the cell. Mutants that are unable to grow on nitrate and hypoxanthine show a pleiotropic loss of NR and xanthine dehydrogenase enzymes activity, respectively. These mutants are defective on a common cofactor synthesis, which involved genes on the synthesis of this cofactor and are called as common component for NR to xanthine dehydrogenases, *cnx* (Pateman et al. 1964; Cove 1979).

In filamentous fungi, the nitrate assimilation pathway offers many advantages mainly for the development of transformation systems. The mutants can be easily obtained by positive selection via resistance to chlorate; it can be done by almost spontaneous means, reducing the possibility of secondary mutations in genes of commercial interest or in genes encoding essential catalytic types. The desired mutants have a simple growth phenotype, i.e., inability to use nitrate as the sole nitrogen source. This pathway is dispensable and, therefore, mutations in the nitrate pathway should not alter the growth or the metabolic fluxes through the important pathways. And, most wildtype will use nitrate as the sole nitrogen source (Unkles et al. 1989).

2. Materials and methods

2.1. Mechanism approach of biosynthesis of AgNPs

2.1.1. Mutagenesis

The fungal strain *Penicillium chrysogenum* MUM 92.11 was used for the mutagenesis study. Table 7 shows the different media used for the mutagenesis procedure and for the selection of mutants.

Mutants with the responsible defective gene of nitrate assimilation (*nia*D-) were obtained by positive selection with resistance to chlorate. For this purpose, a concentration of about 10^7 spores/mL of the fungal strain was inoculated by plate spreading in agar-Glu/ClO_3^- and incubated at 30 °C for 5 days.

The wildtype (WT) strain of *Penicillium chrysogenum* MUM 92.11 will be referred in the text as WT.

2.1.1.1. Selection of *nia*D- spontaneous mutants of *Penicillium chrysogenum* MUM 92.11.

The fungal strain was inoculated on Czapeck-Dock minimal medium (agar-CD or agar-NO_3^-) supplemented when required with a different source of nitrogen (nitrite, ammonium, glutamate and hypoxanthine) at 30 °C for 5 to 7 days.

Table 7 - Czapeck-Dock minimal medium (agar-CD or agar-NO_3^-) composition supplemented with different source of nitrogen (nitrite, ammonium, glutamate and hypoxanthine) as required.

Culture medium	Composition
agar-CD = agar-NO_3^-	3 % Saccharose (Merck, Germany); 0.3 % $NaNO_3$ (Merck, Germany); 0.1 % K_2HPO_4 (Merck, Germany); 0.05 % $MgSO_4.7H_2O$ (Merck, Germany); 0.05 % KCl (VWR, Portugal); 0.001 % $FeSO_4.7H_2O$ (Merck, Germany); 1.5 % Agar (Oxoid, UK)
agar-NO_2^-	Same composition of agar-CD but replace 0.3 % $NaNO_3$ for 0.3 % $NaNO_2$ (Merck, Germany)
agar-NH_4^+	Same composition of agar-CD but replace 0.3 $NaNO_3$ for 0.3 % Ammonia Sulphate (Merck, Germany)
agar-Glu	Same composition of agar-CD but replace 0.3 $NaNO_3$ for 0.3 % Glutamate.H_2O (Merck, Germany)
agar-Hyp	Same composition of agar-CD but replace 0.3 % $NaNO_3$ for 0.3 % Hypoxanthine (Merck, Germany)
agar- Glu/ClO_3^-	Same composition of agar-CD but replace 0.3 % $NaNO_3$ for 0.3 % Glutamate; add $NaClO_3$ at the final concentration of 470 mM (Merck, Germany)

Chlorate-resistant mutants were selected from agar-Glu/ClO_3^- plate. Using the grid in figure 22 and by multiple prinking out, each colony of chlorate-resistant mutant was inoculated in supplemented medium agar-CD, agar-NO_2^-, agar-NH_4^+, agar-Glu and agar-Hyp and incubated at 30 °C for 5 to 7 days. Positive or negative growth of each mutant on each supplemented medium allowed its characterisation.

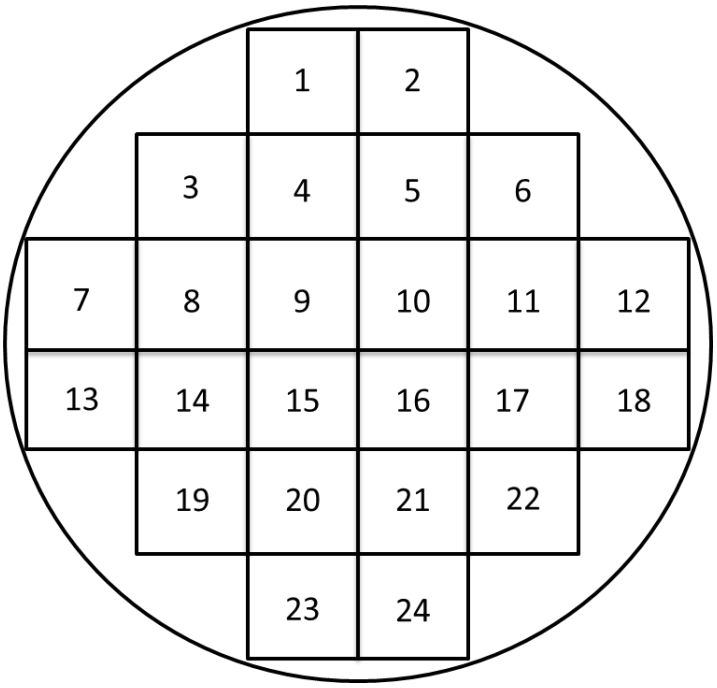


Figure 22 - Inoculation grid

In order to prevent reverse mutation, each colony grown on agar-NH_4^+ plate was re-inoculated in the former order on inoculation grid and confirmed the previous positive or negative growth on different nitrogen supplemented media.

Each colony of mutant grown on agar-NH_4^+ plate was individually stored into aliquots in the spore form. For this purpose each colony was gently removed with a micro-spreader immerse in TWS solution and transferred to microtubes filled with 400 µL of 50 % of glycerol (Merck, Germany) and stored at -21 °C.

2.1.2. Biosynthesis of AgNPs produced by *Penicillium chrysogenum* MUM 92.11 WT and mutants

Biosynthesis of AgNPs was undergone following the same procedure as point 2.3 (chapter I) for *Penicillium chrysogenum* strains. Fresh filtrate was used for UV-Visible spectroscopy, DLS and Zeta-potential analysis. Approximately a volume of 25 mL of filtrate was

stored in microtubes at -20 °C for further determination of total protein concentration, determination of nitrite concentration and SDS-PAGE. Remaining volume of filtrate was freeze dried for EDS, SEM and STEM analysis.

2.1.2.1. Physicochemical characterisation of the Bio-AgNPs produced by *Penicillium chrysogenum* MUM 92.11 WT and mutants

The analysis of UV-Visible spectroscopy, DLS, Zeta-potential, EDS and SEM were carried out according to described on the point 2.3 chapter I.

In addition the size and morphology analysis of the nanoparticles with an Ultra-high resolution Field Emission Gun Scanning Electron Microscopy (FEG-SEM), NOVA 200 Nano SEM, FEI Company. Scanning Transmission Electron Microscope (STEM) images were performed with an acceleration voltage of 15 kV. The samples were covered with a very thin film of Au-Pd (80-20 weight %); in a high resolution sputter coater, 208HR Cressington Company, coupled to a MTM-20 Cressington High Resolution Thickness Controller.

2.1.3. Enzymatic assay

2.1.3.1. Determination of total protein concentration

The total protein concentration (mg/mL) was determinate by Bradford method. For that purpose an aliquot of 1 mL of WT and mutant filtrate was used. One mililiter of Bradford reagent (Sigma-Aldrich, Germany) was added to 100 µL of the filtrate for 2 to 60 min. Negative control was prepared with 0.02 M phosphate buffer (Sigma-Aldrich, Germany) at pH=8. The solutions were measured at an absorbance of 595 nm. The standard curve was calculated with seven standard BSA solutions (Pierce, USA) at following concentrations: 0.00, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg BSA/mL.

2.1.3.2. Electrophoresis with SDS-Polyacrylamide Gel (SDS-PAGE)

The WT and mutant filtrate were previously concentrated with centrifugal filter devices (Centricon; Millipore, USA) with a molecular weight cut-off of 30 000 MWCO for a suitable amount of protein. A volume of 10 µL was added to 10 µL 2X Buffer-A (140 mM Tris (Sigma-Aldrich, Germany) at pH=6.8, 30 % glycerol, (Merck, Germany), 4 % SDS (Sigma-Aldrich, Germany), 20 % β-mercaptoethanol (Sigma-Aldrich, Germany), and 0.05 % bromophenol blue (Merck, Germany)). The solution was heated for 5 min at 100 °C for protein denaturation. After

that separating gel was prepared following composition on table 8. Degassing of the separating solution was performed under vacuum at least for 15 min before the addition of APS (Sigma-Aldrich) and TEMED (Sigma-Aldrich).

Table 8 – Composition of the separating gel.

Reagents	Volumes
29.25 % Acrylamide/0.75 % Bis-acrylamide (Sigma-Aldrich, Germany)	5.21 mL
1M Tris/HCl pH 8.8	4.7 mL
ddH_2O	2.46 mL
10 % SDS	250 µL
10 % APS	200 µL
TEMED	15 µL

Separating gel was poured on 12 x 12 x 0.1 cm glass plate sandwiches device Mini-PROTEAN® II Electrophoresis cell previously cleaned with absolute EtOH. The solution was immediately overlayed with ddH_2O. The gel was left to polymerize for 30 to 45 min. Afterwards the ddH_2O was removed. Before adding the stacking gel the glass area was dried with filter paper. The staking gel was prepared following composition on table 9, including the degassing step, and was added over the separating gel.

Table 9 – Composition of staking gel.

Reagents	Volumes
29.25 % Acrylamide/0.75 Bis-acrylamide	1.65 mL
1M Tri/HCl (Panreac, Germany) pH 6.8	1.25 mL
ddH_2O	2.46 mL
10 % SDS	100 µL
10 % APS	100 µL
TEMED	10 µL

The comb was immediately placed into the gel sandwich. The gel was allowed to polymerize for 30 to 45 min. The comb was removed by pulling it straight up slowly and gently and the gel was attached to the inner cooling core of the device with Electrode Running Buffer (50 mM Tris/HCl pH 8.3, 192 mM Glycine (Merck, Germany), and 0.1 % SDS). The sample was then loaded and the gel was left to run at 100 to 150 V until the blue mark achieved the bottom gel.

After running the gel was subjected to silver staining. The gel was fixed for ten min with 20 % of EtOH and washed with ddH_2O for the same period of time. Then, the gel was treated with a volume of 200 mL of sensibilization solution (0.2 mg/mL Sodium Thiosulfate (Sigma-Aldrich, Germany) in ddH_2O) between 2 to 5 min and washed again twice with ddH_2O for 20 seconds

each time. A volume of 200 mL of staining solution (2 mg/mL Silver Nitrate in ddH_2O) was used afterwards for the period of 30 min and, then the gel was washed 5 times with ddH_2O for 10 sec. Develop gel with the development solution (15.7 µg/mL Sodium Thiosulfate (Sigma-Aldrich, Germany); 30 mg/mL Potassium Carbonate (Sigma-Aldrich, Germany); 140 µL 37 % Formaldehyde (Sigma- Aldrich, Germany)) in 200 mL ddH_2O was added to the gel until the protein markers started to appeared in the solution. After that the development solution was discarded and replaced with 200 mL of stopping solution (50 mg/mL Tris; 5 mL 25 % acetic acid (Parneac, Germany)) for 1 min.

2.1.3.3. Determination of nitrites concentration

Concentration of nitrites (10^3 mg/mL) was determined on the negative control (filtrate without silver nitrate), time (t) = 0 h and t= 96 h of incubation using WT and mutant filtrates. The Colorimetric Method nº 4500-NO_2B (Clesceri et al. 1999) was followed. The standard curve was determinate from six standard solutions of sodium nitrite ($NaNO_2$): 0.00, 0.05, 0.10, 0.15, 0.20 and 0.25 • 10^3 mg/mL. A volume of 1 mL of WT and mutant filtrate was previously filtrated by a syringe cellulose membrane filter (Whatman, USA) with a pore size of 0.2 µm. A volume of 20 µL of 1 % (v/v) sulfanilamide solution (Sigma-Aldrich, Germany) was added to the microtube and the solution was vortexed. After 2 min of reaction, 20 µL of 0.02 % (v/v) N (l-naftil) ethylenediamine hydrochloride - NED (Sigma-Aldrich, Germany) solution was added, vortexed, and left to react for 10 min. The blank control was prepared as described above but with Milli-Q water in replacement of the filtrate. Nitrite (NO_2^-) is determined through formation of a reddish purple dye by coupling diazotized sulfanilamide with NED. The final colour is positively correlated to the nitrite concentration. All the samples, controls and blanks were transferred to a 96-well microplate and the absorbance was read at 543 nm on a Multi-Mode Microplate Reader (Biotek Synergy HT, US). Three replicates were carried out for each assay.

Data analysis was performed using the statistical program IBM ® SPSS (Statistical Package for the Social Sciences). The wildtype and mutant results were compared using the parametric student t-test, at a 95 % confidence level.

2.1.3.4. Determination of NR specific enzymatic activity

The determination of NR specific enzymatic activity (U/mg total protein) was calculated according to Cove (1966) and a volume of 1 mL of filtrate WT and mutant was used. The NR ability to produce nitrites was tested through a colourimetric method. To obtain a final sample volume of 3 mL, accounting with 1 mL of filtrate, a solution of 22.5 mg $NaNO_3$, 0.5 mg NADPH, 3.5 µg FAD in 0.05 M buffer phosphate at pH=7.75 was prepared. After 20 to 30 min of incubation at 30 °C in the dark, 0.5 mL of 1 % sulfanilamide solution 3:1 (water : concentrated HCl) and 0.5 mL of 0.02 % NED solution. The resultant colour is positively correlated with NO_2^- concentration. All the samples, controls and blanks were transferred to a 96-well microplate and the absorbance was read at 540 nm on a Multi-Mode Microplate Reader. To correct the presence of turbidity due the presence of nitrites on the sample a negative control with all the reagents except NADPH was prepared. Three replicates were carried out for each assay.

2.2. *In-vitro* assay

In-vitro assays were performed according to the table 10 representing the experimental conditions assigned for each assay using a final volume of 1 mL.

Table 10 – UV-Vis. spectra ranging 250 to 800 nm were carried out for each condition of the different assays after 96 h of incubation. In assay I (A-E) different reaction solutions were prepared in microtubes adding the following compounds: β-NADPH, FAD, NR and anthraquinone in phosphate buffer pH = 8. In assay II the same procedure was performed with the WT (A, D and E) and mutant (B and C) filtrate with $AgNO_3$. In assay II E the WT filtrate the proteins were denatured ate 100 °C for 5 min. In assay III and IV same conditions of assay I were used except the addition of the sodium azide as NADPH inhibitor (A-D) and egg-albumin protein (A-D) respectively in the microtubes.

		β-NADPH (0.587 mM; Sigma-Aldrich)	**FAD** (1.39 mM; Sigma-Aldrich)	**$AgNO_3$** (10 mM)	**Phosphate Buffer** (0.5 mM; Merck)	**NR** (500 U/L; Sigma-Aldrich)	**Anthraquinone** (0.5 mM; Sigma-Aldrich)	**Filtrate WT**	**Filtrate MT**	**NADPH inhibitor** (0.5 mM; Merck)	**Egg Albumine Protein** (500 mg/L; Sigma-Aldrich)
I	**A**	-	-	100 µL	900 µL	-	-	-	-	-	-
	B	100 µL	1 µL	100 µL	799 µL	-	-	-	-	-	-
	C	100 µL	1 µL	100 µL	719 µL	80 µL	-	-	-	-	-
	D	100 µL	1 µL	100 µL	619 µL	80 µL	100 µL	-	-	-	-
	E	-	-	100 µL	720 µL	80 µL	-	-	-	-	-
II	**A**	-	-	100 µL	-	-	-	900 µL	-	-	-
	B	-	-	100 µL	-	-	-	-	900 µL	-	-
	C	-	-	100 µL	-	80 µL	-	-	720 µL	-	-
	D	-	-	100 µL	-	-	-	800 µL	-	100 µL	-
	E	-	-	100 µL	-	-	-	900 µL	-	-	-
III	**A**	-	-	100 µL	800 µL	-	-	-	-	100 µL	-
	B	100 µL	1 µL	100 µL	699 µL	-	-	-	-	100 µL	-
	C	100 µL	1 µL	100 µL	619 µL	80 µL	-	-	-	100 µL	-
	D	100 µL	1 µL	100 µL	519 µL	80 µL	100 µL	-	-	100 µL	-
IV	**A**	-	-	100 µL	800 µL	-	-	-	-	-	100 µL
	B	100 µL	1 µL	100 µL	699 µL	-	-	-	-	-	100 µL
	C	100 µL	1 µL	100 µL	619 µL	80 µL	-	-	-	-	100 µL
	D	100 µL	1 µL	100 µL	519 µL	80 µL	100 µL	-	-	-	100 µL

3. Results

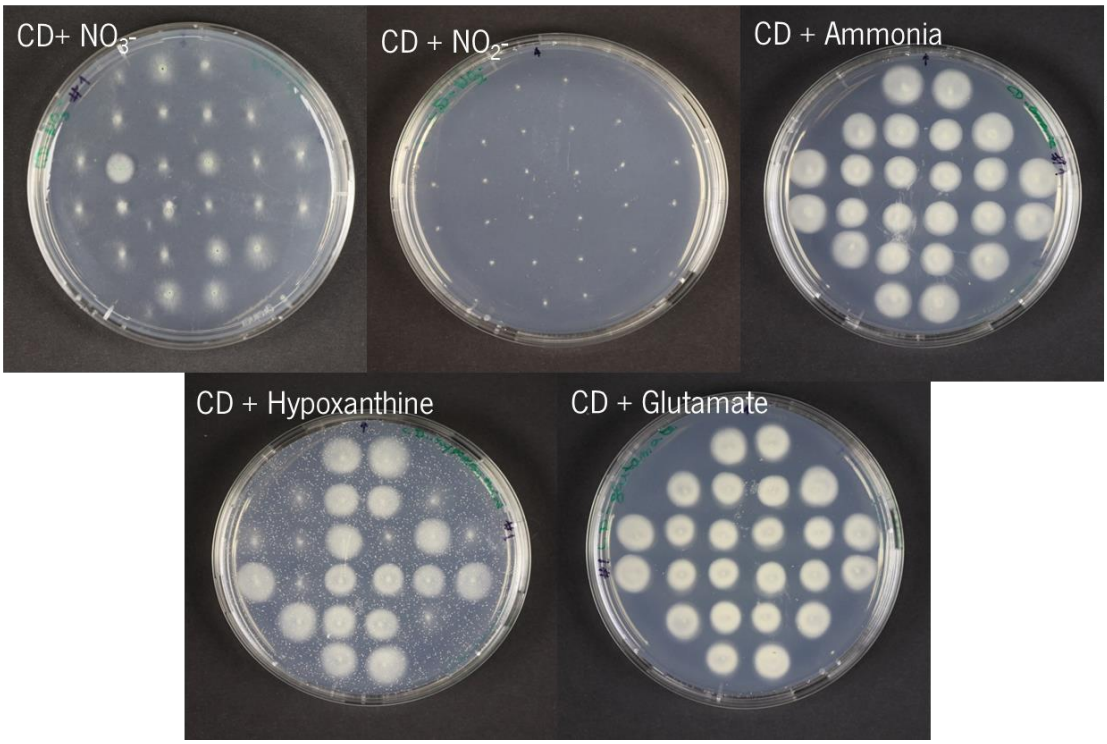
This chapter is focused on modulation of the biochemical mechanism of silver reduction for biosynthesis of silver nanoparticles by the enzyme NR. It has the purpose to define the role of NR on the reduction mechanism of biosynthesis of silver nanoparticles and confirmed by the use of defective mutants on the NR structural gene *nia*D^{-}.

3.1. Mutagenesis and isolation of *nia*D^{-} mutants

Mutagenesis base on positive selection of chlorate-resistant mutants to 470 mM chlorate on minimal medium with 10 mM glutamate (as the sole source of nitrogen) was carried out for *Penicillium chrysogenum* MUM 92.11 and *Neurospora crassa* MUM 92.08. Although several attempts were performed for the strain *N. crassa* MUM 92.08 the mutagenesis was not successful and none of the mutants have grown. For the strain *P. chrysogenum* MUM 92.11, up to thirty-four spontaneous mutants have grown on chlorate medium.

Afterwards, mutants were characterized based on their inability to grow on different nitrogen sources (CD-NO_3^-; CD-NO_2^-; CD-NH_4^+; CD-Hyp and CD-Glu). Figure 23 represents macroscopic characterisation of each of 34 mutants grown in different nitrogen sources Czapec-Dox medium. Characterisation based on the phenotypic properties of chlorate-resistant mutants of *P. chrysogenum* MUM 92.11 (Table 11) followed the classification of mutants proposed by Unkles et al. (1989) using *Aspergillus nidullans* mutants defective in nitrate assimilation. The ability to grow was determined with respect to the wildtype strain and other mutants, since for an individual strain growth rates on different sources vary significantly.

First set



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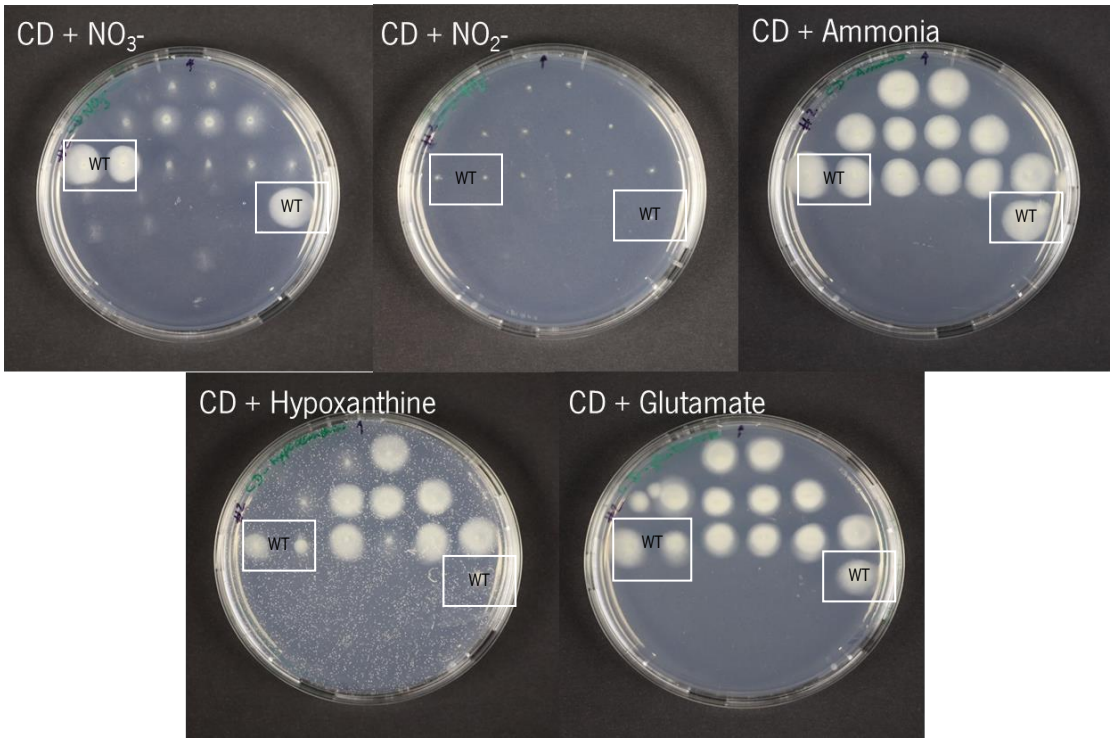

Figure 23 – Macroscopic characterisation based on the assimilation of *P. chrysogenum* MUM 92.11 wildtype (WT) and mutants on different nitrogen source plates with 3 days of growth.

Table 11 – Phenotypic properties of chlorate-resistant mutants of *P. chrysogenum* MUM 92.11.

Set	Mutant	Chlorate Resistant	Only source of nitrogen					Genetic Mutation
			NO_3^-	NO_2^-	NH_4^+	Hyp	Glu	
First	P1	R	+	-	+	+	+	*crn*A
	P2	R	-	-	+	+	+	*nia*D;*nir*A
	P3	R	-	-	+	-	+	*cnx*A-J
	P4	R	-	-	+	+	+	*nia*D;*nir*A
	P5	R	-	-	+	+	+	*nia*D;*nir*A
	P6	R	-	-	+	-	+	*cnx*A-J
	P7	R	-	-	+	-	+	*cnx*A-J
	P8	R	+	-	+	+	+	*crn*A
	P9	R	-	-	+	-	+	*cnx*A-J
	P10	R	+	-	+	+	+	*crn*A
	P11	R	-	-	+	-	+	*cnx*A-J
	P12	R	-	-	+	-	+	*cnx*A-J
	P13	R	-	-	+	+	+	*nia*D;*nir*A
	P14	R	+	-	+	+	+	*crn*A
	P15	R	+	-	+	+	+	*crn*A
	P16	R	-	-	+	+	+	*nia*D;*nir*A
	P17	R	-	-	+	-	+	*cnx*A-J
	P18	R	-	-	+	+	+	*nia*D;*nir*A
	P19	R	-	-	+	-	+	*cnx*A-J
	P20	R	-	-	+	+	+	*nia*D;*nir*A
	P21	R	+	-	+	+	+	*crn*A
	P22	R	+	-	+	+	+	*crn*A
	P23	R	+	-	+	+	+	*crn*A
	P24	R	+	-	+	+	+	*crn*A
Second	P25	R	-	-	+	+	+	*nia*D;*nir*A
	P26	R	-	-	+	-	+	*cnx*A-J
	P27	R	+	-	+	+	+	*crn*A
	P28	R	+	-	+	+	+	*crn*A
	P29	R	+	-	+	+	+	*crn*A
	P30	R	-	-	+	-	+	*cnx*A-J
	P31	R	-	-	+	+	+	*nia*D;*nir*A
	P32	R	-	-	+	+	+	*nia*D;*nir*A
	P33	R	-	-	+	-	+	*cnx*A-J
	P34	R	-	-	+	+	+	*nia*D;*nir*A

R – Resistant; (+) Positive growth; (-) Negative growth

Of the thirty-four chlorate-resistant mutants isolates it was observed that none of them could grow on CD minimal medium with nitrite as the sole nitrogen source. As a consequence, the phenotypic group of defective mutants of both nitrate and nitrite reductase gene was designated as *nia*D$^-$/*nir*A$^-$ (Alekesenco et al. 1995). Moreover, eleven had a phenotype indicative of *nia*D$^-$/*nir*A$^-$, twelve mutants had a phenotype *crn*A$^-$ and eleven mutants had a phenotype *cnx*A-J$^-$. From the *P. chrysogenum* with *nia*D$^-$/*nir*A$^-$ phenotype, the P2 mutant was selected for the production of AgNPs.

The wildtype strain of *Penicillium chrysogenum* MUM 92.11 was tested relatively to the use of the metabolic pathway of nitrate. The obtained results are represented on the table 12.

Table 12 - *Penicillium Chrysogenum* MUM 92.11 metabolic pathway of nitrate assimilation.

Fungus	agar-NO_3^-	agar-NO_2^-	agar-NH_4^+
P. chrysogenum MUM 92.11	+	-	+

(+) Positive grow; (-) Negative grow

3.2. Mechanism approach of biosynthesis of AgNPs

To analyse if the mutant (P2) *nia*D$^-$/*nir*A$^-$ was able to produce AgNPs, the same procedure of biosynthesis as described on 2.3 point of the chapter I was adopted.

For this proposal, wildtype and mutant P2 of *Penicillium chrysogenum* MUM 92.11 were used. The production of silver nanoparticles was monitored through the change of color of the filtrate treated with 1 mM of $AgNO_3$. A brownish color of the filtrate was observed after 96 h of incubation for both wildtype and mutant *P. chrysogenum* MUM 92.11 (Figure 24). A negative control without $AgNO_3$ was prepared.

No visible differences between the wildtype and the mutant filtrate at 96 h of incubation were found. Wildtype and mutant filtrate developed a brownish reaction solution comparing to their negative control.

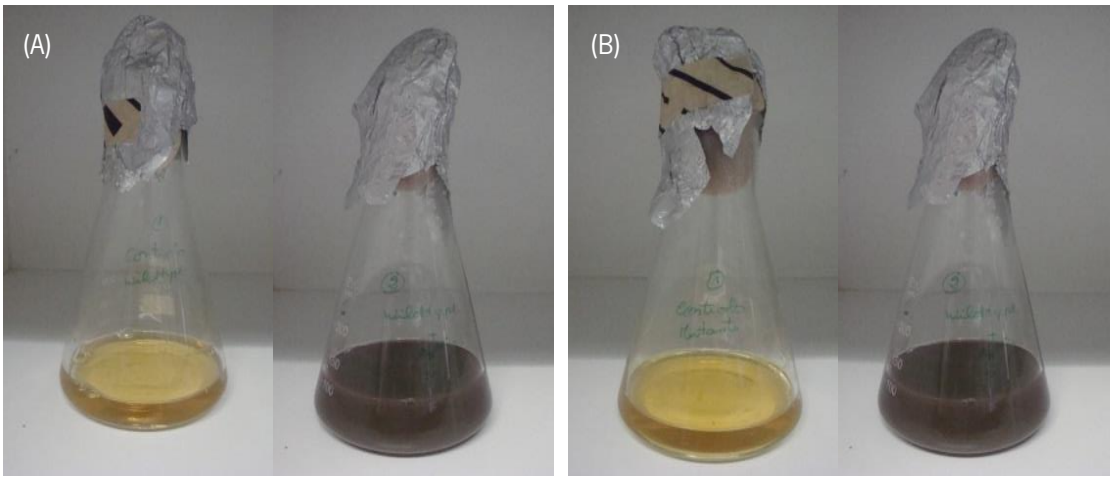

Figure 24 - Cellular filtrate of *Penicillium chrysogenum* wildtype (A) and mutant (B), treated with 1 mM of $AgNO_3$ after 96 h of incubation in the dark. Corresponding negative controls are shown on the left of each image.

The production of AgNPs was monitored with the UV-Vis spectroscopy based on the excitation of surface plasmon vibrations of Ag^0 between 380 - 420 nm, 96 h after the addiction of $AgNO_3$ on the filtrate (Figure 25).

The WT showed a lowest absorbance peak when comparing to mutant filtrate with $AgNO_3$. That result also is observed for the respective controls.

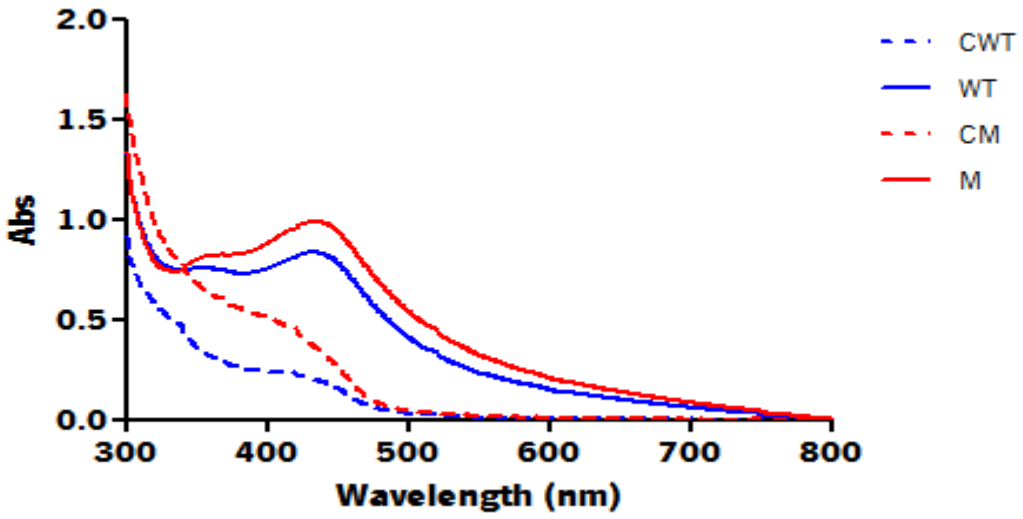

Figure 25 – UV-Visible spectrum of *P. chrysogenum* MUM 92.11 (WT) with its respective mutant (M) with 1 mM of $AgNO_3$ aqueous solution, after 96 h of incubation and their negative controls, CWT and CM, respectively.

The size distribution of AgNPs produced by WT and mutant filtrate of *P. chrysogenum* MUM 92.11 (Figure 26) was determined by DLS. Table 13 represents the average size (nm) and polydispersity of AgNPs produced by WT and mutant filtrate. In terms of these physicochemical

features same size distribution curves, average size and polydispersity values were obtained for AgNPs produced by both strains.

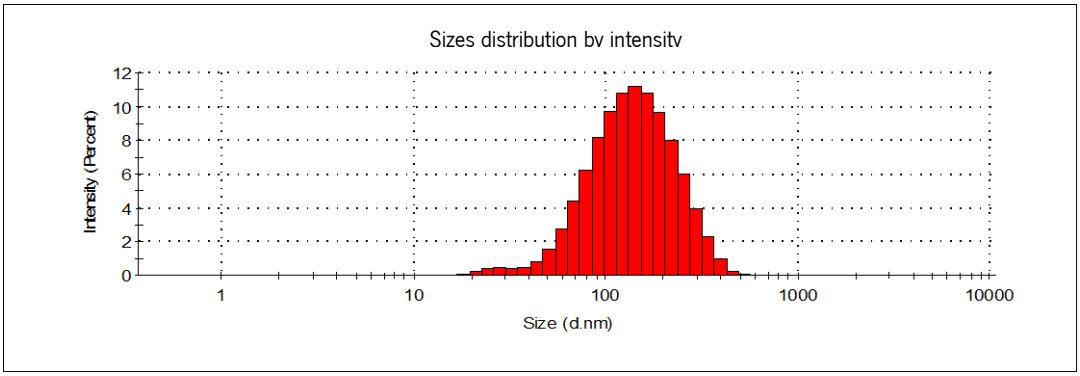

Figure 26 – AgNPs sizes distribution by intensity of *Penicillium chrysogenum* MUM 03.50 mutant.

Table 13 - Average of size (nm) and polydispersity of the fungus *P.* chrysogenum MUM 92.11 wildtype and its respective mutant analyse by DLS.

Fungus	Average size (nm)	Polydispersity
Wildtype (WT)	141.8	0.242
Mutant	141.8	0.211

The Zeta-potential analysis (Table 14) clearly shown no difference of the surface charge produced by AgNPs for both WT and mutant filtrate *P. chrysogenum* MUM 92.11. In addition, WT and mutant filtrate were highly negative indicating that the AgNPs surface had a strong anionic charge.

Table 14 - Solution charge (mV) of *P. chrysogenum* MUM 92.11 WT and mutant fungus analyse by Zeta-Potential.

Fungus	Charge (mV)
WT	- 25.1
Mutant	- 25.8

Real charge (mV) of ddH_2O with $AgNO_3$ = -1.1

The morphology, size range and the elemental composition of AgNPs in the freeze-dried filtrate of *P. chrysogenum* MUM 92.11 WT and mutant were analysed by SEM/STEM and EDS, respectively. A representative micrograph of the AgNPs produced by WT and mutant filtrate was shown by the SEM/STEM microscopy in figure 27. Variable shapes of AgNPs were observed, most of them were spherical, although in aggregates. Besides that a size range of 21.5 nm to 52.5 nm for AgNPs produced by WT (Figure 27 (A) and (C)) and a size range of 20.6 nm to

561.0 nm was observed for AgNPs produced by the filtrate of the mutant strain (Figure 27 (B) and (D)).

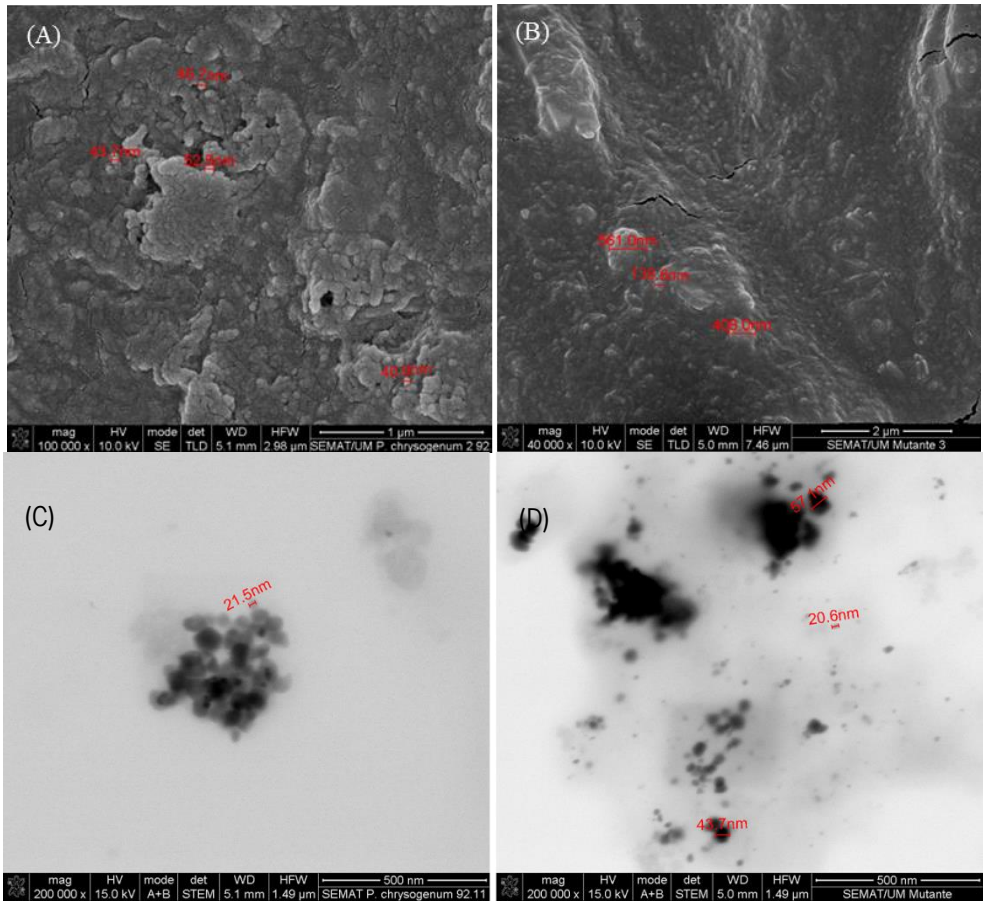

Figure 27 – SEM and STEM images of AgNPs synthetized by *P. chrysogenum* MUM 92.11: WT (A) and C)) and mutant (B) and D)).

Elemental composition of the AgNPs in the freeze-dried filtrate of WT and mutant were determined by EDS (Figure 28). It can be observed that the WT *P. chrysogenum* MUM 92.11 shown the highest signal on the silver region (proximally at 3 KeV) comparing with mutant. Other elements such as C, N, O, P, S and K were also found in the freeze-dried filtrate of WT and mutant, indicating the compounds heterogeneity of both of filtrates. In addition, the WT filtrate shows the presence of Mg and mutant filtrate exhibited the presence of Na and higher percentage of O.

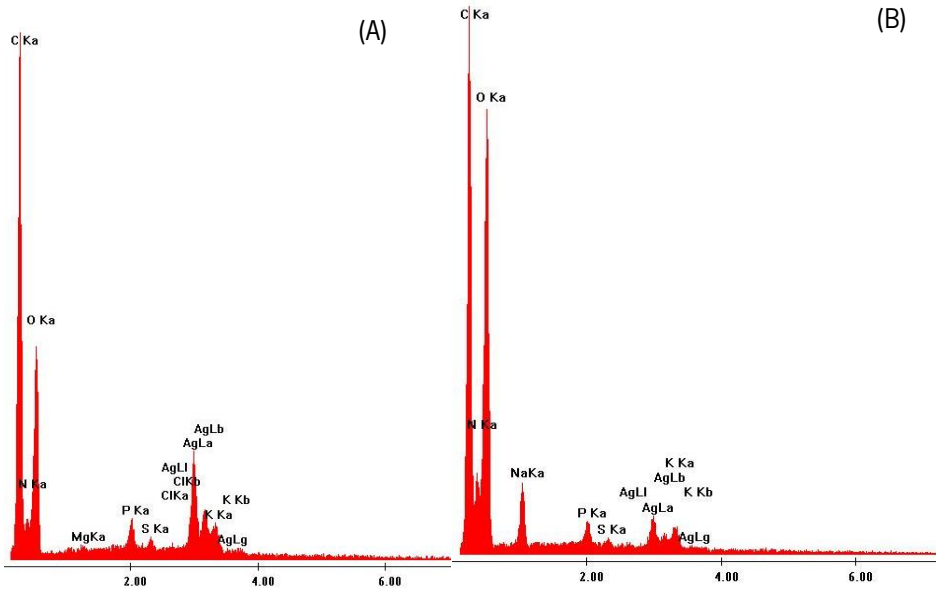

Figure 28 - EDS spectrum of the *P. chrysogenum* MUM 92.11 filtrate after 96 h of incubation: (A) wildtype and (B) mutant.

3.3. Analysis of the NR enzyme

The obtained mutant filtrate where subject to a characterisation relatively to the presence of the NR enzyme and its respective activity.

3.3.1. Electrophoresis with SDS-Polyacrylamide Gel (SDS-PAGE)

The presence of NR enzyme on the mutant filtrate was analysed through the SDS-PAGE. The figure 29 exhibit a single band in WT and mutant filtrate at the 50 KDa suggesting the presence of a protein with approximately the molecular weight of NR enzyme. In addition, protein band at 20 KDa was also showed in both of filtrates. Nevertheless in the WT filtrate the protein bands was more concentrated and other proteins bands were found comparing to the mutant filtrate.

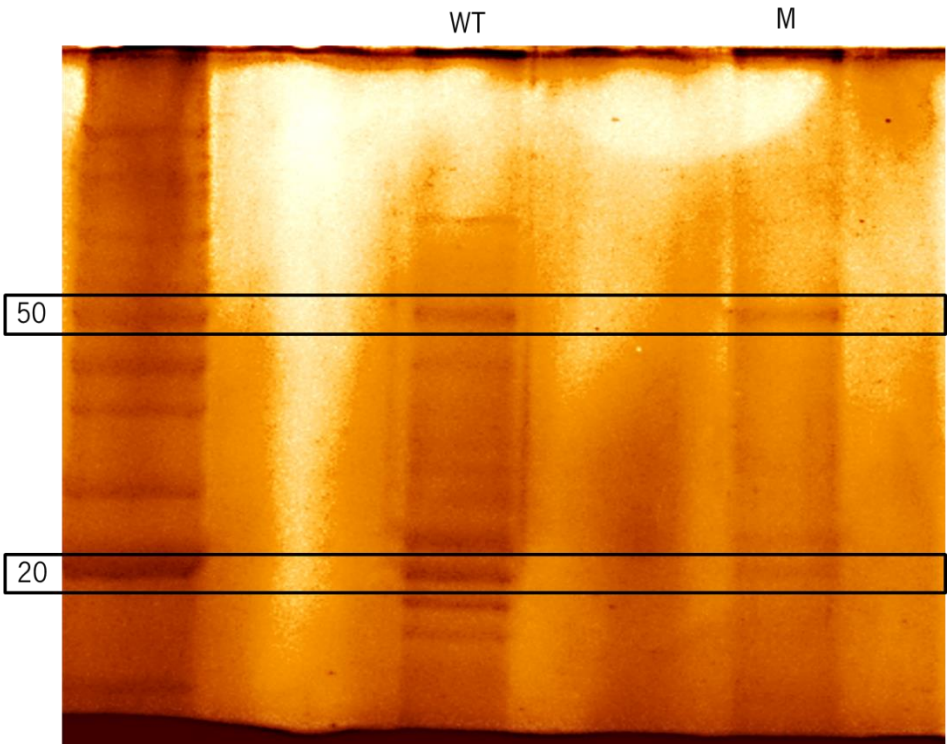

Figure 29 – SDS-PAGE of the filtrates WT and mutant (M) of the fungus *P. chrysogenum* MUM 92.11.

3.3.2. Determination of nitrite concentration

The concentration of nitrites present in both WT and mutant filtrates, without (negative control) and with silver nitrate at t = 0 h and t = 96 h was determined through the method described on the section 2.1.3.3 (Table 15). A significant increase of the concentration of nitrites from control to the time point of the addition of $AgNO_3$ at t = 0 h and consequently to t = 96 h was found for both WT and mutant filtrate of *P. chrysogenum* MUM 92.11. In the WT and mutant filtrate an increase of 0.044 and 0.013 $\bullet 10^3$ mg/mL, respectively was found for 96 h of incubation with $AgNO_3$. Although the concentration of nitrites was higher in the WT filtrate at t = 96 h comparing to the same time of incubation for the mutant filtrate no statistically significant differences ($P > 0.05$) were found (data not show).

Table 15 – Nitrites concentration.

Fungus	Control (10^3 mg/mL)*	t = 0 h (10^3 mg/mL)*	t = 96 h (10^3 mg/mL)
Wildtype	0.003	0.106*	0.150
Mutant	0.001	0.126*	0.139

(*) Statistically different values ($P < 0.05$)

3.3.3. Determination of NR specific enzymatic activity

The specific enzymatic activity of NR enzyme was determined by measuring the production of nitrites in a defined period of time, as the product of the enzymatic reaction, and calculating the total protein concentration. The specific activity of NR enzyme was defined as Unit per milligram of total protein (Table 16). Slightly high activity of NR was found in WT filtrate comparing to the mutant filtrate.

Table 16 – Determination of NR enzymatic activity.

Fungus	U/mg total protein
WT	2.852E-04
Mutant	2.730E-04

3.4. *In-vitro* assay

An attempt to modulate simulating the biochemical conditions of the production of $AgNO_3$ proposed by Durán et al. (2005) for *Fusarium oxysporum* was carried out in this part of the study. The experimental conditions of *in-vitro* assays were performed according to the table 10 (see chapter II, section 2.2).

The *in-vitro* assays were divided in four assays I, II, III, IV. In assay I different reaction solutions were prepared in microtubes adding the following compounds: β-NADPH and FAD (as electron donors), NR (enzyme involved in Ag^+ reduction) and anthraquinone (as electron shuttle) in phosphate buffer pH = 8. In assay II the same procedure was performed with the WT and mutant filtrate with $AgNO_3$. The enzyme NR was added to mutant filtrate in order to stimulate; the NADPH inhibitor (sodium azide) was used to prevent AgNPs production in the WT filtrate. In another microtube the WT filtrate was heated at 100 °C for 5 min to denature proteins and consequently the NR enzyme. In assay III and IV same conditions of assay I were used except the addition of the sodium azide as NADPH inhibitor and egg-albumin protein (about 50 KDa), respectively in the microtubes. These two parts had the purpose to detect the presence of surface plasmon vibrations when added NADPH inhibitor and the protein, respectively. The UV-Vis. spectra ranging 250 to 800 nm were recorded for each condition of the different assays at 96 h of incubation.

According to the obtained data (table 17), no surface plasmon resonance between 380 and 420 nm was detected in none of the different assays except on assay II (WT and mutant

filtrate). In this assay, the surface plasmon resonance was always observed even with the addition of NADPH inhibitor or the denaturation of the proteins in the WT filtrate. No increase in the intensity of the plasmon resonance in the UV-Vis. spectra was detected with the addition of NR to the mutant filtrate. These compounds (NR, sodium azide or denatured proteins) showed all the same response which means that did not occur any alteration on the production of AgNPs and, consequently, did not influence the total AgNPs production.

Table 17 – The obtained results of UV-Vis. spectra ranging 250 to 800 nm for each condition of the different assays after 96 h of incubation.

Assay		Result
I	A	-
	B	-
	C	-
	D	-
	E	-
II	A	+
	B	+
	C	+
	D	+
	E	+
III	A	-
	B	-
	C	-
	D	-
IV	A	-
	B	-
	C	-
	D	-

(+) Presence of excitation of surface plasmon vibrations at the 380 - 420 nm, approximately, with the UV-Vis analyse
(-) Absence of excitation of surface plasmon vibrations at the 380 - 420 nm, approximately, with the UV-Vis analyse

The figure 30 described the II assay. This is the only assay that used filtrates and had a different response compared to the others assays.

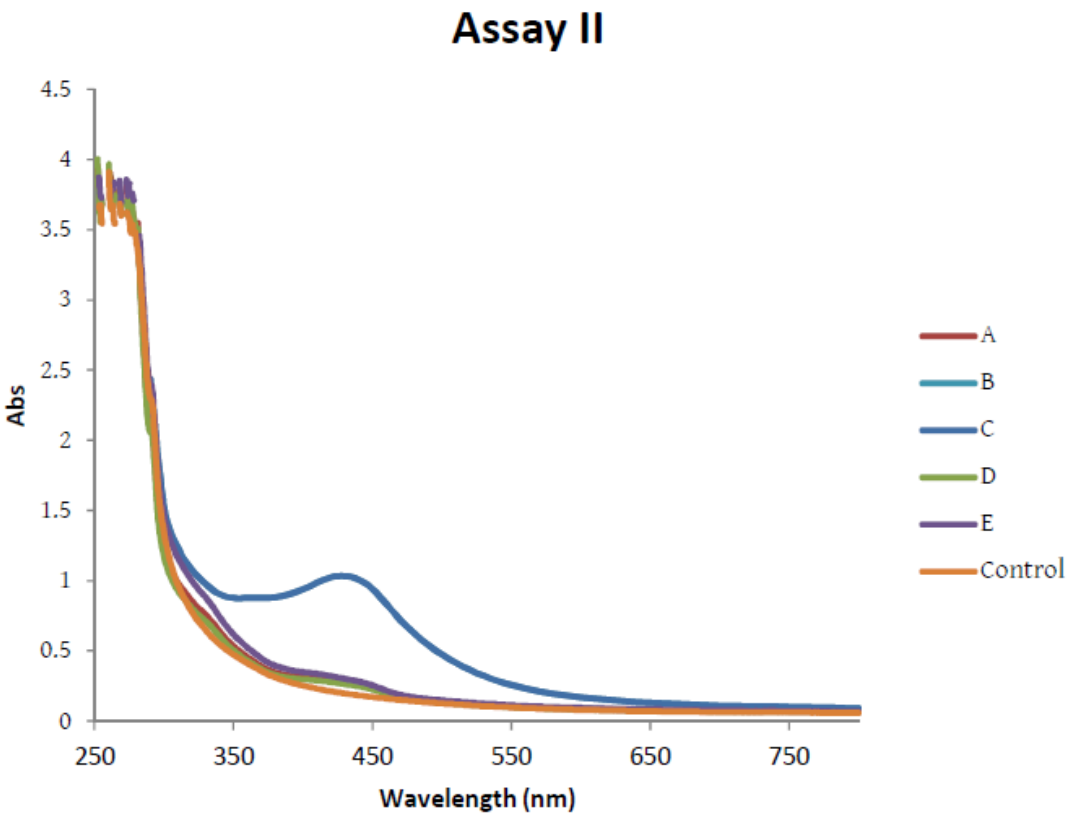

Figure 30 – The obtained results of UV-Vis. spectra ranging 250 to 800 nm for each condition of the II assay after 96 h of incubation. The III assay was used as control.

4. Discussion and Conclusion

Mutant deficient in the genes required for nitrate assimilation were obtained directly, in a spontaneous way, by positive selection on the basis on the chlorate resistance. The basic idea of this conventional method of mutagenesis is originated by the presence of molecular mimesis between nitrate and chlorate ion associated to the cellular toxicity of the chlorate ion (Cove 1979). Other methods of mutant selection can introduce pleiotropic and undesirable mutations (Unkles et al. 1989). The mutation in different genes related to the nitrate assimilation can be easily assessed allowing the differentiation of the generated mutants on the basis of growth tests (Unkles et al. 1989). Therefore, since no mutagenic agents are used, the possibility of secondary mutations that would affect important genes is reduced (Varallo et al. 2005).

In this study, 34 chlorate-resistant mutants generated by the above mentioned strategy arose from inoculation of 10^7 spores/mL and from those 3 were found to be revertants to nitrate prototrophy. Of the mutants obtained from the fungus *P. chrysogenum* MUM 92.11 different phenotype results were found comparing to those reported for *Aspergillus* by Cove (1979) and Unkles et al. (1989). When observed the metabolic pathway of nitrite assimilation neither wildtype nor mutants grew on nitrite medium (*nia*D$^-$, *nir*A). The same results were found by Aleksenko et al. (1995) for *Penicillium canescens*. Randomly selected mutant (P2) was used for the biosynthesis of AgNPs (point 3.2), analysis of NR enzyme activity (point 3.3), and the *in-vitro* assay (point 3.4).

The reduction of Ag^+ ions was monitored by the change of colour of the WT and mutant filtrate from yellow to brown after 96 h of incubation. The presence of an absorbance peak on UV-Vis spectroscopy analysis at wavelengths ranging from 380 to 420 nm indicated the production of spherical AgNPs for WT and the mutant isolate. The analysis of the size and polydispersity of the nanoparticles as well as the filtrate charge (Zeta-potential) confirmed the production of AgNPs from WT and from the mutant filtrate.

The SEM micrographs show the high density of nanostructures indicating the presence of AgNPs in the mutant *P. chrysogenum* MUM 92.11 with characteristic silver nanocrystals found at 3 KV (Kumar and Mamidyla, 2011; Jain et al. 2011). Therefore the peaks found for C, N, O and S indicated the presence of fungal proteins as AgNPs capping material in both filtrates (Durán et al. 2010; Jain et al. 2011). The results indicated that mutants with defective nitrate

assimilation system are effectively able to produce AgNPs. The present results are in discordance of Durán (2005; 2011) who proposed in its model the preponderant role of the NR in the reduction of Ag^{+} for the subsequent production of AgNPs. The results in this study indicated that NR might not be the most important enzyme involved in the biochemical pathway of the production of AgNPs in filamentous fungi *P. chrysogenum*.

NR enzyme assays were undertaken to determinate the activity of this enzyme was present in WT and mutant filtrate. From SDS-PAGE results a 50 KDa band was obtained in both filtrates indicating the presence of a protein with the same molecular weight as reported by some authors (Douglas et al. 1998; Einsle et al. 1999; Kumar et al. 2007) and described as 40 - 50 KDa. The lowest concentration of the protein band at 50 KDa on the mutant filtrate comparing to WT can be explained by Patemand et al. (1967). In fact mutants with phenotype *nia*D^{-};*nir*A^{-} produce low levels of both nitrate and nitrite reductase (Cove 1979).These data were corroborated with the determination of NR enzymatic activity of WT and mutant filtrates. Birkett and Rowlands (1981) discussed that lack of NR activity was identified as one cause of chlorate-resistance in mutants. Besides that, it was proposed that *nir*A^{-} gene product was involved in the mediation of the induction of nitrate and nitrite reductase by nitrate (Patemand and Cove 1967). The presence of the NR enzyme activity and the concentration of nitrites in the mutant filtrate indicated a more complex system of production of AgNPs than the one proposed by Durán et al. (2005) and adopted by other authors.

In order to verify if each compound identified as key-compound proposed by Durán et al. (2005) influence the production of nanoparticles an *in-vitro* assay was developed. No change in the colour of the reaction solution and consequently no production of AgNPs was detected in assays I, III and IV. In assay II the presence of surface resonance plasmon peak was observed on all the microtubes containing the fungal filtrates. Several studies showed the involvement of NR enzyme in the synthesis of AgNPs (Ahmad et al. 2003; Durán et al. 2005; Kumar et al. 2007; Ingle et al. 2008; Durán et al 2011; Jain et al. 2011). In our study, the presence of an UV-Vis. absorbance peak at wavelengths ranging from 380 to 420 nm in the fungal filtrate with addition of an inhibitor of NADPH (assay II D) and denatured proteins by heat (assay II E) confirmed the production of AgNPs. This result suggested that other compounds may be involved in the biosynthesis of nanoparticles. Recently, Birla et al. (2013) proposed that free amino acids are responsible for the synthesis of silver nanoparticles in *Fusarium oxysporium*. In their study, they

found that AgNPs were synthesised after the heat denaturation of fungal filtrate in the dark and in the sunlight conditions. Moreover, they confirm the presence of free amino acids in the heat denatured fungal filtrate using the Ninhydrine test. They suggested that synthesis of AgNPs was due to transfer of electrons from free amino acids to silver ions. The involvement of hydroxyl and carboxyl groups of amino acids residues have been also reported by other authors (Naik et al. 2002; Xie et al. 2007; Birla et al. 2013). Due to the fact that no production of AgNPs was obtained in the in-vitro assays with exception to the microtubes with fungal filtrate, it can be concluded that other compounds present in the filtrate might be also involved with earlier described key-compounds in the production of AgNPs for the strain *P. chrysogenum* MUM 92.11.

General conclusions and futures perspectives

General conclusions and futures perspectives

1.1. General conclusions

Selection of the best producer of stable biosynthesised AgNPs was performed through the characterisation of their physicochemical features: change of colour, presence of a surface plasmon resonance, DLS, ICP-OES, Zeta potential analysis, EDS and SEM, nanoparticles of selected fungi specie. In this study the fungal strains of *P. chrysogenum* MUM 92.11 and *N. crassa* MUM 92.08 were selected as the best producers of AgNPs.

Both strains were used for mutagenesis and modulation studies of biochemical mechanisms for the production of AgNPs. Several unsuccessful attempts were performed for the mutagenesis of the strain *N. crassa* MUM 92.08 and none of the mutants have grown. The mutagenesis studies carried on with *P. chrysogenum* MUM 92.11. Irregular shaped nanoparticles where produced by mutants. However, so far, there is no report on the study of the production of biological synthesis of AgNPs with mutants. In this study the involvement of nitrate reductase enzyme in the production of AgNPs was inconclusive. Experimentation also demonstrated that without fungal cell filtrate synthesis of AgNPs did not occur. However it did occur in cell filtrate even after the heat denaturation of fungal filtrate protein, indicating that amino acids could be involved in the production of AgNPs.

1.2. Futures perspectives

In a future work, one of the points to improve the unidispersion and the uniform shape of the AgNPs it will be important to consider the optimization of the experimental conditions, such as, pH, temperatures and Ag^{+} ions concentration. Besides that, in manner to obtain a more exact result of the charge of the AgNPs by zeta potential, it could be carried out the separation of nanoparticles from the medium by centrifugation and rinsed with deionized water before the measure. Furthermore, to complement the physicochemical analysis of the AgNPs, X-ray diffraction (XRD) and Fourier Transform Infrared (FT-IR) could be carried out. These analyses will allow to identify the periodic atomic structure of crystals (XRD) and interaction between the proteins and de AgNPs (FT-IR) and determining the AgNPs stability. Therefore, optimization of the parameters would lead to the rapid and large scale production of AgNPs at industrial level which may use as novel antimicrobials against multidrug resistant microorganisms.

Fairly to the mutagenesis and modulation of biochemical mechanisms for the studies of the production of AgNPs some aspects were left open in this work, which are likely to be taken up into future research to further clarify, such as the understanding of the compounds that are involved on the AgNPs. Also, due to the failure of obtaining spontaneous mutants through the resistance to chlorate medium for the strain *N. crassa* MUM 92.08 it could be important to analyse its response to the mutagenesis and could be carried out through other ways of mutation, like: site-directed mutagenesis.

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