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Antimicrobial activity of biostabilized palladium nanoparticles against bacterial pathogens.

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ABSTRACT

In the present work, we reported the use of biosurfactant isolated from *C. krusei* for stabilizing palladium nanoparticles. The formation and characterization of palladium nanoparticles were done by UV-vis spectroscopy, XRD, FTIR analyses. The biologically stabilized palladium nanoparticles were characterized by a peak at 417 nm in the UV-vis spectrum. The crystallinity with an average size of 50 nm for palladium nanoparticles was assured by XRD analysis. SEM and TEM showed the spherical morphology of the nanoparticle. The antibacterial activities of palladium nanoparticles were compared based on diameter of inhibition zone in disc diffusion test and minimum inhibitory concentration of nanoparticles dispersed in batch cultures. *Staphylococcus aureus* and *Streptococcus mutans* showed the highest sensitivity to palladium nanoparticles compared to the other strains and was adversely affected by the palladium nanoparticles. Mechanisms of antimicrobial activity of palladium nanoparticles against the pathogens were studied by Chemiluminescence assay with the release of hydrogen peroxide with increase in palladium nanoparticles concentration. Maximum protein leakage and growth inhibition were observed in case of *Staphylococcus aureus* and *Streptococcus mutans.* Thus biostabilized palladium nanoparticles were synthesized and proved to be a good novel antimicrobial material.

Keywords: Palladium, nanoparticles, *Staphylococcus aureus, Streptococcus mutans*, antimicrobial activity.

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INTRODUCTION

Over the last two decades metal nanoparticles have been extensively studied because of their electronic, catalytic and unique optical properties. This imparts a promising feature in the application of nanoparticles in various field *viz.* optoelectronics, optics, nanostructure fabrication biochemical sensing [1]. Various approaches have been recorded for the synthesis of nanoparticles such as chemical reduction [2; 3], photochemical reduction and aerosol method [4; 5]. However these methods may have adverse effects in medical applications [6], therefore an alternative and environmentally benign method of biological synthesis of nanoparticles have been developed. The use of biological agents *viz.* leaf extract [7] for the synthesis of palladium nanoparticles (PdNPs) serves as an advantage over the chemical process due to its compatibility for pharmaceutical and eco-friendliness.

Thin coating of palladium nanoparticles can be used for preparation of microbial resistant objects as it possesses antimicrobial property even at low concentrations. Various mechanisms have been involved such as release of H_2O_2 from the surface of palladium nanoparticles that inhibited the microbial growth [8]. However, only few studies have reported to assess the antibacterial activity against pathogenic species *viz. Staphylococcus sp.* and *Eschericia coli* using palladium nanoparticles [9]. Moreover, no works have reported about the analysis of antimicrobial activity against bacteria such as *Streptococcus sp.*

The present study deals with the use of biosurfactant isolated from *C. krusei* for the stabilization of palladium nanoparticles. Biologically stabilized nanoparticles were characterized by UV-vis, FTIR, SEM and TEM. Also, we have investigated the antimicrobial activity against three bacterial species *viz*. *S. aureus, S.mutans* and *E. coli*. Our result showed that biologically stabilized palladium nanoparticles produced a high bactericidal activity.

MATERIAL AND METHODS

Chemicals

All the chemicals were of the highest purity grade. $PdCl_2$, $NaBH_4$ were obtained from Sigma-Aldrich .Chemicals for minimal media preparation including NH_4NO_3, KH_2PO_4, $MgSO_4$. $7H_2O$, yeast extract, glucose, were obtained from Hi Media, Mumbai, India.

Yeast growth and Biosurfactant production

The yeast species used in the present study i.e., *Candida krusei* was isolated from electronic industrial wastewater, Elambur, Perambalur, Tamilnadu, India. The yeasts were phenotypically characterized and identified to species levels by Vitek 2 Compact Yeast card reader with software version V2C 03.01 from Council for Food Research and Development (CFRD), Kerela, India. The isolate was subcultured in YEPD (yeast extract: 10g/L; peptone: 20 g/L; dextrose: 20 g/L) agar slant and maintained at 4°C. Synthesis and Characterization of the biosurfactants produced by yeast species was done following the methodology of Basak et al., 2014 [10].

Preparation of Palladium nanoparticles

Palladium nanoparticles were synthesized following the procedure of Wang, 2008 [11] using yeast biosurfactants for particle stabilization. The PdNPs was characterized by UV-Vis spectral ananlysis scanning electron microscopy (SEM) analysis and TEM analysis.

Applications of biofunctionalized PdNPs as an antimicrobial agent

Antimicrobial assay was performed by Disk diffusion assay against the target pathogenic bacteria collected from clinical microbiology laboratory nearby hospital in Perambalur. Pathogenic bacterial sensitivity to antibiotics is commonly tested using a disk diffusion test, using antibiotic infused disks [12]. A similar test with biostabilized nanoparticle loaded disks was used in this study. The disks were made following the methodology of Johnson and Washington, 1976. Each disk were punched out containing 10 mg/ml nanoparticles and stored in a desiccator at room temperature. The bacterial suspension (100 µl of 10-4 to 10-5 CFU/ ml) was applied uniformly on the surface of a Mueller Hinton (MH agar) plate before placing the disks on

the plate (3 cultures for each plate). The plates were incubated at 37° C for 24 h, after which the average diameter of the inhibition PdNPs surrounding the disk was measured with a ruler with up to 1 mm resolution. The mean and standard deviation (SD) reported for each type of biosurfactant stabilized nanoparticle and with each microbial strain were based on three triplicates.

To determine the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC), appropriate volume of pathogens were inoculated in NB and SD broth at different concentrations of PDNPS varying from 0.2-10.0 mg/ml and kept for incubation at 24 h and 72 h respectively for bacteria and fungi at 37° C. Control tubes containing only inoculated broth and PDNPS solution were kept. The MIC and MBC were determined following the procedure of Hammond and Lambert 1978 [13].

Mechanism of the antimicrobial activity of the particles

Intracellular protein leakage

The effect of the PdNPs in the intracellular protein level was monitored. The intracellular protein leakage was determined from the method specified [14].

Lactate dehydrogenase (LDH) quantification

Lactate dehydrogenase (LDH) is present in the cytoplasm of the bacteria. LDH level was analyzed to determine the damage caused by the particles to the pathogenic bacteria. The LDH level was determined by the method specified [14].

Alkaline phosphtase (ALP) quantification

The amount alkaline phosphatase (ALP) was measured by slightly modifying methods specified [14].

RESULT AND DISCUSSION

In the present study, *Candida krusei* was the model organism for the synthesis of biosurfactant. The yeast species *Candida krusei* showed the biosurfactant production. The drop collapse test and oil displacement test were also conducted for the primary screening of biosurfactant production. The oil displacement test is an indirect measurement of surface activity of a surfactant sample tested against oil. The biosurfactant produced by *C. krusei* reduced the surface tension of distilled water to a minimum value of 35.7 ± 0.6 mN/m with low value of CMC. The results suggested that the biosurfactant from the yeast species provided excellent properties in terms of reduction of surface tension and a low value of CMC. The emulsification index of the biosurfactant was evaluated by determining the emulsifying activity with respect to vegetable oil. The biosurfactant exhibited different stabilization property with the vegetable oil tested as expressed in terms of emulsification index of 72 ± 0.4 %.

Biochemical and analytical characterization of biosurfactants: Carbohydrate, protein, and lipid estimation of the biosurfactant produced by yeast characterized the biosurfactant as a glycolipid with carbohydrate (35.29 %) and lipid (53.42 %) as major constituents.

The synthesis of PdNPs using yeast biosurfactant as a biostabilizer was confirmed by UV-Vis spectrum (Fig.1). The color of the solution of $PdCl_2$ gradually changed from light yellow to dark brown following the addition of the stabilizing agent with stirring for 2 h at 60° C, indicating the formation of PdNPs. The reference sample $PdCl_2$ showed a peak at 417 nm due to the absorption of Pd(II) ions. For the reduced samples, the peak at 417 nm was absent, and a broad continuous absorption was observed, indicating a complete reduction of Pd(II) ions to PdNPs.

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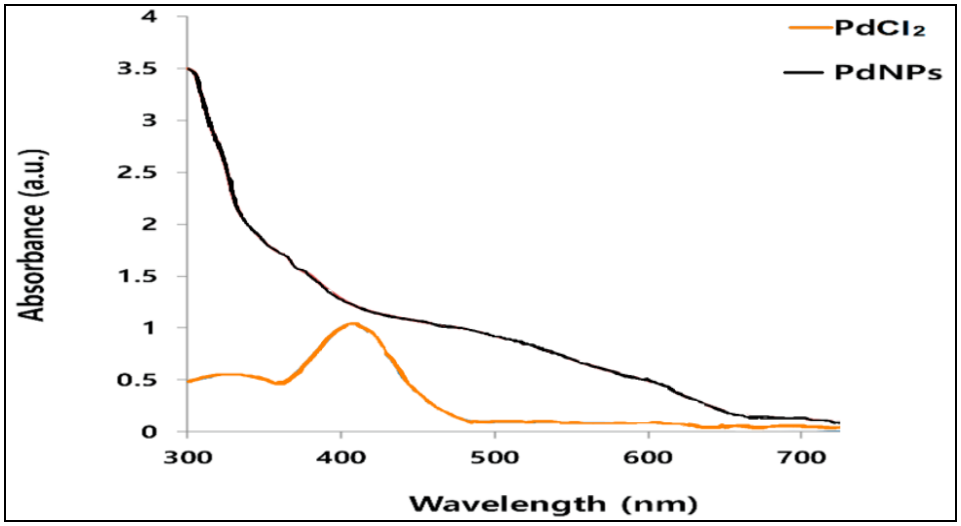

Fig. 1. UV-Vis spectra of $PdCl_2$ solution and biostabilized PdNPs

Scanning electron microscopy

Surface morphology of PdNPs and yeast biosurfactant stabilized PdNPs were studied using SEM (Fig 2a, 2b). A difference in surface smoothness was noted. Biostabilization by yeast biosurfactant resulted in an enhancement of surface smoothness of the nanoparticles.

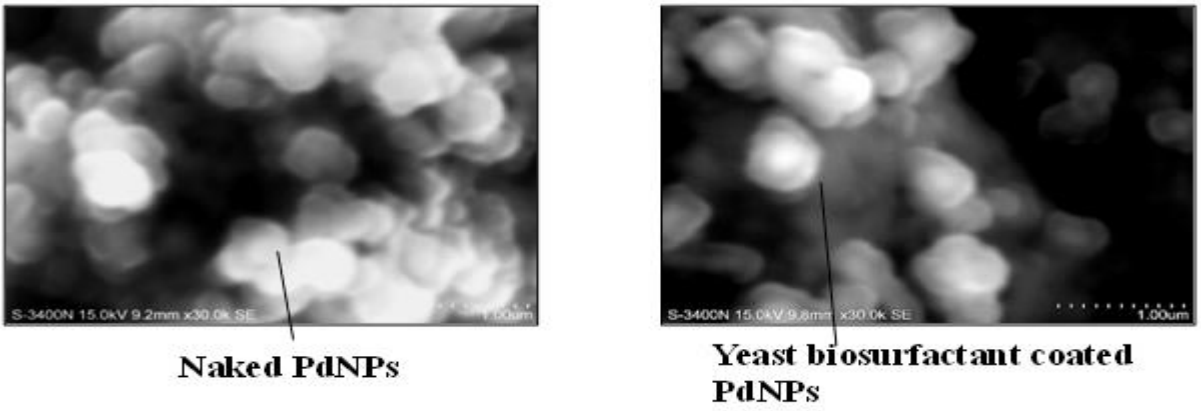

Fig. 2. Scanning electron micrograph of (a) naked PdNPs and (b) biostabilized PdNPs synthesized at 60° C.

These pictures substantiate the approximate spherical shape to the nanoparticles, and most of the particles exhibit some faceting. From the pictures, it also can be seen that the size of the nanoparticle is less than 50 nm (fig. 3).

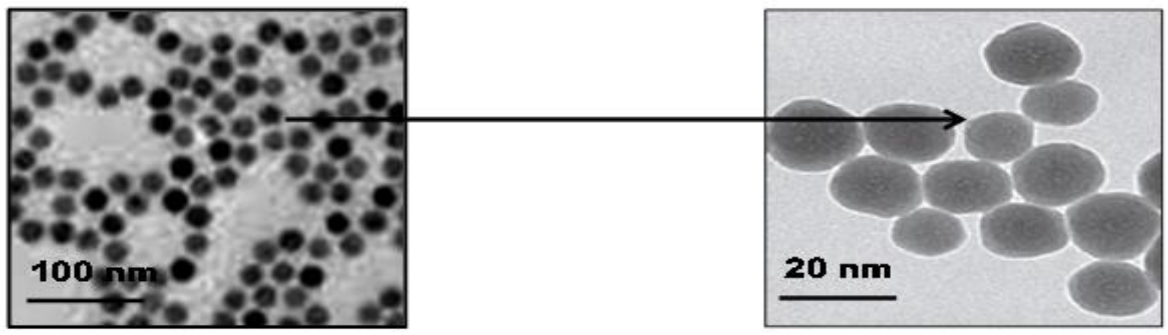

Fig. 3. Transmission electron micrograph of biostabilized PdNPs

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FTIR spectra of biostabilized PdNP

The peaks obtained at 3401cm^{-1} represents O-H stretching vibration. The peak obtained at 1652 cm^{-1} indicated C=O stretching. The absorption peat at 1241 cm^{-1}and 1029 cm^{-1} indicated C-O-C antisym stretch in esters and lactones and C-N stretching vibrations of aliphatic and aromatic amines respectively. The FTIR spectra of the polymer evidenced the presence of carboxyl groups, which may serve as binding sites for divalent cations. A comparison of functional groups presents that EPS having a higher number of variable functional groups was more complex than the other EPSs reported previously (fig. 4.).

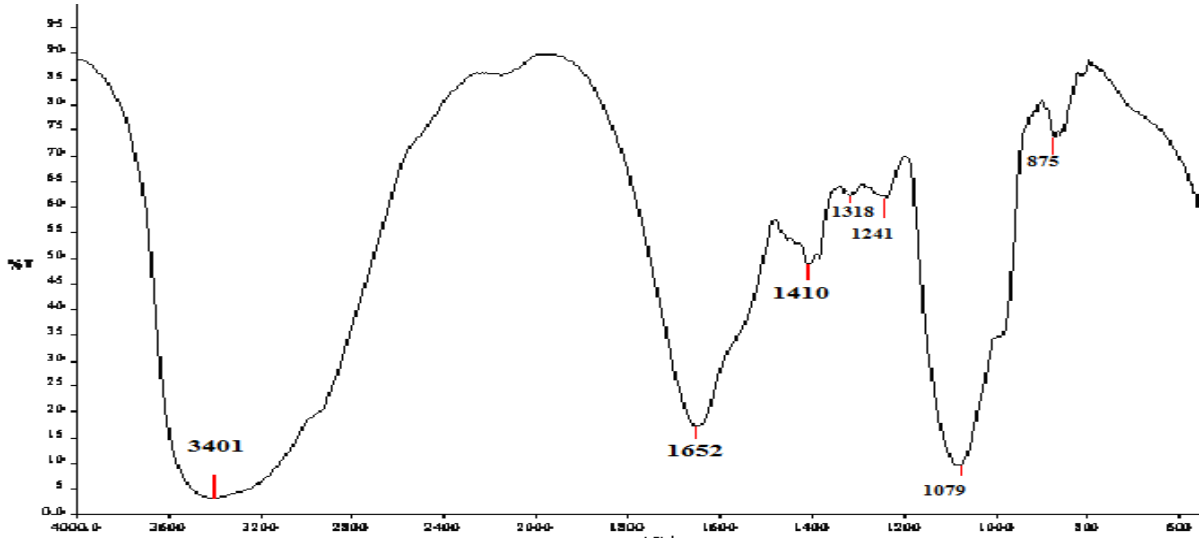

Fig. 4. FTIR spectra of biostabilized PdNPs

Applications of biostabilized Pd NPs as an antimicrobial agent

Determination of minimum inhibitory and microbicidal concentration

The MIC and MBC showing the antimicrobial activity of the biologically stabilized palladium nanoparticles dispersed in batch cultures is represented in Table 1. The concentration range taken for this study was 0.2-1.0 mg/ml for the bacterial species. In order to understand the microbicidal effect of biostabilized PdNPs, 100µl of aliquot from the incubated broth each containing pathogens were plated on NA and incubated for 24h. It was observed that the microbicidal effect of biostabilized PdNPs is dependent on the concentration of palladium nanoparticle that inhibits the microbial growth. Significant antimicrobial growth was not seen at concentrations below 0.4 mg/ml for all the biostabilized PdNPs samples. This might be due to the presence of limited Pd^{++} ions that act as micronutrient for the growth and metabolism of the microbes [15].

Table 1. MIC and MBC of biologically stabilized PdNPs for various pathogenic bacteria

Activity	Test Organisms	MIC (mg/ml)	MBC (mg/ml)
Antibacterial	*S. aureus*	0.6	6.8
	S. mutans	0.82	7.1
	E. coli	1.0	8.4

Disk diffusion assay

The antibacterial activity of biostabilized palladium nanoparticle was compared for various microorganisms based on the diameter of inhibition PdNPs in disk diffusion test. The diameters of the zone of inhibition reflects the extent of susceptibility of the microorganisms . The strains susceptible to biostabilized palladium nanoparticle exhibit larger diameter for the PdNPs of inhibition, whereas resistant strains exhibit smaller diameter. The disks with palladium nanoparticles exhibited a larger PdNPs of inhibition for *S. aureus* compared to *E. coli* and *S.mutans* and strains selected for this study (Figs. 5). The diameter of the PdNPs of

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inhibition for palladium nanoparticle impregnated disks for *S. aureus* was almost 20–30% greater than that observed with the *S. mutans* strains selected for this study. Similarly, for S. aureus the palladium nanoparticle impregnated disks were found to be significantly more effective compared to E.coli strain by 40-50 % (Table 2). Since the diameter for the PdNPs of inhibition was measured on agar plates using a ruler with 1 mm resolution, the possibility of measurement errors exist; however, the method illustrates the potential bacteriocidal effect of biostabilized palladium nanoparticles to different microbial strains.

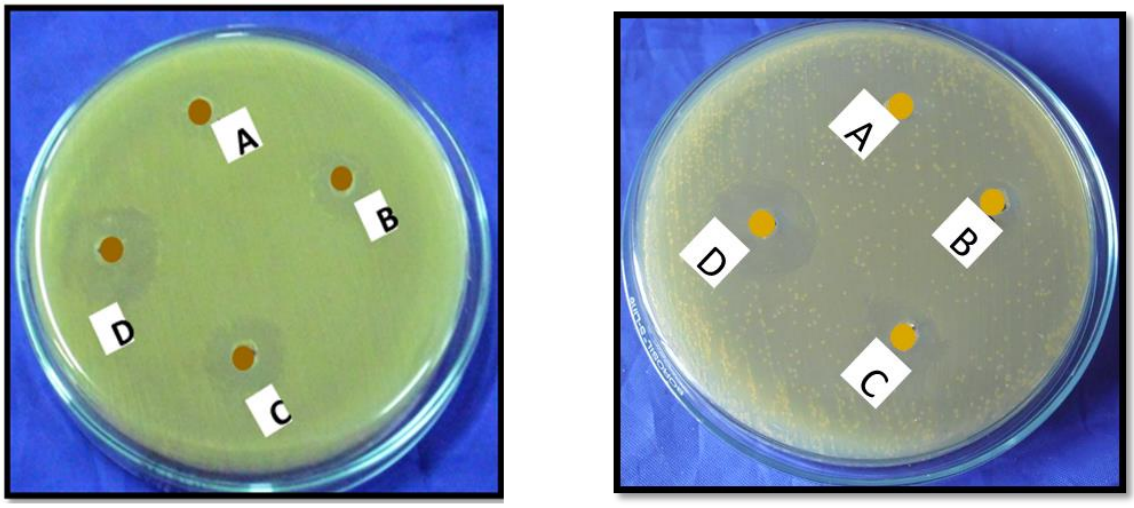

Fig. 5. Zone of inhibition against (A) Water (control) (B) *E.coli* (C) *S. mutans* and (D) S*. aureus* with respect to 10 mg/ml PdNP

Table 2. Antimicrobial activity of biostabilized PdNP solution

Treatment (w/w)	Inhibition zone at specified concentration (mm ± SD)			
Bacterial species	Water	*S. aureus*	*S. mutans*	*E. coli*
	0.0 ± 0.1	11 ± 0.2	8.5± 0.3	62 ± 0.1

The mechanism of antibacterial activity was better understood on the basis of generation of highly reactive species such as H_2O_2 from the surface of biostabilized PdNPs that penetrate the cell membrane and kill the bacterial cells [16, 17]. The amount of H_2O_2 released was related with the increasing concentration of biostabilized PdNPs. It was observed that biostabilized PdNPs at concentrations of 0.6 mg/ml, 0.8 mg/ml and 1 mg/ml released H_2O_2 at concentrations of 0.04µg/l, 0.37µg/l and 3.1µg/l respectively. Once the cell membrane got captured by biostabilized PdNPs, it remained tightly adsorbed on to the surface of the bacteria thereby preventing further bacterial action and increasing bactericidal efficiency [18].

Analysis of protein leakage and effect of PdNPs on microbial growth

Proteins, minerals and genetic material release out through damaged cell membrane leading to cell death. The amount of protein discharged from the cells increased with the increase in concentration and contact period of PdNPS. The amount of protein released in the suspension by the bacterial pathogens *S. aureus* and *E.coli* respectively, analyzed through Bradford assay. It was noted that the amount of protein released through *S. aureus* was higher than the *E. coli*. The protein leakage for bacteria with varying treatments and concentration showed significant values of 1.112 and 1.220 at 6th and 12th h respectively whereas, the protein released for *E. coli* were 0.645 and 1.018 at the 6th and 12th h. The results indicated that the antibacterial effect of biologically stabilized PdNPS was maximum in *S. aureus* compared to *E. coli* through the highest protein leakage compared to other species. Once the intracellular content released into the cell suspension, it results to stress in the cell wall thereby producing more lactate dehydrogenase and leading to cell damage with prolonged exposure time [19]. This has been shown in fig. 6.

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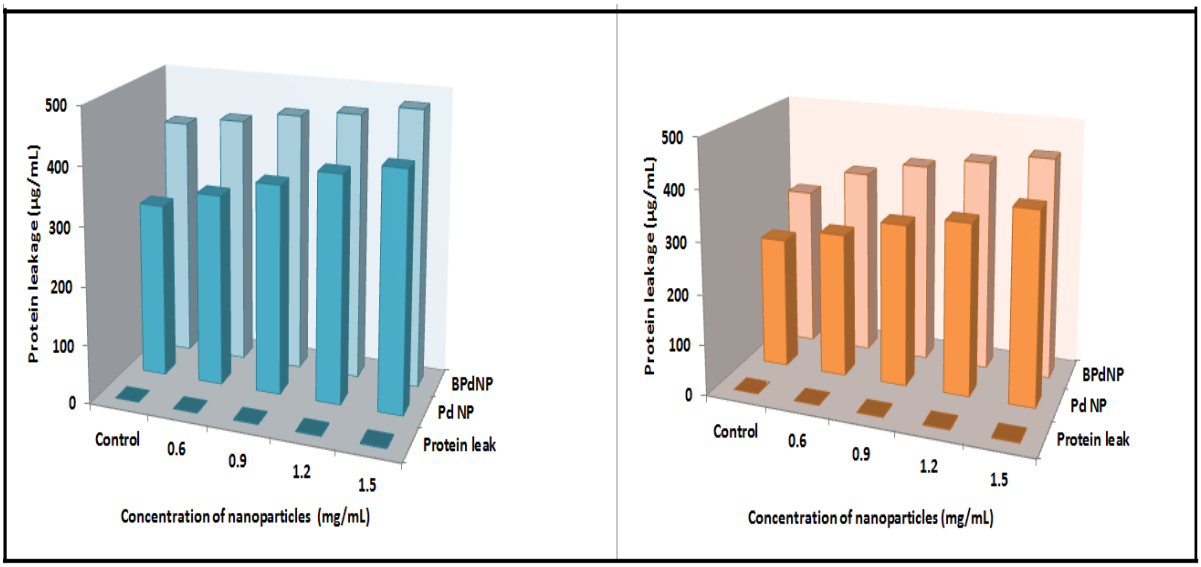

Fig. 6. Protein leakage analysis on interaction of pathogens with different concentrations of naked PdNPs and biostabilized PdNPs.

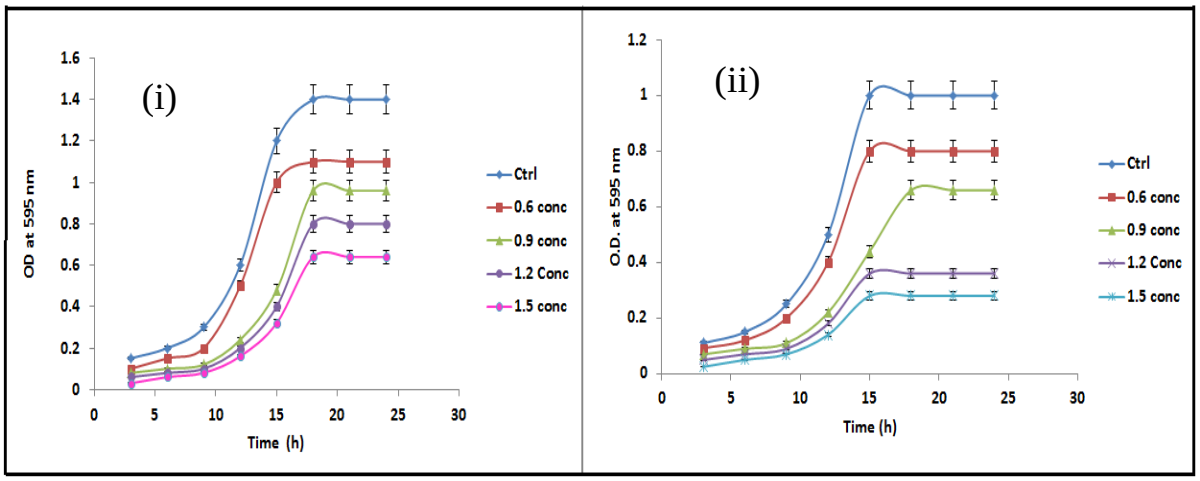

Fig. 7. Growth curves of (i) *S. aureus* and (ii) *S. mutans* treated with biostabilized PdNPs

Fig.7. represented the effect of biologically stabilized PdNPS on the growth of bacterial pathogens with respect to time studied by measuring OD at 595 nm. The growth inhibitions of the pathogens were noted as a function of time, suggesting significant differences in antimicrobial activity of the PdNPS. Both the microbial strains showed significant growth inhibition at a representative PdNPS concentration of 0.9 mg/ml during 24 h of incubation for bacterial pathogens. This might be due to the electrostatic attraction achieved between positively charged PdNPS and negatively charged cell membrane [20], interaction of palladium ions with microbes [21] and location of PdNPS [11]. The OD at 595 nm illustrated the function of cell density that correlated with the growth of the colonies. The growth of the microbes decreased with the increase in PdNPS concentration. At low concentrations of PdNPS significant inhibition in the growth was not observed whereas, at a concentration of 1 mg/ml and 1.5 mg/ml respectively for bacterial pathogens remarkable growth of inhibition was noticed.

Lactate Dehydrogenase activity

The amount of cell membrane damage of *S. aureus* and *S. mutans* cells were referred by LDH levels in cell medium. The LDH levels in the cell culture were increased in all treatment groups after exposure to biostabilized PdNPs for a period of 24 h in case of both the pathogens. The LDH levels were found to be maximum at a concentration of 1.5 mg/ml of biostabilized PdNPs for both the pathogens (fig. 8). The damage

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to the cell membrane directly led to the loss of minerals, proteins and genetic material causing stress in the cell wall. As a consequence, the cell walls produced more lactate dehydrogenase enzymes leading to cell membrane damage with respect to time [19].

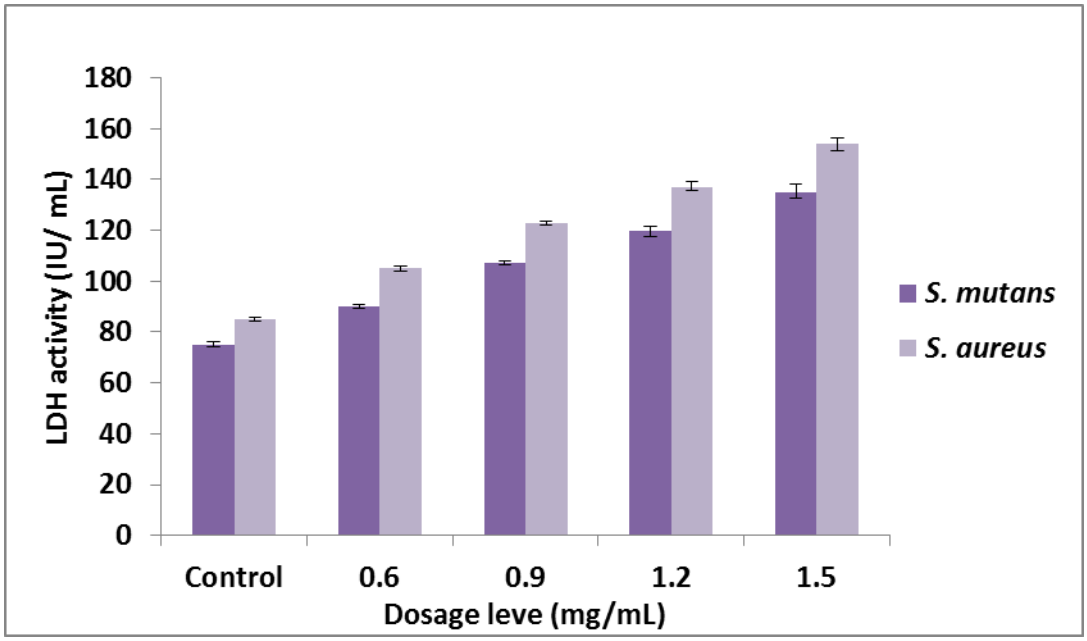

Fig. 8. LDH activities of pathogens in the cell culture medium after 24 h exposure to biostabilized PdNPs at different concentrations

Alkaline phosphatase is an enzyme present in the periplasmic space of the bacteria. The enzyme activity increases during phosphate starvation and sporulation [22]. In the present study, the ALP levels of *S. aureus* and S*. mutans* were increased upon treatment with PdNPs (Fig. 9).

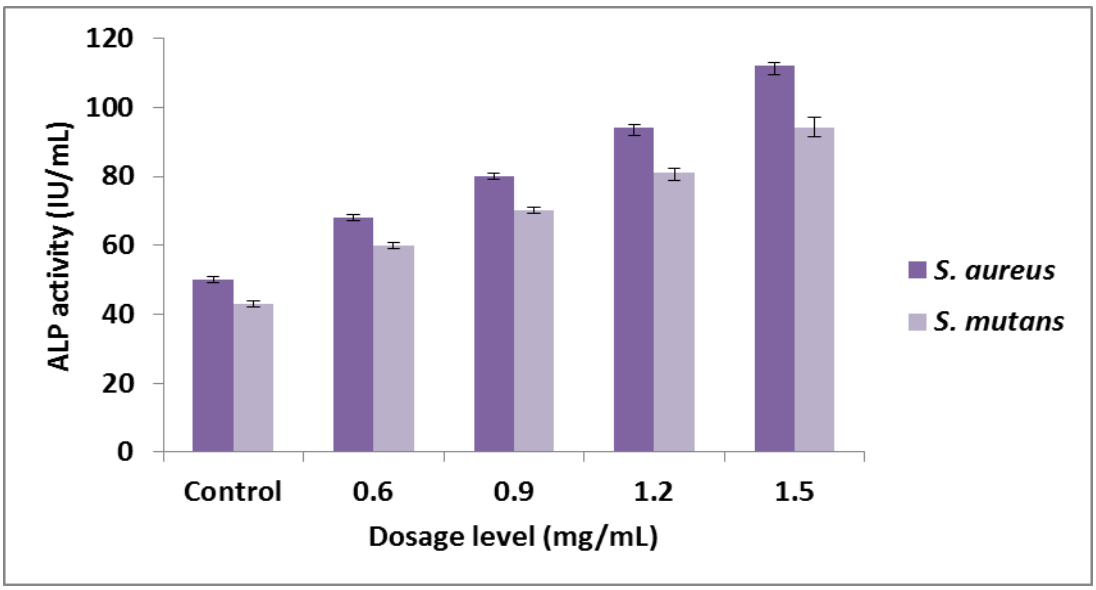

Fig. 9. ALP activities of pathogens in the cell culture medium after 24 h exposure to biostabilized PdNPs at different concentrations

This may be due to the oxidative stress imposed on the bacteria by the nanoparticles and in order to overcome the phosphate starvation, bacteria produce higher amounts of ALP. In the present results, *S. aureus* ($p < 0.05$) produced higher amounts of ALP when compared with gram negative bacteria.

CONCLUSION

Biosurfactant stabilized nanoparticles of size less than 50 nm were synthesized by a cost effective, ecologically benign biologically stabilizing agent, isolated from *Candida krusei*. The nanoparticles were characterized by UV-vis, FTIR, SEM and TEM analysis. The biosynthesized palladium nanoparticles were found to have significant antimicrobial activity against different type of microbes. Application of palladium nanoparticles based on this findings may lead to valuable discoveries in various sections like medical devices and antimicrobial agents.

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