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RESEARCH ARTICLE

BIOSYNTHESIS OF SILVER NANO PARTICLES FROM ENDOPHYTIC BACTERIA, ANTIBACTERIAL ACTIVITY AND MOLECULAR CHARACTERIZATION OF *Bacillus subtilis.*

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Abstract

This study highlights the ability of nitrate reducing *Bacillus subtilis* STBBP1 cell free extract used for the preparation of silver nanoparticles . The SEM graph revealed spherical shaped Nanoparticle in the range of 40-60mm. The antibacterial activity of silver Nanoparticles by *Bacillus* was investigated against various pathogenic organisms. Highest antibacterial activity was found in *Escherichia coli* (15mm) followed by *Streptococcus pyogenes* (11mm). The formation of silver Nanoparticles was monitored by UV-Vis spectra showed surface Plasmon resonance peak at 430 nanometer, SEM-EDX spectra showed the presence of element silver in pure form. FTIR proved the the presence of biomolecule responsible for the reduction of silver ions and X-ray diffraction analysis confirmed that the obtained silver Nanoparticles were in crystalline form. It showed an array of absorbance bands in 600cm -1. The molecular characteristic of *Bacillus subtilis* STBBP1 was evaluated by PCR amplification of 16srRNA and sequences were deposited in gene bank . This opens a new avenue of result were the Endophytic bacteria can also be used in the synthesis of Nanoparticles.

Introduction:-

Nanotechnology is the biosynthesis of Nanoparticles such as Nanosilver. Nanoparticles has dynamically developed as an important field of modern research with potentially effects in electronic and medicine.It isdefined as a research for the design, synthesis and manipulation of structure with dimension smaller than 100nm.(Mansuya *et al*,2010). Biologically synthesized silver Nanoparticles could have many applications in various fields such as solar energy absorption and intercalation material for electrical batteries, as optical receptors .Recently biosynthetic methods employing microorganisms such as bacteria, fungus and plant extract emerged as a simple and viable alternative to more complex chemical synthetic procedures to obtain Nanomaterials. (Pum and sleytr,1999).Many Nanoparticles come up but silver Nanoparticles play a major role in the field of Nanotechnology and Nanoparticles.(Ahmad *et al*,2005).Microbial endophytes are organisms which colonize living tissue of plants and live in a symbiotic relationship with the host. It is ubiquitous in all plant species on earth and contribute to the host plants by producing abundant metabolites that are harnessed for plant defense and endurance. Since they occupy unique biological niche.

Endophytes act as a novel bioactive products for explotation of Medicine, agriculture and industry. Screening of new endophytes for their functional role in a promising way to overcome the increasing threat of drug resistant strains of human and plant pathogen,(Strobel *et al*,2004). Nanoparticles produced by a biogenic enzymatic process is so superior than chemical methods. Hence Endophytic bacteria have the natural ability for degradation of xenobiotics or may act as a vector. (Kato, 2011).

Materials and methods:-

Isolation of bacteria:-

The marine soil was collected from Athirampattinam .1gm marine soil sample was taken for the isolation of bacteria by serial dilution spread plate technique.

Identification of bacteria:-

The colonies were identified based upon their morphological characteristics by differential staining technique. Motility test were performed by hanging drop technique .Various Biochemical tests was done to determine metabolic characteristics of an organism.

Production of biomass:-

The isolated colony was subcultured in Nutrient broth and incubated for 24-48 hours at 37 c. After 24 hours the biomass was centrifuged and supernatant was collected for the synthesis of Nanoparcticles.

Biosynthesis of silver Nanoparticles (Kalimuthu *et al,* 2008):-

The sample was added to the silver nitrate solution at 1mM concentration control(without silver nitrate).The reaction carried out in bright conditions for 24 hours.

Characterization of silver Nanoparticles:-

The bioreduction of Ag+ ions in solution was withdrawn at different time intervals and absorbance at 200-1100 nm using UV vis spectroscopy. The sample pellet was then subjected to FTIR spectra were recorded in the range of 4000-450 cm_1 at a resolution of 4 cm-1.Then dried bacterial culture were scanned on Scanning Electron microscope.(fig.2)

Antibacterial activity by agar well diffusion method:-

The silver Nanoparticles synthesized from *Bacillus subtilis* was tested for their antibacterial activity against *Enterobacter aerogenes ,Escherichia coli, Klebsiella pneumonia ,Streptococcus pyogenes*. Samples were poured into the wells and incubated at 35c for 18 hours and zones were measured.(Kim *et al*.,2007)

Molecular characterization of bacteria:-

Genomic DNA extraction was done. Then 16s rRNA gene fragments were amplified by PCR kit (Genei pvt Ltd, India) (Plate 2)..16srRNA gene sequences were obtained in this study aligned on Bioedit software and deposited in gene bank such as NCBI, DDBJ ,EMBL (Plate3). Phylogenetic analysis was constructed using Neighbour joining method.(Saitou and Nei,1987) and restriction sites were analysed by using NEB cutter program version2.0 tools in online.(Fig.1).

Results:-

In this present study bacteria was isolated from marine soil and identified by various biochemical test. Then silver Nanoparticles were formed by reduction of Ag+ into Aa0 with addition of *Bacillus subtilis* extract to the solution in 1mM AgNo3 (Plate 1).The SEM graph revealed relatively spherical shaped Nanoparticles with the diameter in the range of40-60nm.The antibacterial activity of AgNPs synthesized by *Bacillus subtilis* was investigated against various pathogenic organisms such as *Enterobacter aerogenes,Escherichia coli, Klebsiella pneumonia ,Streptococcus pyogenes* and *Marinococcus halophiles*.The antibacterial activity was found against *Escherichia coli*(15mm) followed by *Streptococcus pyogenes* (1mm)(Table 1)..FTIR spectral analysis showed an array of absorbance band in 600cm-1 .The FTIR spectrum of *Bacillus subtilis* shows peaks at shown the presence of 780.45, 3370.61, 2918.40, 2399.62, 1272.16, 1609.22, 1377.16,1252.42,1041.49,537.56 respectively.(Table 2&Figure 2).The formation of silver Nanoparticles was monitored by UV-visible absorption spectra at 200 and 600nm where the band was clearly detected at 430nm.

Discussion:-

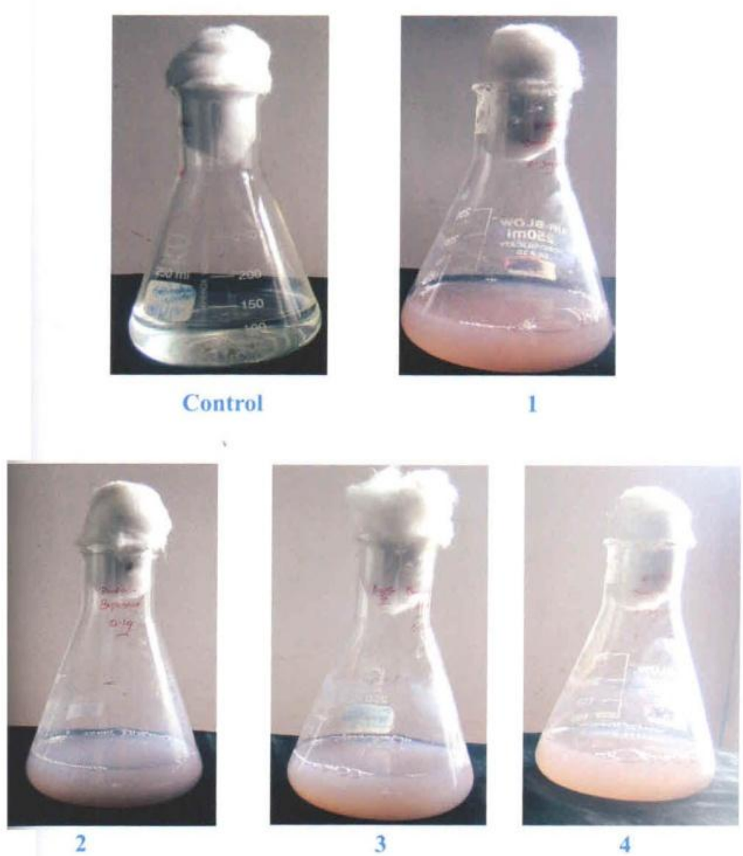
Silver Nanoparticles have been synthesized using *Bacillus subtilis* supertanant alone, In the presence of Glucose it increased the synthesis of silver nitrate particles. Similar studies were conducted by Travan *et al*,2009 suggested that the solution turned from whitish to brown after 24 hours formation of silver Nanoparticles.

The enzyme involved may be the nitrate reductase present in *Bacillus subtilis* then the solution colour changed to brownish color. Silambarasan and Abhram 2012 investigated that the AFM gave the the shape and 62.8 nm size of silver nanoparticles produced by *Bacillus subtilis*. Kalimoothu *et al* 2008 investigated that formation of nanoparticle is observed by the color change from pale to white from brown and it further confirmed by UV vis spectra at around 432nm.the SEM micrographs recorded showed comparatively spherical nanoparticles were observed.

In our study this change led to the quest of novel antimicrobials from various sources. Silver and its derivatives are widely used in medicine for a long time in the treatment of infections. Thus it is fitting to inveigate the antibacterial activity of synthesized nanoparticles.

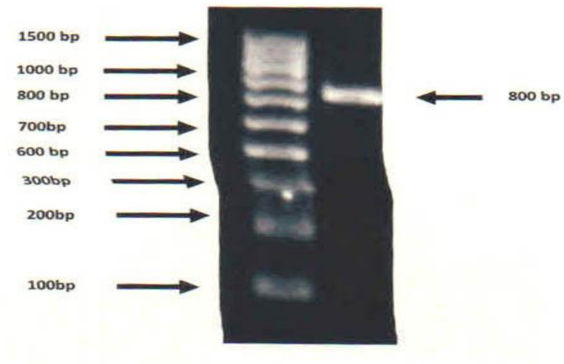
Souza et al 2004 the extracellular synthesis of nanoparticles by *Klebsiella pneumonia, E.coli, Enterobacter clocaca*. Biologically synthesized silver nanoparticles could have many applications in areas such as nonlinear optics spectrally selective coatings for electrical batteries, as optical receptors, catalyst in chemical reactions and as antibacterial capacity.

Plate 1 – Synthesis of silver Nanoparticles from Endophytic bacteria



1 - $0.1gAgNO_3$ + *Bacillus subtilis*
2 - $0.2gAgNO_3$ + *Bacillus subtilis*
3 - $0.3gAgNO_3$ + *Bacillus subtilis*
4 - $0.4gAgNO_3$ + *Bacillus subtilis*

Plate 2 – Isolation of Genomic DNA



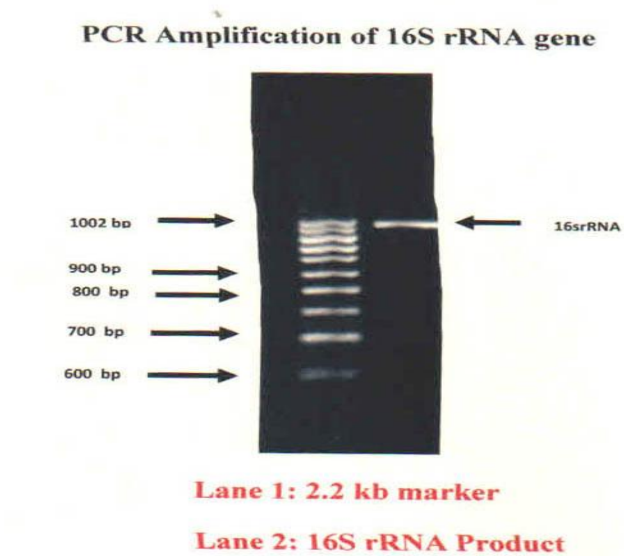


Fig 1 – Phylogenetic tree analysis

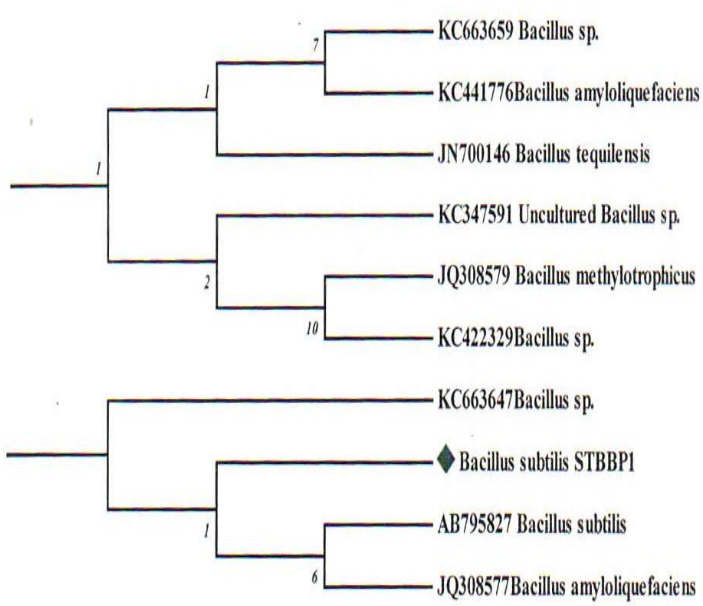


Fig 2 - Detection of various functional groups by FTIR

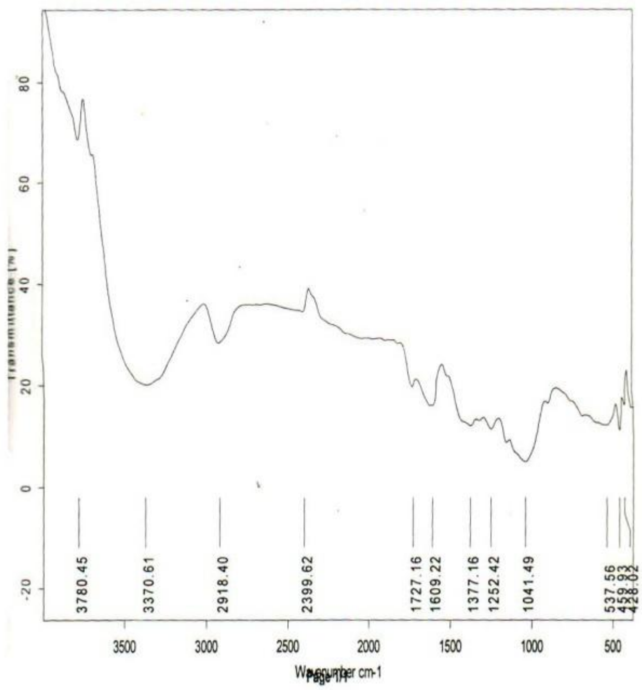


Table 1 – Antibacterial activity of synthesis of silver nano particle

S. No	Bacterial cultures	Different concentration of $AgNO_3$ *Bacillus subtilis* (Zone of inhibition in mm)				
		0.1g	0.2g	0.3g	Pellet (B.C)	Control
1.	*Enterobacter aerogenes*	10	10	12	13	-
2.	*Escherichia coli*	7	10	15	5	-
3.	*Klebsiella pneumoniae*	5	7	5	5	-
4.	*Marinococcus halophilus*	5	7	7	9	-
5.	*Staphylococcus pyogenes*	5	10	11	5	-

Table 2 – Detection of various functional groups of silver nano particle

S. No	Functional group assignment	Group frequency cm^{-1} of the sample
1.	3780.45	N-H stretching vibrations primary fee two bands
2.	3370.61	Secondary, free one band imines (=N-H); one band Amine salts
3.	2918.40	C-H stretching
4.	2399.62	Isocyanides
5.	1272.16	Unsaturated; vinyl ester type
6.	1609.22	Amines primary dilute solutions
7.	1377.16	$C-NO_2$ nitro compounds aromatic
8.	1252.42	C-vibrations, Aromatic primary
9.	1041.49	O-H bending & C-O stretching vibrations primary alcohols
10.	537.56	Halogen compounds C-X stretching vibrations C-Br

Plate 3 – 16srRNA gene sequences of *Bacillus subtilis*

```
LOCUS       Bacillus_subtilis       1002 bp    DNA     linear   BCT 30-MAR-2013
DEFINITION  strain STBBP1 16S ribosomal RNA gene, partial sequence.
ACCESSION   ST631640
VERSION :
KEYWORDS    .
SOURCE      Bacillus subtilis
  ORGANISM  Bacillus subtilis
            Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1002)
  AUTHORS   Srividhya, M., Tamil Selvi,S., Bharathidasan,R., Bhuwaneswari, S. and
            Prabakaran, M.
  TITLE     Silver nano particles synthesis from endophytic Bacteria
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1002)
  AUTHORS   Srividhya,M., Tamil Selvi, S., Bharathidasan,R., Bhuwaneswari,S. and
            Prabakaran,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-MAR-2013) Department of Microbiology, Shrimati Indira
            Gandhi College, Trichy, Trichy, Tamilnadu 620002, India
COMMENT     Bankit Comment: ALT EMAIL:mprabu_sgbra@yahoo.com.
            Bankit Comment: TOTAL # OF SEQS:1.

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            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     /strain="STBBP1"
                     /db_xref="taxon:1423"
                     /note="[cultured bacterial source]"
     rRNA            <1..>1002
                     /product="16S ribosomal RNA"
BASE COUNT      247 a    229 c    323 g    203 t
ORIGIN
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       61 gctaccactt acagatggac ccgcggcgca ttagctagtt ggtgaggtaa cggctcacca
      121 aggcgacgat gcgtagccga cctgagaggg tgatcggcca cactgggact gagacacggc
      181 ccagactcct acgggaggca gcagtaggga atcttccgca atggacgaaa gtctgacgga
      241 gcaacgccgc gtgagtgatg aaggttttcg gatcgtaaag ctctgttgtt agggaagaac
      301 aagtgccgtt caaatagggc ggcaccttga cggtacctaa ccagaaagcc acggctaact
      361 agtgccagca gccgcggtaa tacgtaggtg gcaagcgttg tccggaatta ttgggcgtaa
      421 agggctcgca ggcggtttct taagtctgat gtgaaagccc ccggctcaac cggggagggt
      481 cattggaaac tggggaactt gagtgcagaa gaggagagtg gaattccacg tgtagcggtg
      541 aaatgcgtag agatgtggag gaacaccagt ggcgaaggcg actctctggt ctgtaactga
      601 cgctgaggag cgaaagcgtg gggagcgaac aggattagat accctggtag tccacgccgt
      661 aaacgatgag tgctaagtgt tagggggttt ccgcccctta gtgctgcagc taacgcatta
      721 agcactccgc ctggggagta cggtcgcaag actgaaactc aaaggaattg acgggggccc
      781 gcacaagcgg tggagcatgt ggtttaattc gaagcaacgc gaagaacctt accaggtctt
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      901 gcatggttgt cgtcagctcg tgtcgtgaga tgttgggtta agtcccgcaa cgagcgcaac
      961 ccttgatctt agttgccagc attcagttgg gcactctaag gt
//
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Conclusion:-

Bacillus subtilis displayed the ability to reduce Ag+ to Ag0, with the size of Nanoparticle ranging between 40-60 nm that was found to be slightly aggregated drug resistance of microorganisms to chemical antibacterial agents is on the rise and their use in medical applications is being limited.

Therefore on alternative way to overcome the drug resistance of various microorganisms is need desperately. The limited usefulness of silver and its derivatives against microorganisms due to their interfering effects of salts is surpassed by the use of Ag Nanoparticles that are proving to be effective antibacterial agents.

This opens a new avenue of research were the Endophytic bacteria can also be used in the synthesis of Nanoparticles.

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