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RESEARCH ARTICLE

SYNTHESIS OF HEAVY METAL NANOPARTICLE BY BIOTECHNOLOGICAL APPROACHES FOR ENVIRONMENTAL APPLICATION

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ABSTRACT

The present study is based on the synthesis of silver nanoparticles (Ag NPs) by "cleaner and greener" approach for environmental clean-up. Our way of Green synthesis methods include hydrolysis of protein solid waste, extraction of bio molecule, Synthesis of silver nanoparticle and anti-bacterial application. Proteolysis bacteria isolated from marine environment was identified as *Bacillus* sp. CJ2 and used for the hydrolysis of protein solid waste, which resulted in bio-molecule rich broth production. From the bio-molecule rich broth, enzymes were separated out by ammonium sulphate precipitation method. Enzyme molecular weight was found to be around 60KDa. Later remaining bio-molecule was utilized for synthesis of silver nanoparticle. Synthesized Nano-particle was found to be stable, which shows the presence of both reducing and stabilizing agent in bio-molecule. The mechanism of the Ag NP bactericidal activity is discussed in terms of Ag NP interaction with the cell membranes of bacteria. The property was found to be positive for the sewage water isolated *E coli*.

INTRODUCTION

The biosynthesis of nanoparticles is an emerging highlight of the intersection of nanotechnology and biotechnology. It has received increasing attention due to a growing need to develop environmentally-benign technologies. Nanotechnology includes synthesis, characterization, exploration and application of Nano-sized (1-100nm) materials for the development of science. It deals with the materials whose structures exhibit significantly novel and improved physical, chemical, and biological properties, phenomena, and functionality due to their nano scaled size. Because of their size, nanoparticles have a larger surface area than macro-sized materials. Nano-biotechnology describes an application of biological systems for the production of new functional materials such as nanoparticles. It combines biological principles with physical and chemical procedures to generate Nano-sized particles with specific functions. It represents an economic alternative for chemical and physical methods of nanoparticles formation. Nanoparticles, because of their small size, have distinct properties compared to the bulk form of the same material, thus offering many new developments in the fields of biosensors, biomedicine, and bio nanotechnology. Silver nanoparticles are one of the promising products in the nanotechnology industry. The development of consistent processes for the synthesis of silver Nano-materials is an important aspect of current nanotechnology research. One of such promising process is green synthesis. Silver nano particles can be synthesized by several physical, chemical and biological methods. Current research in inorganic Nano-materials having good antimicrobial properties has opened a new era in pharmaceutical and medical industries. Silver is the metal of choice as they hold the promise to kill microbes effectively. Silver nanoparticles have been recently known to be a promising antimicrobial agent that acts on a broad range of target sites both extra cellular as well as intracellular. Silver nanoparticles shows very strong bactericidal activity against gram positive as well as gram negative bacteria including multi-resistant strains and also it was found to be in few studies. Both the top-down and bottom-up approaches have been followed to synthesize nanoparticles. Here, chemical and biological methods have been successfully applied to synthesize silver nanoparticles.

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MATERIALS AND METHODS

Based on morphological and biochemical tests bacteria were isolated and incubated for 24 hours at 30ºC. After 24 hours of incubation, a loop of culture was inoculated in 100 ml of nutrient broth in a 500ml Erlenmeyer flask and incubated in rotary shaker at 120 rpm and 35ºC for 24 h.

Hydrolyzation of protein solid waste

The protein waste particles of dimensions approximately 1.0 to1.5 cm were obtained by manual scissoring and maintain the pH at 7.0±0.2 The solid waste were packed and stored at 4°C.20 g protein waste is added in 100 ml minimal media with 1ml trace element, An aliquot of 10 ml of inoculum was transferred to 100 ml cooled minimal.

Extraction of bio molecule from hydrolysed protein solid waste

The hydrolysed broth is centrifuged in cooling centrifuged at 4°C.The bio molecule is analysed for HPLC and used for the production of nanoparticles.

BIOCHEMICAL ANALYSIS

Fermented Protein Waste: By Lowry's method protein absorbed read at 650 nm.

Protease estimation: Sample along with1 ml of phosphate buffer was added followed by addition of 1ml substrate. Then 3ml of 5% TCA was added and filtered for absorbance at 280 nm.

Lipid Estimation: 0.5ml sample mixed with 6ml of phosphovallin reagent and incubated in dark at 70°C for 45minutes and absorbance was read at 533nm.

Lipase Assay: 2ml of Triton X-100, 2ml of $CaCl_2$, 1ml of NaCl, 4ml of phosphate buffer and 5ml of substrate incubated for 5 min.1ml of supernatant containing enzyme was incubated .10ml of ethanol: acetone mixture (1:1) was added to arrest the reaction. Few drops of phenolphthalein was added and titrated against 0.02 NaOH. End point is the appearance of pink colour.

Estimation of Ammonia: 5ml of sample with 25ml of distilled H_2O, 25ml of boric acid added and kept for distillation. Free ammonia released was collected. From this 50ml of distillate was collected and titrated against 0.02N H_2SO_4. End point is the conversion of violet colour to green colour.

Amino Acid Estimation assay: 5ml of sample, 1ml of ninhydrin solution was added with the sample. Incubated for 3-5 minutes and absorbed at 570nm.

Determination of amino acid composition of bio molecule rich broth by HPLC

The total free amino acid composition of fermentation medium was determined, using C18 column (Column size: l = 0.10 m, Ø = 4.6 mm) in Agilent model 1100 HPLC analyser.

Extraction of enzyme from hydrolysed solid waste ammonium sulphate precipitation

100 ml of bio molecule was sheared. Ammonium sulphate is added slowly till dissolved and formed precipitate, overnight incubated and centrifuged. The supernatant is separated and again repeated the procedure with ammonium sulphate. Again it was centrifuged and the pellet is collected to run SDS-PAGE.

Dialysis of bio molecule

The bio molecule obtained from the hydrolysed solid waste is poured in the washed membrane and placed in beaker containing distilled H_2O.

Molecular weight analysis of enzyme -SDS – page

Separating gel 12% (w/v)prepared and polymerised the stacking gel .The wells were loaded with sample and electrophoresed at 50volts. The gel was stained to visualize the proteins bands.

Synthesis of silver nanoparticle and its characterization

1M of Silver nitrate mixed with 30 ml of bio molecule is added to observe different time interval 12h, 24h, 48h using UV-visible spectrophotometer. The change in colour is observed from white to yellow and later brown colour shows the preliminary results on formation of silver nanoparticle. The stable silver nanoparticle collected by centrifugation (10,000 rpm,15 min), and was dried at 50°c for 5 h in hot air oven which is stored in a sterile for further use.

Antimicrobial activites of silver nano particles

10mg of silver nanoparticle was added in the 24 hours grown *E coli* culture. Growth turbidity measurement of *E. coli* was read on UV-visible spectrophotometer at 600nm from zeroth hour to 48 hours for all the conditions. Readings were plotted to analyse the growth nature of *E coli* in various condition.

Standardization of genomic DNA

By slandered procedure bacterial genomic DNA extracted and electrophoresed at 0.25% of agarose. The gel was viewed under an UV Trans-illuminator.

RESULT AND DISCUSSION

Screening of proteolytic bacteria

Different colonies were isolated (Fig 1) and separated by quadrant streaking on nutrient agar (Fig 2). Among the 15 isolates C2 showed highest zone of clearance (Fig 3). Therefore C2 was used for the hydrolysis of protein solid waste. Later C2 was identified as gram positive rods and positive (Fig 4) and biochemical test performed which shows C2 has to be of *Bacillus* sp.

Hydrolysis of protein waste

The results of proximate composition and pH of protein solid waste and hydrolysed under optimized condition by using C2 were given (Table 1)The maximum protease activity was observed in 78h of hydrolysis period at the end of exponential cell growth phase (Graph 1). The repression of protease after 72 h was due to excessive release of amino acids from protein waste (Graph 2). The ability of *Bacillus* sp.

The Biomolecules present in the hydrolysed protein waste specifically protein, amino acids, lipid influenced the growth of bacteria shown (Graph 1), therefore the OD value is observed at high range.(Graph 2) and (Graph 3) shows the hydrolysis of protein and lipid against protease and lipase respectively of solid waste. Physical and biochemical characteristics of *Bacillus* sp. C2 given (Table 2).

Determination of amino acid composition of bio molecule rich broth by HPLC

Graph 5 and Table 3 represent the composition of amino acid in Biomolecule. Each peak represents the retention time of the amino acids present in the biomolecule rich broth.

Extraction of Enzyme From Bio-Molecule Solution SDS-PAGE

Extracted crude enzyme's molecular weight was found by SDS gel electrophoresis as around 68kDa (Fig 5)

Synthesis of silver nanoparticle and its characterisation

The change in colour is observed (Fig 6) from white to yellow and later brown colour shows the preliminary results on formation of silver nanoparticle. The UV-visible spectra of silver nanoparticle solution exhibited absorption maximum at about 420 nm to 430 nm the band of the silver nanoparticles (Graph 6). This confirms the synthesis of silver nanoparticle using bio-molecules.

Antibacterial Activity of Silver Nanoparticle

Decrease in the *E coli* concentration found when 24 hours grown medium of *E coli* added with 10mg of silver Nano-particle (Fig 7),(Fig 8) and in (Graph 7) indicates the concentration of *E coli* grown over the medium is very less which implies the effective action of silver nanoparticle that shows the positive result. The OD has been taken mentioned in (Table 4) which shows the activity of Ag NP on *E coli* at different interval and found that the culture containing *E coli* alone was grown in normal growth phase as shown in (Graph 7- series 1) and another culture containing Ag NP - *E coli* culture shown less growth with 24h (Graph 7- series 2). At the same time Ag NP was added to 24h grown culture of *E coli* shown resistant in growth after 6h in (Graph 7- series 3).
Action of silver nanoparticle on *E coli*

***A*garosegelelectrophoresis**

Genomic DNA analysis was carried out to find out the action of silver nanoparticle on *E coli* and its ability to disturb genomic DNA (Fig 9). It was found as DNA is not affected by the silver Nanoparticle and hence therefore we predict its action has to be on its cell wall alone.

Fig .1-Different colonies streaking plate

Fig.2-Colonies separated by quadrant Plate

Fig.3-Zone of clearance

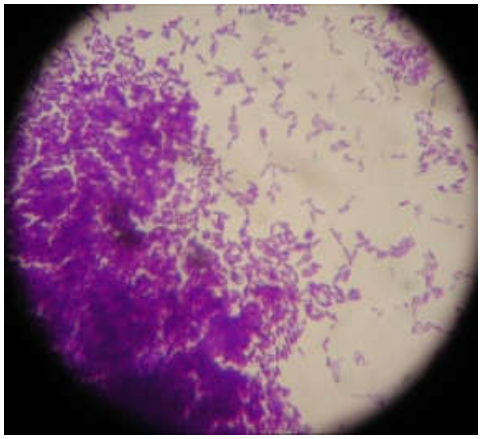
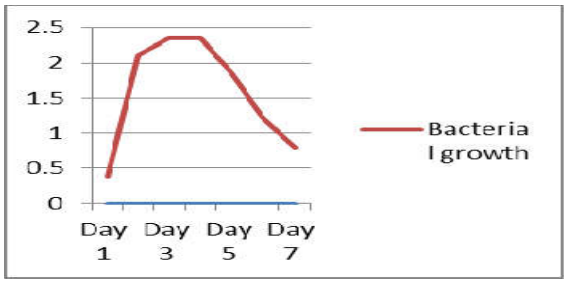
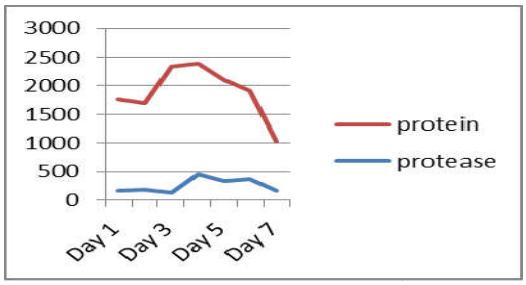


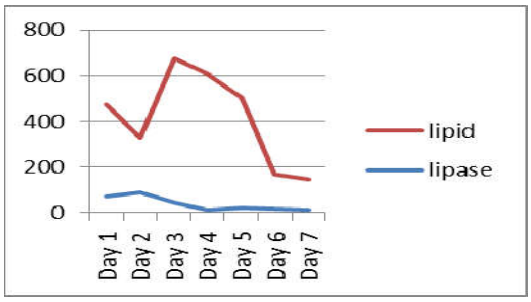
Fig.4- Gram positive rod shaped *Bacillus* sp. C



Graph 1- Bacterial growth curve



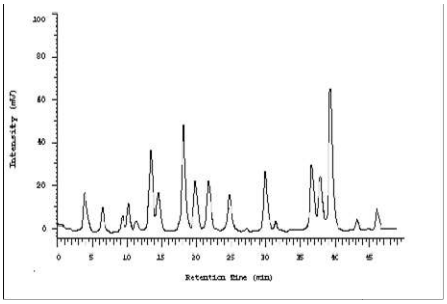
Graph 2-Hydrolysis of protein against protease



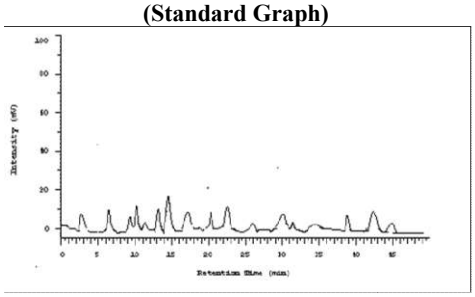
Graph 3- Hydrolysis of lipid against lipase

Table 2 –Characterization of *Bacillus Sps*

S.No	Biochemical tests	Results
1.	Oxidase	-ve
2.	Catalase	+ve
3.	Nitrate reduction	+ve
4.	Urease	+ve
5.	H2S production	-ve
6.	Methyl red	-ve
7.	Vogues proskaeur	+ve
8.	Citrate utilization	-ve
9.	Indole production	-ve
10.	Casein hydrolysis	+ve
11.	Lipid hydrolysis	+ve



Graph 4- Analysis of amino acid by HPLC (Standard Graph)



Graph 5- Analysis of amino acid by HPLC

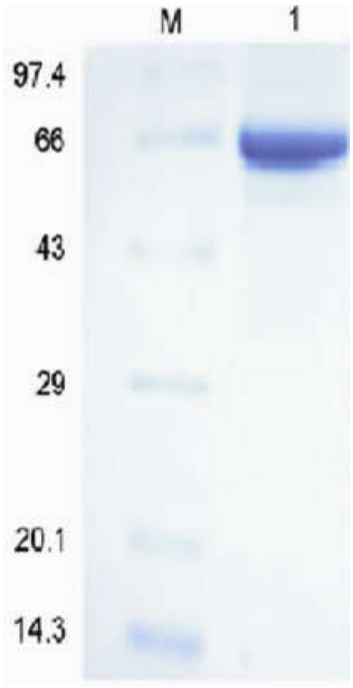


Fig. 5- Molecular weight analysis of enzyme

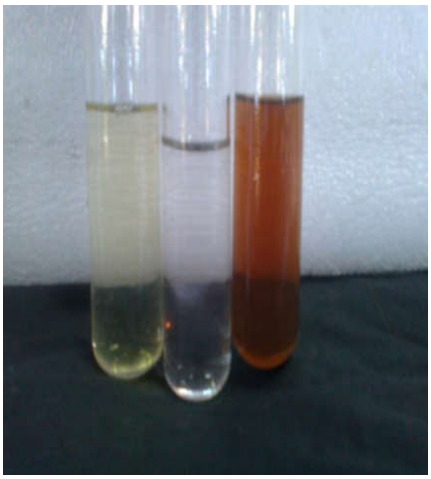


Fig.6- Synthesis of Ag NP

***Fig.7-E coli* inhibition by Ag NP**

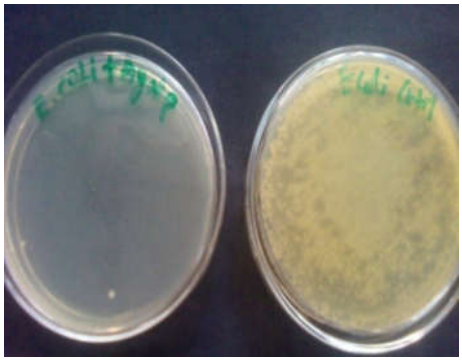
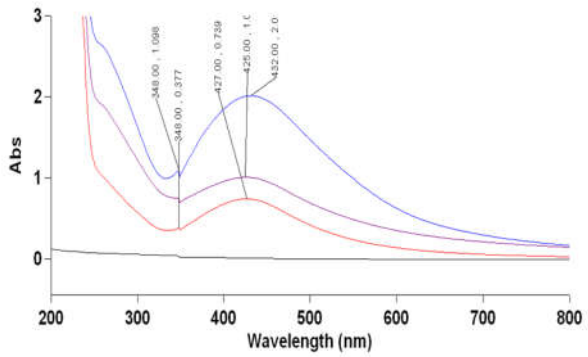


Fig.8- Pour plate of *E coli* grown with Ag NP

Fig.9-Genomic DNA analysis

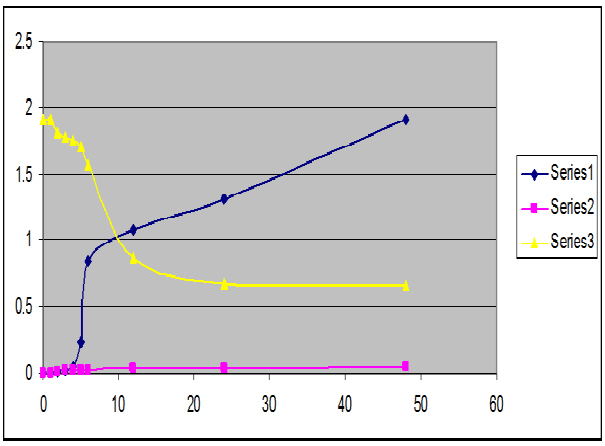


Graph 6: UV visible spectra of Ag NP solution

Where, --- Absorption spectra of Ag NP at 12 hours, --- absorption spectra of Ag NP at 24 hours,--- Absorption spectra of Ag NP at 48 hours

Conclusion

In this report, we demonstrated greener bioprocess, where Bio-based hydrolysis was carried out for conversion of proteinaceous leather industry solid waste to bio-functionalities product (Bio molecules). The proteolytic strain *Bacillus* sp. CJ2 was isolated from the sea sediment showed better hydrolytic efficiency on the proteinaceous solid waste.



Graph.7-Antibacterial activity of Ag NP against *E coli*

Series1: *E coli* growth in nutrient broth.
Series2: *E coli* growth in nutrient broth with silver nanoparticle.
Series3: Effect of silver nanoparticle on 24 hours grown *E coli* Culture.

Table.3:D-7000 HPLC System

NO.	COMPONENT NAME	R.T.	AREA	AREA %
1	ASPARTIC ACID	4.02	93452.6	5.326
2	GLUTAMIC ACID	6.49	3045.6	0.2014
3	ASPARAGINE	9.2	465	0.0091
4	SERINE	10.21	419	0.004
5	GULTAMINE	11.56	1289	0.119
6	GLYCINE	13.4	22875	1.359
7	THREONINE	14.92	164245	7.284
8	ARGININE	18.05	316574	18.699
9	ALANINE	19.56	264	0.002
10	CYSTINE	21.74	361524	19.349
11	TYROSINE	24.93	1648	0.112
12	HISTIDINE	27.25	869	0.105
13	VALINE	29.92	319545	20.508
14	METHIONINE	31.46	1169	0.084
15	ISO-LEUCINE	36.66	33526	1.688
16	PHENYL ALANINE	37.95	35975	1.880
17	LEUCINE	39.32	2799	0.141
18	LYSINE	43.46	46821	2.583
19	PROLINE	45.1	92	0.002
20	TRYPTOPHAN	46.2	369125	20.542
21	TAURINE	47.33	103	0.002

The enzymes and hydrolysed products were characterized. The bio molecules were used as a reducing and capping agent for the synthesis of silver nanoparticles. Synthesized nanoparticle was found to be stable for long duration. The nanoparticle showed good antibacterial property against pathogenic strain *E coli*. This property can be implemented in medicinal and environmental fields for wide variety of applications. Therefore, the study confirms the hydrolysis of

proteinaceous solid waste and its bio-conversion to produce value added bio-functionalities product, which can be consider as "green approach for environmental clean-up". The green synthesis of nanoparticle also can be considered as a novel approach in the Nano-biotechnology.

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