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Biosynthesis of CdS Nanoparticle using Metal Resistant Bacteria

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ABSTRACT

The metal resistant microorganism may possess the mechanisms to tolerate toxic metal concentrations one of them is to precipitate the metals using a counter ion present in the surrounding medium. In the present study, cadmium (Cd) resistant organisms were isolated from contaminated soil using enrichment and further screened using gradient plate technique for highest Cd resistant organism. Based on the biochemical test, the selected strain CdR3 was identified and belonging to genus *Gluconobacter*. The CdR3 was able to biosynthesize monodisperse Cd nanoparticles in almost all the conditions tested however the smallest sized obtained at pH 7 and 42˚C. FTIR and XRD of nanocrystals formed by *Gluconobacter* confirm that it was CdS nanoparticle. The particle size distribution of nanoparticle confirms the size of crystals ranging from 0.638 to 1.926 nm which was pH dependent. In addition monodisperse particle synthesis at ambient condition can be achieved using this organism.

Keywords: CdS nanoparticles, Particle size analysis, monodisperse, XRD, metal resistance

I. INTRODUCTION

Nanotechnology has recently emerged as an elementary division of science and technology for investigation and regulation of the interaction at cell level between synthetic and biological materials with the help of nanoparticles [1]. Due to unusual optical, chemical, and electrical properties of nanoparticles, interest in synthesis of nanoparticle grew over the past decade. Nanoparticles are used in the fields of catalysts, optics, life sciences, pharmacy, medicine, mechanics, magnetism and energy science [2, 3]. Variety of techniques are used for synthesis of metal nanoparticles viz. chemical recovery using regenerative materials, aerosol technique, electrochemical deposition, photochemical recovery, laser exposure. These techniques are limited by generation of a toxic waste and particle size is greatly varied [4]. A wide range of nanophasic and nanostructured particles are being fabricated globally with the aim of developing a clean, nontoxic, and eco-friendly technologies [1]. The term of a green nanotechnology emerged with a lot of attention has attracted. In the green nanotechnology, biological synthesis of nanoparticles is focused. Technology based on the use of microbes for the synthesis of nanomaterials is relatively new and largely unexplored area of research in material synthesis, which has a potential to develop a simple, practical, inexpensive ways to produce novel metallic nanoparticles possessing unique chemical and physical properties [4]. Many microorganisms have ability to aggregate an inorganic material within or outside the cell to form nanoparticles. For example, single cell organisms like magnetic bacteria produce magnetic nanoparticles and nanoparticles of quartz and other bacteria, viruses, fungi and yeast produce various kind of nanoparticles i.e. silver, gold, CdS, Bismut, TiO_2, SiO_2 and so on [1, 4-6]. To produce semiconductor nanocrystals cheaply and efficiently, biological methods of synthesis are being explored using *E. coli* to synthesize intracellular cadmium sulfide (CdS) nanocrystals. The nanocrystals are composed of awurtzite crystal phase with a size distribution of 2–5 nm [2]. Even M13 bacteriophage was reported to produce semiconductor nanowires made up of CdS [7].

Pre-treatment of *Ralstonia sp.*TAK1 with an inducing concentration of Cd conferred a cross protective response against subsequent exposure to the lethal concentrations of Zn [8]. There are various mechanisms of metal resistance reported. One of them is to precipitate metal after reduction. This could lead to formation of nanoparticles.

Present study aimed to isolate, screen and identify Cd resistant bacteria from the metal contaminated soil and characterized their Cd nanoparticle synthesis potential under various physicochemical conditions. The Cd nanoparticles produce were characterized using UV spectroscopy, FTIR, X-RD, and particle size distribution analysis.

II. METHODS AND MATERIAL

The sources of the chemicals and related reagents used in the study were of analytical grade and were purchased from standard companies**;** nutrient broth sodium chloride, ethyl acetate, methyl red, Gentian violet, safranine, congo red Na_2S from Hi-media. $CdCl_2$ was from SuLab chemicals.

A. Isolation of Cd resistant bacteria

Soil sample was collected from surrounding the chemical polluted zone at Naroda GIDC, Ahmedabad. One gm of soil sample was added to 10 ml distilled water. Mixed well, and allowed soil to settle. The soil suspension was enriched by inoculated it in nutrient broth amended with 300 mM $CdCl_2$ in a flask and kept it on shaker (110 RPM) at room temperature for 5 days. 0.1 ml of sample from the flask was spread on nutrient agar containing 10 ppm $CdCl_2$ and incubated at 37 ˚C for 48 hours.

B. Screening of Cd resistant bacteria

The colonies developed on 10 ppm nutrient agar plates were streaked on the nutrient agar plates containing 300 and 400 ppm concentration of $CdCl_2$ followed by incubation at 37℃ for 48 hours. The colonies showed growth on highest concentration of $CdCl_2$-nutrient agar plates were screened further using gradient plate technique. For gradient plate technique colonies that showed higher growth were transferred in nutrient broth and incubated at for 37^{o}C for 24-48 hours. The isolate was streaked in line form on gradient of 100 -300 ppm concentrations and the highest tolerant colony was selected for further study. Selected colony was streaked on nutrient agar slants amended with 50 ppm cadmium and stored at 4˚C. Periodic transfer was given at every month.

C. Identification of Cd resistance bacteria

Selected colony was identified based on colony morphology, spore staining and biochemical tests. Different types of biochemical test such as carbohydrate fermentation, methyl-red test, Voges-proskauer test, Citrate utilization test, H_2S production test, Nitrate reduction test, Indole production test, Starch hydrolysis test, and Triple sugar iron agar test were performed [9].

C. Production of Nanocrystals

To 50 ml nutrient broth amended with 400 ppm $CdCl_2$ solution after autoclaving was inoculated with isolate showed growth on the nutrient agar plate containing 300 ppm, 400 ppm $CdCl_2$ and incubated at room temperature for 48 hours on shaker. Periodically (5, 10, 15, 30, 60, 120 minutes), 1ml of sample was withdrawn and mixed with 8 mM Na_2S. The spectral scan in range of 200-700 nm was recorded.

1) Effect of pH on nanocrystal formation

Nutrient broth amended with 400 ppm $CdCl_2$ adjusted to pH 1, 3, 5, 7, and 9 was inculated from master plate and keep on shaker for 48 hrs. Equamolar concentration of Na_2S was added to the sample withdrawn periodically (0, ½, 1, 2, 3, 5, 12, and 24 hrs) and U.V spectrum was recorded for analyzing Surface Plasmon Resonance of CdS nanoparticles.

2) Effect of temperature on nanocrystal formation

Nutrient broth amended with 400 ppm $CdCl_2$ was inoculated as describe earlier and flasks were incubated at different temperature 27 ˚C, 37˚C, 45˚C. Equamolar concentration of Na_2S was added to the samples withdrawn periodically (0, ½, 1, 2, 3, 5, 12, and 24 hrs) and U.V spectrum was recorded for analyzing Surface Plasmon Resonance of CdS nanoparticles.

3) Sample preparation for analytical test

Sample was prepared and sent for the X-ray diffraction method, Fourier Transform Infrared spectroscopy (FTIR) and particle size analysis at SICART, New Vallbh Vidyangar.

III. RESULTS AND DISCUSSION

Cadmium (Cd), usually detected in its ionic form of Cd^{2+}, is a major environmental hazard causes adverse toxic effects on human health and other living organisms. However, Biological systems, masters of ambient condition chemistry, synthesize inorganic materials that are hierarchically organized from the nano- to the macroscale. These biominerals are composite materials and consist of an inorganic component and a special organic matrix (proteins, lipids, or polysasharides). Microbial systems may be used as templates for organizing nanoparticles or be programmed to express a set of worker proteins that can synthesize nanoparticles [4] (Tsibakhashvili 2010). Cd-resistant bacteria were isolated from Cd-contaminated soils [9] (Prapagdee 2009). Microorganisms such as bacteria, fungi, and yeast are recently found as possible eco-friendly nanofactories.

A. Isolation and Characterization of Cd Resistant Bacteria

After 5 days, growth was observved in a flask containing nutrient broth amended with 300 ppm $CdCl_2$, hence, the organisms that survived were Cd-resistant. Further, isolated colonies were seen on nutrient agar containing 10 ppm $CdCl_2$ when spreading was done using inoculums from the flask.

Total 12 different microorganisms were grown on the nutrient agar containing 10 ppm $CdCl_2$. Microorganism have several resistant mechanisms that can prevent heavy metal toxicity either by inducing development of tolerance or resistance. Heavy metals are able to induce increased resistance levels in soil bacteria and modify bacterial responses to environmental conditions either by inducing mutations or by altering physiological responses [10].

1) Screening of Cd Resistance Organism

Isolated colonies were seen on nutrient agar plate containing 300 ppm and 400 ppm concentration of $CdCl_2$. No growth of organisms was observed on 400 ppm concentration however colonies were observed on 300 ppm $CdCl_2$ (Fig. 1). Isolates from nutrient agar plates were further screened using gradient plate technique containing gradient of 300 ppm to 400 ppm concentration of $CdCl_2$.

Colonies were streaked on gradient plate shows lowest growth at highest gradient and highest growth at lowest gradient. Four different Cd resistance cultures were isolated and labelled as CdR1, to CdR4 respectively. The highest tolerance toward Cd was shown by CdR3 hence it was used for further study. Some Cd-resistant bacterial strains increase resistance under higher levels of Cd toxicity because Cd could induce the alteration in the bacterial response or resistance mechanisms [8] (Prapagdee, 2009).

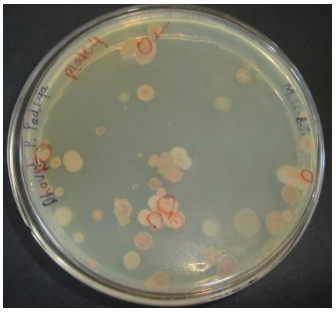

Figure 1: Cd resistance cultures isolated from enrichment flask.

2) Gram Staining and Colony Characteristics

From the morphological study, the CdR3 is Gram-negative and short-rod shape. Its colony on nutrient agar plate was brown colored, smooth, rounded, convex. The colony diameter was 2-3 mm after incubation for 48 h.

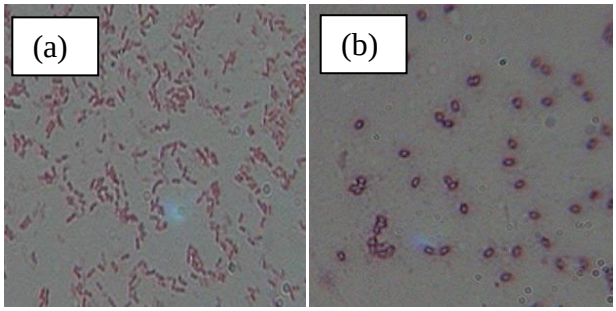

Figure 2: Phynotypic characteristics of CdR3 (a) Gram staining of the isolated culture and observe the slide at 100X under microscope. (b) Endospore staining.

The endospore appears as colourless beads in a colorless cytoplasm against the black background (Fig. 2).

To confirm the Gram's nature, the isolate was further grown on the selective medium for gram negative organisms (Fig. 3).

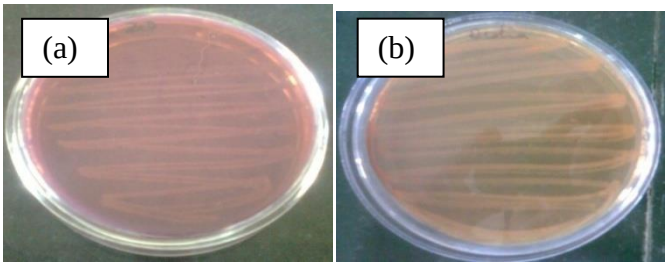

Figure 3: Growths of CdR3 on (a) EMB agar (b) MacConkeys agar plate indicate that it is Gram negative.

3) Biochemical tests

Glucose fermentation and acid production change the colour of the medium to pink and gas produced was collected in Durhum's tube as a small bubble all the isolated organism ferment glucose because after incubation develops pink colour and produce gas. All of the four colonies give positive result. Methyl red test the development of stable red colour after addition of methyl red indicator indicates positive test. All the isolated culture all the test tube showed negative result. Voges-Proskuer test Development of red colour within 15 minutes or more, after addition of α-napthaol and 40% KOH reagents indicate the presence of diacetyl. All the isolated culture showed diacetyl present in tubes indicated positive result. In citrate utilization test, positive test was represented by development of a deep blue colour within 24-48 hours indicating that the organism has been able to utilize the citrate contained in the medium, with the production of alkaline products. All the four isolates showed development of blue colour indicates citrate utilization

TABLE I. RESULTS OF BIOCHEMICAL TESTS

Test		**Isolates**			
		CdR1	**CdR2**	**CdR3**	**CdR4**
Carbohydrate fermentation	G	+	+	+	+
	M	-	-	-	-
	L	-	-	-	-
M-R Test		+	+	+	+
V-P test		-	-	-	-
Ammonia Production		-	-	+	-
Nitrate Reductase		+	+	+	+
H_2S Production		+	+	+	+
Indol Production		-	-	-	-
Starch Degradation		+	+	+	+
TSI		+	-	-	-
Simon Citrate test		+	+	+	+
Phosphate solubilisation		+	+	+	+

Key, '+'-Positive test, '-'- Negative Test, G – glucose, M – maltose, L-lactose, NR- nitrate reduction, MR- methyl red, vp- vogesproskauer, H_2S- hydrogen sulphide, TSI- triple sugar assay.

Indole production test development of bright red fuchsia red colour at the interface of the reagent and the broth within the seconds after adding the Ehrilich's reagent is the indicative of the presence of indole and was a positive test. No isolates development of bright red fuchsia red colour at the interface of the reagent and the broth. Nitrate reduction test showed development of red colour within 30 seconds after adding the test reagent indicates the presence of nitrates and was positive nitrate reduction test. Blackening of filter paper strip indicates positive test for sulfate reduction which was due to formation of lead sulfide, which indicates H_2S production. All four isolates showed blackening of filter paper strip. The change of red litmus to purple or blue indicates the ammonia production and can be read as positive test. Results are summarized in the Table 1. All the isolates were tested for phosphate solubilizing activity on Pikovaskys agar plate containing methyl red which acts as an indicator. Isolates showed phosphate solubilizing activity via formation of pink colour zone because organic acid production. Production of acid lead to change in pH from 7.5 to 6 or 5. By the biochemical test result and Gram's staining result, the organisms belonging to the *Gluconobacter* genus (Bergy's manual's addition 9^{th}).

B. Production of nano particles of CdS

For produciton of nano particles of CdS, the CdR3 isolate was grown in the presence of $CdCl_2$ and over the period of time the samples were withdrawn and mixed with Na_2S for formation of CdS nanoparticles.

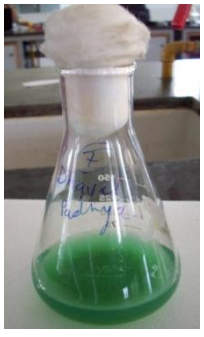

Figure 4: Flask showing growth and nanoparticle synthesis using selected culture CdR3

1) Effect of Temperature

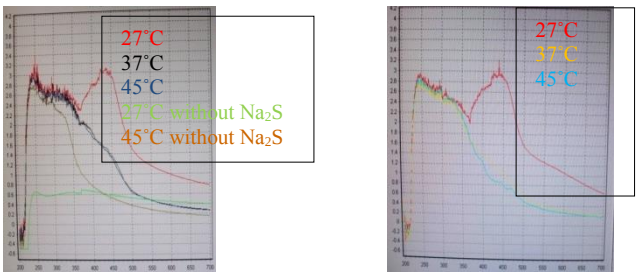

Figure 5: (a) 0 hr: Nanocrystal formation was seen at 27˚C, 37˚C,45˚C as peak was seen in the graph, when Na2S was added. Even nanocrystal formation was seen at 45˚C without addition of Na_2S. But no nanocrystal formation was seen at 27˚C without Na_2S. (b) 30 min: Nanocrystal formation was seen at 27˚C, 37˚C, 45˚C when Na2S was added even after 30 min. By comparing (a) and (b), we can see that there is no increase on nanocrystal formation as peaks are similar in both graphs.

2) Effect of pH

Nonanocrystal formation was seen at pH 3 (b) nanocrystal formation was seen at pH 5 but lesser compared to 7. (c) nanocrystal formation was seen maximum at pH 7. (d) nanocrystal formation declines at pH 9.

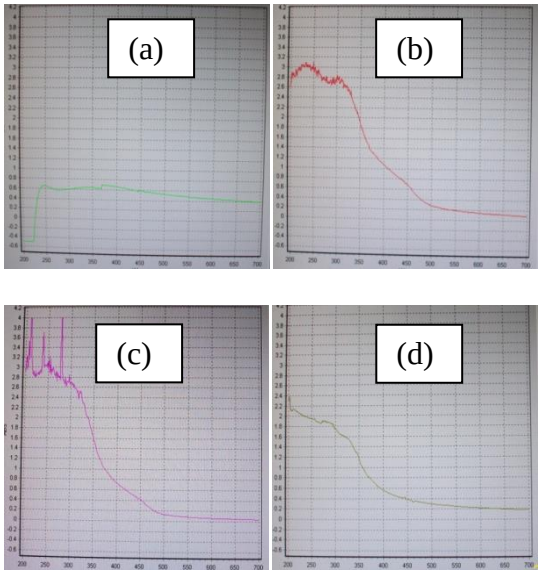

Figure 6: Spectral scan of the of nanocrystals formed at pH 3 (a), 5 (b), 7 (c), and 9(d).

Nanocrystal formation was seen maximum at pH 7 and declines at pH 9 (Fig. 6).

3) FTIR Analysis of nanoparticles

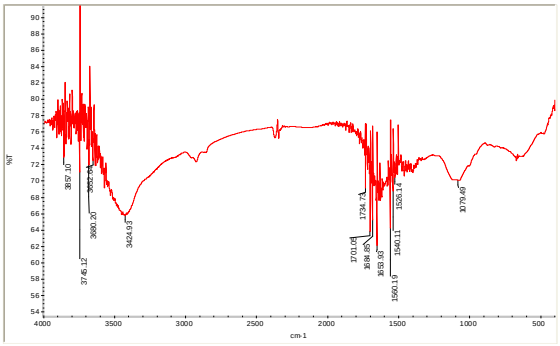

Figure 7: IR spectra of CdS nanoparticles

IR spectra of CdS nanoparticles is presented in fig. 7. The IR stretching frequencies for CdS nanoparticles observed as two bands, medium to strong, at 660 and 690 cm^{-1}. The band at 3424 cm^{-1} is due to O-H stretching vibrations of water molecules as the nanoparticle synthesis was carried out in aqueous medium hence consider to be green process.

The bending vibrations of $O=C-NH_2$ is appeared at 1684-1653 cm^{-1} indicated the presence of protein or protein like moiety playing a role in biosynthesis CdS nanoparticles. CdS particles showed two stretching bands of C-O at 1120 and 1079 cm^{-1} shown the involvement of carbonyl moieties [5, 6]. Band stretching at frequencies 1701 and 1560-1526 cm^{-1} indicates presence of C-H starching because of several metabolites produces by the organism. The role of this metabolite needs to address in further studies.

4) Particle size distribution analysis

TABLE III. THE PARTICLE SIZE DISTRIBUTION ANALYSIS AT PH 5, 7 AND 9

Parameter	**pH 5**	**pH 7**	**pH 9**	
Diameter (nm)	1.926	0.6389	1.485	5.664
Number (%)	100	100	~100	~0
Width (nm)	0.288	0.074	0.2024	0.9345
M.Wt. (kDa)	3.08	0.233	1.5	38.5

Cultivating *E. coli* and a strain of *Klebsiella pneumoniae* (formerly *K. aerogenes*) in the presence of $CdCl_2$ and

Na_2S results in the intracellular formation of CdS [11-13]. However, present isolate CdR3 shows extracellular synthesis of CdS nanoparticles. Particle size distribution data showed pH dependence of particle size distribution. The results obtained were presented in Table 3 for CdS nanoparticle biosynthesis. The particles synthesized at the pH 5, 7 and 9 showed CdS nanoparticle diameter of 1.966, 0.6389, and 1.415 nm respectively. This shows at neutral pH CdS nanoparticles synthesized were small in size compared to other two pH tested. In addition, nanoparticles synthesized at pH 9 showed synthesis of larger size of particles however in very few numbers.

Bravibacterium casei biosynthesized the nanoparticles of 10-30 nm diameter at pH 9 [14]. Magnetic octahedral nanoparticles of sizes below 12 nm are formed extracellularly on the surface of the thermophilic iron reducing bacterial strain *Thermoanaerobacter ethanolicus* (TOR-39) [11, 15] within 24–48h after the addition of $CdCl_2$ and 0.05% cysteine hydrochloride, *Clostridium thermoaceticum* in late exponential- to early-stationary phase precipitated bright yellow CdS crystals on the surfaces of the cells as well as in the medium [16-18]. A facultative anaerobic bacterium, *Enterobactercloacea* also can bioreduce selenite to selenium both inside and outside aggregates. A simple route for the synthesis of cadmium sulfide nanoparticles by photosynthetic bacteria *Rhodopseudomonas palustris* has been demonstrated [2, 12]. It was observed that the cysteine desulfhydrase producing S^{2-} in the *R. palustris* was located in cytoplasm, and the content of cysteine desulfhydrase depending on the growth phase of cells was responsible for the formation of CdS nanocrystal, while protein secreted by the *R. palustris* stabilized the cadmium sulfide nanoparticles. In addition, *R. palustris* was able to efficiently transport CdS nanoparticles out of the cell [12].

Bacterium *Clostridiumm thermoaceticum* precipitates CdS at the cell surface as well as in the medium from $CdCl_2$ in the presence of cysteine hydrochloride in the growth medium [19]. Most probably, cysteine acts as the source of sulfide.

4) X-RD analysis of biosynthesized CdS nanoparticles

Results of the X-ray diffraction studies were carried out for CdS samples and a typical pattern for sample is presented in Fig 8.

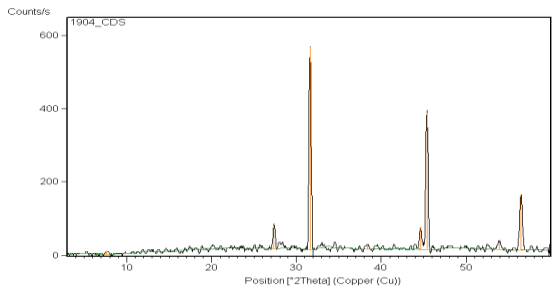

Figure 8: X-RD of CdS nanoparticles

The XRD patterns revels that, the particle size is dependent on the biomass. The particle sizes (d) were calculated using Debye Scherrer equation [20].

$$d = 0.9\lambda/B\cos\theta$$

Where, λ is the wavelength of the X-ray used, θ is the angle of reflection and B is the full width at half maximum. From Fig. it can be seen that broad peaks that observed in the XRD patterns of CdS are belonging to cubical structure of CdS nanocrystals because it has three main peaks at 31.65°, 44.62° and 56.44°. The mean particle diameter is calculated to be 0.6 nm from the Scherrer formula**.** The CdS nanoparticles could possesses the hexagonal phases with (002) plane. Have reported the hexagonal wurtzite structure of CdS for the plane (002) [21]. The hexagonal phase of CdS has two main peaks at 28.30 (101 planes) and 48.10 (103 planes) whereas the cubic phase has three main peaks at 26.50 (111 planes), 43.90 (220 planes) and 51.90 (311planes) [22]. The absence of planes referring to cubic structured CdS in XRD patterns of glucose capped CdS indicates the presence of only hexagonal CdS nanoparticles.

IV.CONCLUSION

The contaminated soil may serve as the potential source for isolation of metal resistant organisms. The isolate could use for green synthesis of nanoparticles of various size. In present study isolated Cd resistant organism *Gluconobacter* sp. was able to synthesize very small monodisperse nanoparticles at ambient conditions ranging from 0.638 to 1.926 nm. The size of particles was found to be pH dependent. This and similar metal resistant organism need to characterized vigorously for a green alternative of nanoparticle synthesis.

V. ACKNOWLEDGEMENT

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