Scholars Academic Journal of Pharmacy (SAJP) **ISSN 2320-4206 (Online)**
Sch. Acad. J. Pharm., 2015; 4(9): 389-397 **ISSN 2347-9531 (Print)**

(An International Publisher for Academic and Scientific Resources)
www.saspublisher.com

Research Article

Biosynthesis of silver nanoparticles using *Bacillus sp.* for Microbial Disease Control: An *in-vitro* and *in-silico* approach

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Abstract: The development of reliable and eco-friendly organisms of silver nanomaterials is an important aspect of current nanotechnology research and application. In current study, we report extracellular biosynthesis of metallic silver nanoparticles at two molar concentrations (1mM and 5mM) of $AgNO_3$ using *Bacillus sp.* isolated from solid waste soil sample. The potential culture was isolated on nutrient agar medium and identified by several staining and biochemical tests. The potential isolate capable of reducing $AgNO_3$ was identified as *Bacillus sp.* The reduction of $AgNO_3$ was monitored by visible colour change of the solution. UV-Vis spectrum of silver nanoparticles (1mM and 5mM) obtained from the above bacteria containing silver ions showed a peak around 393nm and 405nm respectively. These nanoparticles (1mM and 5mM) were processed for antimicrobial activity and analyzed for plastic degradation. 5mM concentration of silver nanoparticles showed better results against *Pseudomonas sp*., *Staphylococcus sp*., and *Aspergillus niger* compared to 1mM concentration. Silver nanoparticles showed good antibacterial effect against *Klebsiella sp.* at 1mM concentration. Whereas, *Candida albicans* was found to be resistant to silver nanoparticles. Efficacy of the silver nanoparticles in degradation of plastics was analyzed by liquid culture method. Silver nanoparticles and bacteria degrade 18% of plastics and silver nitrate 0% of plastics after 15 days period; this reveals that formed silver nanoparticles were not having enhancing role in the plastic biodegradation as compared to microorganism. *In-silico* analysis was performed to reveal the protein-protein network and functional partners of silver nitrate reductase of *Bacillus sp.*
Keywords: Silver nanoparticles, Extracellular, *Bacillus sp.*, Reductase, antimicrobial activity, Plastic degradation, *In-silico*.

INTRODUCTION:

Nanotechnology, a latest technology for the effective control of microbial diseases and against degradation of plastic. Synthesis of nanoparticles of variable shapes, sizes and various chemical composition and their use for human benefits is defined as nanotechnology [1]. Nanoparticles have been used widely in various fields and the nanoparticles synthesized by various chemical processes are toxic in nature. Therefore, there is a growing need to develop cost effective, eco-friendly and conveniently reproducible methods of nanoparticles synthesis [2].

Due to small particle size and enormous specific surface area of nanosilver, facilitates more rapid dissolution of ions than the equivalent bulk material which potentially leads to increased toxicity of nanosilver [3]. It is well known that both unicellular and multicellular microbes produce inorganic materials either intracellular or extracellularly [4]. Additional downstream processing steps such as ultrasound treatment requires intracellularly synthesized nanoparticles and reaction with suitable detergents to release the nanoparticles from cells during their purification. Thus, nanoparticles which are synthesized by extracellular method is economical with simpler downstream processing as compared to intracellular synthesis [5]. The microorganisms such as, bacteria, yeast and fungi act as interesting nanofactories and play an important role in the remediation of toxic metals through reduction of metal ions. These microbes are good candidates in the synthesis of cadmium, gold and silver nanoparticles [4, 6].

Nowadays, resistance to commercially available antimicrobial agents which are synthesized by pathogenic bacteria and fungi has become a serious problem [7]. Pathogenic microbes, such as, bacteria, molds, yeasts and viruses, in living environment cause severe infections in human beings. Therefore, there is a pressing need to search for new antimicrobial agents both from natural and inorganic substances [4, 8].

Silver as an inorganic antimicrobial agent has been employed since ancient times to fight against infections [9]. For these reasons, the present work has been focused on the extracellular biosynthesis of silver nanoparticles using culture supernatant and evaluation of their antimicrobial activity against human and plant pathogenic bacteria as well as fungi.

MATERIAL AND METHODS:

Isolation of Bacteria:

Solid waste soil sample from dumping area of Mavaalipura, Bangalore was collected in sterile polythene bags. The soil sample obtained from solid waste was diluted in sterile saline solution (0.9% w/v) and isolates were obtained by spread plate technique on nutrient agar medium at 37˚C after 24-48 h. The morphological and physiological characterization of the isolates was performed according to Bergey's manual of determinative bacteriology.

Morphological and Biochemical tests:

Morphology of the isolates were assessed by Gram staining, Endospore staining, Capsule staining, Negative staining. Biochemical analysis were performed by Indole test, Methyl red test, Vogues Proskauer test, Starch hydrolysis test, Catalase test, Gelatine hydrolysis test, Triple sugar iron agar test, H_2S test, Citrate utilization test [10].

Extracellular synthesis of Silver nanoparticles using culture supernatant:

The isolated colonies were inoculated in nutrient broth and incubated for 24-48 hrs at 37^0C in an orbital shaker at 150 rpm. After incubation, the broth was centrifuged at 12,000 rpm for 10 min to collect the bacterial culture supernatant.1mM and 5mM silver nitrate solutions were prepared in double distilled water and mixed in an equal amount (1:1 ratio) with bacterial culture supernatant. The whole sample was kept in the shaker at 150 rpm for 3-5 days in dark condition. The reduction of silver nitrate was monitored by visible colour change of the solution [5, 11].

UV-Visible spectroscopy:

The reduction of the Ag+ ions into metallic silver by the bacterial supernatant in the solutions and formation of silver nanoparticles were characterized by UV-visible spectroscopy which is monitored by sampling the 2mL aqueous component and measuring the UV-Vis spectrum of solutions in the range of 200-800nm wavelength at 1nm resolution both for 1mM and 5mM silver nanoparticles.

Antimicrobial activity against different pathogenic microorganisms:

The test microorganisms *Pseudomonas sp*., *Staphylococcus sp*., *Klebsiella sp*., *Aspergillus niger* and *Candida albicans* were obtained from culture collection unit of Department of Biotechnology, Reva Institute of Science and Management, Bangalore. The antimicrobial activity of silver nanoparticles (1mM and 5mM) was carried out against microorganisms by well diffusion method [4].

Nutrient agar medium was used to culture *Pseudomonas sp*., *Klebsiella sp*., *Staphylococcus sp*., Potato dextrose agar to culture *Aspergillus niger* and Sabouraud's agar to culture *Candida albicans*. Wells were made on all agar plates using gel puncture. Sterile cotton swabs were used to spread each strain uniformly on individual petriplate. Silver nitrate is used as control and silver nanoparticles (1mM and 5mM) as test sample. After incubation of *Pseudomonas sp*., *Klebsiella sp*., *Staphylococcus sp*., at 37^0C for 24 hrs and *Aspergillus niger*, *Candida albicans* at room temperature for 4-5 days, the different levels of zone of inhibition were measured.

Degradation of plastic bags by liquid culture method:

Polythene bags were cut into discs of 3cm diameter, discs were weighed and poured into 3 flasks containing $AgNO_3$ solution, nutrient broth inoculated with bacterial culture and AgNPs solution. All the flasks were incubated in an orbital shaker at 150 rpm for 10-15 days. The discs were then taken out from all the 3 flasks aseptically after 10-15 days. Discs were washed properly with double distilled water followed by 70% ethanol then dried, weighed and final weight loss were calculated [12].

PPI (Protein-Protein interaction) prediction:

STRING (http://string.embl.de/) is a biodatabase of known and predicted target protein interaction network by using four sources: Genomic context, High-throughput screening experiments (Conserved), Co-expression and previous knowledge. STRING currently contains the databases of 5,214,234 macromolecules from 1,133 organisms [13]. STRING database was used to predict protein-protein network and functional partners of nitrate reductase target enzyme. Further, the protein-protein networks were analyzed.

RESULTS AND DISCUSSION:

Isolation and identification of potential isolate:

The bacteria were isolated from solid waste soil by serial dilution method. The potential isolate was found to be Gram positive rod shaped bacteria. Isolated bacterial strain has shown morphological and biochemical characteristics of *Bacillus sp.* (Table.1, 2 & 3).

Table1: Morphological characteristics of the isolate

S.No	Colour	Form	Margin	Elevation	Density	Opacity
1	Off-white	Irregular	Entire	Flat	Sparse	Opaque
2	Off- white	Irregular	Entire	Flat	Dense	Opaque
3	Off-white	Irregular	Serrate	Flat	Dense	Opaque
4	Off- white	Regular	Entire	Flat	Sparse	Opaque

Table 2: Staining tests of the isolate capable of synthesizing Silver nanoparticles

Gram staining	Endospore staining	Negative staining	Capsule staining
+ve bacilli	-ve	-	-
+ve bacilli	-ve	-	-
+ve bacilli	+ve	+ve	+ve
+ve bacilli	-ve	-	-

Table 3: Biochemical characterization of the isolate capable of synthesizing Silver nanoparticles

Methyl red test	Citrate utilisation test	Gelatine test	Starch hydrolysis test	H_2S test	Triple sugar iron agar test	Indole test	VP test
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
+ve	-ve	+ve	+ve	-ve	Glucose utilization	-ve	-ve
-	-	-	-	-	-	-	-

Characterization of Silver nanoparticles:

The isolated species was used for 1mM and 5mM silver nanoparticles and incubated for 3-5 days with bacterial supernatant. Both the solutions have shown significant colour change (Fig1a & b). The resulted nanoparticles at the interval of 24 hours were observed for UV-Vis spectrophotometric analysis. From the initial screening of UV-Vis spectrophotometric results and antimicrobial activity, it was obvious that 5mM silver nanoparticles which were incubated for 5 days have shown promising results in UV-Vis spectrophotometric analysis and antimicrobial activity. These nanoparticles were further characterized by UV-Vis spectrophotometric analysis which shows the absorption peak at 393 nm for 1mM AgNPs and 405 nm for 5mM AgNPs (Fig.2) due to plasmon resonance which indicates the synthesis of silver nanoparticles.

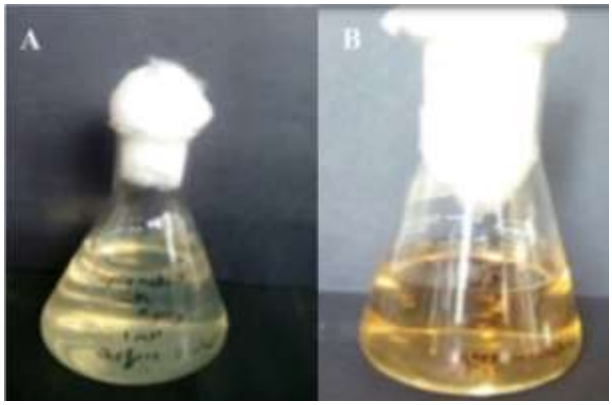

Fig-1a: Conical flasks containing *Bacillus sp.* culture supernatant in aqueous $AgNO_3$ solution at 1mM concentration [A: 0 hour B: 120 hours]

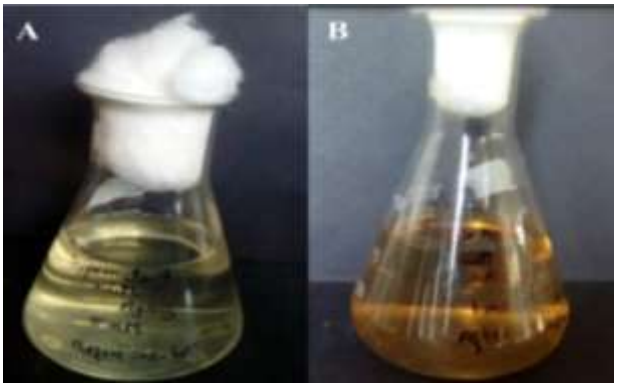

Fig-1b: Conical flasks containing *Bacillus sp.* culture supernatant in aqueous $AgNO_3$ solution at 5 mM concentration [A: 0 hour B: 120 hours.]

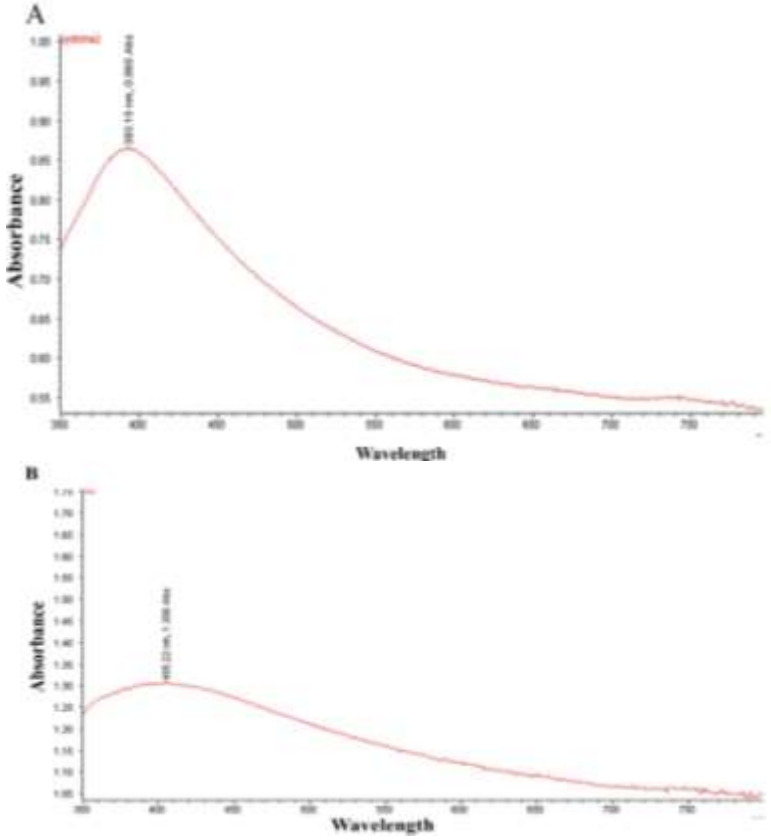

Fig-2: UV-Vis Spectrum of AgNPs [A: 1mMB:5mM]

Antimicrobial activity of Silver nanoparticles:

The antimicrobial activity of silver nanoparticles was investigated against different pathogenic organisms viz; *Pseudomonas sp*., *Staphylococcus sp*., *Klebsiella sp*., *Aspergillus niger* and *Candida albicans* using well diffusion method. The highest antibacterial effect was shown against *Pseudomonas sp*. (10mm). The results showed that the silver nanoparticles showed good antibacterial effect at 5mM concentration and it also showed quarum quenching in 1mM concentration against *Pseudomonas sp*., *Staphylococcus sp*. and *Klebsiella sp*. (Fig3).

The antifungal effect was shown against *Aspergillus niger* (17mm & 20mm) for 1mM & 5mM concentration respectively (Fig4). Whereas, silver nanoparticles have not shown any antifungal effect against *Candida albicans* which indicates that *Candida albicans* is resistant to silver nanoparticles (Fig5).This confirms that 5mM concentration has showed better results against *Staphylococcus sp*., *Pseudomonas sp*. & *Aspergillus niger* (Table 4).

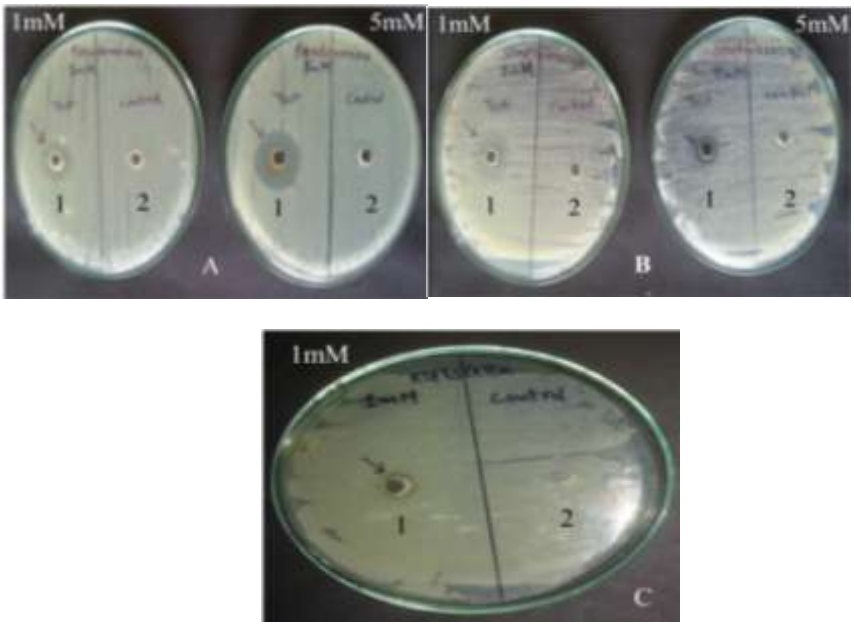

Fig-3:Antibacterial activity of AgNPs against {A-*Pseudomonas sp*. B-*Staphylococcus sp*.& C-*Klebsiella sp*. at 1mM and 5mM concentration shown by well diffusion method. (1. Silver nanoparticles- Test 2. Silver nitrate- Control)}

Fig-4: Antifungal activity of Silver nanoparticles against *Aspergillus niger* at 1mM & 5mM concentration shown by well diffusion method
[1.Silver nanoparticles- Test 2. Silver nitrate- Control]

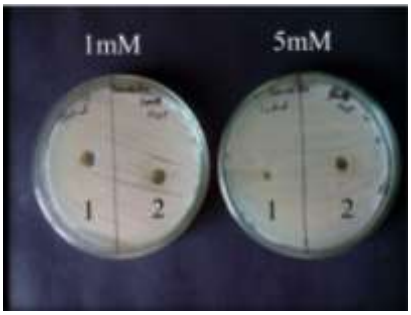

Fig 5: Antifungal activity of Silver nanoparticles against *Candida albicans* at 1mM & 5mM concentration
[1 Silver nitrate- Control 2. Silver nanoparticles- Test]

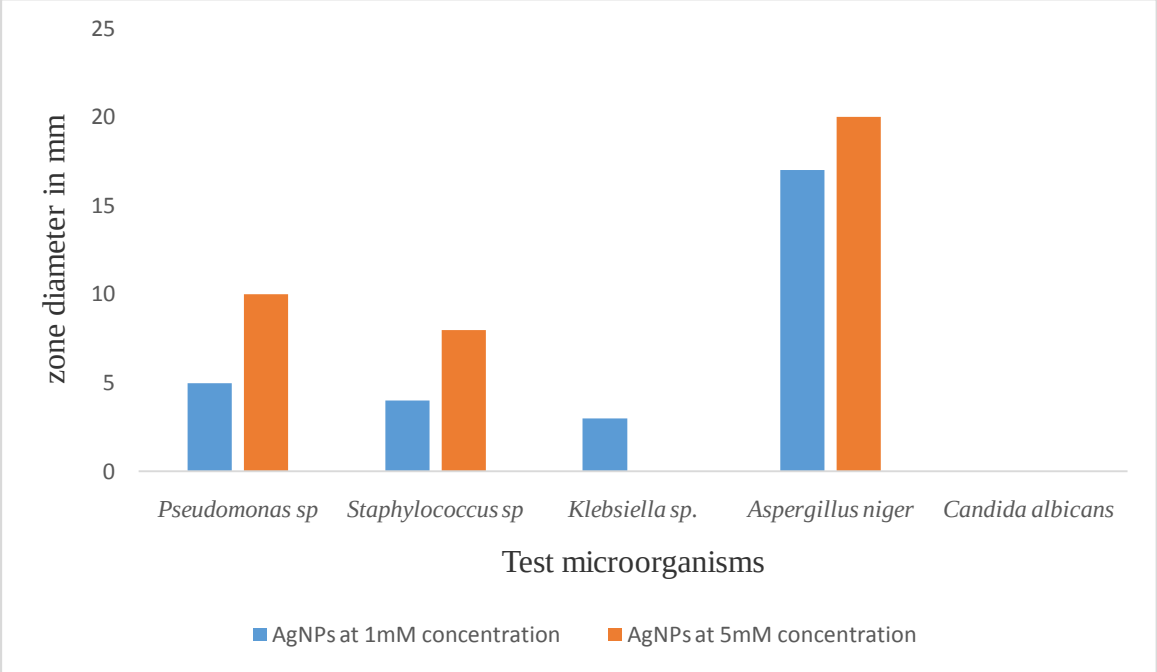

Fig 6: Graph representing the antimicrobial activity of AgNPs against pathogenic microorganisms

Table4: Antimicrobial activity of AgNPs on different microorganisms (Zone of inhibition)

S.No	Tested organisms	AgNPs at 1mM concentration	AgNPs at 5mM concentration
1.	*Pseudomonas sp.*	5mm	10mm
2.	*Staphylococcus sp*	4mm	8mm
3.	*Klebsiella sp.*	3mm	-
4.	*Aspergillus niger*	17mm	20mm
5.	*Candida albicans*	-	-

Degradation of Plastic Bags:-

For the degradation of plastic pieces, 3cm discs of plastics were inoculated in the $AgNO_3$ (5mM) solution, AgNPs (5mM) and liquid culture medium containing bacterial isolate and kept for 15 days to observe the percentage of weight loss (Fig7). The result shows the degradative ability of the bacteria and AgNPs (Fig8). The percentage of weight loss due to degradation by bacteria and AgNPs was found to be 18% (Table 5). This shows that AgNPs was not having enhancing role in the plastic biodegradation as compared to microorganism after 15 days of incubation.

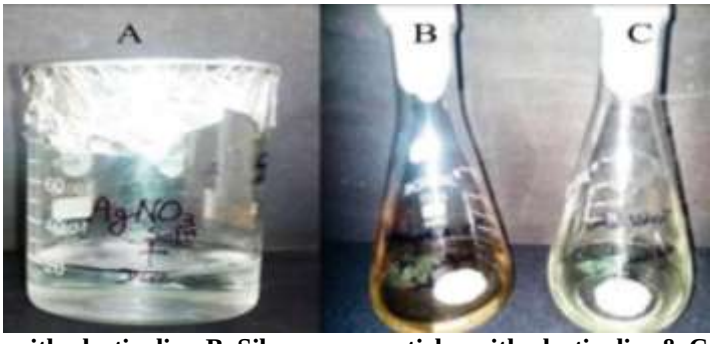

Fig-7: A. Silver nitrate with plastic disc, B. Silver nanoparticles with plastic disc & C. Bacterial culture with plastic disc before incubation

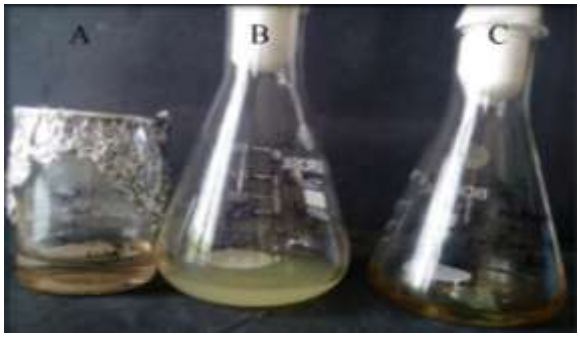

Fig-8: A. Silver nitrate with plastic disc, B. Bacterial culture with plastic disc & C. Silver nanoparticles with plastic disc after 15 days of incubation

Table-5: Degradation of Plastic samples by synthesized AgNPs, Bacterial culture and $AgNO_3$ solution after 15 days

S.No	Samples	Initial wt. (mg)	Final wt. (mg)	Difference (mg)	Weight loss after 15 days (%)
1.	AgNPs	11	9	2	18
2.	Culture with bacterial isolate	11	9	2	18
3.	$AgNO_3$	11	11	0	0

Protein-Protein interaction (PPI) prediction:-

For performing almost all the cellular and molecular functions the PPI are important. Proteins often interact with other closely related proteins in a mutually dependent way to perform a common molecular function. It is understandable that translational factors interact among themselves to carry out the whole translation. The molecular function of protein is predictable from this based on their proteins network. It is very difficult that proteins bring out function with any interactions with other macromolecules. For this reason, in this post metabolomic era PPI databases have turned as a most important and valuable resource for searching biological networks and metabolic pathways in cells. The protein narK was found to have interaction with nitrite extrusion permease, involved in excretion of nitrite. yrpB had interacted with anionic nitro alkane dioxygenase. nasE and nasD showed interaction with assimilatory nitrite reductase subunit, required for nitrite assimilation. hemE protein interacted with uroporphyrinogen decarboxylase, catalyzes the decarboxylation of four acetate group (Table6). Other hypothetical proteins (HPs) do not interact with any other proteins. Protein network and functional partner's details are shown in fig.9. From fig.9, it is observed among the ten functional partners, HemE is distantly related to nitrate reductase. Whereas, all others are closely related with nitrate reductase. Fig.9 indicates the protein-protein interacting networks of HPs, which might have functions of their interacting proteins.

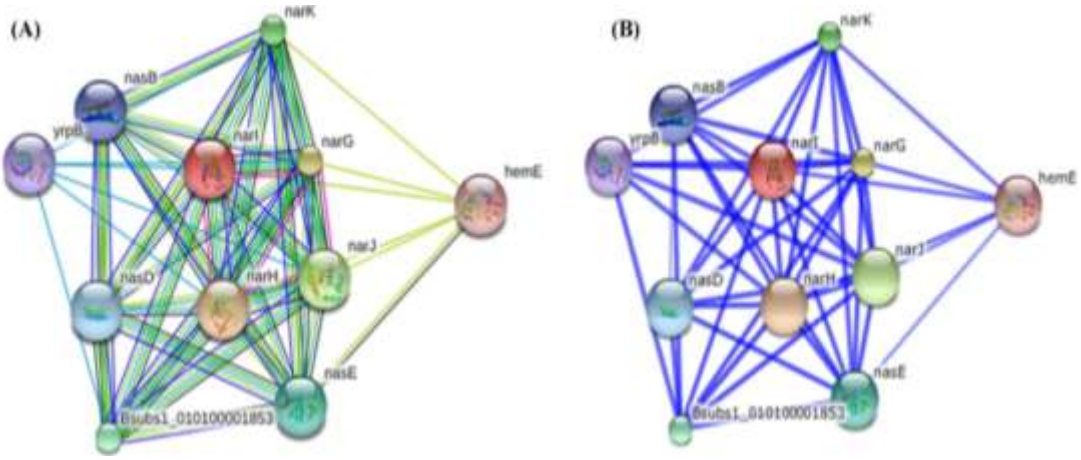

Fig-9: String database results of protein network of nitrate reductase. (A) Evidence view of nitrate reductase functional partners. (B) Confidence view of nitrate reductase functional partners

Table 6: List of closely related proteins associated with nitrate reductase

narH	nitrate reductase subunit beta (EC-1.7.99.4) (487 aa)	0.999
narG	nitrate reductase subunit alpha (EC-1.7.99.4); The alpha chain is the actual site of nitrate re [...] (1161 aa)	0.999
narJ	nitrate reductase J (EC-1.7.99.4); Chaperone required for proper molybdenum cofactor insertion [...] (184 aa)	0.999
narK	nitrite extrusion permease; Involved in excretion of nitrite produced by the dissimilatoryredu [...] (375 aa)	0.999
Bsubs1_010100001853	no annotation available (401 aa)	0.993
nasE	Assimilatory nitrite reductase subunit (EC-1.7.1.4); Required for nitrite assimilation. Require [...] (106 aa)	0.970
nasD	assimilatory nitrite reductase subunit (EC-1.7.1.4); Required for nitrite assimilation (805 aa)	0.949
nasB	assimilatory nitrate reductase electron transfer subunit; Required for nitrate assimilation (770 aa)	0.922
yrpB	anionic nitro alkane dioxygenase (EC-1.13.11.-); Catalyzes the oxidation of alkyl nitronates to [...] (347 aa)	0.900
hemE	uroporphyrinogen decarboxylase (EC-4.1.1.37); Catalyzes the decarboxylation of four acetate gro [...] (353 aa)	0.858

CONCLUSION:

Biosynthesis of silver nanoparticles using *Bacillus sp.* culture from the supernatant was demonstrated. With their unique chemical and physical properties silver nanoparticles are proving as an alternative for the development of new alternative antimicrobial agent. *In-silico* protein network analysis reveals the functional partners which are closely associated with nitrate reductase. Further research is required to fully understand the biochemical and molecular mechanism of nanoparticle formation in order to achieve better control over size and poly dispersity of nanoparticles. Our *in-silico* study reveals that along with silver nitrate reductase, anionic nitro alkane dioxygenase and uroporphyrinogen decarboxylase molecules can increase the efficiency of silver nanoparticles production. Our results also reveal anionic nitro alkane dioxygenase and uroporphyrinogen decarboxylase molecules were found in different organism as well. These results have thrown light in biosynthesis of nanoparticles using different organisms. Our study is probably the first such attempt to predict protein-protein interaction and molecular functional network of nitrate reductase. However, validation of our results through *In-vitro* and *in-silico* experiments will enlighten hope for the future research in nanoparticles synthesis using *Bacillus sp.*

Acknowledgement:

The authors would like to express their gratitude to the management of REVA University for providing us the necessary facilities to carry out this research work. We would also like to acknowledge Dr.Kumar.R, VIT University, and Vellore for his help and support of this work.

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