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Biosynthesis of Zirconium Oxide Nanoparticles using Marine Actinobacteria and its Applications

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ABSTRACT

The aim of the present study was to biosynthesize Zirconium oxide nanoparticles from zirconium oxychloride with the help of marine Actinobacteria. The isolated Actinobacteria were grown in SS broth and the preliminary confirmation of the conversion of $ZrOCl_2$ to ZrO nanoparticles was obtained by UV-Vis Spectrophotometric analysis which showed highest peaks at 240 nm. The crystal nature of the nanoparticles was confirmed by XRD analysis to be Zirconium oxide with a particle size of 35 nm. The biosynthesized ZrO nanoparticle was used for drug delivery studies and antioxidant activity. Drug binding ability was tested with an antacid drug that was naturally coupled with the nanoparticles and the efficiency of binding and bond nature was studied by FT-IR analysis. The biosynthesized Zirconium oxide nanoparticles by the SDS2 Actinobacterial isolate showed high levels of antioxidant activity (64.8%) as opposed to a mere 36.12% activity without nanoparticles with the H_2O_2 scavenging test indicating a significant amount of antioxidant properties by the ZrO nanoparticles. It also showed prevention against free radical mediated damage caused to DNA.

Keywords: Zirconium oxide Nanoparticles, Anti-oxidant, Lipid peroxidation, H_2O_2 peroxidation test, β carotene assay

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INTRODUCTION

Actinobacteria represent the high G+C Gram positive bacteria and they constitute one of the largest phyla's amongst the bacterial group consisting of a wide morphological spectrum that varies from coccoid to hyphal forms and also wide physiological and metabolic spectra. Actinobacteria have been a major source of antibiotics like streptomycin and there is a possibility that these organisms might be producing substances that naturally mediate the conversion of appropriate substrates to nanoparticles under conditions like pH variations or other stress conditions [1].

Nanoparticles have a wide application now in the field of Biotechnology. They are mainly used because of their large surface to mass ratio; nanoparticles are a subject of interest in the medical field [2]. Various studies are now being conducted to see how this property of nanoparticles can be exploited in the field of drug delivery and to check for the stability of these particles in vitro, under the various environmental conditions that they will experience once inside a host.

Nanoparticles have a wide spectrum of applications in human health, right from using their natural cytotoxic activity, to tumor imaging and drug delivery. Radiation therapy is the most widely used cancer therapy currently over anticancer drugs. This is because these drugs are either poorly soluble in body fluids or they are degraded in the GI tract or because they are unstable in the blood. Some are so rapidly cleared from the system that they hardly have the time to migrate to the site of the tumor [2]. Hence they have to be administered in high doses. High doses imply high toxicity, and that includes necrosis of healthy cells along with the cancerous cells. Nano particles have the ability to bind to chemical compounds like drugs and also, they tend to release the drug at a slow rate. By functionalizing the nanoparticles, the whole nanoparticle-drug complex can be made soluble. This solves the above mentioned issues with anticancer drugs. By loading the drug onto the functionalized nanoparticle or simply by adsorbing the drug onto the nanoparticle, a smaller and yet more efficient dose of the drug can be administered, ensuring minimal toxicity. The drug can be targeted onto the tumor site and the slow release rate will control the excess drug diffusion which collectively enhances the efficacy of the drug. Zirconium Oxide nanoparticles are metallic nanoparticles that have been synthesized by chemical and physical processes in the past. Although the chemical and physical methods have been successful, the resultant nanoparticles seem to have harmful effects due to the solvents that have been used for their synthesis [3].

Oxidation of metabolic substrates is a pre requisite for the synthesis of energy molecules in the body. During this process, several free radicals are produced which have an unpaired or nascent electron. Nitrogen or Oxygen species having such nascent and free electron species are called Reactive Nitrogen (RNS) or Oxygen Species (ROS), respectively. Some of these free radicals are relatively long lived in the body. The presences of these ROS or RNS inside the human body are known to have adverse affects. They are known to cause membrane lipid peroxidation, DNA damage, protein damage to name a few [4]. These alterations are implicated in a number of human diseases and it also causes the aging. Lipid peroxidation that leads to adverse alterations is caused by free radical damage and lipid components seem to be highly prone to this action. Damage to proteins is catalyzed by free

radicals and this leads to loss in enzyme activity. This in turn leads to carcinogenesis and mutagenesis [5].

Therefore the focus of our study was to determine the biologically synthesized ZrO nanoparticles that are free from any chemical solvents could be used to prevent free radical damage to the body.

MATERIALS AND METHODS

Sample Collection and Isolation of Actinobacteria

Water samples were collected from Marina Beach (12°53'50N", 80°19'55E"), Chennai, Tamilnadu, India for the isolation of Actinobacteria. The samples were collected in sterile bottles which were then transported to the Molecular Biology and Microbiology Laboratory in VIT University, Vellore, Tamilnadu, India. The samples were processed immediately and it was plated on Starch Casein Agar (SCA) media upon serial dilution. The powdery and leathery colonies of actinobacteria that appeared on the petriplates were counted from the 5th day onwards upto 28^{th} day [6].

Fermentation process

Each culture was grown in SS broth containing 50% marine water and incubated on a rotary shaker at 110 rpm at 28°C for 7 days. The slow, constant shaking was to ensure that no wall precipitation takes place.

Biosynthesis of ZrO Nanoparticles

After incubation, the broth was collected and centrifuged at 10,000 rpm for 10 mins in a cooling centrifuge. The supernatant was used for testing the antioxidant properties and Zirconium oxide nanoparticle synthesis. To 100 ml of the supernatant from each culture, 1 mM of zirconium oxychloride was added in 250 ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker at 110 rpm for 24 hrs.

Characterization of the ZrO nanoparticles

UV Spectrophotometric Analysis

After the ZrO biosynthesis, a portion of the broth was subjected to UV Vis Spectrophotometric analysis (U-2800, Japan). Each supernatant was subjected to spectrophotometric analysis from 200 nm to 800 nm

XRD Analysis

XRD analysis (Bruker, D8 advance, Germany) is a non-destructive analytical technique of using X-rays to determine the crystallographic structure and physical properties of a material and this is used to determine the type of nanoparticles. The nature of the nanoparticle was analyzed after passing the powders through a 100 mesh sieve and then

placing it in the sample holder. The samples were scanned at 25° from 10° to 70° with a count time of 2.0 seconds using 10 mg of each sample [7].

Antioxidant Tests

H_2O_2 scavenging activity

The ability of Zirconium oxide to scavenge hydrogen peroxide was determined by the method developed by Ruch et al, 1989 [8]. A 40 mM solution of hydrogen peroxide in phosphate buffer (pH 7.4) was prepared. Three ml of the broth was added to 0.6 ml hydrogen peroxide solution and incubated for 10 mins at room temperature. The absorbance indicating the hydrogen peroxide concentration was determined by spectrophotometry at 230 nm against phosphate buffer without hydrogen peroxide and this was taken as the blank [9]. The percentage of scavenging of hydrogen peroxide was calculated by the formula,

$$\text{Percent scavenged } [H_2O_2] = \frac{A_0 - A_1}{A_0} X\ 100$$

Where, A_0 was the absorbance of the control reaction and
A_1 was the absorbance of the sample.

DPPH radical scavenging activity

The activity of scavenging of free radicals of 1,1-diphenyl-2-picryl-hydrazil (DPPH) was measured. A 0.1 mM solution of DPPH in ethanol was prepared. To 3 ml of the broth 1ml of the reagent was added and shaken vigorously, followed by incubation for 30 minutes at room temperature. Absorbance at 517 nm was observed spectrophotometrically. Lower the absorbance meant increase in the radical scavenging activity [9]. The unscavenged DPPH free radical concentration in the reaction mixture was calculated by the formula,

$$\text{DPPH scavenging effect (\%)} = 100 - [\frac{A_0 - A_1}{A_0} X\ 100]$$

Where, A_0 was the absorbance of the control reaction and
A_1 was the absorbance of the sample.

Anti - lipid peroxidation assay

For the anti-lipid peroxidation test, goat liver was collected immediately after slay from a slaughter house and the test was conducted about an hour after collection. The liver was rinsed with phosphate buffered saline to get rid of residual blood. To 100 mg of the liver tissue 0.9 ml of 1.5g/l KCl was added to make a 100g/L liver homogenate that was stored at -20°C. Hundred micro liter of 50 mM $FeSO_4$ and 2.8 ml of the goat liver homogenate, 1 ml of the sample was added. The mixture was incubated for 30 minutes at 37°C. After incubation, the reaction was stopped by adding 10% TCA-0.67% TBA in Acetic acid (50%). This mixture was then boiled for 1 hour at 100° C in a water bath. A separate tube containing 1 ml of the homogenate and the TCA-TBA mixture in Acetic acid serves as

the control. The mixture was centrifuged at 10,000 rpm and the absorbance of the supernatant was measured at 535 nm [10].

The percentage activity of anti- lipid peroxidation was calculated by the formula,

$$\%ALP = \frac{\text{absorbance of } Fe2^{+} \text{induced peroxidation} - \text{absorbance of sample}}{\text{absorbance of } Fe2^{+} \text{induced peroxidation} - \text{absorbance of control}} \text{ X } 100$$

β-carotene Bleaching Assay

For the β-carotene bleaching assay, 10 ml solution of Beta Carotene dissolved in acetone (2mg/ml) and a 10 ml solution of Linoleic acid in ethanol (2mg/ml) was prepared separately treand then mixed together with 10 ml of 2% molten agar and mixed thoroughly to get an orange mixture that was poured into petri plates and left to solidify in the dark. Using a sterile agar borer, wells were bored into each plate. With a micropipette, 100 µl of extract was pipetted into the wells and the plates were incubated in the dark for 4 hours at 45°C. Antioxidant activity is indicated by colour retention around the wells. The size of the zone of colour retention was also measured [11].

DNA Damage Inhibition Test

DNA damage inhibition was estimated by pBR322 which was prevented by the free radicals produced by the oxidation of H_2O_2. One microliter of the samples was added into a microcentrifuge tube containing one microliter of pBR322, followed by four microliter of 3% H_2O_2. The tubes were placed in a transilluminator for ten minutes. Four microlitres of tracking dye (bromophenol blue) was added into each tube and then transferred into a 1% agarose gel slab and electrophoresed. The electrophoresed samples were then observed on a UV transilluminator. If the sample has DNA damage inhibition properties then it would be observed by the intact DNA band pattern showing protection from free radical damage [12].

Drug Loading

An antacid drug was used to check for the drug loading capability. The SS broth was centrifuged and the supernatant thus obtained was processed further. Twenty five mg of the antacid drug was added to 100 ml of the supernatant of each culture in Erlenmeyer flasks and incubated for 10 minutes at room temperature. Following this 1 mM $ZrOCl_2$ was added and the flasks were incubated for 24 hrs again at room temperature.

Characterization of Actinobacteria

16s RNA sequencing was carried out to characterize the Actinobacteria sample SDS2.

RESULTS AND DISCUSSION

Isolation and characterization of Actinobacteria

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From the Marine sediment samples collected, 15 actinobacterial cultures were isolated. Of the fifteen isolates, only four isolates showed anti-oxidant activity. In another study by Zhong et al [13], a single strain of *Streptomyces* that they isolated was observed to have anti-oxidant property.

Identification of Actinobacteria

Partial 16S rRNA sequence of the bacteria was obtained and a neighbour-joined phylogenetic tree was obtained by omitting the unaligned regions [14]. Figure 1 shows the relationship between the species VITSDSB and related representatives of the Streptomyces genus**.**

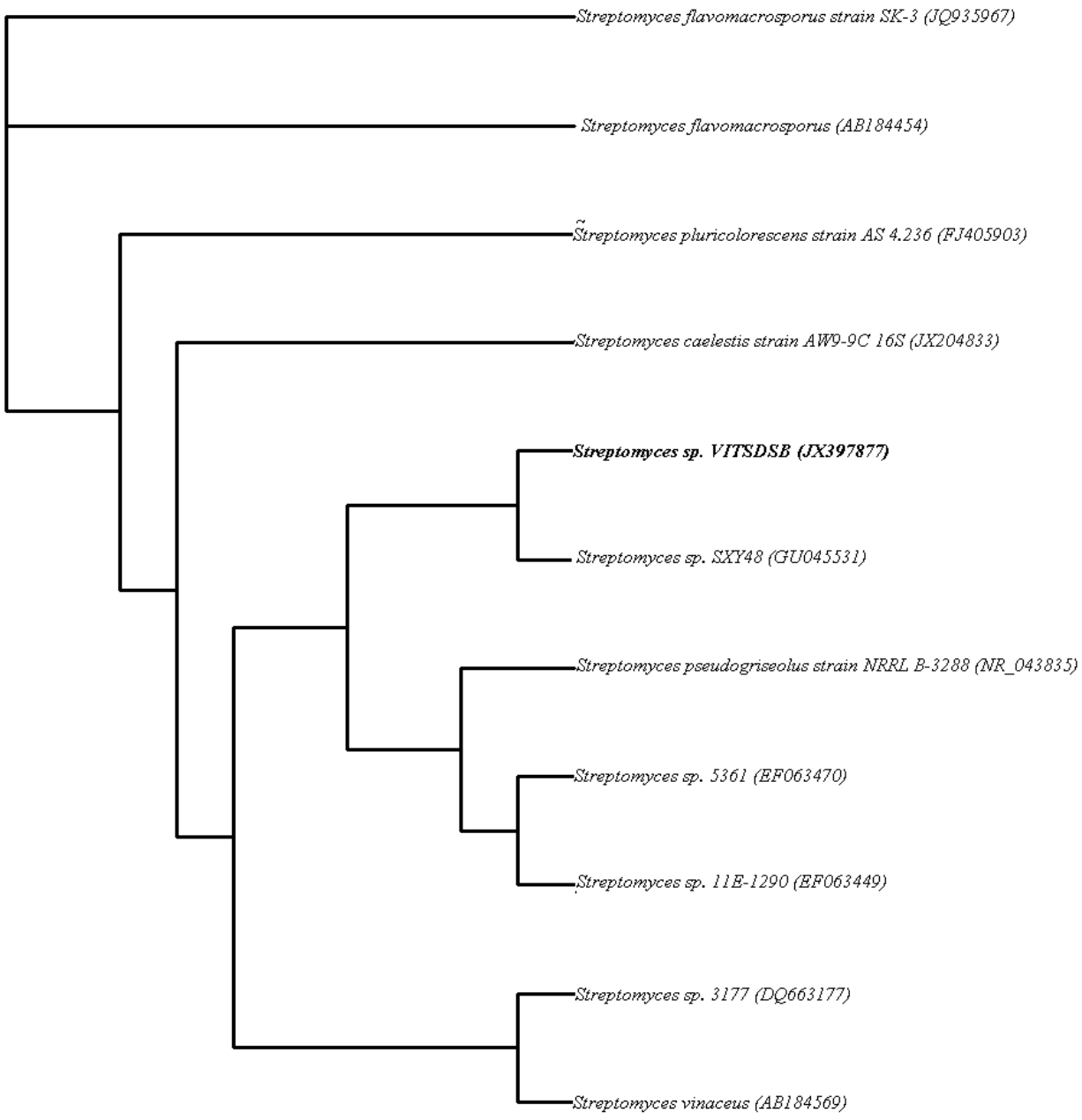

Fig. 1: The relationship between the species VITSDSB and related representatives of the Streptomyces genus.

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ZrO nanoparticle Biosynthesis and characterization

The bioconversion of Zirconium oxychloride to Zirconium oxide nanoparticles was detected by UV-Vis Spectrophotometric analysis and X-Ray diffraction studies. The results obtained are as follows.

UV Spectrophotometry

A prominent peak was observed in the range of 235 nm – 245 nm of the spectrum, with the highest absorbance at 240 nm seen for SDS-2 (Fig. 2)

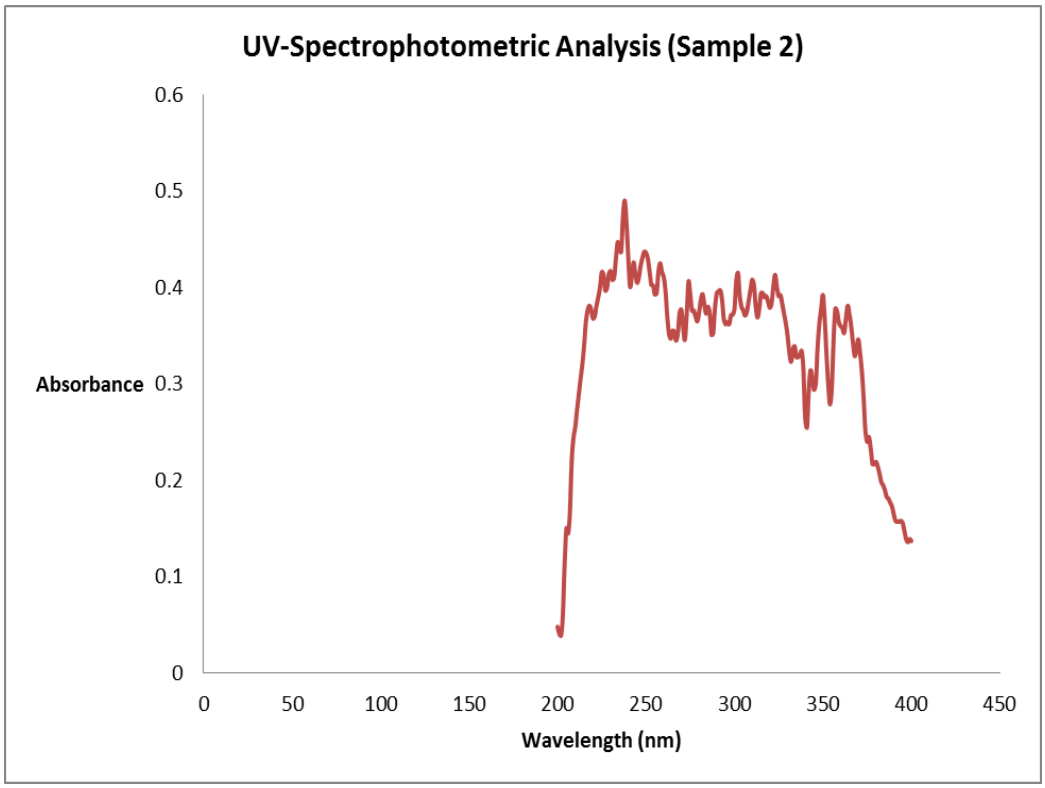

Fig. 2: UV Spectrophotometric analysis - SDS2

XRD Analysis

The XRD patterns were found to be coinciding with the JCPDS (89-9066) pattern for the zirconium oxide. The 2 ɵ peaks were 31.54, 44.90, 56.21 and 66.57 were corresponding to the planes for diffraction with 111, 112, 310 and 320. The particle size was calculated from the XRD pattern to be 35nm (Fig. 3).

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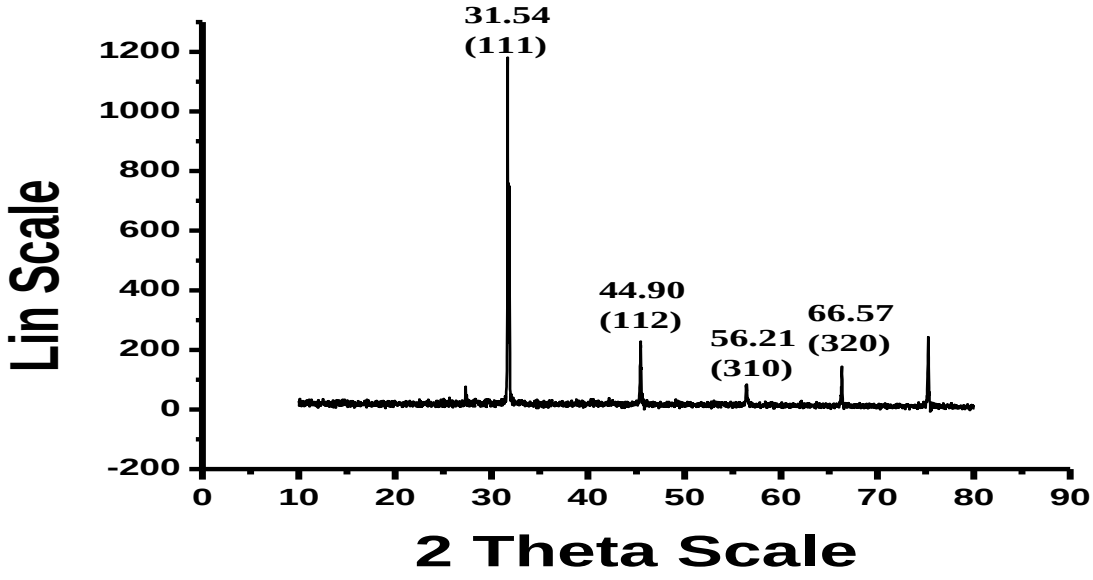

Fig. 3: XRD Graph confirming the presence of ZrO Nanoparticles

Antioxidant Activity

H_2O_2 peroxidation Test

SDS 2 had a lower anti-oxidant activity in the extract without ZrO. After synthesis of the nanoparticle, the activity of SDS 2 increased greatly. It can be inferred that presence of the nanoparticle had an effect on the culture's ability to scavenge the free radicals produced by H_2O_2 (Table 1).

Strains	Without ZrO	With ZrO
SDS 1	66.99%	64.21%
SDS 2	36.12%	64.8%
SDS 3	67.46%	46.72%
SDS 4	67.39%	64.5%

Table 1. H_2O_2 peroxidation activity

DPPH Scavenging activity

The extract from SDS 2 showed highest anti-oxidant activity of 32.347% and showed better DPPH scavenging activity compared to cultures SDS1 and SDS3 (Table 2). In another study by Thenmozhi et al (2010) [15], intracellular extracts from *Streptomyces* VITTK3 showed 22% activity.

SDS 1	31.045%
SDS2	32.347%
SDS 3	26.429%
SDS 4	25.251%

Table 2: DPPH scavenging activity (with ZrO)

Anti- Lipid peroxidation Test

SDS2 had comparatively lesser activity than the other cultures. After the ZrO nanoparticles were synthesized, the culture SDS2 showed an increase in the activity. This may suggest a possible effect of the nanoparticles on the anti lipid peroxidation activity (Table 3).

Strain	Without ZrO	With ZrO
SDS 1	9.95%	21.223%
SDS 2	9.36%	37.61%
SDS 3	9.657%	34.88%
SDS 4	9.76%	26.66%

Table 3: Anti- Lipid peroxidation activity

β-carotene Bleaching Test

The region surrounding the well containing the SDS1 extract having Zirconium oxide nanoparticles showed color retention. This indicates that the extract with the nanoparticles had a protective effect in preventing the discoloration of β-carotene caused by free radicals derived from oxidation of linoleic acid [11].

DNA Damage inhibition

After electrophoresis, the gel was observed on a UV transilluminator. Only culture SDS2 showed a single band which indicated its DNA protecting ability (Figure 3).

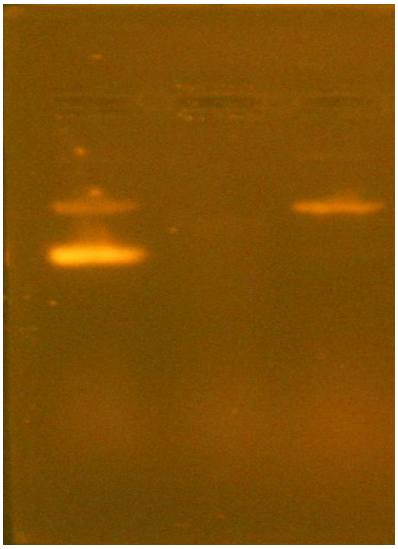

Fig. 4: Band of Protected DNA

Drug Loading

The preliminary results of drug loading were obtained from FT-IR analysis and they showed that the drug by adsorption had formed a complex with the ZrO nanoparticles by natural coupling.

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Fig.5: ZrO nanoparticles

Fig. 6: Drug loaded ZrO nanoparticles

CONCLUSION

Zirconium oxide nanoparticles have a variety of properties that can be exploited for various practical applications. By FT-IR analysis, we were able to show that by natural coupling, the drug we used to check for drug binding was successfully adsorbed onto the nanoparticles and further tests need to be conducted to study drug release kinetics. In further research we are making efforts to study the in vitro and in vivo drug release kinetics to determine the rate of drug release from the matrix, which in this case is the ZrO nanoparticles, thus determining nature of the ZrO nanoparticle's sustained and slow drug release properties. From our tests we have shown that the nanoparticles themselves showed high levels of antioxidant activity, antioxidant drugs could be coupled with the nanoparticles thus forming a complex with elevated levels of antioxidant activity. It has been proved that lipid peroxidation effects might ultimately lead to mutagenesis and carcinogenesis [16]. So, since the nanoparticles are free radical scavengers they can be used as agents to prevent the development of cancerous growths. The properties of ZrO nanoparticles can be explored further by performing various other chemical and biological confirmatory tests. These aspects can be engineered in such a way as to benefit medical as well as industrial applications that are already widespread but not economically feasible or still very expensive to be readily available to the general public. The particle size being 35nm means that the nanoparticles have large surface area to volume ratio. This means that for its volume, a higher ratio of drug to nanoparticle formulation can be constructed enabling a higher dose per milligram and the prospects of this can be further studied.

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