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Implementation of copper nanoparticles (CuNP) in the composition of a chitosan bioplastic film to compare antimicrobial activity with polyethylene terephthalate (PET)

Implementación de nanopartículas de cobre (CuNP) en la composición de una película bioplástica de quitosano para comparar la actividad antimicrobiana con tereftalato de polietileno (PET)

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Abstract
In this research, NPCu were synthesized to be used in the composition of a chitosan bioplastic film and to compare the antimicrobial activity of this bioplastic with a commonly used plastic (PET). These nanoparticles were obtained by chemical reaction of copper sulfate with ascorbic acid using microwaves. They were characterized by scanning electron microscopy (SEM) giving an average nanoparticle size of 15 nm; the chitosan film enriched with NPCu was subjected to microbiological study in which the formation of bacterial inhibition halos with a size of 15.5 mm and 14.5 mm in diameter was observed in the samples of the bioplastic with strains of E. Coli and Staphylococcus strains, respectively, while in the PET samples no bacterial inhibition could be visualized, thus consolidating the main objective of the research. In addition, the degradability of the bioplastic was 98% in a period of 8 weeks.

Key words
Antimicrobial activity, nanotechnology, copper nanoparticles, chitosan, bioplastic.

Resumen
En esta investigación se sintetizaron NPCu para ser utilizadas en la composición de una película bioplástica de quitosano y comparar la actividad antimicrobiana de éste bioplástico con un plástico de uso común (PET). Estas nanopartículas fueron obtenidas mediante reacción química del sulfato de cobre con ácido ascórbico utilizando microondas. Las mismas fueron caracterizadas por microscopía electrónica de scanning (SEM) dando un tamaño de nanopartículas promedio de 15nm; la película de quitosano enriquecida con NPCu se sometió a estudio microbiológico en el cual se observó la formación de halos de inhibición bacteriana con un tamaño de 15,5mm y 14,5mm de diámetro en las muestras del bioplástico con las cepas de E. Coli y Staphylococcus respectivamente, mientras que en las muestras de PET no se pudo visualizar inhibición a las bacterias; consolidando con esto, el objetivo principal planteado en la investigación. Además, la degradabilidad del bioplástico fue de un 98% en un lapso de 8 semanas.

Palabras clave
Actividad antimicrobiana, nanotecnología, nanopartículas de cobre, quitosano, bioplástico.

1. Introduction

Humanity and the environment surrounding it have been exposed since its origins to nanoparticles of natural origin resulting from volcanic eruptions, combustion and others; which have increased dramatically since the so-called industrial revolution with the development of other energy sources such as combustion engines, thermal and atomic power plants, and other sources that have been the beginning of the extremely rapid progress of nanotechnology whose interest lies in its size, which being so small can behave very differently from the same material in its mostly visible state.

There are currently thousands of products containing nanomaterials and nanocomposites that have become key to innovative technologies, generating the creation of new businesses and the possibility for the economic system to progress in various industries such as food, medicine, textile, petrochemicals, etc.

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In the case of the use of nanoparticles in the food industry, only polymers based on lipids, polysaccharides and proteins can be used, although there are nanoparticles of some metals that are included as additives containing antimicrobial properties very useful for coatings of refrigeration systems, casings and food packaging containers in which we can find nanoparticles of copper, silver, gold, etc., reinforcing the innocuousness of industrial processes, maintaining and assuring the quality of the product that will go out to the market, providing it with a longer shelf life.

The incorporation of nanoparticles in the industry continues to increase and, due to new applications, it mainly offers protection against the early degradation of biological products, generating a higher level of quality in the elaboration of more resistant, longer lasting, and healthier foods.

This research work focuses on the use and utilization of the properties contained in copper nanoparticles by implementing them in the formulation of a chitosan biopolymer, in order to test the antimicrobial effect of copper and chitosan compared to a common plastic derived from petrochemical processes, the two plastics will be evaluated against two common strains that are part of the process of quality control analysis of industries: Escherichia Coli, bacteria that can produce diseases and cause diarrhea (Traveler's Diarrhea) and Staphylococcus aureus, invades tissues, causes skin and soft tissue infections and food poisoning.

1.1. Nanotechnology

The ideas and concepts behind Nanoscience and Nanotechnology began with a lecture by physicist Richard Feynman entitled "There's Plenty of Room at the Bottom" at a meeting of the American Physical Society in December 1959, long before the term nanotechnology was used. In his speech, Feynman described a process in which scientists could manipulate and control individual atoms and molecules. [1]

The development of Nanoscience and Nanotechnology has brought about a new framework within science and technology. Analytical Chemistry has experienced, as other areas of science, a great change thanks to the needs and opportunities provided by Analytical Nanoscience and Nanotechnology. On the one hand, the exponential growth in the use of nanoparticles in industry that has been taking place in recent years is causing and demanding the appearance of methods of analysis at the nanoscale for the characterization and analysis of products, environmental control and toxicological studies. On the other hand, the possibility of using nanoparticles with exceptional properties allows the development of new analysis strategies or improvement of the existing ones, for the analytical control of compounds of environmental, food, clinical or toxicological interest. These two aspects are addressed in what we call today "Analytical Nanoscience and Nanotechnology". [2]

Table 1. Shows different criteria for defining nanotechnology:

Table 1. Nanotechnology Definitions.

Source	Definition
International patent classification (IPC) subclase B82B	"Nanostructure" is "a precise atomic arrangement of matter having a particular configuration of shape including at least one essential integral element that: 1. Is formed exclusively from one atom, one molecule, or a very limited collection of atoms or molecules, which collection is in its entirety undetectable by an optical microscope; and 2. has been formed by having its atoms or molecules individually manipulated as discrete units during its manufacture."
physics.about.com	"The development and use of devices that are only a few nanometers in size"
hyperdictionary.com	"The branch of engineering that deals with things smaller than 100 nm (especially with the manipulation of individual molecules)"
NASA	"Nanotechnology is the creation of functional materials, devices and systems through the control of matter at the nanometer scale (1-100 nm), and the exploitation of new phenomena and properties (physical, chemical, biological, mechanical, electrical...) at this same scale"

International standard organization (ISO, 2010)	"Application of scientific knowledge to manipulate and control matter at the nanometer scale in order to make use of its properties and phenomena related to its size and structure, as opposed to those associated with individual atoms or molecules or bulk materials."
Fundación española para la ciencia y tecnología (FECYT)	"Technology generated with objects ranging in size from a tenth of a nanometer to a hundredth of a nanometer."

Source: [3]

Currently, the term Nanotechnology is used very frequently in the field of research, however, it is highly debatable whether Nanotechnology is a reality today. The current progress in this field can rather be described as nanoscience, meaning the body of knowledge that lays the foundation for the future development of a technology based on the detailed manipulation of molecular structures. [4]

The true potential of nanomaterials, or nanoscale materials, lies in the exceptional behavior they exhibit because of their small size. By way of example, here are some of the properties that are most influenced by the size of the material:

- Electrical conductivity.
- Thermal conductivity.
- Color.
- Chemical reactivity.
- Elasticity.
- Resistance.

It can be considered that Nanotechnology is in its infancy, since in recent years it has been developing mainly Nanoscience, which is the mainstay for the development and specific applications of Nanotechnology itself. [4]
Figure 1 shows the main scientific and technical areas of research in Nanotechnology.

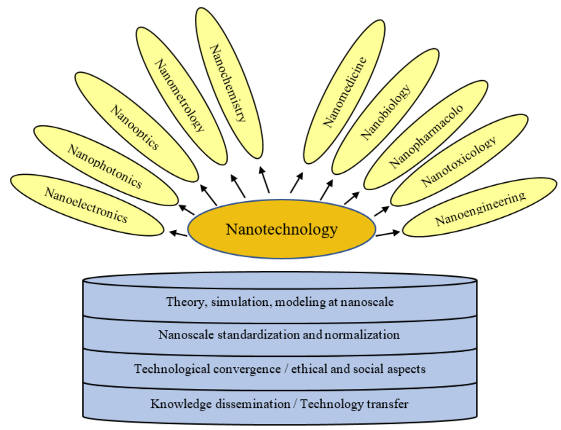


Fig 1. *Main scientific-technical areas of research in Nanotechnology*
Source: [2]

1.2. Characteristics of nanotechnology

The following items describe some of the characteristics of nanotechnology:

➢ TOP-DOWN and BOTTOM-UP methods: TOP-DOWN are those that use structures larger than nanometers, such as micrometers20, to reach nanometric structures. On the other hand, BOTTOM-UP, in which physical and chemical processes, such as synthesis, are used to control and manipulate atoms and molecules to form and grow nanostructures. [5]

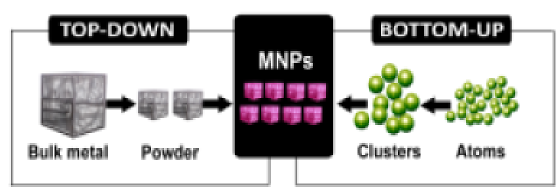


Fig. 2. *Ways to create top-down and bottom-up "nanocoses".*
Source: [6]

Interdisciplinary: This relates to other scientific macro disciplines such as materials science, biochemistry, chemistry, physics, mathematics, etc.

1.3. Risks of nanotechnology

There is hardly any specific legislation on nanotechnology and nanomaterials, although they fall within the definition of "substance" included in the European Regulation on Chemicals (known as REACH; acronym for Registration, Evaluation, Authorization and Restriction of Chemicals). In this regard, EFSA published guidance on assessing the risks of nanoscience and nanotechnology applications in food and the food chain. One of its recommendations was the need to develop, improve and validate routine methodologies to study the toxicity of nanoparticles in depth, given the growing number of new nanomaterials on the market. The international interest aroused is shown in the number of publications that have appeared on the different detection techniques; in 2014 a study promoted by the United States Environmental Protection Agency (EPA) proposes conductivity measurements, while a second study opts for flow cytometry and light scattering and red fluorescence, although the Agency's interest in these subject dates to 2007. [7]

1.4. Applications of nanoparticles in industry

The possible multiple applications of the advances and discoveries in Nanosciences and Nanotechnologies are the main basis on which the importance of the field is based. Likewise, there is a tendency in the literature to assign new names to already known disciplines that add the prefix "nano" to their field of study, to identify the traditional lines that study phenomena and applications at the nanoscale. Such is the case of nanoelectronics, considered to be the future of microelectronics, which will not only allow further miniaturization of the various parts of electronic instruments, but will also provide new advances based on the properties that arise at the nanoscale. Another case is that of nanomaterials, which refers to the field of materials science that focuses on the study of their properties at the nanoscale. [5]

The applications of nanotechnology and the incorporation of NMs in the agri-food industry are growing, highlighting in the short term their majority use in food contact materials, while in the long term the use of nanoencapsulates is expected. Due to the increase of these new applications, the population is more and more exposed to these NMs, so a correct risk assessment is essential, as well as the development of a specific legislative framework to guarantee consumer safety. [3]

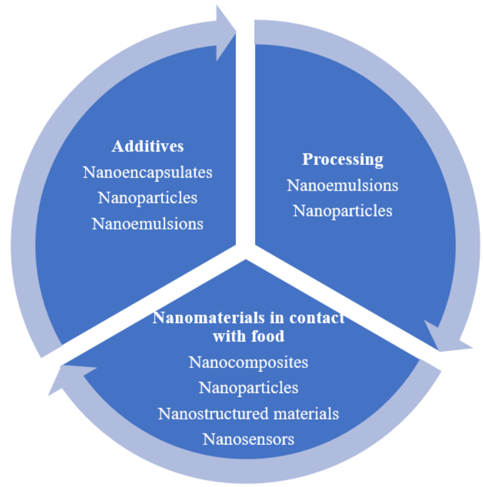


Fig. 3. *Nanoparticles applications in industry.* Source: [8]

1.5. Nanoparticles in food processing

Organic components that are present in foods such as proteins, carbohydrates and fats can range in size from large polymeric chains to simpler molecules in the nanometer range. Organic nanomaterials can be synthesized for specific purposes, such as encapsulation of nutrients to increase their bioavailability, improve the taste, texture and consistency of food products, or mask an undesirable taste or odor. The beverage industry is looking to use nanoemulsions (lipid-based), as these have droplet sizes much smaller than the visible wavelengths; therefore, most nanoemulsions appear optically transparent. This is a very favorable characteristic for their application as nutrient carriers, without compromising the color and flavor of these products. [8]

1.6. SEM applications

Scanning electron microscopy equipment is used in any area that works with solid materials and where a microscale view is useful, such as chemical, biological, environmental, metallurgical, ceramics, among others.

The main applications of SEM are detailed below:

- Characterization of morphology, chemistry, and texture in general mining, petroleum and more.
- Composition of polymer surfaces, semiconductor compounds.

- In dentistry it is used for the study of the alterations caused by acids and food debris on the surface of teeth.
- Quality control in products for human consumption such as: fibers and expertise.
- The requirements for the samples to be analyzed are shown below:
- They should be delivered properly labeled, conditioned, and packaged to ensure the quality of the visualizations.
- They must be conductive, solid and free of humidity or exposed to any chemical.
- Samples should be as small as possible, with a diameter of 4 cm and a height of 1 cm.
- In case the samples are not conductive, they will be coated with gold or carbon.
- If necessary, the samples will be subjected to a vacuum treatment.

1.7. Chitosan-copper nanocomposite films (QS-CuNP)

It is possible that formulating chitosan/copper nanoparticle (QS/nCu) nanocomposites could increase their physicochemical and antibacterial properties while maintaining the biocompatibility of the biopolymer. [9]

The antimicrobial activity of metal nanoparticles is known to be a function of the surface area in contact with microorganisms. An inverse relationship between nanoparticle size and antimicrobial activity has been demonstrated, where nanoparticles in the 1-10 nm size range have been shown to have the highest antibacterial activity. The transition from microparticles to nanoparticles involves an increase in the contact area/volume ratio (aspect ratio), where the contact area increases dramatically allowing a greater number of interactions with organic and inorganic molecules and bacteria. There is evidence that nanoparticles made from natural polymers such as chitosan have bactericidal properties. The effectiveness of these nanoparticles against Staphylococcus aureus, Listeria monocytogenes, Salmonella typhimurium, Escherichia coli and Streptococcus mutans has been evaluated. [10]

Every organism needs to find in its environment all the substances necessary for energy generation and cell biosynthesis. The elements of that environment that are used for cell growth are referred to as nutrients. The nutritional requirements of a bacterial cell are revealed by its elemental composition, which is composed of C, H, O, N, S, P, K, Mg, Fe, Ca, Mn and traces of Zn, Co, Cu and Mo. These elements are found in aqueous form, inorganic ions, small molecules, and macromolecules, which play a structural or functional role in the cell. The culture media, whether solid, semi-solid or liquid preparations, constitute the micro-world of microorganisms in laboratory conditions, trying to reflect their natural habitat in relation to the satisfaction of their most vital and main needs as living beings. The design of a culture medium will then respond to the requirements of the microorganism in question and to the purpose of its multiplication. Depending on the purpose to be pursued, the media to be used for the cultivation of these microorganisms can be either complex or defined. [11]

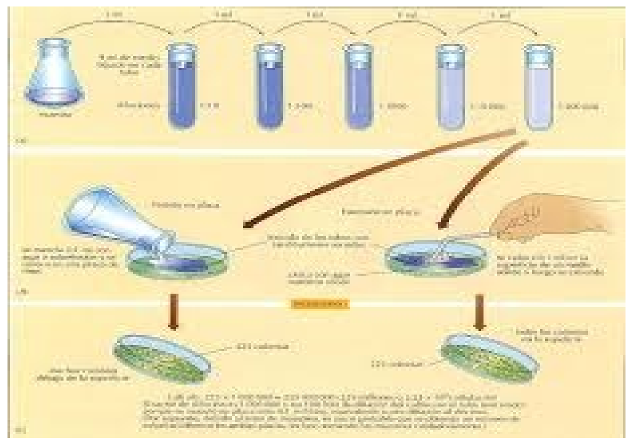


Fig. 4. *Means of seeding*
Source: [12]

1.8. Isolation/transfer techniques

Isolation can be achieved directly from a sample with appropriate proportions of microorganisms using the following methods:

- By diffusion on the surface of a solid medium in Petri dishes.
- By dilution in Agar
- Macro and micro dilution in broth.
- Epsilon test.
- Automated methods and special tests.

The Agar diffusion technique is qualitative, and its results can be interpreted only as sensitive, intermediate or resistant, and is specifically designed for fast-growing bacteria such as Staphylococcus sp. or members of the Enterobacteriaceae family. It is applied to the surface of a dry agar plate. The strain should be streaked onto the surface of the medium in such a way that confluent growth is achieved. Each plate is observed in an indirect light and each inhibition halo is measured using a caliper or in its absence a ruler graduated in the appropriate way. If no halo is present, 0 mm should not be reported, but 6 mm, as this is the diameter of the disk. [13]

Table 2. Advantages and disadvantages of Agar diffusion

Agar diffusion technique	
Advantages	**Disadvantages**
It is easy to perform and highly reproducible	It provides only qualitative information
Low cost	This technique must be modified for use on fastidious or slow growing organisms.
Does not require special equipment	
Results are easy to interpret	
It is very flexible when choosing the antibiotic to test for.	

By dilution in Agar the samples that are already diluted are mixed with the agar and sown directly on the surface of the agar plate, as in the previous technique, the plates are incubated until the appearance of colonies.

In the macro and micro dilution in broth, the test tube with the liquid culture medium, such as simple broth, is taken. The material to be diluted is taken and then seeded in the test tube using the loop loaded with the bacteriological material and shaken with moderate movements.

Epsilon test, this method is a combination of agar diffusion and agar dilution, and the minimum inhibitory concentration can be seen by direct reading with strips composed of antibiotics (Amoxicillin).

Automatic methods and special tests are based on the characteristics of the microorganisms to be studied. But it should be borne in mind that there may be bacteria that usually have the same behavior before a physical or chemical agent.

2. Materials and methods

The synthesis of NPCu was carried out at the Organic Chemistry Laboratory of the Chemical Engineering Faculty of the University of Guayaquil, for which the following methodology was followed:

a. First the reagents for the solution are weighed separately which we will call "solution 1" and "solution 2" for Copper Sulfate and Ascorbic Acid (Vitamin C) respectively:
b. For solution 1, 40g of $CuSO_4$ were weighed and dissolved in a beaker with 120ml of distilled H_2O to obtain a saturated solution at room temperature (25°C).
c. For solution 2, 96g of $C_6 H_8 O_6$ (Vitamin C) are weighed to be dissolved in 340ml of distilled H_2O at room temperature (25°C).
d. Once the two solutions are obtained, proceed to mix them, and accelerate the sedimentation reaction of the copper nanoparticles with the help of microwave wave irradiation in 1-minute cycles until the solution boils and the agglomerated NPCu are visualized at the bottom of the beaker.
e. The solution is cooled so that it can be vacuum filtered using a microfilter that will help to retain the nanoparticles and prevent loss of nanoparticles.
f. When all the product has been drained, the copper is washed with 80 ml of absolute alcohol while stirring with a stirrer or spatula.
g. The raw material (NPCu) is subjected to drying in an oven for 15 minutes at 45°C.
h. The yield of nanoparticles is weighed and calculated in relation to the amount of copper sulfate used.

The reagents used in the synthesis of copper nanoparticles were obtained by purchasing them in the city of Guayaquil from Laboratorio Cevallos, a supplier of chemical products and laboratory equipment.

The other instruments were used from the Organic Chemistry laboratory of the Chemical Engineering Faculty of the University of Guayaquil.

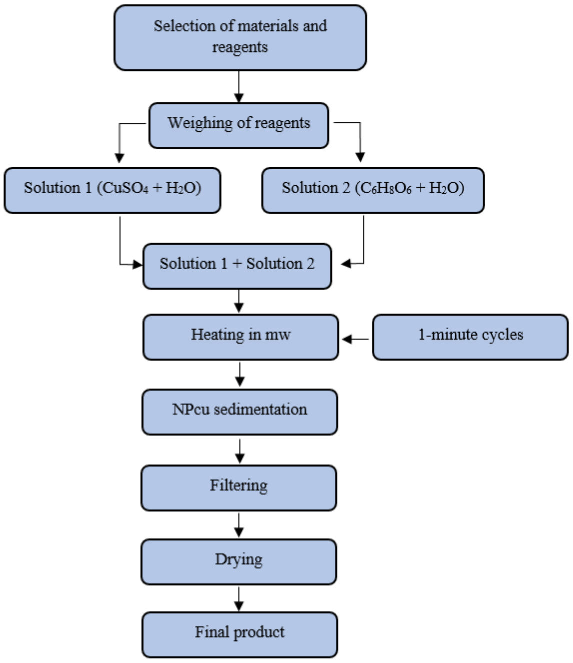


Fig. 5. *Copper Nanoparticle Synthesis Flowchart*

2.4. Procedure for calculating the degradation area of the biopolymer.

To calculate the degradation area of the plastic, the Triangulation Method will be used as a reference, which is used to calculate the area of polygons and irregular figures, as is the case of our bioplastic. Below is detailed how to calculate the area of an irregular polygon:

a) We divide the figure into N triangles, according to the number of sides of the figure and with the condition that all sides converge to the same interior point.
b) Measure the height of the triangles. Each of the heights of the triangles will be the segment perpendicular to the side of the polygon that goes to the center.
c) Calculate each of the areas of the triangles by adding the N areas of the plastic with the following equation:

Irregular polygon area

$$= \frac{L_1 * h_1}{2} + \frac{L_2 * h_2}{2} + \cdots + \frac{L_n * h_n}{2} \qquad (1)$$

Where L_i are each of the sides and h_i are the heights of the triangles.

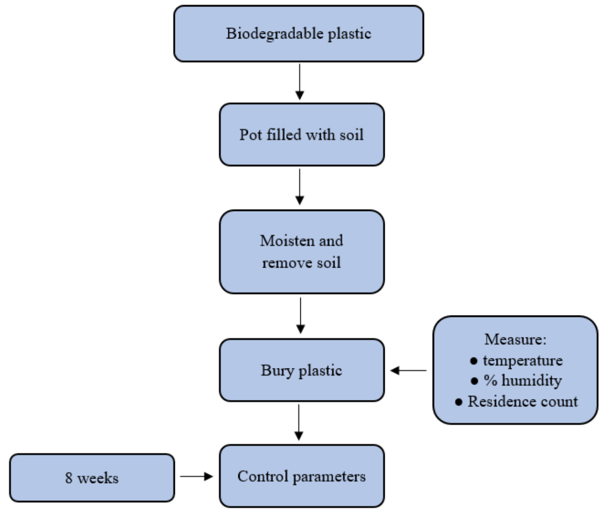


Fig. 6. *Flow chart of the development of the biodegradable plastic degradability test.*

3. Results

Calculation of moles of copper sulfate: $CuSO_4$
$Cu = 63{,}546g$
$S = 32{,}065g$
$O = 15{,}999g * 4 = 63{,}996g$

$$40g\ CuSO_4 * \frac{1\ mol}{159{,}607g\ CuSO_4} * \frac{63{,}546g\ Cu}{1\ mol\ CuSO_4} = 15{,}9256g\ Cu \qquad (2)$$

Table 3. Average number of NPCu synthesized in the laboratory.

NPCu synthesized in the laboratory		
	NPCu (g) sample	**Average**
Solution # 1	7,88	
Solution # 2	7,75	7,75
Solution # 3	7,61	
TOTAL	23,24	

Source: Authors

Then, the % yield is as follows:

$$\%Yield = \frac{Actual\ Yield}{Theoretical\ Yield} * 100 \quad (3)$$

$$\%Yield = \frac{7{,}75g}{15{,}9256g} * 100 \quad (4)$$

$$\%Yield = 48{,}643\ \% \quad (5)$$

As a result of the stoichiometric calculation to obtain the theoretical yield, 15.9256g Cu was obtained, which is the amount of copper contained in 40g of Copper Sulfate used for the synthesis reaction of nanoparticles. The weight of the nanoparticles synthesized in the laboratory had an average result of 7.75g expressed in Table #4, which contains 3 different weights resulting from various tests to obtain NPCu; which would be the actual yield obtained from the synthesis.

Applying Equation 3, to calculate the yield of the Copper Nanoparticles gave an estimated 48% total yield in the synthesis of obtaining copper nanoparticles to be used in the manufacture of a biodegradable plastic.

3.1. Biodegradable Plastic

The characteristics of the biodegradable plastic obtained by the authors are shown in the table below:

Table 4. Bioplastic Characteristics

Bioplastic Characteristics	
Thickness	1mm
Color	Light amber
Flexibility	Moderate
Elasticity	Little
Texture	Smooth/Soft

3.2. Bioplastic Degradability.

The bioplastic was exposed to natural conditions which was buried in soil and observed every week for about 8 weeks to evaluate the degradability of this and the time it takes to degrade; with this we could visualize and verify the degradative properties of the plastic obtained, which is beneficial to the environment by having a short decomposition time. Next, the degradation process of the bioplastic is detailed by calculating its area over time.

3.3. Calculation of the degraded area of bioplastics

To calculate the area of the plastic we will use the triangulation method that is used to calculate the area of an irregular figure, which consists of dividing the polygon into N triangles and the area of the polygon will be the sum of the area of these N triangles.

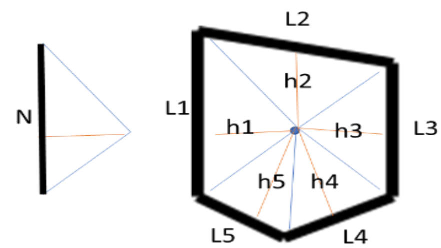


Fig. 7. Area of an irregular figure - Triangulation Method

Table 5. Bioplastic degradability control.

Bioplastic degradability control.			
Week	Temperature °C	% Humidity	Bioplastics area (cm²)
0	28	88	8,125
1	27	87	3,905
2	29	85	2,265
3	31	92	2,185
4	24	89	1,175
5	28	80	0,8575
6	32	87	0,725
7	29	87	0,455
8	30	82	0,1625

When calculating the area of the biodegradable plastic to determine the degraded surface of the bioplastic sample, the detailed weekly data were taken; once the results were obtained, they were grouped in Table 6 in which the change in the size of the bioplastic in the degradability control of the bioplastic can be observed, this shows the variations of the degradation from week 0 to week 8 with their respective conditions of temperature and % of humidity.

Figure 8 shows the graph of the degradability control of the bioplastic in which the increase in the biodegradation of the plastic and the reduction of its size by 98% in only 8 weeks can be more clearly evidenced.

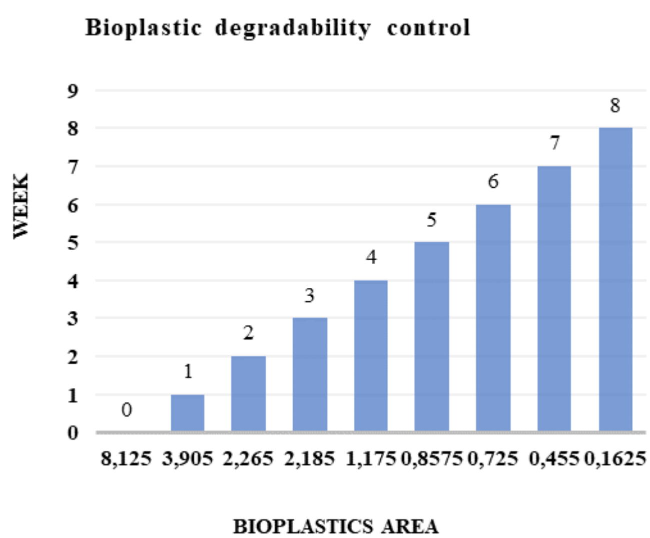


Fig. 8. *Bioplastic Degradability Control Chart*

3.4. Antimicrobial Activity

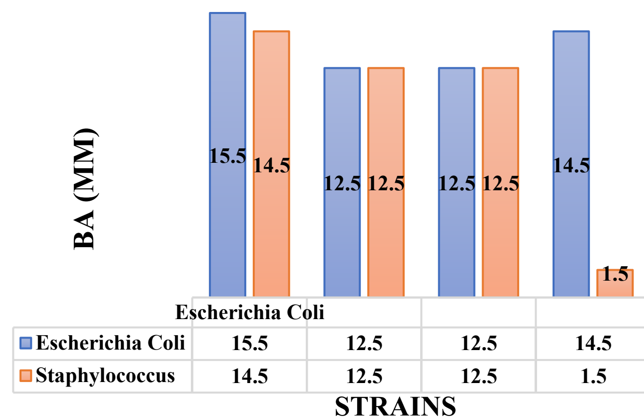


Fig. 9. *Comparison graph of the size of the Ba samples analyzed.*

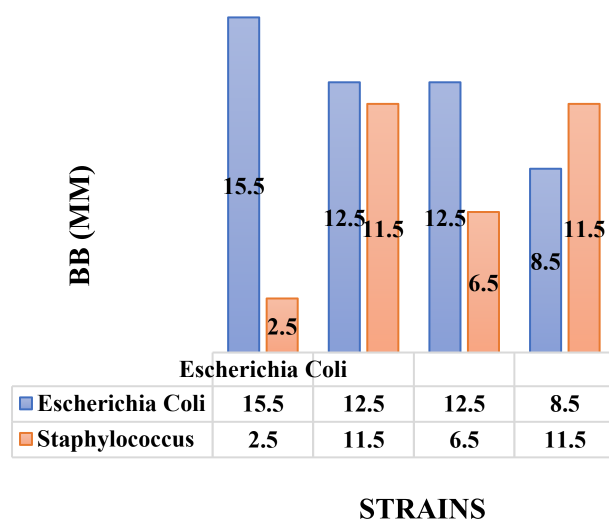
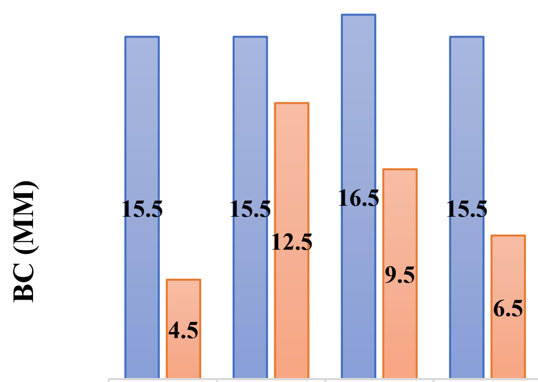


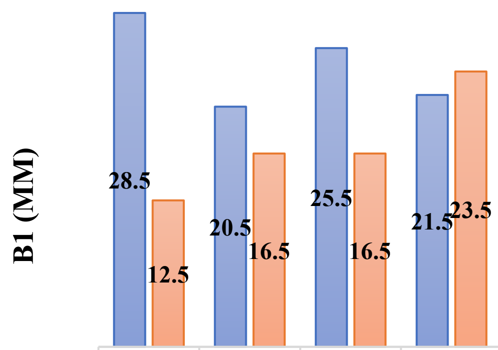
Fig. 10. *Comparison graph of the size of the Bb samples analyzed.*



Escherichia Coli				
Escherichia Coli	15.5	15.5	16.5	15.5
Staphylococcus	4.5	12.5	9.5	6.5

STRAINS

Fig. 11. *Comparison graph of the size of the analyzed Bc samples.*



Escherichia Coli				
Escherichia Coli	28.5	20.5	25.5	21.5
Staphylococcus	12.5	16.5	16.5	23.5

STRAINS

Fig. 12. *Comparison graph of the size of the haloes of the B1 samples analyzed.*

The samples analyzed with each of the bacterial strains used to test the inhibitory power of the copper nanoparticles in a biodegradable plastic based on chitosan with NPCu showed positive results by observing the formation of inhibition halos around the plastic disc containing the nanoparticles, unlike the normal plastic (PET), which did not show the presence of halos.

Figures 9, 10 and 11 show the sizes of the inhibition halos of the discs of samples enriched with NPCu that are being compared between Escherichia Coli and Staphylococcus strains; the difference in the growth of the halos and the effectiveness exerted on each of the bacteria is clearly evident; the inhibitory effect of the copper nanoparticles on Escherichia Coli is greater than on the Staphylococcus samples, their maximum value being 15.5 mm and 14.5 mm in diameter, respectively.

4. Conclusions

From the results obtained and described in the report of this research work, it has been possible to reach the following conclusions:

- Copper nanoparticles were prepared from a solution of copper sulfate and ascorbic acid using microwaves in which a 48.643% yield was obtained.
- In the scanning electron microscopy analysis, the nanoparticles observed under the light beam showed a minimum size of 4nm and a maximum of 25nm with an average of 15nm.
- Bioplastic chitosan film of Chinese origin with 86.7% purity was obtained, incorporating copper nanoparticles with an average size of 15 nm.
- Bacterial inhibition of the bioplastic with NPCu was obtained, with diameters of 15.5mm against Escherichia Coli and 14.4mm against Staphylococcus.
- No inhibition against E. coli and Staphylococcus strains was obtained in the common plastic samples.
- The degradability of the bioplastic reached 98% within 8 weeks in seeded soil.

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