Jagua Publication

www.jpub.org

Journal of Applied Health Sciences and Medicine 4(3) (2024): 1 –11

Research Article

Estimation Activity of Chitosan Nanoparticles Biosynthesized Locally by *Bacillus subtilis* against Bacterial Pneumococci Activity

Hashim Mohammed Hashim Al Araji[1*], Baydaa A. Hassan[2]

[1]University of Kufa, Faculty of Sciences, Department of Biology, Najaf/ Iraq, and Kerbala University, College of Applied Medical Sciences, Kerbala, Iraq.
[2]University of Kufa, Faculty of Sciences, Department of Biology, Najaf/ Iraq.
*Corresponding author email: hashim.mohammed[@]uokerbala.edu.iq

How to cite:
Araji et al 2024. "Estimation Activity of Chitosan Nanoparticles Biosynthesized Locally by *Bacillus subtilis* against Bacterial Pneumococci Activity". *Journal of Applied Health Sciences and Medicine* 4(3): 1 – 11. https://doi.org/10.58614/jahsm431

Received: December 1, 2023
Accepted: April 2, 2024
Published: April 16, 2024

Abstract:

Bacillus subtilis used as a biogenic bacterium to biosynthesized CNPs after selection an efficient isolate from twenty isolates (S_1-S_{20}) examined the ability to biosynthesis of CNPs based on morphological characters as altering of color, microscopic examination and it was confirmed diagnosis by VITEK 2 Compact System apparatus. The biosynthesis of CNPS were prepared using S_2 during an addition of CS to the bacterial culture of free cells at a concentration 2 mg/ml, then centrifugation at 4200 rpm for 15 minutes to separate supernatant that contains NPs from free cells then used for synthesis. The characterization was performed by spectrophotometry of UV, Scanning Electron Microscope (SEM), Energy Dispersive Spectroscopy (EDS). UV-visible spectroscopy analysis was utilized to know CNPs synthesized via absorption at peak 280 nm, analysis by SEM referred to spherical shape with a rough surface of synthesized NPs and characterized with a small size (10-60) nm .The biosynthesized CNPs were analyzed by EDS to estimate elemental percent which was 58.03% for carbon, 36.57% for oxygen, 2.06 % for phosphate, 1.15 % for magnesium, 0.11 % for ferrous, 0.32 % for silicon, 0.47 % for potassium, and 0.76% for sodium. The research showed the antibacterial role of synthesized CNPs when it used different concentrations and it tested against different isolates of *S. pneumonia* that were collected 120 samples from lung cancer patients and selected as 5, 33, 35, 38, and 61 where gave the greatest inhibition zone on Muller Hinton agar with a diameter 21 mm and the lowest diameter 14 mm as well as our study showed antibiofilm activity of prepared NPs at different concentrations (100, 200, 300, and 400) µg/ml as against all isolates that form biofilms such as isolate 5 and isolate 61, where the greatest inhibition zone with a diameter of 21 mm on Congo red agar.

Keywords: *Bacillus subtilis*, Chitosan, Biosynthesized Chitosan Nanoparticles.

Introduction

Chitosan (CS) represents a structure composed of polysaccharides or material fibrous that can be synthesized through a deacetylation process that occurs on atom (N) in chitin structure, while chitin considered a biopolymer structure that is produced naturally in the shells of crustaceans is similar to those found in other organisms as crabs, crannog , and crayfish as well as CS obtained from microorganisms as fungi and bacteria, while CNPs are manufactured with suitable features of Nano scale including the smallest size, influences of certain interface and surface, and effects of a quantum size in addition to the huge biomedical application of biosynthesized CNPs as delivery for drugs and genes, different diseases treatment as therapy for cancer, and engineering's for tissues (Jha and Mayanovic,2023). The nanomaterial can be used to treat diseases, diagnose and prevent diseases where the nanotechnology may be used to assist in the cell monitoring in the human body as nano-probes that associated with biological markers to help identify early and enable nano-biosensors in the science of genomics and proteome that lead to possible to prevent and control of diseases so, some countries were utelized of nanoparticles as to deliver drugs in the diseases treatment of cancer (Dilnawaz *et al*.,2023). CS exhibits a wide range of antimicrobial activity against bacteria, filamentous fungi, yeast and even virus, studies have reported antibacterial activity of CNPs against both gram positive and gram-negative bacteria such as in the current study pneumococcal bacteria (Wang, *et al.*, 2020; Saad, *et al.*, 2021 and Li, *et al.*, 2023).

B. subtilis considered one, of the major sources and is classified as Generalized Recognized as Safe (GRAS) according to Food and Drug Administration (FDA) for probiotics, a species that represents a biosynthetic remarkable potentially (Iqbal *et al*.,2023) also secretions different secondary metabolites as lipopeptides and enzymes which give wide spectrum activities of anticancer, antimicrobial, and antinematicidal (Gamal *et al*.,2021, Awan *et al*.,2022, Chavarria-Quicano *et al*.,2023) as well as activation of immunity, lipopeptides play a role as modulators in immunity via binding to the pattern recognition receptors (PRRs) especially Toll-like receptors (TLRs) which expressed on the surfaces of dendritic and macrophage immune cells to achieve functions of the immune system (Cao *et al*.,2023).

Materials and Methods

Detection of Effective Isolate for Biosynthesis of CNPs

An efficient isolate was diagnosed based on morphological features, and direct microscopic examination, twenty isolates were cultured on nutrient agar and the colonies appeared as waxy, creamy as well as the edges were irregular and opaque while the bacterial smear was observed as gram-positive bacilli with short rod shape with purple color when used oil immersion for objective lens under a microscope as well as the isolates were detected by confirmatory diagnosis via VITEK 2 Compact System where examined of a twenty isolates (S_1-S_{20}) and it was selected an efficient isolate (S_2) that was based on the criteria as activity against pneumococci, the altered color of culture, and measured of the absorption spectrum.

Large-Scale of CNPs Production by *B. subtilis*

The supernatant with free cells was taken after the culturing of an efficient bacterial isolate (S_2) on BHI broth in shaking incubator for 72 hours at 37°C under sterilized condition, more incubation to permit the synthesis of biomass, then it was used cooling centrifugation with 4200 rpm of specific gravity for 15 .The supernatant was utilized in biosynthesized CNPs, it was added chitosan and acetic acid, the supernatant was aggregated in a flask then was inoculated with efficient isolate under sterilized conditions and then was added BHI broth with 800 ml, 1g of CS as basic nutrients and 8 ml of acetic acid, the flask was placed in shaking incubator 150 rpm for 24 hours at 37 °C and it was used for more one times in periods different.

Characterization of CNPs Manufactured Locally

Ultra-Violet Spectroscopy for CNPs

Biosynthesized CNPs molecules was measured and confirmed by an spectrophotometer apparatus (Shimadzu UV- visible 1800) in AL-amen Center to Advanced Research and Biotechnology.

Scanning Electron Microscope (SEM) for CNPs

CNPs were analyzed by SEM at the University of Tehran to detect the size and morphological surfaces of its.

Energy Dispersive x-ray Spectroscopic (EDS) Analysis for Biosynthesized CNPs

Chitosan NPs were analyzed by using EDS to measure the amount of each element where it was performed at Tehran University.

Antibacterial Role of Biosynthesized CNPs

The activity of biosynthesized CNPs against bacterial isolates of *S. pneumonia* resistant to antibiotics that were isolated from lung cancer patients by using a diffusion method on agar and after isolation, the swabs from tonsillitis and sputum of patients were suspended in 5 ml of BHI broth to support growth isolates of *S. pneumonia* and incubation for 24 hours at 37°C, then swabs were streaking on the surface of Mueller Hinton Agar and others agars that was contained 5% of blood to encourage the growth then it was to pore with cork poorer sterilized at 8 mm in diameter . The biosynthesized CNPs were placed in each well with different concentrations (100,200,300, and 400) μg/ml. Media were placed in an incubator for 24 hours and 37°C (5% CO_2) and after incubation it was measured the diameter by millimeters of inhibition zone around each well.

Antibiofilm Role of Biosynthesized CNPs.

According to (Cockeran *et al*.,2020).

Results and Discussion

Detection of Effective Isolate for Biosynthesis of Chitosan NPs

Three isolates were selected as effective and then it was chosen isolate S_2 was more effective than the other two isolates in the biosynthesis of CNPs as in figure 1. All isolates were not able to biosynthesis or had different activities due to containing various activities for enzymes and processes of metabolism (Yusof *et al*., 2019; Bhardwaj *et al*., 2020), also the wall in the bacterial cell contains enzymes that cause to reduce the metallic ions into their nanoparticle molecules where the bacterial cell leads to the secretion of reductase enzymes which play a role in the biological reduction process for metallic ions into nanoparticles via extracellularly (Ovais *et al*., 2018).

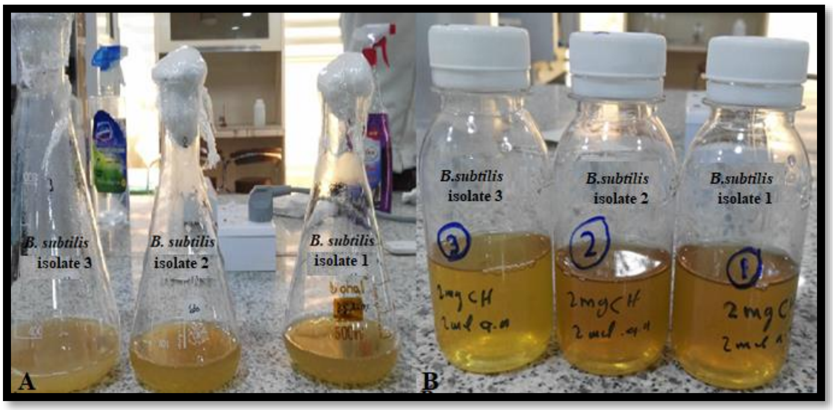

Figure 1. The effective isolate (S_2) of *B. subtilis*. A: Before synthesis of nanomaterial. B: After synthesis of nanomaterial.

The ability of B. subtilis to the production of various enzymes led to it was considered as a factory to the synthesis of enzymes, chemical materials, and other materials act as antimicrobial in various fields as medicine, agricultural, and industry (Fang and Zhang, 2020; Abd ElHack *et al*., 2020).

Large-Scale of Chitosan NPs Production by B. subtilis

B. subtilis was utilized for the biosynthesis of CNPs and it caught an efficient isolate illustrated the competence for synthesis extracellular after using a supernatant that contained free cells-only as well as added CS in form of the synthesized substrate under appropriate conditions previously controlled ,where it was seen alters of features of the medium used in the biosynthesis, especially from light color to dark color after it was placed in an incubator and shaking to assist in the synthesis and in speed as 150 rpm , as shown in figure 2. CS was not toxic and used as an antimicrobial agents while micro-particles of CS have an interesting role in many applications as industrial food and synthesis of pharmaceutics, treatment of wastewater and water, and delivery of drugs (Van Bavel *et al* .,2023), the most common ways to prepare CNPs comprise evaporation of solvent that based on the

https://doi.org/10.58614/jahsm431

emulsion, gel of ionic, micro-emulsion, and diffusion of emulsification solvent, these methods in addition to way in the research were considered noncomplex methods and organic solvents used in methods of preparation as less. Furthermore, these techniques were based on the main characteristics of CNPs such as concentration used, molecular weight, deacetylation degree of CS, these directly affect each size, charge of surface and crystalline form (Jha and Mayanovic, 2023)

Figure 2. Biosynthesis of Chitosan NPs via utilized *B. subtilis* on BHIB Medium.

Characterization of CNPs Manufactured Locally according to physical and biological characters

Ultra-Violet Spectroscopy for CNPs

The research was taken of CNPs after it was extracted, and dried as well as it was acted a suspension of CNPs that extracted then, the mixture of reaction placed in an incubator at 37℃ for 24 hours where showed was altered of color that referred to presence of NPs in the mixture of reaction, it was observed alteration color of the mixture reaction for CNPs by visually and it was measured absorbance at the wavelength between 190 nm and 900 nm via the utilization of a spectrophotometer that was given a peak spectrum of absorption for biosynthesized CNPs in the mixture at 280 nm as in figure 3, our results identical with another study by Ali and Aldujaili, (2022) and Gabriel *et al*., (2017) when they found the presence of CNPs that can be seen in a single peak at 280 nm.

https://doi.org/10.58614/jahsm431

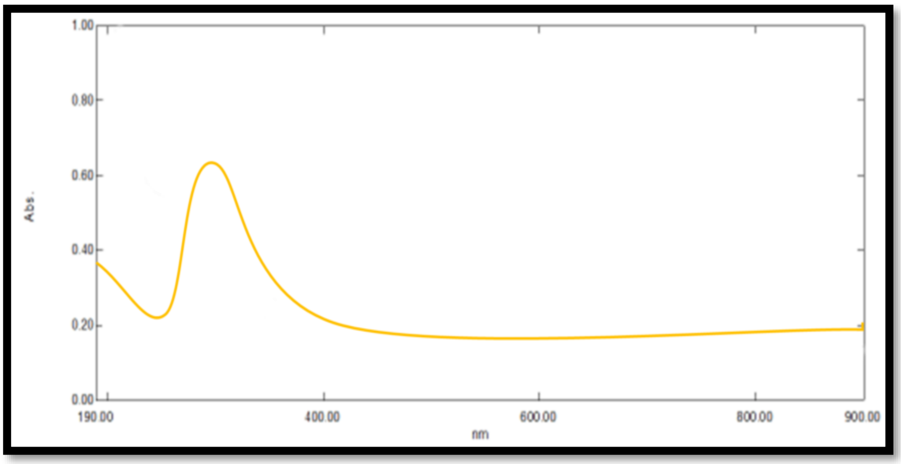

Figure 3. Spectrophotometric analysis of CNPs synthesized by *B.subtilis*.

Scanning Electron Microscope (SEM) for CNPs

The results were identical study by El-Naggar *et al*. (2022) they found the size of NPs were distributed between 10 nm to 60 nm where the most NPs with the same size but a few numbers from it appeared with various sizes that reached more than 60 nm.

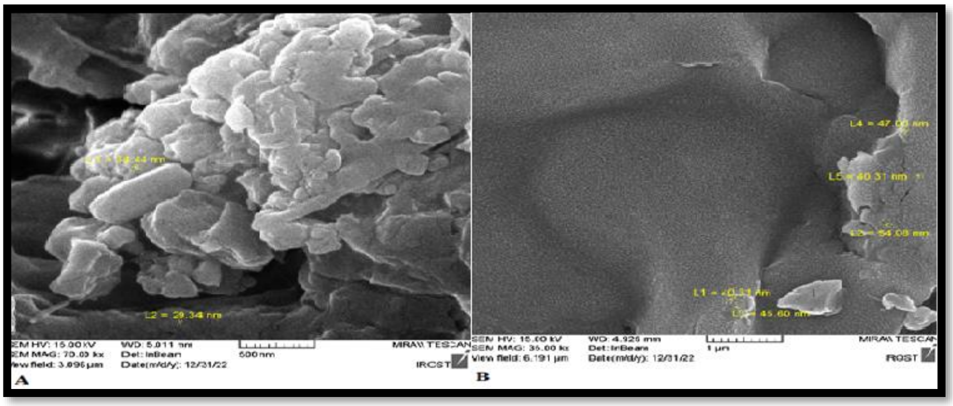

Figure 4. Biosynthesized CNPs by SEM A: 500 nm of distribution size (34.44 -29.34) nm,B: 1 µm of distribution size (40.31 – 54.08) nm.

Energy Dispersive x-ray Spectroscopic (EDS) Analysis for Biosynthesized CNPs

The results showed a percent for each element in the sample of CNPs with higher peaks where it was 58.03% for carbon, 36.57% for oxygen, 2.06 % for phosphate, 1.15 % for magnesium, 0.11 % for ferrous, 0.32 % for silicon, 0.47 % for potassium and 0.76% for sodium as shown in figure 5 and table 1. The elements of CNPs can be analyzed by using spectroscopic dispersive of energy also called analysis of mapping and points and that be used to detect the quantification of elements for biosynthesized CNPs via the reading of peaks for elemental optical absorption (Ali *et al* ,2022).

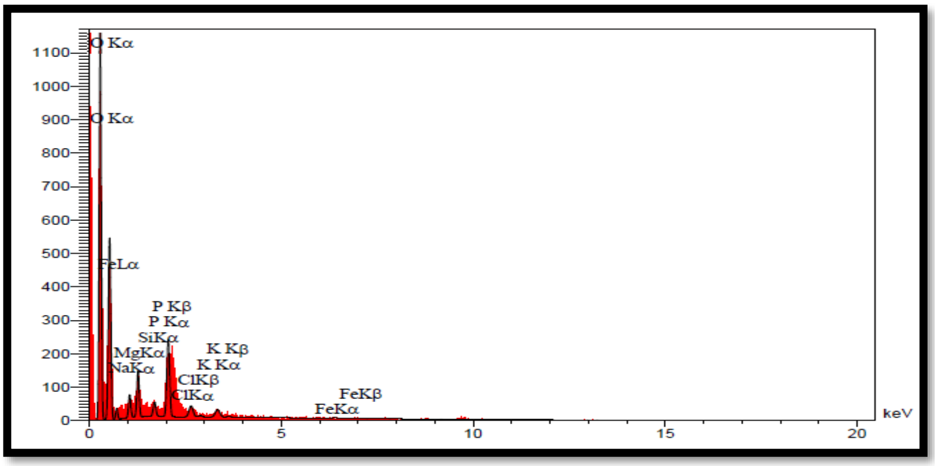

Figure .5 EDS Analysis for biosynthesized CNPs by *B. subtilis*.

Table 1. Percentage of elements to constitute of biosynthesized CNPs

Elt	Line	Int	Error	K	Kr	W%	A%	ZAF
C	Ka	243.7	32.1473	0.7108	0.2904	58.03	66.14	0.5004
O	Ka	133.6	32.1473	0.1941	0.0793	36.57	31.30	0.2168
Na	Ka	15.7	32.1473	0.0087	0.0036	0.76	0.45	0.4716
Mg	Ka	33.5	32.1473	0.0174	0.0071	1.15	0.65	0.6178
Si	Ka	11.6	37.7970	0.0062	0.0025	0.32	0.16	0.7883
P	Ka	66.8	37.7970	0.0405	0.0165	2.06	0.91	0.8035
Cl	Ka	14.3	4.2330	0.0108	0.0044	0.54	0.21	0.8203
K	Ka	10.4	1.1877	0.0096	0.0039	0.47	0.16	0.8344
Fe	Ka	0.8	0.2592	0.0021	0.0008	0.11	0.03	0.7530
				1.0000	0.4085	100.00	100.00	

Antibacterial Role of Biosynthesized CNPs

The research showed its role against all isolates collected of *S. pneumonia* that resistant to antibiotics such as isolates 5,32,33,35,38, and 61 and these isolates highly resistant to optochin disk were obviously when it was cultured on Muller Hinton agar medium that contained 5% of blood according to Mekuria and his colleagues in 2023 but, it can be seen inhibition zone around the colonies of the same isolates with it using various concentrations of biosynthesized CNPs (100,200,300, 400) µg/ml and the diameter was reached of the highest inhibition zone as 21 mm at 400 µg/ml concentration and 14 mm at 100 µg/ml concentration with the lowest inhibition zone as in the figure 6 and figure 7 . The most prevalent proposed antibacterial activity of CS is by binding to the negatively charged bacterial cell wall causing disruption of the cell, thus altering the membrane permeability, followed by attachment to DNA causing inhibition of DNA replication and subsequently cell death, another possible mechanism is that CS acts as a chelating agent that electively binds to trace metal elements causing toxin production and inhibiting microbial growth (Divya *et al.*,2017).The results agree with Ali *et al.*, (2022) when who exposed that the chitosan's nanoparticle could inhibit the growth of, *K. pneumonia* at the same concentrations.

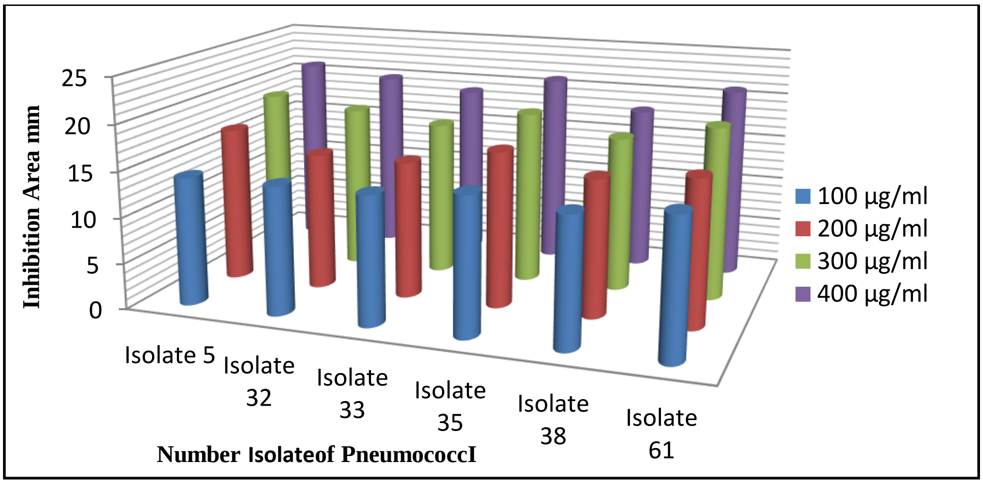

Figure 6. Inhibition zone of biosynthesized CNPs by *B. subtilis* against different isolates of *S. pneumonia*.

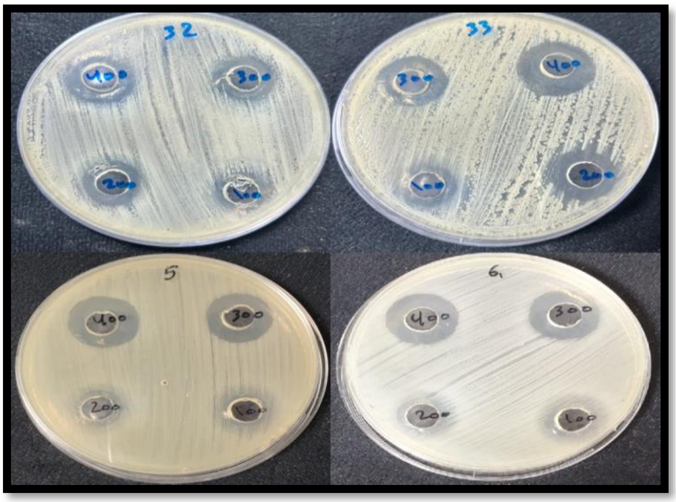

Figure 7. Antibacterial activity of biosynthesized CNPs by *B. subtilis* at different concentrations against various isolates of *S. pneumonia*.

Antibiofilm Role of Biosynthesized CNPs.

The research observed that all collected isolates from patients such as 5 and 61 have the ability to form biofilms through culturing on Congo red agar medium via it altered from pink color to black color indicating to form biofilms where used different concentrations of biosynthesized nanoparticles at (100,200, 300, 400) µg/ml and these were used to test on these isolates as in the figure 8.

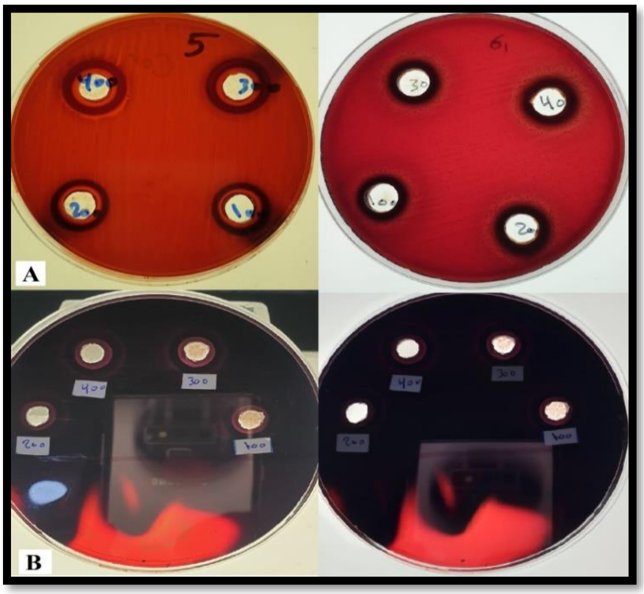

Figure 8. Antibiofilm activity of biosynthesized CNPs by *B. subtilis* at different concentrations against isolate 5 and 6 of *S. pneumonia* on Congo red agar Medium. A: After 24, B: After 48 hours at 37 °C.

The results showed its activity role to prevent the formation of biofilms by these nanoparticles for the isolates selected and it was the largest diameter of inhibition zone 21 mm, a biofilm is considered multicellular aggregation and also it represents a way of connection among bacterial cells and is used to avoid various agents exposure as antibiotics and immune defenses by the human body, there are no antibiotics that have the ability to overcome the biofilms of bacterial cells, so the biosynthesized nanoparticles in our study showed to have an antibiofilm activity where act as reduction the biofilm formation in the figure 8, it was observed obviously an inhibition zone around colony of pathogenic bacteria when culturing on Congo red Medium as well as it can be used as therapy that candidate promising and replaced by antibiotics (Roy *et al*.,2018; *Ali et al*.,2022) . The role of activity for antibiofilm can be increased with increasing of biosynthesized CNPs concentrations where identical with another study by Sarfraz *et al*., (2023) who have obtained the results referred to a significant reduction in the formation of biofilms by pathogenic bacterial isolates with the increasing concentrations of biosynthesized CNPs that used against it.

Conclusion

The biogenic bacteria *Bacillus subtilis* has the ability to biosynthesis CNPs and those NPs showed the activity as antibacterial to inhibit pathogenic isolates of *S. pneumonia* with a high rate at a concentration of 400 µm/ml in addition to the activity as antibiofilm to inhibit the S.pneumonia isolates that form biofilm and cause a higher resistance to antibiotics that through the study of characteristics NPs and detections optimal features by used of Ultra-Violet Spectroscopy to confirmed the presence of

CNPs at single peak of absorbency, SEM to detect the size of NPs and EDS to know the quantities of each element that constituent to NPs.

References

[1] Abd El-Hack, M. E., El-Saadony, M. T., Shafi, M. E., Qattan, S. Y. A., Batiha, G. E., Khafaga, A. F., Abdel-Moneim, A. E., and Alagawany, M. (2020). Probiotics in poultry feed: A comprehensive review. Journal of Animal Physiology and Animal Nutrition, 104(6), 1835–1850.

[2] Ali, Z. H., & Aldujaili, N. H. (2022). Bio-Environmental preparation of chitosan nanoparticle using Bacillus subtilis and their biomedical activity. In *IOP Conference Series: Earth and Environmental Science* (Vol. 1029, No. 1, p. 012023). IOP Publishing.

[3] Ali, Z. H., Al-Saady, M. A. A. J., Aldujaili, N. H., Rabeea Banoon, S., & Abboodi, A. (2022). Evaluation of the Antibacterial Inhibitory Activity of Chitosan Nanoparticles Biosynthesized by Streptococcus thermophilus. *Journal of Nanostructures*, *12*(3), 675-685.

[4] Ali, Z. H., Al-Saady, M. A. A. J., Aldujaili, N. H., Rabeea Banoon, S., & Abboodi, A. (2022). Evaluation of the Antibacterial Inhibitory Activity of Chitosan Nanoparticles Biosynthesized by Streptococcus thermophilus. *Journal of Nanostructures*, *12*(3), 675-685.

[5] Awan, Z. A., Shoaib, A., Schenk, P. M., Ahmad, A., Alansi, S., & Paray, B. A. (2022). Antifungal potential of volatiles produced by Bacillus subtilis BS-01 against Alternaria solani in Solanum lycopersicum. *Frontiers in Plant Science*, *13*.

[6] Bhardwaj, K., Sharma, A., Tejwan, N., Bhardwaj, S., Bhardwaj, P.,Nepovimova, E., Shami, A., Kalia, A., Kumar, A., and Abd-Elsalam, K. A. (2020). Pleurotus Macrofungi-Assisted Nanoparticle Synthesis and Its Potential Applications: A Review. Journal of Fungi, 6(4), 351.

[7] Cao, X. Y., Aimaier, R., Yang, J., Yang, J., Chen, Z. Y., Zhao, J. J., ... & Zhao, H. X. (2023). Effect of bacillus subtilis strain Z15 secondary metabolites on immune function in mice. *BMC genomics*, *24*(1), 273.

[8] Chavarria-Quicano, E., De la Torre-González, F., González-Riojas, M., Rodríguez-González, J., & Asaff-Torres, A. (2023). Nematicidal lipopeptides from Bacillus paralicheniformis and Bacillus subtilis: a comparative study. *Applied Microbiology and Biotechnology*, *107*(5-6), 1537-1549.

[9] Dilnawaz, F., Acharya, S., & Kanungo, A. (2023). A clinical perspective of chitosan nanoparticles for infectious disease management. *Polymer Bulletin*, 1-25.

[10] Divya, K., Vijayan, S., George, T. K., & Jisha, M. S. (2017). Antimicrobial properties of chitosan nanoparticles: Mode of action and factors affecting activity. *Fibers and polymers*, *18*, 221-230.

[11] El-Naggar, N. E. A., Shiha, A. M., Mahrous, H., & Mohammed, A. A. (2022). Green synthesis of chitosan nanoparticles, optimization, characterization and antibacterial efficacy against multi drug resistant biofilm-forming Acinetobacter baumannii. *Scientific Reports*, *12*(1), 19869.

[12] Fang, H., and Zhang, D. (2020). Bacillus subtilis: a universal cell factory for industry, agriculture, biomaterials and medicine. Microbial Cell Factories, 19(1), 1–12.

[13] Gabriel, J. S., Gonzaga, V. A. M., Poli, A. L., & Schmitt, C. C. (2017). Photochemical synthesis of silver nanoparticles on chitosans/montmorillonite nanocomposite films and antibacterial activity. Carbohydrate Polymers, 171, 202–210.

[14] Gamal, A. A., Abbas, H. Y., Abdelwahed, N. A., Kashef, M. T., Mahmoud, K., Esawy, M. A., & Ramadan, M. A. (2021). Optimization strategy of Bacillus subtilis MT453867 levansucrase and evaluation of levan role in pancreatic cancer treatment. *International Journal of Biological Macromolecules*, *182*, 1590-1601.

[15] Iqbal, S., Begum, F., Rabaan, A. A., Aljeldah, M., Al Shammari, B. R., Alawfi, A., ... & Khan, A. (2023). Classification and Multifaceted Potential of Secondary Metabolites Produced by Bacillus subtilis Group: A Comprehensive Review. *Molecules*, *28*(3), 927.

[16] Jha, R., & Mayanovic, R. A. (2023). A Review of the Preparation, Characterization, and Applications of Chitosan Nanoparticles in Nanomedicine. *Nanomaterials*, *13*(8), 1302.

[17] Jha, R., & Mayanovic, R. A. (2023). A Review of the Preparation, Characterization, and Applications of Chitosan Nanoparticles in Nanomedicine. *Nanomaterials*, *13*(8), 1302.

[18] Li, R., Liu, J., Liu, Y., Cao, L., Qiu, W., & Qin, M. (2023). Probiotic Effects of Bacillus subtilis on Growth Performance and Intestinal Microecological Balance of Growing-to-Finishing Pigs. *Journal of Food Biochemistry*, *2023*.

[19] Mekuria, S., Tolossa, D., Abebe, T., Nour, T. Y., Tesfaye, A., & Roble, A. K. (2023). Prevalence, Antimicrobial Drug Resistance and Associated Risk Factors of Streptococcus Pneumoniae Bacteria Infection Among Under-Five Children With Acute Lower Respiratory Tract Infection Attending Sheik Hassan Yebere Referral Hospital, Jig-Jiga, Ethiopia. *Infection and Drug Resistance*, 3511-3523.

[20] Ovais, M., Khalil, A. T., Ayaz, M., Ahmad, I., Nethi, S. K., and Mukherjee, S. (2018). Biosynthesis of metal nanoparticles via microbial enzymes: a mechanistic approach. International Journal of Molecular Sciences, 19(12), 4100.

[21] Roy, R., Tiwari, M., Donelli, G., & Tiwari, V. (2018). Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. *Virulence*, *9*(1), 522-554.

[22] Saad, A. M., El-Saadony, M. T., El-Tahan, A. M., Sayed, S., Moustafa, M. A., Taha, A. E., ... & Ramadan, M. M. (2021). Polyphenolic extracts from pomegranate and watermelon wastes as substrate to fabricate sustainable silver nanoparticles with larvicidal effect against Spodoptera littoralis. *Saudi Journal of Biological Sciences*, *28*(10), 5674-5683.

[23] Sarfraz, M. H., Muzammil, S., Hayat, S., Khurshid, M., & Sayyid, A. H. (2023). Fabrication of chitosan and Trianthema portulacastrum mediated copper oxide nanoparticles: Antimicrobial potential against MDR bacteria and biological efficacy for antioxidant, antidiabetic and photocatalytic activities. *International Journal of Biological Macromolecules*, *242*, 124954.

[24] Van Bavel, N., Issler, T., Pang, L., Anikovskiy, M., & Prenner, E. J. (2023). A Simple Method for Synthesis of Chitosan Nanoparticles with Ionic Gelation and Homogenization. *Molecules*, *28*(11), 4328.

[25] Wang, Z., Hu, L., Li, J., Wei, L., Zhang, J., & Zhou, J. (2020). Magnitude, temporal trends and inequality in global burden of tracheal, bronchus and lung cancer: findings from the Global Burden of Disease Study 2017. *BMJ Global Health*, *5*(10), e002788.

[26] Yusof, H. M., Mohamad, R., and Zaidan, U. H. (2019). Microbial synthesis of zinc oxide nanoparticles and their potential application as an antimicrobial agent and a feed supplement in animal industry: a review. Journal of Animal Science and Biotechnology, 10(1), 1–22.