Biosynthesis of selenium nanoparticles from halophilic bacteria

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DECLARATION

I hereby declare that the data presented in this dissertation report entitled, "Biosynthesis of selenium nanoparticles from halophilic bacteria" is based on the results of investigations carried out by me in the Biotechnology Discipline at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Sanika Samant, Assistant Professor, SBSB and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report "Biosynthesis of selenium nanoparticles using halophilic bacteria" is a bona fide work carried out by Mr. Gourab Dutta under my supervision in partial fulfilment of the requirements for the award of the degree of Master of Science in Marine Biotechnology in the Discipline of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

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PREFACE

This dissertation explores the study of the biosynthesis of selenium nanoparticles using halophilic bacteria. Halophilic bacteria have not been used widely for the synthesis of selenium nanoparticles. These nanoparticles have good antioxidant activity and antagonistic activity against various organisms.

Motivated by different bacteria isolated from the salt pans from Goa and the activity of these organisms, this journey of discovery has been guided by the expertise and support of my supervisor, Dr. Sanika Samant. Additionally, collaboration with colleagues and the unwavering encouragement of family and friends have been instrumental in shaping this research.

As this dissertation contributes to our understanding of bacterial contribution in the biosynthesis of selenium nanoparticles. This approach can help to reduce the dependency of physical and chemical processes in the synthesis of selenium nanoparticles also harness the power of halophilic bacteria that are found in abundance in Goa.

Gourab Dutta

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Abbreviations used

Abbreviations used	Full form
C	Celsius
DNA	De- oxy ribose Nucleic Acid
DW	Distilled water
E. Coli	*Escherechia Coli*
EDX	Energy dispersive X-ray
Fig	Figure
FTIR	Fourier Transform Infrared Spectroscopy
g	Gravitational Force or RCF
mL	Mililiter
MHA	Mueller Hinton agar
nm	Nano meter
NTYE	Nacl tryptone yeast extract
RCF	Relative Centrifugal Force
RNA	Ribose Nucleic Acid
rpm	Revolutions Per Minute
rRNA	Ribosomal RNA
RT	Room temperature
SEM	Scanning Electron Microscope
sp	Species
SeNP	Selenium nanoparticles
UV	Ultra Violet
XRD	X-ray diffraction

°	Degree
µg	Micro Gram
µL	Micro Liter
%	Percent

Abstract

Selenium nanoparticles (SeNPs) are gaining significant attention due to their unique properties and potential applications in biomedical and environmental fields. This study presents the successful biosynthesis of SeNPs using halophilic bacteria. The biosynthesized SeNPs were thoroughly characterized using various techniques, including X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), Energy Dispersive X-ray analysis (EDX), Raman spectroscopy, and UV-Visible spectroscopy (UV-Vis). XRD analysis confirmed the crystalline nature of the SeNPs, while FTIR revealed the presence of functional groups involved in their biosynthesis and stabilization. EDX analysis verified the elemental composition of the nanoparticles. Raman spectroscopy provided further insights into the structural characteristics of the SeNPs. Finally, UV-vis spectroscopy demonstrated the characteristic absorption peak of SeNPs. Some applications of these synthesized nanoparticles were also analysed. This study highlights the potential of halophilic bacteria as an eco-friendly and sustainable source for producing selenium nanoparticles.

CHAPTER - 1
INTRODUCTION

1.1. Background

Life can survive across a wide range of salt concentrations found in natural ecosystems, including freshwater settings and hypersaline environments such as the Dead Sea, saltern crystallizer ponds, and other sodium chloride-saturated environments. The microbial communities that have adapted to these conditions exhibit great diversity, reflecting the varying features of salty and hypersaline environments on Earth (Oren A.1988).

Halophiles can be classified as slight, moderate, or extreme depending on their salt requirements. They are commonly found in marine and hypersaline environments. These organisms are exposed to metals in their environment as the ecological niches they inhabit serve as sinks for metals. Therefore, they are highly valued for their ability to synthesize nanoparticles (Srivastava et al, 2015).

The science and engineering field of nanotechnology is concerned with manipulating matter at the nanoscale, or the minuscule scale of atoms and molecules. This study is conducted on a very small particle size. The majority of the materials used in this investigation are nanoparticles, which are incredibly small particles. There are 100 nm to 1 nm in this size range. They are also available in size of 400 nm (Varlamova et al., 2022).

These nanoparticles have a set of unique properties which differ significantly from their large forms. The main reasons are increased surface area to volume ratio and quantum effects.

The concept of nanoparticles gained scientific traction in the 19th century with Michael Faraday's work on colloidal gold. The 20th century saw significant advancements. The invention of the scanning tunneling microscope in 1981 fueled research and development by making it possible to see and manipulate nanoparticles (Bayda et al., 2020).

Nanoparticles are categorized via different classifications. Some common classifications are-

- **Material-based –** In this classification**,** the composition of nanoparticles plays the most important role. Like metallic nanoparticles (Gold, Silver, Iron oxide), carbon nanoparticles, Ceramic nanoparticles (Silica, titanium dioxide)
- **Shape-based – In this classification, there are different shapes that form** the basis of classification. Like nanospheres, nanorods, nanotubes, and nanowires.
- **Functionality-based** – The function of a particular nanoparticle plays the most pivotal role in this classification. Such as magnetic nanoparticles, fluorescent nanoparticles, and catalytic nanoparticles (Joudeh & Linke, 2022)

There are three main processes used to synthesize nanoparticles: physical, chemical, and biological. Physical techniques include laser ablation and evaporation condensation. However, the physical synthesis of MNPs using a tube furnace at normal pressure has some disadvantages, such as the need for a large space and high energy consumption. (Dhand C et al,2015). Furthermore, physical methods can be time-consuming, which is another disadvantage. Chemical methods involve the use of reducing agents to reduce metal ions, resulting in the formation of MNPs. However, these methods have the disadvantage of using toxic and expensive chemicals in the synthesis process (Thakkar et al,2010).

Biological methods are increasingly favored over physical and chemical methods due to their advantages like cost-effectiveness, eco-friendliness, non-toxicity etc. Bacteria, archaebacteria, fungi, and plants can be used for MNP preparation without the need for toxic and expensive materials (Iravani et al, 2013).

These nanoparticles have many applications due to their unique properties. Some of them are-

- **Electronics** – due to smaller size and energy efficiency nanoparticles are being used as transistors in various electronic devices.
- **Energy**- nanoparticles are used to make solar cells, batteries, fuel cells.

- **Material science** – they are very important in creating stronger, lighter and more durable materials for construction, aerospace, and more.
- **Cosmetics and textiles** – Nanoparticles are being used in the production of sunscreens, wrinkle-resistant fabrics, and more.
- **Environmental** – Nanoparticles are very useful in the construction of nano-remediation techniques for air and water purification, hazardous waste treatment (Altammar, 2023).

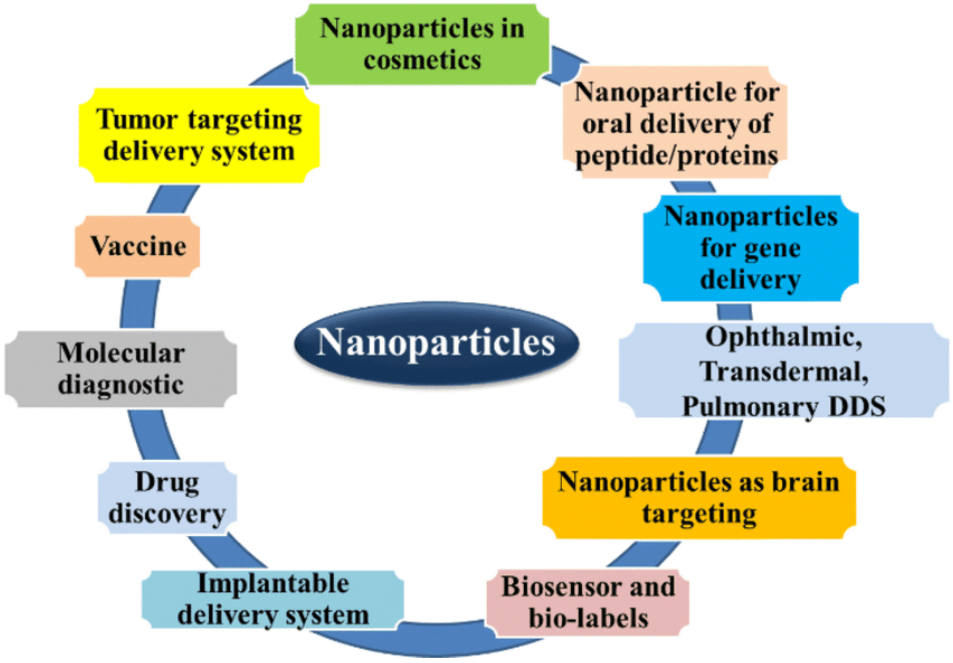


Fig: 1.1

Source: (Khan et al. 2020)

In addition to the aforementioned applications, nanoparticles play a crucial role in drug delivery. They are often referred to as the 'magic bullet' of the 21st century due to their ability to deliver drugs directly to diseased sites, such as tumors while minimizing side effects (Tewabe et al., 2021).

Nanoparticles also protect drugs from degradation in the body, thereby improving their shelf life and effectiveness. Nanoparticles facilitate drug release in a gradual and continuous manner, allowing for less frequent drug use (Altammar, 2023)

They can also aid in crossing barriers such as the blood-brain barrier, expanding treatment options (Hersh et al., 2022)

While nanotechnology is still in its early stages, the potential of nanoparticles will soon be fully realized. Selenium nanoparticles are a specific type of nanoparticle composed of the element selenium at the nanoscale. Nanoparticles range in size from 1 to 100 nm some are even 400 nm in size, making them extremely small. Due to their microscopic size, they have a relatively high surface area-to-volume ratio, which enhances their reactivity and biological activity (Bisht et al., 2022)

Compared to other metal-based nanoparticles, they are considered less toxic. (Nie et al., 2023) The project topic involves the use of a halophilic bacterium to produce selenium nanoparticles from a selenium salt precursor, sodium selenite (Alvares & Furtado, 2022)

These nanoparticles have various applications, including as antioxidants that scavenge harmful free radicals, protecting against conditions such as cancer and cardiovascular disease. Additionally, they exhibit antibacterial, antifungal, and antiviral properties.

To further describe the antibacterial properties of these nanoparticles, they can damage the bacterial cell membrane, leading to the release of cellular contents and, ultimately, cell death. Additionally, they can induce oxidative stress within bacterial cells, causing damage to DNA, proteins, and lipids. Furthermore, these nanoparticles can interfere with metabolic pathways necessary for bacterial growth and survival (Medina-Cruz et al., 2023)

Various methods can be used to characterize these nanoparticles, including UV-vis spectroscopy, XRD, SEM, TEM, FTIR, and AFM. Nanoparticle composition, size, shape, and functional groups present on the surface of nanoparticles can be determined using these methods (Alvares & Furtado, 2022)

1.2. Aim and objectives

a) Determination of metal tolerance/resistance of halophilic bacterial isolate
b) Identification of the given halophilic bacterial isolate
c) Biosynthesis of Selenium nanoparticles
d) Physicochemical characterization of synthesized nanoparticles
e) Evaluating the applications of selenium nanoparticles.

1.3. Hypothesis

Halophiles live in extreme conditions where metal toxicity is prominent due to waste water coming from various industries. Therefore, halophiles can be a good source to produce various nanoparticles (Srivastava et al, 2015). Chemically (Sol-gel method, chemical reduction, microemulsion, etc.) synthesizing nanoparticles requires the use of toxic components (Thakkar et al.,2010), and the physical (Evaporation, sputtering, mechanical milling) synthesis process is very time-consuming and has very high-power consumption (Dhand et al.,2015). That's why the biosynthesis of nanoparticles should be improvised so that the effectiveness of production can be increased. Halophilic bacteria have enhanced tolerance towards the metal selenium. Therefore, the susceptibility and sensitivity of halophilic bacteria towards selenium is low and gives a high survival rate to the cultures. Selenium nanoparticles (SeNPs) possess distinctive physicochemical properties that render them valuable in a multitude of scientific and industrial fields. Their nanoscale dimensions result in an augmented surface area-to-volume ratio, thereby promoting enhanced reactivity and potential for surface modifications. SeNPs exhibit superior biological activity in comparison to traditional selenium compounds, including antioxidant properties, anti-inflammatory effects, and potential anticancer activity. In biomedical applications, SeNPs demonstrate considerable potential as drug delivery vehicles, biosensors,

and therapeutic agents. Furthermore, their distinctive optical and electrical properties render SeNPs a valuable resource in fields such as electronics, photovoltaics, and environmental remediation.

1.4. Scope

Synthesis of selenium nanoparticles from halophilic bacteria has a promising future. There is vast literature published on the synthesis of selenium nanoparticles by halophilic bacteria. Diverse applications of selenium nanoparticles are also being discovered which have profound keynote potential to evolve as a very important biotechnological product of global interest.

CHAPTER - 2
LITERATURE REVIEW

2.1. Different organisms used for production

Selenium nanoparticles have been synthesized using a variety of organisms in an environmentally friendly manner. These organisms include common bacterial species such as *Bacillus subtilis, Pseudomonas aeruginosa, Zooglea ramigera* and *Lactobacillus* sp. The mechanism by which these bacteria produce selenium nanoparticles is that they utilize reducing agents (such as ascorbic acid) to convert selenium salts (such as sodium selenite) into SeNPs. Some bacteria employ a reductase enzyme to reduce selenium salts. For example, *Bacillus subtilis* is capable of reducing selenite intracellularly (Kulkarni et al., 2023).

The production of selenium nanoparticles also involves fungi. Among the species are *Aspergillus sp., Fusarium sp*., and *Alternaria alternata*. They function much like bacteria do. In order to lower selenium salts and create selenium nanoparticles (Kulkarni et al., 2023)

Yeasts employ their cellular machinery for the reduction of selenium. They utilize reductase enzymes to reduce selenium salts into elemental selenium. They offer an easy cultivation process and the potential for the controlled synthesis of SeNPs. Some of the species include *Saccharomyces cerevisiae* and *Candida utilis* (Kulkarni et al., 2023)

For the production of SeNP, plants and plant extracts are also widely used. Garlic, broccoli, tea leaves, fenugreek, grape seed extract, lemon leaves, and many more plants are among the sources. Phytochemicals (flavonoids, polyphenols) found in plant extracts function as stabilizing and reducing agents for the production of SeNP (Pyrzynska & Sentkowska, 2022).

Algae like *Spirulina platensis and Chlorella vulgaris* are also used for biosynthesis for SeNP. These algae possess reductase enzymes and bioactive compounds for the biosynthesis of SeNPs within their cells (Kulkarni et al., 2023).

2.2. Precursors used for synthesis

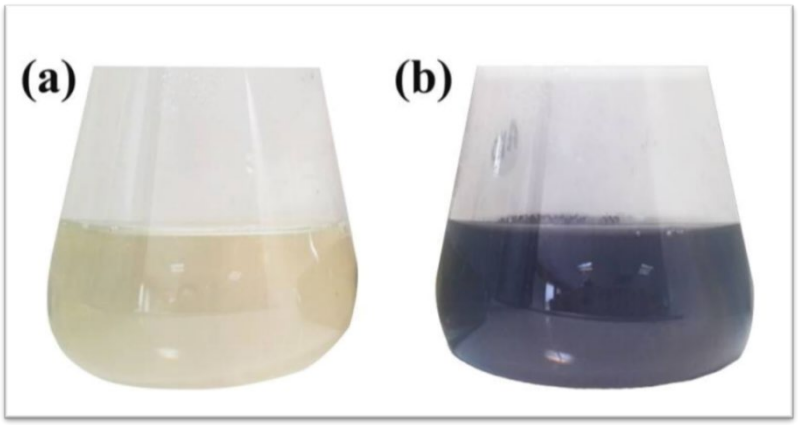
Precursors are necessary for the biogenic production of nanoparticles. The choice of precursor is crucial for the synthesis process to proceed smoothly. Sodium selenite and sodium selenate are the most commonly used precursors for the biosynthesis of SeNPs. Selenous acid and selenium dioxide are also used as precursors, although they are less common (Bisht et al., 2022).

2.3. Halophiles in the production of nanoparticles

Halophiles survive and live in extremely harsh environments where other bacteria might not survive; thus, using them for the biosynthesis process offers robustness in the synthesis process. They have unique set of enzymes and biomolecules which can effectively reduce selenium salts to SeNPs. Literature exists on the conversion of selenite to SeNPs using *Haloferax alexandrinus*. This halophilic bacterium has been utilized to produce SeNPs. This process is extracellular (Alvares & Furtado, 2022). The production of selenium nanoparticles using *Halomonas venusta*, another halophilic bacterium, in an extracellular process has also been reported in existing literature (Vaigankar et al., 2022). Selenium nanoparticles were synthesized extracellularly using the halophilic bacterium *Halococcus salifodinae* also (Srivastava et al., 2014).

2.4. Observation process

There are several methods to spot the production of nanoparticles. Some give a black color in the media (e.g. Reduction of tellurite), some give white precipitate (e.g. zinc nanoparticles). The reduction of selenium nanoparticles results in a brick-red color in the sample (Alvares & Furtado, 2022).



Synthesis of tellurium nanoparticles in flask (b) where no tellurium nanoparticles production observed in flask (a)

Fig: 2.1

Image source – (Barabadi et al. 2018)

2.5. Optimal precursor

The use of different precursors can affect the production of nanoparticles. When selenite salt is used in the media, it increases the growth rate of the bacteria (Kousha et al., 2017).

2.6. Tolerance of selenium

Some bacterial endophytes have been found to exhibit significant tolerance to selenium, with some tolerating up to 180 mM (Acuña et al., 2013). Some bacteria can tolerate very less amounts of selenite content (up to 6mM) (Srivastava et al., 2014). A bacterial strain, *Proteus mirabilis* QZB-2, was isolated from a lateritic red soil and identified as having high selenite resistance, up to 300 mM. This strain could be used for synthesizing SeNPs due to its high SeO_3^{2-} tolerance and rapid reduction rate (Jin-Rong et al., 2022). *Lysinibacillus xylanilyticus* and *Lysinibacillus macrolides*, which were isolated from a selenium-rich environment, demonstrated high selenium tolerance and effectively reduced selenite to SeNPs (Zhang et al., 2019).

2.7. Characterization

2.7.1. UV-vis spectroscopy

Chemically prepared selenium nanoparticle showed an absorption peak at 265nm. (Vahdati et al., 2020). Biogenically produced selenium nanoparticles may be characterized through the use of UV-Vis spectroscopy, which displays an absorption peak at approximately 240 nm. (Srivastava et al., 2014). Brick red powder produced using *Haloferax alexandrinus* GUSF-1, dispersed in methanol showed a characteristic peak at 239 nm (Alvares & Furtado, 2022)

2.7.2. XRD analysis

Every molecule has a characteristic X-ray diffraction profile. The XRD of biogenically produced selenium nanoparticles are reported to exhibit band peaks of 2θ at 23.40°, 29.66°, 41.26°, 43.68°, 45.24°, 51.62°, 55.93° and 61.47° with the highest peak at 29.66º (Alvares & Furtado, 2022).

2.7.3. SEM and EDX analysis

Wang et al (2023) demonstrated that selenium nanoparticles (SeNPs) synthesized by *Pediococcus acidilactici* were spherical, as confirmed by transmission electron microscopy (TEM). Scanning electron microscopy (SEM) with energy-dispersive X-ray spectroscopy (EDX) analysis showed that selenium was the primary component. Shagdarova et al. (2023) highlighted that SeNPs synthesized using a quaternary chitosan derivative exhibited a positive charge and sizes ranging from 119.5 to 238.6 nm. SEM analysis confirmed stable nanoparticle formation. Hariprasath and Selvakumar's study on biogenic selenium nanoparticles (SeNPs) using *Syzygium aromaticum* demonstrated the formation of agglomerated spherical particles with

diameters ranging from 30 to 55 nm, as shown by SEM-EDX imaging (Selvakumar et al. 2023).

2.7.4. Raman analysis

Raman analysis is a valuable tool for characterizing selenium nanoparticles, as it allows for the study of their vibrational properties and stability (Carla et al. 2023). Various studies have employed Raman spectroscopy to analyze biosynthesized selenium nanoparticles from bacterial strains, revealing the involvement of bacterial proteins in the reduction of selenite and stabilization of nanoparticles (Aruna et al. 2017). Raman spectroscopy has been used to investigate the transformation of selenium nanoparticles into various structures, including t-Se, m-Se, Se-nanoballs, Se-nanowires, and Se-hollow spheres. This provides insights into their structural changes and properties (Azamal et al. 2014).

2.7.5. FTIR analysis

Fourier transform infrared spectroscopy (FTIR) analysis was used to investigate the composition and structure of selenium nanoparticles (SeNPs) in various studies. The FTIR spectra demonstrates the presence of proteins, polysaccharides, and lipids on the surface of SeNPs, which stabilized their structure (Ying et al. 2023). FTIR analysis of biogenic SeNPs synthesized using plant extracts showed peaks related to phenols, polysaccharides, and proteins, indicating the bioorganic capping of the nanoparticles (Deepa et al. 2023). Additionally, FTIR characterization of SeNPs synthesized from aqueous flower extract exhibited important functional groups contributing to their biological activities, such as anti-diabetic effects and antioxidant properties (Samer et al. 2022). In addition, the FTIR analysis of SeNPs, which were prepared from bacterial biomass, highlighted the impact of sample preparation on the spectroscopic

characteristics. This emphasizes the importance of optimized sample treatment procedures for FTIR analysis of microbiological specimens (Alexander et al. 2021).

2.8. Application of selenium nanoparticles

2.8.1. DPPH test

The effectiveness of selenium nanoparticles (SeNPs) in scavenging free radicals was evaluated in multiple studies using the DPPH test, a common method for assessing antioxidant activity. The results indicated that SeNPs exhibited significant antioxidant properties (Lakshmanan et al. 2023). Several SeNP formulations, including those synthesized using plant extracts, quaternary chitosan derivatives, and D-α-Tocopherol polyethylene glycol 1000 succinate (TPGS) as capping agents, demonstrated promising antioxidant effects in vitro. (Puri et al. 2022) (Mohammad et al. 2022). The DPPH test results highlighted the potential of SeNPs in combating oxidative stress and preventing the growth of cancer cells, showcasing their utility in biomedical applications. Furthermore, the results of the DPPH test highlight the significance of SeNPs as versatile and efficient antioxidants with various therapeutic implications (Mohammad et al. 2022).

2.8.2. Antagonistic test against organisms

Selenium exhibits antagonistic activity against certain yeast, such as *C. albicans*, and inhibits the growth of *E. coli* and *S. aureus* (Nguyen et al., 2017). Selenium nanoparticles (SeNPs) have been found to exhibit antagonistic effects in various biological contexts. In the field of biomedicine, they have shown potential as antioxidants, antidiabetic agents, and anti-parasitic agents (Anuse et al. 2022). SeNPs have been found to inhibit alpha-amylase, a key enzyme in diabetes management,

comparable to standard medications like acarbose, suggesting a potential role in treating diabetes mellitus (Ting et al. 2023). SeNPs synthesized from *Streptomyces fulvissimus* have demonstrated anti-Toxoplasma effects in vivo, increasing survival time and reducing parasite count in infected mice (Nagdallan et al. 2023).

CHAPTER - 3

METHODOLOGY

3.1. Preparation of culture media

All constituents of NTYE media were added to the 18 % salt water and was then shaken to dissolve. pH was then adjusted to 6.8 and autoclaved. Autoclaved media was then poured into petri plates and the broth was dispersed into sterile conical flasks and test tubes.

3.2. Maintenance of culture

The culture ASB12 was streaked on the solidified media in petri plates aseptically. Likewise, the culture was also inoculated in NTYE broth and streaked on slants to grow and maintain the culture. The culture was finally cryopreserved using 25 % glycerol solution. The vials were then stored in -20^{o}C refrigerator.

3.3. Effect of different concentrations sodium selenite on bacterial growth

Different concentrations of selenite tube were made. The concentrations used were (5,10,20,40,80,100, 150, 180, 200, 250, 300, 350) mM. These dilutions were prepared by adding different volumes of sodium selenite (1 M stock) with NTYE media. Total volume of the media was made 5 ml. 500 µl Culture ASB12 was then inoculated into it. Tubes were then kept in RT in a shaker incubator for 4 days. Tubes were then taken out of the incubator and analyzed for tolerance by visualizing the turbidity.

3.4. Gram staining

Culture ASB12 was smeared on a glass slide and dried. Gram staining procedure was performed. Gram staining procedure begins with preparing a thin, even smear of bacteria on a microscope slide and heat-fixing it to ensure the cells stick. Next, the slide is covered with crystal violet dye for about a minute. Both Gram-positive and Gram-negative bacteria take up this purple colour. Afterward, Gram's iodine is added as a mordant, helping Gram-positive bacteria retain the crystal violet stain within their thick cell walls. A decolourizer, often a

mixture of alcohol and acetone, is then applied. This step is critical as it washes away the crystal violet stain from Gram-negative bacteria due to their thinner cell walls. Finally, safranin, a counterstain, is added, giving Gram-negative bacteria a pink or red colour. Gram-positive bacteria, having retained the crystal violet, remain purple. After that slide was observed under microscope at 100x (Tripathi et al. 2023)

3.5. Isolation of bacterial genomic DNA

To identify the bacteria, DNA was needed to be extracted from the culture ASB12 and to be sent for sequencing.

3.5.1. Extraction procedure

1.5 ml of bacterial broth culture was pelleted in a 2.0ml capped collection tube by centrifuging for 2 minutes at 12,000-16,000 x g (≈13,000-16,000 rpm). The culture medium was then removed and discarded. The pellet was resuspended thoroughly in 180µl Resuspension Buffer (MS) (DS0090). 20 µl of the Proteinase K solution (20 mg/ml)was added to the sample. This was mixed and incubated for 30 minutes at 55°C. RNA-free genomic DNA was required, so 20 µl of RNase A Solution (DS0003) was added, mixed, and incubated for 5 minutes at room temperature (15-25°C). Then 200 µl of Lysis Solution (C1) (DS0010) was added and vortexed thoroughly (about 15 seconds) and incubated at 55°C for 10 minutes. Then 200 µl of ethanol (96-100%) added to the lysate obtained from the aforementioned steps for the purpose of preparing the lysate for binding to the spin column. This was followed by thoroughly mixing with gentle pipetting. The lysate obtained was transferred from step II onto the Hi Elute Miniprep Spin Column (Capped) (in DBCA016 Collection Tube) provided. This was then centrifuged at a minimum of 6,500 x g (approximately 10,000 rpm) for one minute. the flow-through liquid was discarded and the column was placed in a 2.0 ml collection tube of the same type. The next step was to add 500 µl of the diluted prewash solution (PW) (DS0011) to the

column and centrifugation at a speed of at least 6,500 x g (approximately 10,000 rpm) for one minute. The flow-through liquid was discarded and the same collection tube with the column was reused. 500 µl of diluted Wash Solution (WS) (DS0012) was added to the column and centrifuged at 12,000–16,000 x g (13,000–16,000 rpm) for 3 minutes to dry the column. The flow-through liquid was discarded and the column spun for another minute at the same speed. The collection tube containing the flow-through liquid was discarded and the column was placed in a new 2.0 ml uncapped collection tube. A further 100 µl of the Elution Buffer (ET) (DS0040) was pipetted directly onto the column without spilling to the sides. Incubated for one minute at room temperature (15-25°C) followed by centrifugation at a minimum of 6,500 x g (approximately 10,000 rpm) for one minute to elute the DNA. This step was repeated again with an additional 100 µl of Elution Buffer (ET) for a higher yield of DNA. (HiMedia – MB505)

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3.6. Phylogenetic analysis of sequence

The sequence, as processed by Eurofins India PVT Ltd, was analyzed using BLAST in the NCBI and searched for sequence similarity. After that phylogenetic tree was prepared by using MEGA 11 software.

3.7. Synthesis of nanoparticles

The culture ASB12 was inoculated in NTYE broth with 20mM Sodium selenite concentration. It was then kept on a shaker incubator at room temperature for 4 days. The media was then transferred to 50 ml falcon tubes. The tubes were then centrifuged at 9500 rpm for 5 minutes at 4ºC. The pellet was collected and supernatant was removed. The pellet was then washed

with 18% salt solution. Then the pellet was dissolved in sterile distilled water. The mixture was then sonicated for 10 minutes. The solution was then centrifuged at 9500 rpm for 40 minutes at 4ºC. The pellet was kept and supernatant was discarded. The pellet was then dried at 80ºC overnight. The dried pellet was then grinded to fine powder using pestle and mortar. The powder was then kept and stored in a glass vial.

3.8. Physicochemical characterization of the synthesized nanoparticles

For characterization of the synthesized nanoparticles different techniques were employed. These techniques include XRD, SEM, EDX, Raman, FTIR, UV-vis spectroscopy.

3.8.1. XRD analysis

Powdered sample (20 mg) was used for the analysis of the sample using Rigaku smart lab XRD machine. This sample was put on the holder. The powder was pressed to create a flat and even surface. The sample was scanned in the 2Θ range of 5º to 60º. Standard conditions were used while doing the experiment. Using Origin 8.0 software, the XRD machine's data were plotted to determine the full-width at half maximum, or FWHM, value. Scherrer's equation $D = 0.94 \lambda/\beta$ was utilized to determine the crystallite size of the nanoparticles. Cos θ, where D is the size of the crystallite, λ is the x-ray radiation wavelength, β is the FWHM value, and θ is the diffraction angle (Dudley et al. 2001).

3.8.2. FTIR analysis

FTIR was done in a BRUKER FTIR spectrometer to check for the functional groups which attach to nanoparticles when they are biogenically synthesized. The fine

powdered sample was given in a vial to the technician to do the analysis. JPEG photo was received for analysis. The analysis was done using the standard chart for IR spectrum in the range of 3500 cm^{-1} to 500 cm^{-1}.

3.8.3. Raman analysis

The confocal Raman spectrum of the finely ground sample powder was recorded using a LabRAM evolution microscope. These microscopes provide excellent 2D and 3D confocal imaging capabilities, making them perfect for both macro and micro measurements. With confidence and speed, the most detailed images and analysis may be acquired with the real confocal Raman microscope. 2 mg of powdered sample was placed on a clean slide and processed at 785 nm wavelength of laser light. The data is then plotted in Origin pro software to obtain the Raman shift.

3.8.4. UV-vis spectroscopy

To ensure that the nanoparticles were evenly dispersed, the finely ground sample was suspended in methanol and sonicated at 2 second pulse on and 4 second pulse off for two minutes in MRC LTD intelligent ultrasonic processor. The final mixture was compared to methanol as the reference solution and scanned in the UV-visible range using a spectrophotometer.

3.8.5. SEM analysis

SEM analysis was done using Carl-Ziess Scanning Electron Microscope (SEM), The powdered sample was processed using gold sputter coating for analysis. Sample was then visualized using different magnifications (100kx, 50kx, 20kx).

3.8.6. EDX analysis

The powdered sample was given in a vial to CSIF, BITS Pilani K K Birla Goa Campus. The results were obtained through email in pdf format. It was then analysed.

3.9. Evaluation of the applications of selenium nanoparticles

3.9.1. DPPH test

0.1mM DPPH was prepared in absolute ethanol. Standard (Ascorbic acid) was prepared in ethanol as well. Sample (nanoparticle powder) was dispersed in ethanol to make concentration of 1mg/ml. Then 3ml of sample and 1 ml of DPPH was taken as test, 3ml standard and 1ml DPPH was taken as standard, 3ml ethanol and 1ml DPPH was taken as blank. They were then incubated in dark for 30 minutes. After then absorbance was taken at 517nm. RSA% was calculated using the following formula-

$$\% \text{ DPPH radical scavenging activity} = \{(A\,0 - A\,1) / A\,0\} \times 100$$

In this formula, A0 is the absorbance of the control reaction, and A1 is the absorbance of the presence of all of the extract samples and standard (Baliyan et al., 2022)

3.9.2. Antagonism test

Pathogenic cultures (*E. coli, Klebsiella pneumoniae, Candida albicans*) were spread on MHA plates. Wells were then made in those plates with a cork borer. Three wells were made in each plate. A positive control, methanol as negative control, nanoparticles dispersed in methanol served as test. These plates were then incubated at 37ºC for 24 hr. The zone of clearance was measured in millimetres and results were tabulated.

CHAPTER – 4

ANALYSIS AND CONCLUSIONS

4.1. Determination of metal tolerance/ resistance of halophilic bacterial isolate:

4.1.1. Growth and characterization of the given halophilic isolate:

The given halophilic culture ASB12 isolated from sediment sample of Agarwado solar saltern in Goa-India was routinely cultured and maintained at ambient room temperature (RT; 28–32°C) on agar slopes of NTYE medium.

Fig.4.1. Growth of ASB12 on NTYE agar plate

4.1.2. Colony characterization:

Table- 1: Colony Characteristics of ASB12

Shape	Margin	Elevation	Size	Texture	Pigmentation	Optical property
Circular	Entire	Raised	Small	Smooth	Orange	Opaque

4.1.3. Gram staining:

The cells of ASB12 stained Gram negative on Gram reaction and appeared to be coccoid in morphology when observed using a compound microscope (Lawrence and Mayo) (1000X magnification).

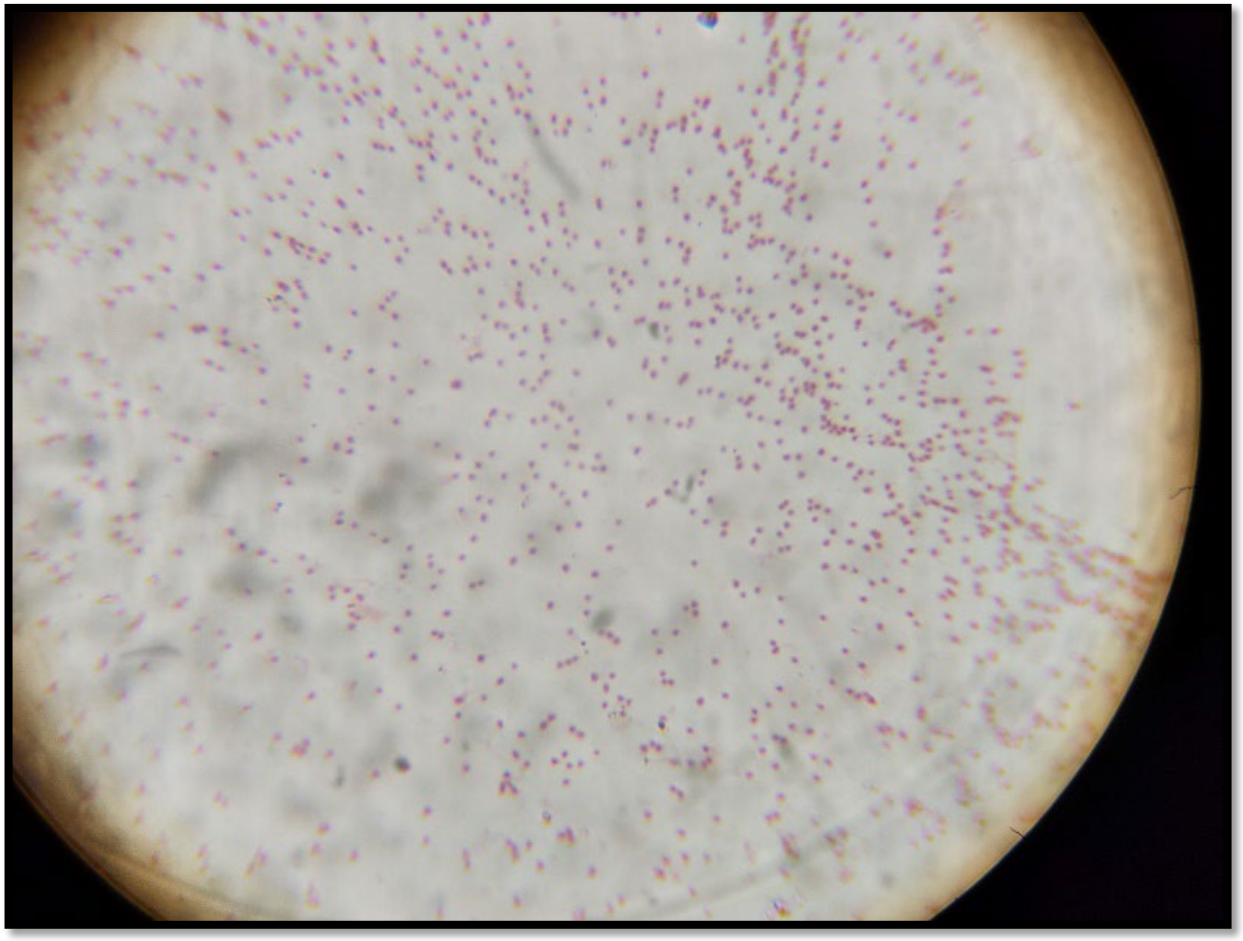

Fig.4.2. ASB12 stained as Gram-negative cocci.

4.1.4. Metal tolerance assay:

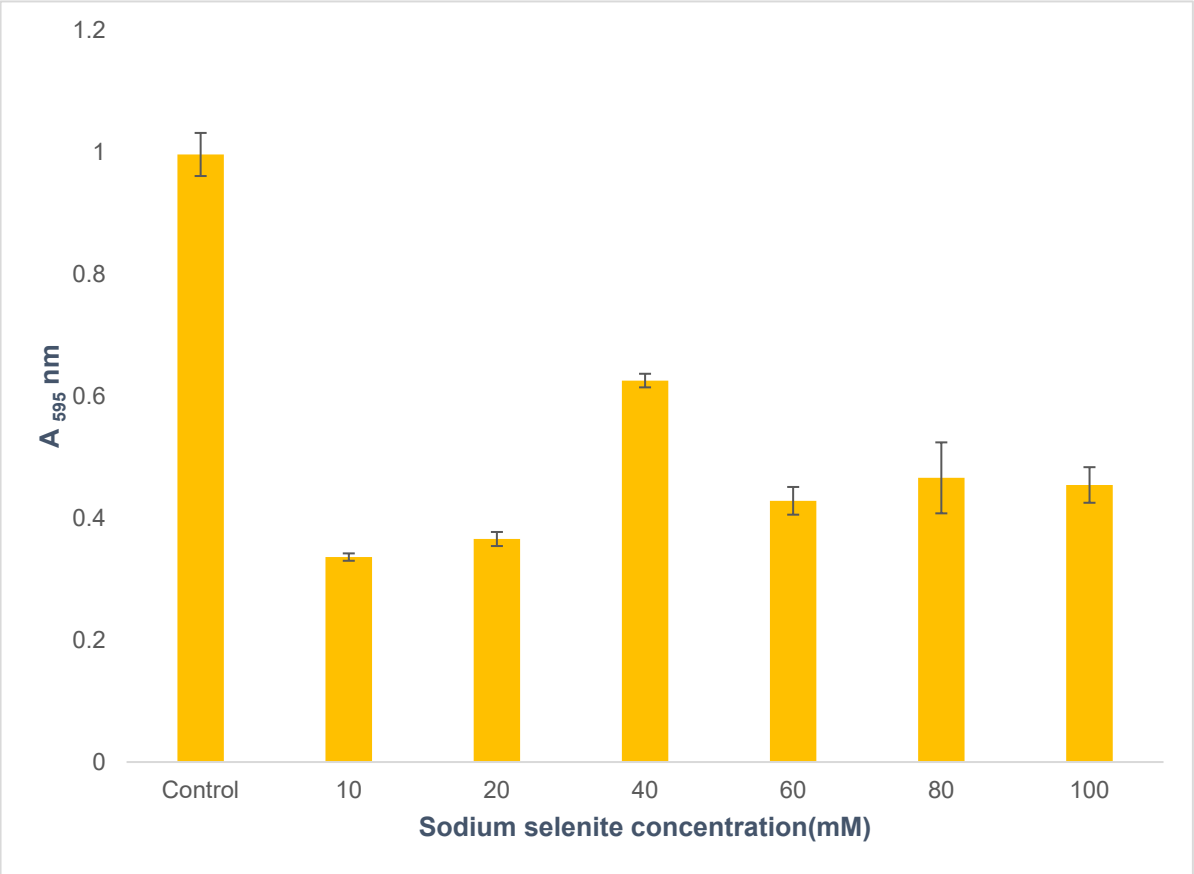

Fig.4.3. Effect of varying sodium selenite concentration on growth of ASB12 in NTYE

Fig.4.4. Tubes containing sodium selenite concentrations up to 250 mM showing effect on growth of ASB12

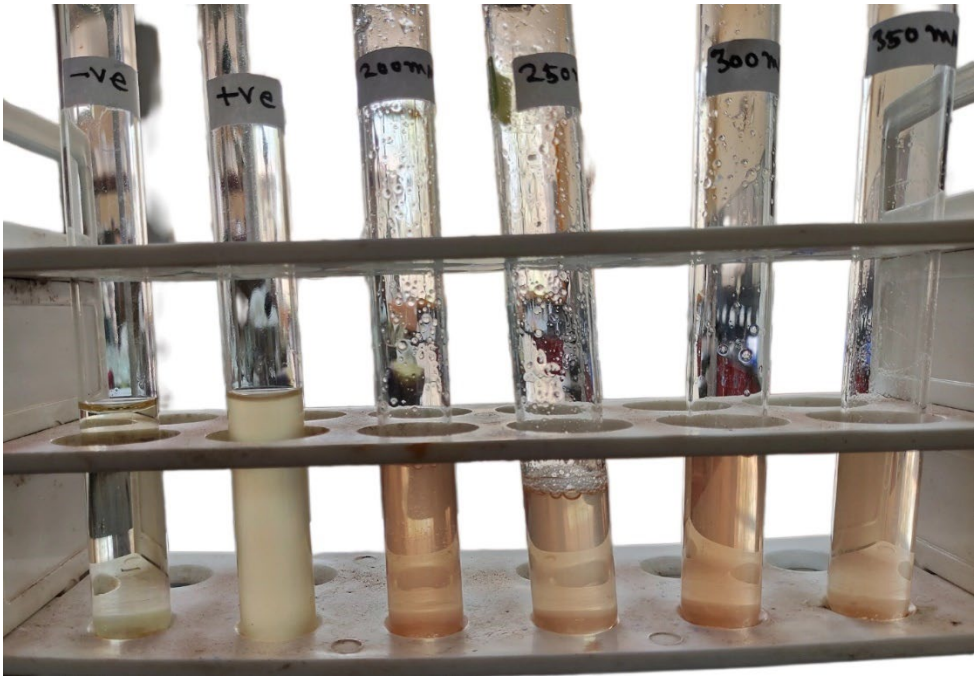

Fig.4.5. Tubes containing sodium selenite concentrations of 200 mM, 250mM, 300 mM and 350 mM showing effect on growth of ASB12

As illustrated by **(Fig.4.3).** the bacteria exhibited the highest growth at a sodium selenite concentration of 40 mM in contrast to other concentrations. It is still lower than control in which no sodium selenite is added. Following this, the growth declined, continuing until 100 mM, up to which absorbance was recorded. ASB12 was observed to tolerate up to 100 mM of sodium selenite, and thus further inoculated with a high concentration of the metalloid salt. It was observed that ASB12 could not grow beyond 160 mM of sodium selenite (**Fig.4.4**).

4.2. Identification of the given halophilic bacterial isolate:

4.2.1. DNA extraction

The DNA extracted using the HiMedia MB505 kit was subjected to electrophoresis on a 0.8% agarose gel. Visualization of the bands using a UV transilluminator indicated the presence of genomic DNA. No evidence of RNA or protein contamination was observed.

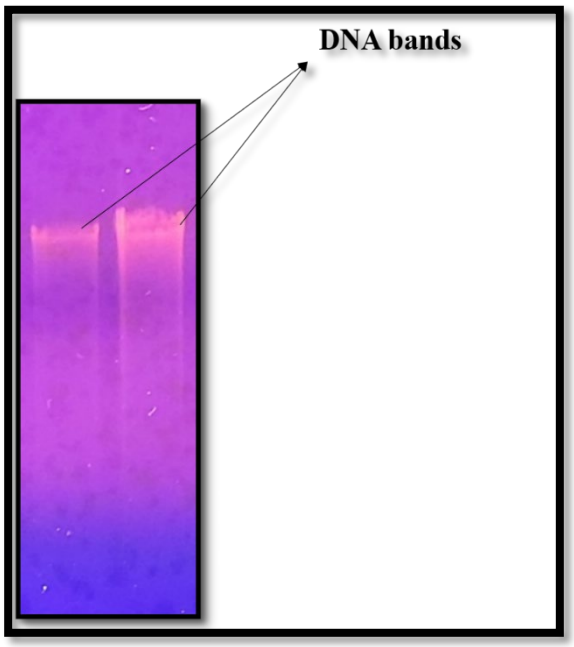

Fig.4.6. Gel electrophoresis profile of ASB12 observed under UV transilluminator

The sequence that was obtained from the company was not satisfactory. The extracted DNA needs to be sent to a different company to get the sequencing results and to make phylogenetic tree to identify the culture ASB12.

4.3. Biosynthesis of selenium nanoparticles:

4.3.1. Growing the culture along with sodium selenite:

Biosynthesis of selenium nanoparticles was done using bacterial cells to grow in NTYE media containing 5 mM concentration of sodium selenite. The flask in which sodium selenite was added turned brick red color while the flask where sodium selenite was not added remained orange in color **(Fig.4.7)**. This is in accordance with literature published by (Alvares & Furtado, 2022).

Fig.4.7. Growth of ASB12 in NTYE supplemented with 5 mM sodium selenite indicating (A) brick-red color and without sodium selenite indicating (B) orange color

4.3.2. UV-vis spectroscopic analysis:

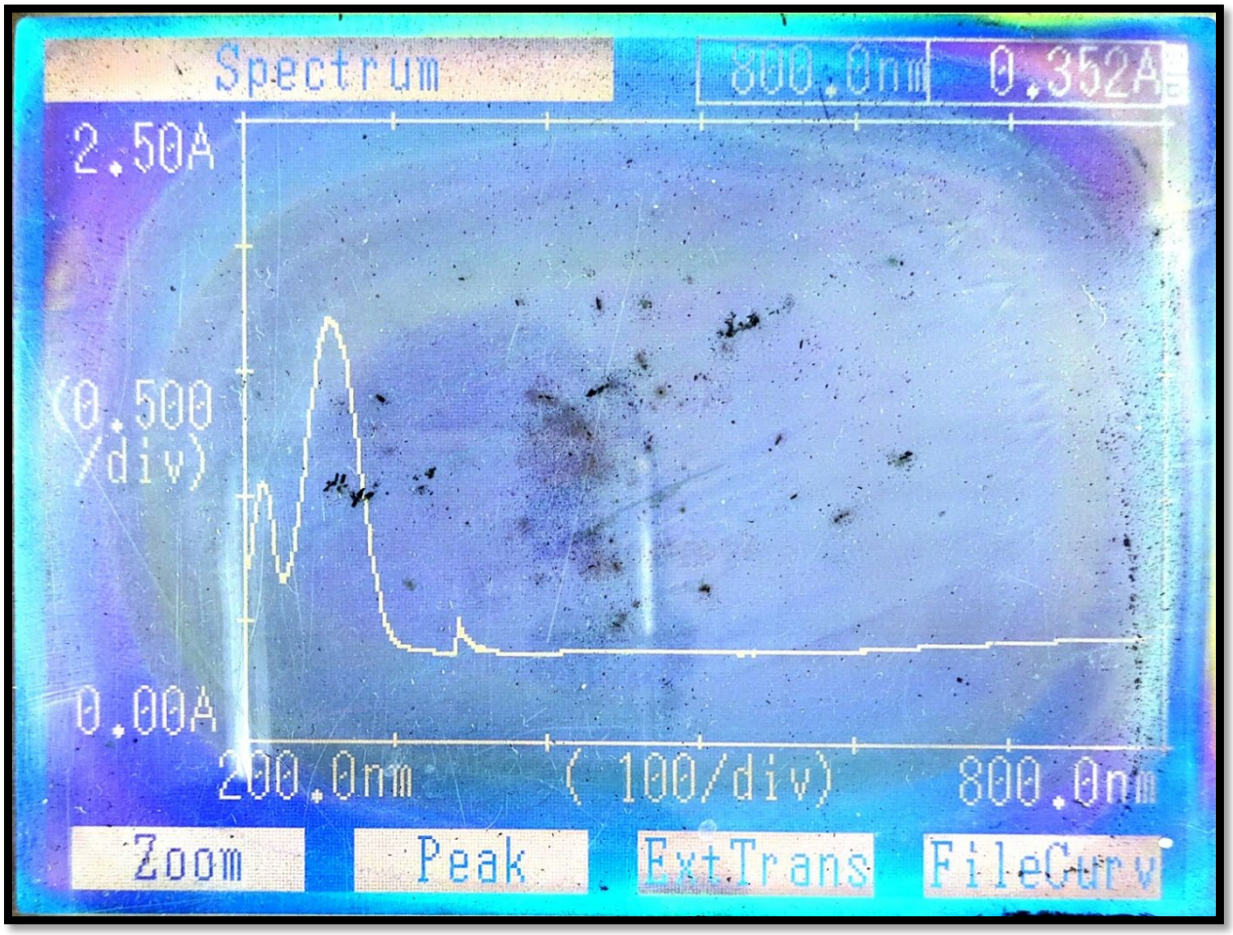

Fig. 4.8. UV-vis spectrum of selenium nanoparticles synthesized from ASB12

Table 2: Absorbance of biosynthesized SeNPs at different wavelengths observed using UV-Visible spectroscopy:

Absorption spectrum (in nm)	Absorbance
314.00	0.505
257.00	1.714
213.00	1.054

Peak at 314 nm: This peak is likely to correspond to the surface plasmon resonance (SPR) of SeNPs. SPR is the collective oscillation of electrons on the nanoparticle surface in resonance with incident light. The exact position of the SPR peak depends on various factors, including the size, shape, and surface chemistry of the SeNPs. The broad nature of the peak suggests a possible distribution of SeNP sizes or morphologies (Yusuf et al. 2020)

Peak at 257 nm: This peak could be attributed to the presence of organic capping agents surrounding the SeNPs. These capping agents, often biomolecules derived from the halophilic bacteria themselves, play a crucial role in stabilizing the nanoparticles and preventing aggregation. The specific biomolecules responsible for this peak would require further analysis using techniques like Fourier-transform infrared spectroscopy (FTIR) to identify their functional groups.

Peak at 213 nm: The presence of a weaker peak at a lower wavelength may be attributed to the presence of residual aromatic compounds or precursor molecules that have not undergone complete degradation during the biosynthesis process. These could be organic molecules present in the bacterial growth medium or intermediates used in selenium reduction.

The range of UV-vis spectra that the selenium nanoparticles should lie between 200 nm to 300 nm according to (Chandramohan et al., 2019; Shar et al., n.d.) . They have confirmed that this range as the characteristic peak for selenium nanoparticles. So, we can assume that the sample is selenium nanoparticles.

4.3.3. Selenium nanoparticles in powder form:

Fig. 4.9. Powdered Selenium nanoparticles in glass vial

Following the extraction of selenium nanoparticles, the resulting pellet is dried at 70°C over the course of one night. This dried pellet is then crushed using a pestle and mortar into a fine powder. This powder is then stored in glass vials **(Fig.4.9)**.

4.4. Physicochemical characterization of synthesized selenium nanoparticles

4.4.1. Raman spectroscopy

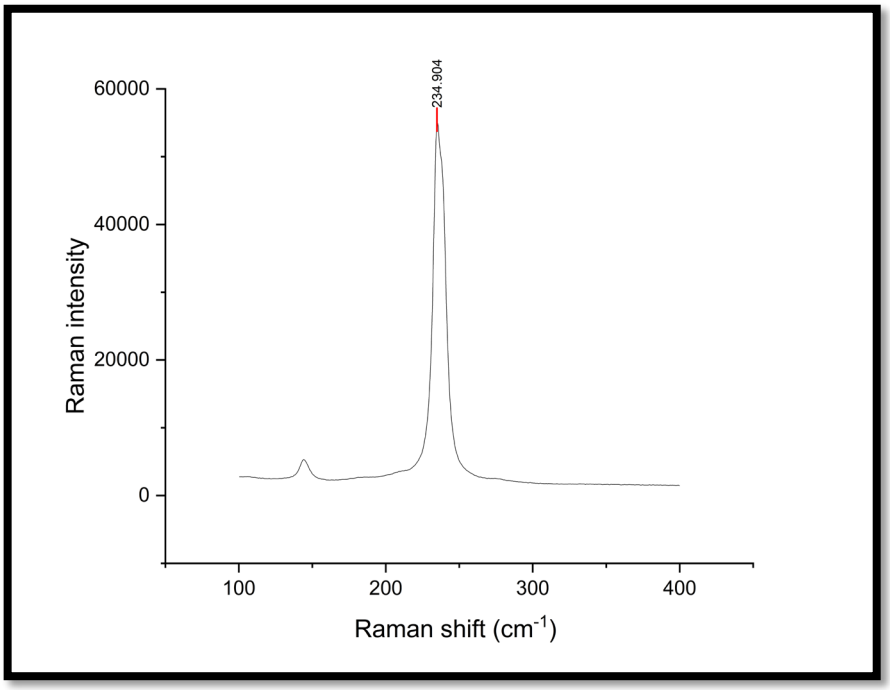

Fig. 4.10. Raman spectrum of selenium nanoparticles synthesized from ASB12

The Raman spectrum displays a pronounced peak at 234.904 cm^{-1} (**Fig.4.10**). This peak position correlates well with the characteristic Raman signature of selenium (Se) in the form of selenium-selenium (Se-Se) bonds. Given that, Raman spectroscopy is a technique that is particularly sensitive to vibrations within a molecule's structure, the presence of this peak strongly suggests the successful incorporation of selenium into the nanoparticles. This is also in accordance with the characteristic peak of trigonal selenium nanoparticles according to (Mellinas et al., 2019)

4.4.2. XRD analysis

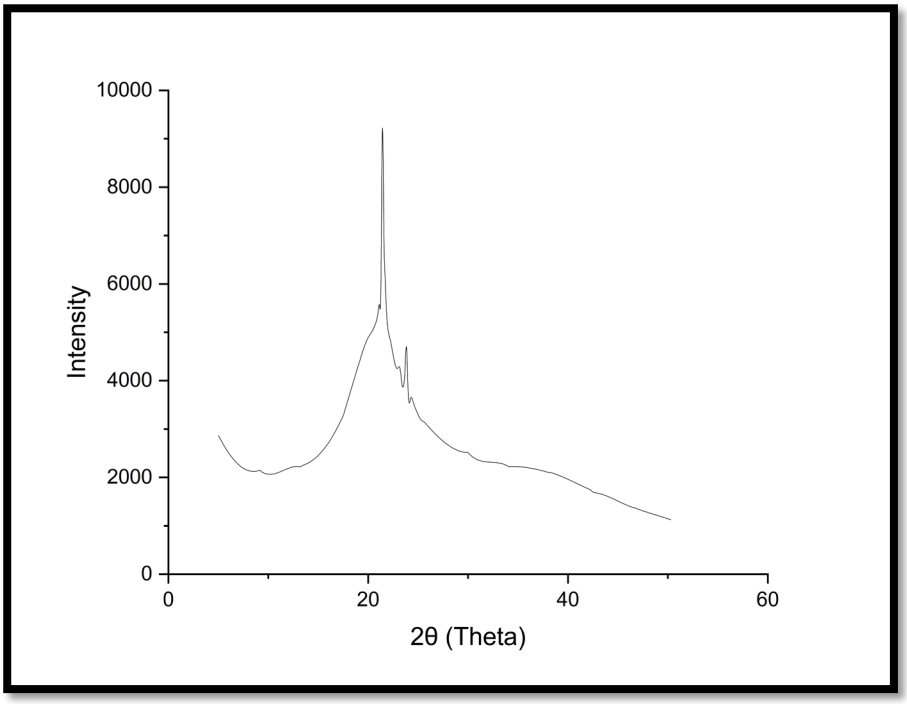

Fig.4.11. X-ray diffraction profile of selenium nanoparticles synthesized from ASB12

The XRD scan was performed on powdered sample from 10°-60° The XRD spectrum (**Fig.4.11**) displayed prominent 2Θ values at 23° and 27°, respectively, which provides a valuable insight into the structure of the biosynthesized selenium nanoparticles (SeNPs). These 2Θ values align reasonably well with the characteristic diffraction peaks of trigonal selenium (t-Se). In trigonal selenium, the Se atoms form helical chains, leading to specific diffraction patterns. The pronounced peaks at 23° and 27° indicate that the SeNPs are likely to possess a considerable degree of crystallinity, with a predominantly trigonal structure. In literature selenium nanoparticles peaks are observed at 23º, 29º, 41º as a characteristic nature of selenium nanoparticles (Alvares & Furtado, 2022); (Zhang et al., 2004).

4.4.3. SEM analysis

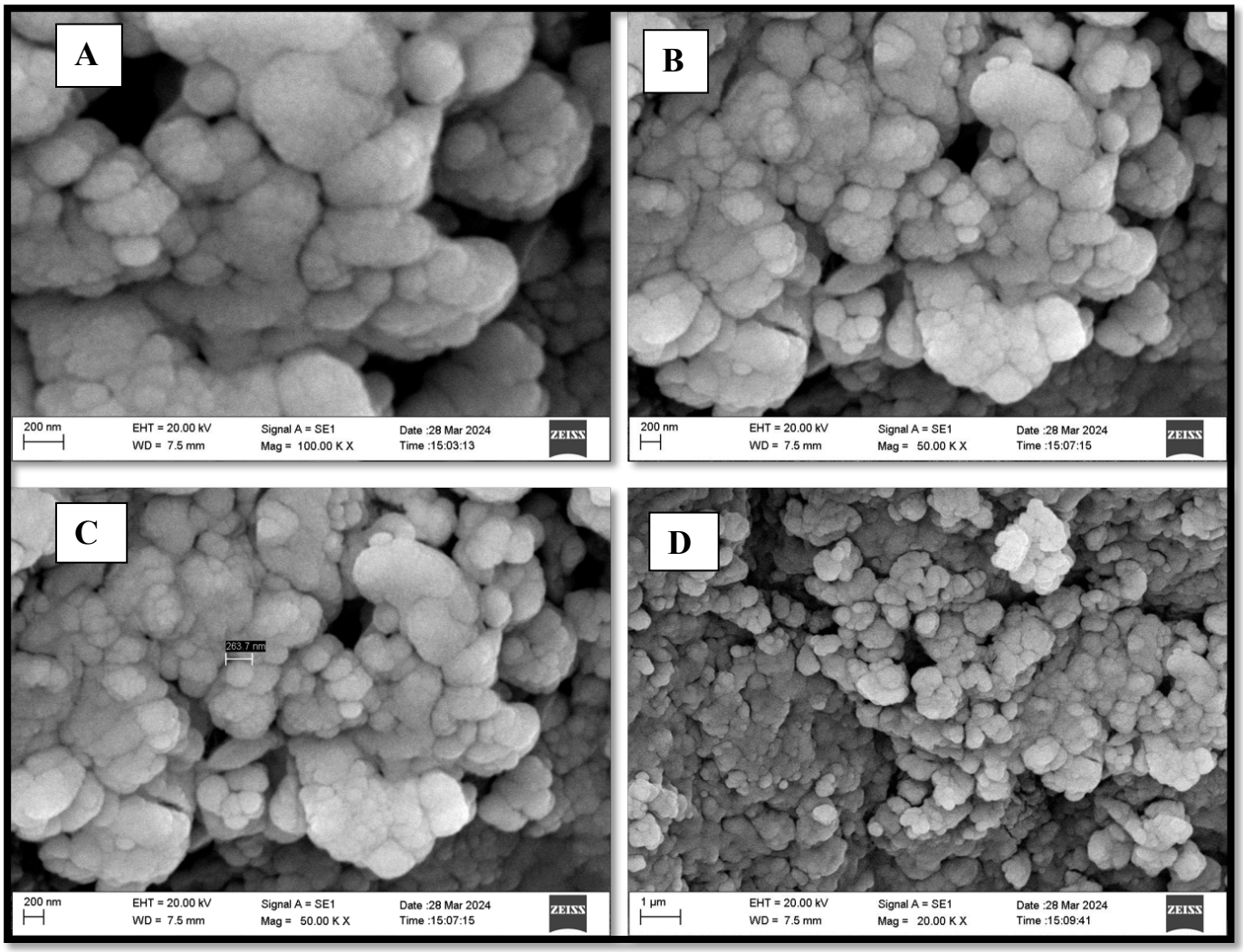

Fig. 4.12. SEM micrographs of selenium nanoparticles synthesized from ASB12 at varying magnifications 100kx (A), 50kx (B, C), 20kx (D)

Spherical Morphology: Spherical shapes are a common morphology observed in biosynthesized selenium nanoparticles. This often arises due to the role of bacteria and their biomolecules in capping the nanoparticle surfaces, guiding their growth (Samrot et al., 2021).

Clumping / Aggregation: The powdered nature of the sample likely contributes to the observed clumping. Drying processes during sample preparation can lead nanoparticles to

aggregate together, especially when biomolecules (from the bacterial synthesis) are present as stabilizing agents (Kalishwaralal et al., 2016).

Size: The 264 nm size (**Fig.4.12C**) falls within a range that is typical for biosynthesized selenium nanoparticles. It should be noted that biosynthesis methods often offer less precise control over size compared to purely chemical synthesis techniques. However, factors such as the specific type of bacteria, growth conditions, and the time of biosynthesis can significantly influence particle size. (Chandramohan et al., 2019)**.**

4.4.4. FTIR analysis

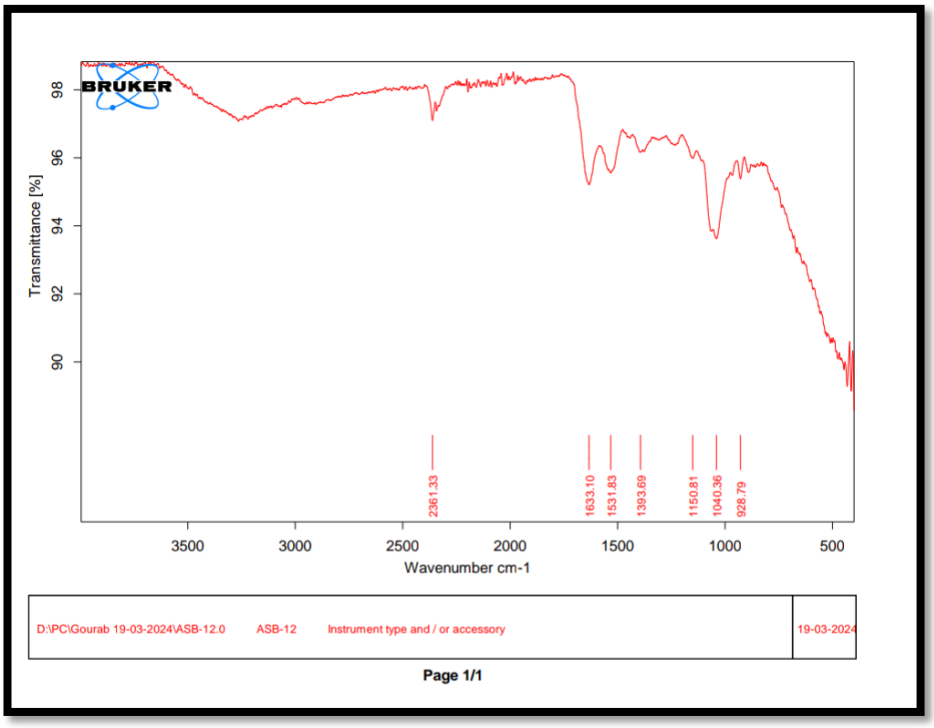

Fig.4.13. Fourier-transform infrared spectrum of powdered selenium nanoparticles synthesized from ASB12

FTIR data was collected. This data displayed peaks at 2361cm^{-1}, 1633cm^{-1}, 1531cm^{-1}, 1393cm^{-1}, 1150cm^{-1}, 1040cm^{-1} and 928cm^{-1}.

2361 cm^{-1}: This peak indicated asymmetric stretching vibrations of C-H bonds or O-H bonds. This could suggest the presence of residual organic compounds or possibly carboxylic acid groups on the surface of the nanoparticles.

1633 cm^{-1} and 1531 cm^{-1}: These robust peaks were highly indicative of proteins, specifically amide I (typically around 1650 cm^{-1}) and amide II (around 1540 cm^{-1}) bands. The presence of these bands strongly suggested that the proteins from halophilic bacteria were acting as capping and stabilizing agents on the surfaces of the nanoparticles.

1393 cm^{-1}: This peak corresponded to the symmetric bending of C-H groups, further supporting the presence of organic components associated with the SeNPs.

1150 cm^{-1} and 1040 cm^{-1}: These peaks align relatively well with the region characteristic of C-O stretching vibrations found in carbohydrates and polysaccharides. The halophilic bacteria might produce exopolysaccharides that participate in the formation and stabilization of the SeNPs (W. Zhang et al., 2011).

928 cm^{-1}: This peak may potentially indicate O-H bending vibrations, which could originate from alcohols, carboxylic acids, or hydroxyl groups present in biomolecules. So, this FTIR spectra has showed that there are various functional groups and capping biomolecules are present at the surface of selenium nanoparticles. This is suggestive of proteins and polysaccharides that might have successfully facilitated the synthesis of selenium nanoparticles. These biomolecules are also known to possibly increase the stability of selenium nanoparticles for long-term storage (Mellinas et al., 2019).

4.4.5. EDX analysis

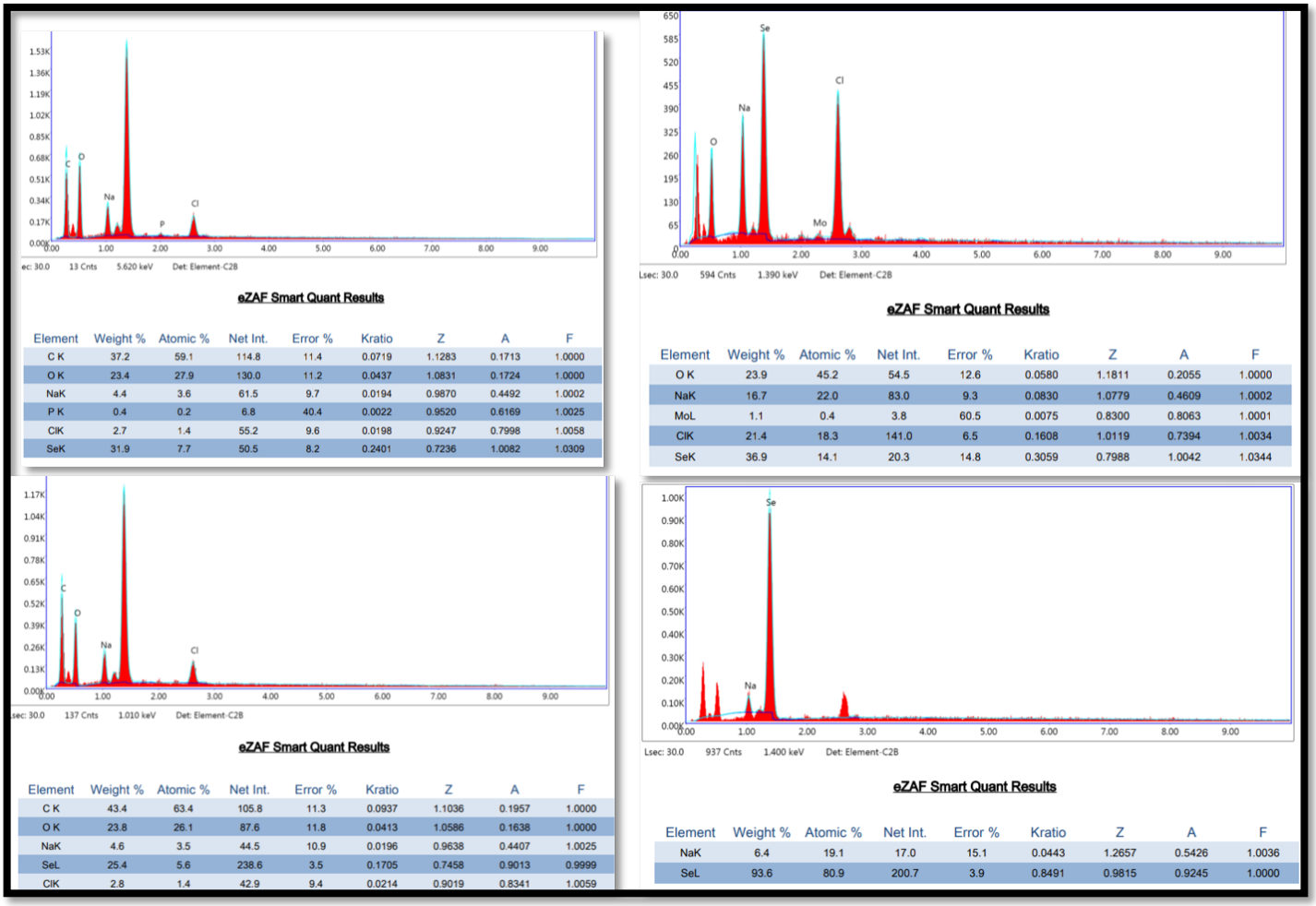

Fig. 4.14. EDX profile image of selenium nanoparticles synthesized from ASB12

The high abundance of selenium in the EDX data provides compelling evidence that the synthesis of SeNPs has been successful. This finding aligns with the objective of the biosynthesis process.

Carbon and Oxygen Traces: The presence of carbon and oxygen signals corroborates the FTIR results, which suggested the association of organic molecules with the SeNPs. These elements are likely to have originated from biomolecules, such as proteins and polysaccharides,

produced by halophilic bacteria. These biomolecules act as capping and stabilizing agents for the nanoparticles.

Sodium and Chlorine: The detection of sodium (Na) and chlorine (Cl) is to be expected, given that salt was used in the growth medium for the halophilic bacteria. These elements may exist as residual salts or may have been incorporated to some extent within the biosynthesized SeNPs. The appearance of trace molybdenum is noteworthy. Its presence could be attributed to accidental contamination during sample handling or preparation for EDX analysis.

Overall, the EDX data corroborates the interpretation derived from FTIR. The combination of these techniques provides a clear picture: the synthesis of SeNPs from halophilic bacteria has been successfully demonstrated, and these nanoparticles are stabilized by a coating of organic biomolecules derived from the bacteria themselves.

4.5. Application

4.5.1. DPPH test

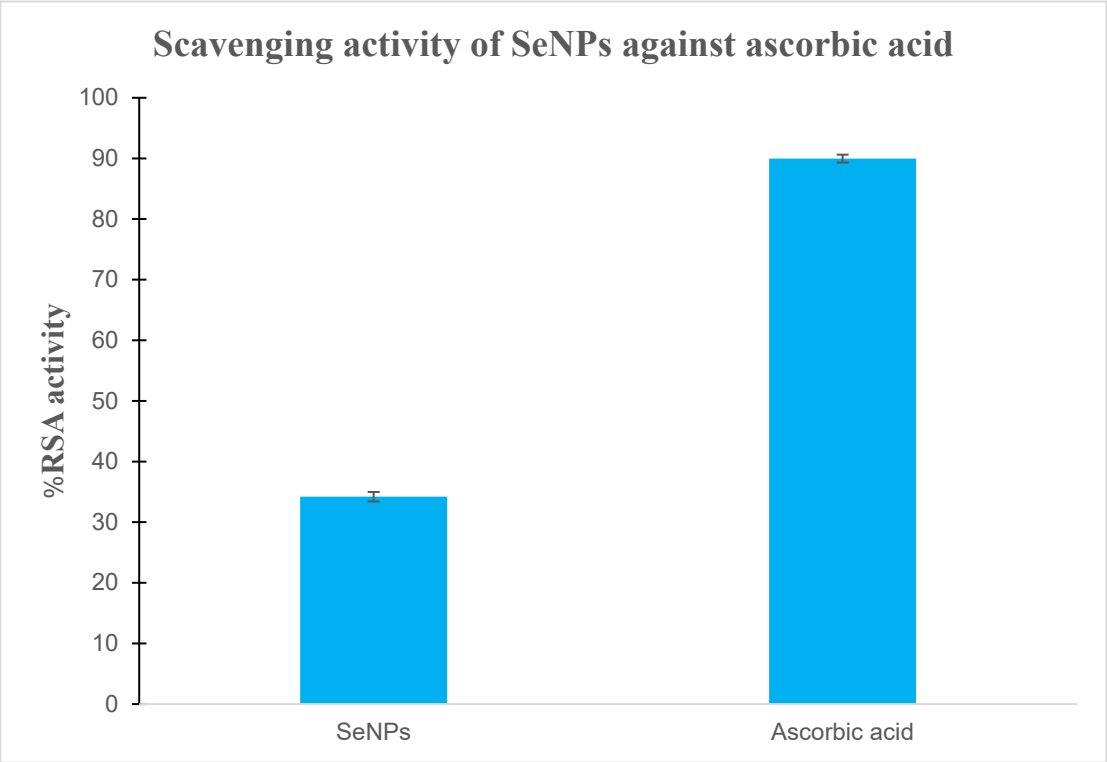

Fig. 4.15. The radical scavenging activity of synthesized selenium nanoparticles against ascorbic acid.

Selenium nanoparticles exhibit radical scavenging activity of 34%, while the established antioxidant, ascorbic acid, demonstrates 90% scavenging activity under the same experimental conditions. This data suggests that selenium nanoparticles possess antioxidant properties, although their radical scavenging capacity appears lower than that of ascorbic acid.

Antioxidants are molecules that counteract the damaging effects of free radicals, which are unstable molecules that can cause cellular damage and contribute to various diseases. Antioxidants can neutralize free radicals by donating electrons or hydrogen atoms, rendering

them less harmful. The percentage values indicate the relative ability of selenium nanoparticles and ascorbic acid to scavenge the specific free radical used in the assay. Ascorbic acid demonstrates a superior ability in this experimental context.

4.5.2. Antagonistic activity of selenium nanoparticles

Fig.4.16. Antimicrobial activity of the biologically synthesized SeNPs

Table 3: Zone of inhibition of microbial growth by control and SeNPs

Sample plate	Control zone (mm)	Test zone (mm)	Difference (mm)
MHA plate - 1	11	13	2
MHA plate - 2	10	12	2

The above table indicates that the sample containing selenium nanoparticles dispersed in methanol demonstrated a greater zone of clearance than the control containing methanol. This

suggests that SeNPs inhibit the growth of *Candida albicans*. The test was also conducted on *E. coli* and *Klebsiella pneumoniae*, but no visual difference in the zone was observed. It can be inferred that biogenically produced SeNPs need to be further purified to be effective against *E. coli* and *Klebsiella pneumoniae*, as compared to that against *Candida albicans*.

CHAPTER – 5

SUMMARY

This dissertation work investigates the biosynthesis of selenium nanoparticles (SeNPs) through the utilization of halophilic bacteria. The use of these extremophilic microorganisms presents a novel approach to the green synthesis of SeNPs, potentially offering nanoparticles with unique properties adapted from the bacteria's salt-tolerant nature. Comprehensive characterization employing techniques like XRD, Raman spectroscopy, UV-vis spectroscopy, SEM, EDX, and FTIR meticulously verified the formation of SeNPs. These analyses provided detailed insights into the crystalline structure, vibrational modes, optical properties, morphology, elemental composition, and surface functional groups of the nanoparticles. The study demonstrated that the halophilic bacteria exhibited tolerance towards sodium selenite, a precursor for SeNP biosynthesis. This tolerance is crucial for efficient bioconversion of the selenium source and highlights the potential for optimising nanoparticle production. Biological assays revealed the promising therapeutic potential of the biosynthesized SeNPs. The DPPH assay demonstrated significant antioxidant activity, indicating the nanoparticles' ability to scavenge harmful free radicals. Additionally, antagonistic activity against Candida albicans was observed, suggesting potential antifungal applications.

In conclusion, this research establishes halophilic bacteria as viable candidates for eco-friendly SeNP synthesis. The resulting nanoparticles exhibit promising antioxidant and antifungal properties, warranting further exploration for applications in biomedical, pharmaceutical, and food-related fields. Future research will focus on optimizing the production yields of SeNPs, conducting in-depth investigations into the mechanisms underlying their biological activities, and expanding the scope of potential applications.

Future prospects

1. Purification of nanoparticles.

2. Phylogenetic analysis of the culture.

3. Pharmaceutical and technological applications of nanoparticles after purification could be explored.

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Appendix- I

Chemicals and Media Used

S. No.	Name of the Chemicals and media used	Company
1.	Magnesium Sulphate heptahydrate	HiMedia
2.	Potassium Chloride	HiMedia
3.	Sodium Chloride	HiMedia
4.	Calcium Chloride	HiMedia
5.	Glycerol	HiMedia
6.	Ethanol	Parisil
7.	Agar	HiMedia
8.	Agarose	HiMedia
9.	Mueller-Hinton agar	HiMedia
10.	Tryptone	HiMedia
11.	Yeast extract	HIMedia
12.	TAE	HiMedia

Preparation of Chemicals and Media

NTYE agar media – For 1000 ml

$MgSO_4.7H_2O$ – 20.0 gm

KCl – 5.0 gm

$CaCl_2$ – 0.2 gm

Tryptone – 5.0 m

Yeast extract – 3.0 gm

Crude salt – 250 gm

Distilled Water – 1000.0 ml

Adjust pH to 6.5-7 using 1M NaOH

For solid media (Agar) 20.0 gm

Digest for 30 minutes, sterilize at 121oC and 15 lbs. pressure for 20 minutes

Agarose gel –

0.4 gm of agarose powder is added in 50 ml 1X TAE buffer. Microwaved for 2 min and after slight cool down of temperature EtBr is added. After that it is poured.

Mueller-Hinton agar –

2.1 gm is dissolved in 100 ml distilled water.

Appendix- III

List of the kits used

S. No.	Name of the kit	Company
1.	Gram Stains- kit K001- KT	HiMedia
2.	HipurA® Bacterial Genomic DNA Purification Kit MB505	HiMedia- HiGenoMB

Appendix- IV

List of Instruments Used

S. No.	List of instruments
1.	Light table
2.	pH meter
3.	Laminar Air Flow
4.	Compound Microscope
5.	Shaker incubator 30°C
6.	Water bath
7.	Weighing balance
8.	Autoclave
9.	Spectrophotometer (UV vis spec mini-1240)
10.	Thermal cycler
11.	Carl Zeiss Scanning Electron Microscope
12.	MRC LTD. ultasonicator
13.	Thermo scientific centrifuge
14.	LAB-i-FUGE ultracentrifuge