International Journal of Advance Research in Science and Engineering
Vol. No.6, Special Issue (01), September 2017, BVCNSCS 2017
www.ijarse.com
IJARSE
ISSN 2319 - 8354

Comparative analysis of microbial Nitrate reductase structure using Bioinformatics

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Abstract: - Nanotechnology is a field that has been prominent in the recent years, making impact in all the spheres of human life. Nano silver (AgNp's), in terms of products, is one of the highly commercialized nanoparticle. Inspite of various attempts to study the mechanism of formation of the AgNp's, in a biological system, not much has been understood. The role of enzyme Nitrate reductase has been assumed in the synthesis of AgNp's. Present study attempted to deduce the possibilities of this enzyme as a means to synthesize the AgNp's. The role of Nitrate reductase is understood by analysing the commonality in the structure and functions. From the available protein data base sequences, some of the strains reported for AgNp's synthesis having this enzyme have been retrieved and has been subjected to sequence analysis.

The role of Nitrate reductase has been understood by performing Multiple Sequence Alignment (MSA) analysis with protein sequences within Bacillus genus and between Bacteria, Fungi and Algae. Protein analysis using Clustal Omega has revealed similarity within the same genus and among different microorganisms. From the cladogram obtained, it is evident that there is linearity in the origin of the nitrate reductase enzyme even among the different groups of microbes. Nitrate reductases among the gram negative have more commonality than gram positive bacteria. From the conserved domain analysis it could be concluded that catalytically active site of alpha subunit has its conserved sequences among the gram positive, gram negative, some thermophiles and archaebacteria. Conserved sequences that were revealed in the catalytic subunits and the percentage similarity analysis is instrumental in predicting the role of specific protein domains in synthesis of silver nano particles.

Key words:- Nitrate reductase, Silver Nanoparticles, Synthesis, Comparison, MSA, Clustal omega

I. INTRODUCTION

The green synthesis approach of silver nanoparticles (AgNp's) by the extracellular production is one of the most economical and simpler procedures. There have been successful attempts for biological synthesis of the AgNp's by using wide range of biomolecules. Among the different natural sources and living systems that have been identified, microbial synthesis delivers a striking diversity in the nature of the particles. As a result, wide ranges of microbes have been identified and are being instrumental in synthesizing AgNp's. With respect to other biological systems, microbes are easier to handle and economical for maintenance and propagation. Researchers have described the nature and properties of AgNp's that are produced either by intracellular or extracellular means .Extracellular productions involve fewer inputs for recovery process and gives scope of more possibilities for range of convenient conditions [(1)]. Whereas intracellular synthesis limits the alterations in conditions as there is a need for the optimal growth of the cells and further, specific procedures need to be employed for the recovery of nanoparticles from the medium containing microbes. Hence majority of the research is to upgrade the methods for extracellular AgNp's synthesis rather than intracellular. Understanding of mechanisms underlying extracellular formation of AgNp's can give greater insight for designing methods that can take up large scale production. Among the different molecules that are identified for reduction of the silver ion to nano silver, role of *nitrate reductase* has been proposed as one of the most significant [(1)].

Nitrate reductase is an integral enzyme in organisms like bacteria,

fungi, algae and in plants. The enzyme is a molybdenum associated protein and is sub-grouped as respiratory nitrate reductases, periplasmic nitrate reductases , assimilatory nitrate reductases . Two classes of assimilatory nitrate reductases have been found in bacteria: ferredoxin- or flavodoxin-dependent *Nas* and NADH-dependent *Nas*, the classification of which is based on the cofactor structure [6] . Majority of studies are done to decipher the role of this enzyme in nitrate reduction. In view of identifying the functional domains of the enzyme in nanosilver synthesis, the present study aims at comparing the similarities in the structure of protein that is enabling many microbes for this process. The comparison of the protein database sequences of this enzyme from various organisms can help in interpretation of the actual molecules or molecules with certain definite properties that help in AgNp's synthesis. In the current scenario of rapid genome and protein sequencing and availability of large amount of data, it would be manually impossible to curate and compare the molecules without the help of computational analysis. Hence it would be appropriate to initiate the comparison of the sequences already available in the database, for the microbes of a particular genus or species, rather than going for the specific studies on the isolated strains in the lab.

II. MATERIALS AND METHODS

i. Extracellular synthesis of AgNp's

Extracellular AgNP's production was screened by incubating the supernatant (250µl) with the filter sterilized 25 ml of 1mM $AgNO_3$ in a 50ml conical flask. Flasks were incubated in presence of light at room temperature for AgNp's synthesis against control which had only 1mM $AgNO_3$. Silver nano particles (AgNP's) produced by isolates were characterised by various standard recommended methods. The primary identification of the AgNp's synthesis was done by the visual colour change. The formation of nanosilver is confirmed by UV-Visible spectroscopy. The FTIR analysis has been done in order to figure out the different protein molecules that may have interacted.

ii. Characterization of AgNp's

In order to characterize the nanoparticles further, SEM images were captured and the morphological features of the particles were determined. The scanning data was obtained after 10 scans. The size and shape of the AgNP's was determined by Scanning Electron Microscopy using SEM S-3700 with accelerating voltage of 30000 Volts, emission current 82000nA and a magnification of 5000.

iii. Qualitative Detection of Nitrate Reductase

Experimentally the culture supernatant has been evaluated for the presence of Nitrate reductase enzyme. Bacillus and Fungal isolates which were characterized for the AgNp's synthesis, were tested for production of the Nitrate reductase by the standard protocol. Cultures were grown in the nitrate broth and the supernatants were tested for the enzyme action. Reagent A (Sulfanilic Acid) and Reagent B (Alpha Naphthalamine) were added into the incubated broth cultures.

iv. Collection of Data Set

From the available protein data base sequences, some of the strains reported for AgNp synthesis having this enzyme have been retrieved and has been subjected to sequence analysis. The enzyme FASTA sequences from the GEN BANK DATABASE was collected and aligned for the BLAST.

v. Multiple sequence alignment

Multiple Sequence Alignment (MSA) is done to align three or more biological sequences of protein or nucleic acid of generally similar length. From the output obtained , homology based studies and evolutionary relationships between sequences can be understood. Clustal Omega is licensed under the GNU Lesser General Public License. Source code as well as precompiled binaries for Linux, FreeBSD, Windows and Mac (Intel and PowerPC) are available at http://www.clustal.org. Clustal Omega is available as a command line program only, which uses GNU- style command line options, and also accepts ClustalW- style command options for backwards compatibility and easy integration into existing pipelines . (

Fabian seivers, Andreas Wilm et al.,2011).Multiple sequence alignment of the FASTA sequences of the Nitrate reductase enzyme among various Bacillus sps.that reported AgNp synthesis had been carried out with the tools in the https://www.ncbi.nlm.nih.gov.The information revealed would help to understand the variations of the enzyme within the same genus. An alignment data that is generated is a hypothesis of positional homology between the amino acids. The analysis has been done by CLUSTALO (Clustal omega) version 1.2.4. MSA has been done among the enzyme sequences within the *Bacillus sps.*(Table 1.) and another among bacteria, fungi and algae (Table2).

vi. Conserved Domain Database (CDD)

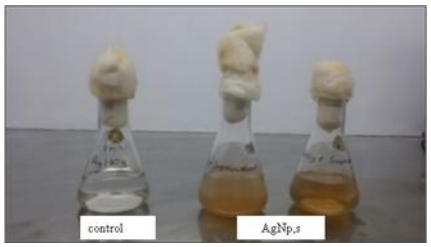
CDD is a resource that consists of a collection of annotated multiple sequence alignment models for domains and full-length proteins. These are available as position-specific score matrices (PSSMs) for fast identification of conserved domains in protein sequences. CDD content includes domains deposited in NCBI, which use 3D-structure information to provide insights into sequence/structure/function relationships. CDD also supports comparative analyses of protein families via conserved domain sequences, and a recent curation effort focuses on providing functional characterizations of a specific subfamily using SPARCLE. (Subfamily Protein Architecture Labeling Engine). (Aron Marchler, Yu Bo et al., 2017). As a response to protein queries submitted to CD-Search by the user , the display of pre-computed domain annotation, the content of the functional label assigned to the corresponding Subfamily Domain architecture(SDA) is shown on the results page. The functional labels are linked to summary pages, which display additional information about a subfamily domain architecture, including evidence for the name and functional label.(https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml)

III. RESULTS AND DISCUSSION

i. Extracellular synthesis of AgNp's

AgNp's production by the micro organisms was identified initially by a colour change in the production media.(.Fig1) AgNP's was confirmed by scanning the test solution at a wavelength range of 300-600nm in a UV-Visible spectrophotometer). FTIR Spectrum was recorded in the range of 4000-500cm^{-1} using a Fourier Transform Infrared Spectrophotometer. The stability of the nanoparticles was attributed to the proteins interacting with the particles in the solution. The FTIR analysis has been done in order to figure out the different protein molecules that may have interacted. FTIR analysis. 361.79, 1384.94, 1635.69, 2852.81, 3423.76, 3444.98 peaks signify the interaction of the groups C-N stretch, Aromatic groups, Primary and Secondary Amines, Aromatic stretch, Carboxylic acids respectively. These interactions of proteins result in the reduction of nanosilver. It has been already reported that the biological molecules are responsible for formation and stabilization of AgNP's in the aqueous medium(3).

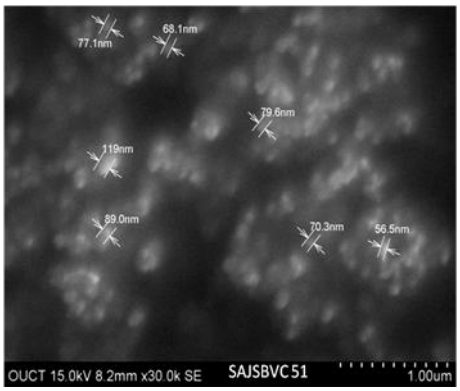
Fig:1Brown color indicating formation of AgNp'S



ii. Characterization of AgNp's

In order to characterize the nanoparticles further, SEM images were captured and the morphological features of the particles were determined. The SEM analysis revealed presence of spherical shaped AgNP's of different sizes ranging between 58.5nm to 119nm.(Fig:2)

Fig:2 SEM picture of AgNp's



iii. Qualitative Detection of Nitrate Reductase

Inspite of various attempts to study the mechanism of the formation of the AgNp in a biological system, not much has been understood. As the role of enzyme Nitrate reductase has been assumed in the synthesis of AgNp's, the present study tried to deduce the possibilities of this enzyme as a means to synthesize the AgNp's. Nitrate reductase test determines whether the microbe produces the enzyme nitrate reductase and nitrite reductase. The two enzymes catalyze two reactions involved in the following conversion. Experimentally the culture supernatant has been evaluated for the presence of Nitrate reductase enzyme. Bacillus and Fungal isolates which were characterized for the AgNp's synthesis were tested for production of the Nitrate reductase. When the bacteria producing Nitrate reductase is grown in the medium containing nitrate, the presence of the enzyme converts nitrate to nitrite. Nitrite formed is made to react with certain chemicals to yield a reddish pink coloured product.(Fig:3[4]. After qualitative confirmation of Nitrate reductase enzyme activity in the culture supernatant, extracellular enzyme was assayed quantitatively by Lowe and Evans method (1964) and the cell bound enzyme was evaluated by Birgit and Kenneth method (1997). Sample with enzyme activity were further checked on SDS PAGE[6]

Fig:3 Qualitative detection of Nitrate reductase

iv. Multiple sequence alignment

The role of Nitrate reductase is better understood by analysing the commonality in the structure and functions of this enzyme which is prevalent in many microbes. Multiple sequence analysis has been done by CLUSTAL O (Clustal omega) version 1.2.4.(Fig.4.) From multiple alignments it was understood that less than 50% identity exists in the enzyme sequence. But there is 100% similarity observed in the KJH58874.1 and AFL23265.1,(Fig 5) *Bacillus licheniformis* Patented sequence of nitrate reductase alpha subunit sequences. This signifies that the catalytic subunit of the enzyme has, highly conserved sequences.The cladogram(fig.6) depicts the evolutionary relationship between the alpha, beta and delta subunits sequences for analysis. The results obtained may be used as a spearhead to analyze the sequences of the isolated strains for the homology that can give the molecular conformation useful for AgNp's synthesis. From the multiple sequence alignment of 5 sequences (Table 2) that has been retrieved from protein database it has been possible to find percentage similarity (Fig 7) among the different microbial nitrate reductases across the genus. *Bacillus subtilis* a gram positive organism enzyme shows similarity of 53.05% with *Pseudomonas stutzeri*, 52.02% with *Escherichia coli* , i.e with an gram negative strains. However, bacillus enzyme had only 15.98% with

Aspergillus niger and 16.49% with *Chlamydomonas reinhardtii* depicting the fact that there could be more unique sequences specific or unique in Fungal and algal strains. Intrestingly, similarity between *Pseudomonas stutzeri* with *Escherichia coli* is 70.58% as both belong to gram negative group and but with *Aspergillus niger* and *Chlamydomonas reinhardtii* is 17.44% and 18.66% respectively. The percentage similarity of *Escherichia coli* with *Aspergillus niger* and *Chlamydomonas reinhardtii* has been observed as 16.70% and 17.65% respectively. *Aspergillus niger* has a similarity of 34.16% with that of *Chlamydomonas reinhardtii* (Fig 8) From the cladogram (Fig 9.) it is evident that there is linearity in the origin of the nitrate reductase enzyme even among the different groups of microbes. Nitrate reductases among the gram negative (KXO78650 and AJF76780) have more commonality than gram positive (AIY99352) bacteria. Also eukaryotic organisms (XP_001391965 and EDP00805) algae and fungi have a common origin[7].

v. Conserved Domain Database (CDD)

Nitrate reductase enzyme complex allows bacteria to use nitrate as an electron acceptor during anaerobic growth. The enzyme complex consists of a tetramer that has an alpha, beta and 2 gamma subunits. The alpha and beta subunits have catalytic activity and the gamma subunits attach the enzyme to the membrane. It is a b-type cytochrome that receives electrons from the quinone pool and transfers them to the beta subunit. From the conserved domain analysis it could be conclude that catalytic active site of alpha subunit has its conserved sequences among the gram positive, gram negative, some thermophiles and archaebacteria. Hence it would be a prospective analysis if one tries to decipher the proteins which have similar sequences to that of alpha subunit in the enzyme for AgNP's synthesis[8].

(Table 1.) *Bacillus sps.* Used for Multiple Sequence Alignment

S.No	Accession	Description
1.	lcl| Query_10001	(KJH58874.1) nitrate reductase [Bacillus licheniformis]
2.	lcl| Query_10002	(CAB82166.2) nitrate reductase beta subunit [Bacillus licheniformis]
3.	lcl| Query_10003	(AGN37220.1) nitrate reductase delta subunit [Bacillus licheniformis9945A]
4.	lcl| Query_10004	(KIU06059.1) nitrate reductase delta subunit [Bacillus subtilis]
5.	lcl| Query_10005	(KIU060064.1) nitrate reductase alpha subunit [Bacillus subtilis]
6.	lcl| Query_10006	(AFL24527.1) Sequence 7427 from patent US 8168417
7.	lcl| Query_10007	(AFL23265.1) Sequence 6165 from patent US 8168417
8.	lcl| Query_10008	(ACP61671.1) Sequence 6165 from patent US 7494798

(Table 2.) Organism source for Nitrate reductase Multiple Sequence Alignment

S.No	Accession	Description
1.	lcl| Query_10001	KXO78650.1 nitrate reductase [Pseudomonas stutzeri]
2.	lcl| Query_10002	AIY99352.1 nitrate reductase [Bacillus subtilis]
3.	lcl| Query_10003	AJF76780.1 nitrate reductase [Escherichia coli]
4.	lcl| Query_10004	XP_001391965.2 nitrate reductase[Aspergillus niger CBS 513.88]
5.	lcl| Query_10005	EDP00805.1 nitrate reductase[Chlamydomonas reinhardtii

International Journal of Advance Research in Science and Engineering
Vol. No.6, Special Issue (01), September 2017, BVCNSCS 2017
www.ijarse.com
IJARSE
ISSN 2319 - 8354

Fig.4 Multiple Sequence Alignment of Bacillus sps. Using Clustal Omega

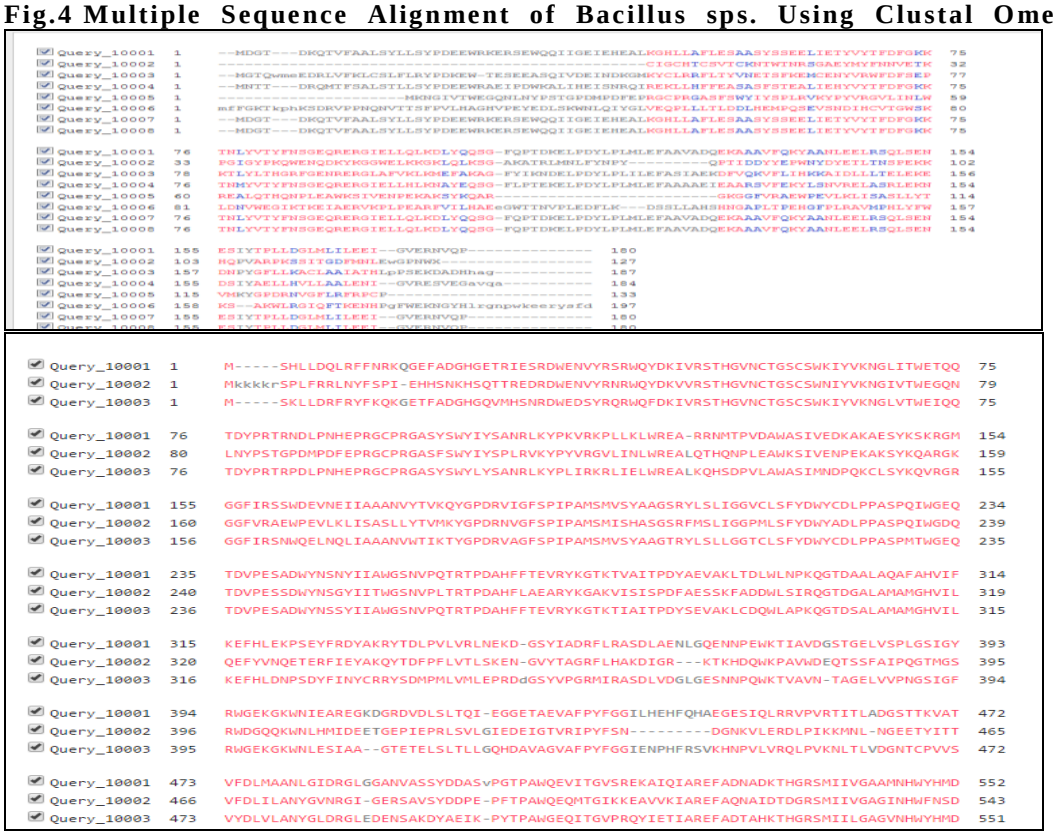
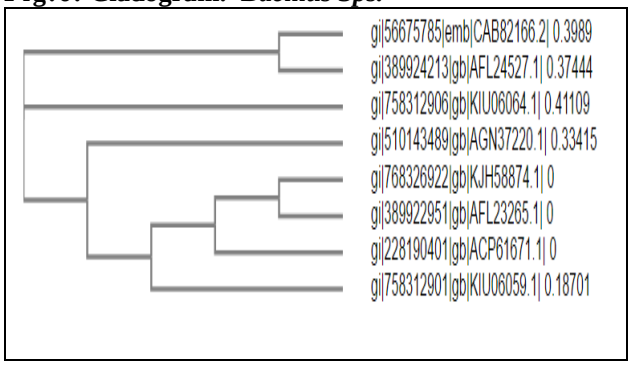


Fig5. Percentage Identity Matrix Clutal2.1- Among different Bacillus sps.

```
#
#
# Percent Identity Matrix - created by Clustal2.1
#
#

  1: gi|56675785|emb|CAB82166.2|  100.00  22.67  16.13  16.47  18.82  18.82  18.82  18.82
  2: gi|389924213|gb|AFL24527.1|   22.67 100.00  18.87  20.41  19.19  19.19  19.19  19.19
  3: gi|758312906|gb|KIU06064.1|   16.13  18.87 100.00  20.43  17.58  17.58  17.58  17.58
  4: gi|510143489|gb|AGN37220.1|   16.47  20.41  20.43 100.00  32.96  32.96  32.96  32.24
  5: gi|768326922|gb|KJH58874.1|   18.82  19.19  17.58  32.96 100.00 100.00 100.00  62.78
  6: gi|389922951|gb|AFL23265.1|   18.82  19.19  17.58  32.96 100.00 100.00 100.00  62.78
  7: gi|228190401|gb|ACP61671.1|   18.82  19.19  17.58  32.96 100.00 100.00 100.00  62.78
  8: gi|758312901|gb|KIU06059.1|   18.82  19.19  17.58  32.24  62.78  62.78  62.78 100.00
```

Fig.6. Cladogram: *Bacillus Sps.*



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Vol. No.6, Special Issue (01), September 2017, BVCNSCS 2017
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ISSN 2319 - 8354

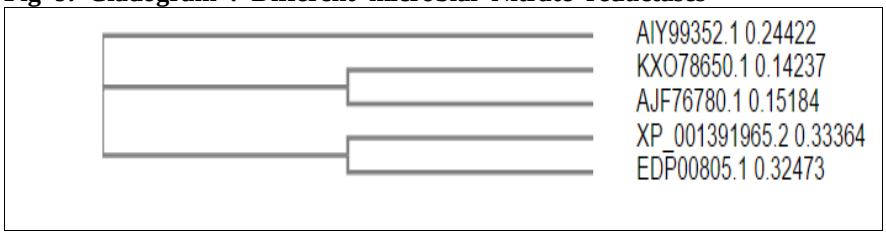
Fig7. Multiple sequence alignment of nitrate reductase from different microbes

Fig8. Percentage Identity Matrix Clutal2.1

```
#
#
# Percent Identity Matrix - created by Clustal2.1
#
#

    1: AIY99352.1          100.00  53.05  52.02  15.98  16.49
    2: KXO78650.1           53.05 100.00  70.58  17.44  18.66
    3: AJF76780.1           52.02  70.58 100.00  16.70  17.65
    4: XP_001391965.2       15.98  17.44  16.70 100.00  34.16
    5: EDP00805.1           16.49  18.66  17.65  34.16 100.00
```

Fig 9. Cladogram : Different microbial Nitrate reductases



IV. CONCLUSION

Bioinformatics tools[11];[12];[13] were used for understanding the mechanism of nitrate reductase in synthesis. Comparative analysis of the enzyme sequences from the database had been carried out among various *Bacillus sps.* using Clustalo (Clustal omega). From the results, 50% similarity exists for β (beta) and γ (gama) regions within the bacillus group, apart from the conserved domains of alpha subunit of the enzyme. Comparison had been done between different AgNp's producers like bacteria, fungi and algae. Further a conserved domain analysis reveals the similarity in the prokaryotic nitrate reductase that can help streamline further studies in the aspect of AgNp's synthesizing mechanism.

V. ACKNOWLEDGEMENT

I am thankful to UGC SERO for the financial support to procure three instruments and required consumables for this work. I acknowledge Head, Dept. Of Microbiology, Principal and Management of Bhavan's Vivekananda College, Sainikpuri for constant support and encouragement extended

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