Universidade do Minho
Escola de Engenharia

Elda Sabino da Silva

Biosynthesis of silver nanoparticles – process optimization and characterization of production mechanisms

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Biosynthesis of silver nanoparticles – process optimization and characterization of production mechanisms

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Trabalho efetuado sob a orientação do
Prof. Dr. José António C. Teixeira
e do
Prof. Dr. Nelson Lima

dezembro de 2024

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BIOSYNTHESIS OF SILVER NANOPARTICLES – PROCESS OPTIMIZATION AND CHARACTERIZATION OF PRODUCTION MECHANISMS

ABSTRACT

The present work aimed to study the silver nanoparticle synthesis process from an aqueous extract of *Rhizopus arrhizus* NRRL 54538 (IPT 1013) biomass to identify the molecules involved in the synthesis mechanism. This process consists of three stages: obtaining biomass, extracting biomolecules, and nanoparticle synthesis. Each process step was studied. It began by exploring the culture medium composition to define a low-cost medium for high biomass production and scale-up. Some carbon sources were tested in a complex medium and glucose showed the best result. However, to reduce the medium cost, it was decided to use waste (corn steep liquor, and molasses) plus some salts. Sugarcane molasses was used as a carbon source and CSL (corn steep liquor) was used as a nitrogen source. With 100 g/L of CSL and 10 g/L of molasses and some salts, microbial growth showed approximately twice the mycelial mass obtained in the complex medium and AgNP synthesis. The extraction step was based on the concentration of total proteins extracted and for this purpose the influence of the extraction temperature and the osmolarity of the solvent was studied. Increasing the molarity of the solvent with NaCL did not provide differentiation in the protein concentration extracted compared to water, as did the increase in temperature. Due to the identification of protein molecules in the aqueous extract composition, identification was carried out by ESI-MS on aqueous extract samples originating from a complex medium, resulting in several enzymes, which were identified as non-reducing and NADH. The nitrate reductase presence was not identified. In the synthesis stage, the alternative medium required a modification in the preparation due to the quantity of solids present in the CSL and molasses that interfered with the particle size measurement. The increase in NADH in the synthesis reaction and the protein inactivation in the extract resulted in an increase in AgNP synthesis compared to the extract, corroborating the NADH action in reducing silver. Increasing the reaction temperature from 30°C to 50°C provided a shorter reaction time from 5 days to around 16 hours for the extract from both mediums. It was not possible to identify the molecules responsible for the synthesis reaction. Still, it was likely through TEM images to verify the presence of the corona protein in the AgNP. When expanding the scale of biomass production, low agitation in the bioreactors did not provide conditions for biomass recovery for the extraction stage. Cultivations carried out at 800 rpm were effectively produced biomass with small hyphae and in recovery conditions, without interfering with the AgNP synthesis condition. At this stage, the cultivation time also went from 5 days in shaken flasks to 40 to 48 hours, in the respective culture media. The antimicrobial activity against *E. coli* and *S. aureus* demonstrated approximately 80% population reduction for AgNP samples from complex medium and 70% for alternative medium samples. The cytotoxic action was observed in AgNPs from complex media at concentrations above 29.6 µg/mL and for AgNPs from alternative media above 59.2 µg/mL. The conclusion is that the process was generally improved in terms of the cost of culture medium and reaction times per step.

KEYWORDS: bioreactor CSTR, corn steep liquor, *Rhizopus arrhizus*, silver nanoparticles, sugarcane molasses.

BIOSSINTESE DE NANOPARTICULA DE PRATA – PROCESSO DE OTIMIZAÇÃO E CARACTERIZAÇÃO DO MECANISMO DE PRODUÇÃO

RESUMO

O presente trabalho se propôs a estudar o processo de síntese de nanopartícula de prata a partir de um extrato aquoso da biomassa de *Rhizopus arrhizus* NRRL 54538 (IPT 1013) com o objetivo de identificar as moléculas envolvidas no mecanismo de síntese. Este processo foi composto por três etapas: obtenção da biomassa, extração de biomoléculas e síntese da nanopartícula. Cada etapa do processo foi estudada individualmente. Iniciou-se pelo estudo da composição do meio de cultura com o propósito de definir um meio de baixo custo visando uma alta produção de biomassa e ampliação de escala. Algumas fontes de carbono foram testadas em meio complexo (referência), a glicose apresentou o melhor resultado. Entretanto para reduzir o custo do meio, optou-se pela utilização de resíduos agroindustriais (licor de milho e melaço de cana) acrescidos de alguns sais. O melaço de cana-de-açúcar foi utilizado como fonte de carbono e o CSL (*corn steep liquor*) principalmente como fonte de nitrogênio. Com 100 g/L de CSL e 10 g/L de melaço e alguns sais, o crescimento microbiano apresentou cerca do dobro da massa micelial obtida no meio referência e síntese de AgNP. A etapa de extração foi baseada na concentração de proteínas totais extraídas onde foi estudada a influência da temperatura de extração e a molaridade do solvente. O aumento da molaridade não proporcionou diferenciação na quantidade de proteínas extraídas em comparação com a água, assim como o aumento da temperatura. Foi realizado a identificação das moléculas proteicas na composição do extrato aquoso originários de meio refereência por ESI-MS, resultando em várias enzimas, que foram identificadas como não redutoras e NADH. Não foi identificado a presença de nitrato redutase. Na etapa de síntese, o meio alternativo exigiu uma modificação na preparação devido a quantidade de sólidos presentes no CSL e melaço que interferiam na medida do tamanho das partículas. A adição de NADH na reação de síntese e a inativação das proteínas do extrato resultou em aumento da síntese de AgNP comparado com o extrato, isso corrobora a ação do NADH na redução da prata. O aumento da temperatura de reação de 30°C para 50°C reduziu o tempo de reação de 5 dias para cerca de 16 horas tanto para o extrato do meio referência quanto do meio alternativo. Não foi possível identificar as moléculas responsáveis pela reação de síntese, mas foi possível por imagens de TEM verificar a presença da proteína corona nas AgNP. Na ampliação de escala de produção de biomassa a baixa agitação nos biorreatores não propiciou condições de recuperação da biomassa para a etapa de extração. Cultivos realizados a 800 rpm foram efetivos na produção de biomassa com pequenas hifas e em condições de recuperação, não interferindo na condição de síntese de AgNP. Nesta etapa, o tempo de cultivo reduziu de 5 dias em frascos agitados para 40 a 48 horas em biorreator, nos respectivos meios de cultura. A atividade antimicrobiana contra *E. coli* e *S. aureus* demonstrou cerca de 80% de redução da população para a amostras de AgNP advinda de meio referência e 70% para as do meio alternativo. A ação citotóxica foi observada nas AgNP de meio complexo em concentrações acimas de 29,6 µg/mL e para as AgNP de meio alternativo acima de 59,2 µg/mL, esta diferença pode estar relacionada com a proteína corona de cada AgNP. Concluiu-se que, apesar da não identificação dos agentes responsáveis pela redução da prata, o processo foi otimizado em termos de custo de meio de cultura e tempos de reação por etapas.

PALAVRAS-CHAVE: biorreator, CSL, melaço, nanopartícula de prata, *Rhizopus arrhizus*.

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Symbols and abbreviations

Å	Angstrom
Ag	Silver
Au	Gold
Ag°	Reduced silver
Ag+	Silver ion
$AgNO_3$	Silver nitrate
AgNPs	Silver nanoparticles
AM	Alternative medium
ATCC	American Type Culture Collection
ARS	Agriculture Research Service
Co	Cobalt
CSTR	Continuous stirrer tank reactor
CFF	Cell-free filtrate
°C	Degrees Celsius
CSL	Corn steep liquor
cm	Centimeter
C	Corn steep liquor
DLS	Dynamic Light Scattering
DNS	Dinitro salicylic acid
ESEM	Environmental Scanning
FTIR	Fourier-transform infrared spectroscopy
g/L	Gram per liter
g	Gram
GRAS	Generally Recognized as Safe
HMDSO	Hexamethyl disiloxane
HPLC	High Pressure Liquid Chromatography
ISO	International Standard Organization
ITC	isothermal titration calorimetry
IPT	Instituto de Pesquisas Tecnológicas do Estado de São Paulo
L	Liter
MTCC	Microbial Type Culture Collection

Min.	Minimum
m	Meter
m^2	Square meter
M	Molasses
Min.	Minimum
Max.	Maximum
mV	Millivolt
µg/mL	Microliter per milliliter
µg/L	Microliter per liter
mg/L	Microgram per liter
mg/Kg	Microgram per kilo
µM	Micromolar
mMol/L	Millimoles per liter
nm	Nanometer
N	Nitrogen
NADH	Nicotinamide Adenine Dinucleotide
NP	Nanoparticle
NPs	Nanoparticles
NO_3^-	Nitrate ion
NO_2^-	Nitrogen dioxide
Pt	Lead
PVP	Poly N-vinyl-2-pyrrolidone
PMAA	Polymethacrylic
PEG	Polyethylene glycol
PES	Polyester
PMMA	Polymethylmethacrylate
RM	Reference Medium
ROS	Reactive oxygen species
rpm	Rotation per minute
SPR	Surface plasmon resonance
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SiO_2	Silicon oxide

TEM	Transmission Electron Microscopy
Ti	Titanium
TiO_2	Titanium oxide
USA	United States of America
UHPLC	Ultra-High-Pressure Liquid Chromatography
UV	Ultraviolet
v/v	Volum per volum
ZP	Zeta potential
%	Percentage

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Dedico este trabalho à minha Família, especialmente aos meus pais

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"As coisas mais insignificantes têm às vezes maior importância e

são geralmente por elas que a gente se perde ...

(Fiódor Dostoiévski)

"A imaginação é mais importante que a ciência, porque a ciência é limitada,

ao passo que a imaginação abrange o mundo inteiro"

(Albert Einstein).

1 INTRODUCTION

Technological development in various science segments has driven the evolution of studies toward larger and more efficient products. Among these products are the so-called “nanostructured products or engineered nanomaterials”, which can be produced from both organic and inorganic materials, however, metals have stood out in this production with metallic nanoparticles (NPs).

Currently, the metallic NPs application is present in a wide variety of products, ranging from the most simplified to those with more noble applications, such as in the medical field, both human and veterinary. The variety of metals used in these products goes through a series of elements on the periodic table, from the simplest to the most noble. The demand to produce these materials has increased in parallel with the knowledge acquired about new (unique) functionalities that they can present depending on their sizes and shapes. One of the functionalities of these nanoparticles is directly related to their physicochemical properties, attributing variations in reactivity and consequently biological activity.

This production has been conducted using different chemical and physical processes that consume a lot of energy and do not meet environmental preservation requirements. In addition, it has provided a production scale to meet market demand, which has increased significantly in recent years.

The search for alternatives to replace these processes led the scientific community to invest in green chemistry applications, using natural and biological products to develop cleaner and less expensive processes, including the use of microorganisms and plant extracts. The biosynthesis of metallic NPs by microorganisms has been known to researchers since 1960 when the observation of these particles inside the microorganism was published (Temple & LeRoux, 1964). Since then, there has been an investment in this innovative technology, which in recent years, with the development of innovative technologies applied to nanotechnology, has boosted the development of products and applications.

The advantages of producing NPs through a biological process are not only economic and ecological, but also the ability of the microorganism to produce particles with distinct physical characteristics, such as size and shape. These characteristics are extremely important in terms of surface area and interactions, especially when intended for certain applications. More recent studies have shown that the properties of NPs obtained from green processes have some properties not found in those from chemical synthesis, such as the protein crown that can differentiate applications.

Numerous microorganisms and plants have been studied to produce metallic NPs through biological or green synthesis processes. However, despite the considerable number of works developed and in progress to make this technology a market reality, there is still a lack of information to improve these systems and provide an industrial process to produce these nanoparticles on a large scale. One of the most studied metals is silver, known for thousands of years and to this day for its antimicrobial properties. For example, Persians, Egyptians, Greeks, and Romans used silver in utensils to preserve food and water, thus reducing degradation caused by microorganisms and prolonging their preservation (Srikar, 2016).

Several genera and species of microorganisms and plants have been used in research reported in scientific circles for the NPs metallic synthesis. Among the varieties of microorganisms identified as producers of silver nanoparticles (AgNPs) are fungi, which have very particular characteristics for this purpose. These microorganisms are easily manipulated, do not present high nutritional and environmental requirements, and can produce several metabolites. Therefore, fungi are considered major players in developing biotechnological processes that focus on AgNP production and possibly other metals.

The protocols for the production of AgNPs by fungi used and reported in the literature have the advantage of using biomass extracts for the nanoparticle synthesis, as it is an extracellular synthesis, allowing the obtaining of a colloidal solution of AgNP, this process is characteristic proves to be interesting display from the scaling up and product recovery point of view.

Several fungi species have already been identified for the biosynthesis of NPs, however, the clarification of the synthesis mechanism and the identification of the biomolecules and mechanisms that engage in this process are not yet clear. Many hypothesized biomolecules (enzymes, NADH) have been considered as silver reduction agents and corona-forming components. protein as stabilizers, but the reported results are still disparate and do not have explanations or evidence to support them. The nitrate reductase enzyme has been recognized for some time as a key element for biosynthesis. However, many other biomolecules have already been mentioned as being involved in the formation of NPs or as stabilizing agents.

The wide variety of organisms used in metallic nanoparticle biosynthesis offers an abundance of different molecules that can be involved in the synthesis. Only the different culture media used in distinct species can generate a diversity of molecules that make this generalized identification difficult. In this

way, the mechanisms of nanoparticle formation elucidation and the identification of the biological or non-biological components involved have been identified as a bottleneck for the complete development of the biotechnological production of metallic NPs.

The various studies reported on microbial growth, whether by bacteria or fungi, have not considered the cost of the process, especially in the composition of the culture media. The use of agro-industrial waste can contribute to cost reduction and provide a competitive scale. Using agro-industrial waste can contribute to cost reduction and provide a competitive scale. The reuse of agro-industrial waste is an alternative to add value to what would otherwise be discarded, and it can be used in biotechnological processes as nutrients in the composition of culture media for the production of biomass or products. The use of waste rich in carbon or nitrogen sources can result in a reduction in the cost of the process without altering productivity. Availability is also an important factor to be considered.

Waste such as corn steep liquor (CSL) resulting from the processing of corn for starch extraction has a composition rich in nitrogen and other nutritious components for microorganisms. Sugarcane molasses, resulting from alcohol production, has a high concentration of sugars and minerals that most microorganisms metabolize. These residues are available for application in biotechnological processes and are already used in various fermentation processes to produce organic acids, enzymes, and biomass (Zvonareva *et al.*, 2018, Gao, *et al.*, 2016, Shen *et al*, 2015, Franco-Cirigliano *et al.*,2013).

In the development of this work, the use of different culture media for biomass production was addressed, seeking the lowest cost of the process and alternatives for the next AgNP synthesis stages. Among the studies conducted, the protein molecules in the composition of the fungal extract were identified to determine which were directly related to the nanoparticle synthesis. This work was designed with this in mind, to develop a process for producing AgNPs, finding the compounds taking part in the AgNPs formation reactions and trying to relate them to the possible mechanisms already described, using a fungus as an agent.

The results obtained were satisfactory from the point of view of using waste in the culture medium for biomass production and perfecting the stages of the AgNPs synthesis process; however, it was not possible to correlate the identified molecules with a possible synthesis mechanism by this fungus used.

2 LITERATURE REVIEW

2.1 Nanotechnology

Nanotechnology is a transversal, disruptive, and pervasive scientific-technological field whose concept was first presented by Richard Feynman in a lecture at the American Institute of Technology in 1959 (Figure 1). The term was created in 1974 by Japanese researcher Norio Taniguchi, who defined it as a technique for producing materials measuring 1 nm (10^{-9} m), with high accuracy and precision for application in separation processes, consolidation, or materials deformation. (Kessler & Charles, 2007; Hulkoti & Taranath, 2014). As the size of the atoms is about 0.1 to 0.4 nm, the nanomaterial can result from the manipulation of just a few atoms. The popularization of the term was achieved in the mid-1980s by Drexler (1986) with the concept of molecular manufacturing when nanoscience was spread in several areas of knowledge such as electronics, optics, magnetic, chemistry, pharmaceuticals, cosmetics, energy, textiles, surface finishing materials and many others (ten Wolde, 1998).

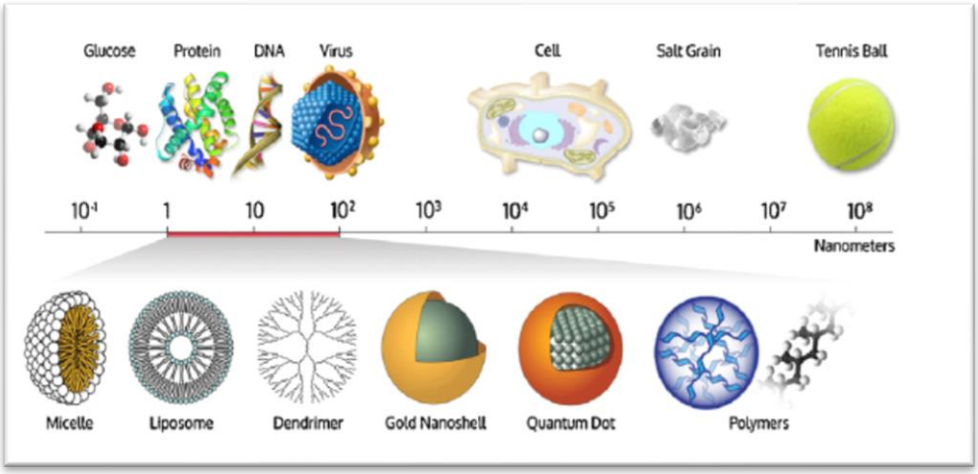


Figure 1 - Nanoscale according to Richard Feynman. (Hulkoti & Taranath, 2014).

The International Organization for Standardization (ISO) defined nanoscale as the range from 1 to 100 nm; nano object as a material with one, two, or three external dimensions within the nanoscale; particle as a small portion of material with defined physical properties, and a nanoparticle (NP) as a nano object with all external dimensions within the nanoscale (ISO/TS 27687, 2008).

According to Sheena Mary et al (2010), a nanoparticle acts as a bridge between bulk materials and molecular structures, as the former have constant physical properties. However, when materials are at the nanoscale, size-dependent properties are observed, such as quantum confinement in

semiconductors, surface plasmon resonance in metallic particles, and supermagnetism in magnetic materials. These are special physical and chemical properties, that arise from the nanocrystalline structure, the high proportion of atoms at the ends of their surfaces, and the interactions between the particles.

Two important and differentiated factors are highlighted in the properties of nanomaterials: surface area and shape of NPs. The surface area size increases significantly when comparing materials with nanometric formation. A cube of 1 cm on a side and 6 cm^2 in area made up of cubic NPs of 1 nm on a side will have a surface area of 6000 m^2, resulting in a greater surface area for chemical interactions, which normally occurs in solid elements, the environment or in reagents specific chemicals (Bell, 2007). In turn, the shape of the NPs influences the crystallinity and optical properties.

NPs can be produced with several types, including organic and inorganic materials. In the organic category (20 to 100 nm) we can find liposomes, micelles, solid lipids, biopolymers, and others, which are mostly biocompatible and biodegradable. Concerning inorganic materials, a series of metallic elements (oxides) are the subject of NP production (1 to 100 nm). NPs of gold, silver, platinum, titanium, aluminum, zinc, iron, cadmium, silicon, copper, and others can be found on the market (Dorcheh & Vahabi, 2016).

In recent years, due to the increased use of nanostructures, various production methodologies have been developed to meet the demands of current market applications. A variety of methods are used to produce metallic nanostructures. Many of these differ in terms of the chemical and physical processes involved. Figure 2 shows the diversity of methodologies used to produce metallic NPs.

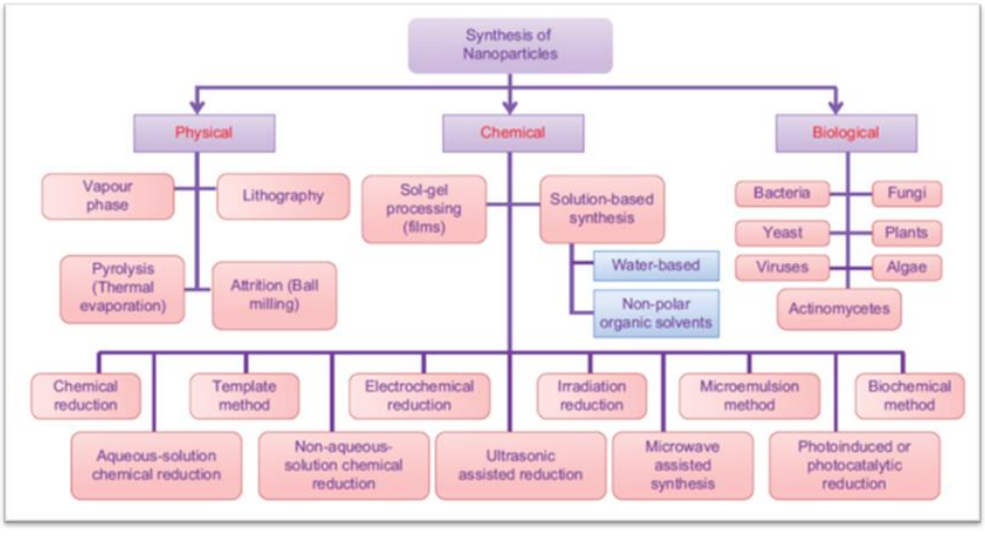


Figure 2 - Methods of metallic NP synthesis (Dhillon *et al.*, 2012).

These methods, mainly chemical, require the consumption of some products such as dispersants, surfactants, or chelating agents to stabilize the NPs. These compounds have toxic characteristics, which generate polluting waste for the environment. In addition to using elevated temperatures and pressures, which are not recommended for increasing production (Durán & Seabra, 2012; Dorcheh & Vahabi, 2016).

As these production techniques do not correspond to current global concerns, the scientific community has been looking for an alternative to replace chemical and harmful methods with biological methodologies (nanotechnology + biotechnology), which presupposes energy savings, reduction of greenhouse gases, biocompatibility and " green processes", which reduces damage to nature. In biological processes, an organism or one or more biomolecules will build, synthesizing NPs (Darroudi, *et al.,* 2011). Among the biological processes for nanoparticle synthesis, we mention bioreduction, biosorption, and synthesis by microorganisms and plant extracts. (Kumar, *et al.*, 2021).

"Nanobiotechnology" offers biology, physics, chemistry, medicine, bioinformatics, and engineering tools to make large-scale NP production a reality, meeting the current requirements of environmental legislation. In addition to the advantages listed above, it is possible to develop biosynthesis methods for other types of metallic NPs that are not synthesized chemically. However, a variety of studies are still needed to elucidate the mechanisms and create the best conditions for a large-scale production process (Durán, *et al.*, 2005; Durán & Seabra, 2012; Hulkoti & Taranath, 2014; Park, *et al.,* 2016; Singh, *et al.,* 2016).

2.2 Metallic Nanoparticles

In the last two decades, nanomaterials have played a particularly significant role in the world of technological applications. Among nanomaterials, metallic NPs have received strong attention in nanoscience due to their different properties and applications in various areas of knowledge. They are fundamental building blocks, as they allow the possibility of preparing nanostructured devices and materials (Vahabi, *et al.*, 2011).

Metallic NPs have unique chemical and physical properties that give them applications in electronics, optics, catalysis, biomedicine, agriculture, cosmetics, and food, among many others. Two main factors for these properties are the surface/area ratio and quantum confinement. Furthermore, they may be related to size, distribution, and morphology, among others, compared to larger particles from the same source (Willems & van den Wildenberg, 2005). The NP metallic morphology can present

different conformations, such as spheres, flakes, dendritic, and others, depending on the element of origin, which can give them different applications (Gúzman, *et al.*, 2009). In addition to the characteristics of size and shape, other properties provide different applications, such as magnetic properties, mechanical resistance, electrical conductivity, optical properties, and thermal stability, and can be controlled more easily and efficiently compared to bulk substances. (Rana & Parmar, 2023).

Among these, AgNPs have been intensely studied due to their great commercial, pharmacological, and therapeutic applicability, attributed to their chemical properties and biocompatibility, in addition to physical characteristics such as morphology, size, and particle agglomeration (Malik, *et al.*,2022). In the medical field, AgNPs are known for their antimicrobial activity and are already used in a considerable number of pharmaceutical and medicinal devices. AgNPs are among the nanomaterials with the greatest diversification, from the most common uses and within the reach of the end consumer to diverse other areas of technology (Qazi & Javaid, 2016). Research conducted in 2012 by Piccinno and collaborators obtained the data presented in Figure 3 (data obtained from industrial companies).

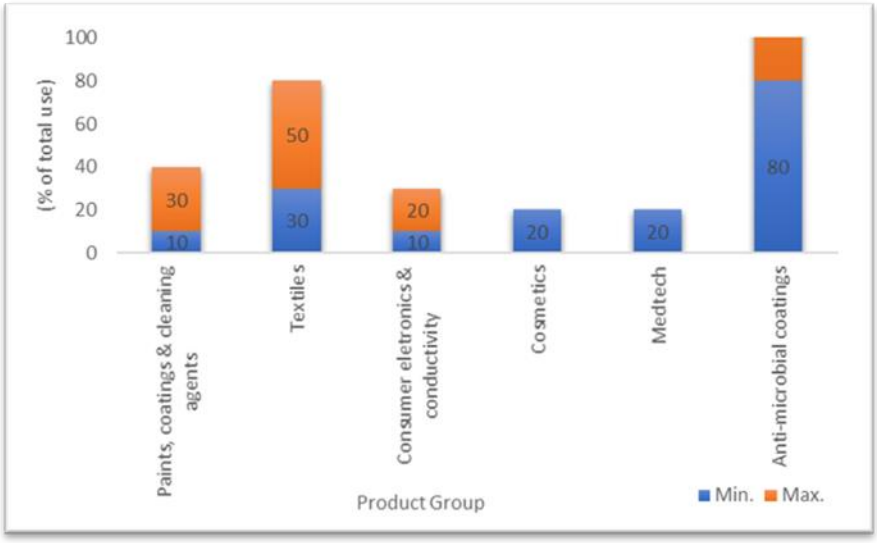


Figure 3 - AgNPs diversification application (adapted from Piccinno *et al.*, 2012).

Historically, since ancient Greece, silver has been used in drinking water storage containers for its antimicrobial properties, and during the Second World War silver nitrate solution was also used as an antimicrobial and healing agent. Even before the NPs use, silver was also composition part of creams and ointments for treating wounds, but the silver ions eventually complexed and the effect time was

reduced (Deepak *et al.*, 2011; Hicks*, et al.*, 2015; Mathur, *et al.*, 2018). With the advent of AgNP production, the potential uses have intensified.

2.3 Production of Silver Nanoparticles

Silver NPs are one of the most nanoparticles produced on the world market, around 1,000 tons/year. Europe produces and uses around 10 tons/year. (Piccinno *et al.,* 2012). Technologies for obtaining AgNPs focus on techniques for controlling size, shape, integrity, and stability (Deepak, *et al.*, 2011; Park*, et al.*, 2016), fundamental for their functionality. Chemical methods employ precursors (e.g., sodium citrate, ascorbate, sodium borohydride, essential hydrogen, polyol measure, Tollens' reagent, N, N-dimethylformamide (DMF), and poly(ethylene glycol) block copolymers (Bouafia, *et al.,* 2021; Gudimalla, *et al.*, 2021) metallic or ionic that is mixed with a reducing agent in the presence of a stabilizing agent (e.g. chitosan, oleylamine gluconic acid, cellulose or polymers such as poly N-vinyl-2-pyrrolidone (PVP), polyethylene glycol (PEG), polymethacrylic acid (PMAA) and polymethylmethacrylate (PMMA) (Bouafia, *et al.*, 2021) for NPs to control size and shape. Generally, these methods are low-cost for large volumes. However, disadvantages include contamination by chemical precursors, the use of toxic solvents, and the dangerous by-product generation, as well as high energy expenditure (Hulkoti & Tanarath, 2014).

The metal NP biosynthesis has been proposed since the 1960s (Temple & LeRoux, 1964; Bansal *et al.*, 2012), and is currently strongly presented as a workable alternative to chemical methods. The favorable points are cost/benefit, absence of toxic reagents, or high energy demand for physicochemical synthesis processes (Park *et al.*, 2016, Srikar, *et al.*, 2016, Sing *et al.*, 2016, Saravanan, *et al.*, 2021). Some nanoparticles produced advantages by green synthesis are easy preparation, less toxicity compared to chemically or physically synthesized nanoparticles, no side effects during the application of medicines due to biological origin, stability, biocompatibility, and low production cost, in addition to high efficiency (Singh, *et al.*, 2018; Kumar*, et al.*, 2021).

In recent years, especially for metallic NPs a large amount of research using microorganisms and plants for NP biosynthesis, has been published. Among the organisms described as potential NP producers are abundant species of bacteria, fungi, yeasts, and algae, in addition to the extracts used from several plant species. (Hulkoti & Taranath, 2014; Rauwel *et al.*, 2015; Park *et al.*, 2016; Srikar, 2016; Alsharif *et al.* 2020; Rai *et al.*, 2021; Salem & Fouda, 2021). Some microorganism species studied are presented in Table 1.

Table 1 - Microorganisms used in the metallic NPs biosynthesis (adapted from Hukolti & Taranath, 2014; Alghuthaymi *et al.*, 2015; Srikar, 2016).

Microorganism	NP	Size (nm)
Bacteria		
Lactobacillus fermentum LMG8900	Ag	6
Bacillus cereus NK1 *Bacillus cereus A1-5*	Ag	50 – 80 6 - 50
Brevibacterium casei	Ag e Au	10 – 50
Staphylococcus aureus	Ag	160 – 180
Ureibacillus thermo sphericus	Ag	10 – 100
Escherichia. coli	Ag	10 – 90
Bacillus cereus A1-5	Ag	6 - 50
Fungi		
Rhizopus oryzae	Ag	~15
Penicillium sp	Ag	52 – 104
Cladosporium cladosporioides	Ag	35
Trichoderma viride	Ag	2 – 100
Fusarium solani	Ag	5 – 35
Aspergillus flavus *Aspergillus oryzae NRRL447*	Ag	8 – 10 68 - 109
Phaenerochaete chrysosporium	Ag	50 – 200
Fusarium oxysporum	Ag	20 – 50
Verticillium sp	Ag	25
Alternaria alternata	Ag	20 – 60
Aspergillus clavatus	Ag	10 – 25
Aspergillus niger	Ag	15 – 20
Neurospora crassa	Ag	60
Phoma glomerata	Ag	60 – 80
Streptomyces noursei H1-1	Ag	6 - 30
Rhizopus stolonifer A6-2	Ag	6 - 40
T. longibrachiatum	Ag	5 – 25
T. viride (cucumber)	Ag	30 – 50
T. harzianum (tomato)	Ag	5 – 40
Aspergillus sydowii	Ag	1 – 24
Phomopsis liquidambaris	Ag	10 - 20

Microorganisms that produce AgNPs require distinct characteristics to be qualified for this purpose. Due to the intrinsic antimicrobial silver activity, these microorganisms must present resistance in contact with silver when the synthesis occurs intrinsically with the microorganism, a characteristic present in a variety wide of fungi. These microorganisms also have a high adaptability, and great resistance, are easy to handle, have few nutritional requirements, and produce several metabolites (Deepak *et al.*, 2011; Durán & Seabra 2012; Sing *et al.*, 2016). Microorganisms for nanotechnological purposes must be recognized as safe (GRAS) on all fronts, without controversy (Adebayo *et al.*,2021).

Fungi are already widely used in various industrial sectors, mainly in the enzyme production in food industry and organic acids production. Fungi can extracellularly break down complex substrates into simplified forms by secreting enzymes for the next metabolization and the various energy sources used. (Adebayo *et al.,* 2021). Given all the characteristics of these microorganisms, it can be said that mycosynthesis is potentially an energetic, economical, and environmentally favorable alternative in metallic nanoparticle production and has shown greater productivity compared to other microorganisms (Singh *et al.*, 2016; Šebesta *et al.,*2022).

In research conducted by Alghuthaymi *et al.* (2015), distinct fungi species were used in 48 studies to obtain metallic NPs of silver, gold, zinc, zirconia, platinum, cobalt, magnetite, and titanium, with the majority of studies presenting the AgNPs synthesis. Several fungi strains within different genera have been used for the NPs biosynthesis, for example, *Fusarium, Aspergillus, Verticillium, Alternaria, Pleorothus, Trichoderma, Penicillium, Rhizopus, Cladosporium*, and others (Kumar *et al.*, 2007; Binupriya *et al.*, 2010; Phanjom & Ahmed, 2012; Das *et al.*, 2012; Bahrulolum *et al.*, 2021; Salem and Fouda, 2021).

Despite the growing prospects for nanostructure use, the increase in investments in the area, and the considerable number of researches on the microorganisms used to produce metallic NPs, the NP formation mechanisms described are still poorly understood in their entirety by the scientific community so that a metallic nanoparticle production process can be precisely determined (Boaufia *et al.*,2021; Malik *et al.*, 2022).

There are many scientific reports of possible reactions with numerous biological origin molecules (microorganisms and plants) for the nanoparticle synthesis, however, there is still no precise description of which enzyme participates or whether any other molecule is preponderant in this reaction metal reduction. (Salem, 2023).

2.4 Metallic NPS Biosynthesis Mechanisms

Several factors are involved in metallic nanoparticle synthesis by microorganisms, but one of them is the organic functional group presence in the cell wall with the capacity to induce the biomineralization of metallic ions, the other factors are chemical and physical, such as temperature, pH, concentration, and composition of the reaction medium (Spagnoletti *et al.*, 2019). The literature described two metallic nanoparticle synthesis forms mediated by fungi: extracellular and intracellular synthesis. Extracellular synthesis is directly linked to the cell wall and the intrinsic fungi capacity to excrete molecules such as peptides, proteins, polysaccharides, oxide reductases, and quinones (anthraquinones and naphthoquinones), which are involved in the metal ions reduction and precipitation, moreover to reductase enzymes which have already demonstrated the capacity to reduce metals, as well as the metalloproteins overexpression when fungi are placed in contact with metals and proteins participate in the reduction process of these metals due to their binding and reduction characteristics. (Park *et al.*, 2016; Adebayo *et al.*, 2021).

Intracellular metallic nanoparticles synthesis in fungi is related to cellular ATPases and hydrogenases (Riddin *et al.*, 2009; Vahabi & Dorcheh, 2014; Adebayo *et al.*, 2021), however, Jha and Prasad, (2016), reported that fungi also use their antioxidant protection system to protect themselves from metal ions that generate reactive oxygen molecules (ROS), resulting in oxidative stress that is harmful to their survival system. The transport system for the main substrates, as well as micronutrients and fungi ions, is composed of enzymes such as catalase, superoxide dismutase, methionine sulfoxide reductase, thioredoxins, peroxiredoxins, glutathione, glutathione peroxidases and transferases (Vitale *et al.*, 2020; Ujam *et al.*, 2021; Ibrahim *et al.*, 2021). Due to metabolic survival, the fungi protection system can be partially obstructed by the toxic ions presence, preventing their access to the cytoplasmic space; however, these ions can have access through other channels, causing a metabolic disturbance and intracellular enzymes can react by precipitating these metals, thus forming nanoparticles. (Gahlawat & Choudhury, 2019).

Despite the metallic nanoparticle formation mechanisms descriptions by fungi, knowledge of the cellular structures and/or biomolecules responsible for and/or involved in the metallic NPs mechanism formation, whether for intracellular or extracellular biosynthesis for the great diversity of organisms used, including the AgNPs formation mechanism by fungi, is still not entirely clear.

There are many molecules, intra and extracellular, besides the inorganic molecules that are reaction medium part, which may be participating in this reducing metal ions process and forming

nanoparticles (Durán *et al.*, 2011; Hamedi *et al.*, 2014; Hulkoti & Taranath, 2014; Dorcheh & Vahabi, 2016; Park *et al.,* 2016; Prasad *et al.*, 2016).

It is known that a large number of microorganisms (bacteria, fungi, including yeast and algae) can reduce metal ions through a metal exposure regulatory mechanism. The biosynthesis of these NPs occurs through the metal ions insoluble complexes formation, both intra and extracellularly (Park *et al.*, 2016).

Many of these biochemical reactions have been described as part of microbial resistance mechanisms for cellular detoxification that involve changes in the inorganic ion solubility by enzymatic reduction and/or precipitation of soluble toxic nanostructures into insoluble non-toxic nanostructures. Extracellular and intracellular biocatalytic synthesis (with possible excretion) mainly involves oxidoreductase enzymes (e.g., NADH-dependent nitrate reductase, flavoprotein α-subunit, NADPH-dependent sulfite reductase, and cysteine desulfhydrase) and cellular transporters (Grasso *et al.*, 2019). The AgNP synthesis can occur in different organisms, whether by microorganism or plant extract, due to the presence of various substances such as carbohydrates, fats, proteins, enzymes and coenzymes, flavonoids, phenols, terpenoids, alkaloids, among others, which can donate electrons and reduce metal ions. This process depends on the organism and the culture medium used. (Srikar *et al.*, 2016; Qamar & Ahmad, 2021).

Several hypotheses are presented for the action of the mechanisms using the metabolites formed during the different fungal strain cultivation, but three components have been identified. The main are nitrate reductase enzyme action, electron transfer by quinones, or both mechanisms (Unkles et al., 2004; Hulkoti & Taranath, 2014; Alghuthaymi *et al.*, 2015, Durán *et al.*, 2015, Srikar, 2016).

NADH-dependent nitrate reductase has been accepted by most researchers as the agent in metallic NP formation. This enzyme catalyzes the nitrate to nitrite conversion in the nitrogen cycle by enzymes from relevant microorganisms. Concerning fungi, nitrate reductase is responsible for NP extracellular synthesis through the metal ions biological reduction (Phanjom & Ahmed, 2015). According to a review by Hulkoti & Tanarath (2014), researchers carried out FTIR analyses and found molecules from the carboxyl group, primary and secondary amines, and differentiated amides, which can be a biological reduction part process and provide stability to NPs.

However, more recent studies have shown that other biomolecules may be present in the NP extracellular synthesis reactions by fungi, such as NADH-dependent reductases, and nitrate-dependent

reductases (Zomorodian *et al.*, 2016; Baymiller *et al.*, 2017), quinones electron transport (Gudikandula *et al.*, 2017), and small molecules such as amino acids, cofactors, and glucose-based substances. Furthermore, there are hypotheses that both the synthesis and nanoparticle stabilization are achieved by molecules secreted (enzymes) by fungal metabolism. Stabilization plays an important role in nanoparticles, preventing aggregation and conferring specific properties, however, the identification of these enzymes and other molecules responsible for synthesis and stabilization in fungi is still incomplete, even because there is a wide variety of genera and species with the capacity to synthesis in different cultivation media (Grasso *et al.*, 2019; Šebesta *et al.*, 2022).

While in intracellular biosynthesis metallic NPs, these compounds are formed by the ions transport into the cell, requiring energy, the extracellular process may involve the metallic ions capture on the cell surface or metabolic interaction, which may be the aforementioned enzymes, or another biological molecule released in cell-free broth or extract. However, the identification of what these biological agents are and how they are present in the system is not clear. (Guilger-Casagrande & de Lima, 2019; Salem, 2023).

The *Fusarium oxysporum* use (Kumar *et al.*, 2007) and *Rhizopus oryzae* (Binupriya *et al.*, 2010) showed preliminary results in the reaction medium, with the nitrate reductase protein presence. The tests were based on the AgNP formation using electron transport inhibitors or not. The research conducted by Jaidev & Narasimha (2010) obtained stable and dispersed NPs by using the aqueous extract of *Aspergillus niger* biomass for the AgNPs biosynthesis. The authors suggest that the proteins present in the aqueous extract are responsible for the AgNP formation and the proteins that are adhered to the NP surface act as coating and stability agents. A similar hypothesis was presented by Ahmad *et al.* (2003) for *Fusarium oxysporum* strains and by Kalimuthu *et al.* (2008) for *Bacillus licheniformis* for NADH-dependent nitrate reductase, responsible for the AgNPs formation.

During a study to verify the nitrate reductase efficiency, Gholami-Shabani *et al.* (2014) purified the enzyme produced by *F. oxysporum* IRAN31C, and obtained biologically active spherical NPs with a 50 nm average size. The authors suggest that this way NPs producing with purified enzymes is more advantageous than producing them with fungal biomass or fungal extract. In this method, the preparation steps are slower; the enzyme can be immobilized and recycled to produce other NPs, and downstream process costs are reduced.

As reported by several authors (Gholami-Shabani *et al.,* 2014; Hulkoti & Taranath, 2014; Alghuthaymi *et al.*, 2015; Qazi & Javaid, 2016; Srikar, 2016), nitrate reductase has been widely used and advocated for metallic NP biosynthesis, in biomass form, fermentative broth or aqueous biomass extract, with different fungi strains. Hamedi *et al.*, 2017 studied nitrate reductase action in AgNP synthesis with the fungus *Fusarium oxysporum* (PTCC 5291), a strain recognized to produce this enzyme, they found that the greatest synthesis was achieved at the beginning growth stationary phase, where it was also determined the highest enzyme activity in the cell-free extract.

A considerable number of studies using different techniques have been carried out to elucidate the enzymatic action mechanisms, proteins, and other substances that may be present and act in the NPs formation.

However, little elucidation has been achieved addressing the mechanisms and applications for the NPs biosynthesis in a purified form with possibilities for production on an industrial scale. The scientific community has made it clear that this is still an open question and a major challenge for science. The proposal still on the agenda is that metallic nanoparticle biosynthesis by microorganisms is briefly defined by three different mechanisms: enzymes and proteins with reducing power, exopolysaccharides, and quinones that act in electron transport (Gahlawat & Choudhury, 2019). Data for sizing is scarce. An example of the proposed mechanism is shown in Figure 4.

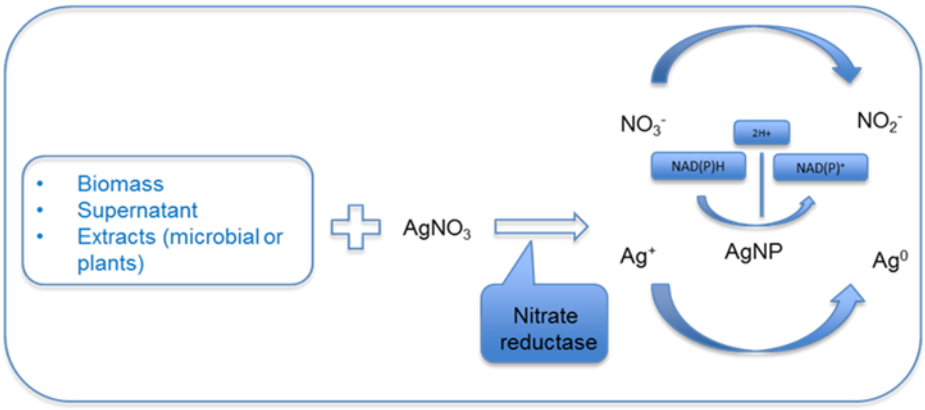


Figure 4 - Synthesis mechanism representation proposed by Alghuthaymi *et al.*, (2015).

Despite a lot of reports on the AgNP biosynthesis efficiency and other metals, especially fungi, there is a consensus that there is still no plausible explanation of the exact action mechanism of different molecules present in reactional media. It is suggested that studies be aimed at a deeper knowledge of molecular biology and genetic aspects involved in microbial biosynthesis, the characterization, and the

identification of the enzyme in the process. A complete understanding of the biosynthesis mechanism can lead development of a large-scale production process to safely obtain quality products. (Grasso *et al.*, 2019; Gahlawat & Choudhury, 2019; Guilger-Casagrande & de Lima, 2019).

2.5 Biological Synthesis Process

Considering that the AgNP biosynthesis is mediated by nitrate reductase and other possible identifiable molecules in fungal extracts or biomass, the cultivation and production conditions of these enzymes are a crucial factor in the productivity, yield, and quality of obtaining AgNPs (Durán *et al.*, 2011; Qazi & Javaid, 2016). However, most published articles focus on NP biosynthesis and not on the process of obtaining the enzyme or identifying other molecules present in the reactional medium, with reducing capacity and that is participating in this synthesis.

Assuming that fungi are major extracellular enzyme secretors and based on other processes of this nature, obtaining high yields in enzyme production is a great possibility (Adebayo *et al.*, 2021). However, as in other biotechnological processes, the NPs biosynthesis can be affected by both chemical and physical parameters involved in process, as well as the pH action, temperature, the cultivation medium composition, reaction time, biomass concentration/ enzyme, metallic species and colloidal conditions interactions that control size, shape, location and dispersion of formed NPs (Durán *et al.*, 2011; Alghuthaymi *et al.*, 2015; Qazi & Javaid, 2016).

It is important to highlight that there is no reference to a scale-up process to produce nitrate reductase or other molecules that are described as reducing agents, as well as for the AgNPs biosynthesis process or other metallic NPs by biotechnological means, independent of the organism used. Due to the challenges reported in (2.4 Metallic NPS Biosynthesis Mechanisms), the biotechnological process AgNP biosynthesis development on a scale has some challenges to be overcome, among which we can highlight that primarily the enzyme complex composition identification and/or molecules involved in the AgNPs biosynthesis and understanding the mechanisms are essential to understand some factors to be studied. After this challenge, the application of reaction complex optimization on a production scale and understanding of physical process parameters influence the AgNPs biosynthesis.

Regarding the biotechnological process development for AgNP production, or any other metallic NP, there are significant advantages for the scientific, industrial, and technological environment. AgNPs are already one of the most industrially produced and have a considerable range of applications, mainly in the health sector.

The NPs extracellular biosynthesis possibility presents operational and economic advantages such as cost reduction, without cell rupture steps and recovery of NPs (intracellular case), as well as obtaining a "green process" with a product with specific properties such as biocompatibility and diverse applications. The process can be carried out using cell culture broth or aqueous biomass extract, under pH, temperature, and agitation, controlled conditions. A succinct presentation of process steps can be seen in Figure 5.

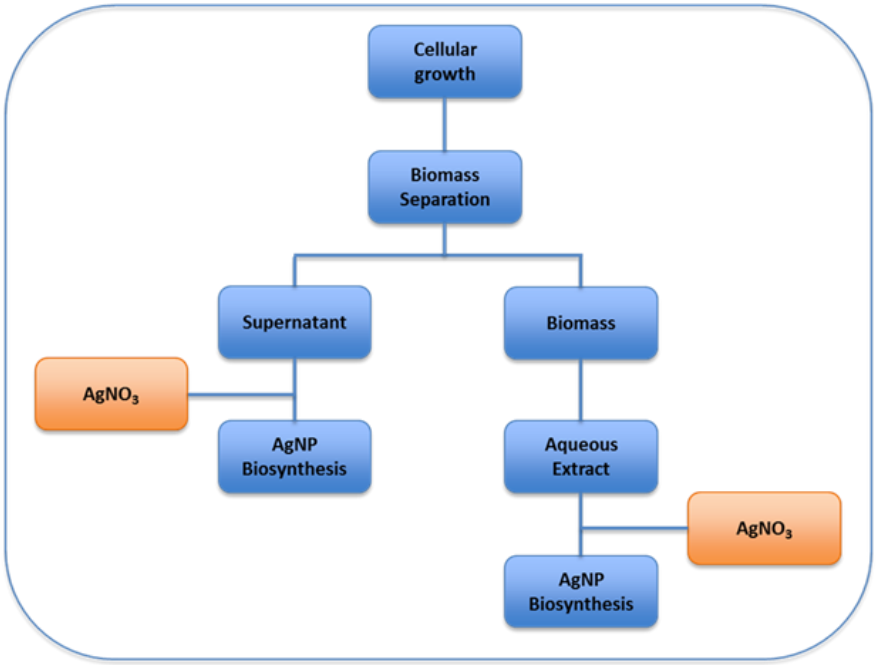


Figure 5 - Simplifies the scheme of biosynthesis process steps using a microorganism.

2.6 Antimicrobial and Cytotoxic Activity

It has long been known that ionic silver exerts antimicrobial activity for microorganisms a wide variety, binding to the bacterial cell wall, causing structural and morphological change, with alteration in DNA/RNA leading to cell death. Its usefulness has already been proven in some applications such as ointments and other formulations with action in minimal concentrations (Galatage *et al.*, 2021). In this way, the AgNPs used for antimicrobial action are no different. Factors such as size, surface area, redox potential, and composition can be relevant in the intended action, in addition to these, the protein corona composition and agglomeration characteristics can also determine the AgNP biological reactivity. (Ahmed *et al.*, 2017)

The AgNP action mechanism on microbial development is described as consisting of two stages, in the first, there is the AgNP stabilization according to the environment through dissolution processes,

aggregation, redox reactions, and release of Ag+ ions, and in the second stage, is where the interaction of Ag+ ions with cell walls occurs, resulting in the functions proteins essential inhibition for microbial growth, leading to cell death. (Kailasa *et al.* 2019). In recent years, work reporting possible antimicrobial action mechanisms has intensified, however, there is still no clarity on these mechanisms, and the action is attributed to previous knowledge acquired. (Durán *et al.*, 2016; Pareek *et al.*, 2018; Estevez *et al.*, 2019; Ahmad *et al.*, 2020).

Hypotheses are inferred for the AgNP's antimicrobial action such as that Ag+ ions have a high affinity to bind to bioactive molecules that contain -NH_2, phosphates, and mercapto, leading to an almost covalent bond. Low levels of Ag+ ions are highly sensitive to interfering with cellular functions thus causing death. The ROS formation (reactive oxygen species) by photochemical oxidation with water, forms highly oxidizing free radicals, causing oxidative stress that also interferes with the cell protein functions, leading to cell death. And the smaller the particle, its surface area – volume ratio modifies the physical-chemical properties and can increase the action. In addition to the attraction of positive and negative charges between Ag ions and the cell wall. (Marambio-Jones & Hoek, 2010; Barngrover & Aikens, 2011; Kailasa *et al.*, 2019; Gumel *et al.*, 2019).

From the point of view of the composition arising from the manufacturing process, it is a fact that AgNPs resulting from biological processes are less likely to have adsorbed toxic compounds than those of chemical origin, contributing to toxicity reduction (Kummara *et al.*, 2016). It is also possible to verify that there is a differentiation in the antibacterial activity between Gram-positive and negative bacteria, which may be related to the cell wall composition of these different microorganisms (Alsharif *et al.*, 2020). Studies indicate that Gram-negative bacteria are more prone to the AgNP action because they have thinner cell walls than Gram-positive bacteria. (Ahmad *et al.*, 2020).

Several procedures to determine antimicrobial activity can be used, however, many results for determining antimicrobial activity have been performed by agar diffusion against target microorganisms. It is known that this type of procedure does not specify the action mechanism and does not consider the physical nanoparticle properties but infers the AgNP antimicrobial action. Estevez *et al.*, (2019) using Transmission Electron Microscopy (TEM), Environmental Scanning (ESEM), and Confocal Raman techniques, verified the presence of AgNPs (produced by *Penicillium expansum*, 15 nm – 40 nm) in the wall of *E. coli* and the wall cell rupture.

A vast number of publications have been reported on the AgNP antimicrobial action on most distinct species of microorganisms, the majority of which are bacteria. Some examples are presented in Table 2 below.

Table 2 - Fungal species used for the biosynthesis of AgNPs and targets for antimicrobial activities.

Microorganism	Size (nm)	Target	Reference
Trichoderma spp.		*S. aureus* ATCC 6538, *E. faecalis* ATCC 29212 *E. coli* ATCC 8939, *P. aeruginosa* ATCC25853	Ramos *et al.,* 2020
F. scirpi	2 – 20	E. coli (Antimicrob.Uropathogenic)	Rodriguez-Serrano *et al.,* 2020
P. kudriavzevii HA-NY2 S. uvarum HA-NY3	12.4 – 30	*B. subtilis* ATCC 6633, *S. aureus* ATCC 29213 *P. aeruginosa* ATCC 27953, *C. tropicalis* ATCC 750, *F. oxysporium* NRC21	Ammar *et al.,* 2021
C. zeylanium	76	*S. aureus* *E. coli* *P. aeruginosa*	Ranjani *et al.*, 2020
P. polonicum	10 – 15	MDR *Acinetobacter baumannii*	Neethu *et al.*, 2018
R. sativus	4 – 30	*B. subtilis (Methicillin resistant)* *S. aureus* *E. coli* *S. marcescens*	Singh *et al.*, 2017
Penicillium aculeatum	4 – 55	*E. coli* *P. aeruginosa* *S. aureus* *B. subtilis* *C. albicans*	Ma *et al.,* 2017
Pleurotus ostreatus	10 – 40	*B. subtilis* *B. cereus* *S. aureus* *E. coli* *P. aeruginosa*	Al-Bahrani *et al.*, 2017
Rhizopus stolonifer	91.24	*B. subtilis* ATCC6633 *S. aureus* ATCC6538 *S. typhimurium* ATCC14028 *P. aeruginosa* ATCC9022 *E. coli* ATCC8739	Alsharif *et al.*, 2020
A. flavus (F10, NCBI accession no. KC911631.1) *E. nidulans* (APF4, NCBI accession no. KC911632.1)	98.92	*E. coli* (MTCC-739) *P. aeruginosa* (MTCC-741) S. *aureus* (MTCC-96)	Barapatre *et al.,* 2016
T. longibrachiatum	24.43	*F. verticillioides* *F. moniliforme* *P. brevicompactum* *H. oryzae* *P. grisea*	Elamawi *et al.*, 2018

Table 2 – continued

Microorganism	**Size (nm)**	***Target***	**Reference**
A. oryzae NRRL447	109.6	*B. mycoides*	Elshafei *et al.*, 2021
G. enigmaticum *T. ljubarskyi*	15 – 25 5 – 40	*B. subtilis* (MTCC 441) *S. aureus* (MTCC 96) *M. luteus* (KUCCC 4) *B. cereus* (KUCCC 7) *B. megaterium* (KUCCC 9) *E. coli* (MTCC 443) *E. aerogens* (KUCCC 12) *K. pneumoniae* (MTCC 109) *S. typhimurium* (MTCC 98) *P. vulgaris* (MTCC 426) *P. aeruginosa* (MTCC 424) *S. paratyphi* (KUCCC 15)	Gudikandula *et al.,* 2017
A. oryzae MTCC No. 3107	40	*S. sclerotiorum* MTCC 8785	Gupta & Saxena *et al.,* 2022
P. radiatolobatum	5.09 – 24.85	*S. aureus* *S. enterica* *B. cereus* *E. coli* *L. monocytogenes*	Kumar *et al.*, 2021
A. terreus BA6	15.82	*E. coli* (ATCC 8739) *P. aeruginosa* (ATCC 9027) *S. typhimurium* (ATCC 14028) *S. aureus* (ATCC 6538) *S. epidermidis* (ATCC 12228) *S. faecalis* (ATCC 10541) *S, aureus* (MRSA) (ATCC 43300) *L. monocytogenes* (ATCC 19111) *A. hydrophila* (ATCC 35654) *C. albicans* (ATCC 10231) *A. niger* (ATCC 16404)	Lofty *et al.,* 2021
T. harzianum *G. sessile*	9.6 - 19.1 5.4 - 8.9	*E. coli* *P. aeruginosa* *S. aureus*	Murillo-Rábago *et al.*, 2022
A. fumigatus DSM819	10 - 34	*E. coli* *B. mycoides* *C. albicans*	Othman *et al.*, 2019
A. fumigatus BTCB10	7.22	*S. aureus* *E. coli,* *K. pneumoniae* *B. subtilis*	Shahzad *et al.*, 2019
B. bassiana	10 - 50	*E. coli* *P. aeruginosa* *S. aureus*	Tyagi *et al.*, 2019

Some authors have also tested antimicrobial action using antibiotic-resistant bacteria as targets (Singh *et al.*, 2017; Ranjani *et al.*, 2021), human pathogens in the biofilms formation (Barapatre *et al.*, 2016; Rodriguez-Serrano *et al.*, 2020); synergistic effect with antibiotics (Hafez *et al.*, 2017); plant pathogens (Almeida *et al.*, 2021), 3rd stage larvae of *Aedes aegypti*, dengue vector (Alsharif *et al.*, 2020), phytopathogenic fungus (Elamawi *et al.*, 2018; Gupta & Saxena, 2023;), human pathogenic fungi (Wang *et al.,* 2021). Elamawi, *et al.,* (2018) and Gupta & Saxena (2023) also tested the action of AgNPs against phytopathogenic fungi. In addition to the variety of challenges with bacteria and fungi, AgNPs have been challenged in in vitro cytotoxicity and genotoxicity tests.

Wypij *et al.*, (2020), using RAW264.6 murine macrophages and MCF-7 breast cancer cells, demonstrated that cytotoxicity was dependent on the nanoparticles concentration used (1 – 64 ug/mL) with viability results for RAW 264.7 cells between 72.1 ± 2.7% and 12.7 ± 3.0% while for MCF-7 cells it was 65.6 ± 1.4% to 16.2 ± 1.3%.

In work carried out by the Alsharif *et al.*, (2020) group, using AgNPs from three different microorganisms (*Bacillus cereus* A1-5, *Streptomyces noursei* H1-1, and *Rhizopus stolonifer* A6-2) they also obtained results similar to those obtained by Wypij *et al.,* (2020), where cytotoxicity was concentration-dependent (0.49 – 1000 ug/mL) in cancer cells (Caco 2) and normal cells (Vero). With different IC50 results for each cell analyzed. The AgNPs synthesized by A1-5, H1-1, and A6-2 presented IC-50 for Vero cells of 290.2 ± 14.7 μg/mL, 511.7 ± 68.5 μg/mL and 259.0 ± 6.4 μg/mL and Caco-2 cells 8.9 ± 0.5 μg/mL, 5.6 ± 3.0 μg/mL, 11.2 ± 0.5 μg/mL, respectively. The cancer cell mitochondrial function was reduced by 50% at the lowest concentrations of AgNPs (5.6–11.2 μg/mL).

Nanoparticles produced by *P. radiatolobatum* were used for cytotoxicity tests against HEK293 cells, which did not show any cytotoxic effect indication, however, it showed effects on human lung carcinoma cells (A549) with IC50 results of 19.67 ± 3 .09 μg/mL. The authors suggest that cell death was induced by ROS (reactive oxygen species) (Kumar *et al.*, 2021).

On the other hand, Wang, *et al.*, (2021) evaluated the proliferative AgNP activity produced by CFF (cell-free fungi) from the fungus *Aspergillus sydowii* against HeLa and MCF-7 cells and the results also show a concentration dependence. At concentrations above 30 μg/mL or 10 μg/mL, proliferation was inhibited for HeLa and MCF-7 cells respectively, thus demonstrating good antiproliferative activity.

Despite so many promising results regarding the biogenic AgNP antimicrobial and cytotoxic action, the actions of these particles are still unclear. Studies that consider, bacterial resistance against

antimicrobial agents have been concerned with the different chemical silver forms (cationic, metallic, and oxides) and their actions. These diverse forms may present differences when in contact with the biological and environmental environment. However, according to Pareek *et al.,* (2018), this contact can reduce AgNP and Ag+ ions efficacy antimicrobial. Current studies have focused on cell interactions with the physicochemical properties of nanoparticles, and a deeper understanding of this action at transcriptomic and proteomic levels has not yet been established. Aiming at products with this specificity, the authors suggest that for a better understanding of the AgNP action in these areas, they are essential for determining the materials composition for antimicrobial action, especially when intended for medical use.

2.7 Impact and Toxicity

A vast number of scientific groups have dedicated themselves to improving the synthesis of nanoparticles both chemically and biologically, however, the issue of toxicity has become present as the results of the technology have advanced. As previously reported (2.5 Biological Synthesis Process), biological synthesis technologies require clarification for the production implementation, as the mechanisms have not been elucidated, and the impact of using these nanoparticles on the environment and living beings remains nebulous.

In recent years, the medical field has faced great antibiotic resistance, opening a window of opportunities for potential products with antimicrobial action. In this way, silver, which has long been used with this property, in association with new nanoscience technologies, has intensified its studies for the synthesis and uses of silver nanoparticles and their actions.

In vitro, tests with chemical-origin nanoparticles are used in most of the work to understand the toxic AgNP actions. The newest and most comprehensive theory to explain the AgNP deleterious effects focuses on the chemical/biochemical and physicochemical reactions in which they can participate. These reactions consider the dissolution, aggregation, and adsorption of other molecules, which are also related to the size, shape, concentration, exposure time, and target cell (Pareek *et al.*, 2018; Jorge de Souza *et al.*, 2019; Rajan *et al.*, 2022). However, there are many reports that many toxicity-inducing reactions point to ROS generation, oxidant protection system collapse, and damage to the mitochondrial membrane, leading to DNA impairment, proteins, and lipids essential for cell survival (Durán *et al.*, 2016; Jaswal & Gupta, 2023).

Akter *et al.*, (2018) in a systematic review to evaluate the induction of AgNP cytotoxicity in various animal, human, and tumor cells, found that particle size, concentration, aggregation, type of coating, and

its physical/chemical properties, and the environment are identified as factors for inducing cytotoxicity and genotoxicity. Each of these characteristics can act independently or together with toxic action. Barbasz *et al.*, (2017), when testing three different types of AgNP nano (positive, negative, and stabilized charges) in tumor cells, histiocytic lymphoma (U-937) and human promyelocytic cells (HL-60), concluded that the nanoparticles surface charge effects combination, the released silver ions, and the corona molecules biological activity act together for the toxic effects on cells. The AgNP with greater dissolution characteristics caused greater toxicity to tumor cells while the stabilized AgNP, despite lower toxicity, still caused irreversible damage to cells.

Several medical and therapeutic devices have been reported to be beneficial to health with antimicrobial, antiviral, antifungal, anticancer, antiproliferative, and several other actions, which can have a positive impact on living beings. However, clinical trial work (Sousa, 2018; Medici *et al.,* 2019) and microbial resistance against AgNP have not yet been reported. Regulations for use dosage are still nonexistent and the application practice in random concentrations may result in the resistance of microorganisms, constituting a concern for public and environmental health (Pareek *et al.*, 2018).

Concerns focus on the lack of AgNP action mechanisms clarification in the most diverse applications, with priority for medical and environmental areas. Although there is vast knowledge of the toxicity of chemical origin AgNP, there is still controversy in the results. (Sharma *et al.,* 2019; Tortella *et al.*, 2020). Several studies have used nanoparticles (chemical origin – NM-300 and NM 300 K) previously characterized and certified by the OECD (Organization for Economic Co-operation and Development), however, biological origin AgNP, despite presenting satisfactory results in several applications and being considered less toxic for use in living beings, have not been compared to those considered references. (Rajan *et al.*, 2022).

So-called biogenic nanoparticles, despite positive and negative results concerning toxicity for well-developed unicellular and multicellular beings, such as mice, and presenting synergistic effects with antibiotics, the results are still too scattered and unconvincing to guarantee application indeed, of actions against bacteria, fungi, and even tissues with different concentrations, different methodologies, and diverse synthesis and reducing agents types, thus demonstrating that there is a gap to be filled in these products application. In this sense, it is also worth highlighting that although biological synthesis produces particles with a protein coating (protein corona), which has resulted in a reduction in toxic effects, however the interactions that may occur in the environment are still worrying, as the products of these processes are unknown reactions, which can be just as harmful (Levard *et al.*, 2012; Jorge de Souza *et al.*,2019).

The complex environments for which they are intended can determine the morphology transformation, bioavailability, and toxicity due to interactions with organic matter, cations, pH, and oxygen differentiation, thus altering their surface properties of Ag+ ions release, increased aggregation in environments with high ionic strength and stabilization by organic molecules (corona protein) adsorption. These changes can be either beneficial or harmful for the organisms/cells to which AgNP activity is proposed (Akter *et al.*, 2018; Zhang *et al.*, 2018; Jorge de Souza *et al*, 2019). Considering the possibilities of nanoparticle action and insertion in today's world, we must pay attention to the different physical/chemical characteristics of these ecosystems, when on a liquid or solid basis. In the biological context, we have extremely complex environments, from the composition and properties of the point of view, and in the environmental area, we also find environments with different complexities, such as fresh water, salt water, mud, oxygenation, anaerobiosis, etc. The interactions between these environments will be diverse, resulting in interference actions for each environment. (Zhai *et al.*, 2016; Calderón-Jimenez *et al.*, 2017; Zhang *et al.*, 2018).

Studies carried out by Ramachandram *et al.*, (2018) demonstrated that biogenic AgNP (plant) may present greater toxicity than biogenic AuNP in adult zebrafish exposed for 14 days with AgNP doses (12.25 μg/L – < 30 nm) and AuNP (20.5 mg/L – 200 – 500 nm). The tissues analyzed showed greater ROS production, morphological changes, and enzymatic changes when exposed to AgNP compared to those with AuNP. On the other hand, studies carried out with AgNP (bacteria) (11 – 75 nm) in male Wistar rats using doses of 97.64 – 132 mg/kg in two application routes, intravenous and intraperitoneal, concluded that the intraperitoneal route presented moderate toxicity up to the 14th day of observation, while intravenous did not show any toxicity. The tissues analyzed by biochemical and histopathological techniques showed no changes when challenged with IC50 doses and total lethal concentration, concluding that the doses used by these routes are not toxic for this animal (Ashajyothi & Chandrakanth, 2019).

The toxic effects were evaluated in HeLa tumor cells (adhesive cells) and U937 (cells in suspension) by Kaba & Egorova (2015) with chemical origin AgNPs (13.2 ± 4.72 nm) in the ranges of 0.5 – 8 .0 μg Ag/mL. The results point to toxic effects at concentrations of 0.5 – 2.0 μg Ag/mL for both cells that were tested. The authors concluded the toxic effects are surface charge reactions combination, released Ag+ ions, and biological particle coating activity.

Using a genomic next-generation sequencing approach on an Illumina platform (High-Throughput SuperSAGE), the silver toxicity in different forms, being AgNP, bulk, and ionic, was evaluated in zebrafish

(*Danio rerio*), the results indicated that the toxicity caused by AgNP is mainly associated with Ag+ ions, as gene changes were found for all silver forms evaluated, mainly in oxidative phosphorylation and protein synthesis (van Aerle *et al.*, 2013).

Male Wistar rats were subjected to treatment with AgNP (chemistry – 10 nm) and AuNP at 1 μM and 2 μM concentrations orally for 14 days to investigate the toxic effects on erythrocytes and tissues. The results demonstrated that there was an increase in ROS formation and the oxidative enzymes absence in erythrocytes and tissues with dose-dependent effects, with AgNP being more toxic than AuNP (Shrivastava *et al.,* 2016)

Salas *et al.*, (2019) evaluated the impact of toxically active ionic silver in vivo assays of *Chlamydomonas reinhardtii* (unicellular green algae) looking for changes in photosynthetic performance. Three silver levels were tested: 59%, 34%, and 7% of total Ag stabilized with casein. The results obtained suggest that casein played a significant role in mitigating silver toxicity in all experiments. The best results were obtained with an intermediate concentration of 34%.

Vogt *et al.*, (2022) sought to understand the toxicological effects of dissolving AgNP (NM-300 K - 14.4 ± 2.5 nm) in three different media (ASTM very hard water, ISO standard dilution, and lake water) and the effects on embryos whitefish (*Coregonus viveretus*) and cockroach (*Rutilus rutilus*). It was observed that the silver dissolution in the test media was not as reported in other experiments, despite the ionic strength of the media and medium regardless, *C. lavaretus* showed greater sensitivity after 8 weeks at concentrations of 0.51 – 0.73 mg/L compared to *R. rutilus* which only showed adverse effects at 5 mg/L in lake water. Like these results, other authors also found that there is a differentiation in the AgNP different particle sizes dissolution and water types (Dobias & Bernier-Latmani, 2013; Zhang *et al.*, 2018).

Zhang & Wang (2019) used the unicellular alga *Euglena gracilis* as a study model to verify the Ag dissolved toxic influence from AgNP (20 and 60 nm). It was observed that the smaller particles dissolved quickly, exhibiting greater toxicity, evidenced by the lower EC50 concentration. However, the Ag+ concentration was comparable in all situations, including when using $AgNO_3$ with similar subcellular distribution patterns in AgNP and algae exposed to $AgNO_3$. The authors suggest that Ag+ dominated the toxicity extracellularly.

Many results presented on the AgNP toxic effects attempt to explain their effects by the ions released by reactions with the environment in which they are found, however, it is also suggested that

nanoparticles functionalized or stabilized with protein molecules or others provide stability, reducing the agglomeration actions and can restrict toxicity actions towards microorganisms or target cells.

Hashmi *et al.*, (2019) were able to verify in vivo tests with mice that particle size had an impact on animals. Particles larger than 50 nm accumulate in the spleen and liver causing long-term cytotoxicity, while smaller particles can be eliminated in the urine according to Gurunathan *et al.*, (2013). On the other hand, the bionanoparticles used for the tests had favorable results in decreasing toxicity, as they were synthesized from plant extracts with an abundance of phenolic compounds that gave these AgNP antioxidant properties. AjNP (*Aerva javanica*) presented 76.1% antioxidant potential, attributed to the abundant flavonoid coverage, with only 16% mortality, presenting the potential for use in the medical field. These results corroborate the theory that the corona composition in biogenic AgNP can determine the interaction with organisms and their applications.

Considering the results of several investigations and several years of silver nanoparticle use in various environments, toxicity still has a knowledge gap due to contradictions. The Ag+ toxicity release remains debatable (health and the environment are still unclear, regardless of the number of publications existing (Akter *et al.*, 2018). The unregulated different concentrations used in commercial products, especially in the medical field, can lead to microbial resistance development against AgNP, in addition to the need for systematic studies to clarify the antibacterial mechanism of AgNP (Pareek *et al.,* 2018).

The properties inherent to AgNP provide an enormous range of applications in the different engineering worlds, however, the look at the application in the medical area has gained greater attention. Therefore, associated nanotoxicology studies are important, requiring appropriate models, analyses, efficient techniques, and above all, relevant regulations, and protective measures. The coating (protein corona), understanding both for biogenic and chemical AgNP, also needs to be better understood, as its interactions with the application environments, despite the hypothesis that biogenic AgNP is biocompatible due to the composition of their crown and provides lower toxicity (Goel *et al.*, 2023).

2.8 Protein Corona - Coating

The thermodynamic stability (lower aggregation potential) and biogenic nanoparticles biological activity are associated with a surface coating layer composed of biological molecules (peptides such as glutathione, metallothionein, membrane-associated proteins, etc.), which may have participated in synthesis, as well as in adsorption on their surfaces called “protein corona” (Bulgarini *et al.*, 2021). In nanoparticles obtained by chemical and physical methods, this coating is carried out in an additional step

using surfactants and polymers to nanoparticles functionalize (Mout *et al.*, 2012). In nanoparticles obtained by the biogenic method, this step occurs simultaneously with the protein layer formation on the nanoparticle surface, which is an advantage from the point of view of the production process, in addition to the molecule biocompatibility. (Mohanta *et al.,* 2018). Although several studies have been published trying to identify and characterize these biomolecules and their interactions with nanoparticles, there is still not enough data for a broad understanding of this composition. (Chowdhury *et al.*, 2014; Grasso *et al.*, 2019; Guilger-Casagrande & Lima, 2019; Dúran *et al.*, 2023).

With the increase in the search for economical and environmentally correct methods for AgNP synthesis, one of the most used in various economic sectors, studies on silver nanoparticle biosynthesis have stood out using the most varied of biological species for this synthesis, such as bacteria, yeasts, various fungi, and plant extracts. Several of these studies attempt to elucidate the synthesis mechanism and the so-called protein corona. As fungi are considered to hold great promise for AgNP biological synthesis, their biomolecules also potential for providing a coating to nanoparticles, providing increased stability, inhibiting agglomerations, and helping to increase biological activity (Guilger-Casagrande & Lima, 2019; Gahlawat & Choudhury, 2019; Priya *et al.*, 2021).

One of the first studies to present the possible protein presence in the AgNPs surface layer was by Chowdhury *et al.*, (2014) which presents a band in gel electrophoresis measuring 85 KDa, attributed to the corona protein of nanoparticles synthesized by *Macrophomina phaseolina*. In a similar work, Fageria *et al.*, (2017) also identified three bands in gel electrophoresis at 65, 55, and 50 kDa as proteins in the AgNP coating synthesized by CFF from *P. shear*. Other authors have also presented the identification of proteins/enzymes participating in the synthesis and coating process of AgNPs in distinct species of fungi (Guilger-Casagrande & Lima, 2019; Priya *et al.*, 2021).

Guilger-Casagrande & Lima (2019), to demonstrate the protein corona importance in AgNP synthesized from CFF of *Trichoderma harzianum*, subjected the NP to a procedure to eliminate the coating and found that NP without the coating was smaller in size compared to those coated ones, and by FTIR, biomolecules functional groups, protein bands and the hydrolytic enzymes NAGase, β-1,3-glucanase, chitinase and acid protease from *T. harzianum* were identified in the coating, indicating that these compounds originate from the microbial growth metabolic reactions.

Gudikandula and collaborators (2017) synthesized silver nanoparticles with the strains *Trametes ljubarskyi* and *Ganoderma enigmaticum* and through FTIR identified primary amine and amide, aromatic

amine, on the surface of AgNP, confirming the presence of proteins. Sanghi & Verma (2009) with AgNP mediated by *Coriolus versicolor* also obtained equivalent results with FTIR spectroscopy in identifying compounds that confirm the presence of proteins on the surface of the nanoparticles.

Rhizopus stolonifer A6-2, *Bacillus cereus* A1-5, and *Streptomyces nourcei* H1-1 isolated from soil samples (Egypt) were challenged in CFF in AgNPs synthesis and presented UV-vis bands at 420 nm, AgNPs formation characteristic. In these same nanoparticles, they verified by FTIR that distinct species present different compositions in protein corona, depending on their excretion metabolism or wall conformation, in TEM images, the protein formation on the nanos surface was visible (Alsharif *et al.*, 2020).

Spagnoletti *et al.*, (2021) used proteomics to determine the proteins present in the NP coating synthesized by CFF from the *Macrophomina phaseolina* strain. It was identified that 60% of this strain's proteins are hydrolytic and form the nanoparticle protein corona. The proteins detected in the CFF showed 53% correspondence with the hydrolytic enzymes, corroborating the corona composition results.

Some authors also used the Raman spectrometry technique and verified the amino group presence, and covalent bonds of N-Ag, S-Ag, and O, in addition to other bands that can be attributed to amino acid residues such as L-alanine, L-valine, and cysteine in nanoparticle protein corona synthesized by biological material from various fungi species. (Ballottin *et al.,* 2016; Estevez *et al.,* 2019; Estevez *et al.,* 2020). In the AgNPs characterization synthesized by *Aspergillus tubingensis*, it was observed that in addition to the plasmonic resonance band at 440 nm, it also presented a band at 280 nm that is characteristic of tyrosine, tryptophan, and/or phenylalanine residues in fungal proteins (Ballottin *et al.*, 2016).

Many other studies report the presence of substances characteristic of proteins (primary amines, amides, aromatic and aliphatic amides, polysaccharides) linked to AgNP surface, in the most diverse microorganism species. *A. flavus* (F10, NCBI accession no. KC911631.1) and *E. nidulans* (APF4, NCBI accession no. KC911632.1) were reported to degrade lignin (Barapatre *et al.*, 2016); *Ganoderma enigmaticum* and *Trametes ljubarskyi* (Gudikandula *et al.*, 2017) *A. oryzae* MTCC 3107 and *S. sclerotiorum* MTCC 8785 (Gupta & Saxena, 2023); *G. sessile* (Murillo-Rábago *et al.*, 2022); *A. fumigatus* DSM819 (Othman *et al.* 2019); *Phomopsis liquidambaris*, an endophytic fungus isolated from *Salacia chinensis* (Seetharaman *et al.*, 2018); *Aspergillus fumigatus* BTCB10, isolated from an industrial drain

(Shahzad *et al.*, 2019); *Fusarium oxysporum* isolated from the medicinal plant *Withania somnifera* (Vijayan *et al.*, 2016); *Penicillium radiatolobatum* (Kumar *et al.*, 2021).

Despite the substantial number of reports of substances extracted from fungal biomasses presence that can reduce silver and form varying sizes of nanoparticles, it can be deduced that these molecules are different for each microorganism and that they have their characteristics for both reductions as well as protein surface formation. Some authors attribute covalent bonds to the molecules, but in short, there is no clarification of who these molecules are and what these bonds are like between the metallic particle and the molecules present in the synthesis substrate.

Studies carried out on AgNPs chemically synthesized (defined medium/substrate) to verify the protein corona formation (functionalization) from known proteins indicate that there is constant competition between proteins for adsorption on the particle surface, where high-affinity biomolecules will replace those with lower mobility and affinity in surface binding, based on the Vroman effect (Nguyen & Lee, 2017). In this way, it can be said that protein affinity is crucial for the protein corona formation and composition (Baimanov *et al.*, 2019). From the point of view of biogenic nanoparticles, this knowledge becomes more complex as we do not know the protein composition of the substrate and which molecules participate in protein corona synthesis and formation since the agents are varied.

In studies on chemical nanoparticle synthesis, techniques such as isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) have been used to determine the bonds and quantities of molecules adsorbed on the particle surface. To identify adsorbed proteins, SDS-PAGE and mass spectrometry techniques have been used (Baimanov *et al.*, 2019).

This coating is attributed to increasing the nanoparticle size and dispersion depending on the protein layer formed size (Piella *et al.*, 2017) and as protection in antimicrobial, cytotoxic, and phytotoxic actions, which ensures that the cell does not have contact directly with the metal but only suffering an action from the metal itself.

In studies for biomedical applications (synthetic nanos), there is a concern with the protein layer, as this may interfere with the expected results. There are two coating types: hard and soft coating. There are still no established criteria to distinguish them due to the lack of specific techniques (Piella *et al.*, 2017; Weiss *et al.*, 2019), however, some studies indicate that the proteins that make up the soft coating may be proteins that are not linked to the particle surface but with less rigid bonds in the molecules that

are hard coating part, which can be switched off depending on the medium they are in, including changes in the zeta potential. (Blundell *et al.*, 2016).

2.9 Applications and Perspectives

As previously reported, silver had been used for hundreds of years before the nanotechnology advent. Its applications were and still are widely used in several areas, mainly in the medical field due to its potential for antimicrobial activity. However, with AgNP emergence, these metallic applications increased substantially, covering several knowledge areas and cutting-edge technology. This increase in the potential silver use with nanotechnology is directly related to the size, surface area, quantum confinement, and shape of NPs, which give them different chemical and physical properties, such as electromagnetism, optical and catalytic properties (Verma & Maheshwari, 2019; Adebayo *et al.*, 2021).

AgNP is used as intercalation (i.e., inserted) materials for electrical batteries, optical receptors, polarizing filters, catalysts in chemical reactions, sensors, bioactive materials, biolabeling, enzyme enhancers in immunoassays, controlled release of drugs, medical devices, cosmetics, food, textile industry, and many others. In the medical field, AgNPs have been used for various antimicrobial purposes (Pereira *et al.*, 2014; Akter *et al.*, 2018; Verma & Maheshwari, 2019; Rana & Parmar, 2022), such as anticancer, anti-inflammatory, wound treatment, and various devices. (Morais *et al.*, 2014; Yadav *et al.*, 2015; Singh *et al.*, 2016; Barabadi *et al.*, 2019; Bamal *et al.*, 2021; Dawadi *et al.*, 2021).

Water treatment is still a major difficulty for less developed countries and can become a public health problem if not carried out properly. AgNPs have been applied in water treatment to carry out the disinfection step in the purification process, whether in a treatment unit (large scale) or water purifiers (small scale) (Fernández *et al.*, 2016; Haider *et al.*, 2016; Rana & Parmar, 2023).

Yu and collaborators (2022) studied the membranes incorporated with AgNP for purification and wastewater treatment. They pointed out the great development achieved in recent years regarding the benefits of using membranes incorporated with AgNP, which when combined with other functional materials act synergistically with a prolonged antimicrobial effect through the controlled release of AgNP and Ag^+, improved hydrophilicity and greater photocatalytic effect.

Other authors have worked to advance AgNP application in wastewater and drinking water treatments, however, these applications have only been tested on a laboratory scale (Moustafa, 2017; Nasrollahzadeh *et al.*, 2021; Khan *et al.*, 2022). There is still concern about the accumulation and residues in systems that could harm human and animal health and the environment. Therefore, one of

the alternatives for wastewater treatment systems has been silver sulfidation to mitigate its toxicity (Khan *et al.*, 2022).

In wastewater, another concern is contamination by dyes used in many industrial processes such as the textile, food, and leather industries. In recent years, legislation has been more stringent, prompting the search for recent technologies to remediate these contaminants so that nanotechnology has stood out in some processes, which have proven to be innovative, efficient, and low-cost. The AgNP is used to remove dyes using the adsorption mechanisms on activated carbon loaded with nanoparticles or photocatalytic degradation, and a combination of both mechanisms can also be used. In a detailed review of AgNP action in dye removal application, Marimuthu *et al.*, 2020 presented a list of 41 publications on AgNP biogenic (plant) demonstrating high-efficiency results in removing various dyes.

Bogireddy *et al*, 2016 studied organic dye degradation, phenol red (PR), and direct blue 24 (DB24) using AgNP biogenic (plant) and obtained satisfactory results. Kulkarni *et al.*, 2018 also used AgNP biogenic with peptide stabilization for Congo red degradation with an efficiency of 92%. Ayele *et al.*, (2021) obtained a degradation of 95.4% with AgNP synthesized by *Acremonium kiliense* in malachite green dye.

With the results presented, AgNP biogenic, compared to synthetic ones has proven to be highly effective as catalysts in the degradation process of dangerous dyes. The degradation efficiency is attributed to its high surface area, high rate of electron migration to NPs surface, accelerated kinetics, and configuration of a more economical process (Jyoti & Singh 2016; Akter *et al.*, 2018).

The textile industry segment has also stood out in the AgNP application to benefit from the antimicrobial properties of these nanoparticles. Principally in the medical field, fabrics with antimicrobial properties are useful against multi-resistant pathogens and opportunistic infections. Due to textile composition characteristics not having functional groups, material composition is necessary for functionalization (dos Santos *et al.*, 2021).

Polyester functionalization studies (PES) with chitosan layers or hexamethyl disiloxane (HMDSO) added with AgNP, obtained stabilization and protection from silver oxidation, also demonstrating control in the ions release and were efficient in the antimicrobial action against *Staphylococcus aureus* and *Escherichia coli* (Ribeiro *et al.*, 2022). Other materials have been reported to have antimicrobial and antiviral properties, such as cotton surgical gowns (Irfan *et al.*, 2017); disinfectant wipes made of polyester, cotton or viscose (Hamouda *et al.*, 2021); face masks, PPE and air filters in polyester/cotton,

cotton, and blends (Ramaiah *et al.*, 2021); N95 face mask (3M 8201 respirators) (Zhong *et al.*,2020); multifunctional nanofiber mats (Karagoz *et al.*, 2021) and polyester athletic clothing (Reed *et al.*, 2016).

AgNP can be incorporated into biodegradable polymers (cellulose, starch, chitosan, agarose) and non-biodegradable polymers (polyethylene, polyvinyl chloride, vinyl alcohol) to obtain different t interesting products. In the food industry, its use has been focused on food preservation and packaging protection, due to its properties against pathogenic bacteria and spoilage fungi (Simbine *et al.,* 2019; Moond *et al.*, 2022; Bamal *et al.*, 2021). Incorporating AgNP into the food composition packaging by a Brazilian company resulted in extending the shelf life of perishable products, doubling the shelf life of pasteurized milk, and approval for the use of technology in the Brazilian market (Alisson, 2015).

Studies have been carried out on various packaging impregnated with AgNP to determine their effectiveness in terms of food durability and migration into the food and potential problems with consumer health (Bajpai *et al.*, 2018; Xing *et al.*, 2019). Gallocchio *et al.*, (2016) verified the nanosilver antimicrobial efficacy and migration in chicken packaging under home storage conditions. No nanosilver was detected in food by European Legislation (maximum level of 0.010 mg kg^{-1}) in 7 days of storage, however, in microbial evaluation, no differences were found between the material with nanosilver and conventional plastic bags.

Other authors have also verified the AgNP use of packaging for various foods and obtained a reduction result in microorganisms number such as ultrafiltered cheese, a reduction in coliforms number (Beigmohammadi *et al.*, 2016); Walnut, hazelnut, pistachio kernel, decreased total and coliform bacteria counts (Tavakoli *et al.*, 2017); cottage cheese, inhibition of total bacteria, molds, and yeasts (Li *et al.*, 2018); mangoes, effectiveness against total bacteria (Chi *et al.*, 2019), all these works sought to simulate the storage used for different products in time and temperature.

When it was not possible to use packaging, there was another approach used by Gemail *et a*l., (2022) in fruits. A wax composition with 100 mg/L of nanosilver was made as a coating on Murcott tangerine (*Citrus reticulata L. Osbeck*) to maintain the fruit characteristics during storage. The experiment results evaluated for 4 months demonstrated no loss of the main fruit characteristics with the treatment, reducing losses due to discarding or fresh weight, with the catalase activity maintenance, pulp firmness, and vitamin C content maintenance.

Nanocomposites have also been widely used in the food industry. TiO_2 is already applied food additive, whitening agent, and antibacterial. Peter *et al.,* (2015) evaluated packages containing Ag/TiO_2-SiO_2 composites to inactivate *Botrytis cinerea* during lettuce storage, increasing the product's shelf life. Another research group also examined the chemical and microbiological aspects of paper packaging with Ag/TiO_2-SiO_2, $Ag/N/TiO_2$, or Ag/TiO_2 NP composites for white bread.

In an attempt to preserve fresh leaves of *Morus alba*, used to feed silk-producing larvae, there are substantial losses of their nutrients during post-harvest and storage, impairing the development of the larvae. Das & Mandal, (2020), subjected the leaves to treatment with a nanosilver solution and showed conservation of primary metabolites such as pigments, proteins, and sugar, in addition to positive results in larvae fed with the treated leaves.

In the agricultural sector, nanotechnology has been intensifying in parallel with the development of food production to increase agricultural production yields and reduce chemical agents that are harmful to the environment. Thus, nanomaterials such as nano pesticides, nano fungicides, nano fertilizers, and nano biosensors are now being used (Bahrulolum *et al.*, 2021; Salem, 2023).

Due to the antimicrobial properties presented with a focus on the medical sector, researchers in the agricultural field saw AgNPs as a potential application against agricultural pathogens that cause agricultural damage. This vision was based on improving seed germination, development, and growth of plants under environmental stress. The methods used for application are aimed at seed preparation, leaf system, and soil mixture (Balusamy *et al.*, 2023).

Mahakham *et al.*, (2017), evaluated the germination of ancient rice seeds treated with biogenic AgNPs (kaffir lime leaf) at a concentration of 5 to 10 mg/L. The seeds aged with the treatment showed greater germination vigor compared to the controls ($AgNO_3$ and hydro conditioning). According to the authors, there was an increase in alpha-amylase activity, making more sugars available, upregulation of aquaporin genes, and increased ROS formation, providing better development in seed germination. In another treatment of sunflower seeds and plants, biogenic AgNPs (*Euphorbia heliescola*) were tested with seed and foliar applications. The results showed that seed and foliar application affected plant characteristics, with larger flower head diameter, and consequently greater weight and number of seeds (Batool *et al*, 2021).

The use as a growth stimulant was tested on plants of *Triticum aestivum* (wheat), *Brassica juncea* (mustard), and *Vigna sinensis* (black-eyed peas), with an improvement in the plant photosynthetic activity and consequently with an increase in the aerial part and plants roots. (Pallavi *et al.*, 2016; Jhanzab *et al.*, 2019).

As previously reported in 2.6, antimicrobial activity may be related to the distinct AgNP physicochemical characteristics, besides interaction with the environment. Microorganisms of different species have already been tested as targets for AgNPs, with results ranging from inhibitory to biocidal action (Moond *et al.*, 2022). Yousef *et al.*, (2021) isolated and identified the bacteria *Escherichia coli, Staphylococcus aureus*, and *Pseudomonas aeruginosa* in fresh cattle semen and used them as an antibiotic synthesized silver nanoparticle with melamine binding (1 to 5 nm). The results were efficient in bactericidal action and did not present changes in the sperm properties of the tested semen, despite the satisfactory result, the authors suggest further studies for the subsequent stages of semen treatment.

The wound treatment with silver ions dates back a long time, nowadays, with the advent of nanotechnology, it has been extended to material manipulation with targeted applications. The first dressing manufactured using nanoparticle coating technology is ActicoatTM (Smith & Nephew) for use in wound care. Mehrabani *et al*, 2018 manufactured in the laboratory a dressing composed of biocompatible and biodegradable silk fibroin/chitin with various contents of AgNPs (0.001, 0.01, and 0.1%) and tested against E*. coli*, *S. aureus*, and *C. albicans* resulting in good antimicrobial results in addition to the physical characteristics necessary for dressings, suggesting it to be an alternative for products for treating wounds in vivo. The already proven silver ion antimicrobial action has led to material manufacture of a new category for medical use, such as catheters (biofilm reduction), which contain nanoparticles or silver ions to provide longer usage time with greater patient safety. Silverline (Spiegelberg GmbH and Co. KG, Hamburg, Germany) and ON-Q Silver Soaker™ (I-Flow Corporation, CA, USA), Multicath5 Expert UP - 9.5 Fr (Vygon Value Life – PT) are some commercially available medical catheters which contain silver nanoparticles to prevent infections. (Agrawal *et al.*, 2018).

In addition to the applications already reported, AgNPs have been extensively analyzed for several other applications with activities, such as antiviral (SARS-Cov, influenza A/H5N1, influenza A/H1N1, dengue virus, HIV, HBV, and new encephalitis viruses), anti-larvicide (A. aegypti, Anopheles stephensi), therapeutic applications, antifungal (*A. niger*, *A. flavus*, *B. cereus*, *Fusarium* sp., *Rhizopus* sp., *F. oxysporum*), anticancer (apoptosis induction, HepG2 (human hepatocellular carcinoma) and MCF-7 (invasive human breast ductal adenocarcinoma). All these activities have been widely studied for product

modernization and more effective applications. (Garg *et al.*, 2020; Mikhailova, 2020; Dawadi *et al.*, 2021; Rana & Parmar, 2023).

Several applications for AgNPs are available, but data demonstrate that particle size, shape, and interaction with other materials are directly related to the power of their action on the intended target (Deepak *et al.*, 2011; Park *et al.*, 2016). Literature data show that NPs synthesized by biological processes have less dispersion in size and shape, greater stability, less propensity to form agglomerates, and, in many cases, it is possible to determine the NP shape, thus providing greater flexibility to the process (Durán *et al.*, 2015; Rauwell *et al.*, 2015).

The elucidation of AgNP biosynthesis mechanisms could increase biotechnological process development for large-scale metallic NP production with defined sizes and shapes. The molecule elucidation presented in biosynthesis can leverage the NP production development from other important metals in the technological market. The biotechnological process has its peculiarities, but if the scientific bottlenecks of biological phenomena are elucidated, the study of operational parameters will be more easily established to establish its scalability.

2.10 Nanostructures and Nano Silver Market

In recent years, nanotechnology has established itself in the world market, with a great product diversity. Of these products, nanometals have stood out for their physical, chemical, and mechanical properties, in addition to the diversity of applications in different industry sectors. Development has intensified as knowledge of the countless possibilities for applications becomes concrete.

The global nanomaterials market value in 2022 was valued at US$ 11.99 billion with a forecast of reaching US$ 61.96 billion by 2032. The prospect is that increased knowledge will boost the use of nanomaterials in several other industrial sectors. In 2022, only applications in the medicinal market accounted for 31% of the production of nanomaterials. The global AgNPs market was valued at US$2.2 billion in 2021 and is expected to reach US$7.73 billion by 2030. AgNPs accounted for the second largest market share at 15% (Figure 6) (https://www.grandviewresearch.com/industry-analysis/us-nanomaterials-market-report).

The increase in the AgNPs market is related to increased knowledge of their properties, mainly in the medical field. As reported in the Antimicrobial and Cytotoxic Activity and Applications and Perspectives items, the scope of applications has expanded as more knowledge becomes available. Two sectors have driven the nanosilver market, the pharmaceutical industry with antimicrobial products, and

biomarkers, biosensors, tissue engineering and implants, nanorobots, and image enhancement devices, and electronics sectors.

There are many players in the AgNP market, such as Advanced Nano Products, American Elements, Dune Silver, E. Merck KG, Nanocomposix, Nanocs Inc, Bayer, BASF, BBI Solutions, Sukgyung AT Co, and many other companies around the world. Although large companies are in the market for these materials production, awareness of the environmental impact of physical and chemical methods has boosted the developing sector of biological synthesis. This development is expected to promote this market for the application of eco-based AgNPs, making the process environmentally friendly.

The silver nanoparticles market is segmented by geographic region. In 2021, Asia-Pacific has attributed the largest share of the market with 34%, however, there is an estimate that North America will account for the largest share of the market by 2030, attributed to market acceptance, increase in the electronics sector, increase of the population and government investment in development and health plans.



Figure 6 - Estimation of the evolution of the metallic nanoparticles market.

3 OBJECTIVES

This work's main objective was to develop a silver nanoparticle synthesis process with a low-cost culture medium, using a fungal strain of *Rhizopus arrhizus* NRRL 54538, which was previously selected (Ottoni *et al.*, 2017).

As specific objectives:

• Study of the culture medium composition and process parameters in shaken flasks using residues from corn processing (CSL) and alcohol production (sugarcane molasses).

• Biomass production in bioreactors focusing on the agitation influence.

• Development of the process for obtaining the fungal extract and molecules identification present.

• Physical parameters study of AgNP synthesis using *Rhizopus arrhizus* NRRL 54538 fungal extract.

• Assessment of the antimicrobial and cytotoxic activity of AgNPs.

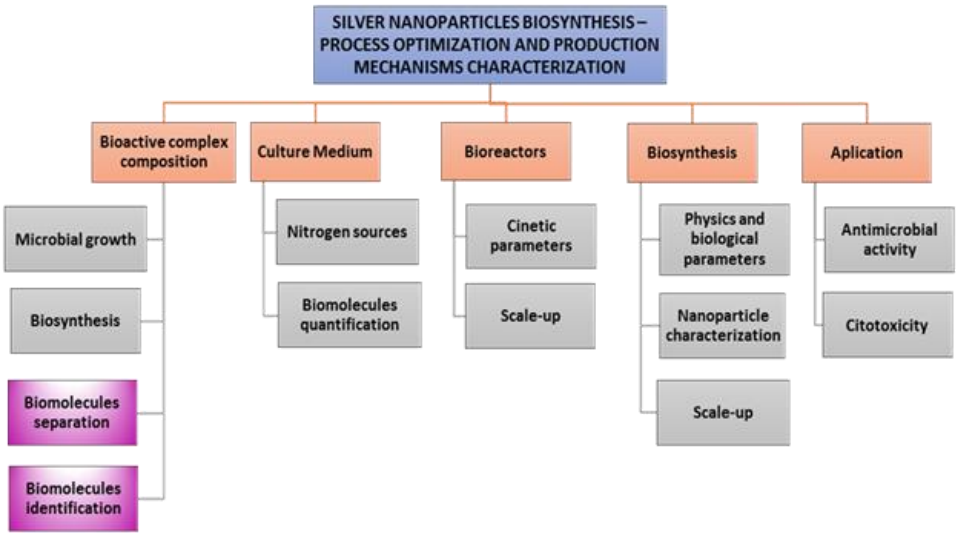


Figure 7 - Flow chart of project task.

4 MATERIALS AND METHODS

4.1 Microorganism

Rhizopus arrhizus NRRL 54538 (IPT 1013) was obtained from ARS Culture Collection, USA. The pure culture was obtained on a potato dextrose agar (PDA), and the spore suspension (glycerol 10%) was stored at -80°C, with a concentration of 10^8 spore/mL.

4.2 Culture medium – fazer uma tabela com a diferenciação dos meios

Reference medium (MGYP – YM)

Malt extract	3 g/L
Yeast extract	3 g/L
Peptone	5 g/L
glucose	10 g/L

Mineral medium (Sukumar *et al*, 2010)

K_2HPO_4	0.5 g/L
NaCl	0.5 g/L
$MgCl_2$	1.0 g/L
NH_4NO_3	0.5 g/L
Yeast extract	0.5 g/L

Alternative medium

K_2HPO_4	0.5 g/L
NaCl	0.5 g/L
$MgCl_2$	1.0 g/L
Yeast extract	0.5 g/L
Corn steep liquor	100.0 g/L
Sugarcane molasse	10.0 g/L

4.3 Fungal cultivation conditions

Frozen *Rhizopus arrhizus* NRRL 54538 spore suspension inoculum at 1% (v/v) was used in all experiments. The CSL was sterilized separately and centrifuged, using only the centrifuge in the medium composition. The flasks were incubated at 35°C with shaking on an orbital shaker at 200 rpm for up to 120 hours.

4.4 Bioreactor assays

The bioreactor assays were carried out in two different equipment: B. Braun mod. B and Bioengineering mod. RALF, both with a useful volume of 2 L, with control over agitation, temperature, and airflow. The pH and dissolved oxygen were measured. The experiments were monitored by the biomass concentration and carbon source consumption, and at the end, the biomass was used for nanoparticle synthesis.

4.5 Extraction assays

The biomass resulting from growth after 5 days was filtered through Whatman filter paper no. 1, washed with sterile distilled water to remove culture medium residues, and placed in a 500 mL Erlenmeyer flask, for every 5 g of washed and wet biomass, 100 mL of sterile distilled water was used. Incubated at 30°C and 150 rpm for 5 days.

4.6 Silver nanoparticles synthesis

After extraction, the flask contents were filtered through Whatman n.1 filter paper, and $AgNO_3$ solution was added to the filtrate to a final concentration of 1mM. The flasks were incubated at 30°C under orbital agitation at 180 - 200 rpm for 5 days.

4.6.1 Effect of temperature on AgNP synthesis

A volume of 3 mL of CFF was added to $AgNO_3$ 1mMol/L (final concentration) and incubated in 8-well microplates at temperatures of (30, 40, 50, and 60) °C for a period of 15 to 20 hours with shaking and absorbance readings at 410 nm at every 1 hour.

4.7 Analytical Methods

4.7.1 Cellular concentration

Cell concentration was determined by gravimetry. The biomass was filtered through Whatman n.1 paper and dried at 105°C for 6 h.

Equation: $$X\ (g/L) = \frac{m_{cel} - m_{memb.} + humidity_{memb.}}{V_{sample}}$$

Were:

$m_{cel.}$ = sample dry mass (g)

$m_{memb.}$ = membrane mass (g)

humidity = humidity membrane mass (g)

V_{sample} = sample volume (L)

4.7.2 Characterization of AgNPs UV-visible analysis

The color changes in appearance in the reaction mixtures were used as the initial evidence of AgNP formation. Samples of 1ml were taken from each flask containing colloidal AgNPs at regular time intervals, and its absorbance was measured using a double beam UV–-visible spectrophotometer (Hitachi mod. U-2000) with a resolution of 1nm in the range of 200–800 nm.

4.7.3 Dynamic Light-scattering Analysis

To analyze the hydrodynamic particle diameter, zeta potential, and PDI of synthesized AgNPs were determined by dynamic light scattering (DLS-PZ Malvern Zetasizer Nano ZS90).

4.7.4 Antibacterial activity

The antimicrobial activity determination was based on the Methodology of Sensitivity Tests to Antimicrobial Agents by Dilution for Aerobically Growing Bacteria (NCCLS/CLSI – 2005). *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538 were used as target microorganisms, previously stored in glycerol suspension at -80°C. 0.5 mL of the microorganism suspension stock was inoculated into Muller-Hinton (MH) broth medium 25 mL. The flask was incubated overnight at 37°C with an agitation rate of 200 rpm. After growth, the cell concentration was determined by optical density at a 625

nm wavelength, and the absorbance was adjusted between 0.08 and 0.1 (5 on the Mac-Farland scale) to prepare the inoculum. The assay was carried out in 96-well microplates with the distribution of nanoparticle dilutions, as shown in the following Figure 8. Each well was inoculated with 10 uL of the bacterial suspension and the plates were incubated in a shaker at 37°C, 150 rpm for 24 hours. After growth, 40 uL of 0.1% rezasurin blue solution was added and incubated for 2 hours at 37°C and 150 rpm. After incubation, microplates were read at 570 nm and 600 nm. The $AgNO_3$ solution was used as a negative control.

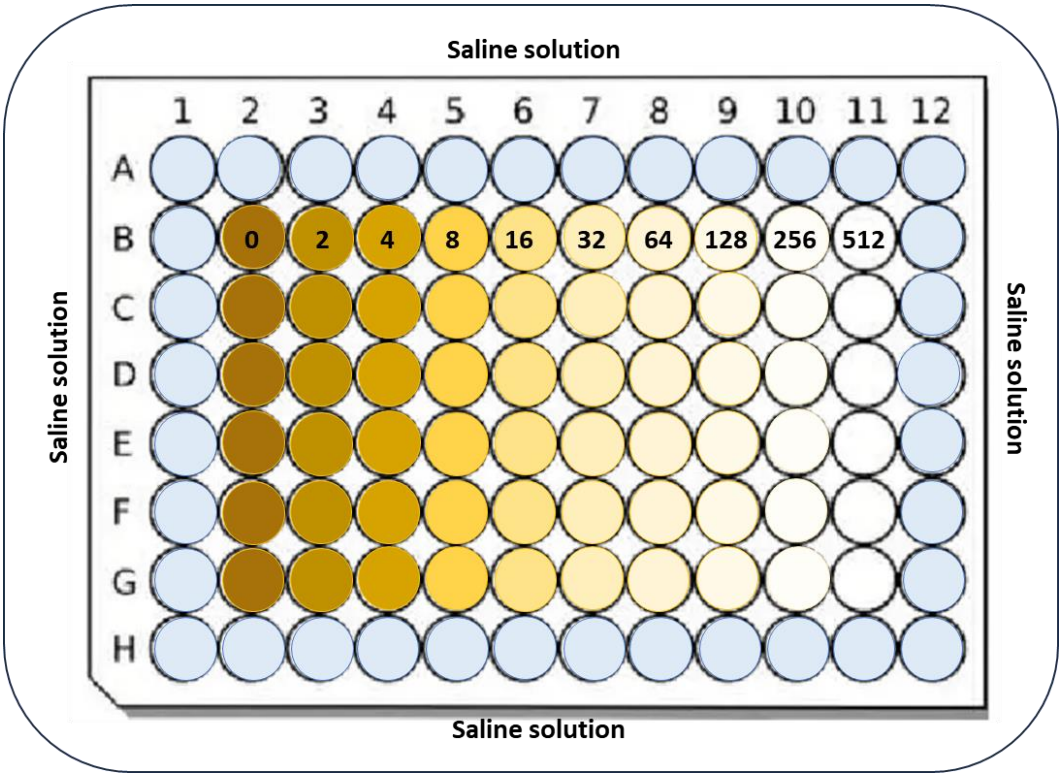


Figure 8 - Representation of the sample dilutions distribution and replicates for antimicrobial action test.

4.7.5 Cytotoxic activity

The cytotoxicity evaluation in vitro was carried out following DIN EN ISO 10.993-5:2009, Part 5, Medical evaluation of medical devices. Part 5. Tests for in vitro cytotoxicity. Mouse connective tissue cells (L929) from the American Type Culture Collection (ATCC) no. CCL1 NCTC clone 929L was used. The cells were cultivated in DMEM culture medium with 10% fetal bovine serum in an oven at 37°C with a 5% CO_2 atmosphere. DMEM medium containing 5% fetal bovine serum was considered the negative control, and SDS was the positive control, for the validation test. The samples were inoculated in a 2^2serial dilution, with six replicates each. The results were evaluated using the replicate average.

4.7.6 Protein Identification by mass spectrometry

The extract proteins were separated by SDS-PAGE capillary electrophoresis and freeze-dried extract. The bands were excised, destained, washed, and digested with trypsin. The resulting hydrolysates were passed through C18 spin tips according to the manufacturer's specifications. Samples were dried in a speed vac and resuspended with 0.1% trifluoracetic acid in 5% acetonitrile: water for LC-MS analysis. The digested peptides were sequenced after chromatographic separation by tandem mass spectrometry. Chromatographic separation was performed on a Dionex Ultimate TM 3000 RS LC nano UHPLC system coupled to an LTQ-XL Orbitrap mass spectrometer equipped with an EASY-nano spray source (Thermo Scientific). The system was controlled by Chromeleon (UHPLC) and Xcalibur (LTQ-XL) software. PepMap EASY-spray C18 (15 cm x 75 µm ID) 3 um 100 Å (Thermo Scientific) was used in the LC separation process. The acquired spectra were submitted to the Proteome Discoverer 1.4 search database (Thermo Electron Corporation) to identify peptides and infer protein assignments from a database consisting of reviewed and unreviewed entries for the family *Rhizopus oryzae* (formerly *Rhizopus arrhizus*). listed in the UniProt database. Search criteria were used following the SEQUEST algorithm.

4.7.7 Determination of Sugars by HPLC

Sugar concentrations were carried out by liquid chromatography using Ultimate 3000 equipment (Dionex®) with an Aminex HPX-87H column (300 x 7.8 mm) using a 0.01 M H_2SO_4 mobile phase under the flow of 0.6 $mL.min^{-1}$ and refractive index detector at 45°C. A component calibration curve was used with a range (0.1 to 10) g/L.

4.7.8 Determination of Total Reducing Sugars by DNS

The determination of total reducing sugars was carried out by the DNS method, using the minimized method according to (Santos *et al.*, 2017) with sample volume modification. In microtubes, 100 µL of sample and 100 µL of DNS were placed and subjected to a boiling bath for 15 minutes. The tubes were cooled in ice water. Subsequently, 1 mL of distilled water was added, homogenized and the reading was performed in 96-well microplates at 540 nm. The calibration curve was made in the range of 0.2 to 2 g/L with glucose.

4.7.9 Determination of Total Proteins

Total protein concentration was performed using the Bradford method (Bradford, 1976). A calibration curve was performed in the range of 0.15 mg/mL to 2.0 mg/mL with bovine albumin. The analysis procedure was: 100 uL of sample and 100 uL of Bradford reagent were pipetted into a 96-well

microplate and homogenized under agitation. Subsequently, it was incubated for 15 minutes at room temperature and the reading was performed at 595 nm.

4.7.10 Determination of ammonia nitrogen

The ammonia nitrogen concentration was determined with a specific electrode for nitrogen detection (Orion - model 95-12) in a potentiometer (Mettler Toledo Seven Compact S220) after sample alkalinization (pH > 9) with 10 M NaOH. Solutions of $(NH_4)_2SO_4$ containing around (10, 25, 50, 100, 250, and 500) mg/L in nitrogen were used to obtain the calibration curve.

4.7.11 Determination of carbon, hydrogen, and nitrogen

The determinations of carbon, hydrogen, and nitrogen were carried out using the elemental analyzer by combustion of elements: C, H, N, and S on the Perkin-Elmer CHN-2400 equipment.

4.7.12 Determination of anions by HPLC

The equipment used was a Thermo Scientific Dionex ICS-3000 HPLC, with Dionex SP 3000 quaternary pump, Thermo Scientific Dionex EG 5000 eluent generator module with Dionex EGC III KOH cartridge, AS-AP automatic injector with temperature conditioning for samples, ion suppression with electrolytic regeneration with a 4.0 mm Dionex AERS 500 suppressor, a Dionex CD-3000 conductivity detector, and a Thermo Scientific Dionex IonPac AS-11HC 4.0 mm precolumn and column under thermal conditioning. Equipment control, data acquisition, and processing were realized using Dionex Chromeleon 6.80 software.

The analytical conditions used were an isocratic flow of 1.5 mL/min of 30 mM KOH mobile phase, produced with automatic generation in the eluent generator with ultrapure water under an inert atmosphere of helium gas, columns at a temperature of 35°C and constant current suppressor 223 mA. The injected volume for both samples and calibration curve points was 10 µL and the run time was 12 min. The samples were stored at 8°C in the automatic injector.

The standard curve was constructed from a standard Specsol Quimlab Multianion anion solution, which contains the anions fluoride (F-), bromide (Br-), chloride (Cl-), nitrate (NO_3^-), sulfate (SO_4^{-2}) and phosphate (PO_4^{-3}).

4.7.13 ICP – OES (Inductively Coupled Plasma Optical Emission spectroscopy)

The silver concentration determinations in AgNP colloidal suspensions were carried out using the ICP OES Mod. Arcos equipment with an axial view (Spectro).

5 RESULTS AND DISCUSSION

The metallic nanoparticle biogenic synthesis has been studied from microorganisms such as bacteria and fungi, which can be intracellular or extracellular, in addition to plant extracts. The process is divided into three stages: microbial growth, biomass extraction, and nanoparticle synthesis. In this work development, studies of the three stages were adopted using the *R. arrhizus* NRRL 54538 (IPT 1013) strain as a biological agent, focusing on producing and identifying reducing agents that act in the AgNP synthesis. The research studies were carried out based on the hypothesis that the metallic nanoparticle's biogenic synthesis is mediated by the presence of enzymes associated with electron transfer. NADH and NADH-nitrate reductase-dependent enzymes are the most important in the biogenic synthesis of metallic NPs. (Baymiller *et al.*, 2017; Jaidev and Narasimha, 2010; Kumar *et al.*, 2007; Durán *et al.*, 2005).

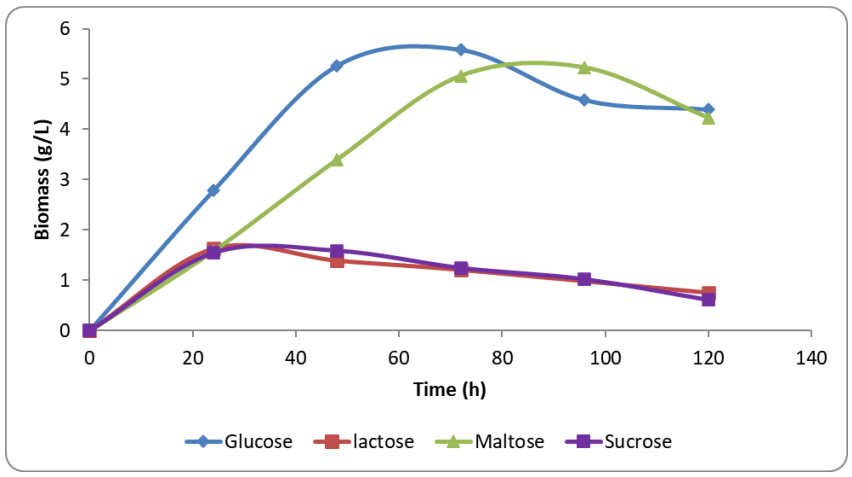
5.1 Study of an alternative culture medium

The biotechnological process development specifically involves defining an appropriate culture medium for the target microorganism growth and stimulating the production of interesting molecules, in addition to the process's physical parameters. The culture medium can metabolically direct the microorganism to produce different metabolic substances, characterizing itself as one of the main components in biotechnological processes (Costa Silva *et al.*, 2017; Tigini *et al.*, 2012). Using a medium with specific substrates for the protein induction that acts in the nanoparticle synthesis can enhance the silver reduction and the nanoparticle formation (Husseiny *et al.,* 2015).

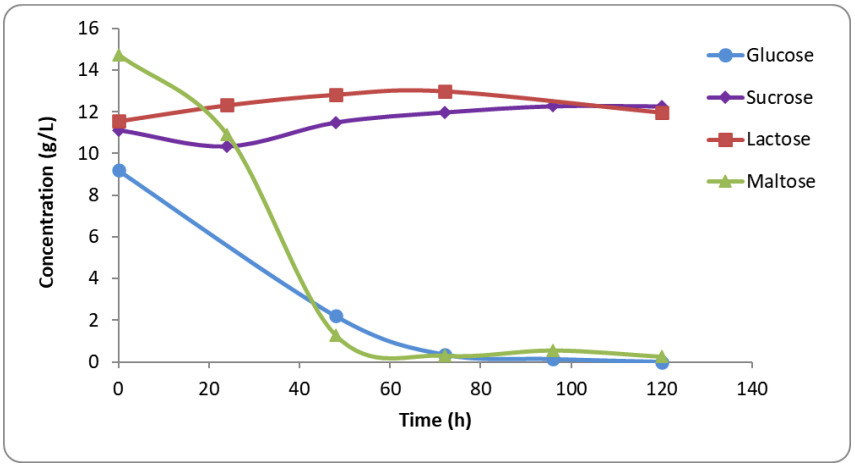
The fungi growth for nanoparticle synthesis is mostly carried out in complex media with a composition containing extracts, favoring microbial growth due to the nutrient richness, however, when the objective is to scale up, the culture medium makes it a relevant factor from a point of view of cost, as these extracts have a high cost. In biotechnological processes, the cost of the culture medium can reach 30% of the process's total cost (Rivas *et al*, 2004; Pitol *et al*, 2017). Taking this factor into account, replacing these compounds (carbon and nitrogen sources) with industrial waste is an alternative evaluated has been in several developments, due to its low cost and availability.

It is known that *R. arrhizus* can metabolize several carbon sources, including hydrolysates with xylose (Kowalczyk *et al*, 2018; Maas *et al,* 2008). To evaluate the ability to metabolize another carbon source, the following were used instead of glucose: lactose, maltose, sucrose, and molasses. The reference medium used was MGYP, which has glucose as the main carbon source and the nitrogen sources are organic compounds, coming from extracts (Ottoni *et al.*, 2017; Hamedi *et al.*, 2017). The

evaluation was carried out by monitoring microbial growth and substrate consumption, the results are presented in Figure 9.



(A)



(B)

Figure 9 - Growth curve with different carbon sources such as glucose, lactose, maltose, and sucrose (A) and monitoring of consumption in g/L of carbon sources (B) over time.

The results presented in Figure 9 demonstrate that the complex medium when used with glucose and maltose provided microbial growth of 5.8 g/L and 5.1 g/L of dry biomass with 72 hours of incubation, respectively, which did not occur with the other sources tested, reaching a mass of 1.5 g/L after 24 h, growth that must have been due to the consumption of the nutrients from the extracts, and a decreasing biomass profile until the test end, probably due to cell lysis. Peixoto *et al.,* (2003) when studying different carbon sources to produce thermostable amylase with *Rhizopus microsporus* var. *rhizopodiformis* achieved good growth when using maltose. A similar result was obtained by Amadioha (1998) with *R. oryzae* to produce amylase. The little growth for the conditions with lactose and sucrose can be attributed to using complex substrates from the extracts and the carbon source non-metabolization offered. Different

Rhizopus species may differ in their ability to metabolize carbon sources, as reported by Rehms and Barz (1995) who studied distinct species challenged by different carbon sources and only *R. microsporus* var. *chinensis* was able to utilize sucrose.

To obtain a culture medium with minimal composition and lower cost, a mineral medium based on Sukumar, et al. 2010 (4.2 Culture medium), with replacement of carbon and nitrogen sources with molasses and CSL, industrial waste with a high sugar and amino acid content (Martínez-Arcos, 2021). The use of industrial waste to develop a nanoparticle synthesis process seeks to apply an economic, ecological, and simple circular economy approach, generating value for the material (Vigneswari *et al.*, 2021). Growth monitoring with biomass measurement is presented in Figure 10.

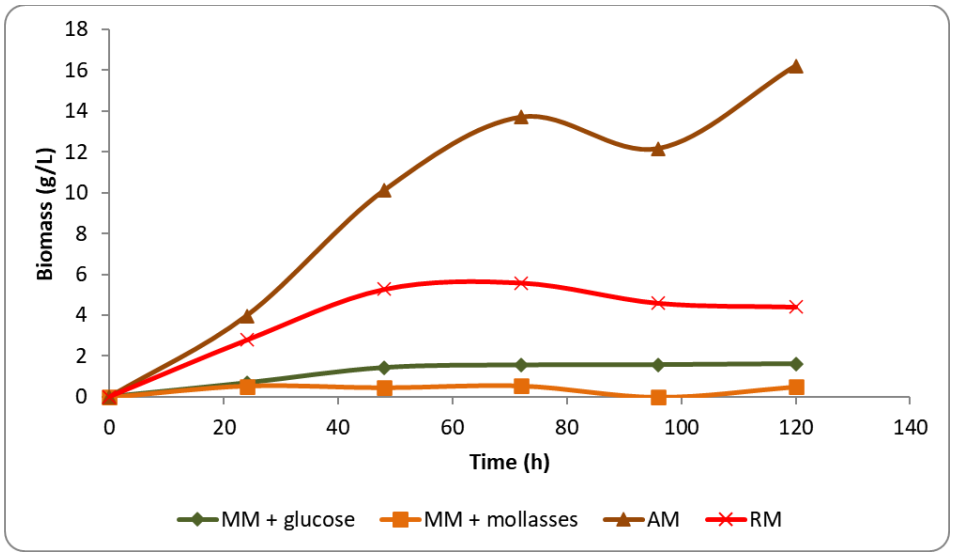
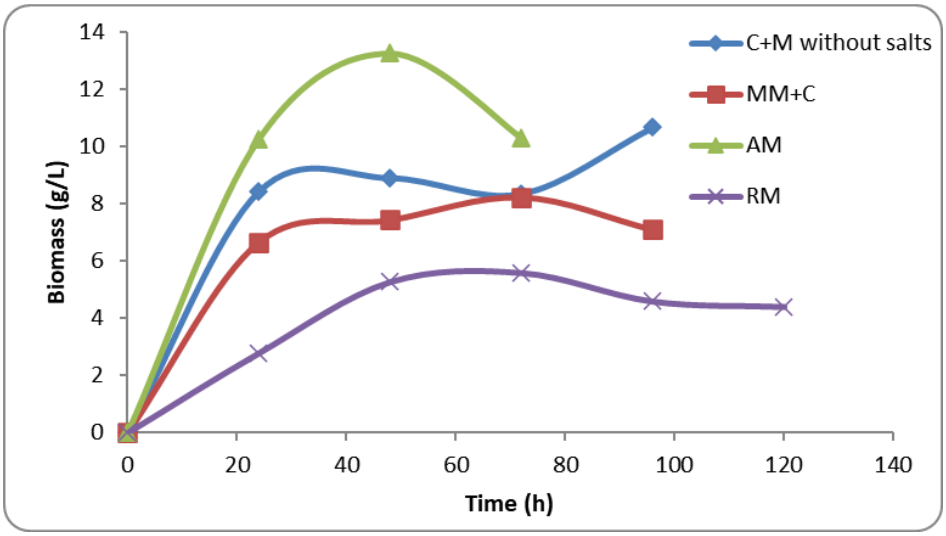


Figure 10 - Comparison of the growth curve concerning the reference medium of the variations of mineral medium with glucose, mineral medium with molasses, and alternative medium.

It was observed that in this medium composition, glucose and molasses did not show growth, however, when CSL and molasses were added, the microorganism reached a biomass around 2.5 times greater than in the reference medium (MGYP) in 72 h of incubation, demonstrating the ability to metabolize the nutrients provided in these wastes. The low growth with glucose and molasses may be related to the non-metabolization of the nitrogen source in the medium, $NaNO_3$, under the studied conditions. The preferred nitrogen sources for fungi are ammonium or glutamine. To use other sources, such as nitrate, they are equipped with catabolism enzymes such as nitrate reductase and nitrite reductase, in a reaction that results in ammonium (Davis & Wong, 2010; Tudzynski, 2014). The condition containing glucose showed a decrease in pH in the first 24 hours of incubation from 6.5 to 3.5, which

may have contributed to low microbial growth, a behavior that did not occur with the other conditions that maintained the pH profile between 6.5 and 7.5. The decrease in pH may also be related to some organic acids production such as fumaric, lactic, or malic acid, which is a metabolism characteristic of this microorganism (Fu *et al.*, 2016; Kowalczyk *et al.*, 2018; Wu *et al.*, 2018).

Based on the results obtained with the mineral medium, the medium study continued using the medium saline composition as a basis Sukumar, *et al.* (2010) and replacing the carbon and nitrogen sources with molasses (Refinarias de Açúcar Reunidas, S.A. (Portugal)) and CSL (COPAM: Companhia Portuguesa de Amidos, S.A. (Portugal)). CSL and molasses have been used as nitrogen and carbon sources in several biotechnological developments to obtain various products, such as antibiotics, glutamic acid, biosurfactants, and others (Zhou *et al.*, 2022). Due to the saline composition of the residues themselves, a condition containing only the two residues was carried out, to verify whether the salts contained were sufficient to meet metabolic needs. The results of microbial growth are shown in Figure 11.



C+M = corn steep liquor + molasses; MM+ C = mineral medium + corn steep liquor

Figure 11 - Comparison of cell growth in the reference medium with the condition without added salts, mineral medium with molasses, and alternative medium.

The preparation without mineral composition addition reached a growth in biomass of 8.2 g/L in 24 hours while with the salt addition, it reached 10.3 g/L and continued to grow until 13.2 g/L in 48 hours. When only CSL was used, 8.2 g/L was obtained after 72 hours of incubation. All conditions using waste presented higher biomass values than the reference medium, thus demonstrating the

microorganism can metabolize the nutrients offered in these substrates. Cardoso *et al.*, (2012) with the *Rhizopus arrhizus* strain UCP 0402/WFCC 0402 also achieved a cellular concentration of 20.61 g/L in the composition of 8% CSL and 6% honey with 96 h of fermentation. Shen *et al.*, (2015), when replacing the yeast extract with a CSL + PM (peanut meal) mixture and molasses, obtained a better condition to produce succinic acid by *A. succinogenes* GXAS 137.

During this study an alternative medium composition for obtaining *R. arrhizus* biomass, 100 g/L of CSL was used without separating solid residues, with clear interference in the measurement of cell concentration (Figure 13). CSL is considered a soluble solid that contains between 40% and 50% (w/w) of the corn dry weight (Zhou *et al.*, 2022). To verify whether this concentration could be reduced and finalize the medium composition, an experiment was carried out with different concentrations (25, 50, and 100) g/L, changing the culture medium preparation. The CSL was sterilized separately and centrifuged, using only the supernatant to compose the culture medium Figure 12.

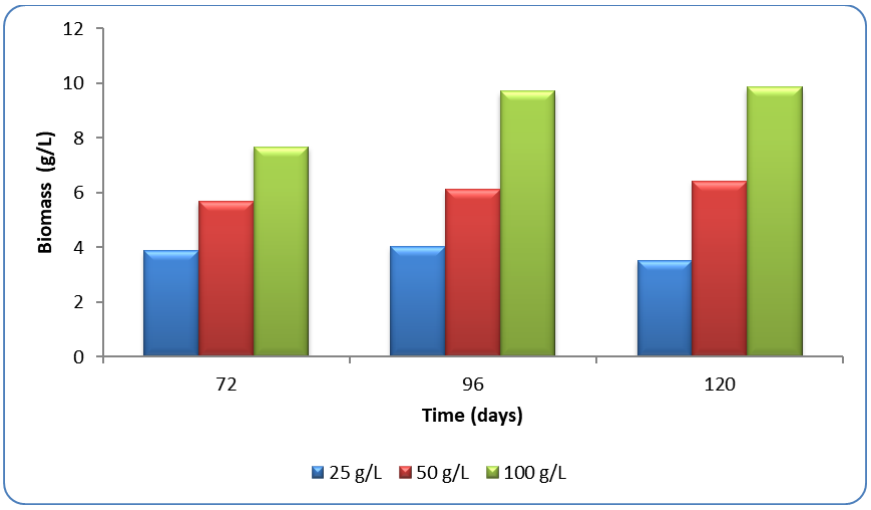


Figure 12 - Cell growth comparison in different corn steep liquor concentrations in an alternative medium.

It was verified that microbial growth reached its maximum after 96 hours of incubation and the CSL concentration of 100 g/L showed the highest biomass. A difference in the final biomass concentration (6.7 g/L) compared to previous tests (~14 g/L) was observed, confirming the solids interference contained in the composite media with the complete CSL (Figure 13).

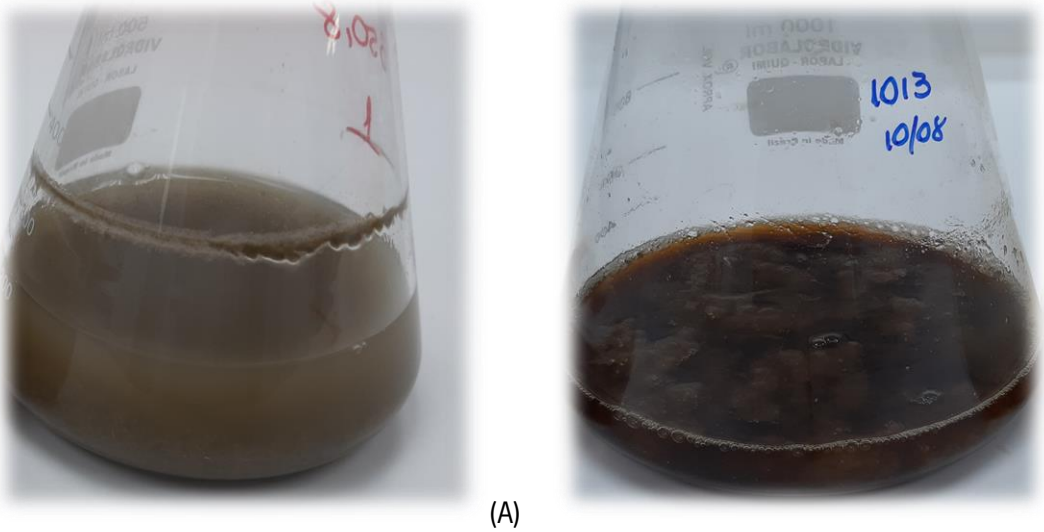


Figure 13 - Difference between the preparation of the culture medium (A) without removing solids and (B) without solids.

With the results obtained, it was possible to compose the cultivation medium for the work development, with the composition in (g/L): 0.5 K_2HPO_4, 0.5 NaCl, 1.0 $MgCl_2$, 10 molasses, and 100 CSL (centrifuged). However, throughout the project, it was necessary to replace the CSL, so an experiment was carried out with different concentrations (0.5; 1; 3; 5; 10; 20; 25, and 30) g/L of corn steep solids (CSS) and also the addition of yeast extract (0.5 g/L) as an increase in essential micronutrients at concentrations of 30 g/L and 35 g/L of CSL, compared with the reference medium and with the CSL (old) at 100 g/L. Results are the average of triplicates with 5 days of incubation (Figure 14).

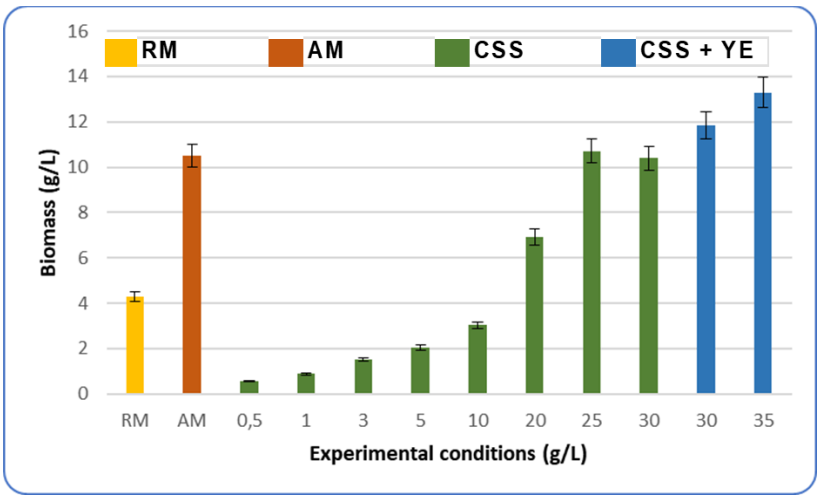


Figure 14 - Comparison of cell concentration of reference medium and alternative medium at different CSS concentrations and addition of yeast extract.

Figure 14 shows that at CSS concentrations of 25 g/L and 30 g/L, the biomass growth is equivalent to the medium with CSL with 10,5 g/L, and when the yeast extract was added the biomass concentration reached 11,9 g/L and 13,3 g/L at the CSS concentrations tested (30 and 35) g/L, respectively. Although the increase in biomass was not substantial, the addition of yeast extract, provided better microbial growth development, certainly supplying essential micronutrients for metabolism. A similar result was obtained by Bulut et al., (2004) by adding yeast extract to the medium with molasses in the process with *R. oryzae*, lactic acid production was increased by 33% compared to the medium with molasses alone.

Another CSL (Ingredion Brasil Ing. Ind. Ltda) was acquired to continue the work and was evaluated in different concentrations and with an increase in yeast extract (Figure 15), as the CSL nutritional composition can vary with the origin and batch, so that can alter the microorganism response (Xiao *et al.*, 2013). In its composition, different concentrations of peptides, amino acids, vitamins, nucleotides, and various trace elements can be found, being considered a rich source of alternative nitrogen for various biotechnological processes (Kuenz *et al.*, 2023).

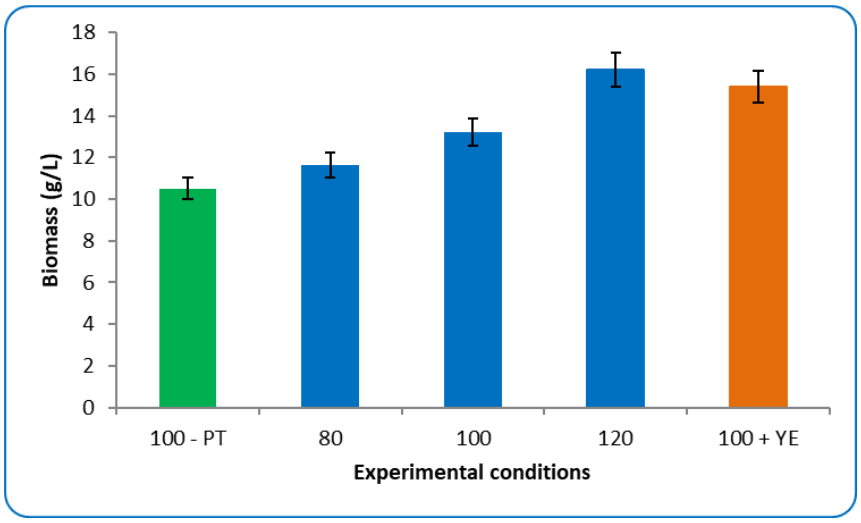


Figure 15 - Cell growth comparison in a new batch of corn steep liquor and with yeast extract addition.

The biomass concentration results after 120 hours of incubation showed no significant difference when compared to the CSL previously used, an increase of 2.7 g/L at a concentration of 100 g/L, which can be attributed to the CSL nutritional change/variation (Figure 15). However, there was an increase of around 5 g/L when 0.5 g/L of yeast extract was added concerning the comparative condition and 2.2 g/L with the new CSL, a result similar to the previous test when tested with CSS, corroborating the need for essential micronutrients for better biomass development. Yeast extract is widely used as the main

nitrogen source for biotechnological processes, however in this case it will only be used to meet essential micronutrient needs. In the lipase production by *R. delemar,* the culture medium optimization was studied, and the best biomass growth conditions were obtained with the yeast extract and beet molasses addition. With a mass of 4.52 g/L while in the basic medium, 2.21 g/L was obtained (Açikel *et al* 2010). A 40% increase in *Monascus purpureus* biomass was achieved with the yeast extract addition to the synthetic medium with a consequent increase in pigment production (Pereira & Kilikian, 2001). A new growth kinetics test was carried out for confirmation, reaching 16.6 g/L of dry biomass after 96 hours of incubation (Figure 16).

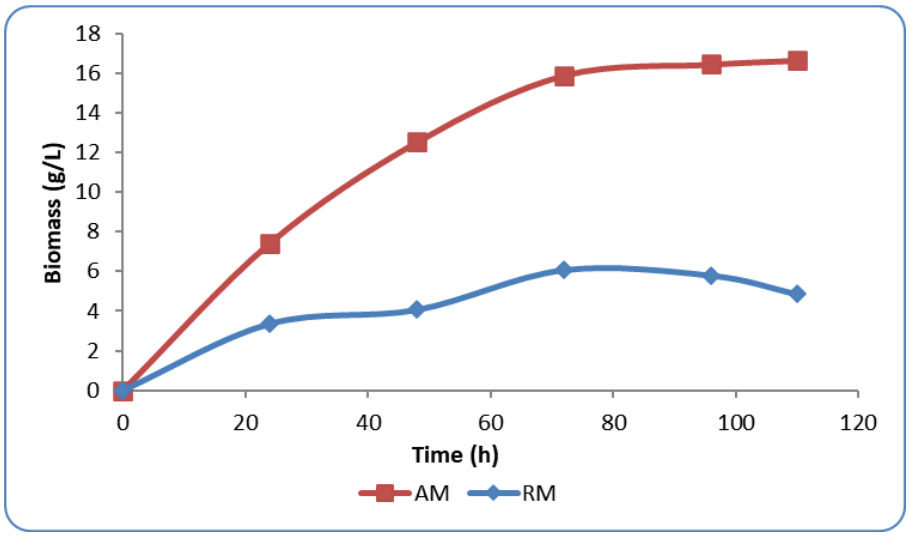


Figure 16 - Growth curve in the alternative medium compared to the reference medium.

The specific growth rate in the two-culture medium did not differ as shown in Figure 17, however, cell productivity was higher in the alternative medium with 0.22 g/L.h and the reference medium with 0.08 g/L.h at 72 h. Considering that the objective was to obtain cellular biomass, the alternative medium presented the best result.

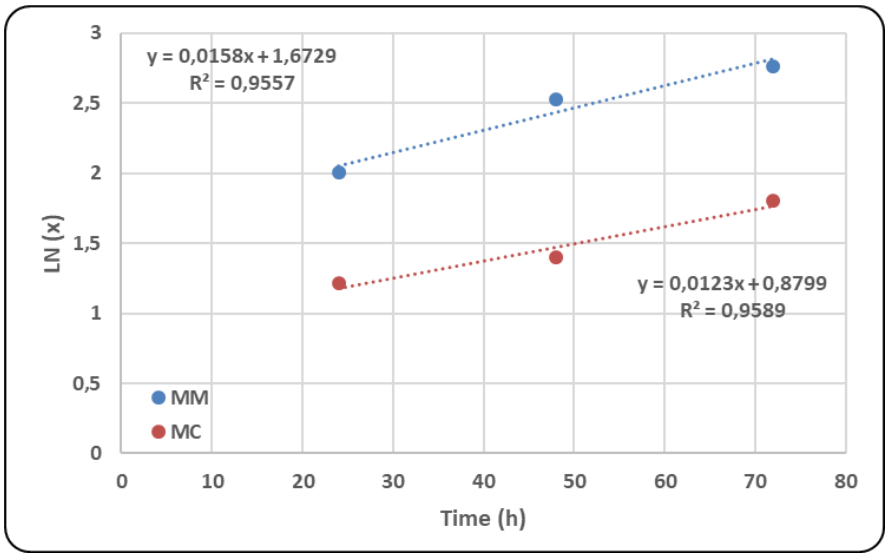


Figure 17 - Specific growth rate in the alternative medium and reference.

With the results obtained in the experiments above, the experimental conditions for obtaining R. oryzae fungal biomass for the synthesis of AgNPs in an alternative cultivation medium were defined as (g/L): 0.5 K_2HPO_4, 0.5 NaCl, 1 .0 $MgCl_2$, 0.5 yeast extract, 10 molasses and 100 CSL (centrifuged), 35°C, 200 rpm, 72 h.

The main elements composition for microbial growth was determined for the two-culture medium, and the results presented in Table 3 show that the carbon and nitrogen source concentrations have little differentiation between the reference medium and the alternative medium composition.

Table 3 - Elementary composition in reference and alternative culture media.

	RM	AM
Carbon (%)	40.04	34.05
Hydrogen (%)	6.83	6.01
Nitrogen (%)	7.84	5.90
Fluorite (mg/L)	0.021	1.770
Chloride (mg/L)	0.328	3.353
Sulfate (mg/L)	0.341	0.941

5.2 Biomass extract

The subsequent step for the AgNP biological synthesis from fungal culture is to obtain the aqueous extract from the biomass. Assuming that the synthesis is mediated by proteins extracted from biomass (Hamedi *et al.*, 2016; Abdel-Hafez *et al.*, 2016), some tests were carried out to obtain an extract

with the highest protein concentration. The extraction method widely described in the literature is a process that consists of subjecting the wet biomass washed to sterile distilled water under controlled agitation and temperature conditions for days. Alsharif and collaborators (2020) studied the AgNP synthesis with 3 different microorganisms (*Bacillus cereus* A1-5, *Streptomyces noursei* H1-1, and *Rhizopus stolonifer* A6-2) and used 10 g of biomass of each for 100 mL of distilled water for the metabolite's extraction. In this work, a proportion of biomass (wet): sterile distilled water was established in 5 g: 100 mL of water. The solubility of proteins in water can be influenced by intrinsic and extrinsic factors (pH, ionic strength, temperature, and solvent additives) and can vary from insoluble to several milligrams per milliliter (Kramer *et al.*, 2012).

Assuming that the molecules that will be dissolved in water are in the cell wall and that a greater quantity of extractives can be obtained due to osmotic difference, a test was carried out increasing the osmotic pressure of the solvent. Arakawa & Timasheff (1991), who presented the interaction of different salts with different proteins, point out that at low concentrations the salts increase solubilization, an effect caused by the interactions between the ions and the protein charges. In the proteins studied, NaCl has greater solubility, has a small effect on the transition temperature, provides molecule stabilization, and has no dissociation effect. The experimental extraction conditions are presented in Table 4. Biomass was obtained by growing the strain in 50 mL Erlenmeyer flasks with a 15 mL reference medium.

Table 4 - Experimental condition, pH values, and cell mass in extraction assay.

Condition	[NaCL] (Molar)	$pH_{initial}$	pH_{final}	$Mass_{inoc.}$ (g)
C1	0	6.9	8.19	0.5257
C2	0.146	5.1	7.23	0.1460
C3	0.154	5.19	7.48	0.5765
C4	0.163	5.24	7.54	0.4475
C5	0.171	5.06	7.33	0.4998
C6	0.205	5.14	7.19	0.3152
C7	0.257	4.93	7.63	0.6883
C8	0.308	4.83	7.29	0.3306
C9	0.342	4.97	6.86	0.2032

The biomass was added to 10 mL of sterile solvent solution according to the table above and incubated at 30°C, 150 rpm for 5 days with daily sampling to determine the protein concentration. The

results obtained in different solvent concentrations showed no differences in extractive power compared to water, which from the second day of incubation already showed an increase in concentration reaching around 35 mg/mL of total protein on the fifth day of extraction, results presented in Figure 18.

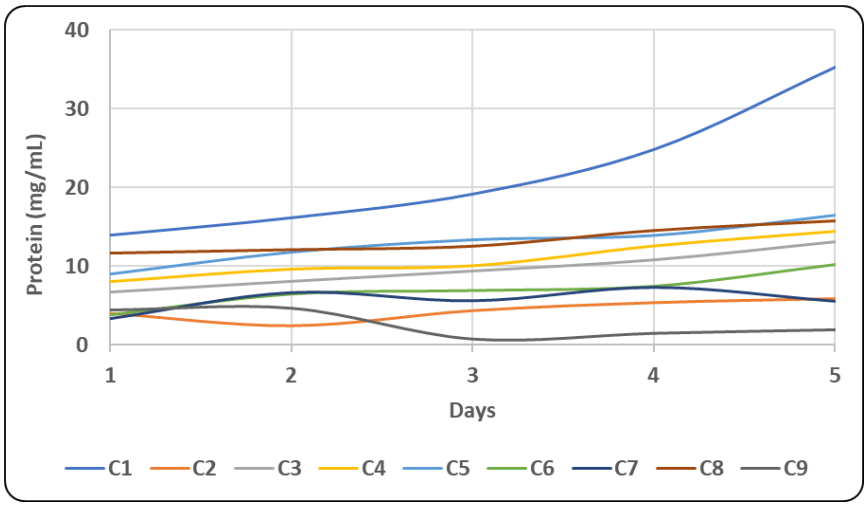


Figure 18 - Total protein concentration over time under extraction conditions with different molar concentrations (NaCl) of the solvent.

The result obtained demonstrates that the osmotic difference did not interfere with the protein molecules extraction that could be extracted from the cell interior, however, the increase in extraction in the water condition and the increase in the pH value in the final solution can be attributed the cell lysis initiation with the release of nitrogenous compounds into the medium, in addition to the hypothesis that the molecules dissolved in the extract may come from the cell wall. Aqueous extraction carried out on other materials such as *Cucurbita maxima* pulp obtained a protein recovery of 1.7% in water and 0.2% in a saline solution (Anastácio *et al.*, 2021). The difference in protein concentration at the extraction end was still between 40% to 45% lower than that obtained with water for the conditions that performed best.

Condition C1 increased total protein concentration by 21.3 mg/mL over the 5 days of extraction, while the best condition in saline solution (C5) only had an increase of 7.4 mg/L. Regardless of the results, the lowest concentrations of NaCl still showed an extraction of around 40% concerning water, which can be attributed to the increase in protein solubility in low concentrations of NaCl.

Another parameter that can interfere with the process extraction is temperature, however, it is known that elevated temperatures can cause protein denaturation, so tests were carried out at different temperatures to optimize extraction and increase the protein concentration in the extract. The tests were carried out with 2 g of biomass in 40 mL of distilled water and incubated at (28, 30, 35, and 40) °C, 150 rpm for 5 days, with periodic sampling to determine the total protein concentration. The results presented

in Figure 19 showed that although there was an increase in the total protein concentration extracted at the different temperatures tested, the best condition achieved was 30°C. In the works reported in the literature, extraction conditions are mostly at temperatures between 28 °C and 30°C, with some exceptions at lower or higher temperatures. (Rai *et al.*, 2015; Wang *et al.*, 2021; Skanda *et al.*, 2022; Gupta & Saxena, 2022).

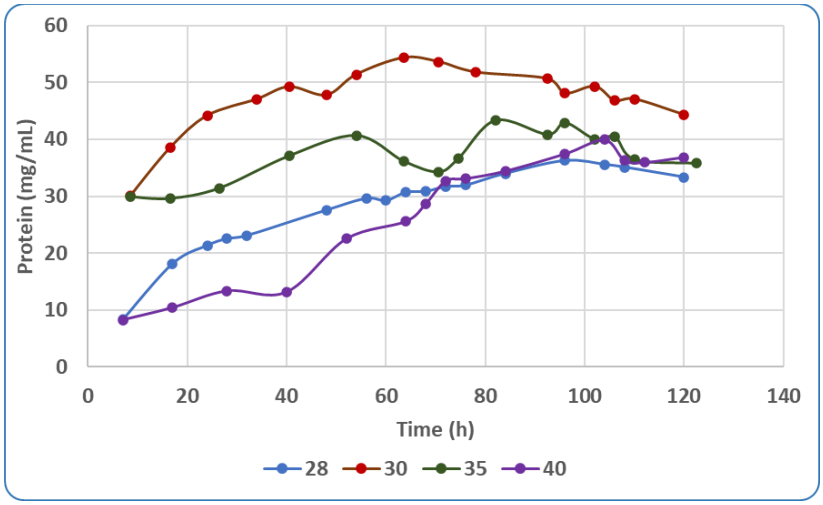


Figure 19 - Total protein concentration over time in water extraction at temperatures of (28, 30, 35, and 40)°C.

It is observed that there is an increase in protein extraction when comparing conditions of 28°C and 30°C. At 30°C the maximum concentration was reached after 63.5 h while at 28°C only after 96 h. However, as the temperature increased to 35°C and 40°C there was an inversion in the extracted concentrations, with a significant impact on the 40°C condition despite reaching a final concentration close to the 28°C and 35°C conditions. Comparing extraction conditions results with other works related to AgNP synthesis becomes complicated because the authors focus on nanoparticle synthesis and not on the extraction step, which is the step to obtain the silver-reducing components.

5.3 Extract Characterization

5.3.1 Enzyme/protein identification

The extract components obtained from microbial biomass are responsible for the silver reduction reaction for the nanoparticle synthesis. A few years ago, the nitrate reductase enzyme was attributed to the function of reducing silver for nanoparticle synthesis (Kumar *et al.*, 2007; Durán *et al*, 2005). Indeed,

some microorganisms can produce nitrate reductase, such as *Fusarium oxysporum*, which is also a natural plant enzyme (Gholami-Shabani *et al.*, 2014; Vijayan *et al.,* 2016).

Rhizopus arrhizus NRRL 54538 (IPT 1013) strain can produce some enzymes depending on the experimental conditions, such as amyloglucosidases, glucoamylases, lipases, isoamylases, proteases, cellulases in addition to organic acids such as lactic, malic and fumaric acids. Therefore, it was necessary to identify the proteins extracted from this biomass, which was carried out using mass spectrometry.

Culture medium extract, and colloidal silver suspension (AgCS) samples were subjected to protein separation by silver-stained SDS-PAGE. For identification by LC-MS-ESI, the bands were cut from the gel, bleached, and subjected to enzymatic digestion to separate the peptides, and subsequently subjected to chromatographic separation. The spectra obtained were submitted to the Proteome Discoverer 1.4 search database (Thermo Electron Corporation) using the database for *R. oryzae* and presented the results listed in Table 5.

Table 5 - Proteins identified in samples of aqueous fungal extract and AgNP colloidal solution.

Sample	Identification	Score	Coverage
Extract	glucoamylase	97.00	88.74
	α- amylase	67.77	89.39
	Aspartyl endopeptidase	30.15	83.29
	Pyruvate decarboxylase	11.97	75.71
	NADH-ubiquinone oxidoreductase	4.66	74.35
	Rhizopuspepsin 2	11.23	82.61
AgCS	glucoamylase	71.64	92.72
	lipase	77.87	95.63
	polygalacturonase	13.38	82.51
	1,3(4)-β-glucanase	43.03	86.35
	Rhizopuspepsin 2	43.58	82.86

In the extract sample, the characteristic proteins of this strain were identified, with a glucoamylase predominance followed by alpha-amylase, in addition to metabolic enzymes. In the AgCS sample, glucoamylase is present and lipase appears, also with a high incidence, in addition to other enzymes not identified in the sample extract. Of the enzymes identified in these samples, none are considered to have oxidoreductive properties, only NADH identified in the extract, despite its low score, is the only molecule identified as capable of reducing silver in the biogenic nanoparticle synthesis.

Another experiment was performed with the lyophilized extract sample to confirm the results. The sample underwent the same preparation procedure for identification by LC-MS/MS-ESI. In this identification, the proteins presented in Table 6 were obtained. The results confirm the presence of some of the previously identified enzymes and NADH, despite the low score (Table 6).

Table 6 - Proteins identified by mass spectrometry in a lyophilized aqueous fungal extract sample.

Identification	Score	Coverage
Lipase OS	10.19	98.98
Fumarate hydratase	9.41	99.80
Endo-1,4-beta-xylanase	6.35	97.69
NADH-ubiquinone oxidoreductase chain 2	5.18	98.28
Orotidine 5'-phosphate decarboxylase	0	100.00
L-lactate dehydrogenase A	0	96.88

All these enzymes are related to the metabolism of this microorganism and are corroborated by work on the corresponding molecule production (Maas *et al.*, 2008; Meussen *et al.*, 2012; Anwar *et al.*, 2023).

These results were obtained based on information available in databases of the *R. oryzae* family, as there are taxonomic complications in the genus *Rhizopus*, with a recent proposal to reclassify *R. oryzae* (formerly *R. arrhizus*) to include two other species, *R. oryzae*, and *R. delemar*. In the classification by Schipper & Stalpers (1984), *Rhizopus oryzae* was recognized as a species with intermediate morphology and physiological characteristics. They investigated 47 strains from 21 species and classified them all as *R. oryzae* because they were fundamentally indistinguishable. In terms of the proteomic database, the Basic Local Alignment Search Tool (BLAST) was used according to the taxonomic characteristics. The protein information available for the taxonomic genus of *Rhyzopus* belongs to *Rhyzopus oryzae*, due to reclassification. BLAST searches for regions of local similarity between sequences. After peptide sequencing from MS/MS fragmentation patterns, the program compares protein sequences to sequence databases and calculates the statistical significance of the correspondences, so that the results obtained can be considered valid as the values are significant.

With these results obtained in the enzyme identification contained in the biomass extract, we can confirm the non-existence of the nitrate reductase enzyme and infer that the possibility of reducing action in the AgNP synthesis for this strain is related to the NADH presence, the only molecule identified with

the oxide reductive characteristic. More recent work also demonstrates that this molecule is one of the possible ones for the biogenic AgNP synthesis reaction (Hietzschold *et al.*, 2019; Mikhailova *et al.*, 2020). Despite many studies performed with a wide range of microorganisms, the diversity of identification of the reducing agents that participate in the reduction reactions of silver or other metals in biogenic synthesis is still a gap in science.

5.3.2 Other elements

Although the biomass was washed abundantly to remove residues from the culture medium and possible metabolic substances released during growth, other soluble elements from the cell mass may have been extracted. These elements may have reduced power and participate in AgNP synthesis. Quantification analyzes of ions and elements were carried out, both in the reference medium extract and in the alternative medium (Table 7).

Table 7 – Ions concentration and elements identified in the fungal extract samples from reference and alternative media.

	Extract RM	Extract AM
Carbon (%)	0.32	0.17
Hydrogen (%)	10.55	10.79
Nitrogen (%)	0.06	0.07
Chloride (mg/L)	1.81	2.29
Sulfate (mg/L)	5.76	3.92
Phosphate (mg/L)	30.23	25.25
Bromide (mg/L)	3.92	-
NH_4+ (mg/L)	79.37	3.5

No preponderant variation was observed in the elemental compositions between the two extracts, regardless of the cultivation medium composition. A scan was also carried out, without quantification (Figure 20), of elements by ICP-OES for both samples and it was observed that the RM extract sample has fewer chemical elements than the AM extract, this variation may be related to the composition of the culture medium waste components.

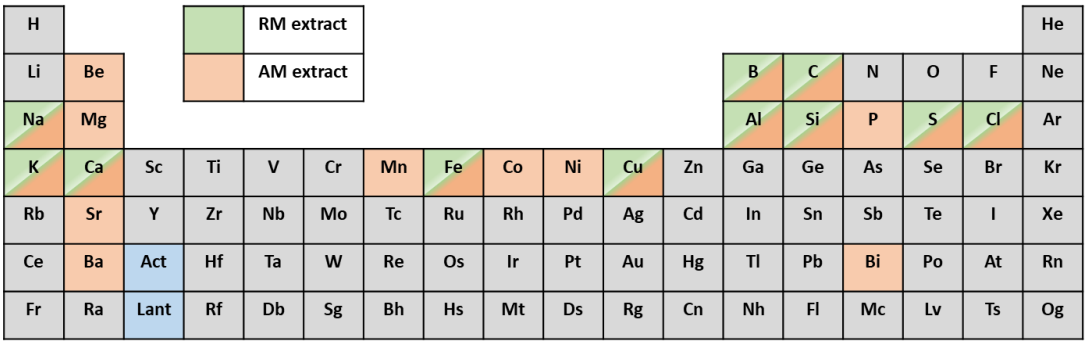
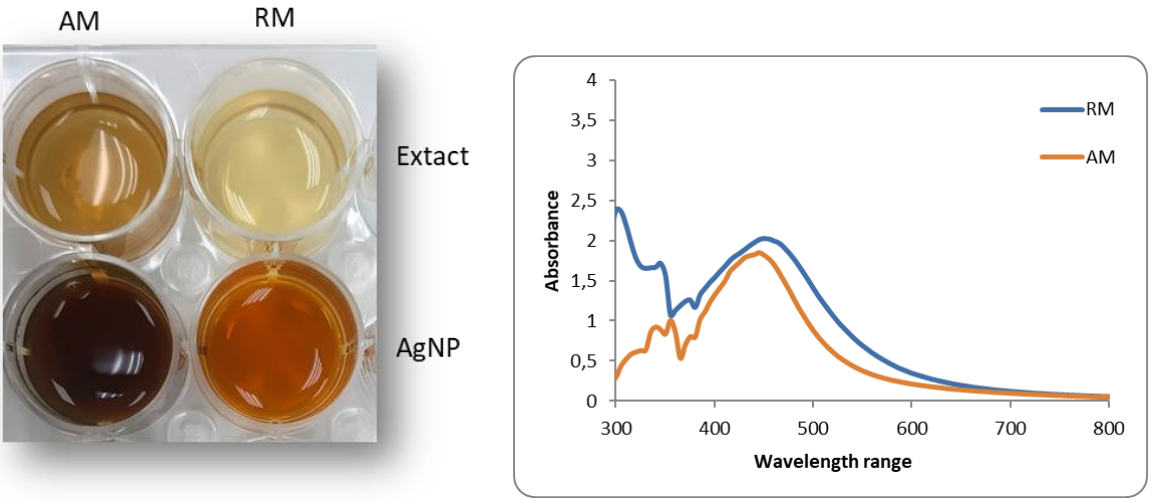


Figure 20 - Chemical elements were identified in the fungal extract samples from RM and AM media.

5.4 Nanoparticle synthesis and characterization

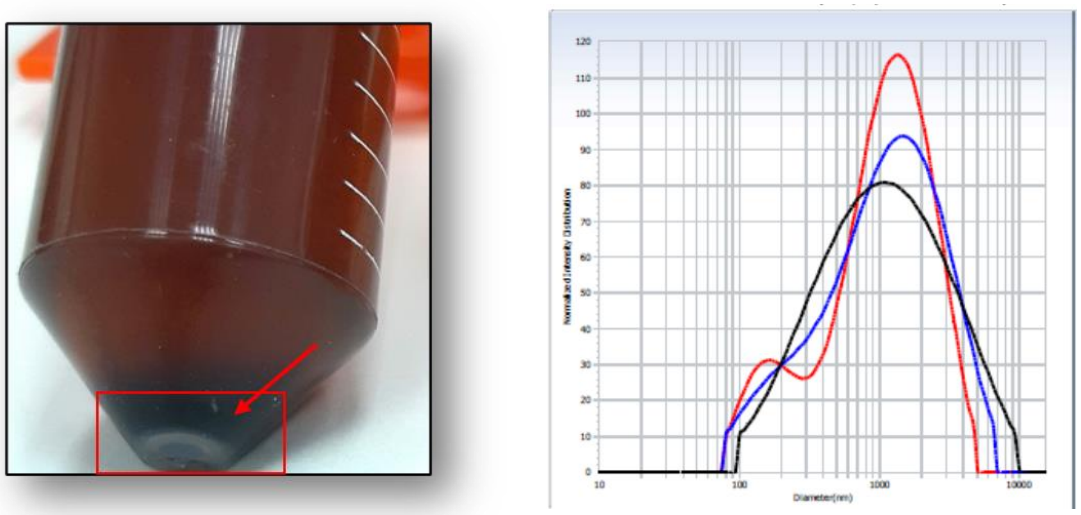
The biogenic synthesis study was obtained from the biomass extract of *R. arrhyzus* in both study media. The extract was added to a silver nitrate solution with a final concentration of 1 mMol/L. All experiments were carried out at 30°C, 150 rpm on a rotary shaker. Monitoring the AgNP formation in the colloidal suspension was done by observing the characteristic color change in this reaction (yellow to brown), indicating the AgNP formation and deposition. This change results from the excitation of the AgNP surface plasmonic vibrations. The light absorption profile by UV-vis spectrometry was carried out in the wavelength range of 300 nm to 800 nm, as the plasmonic bands on the nanometric scale for AgNP are found between 400 nm and 450 nm (Eya'ane Meva *et al.*, 2016; Skanda *et al.,* 2022). The samples that showed a change in color and plasmonic band characteristic of AgNP demonstrated that there was a reduction of silver, which may be in the form of nanoparticles. The absorption spectra in the UV-vis region of the tested AgNPs indicated the surface plasmon resonance peaks at maximum absorbance for RM (455 nm) and AM (450 nm), as shown in Figure 21.



(A) (B)

Figure 21 - Change in color (A) and plasmonic resonance profile (B) of AgNP synthesis from biomass extracts obtained in RM and AM medium.

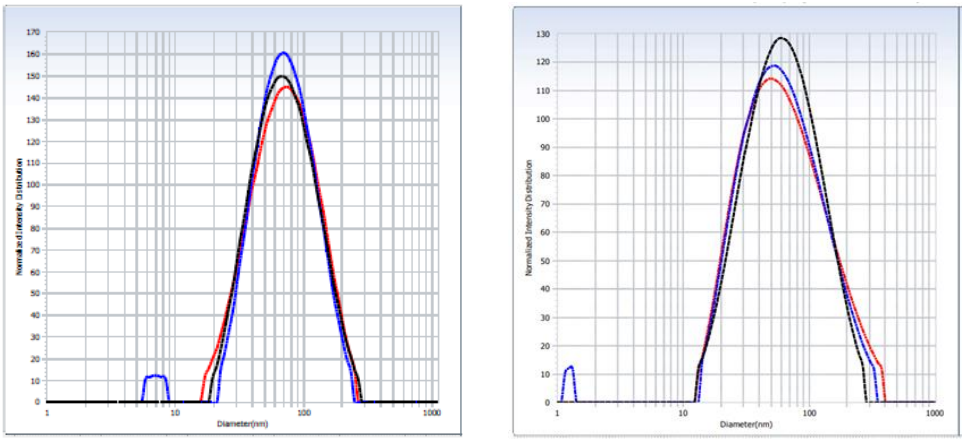
As previously reported, the extracts from the alternative medium initially used in the work presented a very fine solids quantity (Figure 22), where interference was observed in measuring particle size, presenting a considerable dispersion, as the solid particles were evaluated as nanoparticles. This problem was solved with the cultivation medium new preparation, where the AgNP colloidal suspensions showed less size dispersion.



(A) (B)

Figure 22 - Representation of interfering solids in AgNP size measurements (A) and graph of size dispersion variation by DLS (B), the different line colors are repetitions.

The AgNPs were characterized in size by DLS, presenting for the nanoparticles produced by the reference medium an average size that varied from 40 nm to 70 nm with a dispersion of 0.3 to 0.7 nm. Those synthesized by the alternative medium presented a size that ranged from 70 to 100 nm with a dispersion of 0.2 to 0.4 nm (Figure 23). The zeta potential results were - 31.4 mV for RM and -20.3 mV for AM, these values configure more stable nanoparticles. AgNPs are more stable and less likely to agglomerate when the surface charge exceeds +30 mV or less than -30 mV (Saeb *et al.*, 2014; Bruna *et al*, 2021).



(A) (B)

Figure 23 - Graphical representation of the AgNP size dispersion originating from the RM (A) and AM (B) medium, by DLS. The different color lines is repetitions.

The metallic nanoparticle biogenic synthesis is not yet fully understood in scientific circles, precisely due to the diversity of organisms (microorganisms and plants) used in the biosynthesis of various metals, especially silver. As previously reported, there is a diversity of chemical elements and organic molecules, that may be involved in possible reactions. The extract is a complex medium due to its composition and can provide cross-reactions to reduce silver, depending on the conditions offered.

In this work, an attempt was made to identify the components that could have the capacity to reduce silver by forming nanoparticles. Of the compounds/elements identified, NADH showed the greatest potential to be the reducing agent in this synthesis. To verify this potential, an experiment was carried out with different extract conditions and the addition of NADH. The extract used was obtained from biomass grown in the reference medium and underwent some treatments to inactivate NADH (60°C) and the enzymes present (100°C), in addition to being separated by ultrafiltration in a 10KDa membrane,

using both the permeate and the concentrate compared to the pure extract and added NADH. The results of the size of the nanoparticles formed are shown in Figure 24.

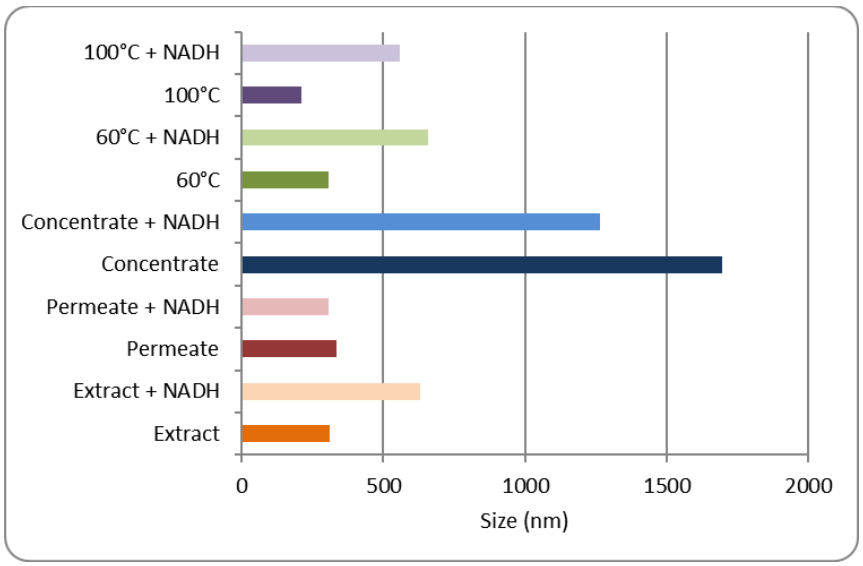


Figure 24 – DLS measured particle size, synthesized in different extract conditions, and extract added with NADH for 120 h.

Although the size values obtained in the experiment do not correspond to the nanoparticle sizes (up to 100 nm), values may have been influenced by the concentration or particle agglomeration (Dinesh *et al.*,2021). It is noted that the NADH addition in all conditions, except for the permeate, showed a larger nanoparticle size. The enzymes and NADH inactivation by temperature (60°C and 100°C without NADH) did not prevent the nanoparticle synthesis, with results like the extract, meaning that the activity of these molecules is not directly related to synthesis. On the other hand, when the extract was subjected to ultrafiltration with the separation of molecules larger than 10 KDa, it was noticed that much larger particles were formed, however, with the NADH addition there may have been an inhibition by the substrate in the reaction. In the permeate where only the smaller molecules remained, it is observed that the difference between the condition with and without the NADH addition does not demonstrate differentiation to the extract.

In the concentrate condition, the increase in particle size may be related to the protein corona or agglomerated nanoparticle formation and not specifically to the size of the reduced silver particle. A similar result was obtained by Hietzschold *et al.*, (2019) when they synthesized AgNP using only NADH. Adding nitrate reductase to the reaction resulted in larger particles. In this same work, the highest synthesis reaction speed was obtained with NADPH at 80 °C, corroborating the results of protein inactivation achieved here.

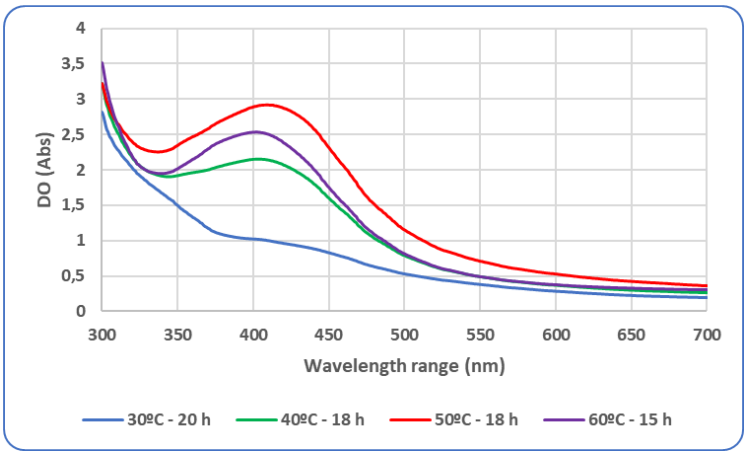
However, several molecules have been identified as reducing agents, but due to the extract composition complexity obtained from fungal, bacterial, or even vegetable biomass, it has not yet been possible to determine precisely which biomolecules are responsible for this reduction. According to some authors, several NADH-dependent molecules such as nitrate reductase, the flavoprotein subunit, and sulfite reductase may be related to metallic nanoparticle synthesis (Bamal *et al.*, 2021; Durán *et al.*, 2023). These results also point to the hypotheses defended about NADH participation as one of the reducing agents in metallic nanoparticle synthesis.

5.4.1 Effect of temperature on AgNP synthesis.

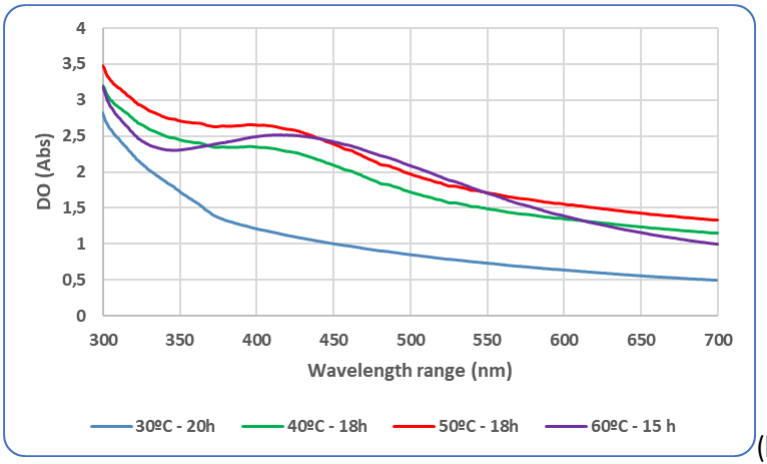
Just as the extract composition is directly related to the synthesis, other factors such as pH and temperature also play a significant role. During the studies carried out in this work, it was observed that the AgNP synthesis for the conditions tested here always occurred in a pH range varied from 7.5 to 9.0. Dinesh *et al.*, (2021) in a study of the parameters that influence the AgNP synthesis from the *Penicillium cinnamopurpureum* aqueous extract, also obtained the best results in the synthesis with pH 9.0 and a temperature of 60°C for 2 h.

In this development, preliminary synthesis studies were carried out at 30°C for 5 days of incubation. In addition to other factors, the reaction time of a process is directly related to its cost, so if the objective is to obtain a process with green technology and at an affordable cost, it is necessary to improve time. The temperature is relevant to the reaction speed in both chemical and biological processes. In this work, assuming that the reducing agents could be the proteins contained in the extract, these certainly have their optimal action temperature. To optimize the reaction time, the synthesis was carried out at different temperatures (30, 40, 50, and 60) °C accompanied by the light absorption reading profile at wavelengths from 300 nm to 700 nm every hour for 15 h to 20 h, depending on the condition.

Figure 25 A and B show the differences in plasmonic resonance profiles for each treated sample. The increase in reaction temperature positively influenced the synthesis time for the two samples tested, since synthesis was obtained within 18 hours compared to the previously studied condition of 120 h. Other authors also achieved equivalent results using other microorganisms. From the *R. stolonifer* biomass extract Abdel-Rahim *et al.*, (2017) and Banu and Rathod (2013) synthesized AgNPs at 40°C and obtained the best result after 48 hours of reaction. The reaction time differentiation by different research can be attributed to the extract composition from different microbial strains, although reducing agents are metabolic substances extracted from biomass, each microorganism has its own metabolism and product differentiation, influencing the synthesis reaction.



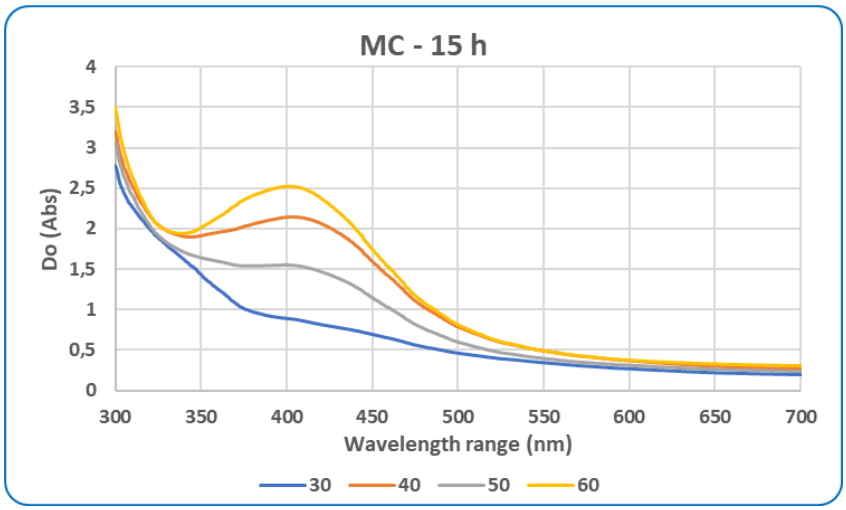
(A)



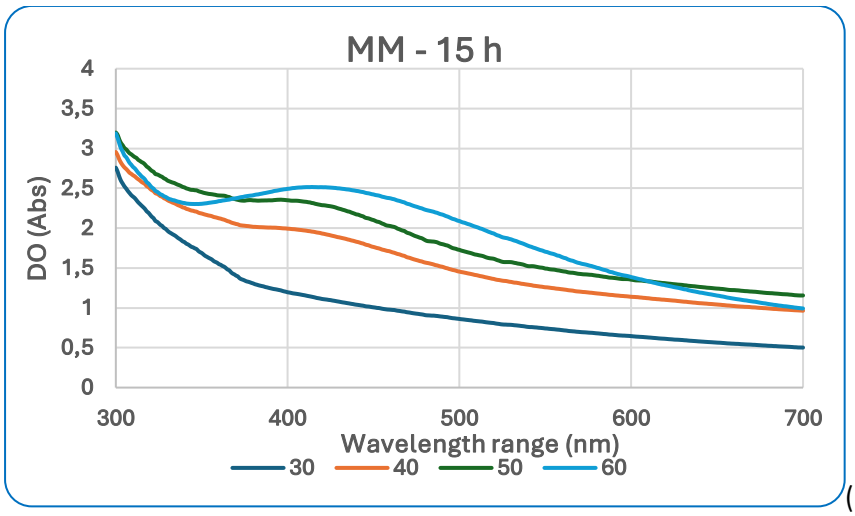
(B)

Figure 25 - Plasmon resonance absorption curves of AgNP synthesis in RM (A) and AM (B) extracts at temperatures of (30, 40, 50, and 60) °C. The curves shown were constructed at the maximum absorption time.

The plasmonic resonance profile behavior of the two samples evaluated was very different when measured at the last reading point, pointing to the best result at 50°C. However, when comparing the two samples after 15 hours of reaction, the best result occurs at a temperature of 60°C, as shown in Figure 26 A and B.



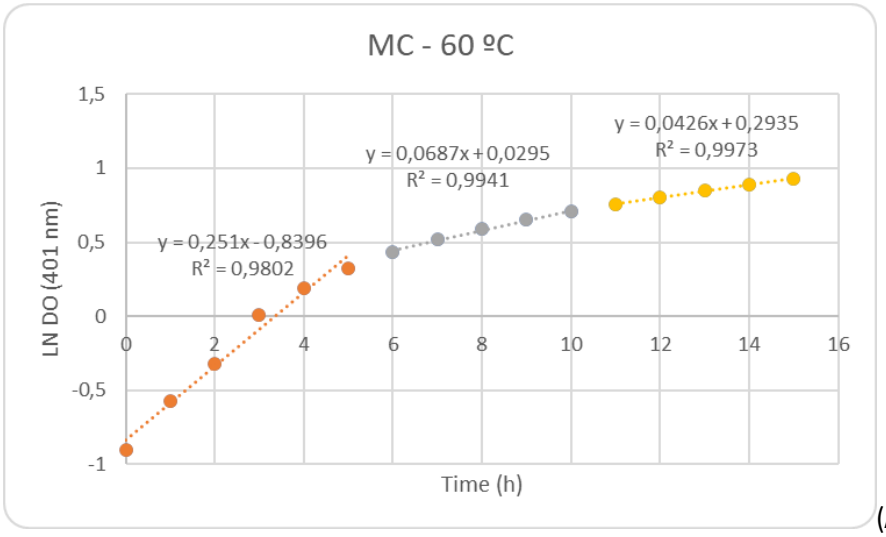
(A)



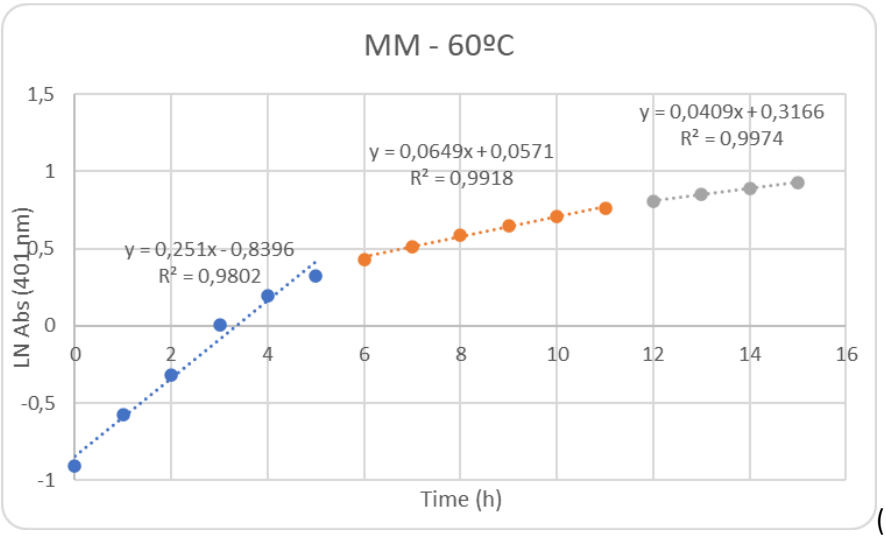
(B)

Figure 26 - Maximum plasmon resonance absorption curves of AgNP synthesis in RM (A) and AM (B) extracts at temperatures of (30, 40, 50, and 60) °C with 15 h of reaction. The curves shown were constructed at the maximum absorption time.

The specific reaction rates in the two samples evaluated at the highest plasmon resonance point (401 nm) do not differ from each other (Figure 27), however, it is clear that there is a greater speed at the beginning of the reaction, which may be related to the particle nucleation and over time the speed decreases, when particle enlargement or the stability coating formation, the so-called protein corona, is suggested. This hypothesis is defended by Godfrey *et al.*, (2020), who studied the AgNP formation followed by protein coating in chemical synthesis. This behavior can also be observed with the plasmon resonance profile of the RM sample presented in Figure 28 A. The peak shift to the right is attributed to the increase in particle size (Dinesh *et al.*, 2021). This sample presented a size of 54.5 nm with 4 h of synthesis and after 4 and 5 days, 57.2 nm and 57.6 nm, respectively, with an increase of approximately 3 nm in particle size. A comparable situation occurred with an alternative medium sample (B) with 51.9 nm within 24 h of synthesis and 95.2 nm after 4 days, with an increase of 43.3 nm.

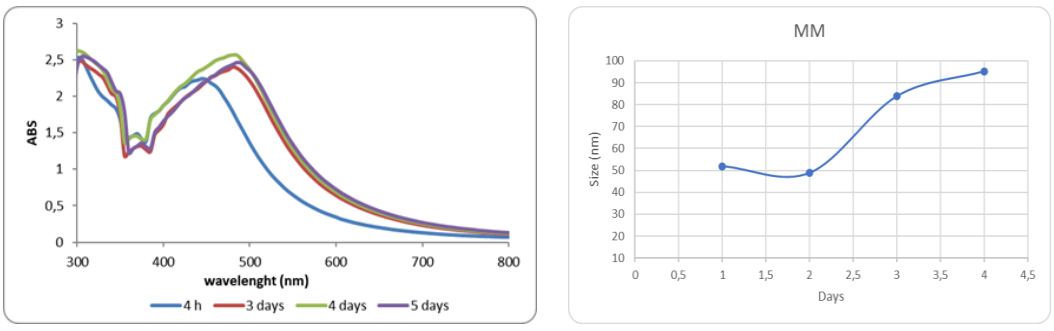


(A)



(B)

Figure 27 - Graphical representation of the synthesis reaction specific rate at 60°C for RM (A) and AM (B) AgNPs.



(A) (B)

Figure 28 - Plasmonic resonance profile of RM AgNP synthesis with increasing size over time (A) and difference size over time of AM AgNP (B).

According to Piella *et al*, (2016), protein coronas can be formed in several ways depending on the proteins available in the medium. They can be larger or smaller, classified as soft or hard corona. The difference between them lies in the balance and dynamics of the bonds with the nanoparticle, providing partial or total coverage and determining the size, which can vary from 3 to 100 nm, also dependent on the exposure time. In the work carried out by Tomak *et al.*, (2022) on nanoparticles of chemical origin with the induction of the protein crown formation through the DMEM medium and fetal bovine serum, the composition it is possible to verify the increase in nanoparticles through plasmonic resonance, DLS (Table 8) and imaging by TEM (not presented).

Table 8 - Particle size results with incubation time in DMEM medium + fetal bovine serum by Tomak *et al.*, 2022.

Incubation time	Size (nm)
Before incubation	65.9 ± 1.3
15 min	149.5 ± 2.2
2 h	196.8 ± 13.0
24 h	287.1 ± 10.3

Source: Tomak *et al.*, 2022

Results similar to those obtained in this work and reported above show that the difference in size is certainly related to the type of proteins and biological material available in each of the systems presented. The TEM images in Figure 29 were acquired from a nanoparticle obtained with RM medium extract and are similar to those demonstrated by the authors, with a coating, which we have not yet identified.

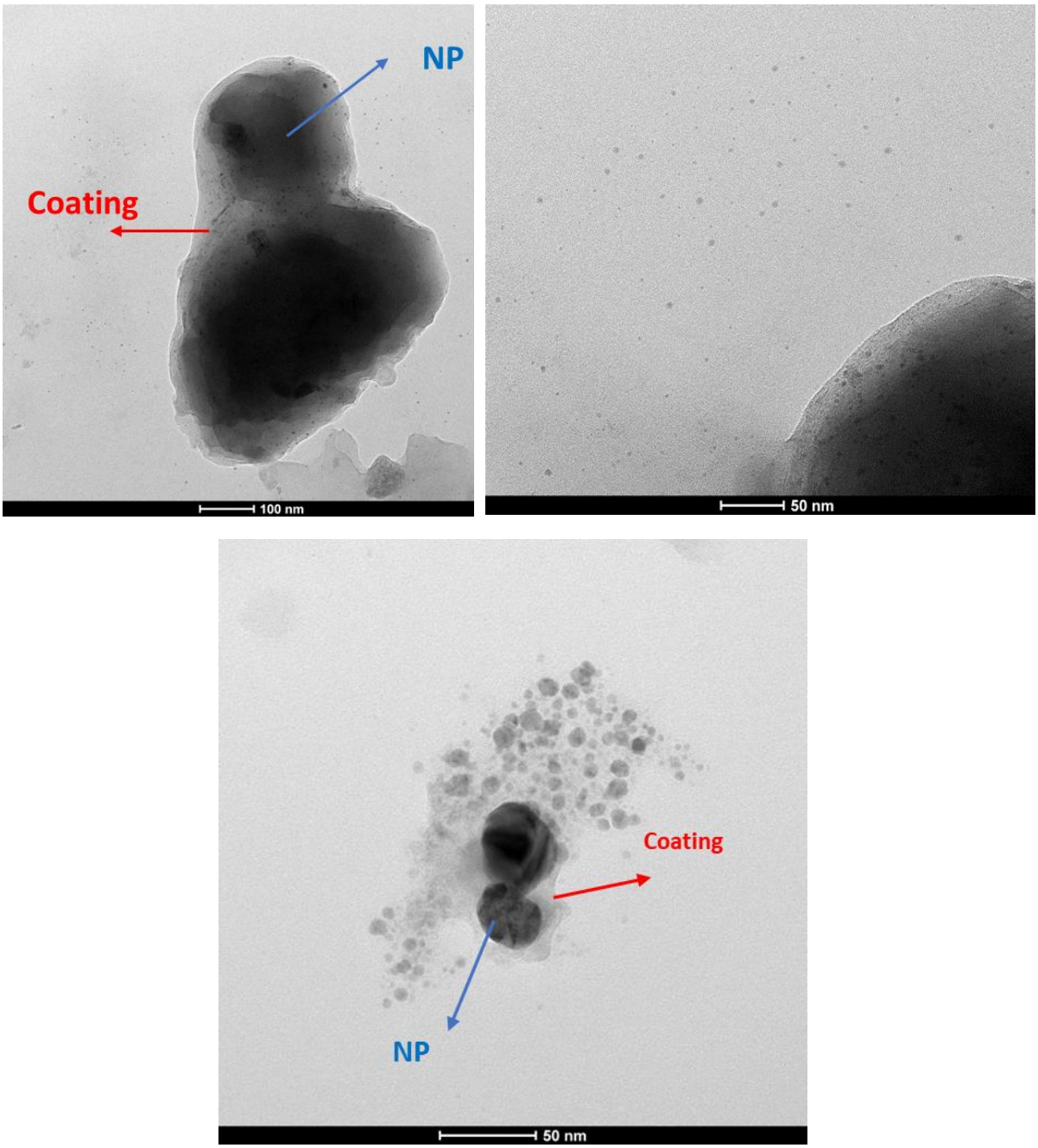


Figure 29 - TEM images of AgNPs synthesized from *R. arrhizus* NRRL 54538 (IPT 1013) extract from RM medium.

The samples were subjected to silver amount analysis by ICP-OES to determine the conversion percentage of ionic silver to metallic silver. The colloidal silver suspensions were centrifuged to separate the particles and the determinations were carried out in both samples, supernatant (RM and AM), and nanoparticle suspension. With the values obtained, a conversion of 55.6% for the RM sample and 53.0% for AM can be attributed, however, these values can be approximate, as there is no guarantee of the total

separation nanoparticle in the centrifugation process, because the suspension is a mixture of various sizes, and the smaller ones may not have been separated during centrifugation.

5.5 Bioreactor Experiments

Since the AgNP synthesis comes from biomass extraction, increasing this is the next step in the search for an increase in scale. Batch tests were carried out in a bioreactor with an operational volume of 2 L, using Rushton-type impellers, where the two culture mediums already studied in the previous stage were tested, even for comparison purposes concerning the biomass produced under different conditions.

Filamentous fungi present different structural forms of growth during their life cycle, with the basic vegetative growth structure, the hyphae, consisting of a tubular filament, resulting from the germination of a single spore (Papagianni, 2004)., when grown in submerged culture, also exhibit morphological forms that can range from dispersed filaments to dense aggregated masses, called pellets. These forms result from the genetic characteristics of the chemical and physical conditions imposed in the process (Kossen, 2000; Znidarsic & Pavko, 2001).

The use of CSTR-type bioreactors for filamentous fungi can be harmful to growth due to the shear caused by the impellers on the fungal hyphae, causing rupture, sometimes even preventing proliferation (Merchuk and Gluz, 1999; Kelly *et al.*, 2006). Because of this particularity, the first experiments were realized with low agitation (300 rpm). The other natural peculiarity of fungi, also related to growth, is the ability to grow anchored to any solid material, which makes it difficult to recover biomass when the process requires it.

5.5.1 Reference medium - RM

The test carried out with the reference medium in a B. Braun bioreactor with a volume of 2L, 300 rpm, and 1vvm of aeration, presented the characteristics of the fungus presented above. The determination of biomass was made impossible because the growth occurred adhered to the parts of the bioreactor, as demonstrated in Figure 30.

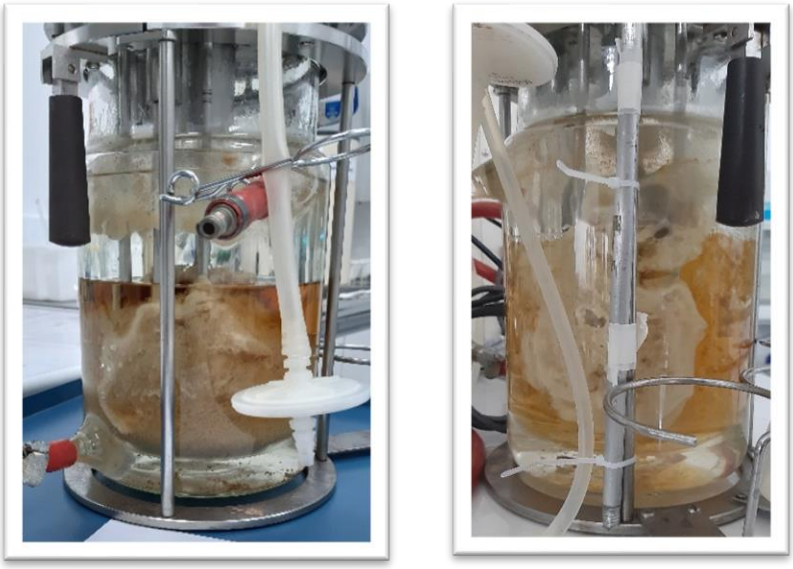


Figure 30 - A representative image of microbial growth of R. arrhizus from two different angles with low agitation in a bioreactor.

Other monitoring parameters were analyzed (Figure 31 A and B), and so was the growth behavior profile of the microorganism in this experimental condition.

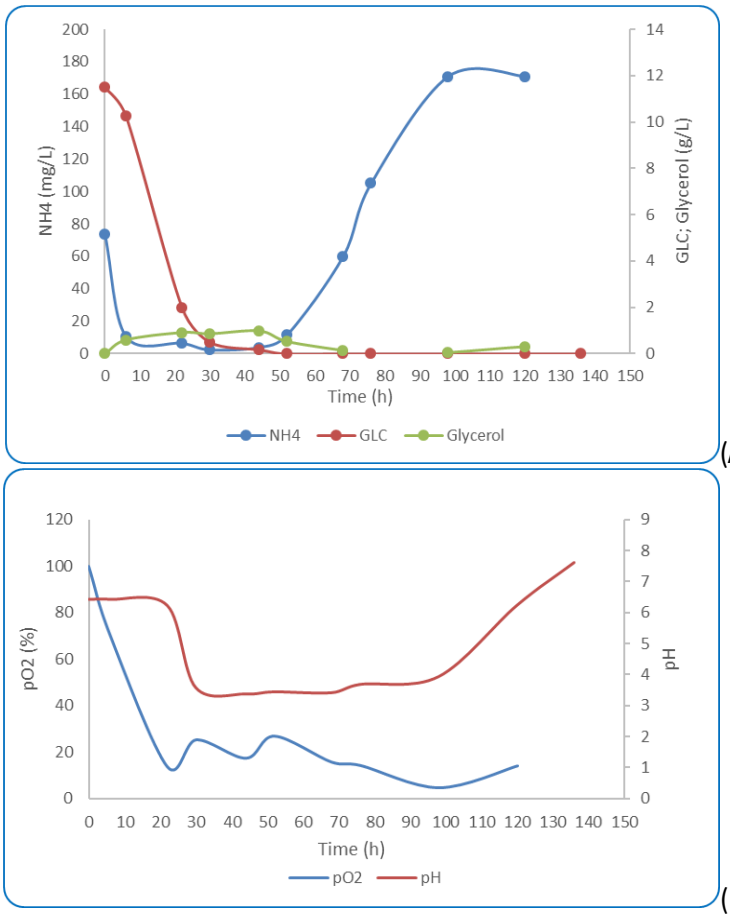


Figure 31 - (A) profile of glucose consumption, formation of ammonia nitrogen and glycerol over time in a bioreactor, and (B) profile of the variation in dissolved oxygen and pH.

Regarding the growth behavior under the tested conditions, it can be observed that the pH remained stable (~6.5) in the first 22 hours, a period also corresponding to the consumption of 80% of the total carbon source. After the end of the carbon source (44 h) over 8 hours, the pH decreased to 3.4, remaining stable for 68 h and increasing at the end to 7.6. At the same time, an increase in ammonia nitrogen was observed after 44 hours of testing. This result may be related to the consumption of indirect carbon sources in the composition of the extracts from the culture medium, such as proteins and amino acids.

At the end of the experiment, the suspended biomass was washed and subjected to the water extraction process for the AgNP synthesis, resulting in particles measuring 44.2 nm by DLS

To minimize the fungus adhesion to the bioreactor parts and facilitate the biomass recovery for the subsequent process stage, tests were carried out with high agitation (800 rpm), despite the incidence of shear on the fungal hyphae. It is worth mentioning that the main objective of increasing agitation was not related to oxygen transfer or improving the system homogenization but rather to reduce the size of the hyphae. The tests were carried out in a Bioengineering mod. RALF with a useful volume of 2 L. The two-culture media studied were tested: reference and alternative. The following Figure 32 shows the cell growth profile in the reference medium and microorganism behavior under agitation at 800 rpm in a bioreactor.

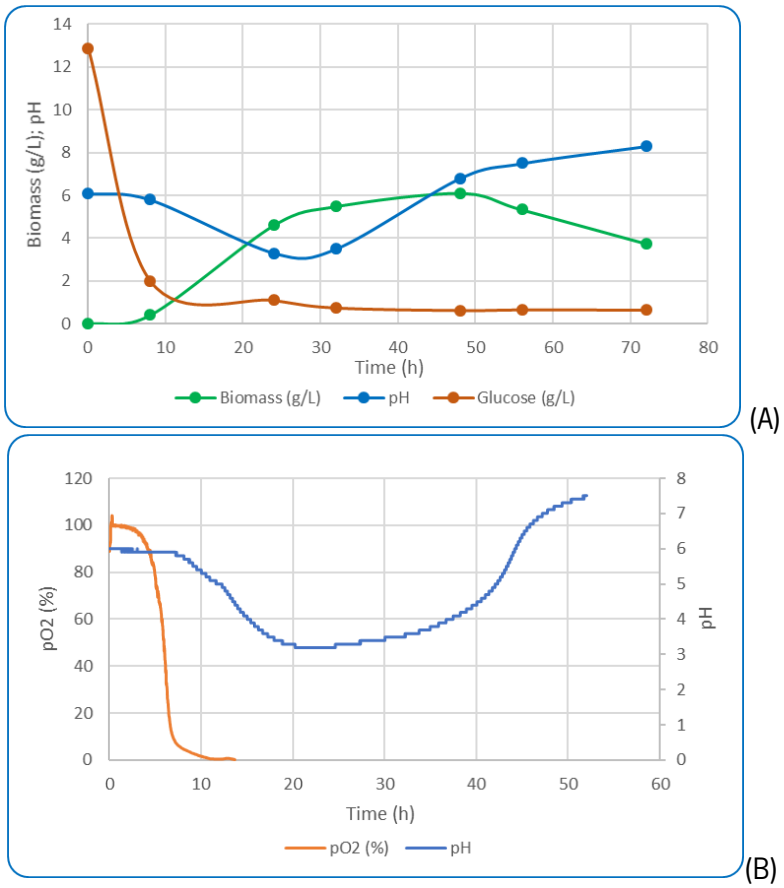


Figure 32 - (A) profile of glucose consumption, cellular growth, and pH over time and (B) profile of variation in dissolved oxygen and pH in a bioreactor with MC medium.

Cell growth behavior under these conditions was similar to the experiment with low agitation concerning pH and dissolved oxygen. The glucose concentration was consumed in the first 20 hours of the experiment, as well as dissolved oxygen, when a reduction in pH and an increase in biomass were also observed. From then on, the biomass stabilized, the pH began rising and the dissolved oxygen remained at 0%. However, it was observed that after 48 hours of the experiment, the biomass reached its maximum concentration (6.1 g/L) and began to decline. The biomass obtained was very fluid (Figure 33) and did not adhere to the reactor parts, allowing its recovery for determination, extraction, and synthesis analysis.



Figure 33 - Appearance of biomass obtained with RM medium in a bioreactor with agitation at 800 rpm.

To determine whether shear interference in the hyphae could have affected the AgNP synthesis capacity, the biomass was collected after 48 h and subjected to the water extraction procedure and subsequently to synthesis. The synthesis result was similar to that of growth in shake flasks, with AgNP size 52.3 nm for the bioreactor and 49.1 nm for the flask.

Due to the larger contact area for extraction in this biomass, an extraction time experiment was carried out to check whether it could be shorter. The biomass from 48 h of the experiment was washed and distributed into several flasks in a proportion of 5 g for 100 mL of water, incubated at 30°C and 150 rpm. The flasks were removed daily until the fifth day and the extraction filtrate was subjected to synthesis. The results obtained are presented in Table 9.

Table 9 - Size and zeta potential of AgNP obtained on extraction different days with biomass from 48 h of growth in a bioreactor test with MC medium at 800 rpm.

Extraction (days)	Size (nm)	PDI (nm)	ZP (mV)
1	71.86	0.28	-27.7
2	45.33	0.28	-11.43
3	48.19	0.29	-19.43
4	55.24	0.28	-24.40
5	47.14	0.43	-19.83

These results demonstrate that with 1 day of extraction, under the conditions tested, AgNPs may present a larger size than the other days, this may be related to a greater activity of the extracted components, however, no differentiation was observed between the results obtained on the other days. It was concluded that 1 day of extraction under these conditions may be sufficient to obtain AgNP synthesis. These results were favorable from the point of view of process time, as obtaining the biomass went from 3 to 5 days to 48 hours and the extraction that used to take 3 to 5 days became just 1 day.

5.5.2 Alternative medium - AM

As mentioned previously, waste utilization in preparing the culture medium can lead to some operational challenges. CSL is a residue from the corn industry and is abundant worldwide, representing around 35% of global corn production. Its composition contains proteins, minerals, vitamins, reducing sugars, organic acids, and other nutrients that make it efficient as a cultivation medium, currently being used in various fermentative processes (Zhou *et al*, 2022).

Due to the test carried out with the reference medium with low agitation that resulted in compact growth in the parts of the bioreactor and difficulty in recovering the biomass, experiments with low agitation were not carried out with the alternative medium. Other factors were addressed when conducting bioreactor experiments, such as the preparation of the culture medium and the initial pH of the process. A first test was carried out without changing the preparation of the culture medium, the initial pH was 4.0, due to the addition of CSL, which has an acidic pH. The experimental conditions were the same as those of the reference medium. This experiment did not show any change in the appearance of hyphal growth; however, it was not possible to monitor it, as foam formed and the mycelium floated to the surface, preventing the removal of samples to continue testing.

As happened previously, it was decided to reproduce the experimental conditions carried out with the reference medium. That is initial pH between 6.3 and 6.5, 800 rpm, and 75 Lh^{-1} (0,625 vvm) of air in 2 L of culture medium. However, the CSL use as a medium component was hampered mainly by increasing the pH value. Precipitation occurred in the culture medium when adjusting the pH to values close to 6.5 (4 Mol/L NaOH). Some attempts to prepare the medium were made to minimize interference, as shown in Table 10. In the meantime, it was noted that the molasses had a quantity of solids that could interfere with the biomass determination and subsequent extraction. Therefore, the molasses was also diluted and centrifuged before preparing the culture medium.

Table 10 - Experimental conditions of the tests carried out with an alternative medium (AM) in a bioreactor with 800 rpm of agitation.

Assay	Condition	Biomass max. (g/L)	Time (h)
1	No pH correction	5.0	32
2	Correct pH in the tank 3.9 – 6.3	5.7	24
3	pH CSL corrected before sterilization	11.3	32
4	No pH correction	11.6	36

Despite the operational problems that occurred in the tests, it was possible to observe that regardless of the pH, the microorganism grew under the conditions offered, reaching biomass values similar to those obtained in flasks. The following test was carried out without pH correction, and it was possible to monitor until the end. The results are presented in Figure 34.

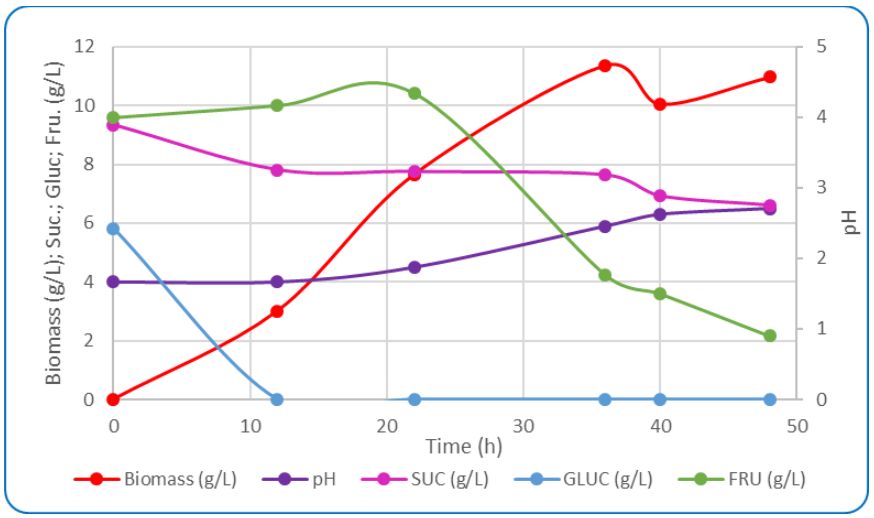
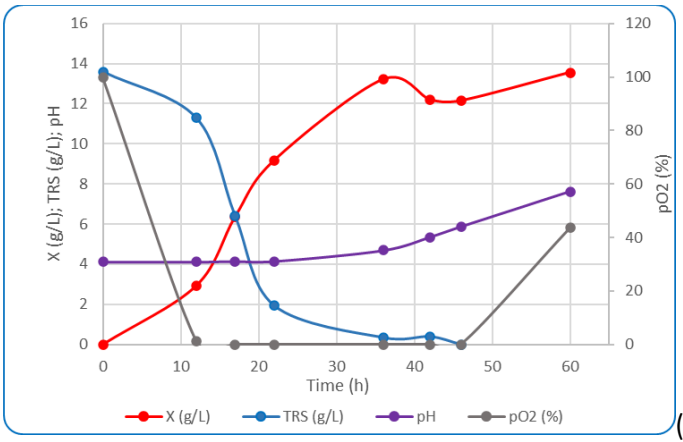


Figure 34 - Growth curve and pH profile and consumption of carbon sources in a bioreactor with AM medium.

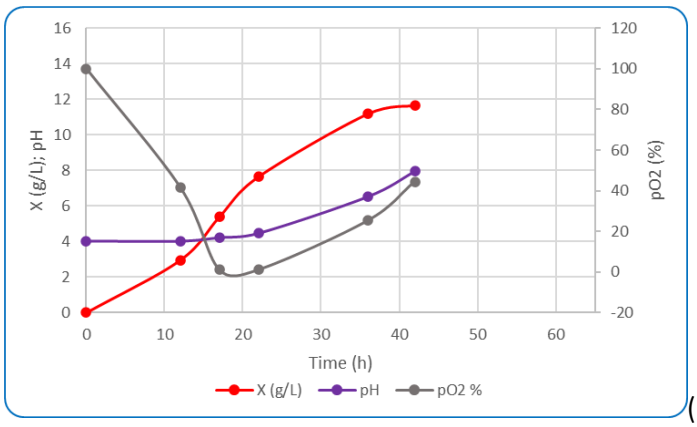
Unlike the condition with the reference medium, the pH varies much less, and the maximum biomass value reached was 11.3 g/L after 36 hours of experiment, with the biomass appearance similar to that obtained with the reference medium, more fluid. When analyzing the carbon source consumption, it was noted that there was no sucrose hydrolysis to make glucose and fructose available to the microorganism, and there was no sucrose consumption, a fact already observed previously in experiments with a carbon source (5.1 Study of an alternative culture medium). A little glucose and practically all the fructose were consumed from the moment there was no more glucose in the medium, however, the biomass amount formed suggests that the microorganism uses carbon sources available in the CSL, in

addition to nitrogen (Hofer *et al.*, 2018; Zhou *et al*, 2022). The analysis of the main nutrients of CSL by HPLC showed in its partial composition 38.4±3.2 g/L of glucose, 29.9±2.6 g/L of fructose, and 195.5±2.5 g/L of lactic acid. These results corroborate the supplementation of essential nutrients for microbial growth and the culture medium pH. The culture medium study carried out by Sekoai *et al.* (2018) for citric acid production by *Aspergillus niger* also obtained satisfactory results at pH 4.54.

Depending on the results obtained previously, two new experiments were carried out to corroborate these data and recompose the culture medium. The same glucose concentration was used to replace molasses and in the other test, no carbon source was added, but both with the same CSL amount (100 g/L). The results obtained in the two experiments are presented in Figure 35.



(A)



(B)

Figure 35 - Growth curve and pH profile, pO_2, and carbon source consumption (TRS – Total reducing sugars) in a bioreactor with medium with CSL and glucose (A) and with only CSL (B).

The two tests were very similar, in the first 12 hours, they obtained the same biomass concentration of 2.9 g/L, from this moment on the glucose test began to show an increase in biomass of around 2 g/L greater until 36 hours of cultivation, a result attributed to the addition of glucose. From 36 hours onwards, a decline in biomass was noted in both tests. From the point of view of cell growth, it

can be said that CSL meets the microorganism's nutritional needs for carbon and nitrogen without the need to add another carbon source, as the difference in biomass observed is not representative under the conditions tested. An analogous situation was seen in the development of Amado *et al.*, (2017) in the production of hyaluronic acid, where the medium composed only of CSL had a higher production than when supplemented with molasses.

Comparing the results of the three experiments carried out with the alternative medium, it can be noted that the medium without the addition of molasses reaches the same biomass concentration as that with molasses, corroborating the perception that CSL is nutritionally complete for these experimental conditions with this microorganism.

From the biomass production point of view, the alternative medium proved to be much more promising than the reference medium, as only with the CSL addition it was possible to achieve a double biomass concentration that with the reference medium, this result confirms the data already obtained with growth in flasks. In addition to the biomass concentration, the experiment time also decreased from 48 to 36 hours (Figure 36).

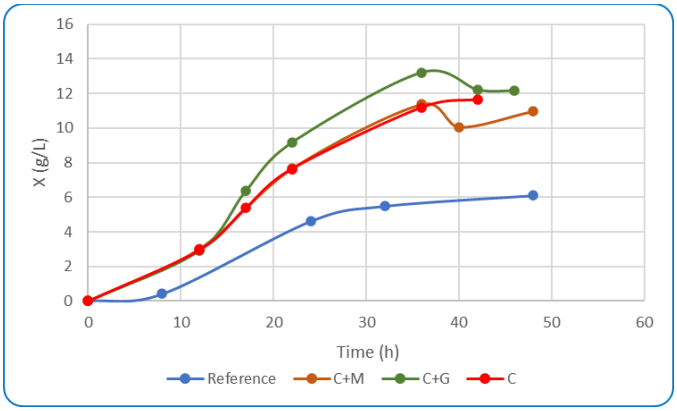


Figure 36 - Comparison of growth curves in a bioreactor with alternative medium (AM), CSL + glucose, CSL, and reference medium (RM).

However, for this work, simply obtaining the highest biomass concentration is not the most important thing. This biomass must have the capacity to promote the synthesis of AgNPs. From the point of view of nanoparticle synthesis, the biomasses of the tests showed differences in the results obtained, as shown in Table 11.

Table 11 - Size and zeta potential of samples from tests with modification of the carbon source at different culture times.

Assay	Growth Time (h)	Size (nm)	PDI (nm)	ZP (mV)
C+M	40	106.77	0.31	-33.8
	48	109.77	0.28	-30.5
C+G	22	38.55	0.35	-19.78
	36	59.78	0.47	-29.4
	46	84.01	0.41	-26.9
C	22	71.65	0.26	-44.67
	36	138.93	0.41	-23.4
	42	802.1	0.74	-26.2

C = corn steep liquor; M = sugarcane molasses; G = glucose

Although the experiment only with CSL as a carbon and nitrogen source presented a cellular concentration similar to the other conditions, the AgNP synthesis did not obtain the same result. Only the biomass with 22 h (7.6 g/L) obtained AgNP within the expected characteristics, while in other conditions with the carbon source addition, both glucose and molasses, the AgNPs were as expected, with sizes up to 100 nm and potential zeta between -20 mV and -30 mV, over the last 20 h of the experiment reaching the maximum biomass concentration. These results demonstrate that despite there being satisfactory growth without the carbon source addition, the cells produced cannot synthesize AgNP within the necessary characteristics.

Although we were unable to identify the molecules that participate in the silver reduction reaction for AgNP synthesis, it is clear from these results that the metabolic adaptation that occurred with the assay without the carbon source addition resulted in cells that, after 22 hours of testing, these produced substances that altered the synthesis reaction. The clear increase in particle size may be related to their agglomeration or the formation of the particles much larger than those considered nano, as the appearance after the reaction time was that of a precipitate.

5.6 Antimicrobial and cytotoxic activity

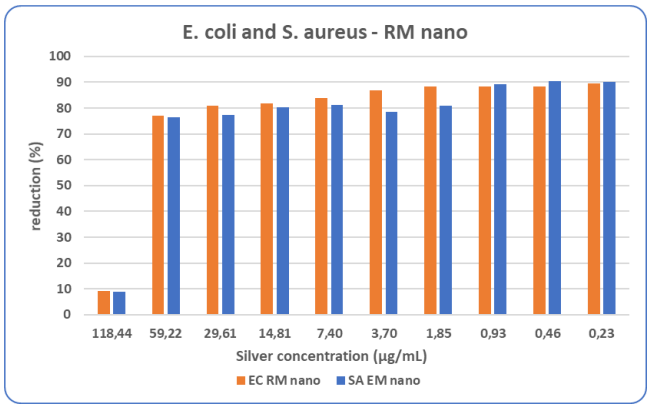
Colloidal silver has long been known for its antimicrobial properties, used as an ointment for wounds, for example. The action of silver nanoparticles has already been extensively tested on several microbial species, including those known for their resistance to antibiotics (2.6 Antimicrobial and Cytotoxic Activity) (Gumel et al., 2019). The action of silver ions works as a highly efficient biocidal antiseptic, preventing enzymatic activity and membrane functions, and causing oxidative stress and damage to the

membrane. It is believed that these reactions in biological environments can induce cytotoxicity and cell death (Zhang et al., 2014; Ahmad et al., 2020), although as in other areas of nanotechnology, the actual mechanisms of action of the silver nanoparticles (Pareek et al., 2018).

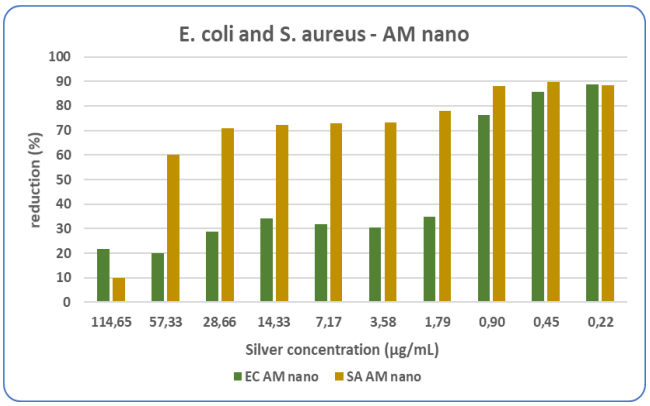
The use of silver nanoparticles for various applications aiming at antimicrobial and cytotoxic action has been widely studied, due to their physical and chemical characteristics (Kailasa, et al., 2019). In vitro cytotoxicity studies are more related to the biological response of cells and the identification of dangers associated with the exposure of these nanoparticles (Ahmed et al., 2017). AgNP is currently used in household products such as washing machines, toys, and packaging, in addition to uses that depend mainly on antimicrobial action such as dressings, fabrics, medical utensils, and others (Zhang et al., 2014). The action of so-called biological nanoparticles, that is, produced from a biological process, is considered promising for applications in the areas of cosmetics, food, and medicine, largely due to their origin.

5.6.1 Antimicrobial activity

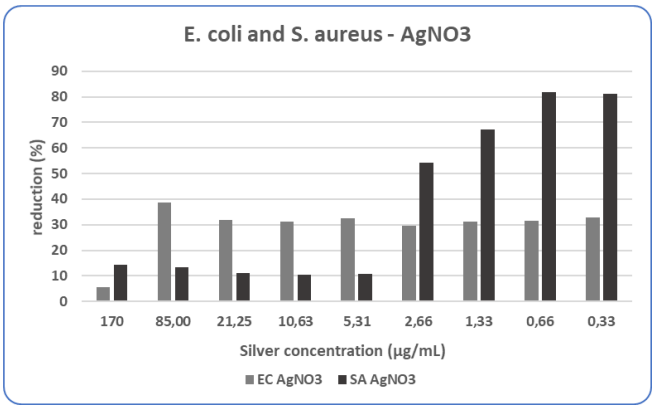
The AgNPs obtained by the two different culture media in this work were tested for antimicrobial action using *E. coli* ATCC 8739 (negative Gram) and *S. aureus* ATCC 6538 (positive Gram) as target microorganisms and as control 1 mMol/L $AgNO_3$ solution. The AgNPs synthesized from biomass grown in reference medium had a size of 60.3 nm and those from alternative medium had a size of 97.6 nm. In Figure 37 (A, B, and C) the results of the nanoparticles and the control antimicrobial activity are presented.



(A)



(B)



(C)

Figure 37 - Reduction of E. coli ATCC 8739 cell viability and S. aureus ATCC 6538 against the antimicrobial activity of AgNP from RM and AM medium and the $AgNO_3$ control.

In all tested conditions, it is observed that the highest concentration of nanoparticles and the $AgNO_3$ solution present a low reduction rate. These results may be due to color interference in the samples and the substances' precipitation in the reaction. We can note that for AgNP obtained in the reference medium, both *E. coli* and *S. aureus* bacteria showed a population reduction rate of around 80% from a concentration of 7.4 µg/mL for *E. coli* and 14.81 µg/mL for *S. aureus*, with a tendency to increase as the concentration of nanoparticles decreases.

With the AgNPs produced by the alternative medium, the response differed for each bacterial lineage. *S. aureus* had an increased population reduction rate from a 57.33 µg/mL concentration while *E. coli* maintains an average reduction rate of 30% at concentrations greater than 1.79 µg/mL and reaches a rate of reduction of more than 70% at the lowest concentrations.

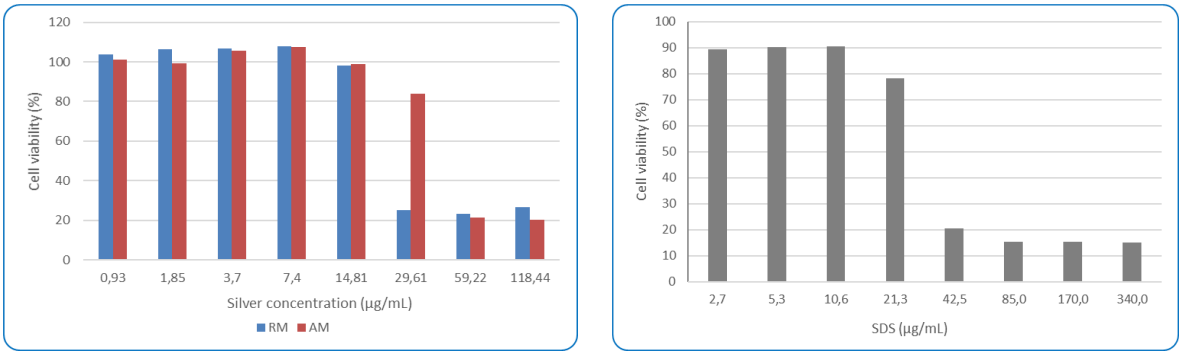
The degree of antimicrobial activity and the differences in the responses of different bacteria depends on several factors such as the environment, physicochemical properties, concentration, cell wall

composition, surface charge, and the size of the AgNPs. Despite all this information on the dependence of the antibacterial action mechanism, there is still no clarity on the AgNP antibacterial activity action mechanism (Kim *et al.*, 2017; Kailasa *et al.*, 2019). Some studies have demonstrated that Gram-negative bacteria are more susceptible than Gram-positive bacteria to the AgNP action, because of the cell wall composition (Noronha *et al.*, 2017; Ahmad *et al.*, 2020).

The biological origin nanoparticles antimicrobial activity can be differentiated as a result of the protein corona structure, which can determine greater or lesser interaction with materials or organisms (Durán *et al*, 2015). The AgNP obtained in this work have not yet been characterized in terms of their protein corona, however, we can infer the hypothesis that they may have different structures, considering that they are generated from cells grown in distinct culture media.

5.6.2 Cytotoxicity activity

In addition to the antimicrobial action, the cytotoxic action is also extremely relevant for certain applications, such as in the pharmaceutical, cosmetic, food, and medical areas that are directly related to human or veterinary use. The same AgNP used for the antimicrobial tests was subjected to cytotoxic activity assay using ATCC mouse connective tissue cells no. CCL1 NCTC clone 929L and DMEM medium plus 5% fetal bovine serum as a negative control. The cell viability results after exposure to nanoparticles are shown in Figure 38.



(A) (B)

Figure 38 - Cellular viability of ATCC mouse connective tissue cells no. CCL1 NCTC clone 929L against the AgNP cytotoxic action from RM and AM medium and SDS control.

A cytotoxic effect was observed at concentrations above 29.6 µg/mL for AgNP obtained by reference medium, with a viability rate below 30%, and for those obtained with AM medium above 59.2 µg/mL with viability below 20%. However, at lower concentrations, viability above 80% can be

observed, demonstrating that there was no cytotoxic effect for these nanoparticles at concentrations lower than 29.3 µg/mL with this mouse connective tissue cell. Ver se os resultados são próximos dos reportados na literatura

Just as in antimicrobial action, the cytotoxic effect is dependent on the same factors already mentioned previously, however, studies indicate that the cytotoxic action is related to the nanoparticle permeation into the cell by endocytosis with the release of silver ions, thus causing several changes internally in cells leading to cell death by apoptosis or necrosis. Numerous studies have been carried out with different human and animal cells and different AgNPs, with or without coating, where the results demonstrate that there is a wide variety of actions, depending on the cell and the nanoparticle, with greater or lesser cytotoxic effect (Ahmed *et al.*, 2017). According to Skóra and collaborators (2021), nanoparticles generated from *Saccharomyces cerevisiae* showed 95% viability in the range of 0.125 – 0.5 mg/mL of nanoparticles after 24 hours of exposure.

Therefore, the results presented are promising, as they indicate that this AgNP synthesis method can produce nanoparticles with potential for applications depending on the purpose and requirements. According to the extensive review carried out by Ahmed et al. (2017), compared to chemically synthesized nanoparticles, biosynthesized nanoparticles have cytotoxic selectivity for immortalized cells at low concentrations and greater antimicrobial activity.

6 CONCLUSIONS

6.1 PARTIAL CONCLUSIONS

6.1.1 Culture mediums

- Glucose was the best option for the complex medium (reference medium) when choosing the carbon source, although growth was observed in other alternative sources.
- Replacing the complex medium with a mineral medium (Sukumar et al. 2010) did not show mycelial development compared to the complex medium.
- Replacing carbon and nitrogen sources with waste (CSL and molasses) in a mineral medium showed better performance from the point of view of microbial growth, approximately doubling the mycelial mass.
- The increase in yeast extract and some salts from the mineral medium composition (Sukumar *et al.*, 2010) proved important for biomass development.

6.1.2 Fungal extract

- Based on protein extraction, increasing the molarity of the solvent (water) did not lead to an increase in the concentration of extracted protein.
- Increasing the extraction temperature also did not provide greater extraction (from the protein point of view).
- Protein identification contained in the extract by mass spectrometry demonstrates that there is no nitrate reductase enzyme presence. The identified enzymes are not classified as oxidoreductive.
- The agent identified as most likely to reduce silver was NADH.
- From the ion determination, the highest concentration was identified as phosphates

6.1.3 Synthesis

- Both the biomass grown in the complex medium and alternative medium could provide the silver nanoparticles synthesis.
- The alternative medium required a new preparation procedure to reduce the quantity of the solid interfering with the process.
- The silver determination in the synthesized samples demonstrated that there was a conversion of 55.6% for the RM condition and 53.0% for AM of the ionic silver supplied.

- Increasing the synthesis temperature had a positive effect on the reaction speed, decreasing the time from 120 hours to around 15 hours at temperatures above 60°C.
- The NADH addition in the synthesis reaction increased the nanoparticle size. With these results, it is possible to consider that NADH was one reducing agent.
- It was possible to verify through images the difference in particle size over the synthesis time indicating the protein corona presence. Although, we were unable to identify them.

6.1.4 Bioreactors

- Agitation at 800 rpm was favorable for the mycelium quality produced, allowing the biomass recovery for subsequent steps.
- Cultivation in a bioreactor provided a time reduction in process time from 40 h to 48 h in both media studied.
- The biomass morphology produced provided a greater contact area for extraction, consequently, the extraction time decreased to just 24 h.
- The change in the hyphae morphology obtained under high agitation did not interfere with the nanoparticle synthesis.
- Biomass produced only with CSL did not perform well in AgNP synthesis.

6.1.5 Antimicrobial and cytotoxic activity

- In the bacteria tested, *E. coli* ATCC 8739 and *S. aureus* ATCC 6538, the AgNP produced by the two-culture medium had antimicrobial action at low concentrations.

- The two nanoparticles obtained in this work did not show cytotoxic activity at concentrations greater than 14.81 μg/mL.

6.2 FINAL CONCLUSIONS

The process development provided the establishment of necessary parameters for laboratory scale production with 2 L bioreactors for the AgNP synthesis from the *Rhizopus arrhizus* NRRL 54538 biomass extract, obtained through alternative culture, using waste from corn maceration industry and sugarcane industry.

The times for each process stage were optimized with the studies realized, establishing a more suitable process for scaling up.

Despite the efforts, it wasn't possible to establish a mechanism for AgNP synthesis from the aqueous extract of *R. arrhizus* NRRL 54538 (IPT 1013), but it provided a wealth of knowledge to search for answers.

At the end of this work, I think there may be some differentiation between the molecules involved in the synthesis of AgNP by fungi or bacteria and their culture medium. The composition of what will be generated in each process may vary, and the synthesis may still be obtained. Therefore, I take the liberty of saying that we may have a generic mechanism in the future, which may involve several molecules as reducing agents.

7 FUTURE PERSPECTIVES

Although there is a huge amount of work studying these processes, understanding the mechanisms of AgNP synthesis using microbial or plant extracts still lacks clarification, therefore, to try to understand the molecules involved and what improvements are involved in silver reduction, more focused studies are needed.

Both biomass production and extraction can be better explored if the molecules are clarified. Knowledge of the existing molecules makes it possible to increase the production of those of interest and optimize extraction.

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