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# Extracellular Synthesis of Zinc Oxide Nanoparticles Using Thermo-Halotolerant *Aeribacillus pallidus* strain SJP 27: Characterization and Antibacterial Potential

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The current work reports the extracellular synthesis of zinc oxide (ZnO) nanoparticles (NPs) using the bacterial isolate *Aeribacillus pallidus* strain SJP 27 (Accession No. MW148443) from soil sample of arid and semi-arid regions of the Great Indian Thar desert. Bacterial cells were grown overnight at 60 °C incorporating a halo-tolerance of 5 % w/v NaCl. Physiochemical characterization of ZnO NPs were carried out using UV-Visible spectroscopy (UV-Vis), Fourier transform infrared spectroscopy (FTIR) and Scanning electron microscopy (SEM). The antimicrobial activity of synthesized ZnO NPs was confirmed by minimum inhibitory concentration (MIC) against *Escherichia coli* (8 mg/ml) and *Staphylococcus aureus* (4 mg/ml). The present study encourages the use of bacterial isolates for the extracellular synthesis of ZnO NPs. To the best of our knowledge, this is the first ever reported study of a thermo-halotolerant, *Aeribacillus pallidus* for extracellular synthesis of ZnO NPs in particular.

**Keywords:** *Aeribacillus pallidus,* Thermo halotolerant, Zinc oxide nanoparticles, FTIR, Energy band gap and extracellular synthesis.

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## 1. INTRODUCTION

Over the last decade, nanotechnology has revolutionized every field of applied sciences [1]. In recent times, the process of green synthesis has further emerged as an alternate to physical and chemical methods of synthesis and has received recognition due to its eco-friendly nature [2]. ZnO being a biocompatible material, has attracted a significant attention not only of materials researchers, but of the nanobiotechnologists as well. Variety of applications like antimicrobial, anti-cancer, bioremediation, biomineralization, bioleaching, and biocorrosion etc. of zinc oxide nanoparticles (NPs) derived by extracellular methods have been reported [3-5]. There is limited evidence of effective ZnO nanoparticle synthesis using plants and microbes. Among methods of microbial synthesis, two main types can be identified intra- and extracellular methods. Intracellular production involves bacteria biomass for the nanocomposite formation, while the extracellular approach excludes microbial cells and uses supernatant rich in biologically active compounds that acts as a strong reducing agent [6]. Green synthesis of NPs from plant source has gained momentum in the recent past. But not all-natural products are non-toxic and even some microorganisms are pathogenic, which constitute the biggest disadvantage for such methods. Microbial agents contain certain enzymes and small molecule that tend to reduce the metal ions into metal NPs. However only a few microbes have the potential to synthesize ZnO NPs. More potential microbes for the synthesis of ZnO NPs must therefore be explored.

In the present study, we have demonstrated the use of the supernatant culture of *Aeribacillus pallidus* for the synthesis of ZnO NPs. Aeribacillus pallidus are gram positive rods, aerobic, motile and endospore forming bacteria, which grow at high temperature of 60 °C and a salt tolerance of 5 % (w/v). This strain also produces innovative exopolysaccharides and flagellin protein that has various advantages [7, 8].

## 2. METHODOLOGY

### 2.1 Sampling, Isolation and Molecular Identification of Bacterial Strain

The bacterial strain, samples were collected from the arid and semi-arid regions of Western India. The samples were quickly transferred to the laboratory for isolation and identification of the bacterial strain. Standard protocols were used for isolation of the strains.

### 2.2 Study of Phenotypic and Biochemical Characterization of the Strain SJP 27

The suspensions of the soil samples were inoculated on nutrient agar (NA), Luria-Bertani broth (LB) by streaking and were incubated overnight at different temperatures. The bacterial strains were then isolated from the soil samples. Basic biochemical tests were performed like Grams staining, shape, colour, endospore, motility test, starch hydrolysis, casein hydrolysis, citrase utilization, indole test, catalase and deaminase test. To test for thermo-halotolerance, growth was tested over temperatures as high as 65 °C and a salt concentration up to 5 % w/v NaCl [9].

### 2.3 16S rRNA Gene Amplification, Sequencing and Phylogenetic Analysis

The molecular characterization of the bacterial isolate SJP27 was carried out using 16S rRNA. DNA was isolated from the pure isolated culture and its quality was evaluated on 1.2 % Agarose Gel; a single band of high-molecular weight DNA has been observed. Frag-

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ment of 16S rDNA gene was amplified by 27F and 1492R primers. A single discrete PCR amplicon band of approximately 1500 bp was observed when resolved on agarose gel. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI GenBank database[10]. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program ClustalW. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7 [11].

### 2.4 Phylogenetic Analysis

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [12]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [13]. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 11 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1445 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [11].

### 2.5 Synthesis of ZnO NPs and Optimization

The selected bacterial isolates were grown in laboratory condition in 500 ml Erlenmeyer flasks containing 100 ml LB medium and incubated in rotatory shaker overnight, with 120 rpm at a temperature of 60 °C and 65 % humidity for 24 h to collect the cell free supernatant of bacteria for extra cellular biosynthesis of ZnO NPs. The supernatant was collected by centrifugation at 10000 rpm for 10 min. 100 ml of Zinc acetate solution (1 mM) was mixed with cell free supernatant and a rigorous magnetic stirring was performed at room temperature for 24 h. The synthesis was monitored for a change in the color of the culture medium by visual inspection. The aqueous culture solution was further kept for drying in oven at 80 °C. The dried sample was crushed in sterile mortar until it was smooth and uniform. Annealing of dried sample was done at 700 °C for 5 h. The dried sample powder was kept in a desiccator at room temperature for further analysis [6]. We have taken control as ZnO nanoparticles which is prepared by chemical method and biosynthesized NPs as the test sample which is obtained by *Aeribacillus pallidus* SJP 27 bacterial strain (thermo halotolerant).

### 2.6 Characterization of ZnO NPs

The transmittance spectra of the resultant ZnO NPs were analyzed using a UV-Vis spectrophotometer (Shimadzu UV2600) with a wavelength ranges from 320-480 nm. The chemical composition of ZnO NPs, and SJP 27 treated ZnO NPs were studied by FTIR examination. The ZnO NPs and SJP 27 treated with ZnO NPs was finely ground and dispersed in double distilled water before performing the FTIR analysis. The FTIR spectrum was recorded in 2000-500 $cm^{-1}$ range using FTIR system (Bruker alpha). Field Emission Scanning electron microscopy (FESEM) (JEOL 7610F Plus) was used to image and evaluate the morphology and size of the ZnO NPs.

### 2.7 Evaluation of Antibacterial Activity of Biosynthesized ZnO NPs (MIC)

The bacterial strains were cultured on Luria Bertani (LB) broth and stored at – 80 °C in 30 % glycerol. The minimal inhibitory concentration (MIC) of ZnO and biosynthesized NPs was determined against Gram-positive (S. aureus) and Gram-negative bacteria (E. coli) by disc diffusion method. Positive and negative controls were included as well.

## 3. RESULTS AND DISCUSSION

### 3.1 Biochemical and Morphological Characterization of Bacterial Strain SJP 27

The isolated bacteria identified as SJP 27 were grown on nutrient agar (NA) and Luria-Bertani broth (LB). The bacterial isolates were Grams positive rods, aerobic, endospore forming, motile and cream in color. All the essential biochemical tests were performed, and the strain was tested negative for amylase, caseinase and citrase activity but showed activity for tryptophanase, catalase and deaminase. The G + C content of the strain was 56 %. The bacterial growth was determined at various temperatures viz. 37, 45, 50, 60 and 65 °C and an optimum temperature of 60 °C for bacterial growth was achieved. The salt, sodium chloride (NaCl), concentration was tested from 0-5 % (w/v) on nutrient agar plates. The optimum pH range for growth was also determined.

### 3.2 Molecular Characterization of Bacterial Strain SJP 27

The extraction of genomic DNA of isolated pure cultures from soil sample SJP 27 was carried out and quality was checked on 1.2 % agarose gel. A single band of high-molecular weight DNA was observed. Isolated DNA from pure culture SJP 27, was amplified with 16S rRNA Specific Primer (8Fand 1492R) using Veriti® 96 well Thermal Cycler (Model No. 9902). Single discrete band of 1445 bp on 1.2 % agarose gel was observed and shown in Fig. 1.

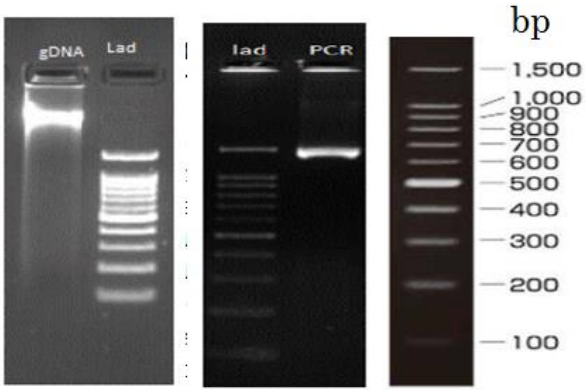


**Fig. 1 –** (a) genomic DNA, (b) 16s DNA amplicon, and (c) DNA ladder

### 3.3 Phylogenetic analysis

Phylogenetic analysis was carried out using neighbour joining method and SJP 27 showed 98 % similarity with Aeribacillus pallidus, strain: DSM 3670T based on nucleotide homology (Fig. 2), Contributing towards the research on Aeribacillus pallidus, which is isolated mainly from hot water springs, deserts, marsh lands and lands contaminated from industrial wastewater [14, 15]. The 16S rDNA sequence isolated from SJP 27 was submitted to NCBI (**MW148443**).

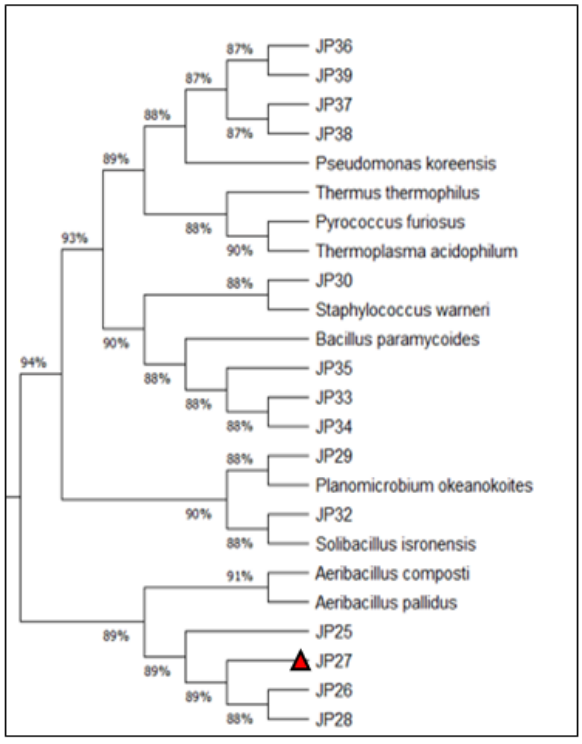


**Fig. 2 –** Evolutionary relationship based on 16S rDNA gene sequences. Strain SJP 27 (**MW148443**) is indicated by red triangle

### 3.4 FTIR Spectra

FTIR offers an impression to the vibrational and rotational modes of the existing molecules in the samples. It is also helping to identify the functional groups possible in the reduction and stabilization of ZnO NPs. The FTIR spectra of controls (ZnO) and SJP 27 are shown in Fig. 3. The control (ZnO) sample has a clear absorption band at 543 $cm^{-1}$ which corresponds to the stretching of Zn–O bond in the tetrahedral coordination [16]. This confirms the formation of ZnO. Other absorption bands are also visible at 602, 1031, 1330, 1407, 1466, 1525 and 1693 $cm^{-1}$ in Fig. 3. The feeble bands close to 600 $cm^{-1}$ are generally assigned to the stretching vibrations of Zn–O bonds in octahedral coordination. Remaining other absorption bands are correlated to the different vibrational states of carbon-based bonds. It is reported that C=C stretching, C–O stretching, COO–Zn, C=O bond respectively give rise to the absorption bands at 1030, 1407, 1466, 1525 and 1693 $cm^{-1}$. As the control (ZnO) samples synthesis is via the route of different organic vehicles like ethanol and propanol and zinc acetate precursor, the presence of such carbon-based absorption bands is plausible. SJP 27 sample notices a reduction in the strength of each absorption band as compared to the control (ZnO) sample. Reduction of the band at 543 $cm^{-1}$ and slight rise at 602 $cm^{-1}$ indicate that ZnO tetrahedral coordination weakens and the octahedral one strengthens due to the involvement of bacterial strain with the synthesis of ZnO. Another, absorption band assigned to COO–Zn bond also observes a strong reduction due to bacterial infusion. Here it can be concluded that bacterial strain affects the Zn based bonds significantly in NPs.

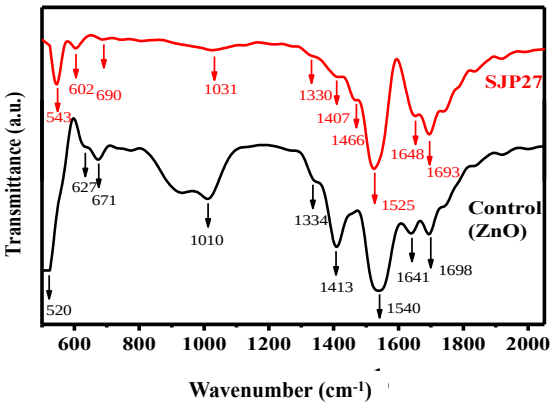


**Fig. 3** – FTIR spectra of ZnO synthesized through chemical and bio-route respectively

### 3.5 UV-Visible Spectra

UV-Vis transmittance spectra as presented in Fig. 4 shows a clear red shift in the absorption dip, from control (ZnO) to SJP 27. The control (ZnO) sample has absorption dip at 373 nm (3.324 eV) which occurs due to the band-to-band transition indicating the corresponding energy to be the band gap. The bacterial strain has been found to reduce the band gap to 3.245 eV (383 nm) eventually triggering a red-shift of the absorption dip. It will be discussed in the further section of FESEM that the particle size enhances due to the inclusion of bacterial strain in the synthesis process. The quantum confinement effects establish a fact that reduction of particle size causes a blue shift of the band gap in ZnO [17]. Here, reverse is happening, so

the red shift is justified following the identical reason of a quantum confinement effects.

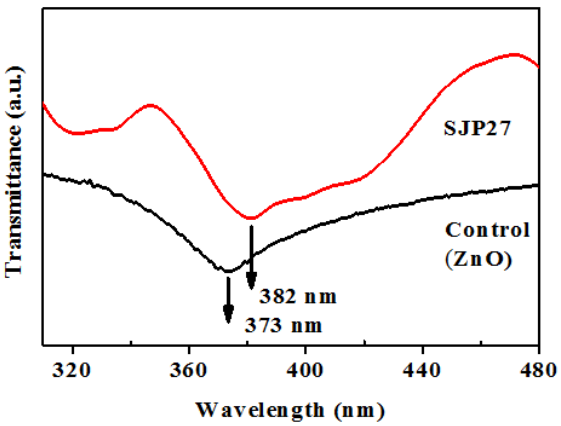


**Fig. 4** – (a) and (b) transmittance spectra of ZnO synthesized through chemical and biosynthesis, respectively

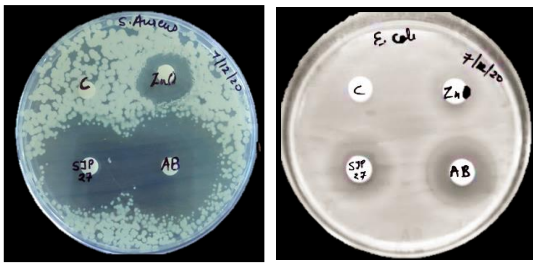


**Fig. 5** – Antimicrobial activity of control (ZnO) and biosynthesized SJP 27, respectively

### 3.6 Evaluation of Antibacterial Activity of Biosynthesized ZnO NPs (MIC)

In our study, the anti-bacterial activity of control (ZnO) and biosynthesized NPs were tested against E. coli and S. aureus was determined by disc diffusion method. The values were 8 and 4 mg/mL (Fig. 5). It was found that the biosynthesized NPs exhibit significant activity by inhibiting the bacterial growth.

### 3.7 Morphological Properties

The morphological images of ZnO NPs (control) recorded at different magnifications are presented in Fig. 6. Evenly distributed and more prominently hexagonal structures are evident there. The high magnification image of hexagonal structures is shown in the inset of Fig. 6b. The average size of the NPs appears to be 80 nm. However, the bacteria based ZnO NPs (SJP 27) have the size around 237 nm and mostly depict agglomeration, as shown in Fig. 7. It is found that high temperature synthesis via bacterial strain forms the agglomerates, which is the underlying reason for the development of larger size of nanoparticles in SJP 27 sample [18]. The size distribution ranges from 55nm to 112 nm for control (ZnO) and biosynthesized nanoparticles from 200 nm to 262 nm SJP 27 is demonstrated and shown in Fig. 8.

## 3. CONCLUSIONS

The present study demonstrated the eco-friendly, facile and extracellular synthesis of ZnO NPs through a thermo-halotolerant bacteria *Aeribacillus pallidus,*

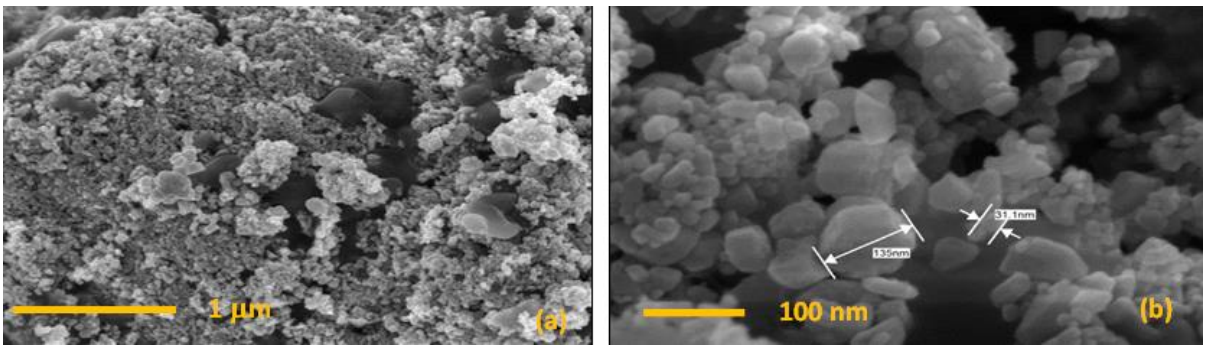


**Fig. 6** – SEM micrographs: (a), (b) ZnO synthesized through chemical route

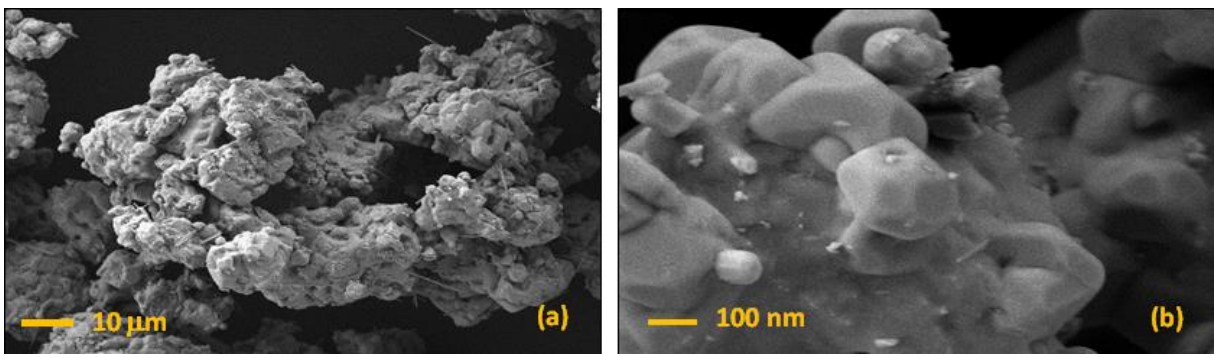


**Fig. 7** – SEM micrographs: (a), (b) ZnO synthesized through biosynthesis route

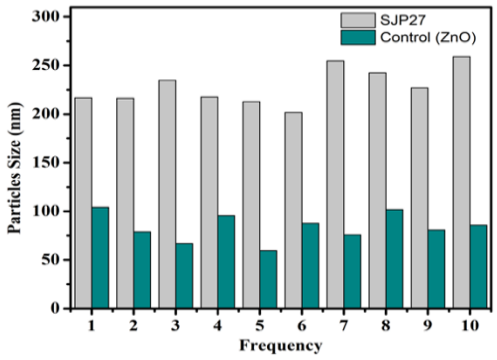


**Fig. 8** – Size distribution of control (ZnO) and SJP 27 NPs

*strain* SJP 27 in the size ranges from 200 nm to 262 nm. The synthesized NPs from bacterial strain are biologically active against the tested Grams positive and Grams negative strains. Therefore, our findings encourage to isolate and screen unexplored thermophiles, which could be considered for utilization in the biosynthesis of NPs for various potential biotechnological applications.

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## Позаклітинний синтез наночастинок оксиду цинку з використанням термогалотолерантного штаму *Aeribacillus pallidus* SJP 27: Характеристика та антибактеріальний потенціал

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В роботі повідомляється про позаклітинний синтез наночастинок (NPs) оксиду цинку (ZnO) з використанням бактеріального ізоляту *Aeribacillus pallidus* штаму SJP 27 (обліковий номер MW148443) із зразка ґрунту посушливих і напівпосушливих районів великої індійської пустелі Тар. Бактеріальні клітини вирощували протягом ночі при 60 °C, включаючи галотолерантність 5 % w/v NaCl. Фізико-хімічні характеристики ZnO NPs вивчалися за допомогою УФ-видимої спектроскопії (UV-Vis), інфрачервоної спектроскопії з перетворенням Фур'є (FTIR) та скануючої електронної мікроскопії (SEM). Антимікробна активність синтезованих ZnO NPs була підтверджена мінімальною інгібуючою концентрацією кишкової палички *Escherichia coli* (8 мг/мл) та золотистого стафілокока *Staphylococcus aureus* (4 мг/мл). Це дослідження стимулює використання бактеріальних ізолятів для позаклітинного синтезу ZnO NPs. Наскільки нам відомо, це перше з коли-небудь опублікованих досліджень термогалотолеранту *Aeribacillus pallidus* для позаклітинного синтезу, зокрема, ZnO NPs.

**Ключові слова:** *Aeribacillus pallidus,* Термогалотолерантний, Наночастинки оксиду цинку, FTIR, Ширина забороненої зони, Позаклітинний синтез.