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RESEARCH ARTICLE

BIOSYNTHESIS AND ANTIBACTERIAL ACTIVITY OF TITANIUM NANOPARTICLES USING LACTOBACILLUS

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ABSTRACT

The development of eco-friendly technologies in material synthesis is of considerable importance to expand their biological applications. A variety of green nanoparticles with well-defined chemical composition, size, and morphology have been synthesized by different methods and their applications in many technological areas have been explored. Efficient and green chemistry approaches has led to the use of microorganisms.

Native Lactobacillus of Iraq derived from some local fermented products was used in this study. Total of 15 Lactobacillus isolates were isolated from local fermented food in Najaf city inoculated on de Man, Rogosa and Sharpe (MRS) agar. Plates were incubated for 24 hrs at 30 °C under anaerobic condition. Titanium nanoparticles had antibacterial activity against indicator strain (Pseudomonas aeruginosa). The efficient isolate (MT14) of Lactobacillus was selected for titanium nanoparticles formation and identification.

Titanium nanoparticles formation by Lactobacillus in some aspects was observed during the study. Characterization of nanoparticles was done by SEM /EDS analysis. Scanning electron Microscope revealed the formation of spherical nanoparticles with size ranging between 50-90nm. Energy dispersive spectroscopy (EDS) was done using SEM instrument equipped with Bruker EDS attachment showed the optical absorption peak.

The efficient isolate (MT14) of Lactobacillus was identified by morphological and biochemical characteristics and confirmed by using species specific primers for the polymerase chain reaction (PCR) that enable specific detection of Lactobacillus rhamnosus. The primers amplify a 122 bp DNA sequence of the genus Lactobacillus rhamnosus.

INTRODUCTION

The use of microbial cells for the synthesis of nanosized materials has emerged as a novel approach for the synthesis of metal nanoparticles. The interactions between microorganisms and metals have been well documented and the ability of microorganisms to extract and/or accumulate metals is employed in commercial biotechnological processes such as bioleaching and bioremediation (1, 2).

Bacteria are known to produce inorganic materials either intra cellular or extra cellular. Microorganisms are considered as a potential biofactory for the synthesis of nanoparticles like gold, silver and cadmium sulphide. Among the microorganisms, prokaryotic bacteria have received the most attention in the area of metal nanoparticle biosynthesis. The formation of extracellular and intracellular metal nanoparticles by bacteria like Escherichia coli, Pseudomonas stutzeri, Pseudomonas aeruginosa, Plectonema boryanum, Salmonella typlus, Staphylococcus currens, Vibrio cholerae, etc., have been reported (1, 3, 4).

Titanium is one of the strongest readily available metal, making it ideal for wide range of practical applications such as in automobiles, missiles, airplanes, helicopters, submarines, cathode ray tubes, batteries and even in jewelry and artificial gemstones, etc. It is 45% lighter than steel with comparable strength, and twice as strong as aluminum while being only 60% heavier (5).

Titanium is suggested for use in desalinization plants because of its strong resistance to corrosion from sea water (particularly when coated with platinum). In medical applications titanium pins are used because of their non-reactive nature when contacting bone and flesh. Many surgical instruments, as well

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as body piercing are made up of titanium for this reason as well. In terms of a mechanism, TiIV binds well to transferrin in human serum, which could deliver it to the cancer cells. This further emphasizes their future role in cancer chemotherapy and gene delivery (5).

The importance of titanium and environmental issues related to the production of nanopowder. The present work reports an eco-friendly biotechnological approach for the synthesis (Lactobacillus assisted) of nanotitanium for possible applications.

MATERIALS AND METHODS

Bacterial isolation

The Lactobacillus isolates were obtained from different fermented food. Each sample (10gm) was aseptically added into 90 ml of sterile 0.9% NaCl solution. Homogenized and serially diluted, 1 ml of the diluents was pour-plated on de Man, Rogosa and Sharpe (MRS) agar. Plates were incubated for 24 hrs at 30 °C under anaerobic condition (anaerobic generating Kit, Merck). Total of 15 representative colonies were randomly picked and sub-cultured to obtained pure culture. The isolates were maintained on MRS agar plates kept at 4°C. The stock cultures were stored at -20°C in MRS broth supplemented with 15% of glycerol for subsequent use (6, 7).

Biosynthesis of Titanium nanoparticles

Single colony from MRS agar plate was inoculated in 5 ml MRS broth and incubated at 30 °C for 18 -24 hr under anaerobic condition. Supernatant was obtained by centrifugation at 10.000 xg for 10 min. The filtrate was diluted 5 times and pH of the culture solution was noted in the range of 2–4 depending upon the strength of the solution. 10% suitable sugar solution was added to the culture solution and this was allowed to incubate overnight. Next morning to each of the culture, around 20 ml 0.025(M) titanium dioxide solution was added.

Culture solution were stirred thoroughly on a magnetic stirrer for 0.5 h and then allowed to incubate in laboratory ambience on a laminar flow. After 3–4 days, the culture solution was observed to have distinctly markable deposits at the bottom of the conical flask. A remarkable change in pH was observed at this stage, which is currently under standardization. Nanoparticles containing culture solution was filtered under the laminar flow through whatman filter paper, allowed drying under blow of hot air (5).

Spectrophotometry

Spectrophotometry experiments was carried out by the times 0, 24, 48, 72 and 120 after incubation of the strains. In the following order, first, (1/50) diluted solutions with concentration of equal 1 was prepared from first tube (bacteria culture plus TiO2), second tube (bacteria only) and culture medium. Then these solutions were exposed to the spectrums with wave lengths of 350-900 nm with the scale of 50 and amount of adsorbed spectrum was measured next (8).

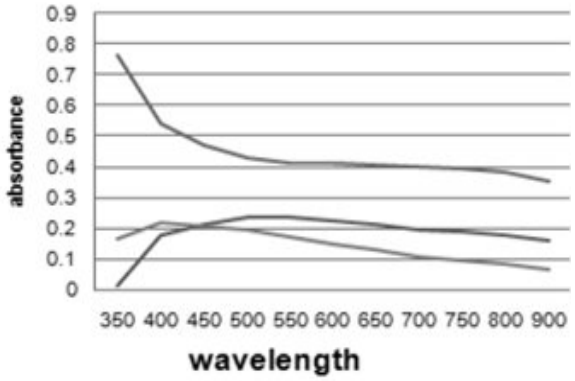

Figure 1 Absorbance spectrum of titanium nanoparticles synthesized by Lactobacillus, the upper line represents Tio_2, the other used as a control.

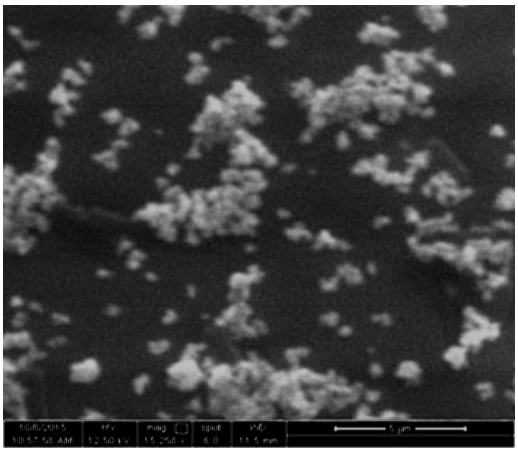

Figure 2 SEM micrograph of the titanium nanoparticles formed using Lactobacillus (MT14)

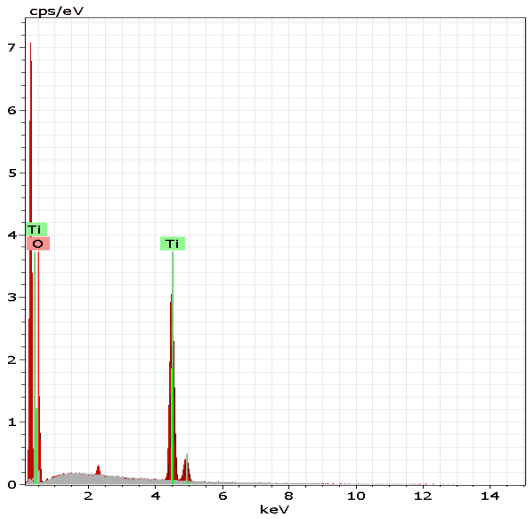

Figure 3 EDS analysis of titanium nanoparticles synthesized by Lactobacillus **(**MT14**)**

SEM/EDS analysis

The thin film of the sample was prepared on a small aluminum plate by just dropping very small amount of the sample on the plate, extra solution were removed using a blotting paper and then the film on the plate was allowed to dry overnight .The SEM/EDS analysis was performed on a Scanning electron microscope (Inspect S50, fei company, Netherland) in Electron microscope unit, Faculty of Science ,University of Kufa (8).

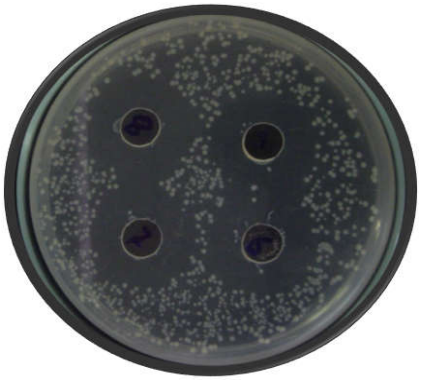

Figure 4 Inhibition zones around titanium nanoparticles synthesized by Lactobacillus (MT14) detected by using the agar well diffusion method, Pseudomonas aeruginosa was used as the indicator strain.

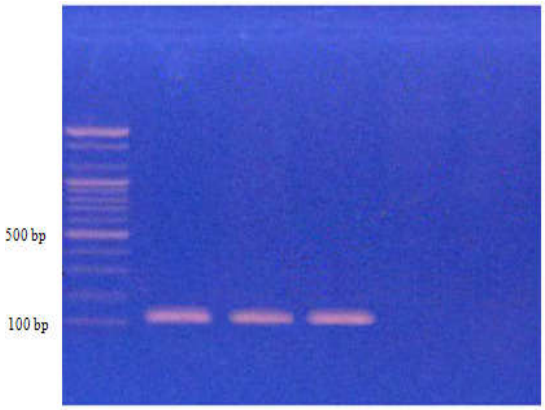

Figure 5 Agarose gel electrophoresis of PCR products of 122 bp using specific primer of Lactobacillus rhamnosus, Lane L, DNA ladder (100bp). The last lines represent negative control.

Detection of antibacterial activity

Inhibitory activity was detected by the agar-well diffusion method of Tagg and McGiven (1971), with some modifications. Portions (100 µl) were added to wells (5mm) cut into the plate, which was inoculated with Pseudomonas aeruginosa (as the indicator strain) and the plate was incubated for 18 hour at 37°C. The isolates that showed clear inhibition zones were purified by streaked from the broth and restreaked for single colony (9).

Identification of the efficient isolate

Isolate with which gave the largest inhibition zone against Pseudomonas aeruginosa was identified to species-level using cultural, biochemical and molecular characteristics. Morphological and cultural characteristics including; colony appearance, cell form, size, and arrangements, Gram stain, spore formation, motility, growth at pH 4.4, 8.0 and 9.6, growth in presence of 6.5% and 8% NaCl , growth at 4°C ,15, 37 and 45 °C were examined using MRS medium and incubation for 18 hr under anaerobic conditions ,Biochemical characteristics like Catalase test, arginine hydrolysis, and production of CO_2 from glucose were investigated as described by (6,7).

Molecular identification of the isolate

Extraction of bacterial DNA

Total DNA was extracted from colonies grown on agar plates by boiling method according to (10) with some modifications. 3-5 bacterial colonies was scraped using sterile toothpick from surface of agar plates and suspended in 300 µl Tris–EDTA buffer. The suspension was heated for 15min at 100°c followed by 15 min on ice rapidly. The suspension containing DNA was stored at-20 C until used as template for PCR.

PCR amplifications

Species-specific primers were used to amplify region of a 122 bp segment of L. rhamnosus. The primer sequences for the forward and reverse oligonucleotides were as follows: forward primer is TGCTTGCATCTTGATTTAATTTTG and the reverse primer is GGTTCTTGGATYTATGCGGTATTAG (11).

A PCR mixture was prepared consisting of 1.25 ul (10µM) of forward and reverse primers, 12.5 ul of 2X KAPA2G Robust Hot Start Ready Mix (KAPA2G Robust Hot Start DNA Polymerase 1 U per 25 µl reaction in a proprietary reaction buffer containing dNTPs, 0.2 mM of each dNTP at 1X, $MgCl_2$ 2 mM at 1X) and 10ul of bacterial DNA per reaction. The reaction was run for initial denaturation at 94 °C for 5 min followed by 35 cycles of 1min at 94 °C (denaturation), 1min at 62 °C (annealing), and 1min at 72 °C (extension) and 5min at 72°C (final extension) in a thermocycler. Products of the PCR were electrophoresed on a 1.5% agarose gel containing ethidium bromide and photographed. Positive results were indicated by the presence of a 122 bp band seen on the gel with an Ultraviolet transilluminator.

RESULTS AND DISCUSSION

Titanium dioxide exposing to the bacteria was reduced and nanoparticles of titanium formed. Solution color changed from light brown to dark brown in samples of capable strains, whereas the incapables demonstrated no color changing as well the solutions of second tube as controls. Black bulk of titanium consists of nanoparticles and bulk particles observed after drying of solutions under controlled situation. Fifteen strains of native *Lactobacillus* were investigated through which one isolate (MT14) from fermented apple have been selected as the best strain during the research (that gave the largest inhibition zone against the indicator strain strain)

UV analysis is being applied to determine concentration of a variant in a solution. Every analytic item has its own adsorbing pattern. Figure (1) shows the adsorption diagram of Tio_2, Blank as Bacteria only and Sample as Bacteria and TiO_2 mixture. Differences in adsorption diagram of the strains in different states demonstrating a material is different from bacteria and TiO_2 which are nanoparticles of titanium.

Figure (2) shows the SEM micrograph of the titanium nanoparticles being formed using MT14 strain. The micrograph clearly illustrates individual nanoparticles as well as a number of aggregates. The measurement of size was performed along the largest diameter of the particles. The particles are found almost spherical in shape having a size of 50-90 nm. EDS analysis of titanium nanoparticles synthesized by Lactobacillus (MT14) was shown in Figure (3).

Detection and isolation of the efficient isolate

A total of bacterial isolates from various samples were screened for antibacterial activity production by the agar well diffusion method using Pseudomonas aeruginosa as the indicator strain. The results showed that 3 isolates gave positive inhibition zones (17-20 mm). One isolate (MT14) from fermented apple that gave the largest inhibition zone against the indicator strain was selected for identification and further study, Figure (4).

Identification of the selected isolate (MT14)

Morphological and cultural characteristics of the selected isolate (MT14) are studied. Colonies of the isolate (MT14) on MRS agar plates were small, grayish white, circular, low convex with entire margin, and non-pigmented .Cells were Gram positive, non-motile, nonspore former, short rods in shape and appeared either singly or in pairs. Growth occured at pH 4.4 and 8.0 but not at pH 9.6. The isolate could not grow in presence of 8% NaCl but grew in 6.5% NaCl .Growth occured at 15, 37 and 45 °C, but not at 4°C. Catalase negative, Arginine is hydrolysed. CO_2 is produced from glucose.

Molecular identification of the isolate (MT14)

Isolate (MT14) were analyzed and identified as Lactobacillus rhamnosus by molecular identification with species specific primer. The results of the specific PCR reactions are shown in Fig (5). Strain (MT14) produced PCR fragments (122bp) with specific primers. A genus-specific diagnosis would provide important information regarding, detection of many species that is importment in treatment, control and prevention of many disease.

Determination of the species has important implications; a highly sensitive and specific test to identify members of this genus might augment investigations. Examples of application of the technique described in this paper to probiotic investigations include determining prevalence in a population where the species has been determined from a sample of the population and providing rapid, highly sensitive identification of materials with Lactobacillus rhamnosus.

Amplification of DNA using PCR can be accomplished rapidly and is of particular value when concentrations of viruses or bacteria are low, when bacteria that are shed are nonviable, or when isolation of an organism is difficult. (12, 13). The PCR can be used as a highly sensitive and specific test for the presence of bacteria in clinical specimens (14). Although other molecular genetic techniques require careful handling of specimens to provide good quality DNA for analysis, sheared or degraded DNA can be used for the PCR (15). Amplification of DNA using the PCR may enable detection of microorganisms below the level that can be detected by microbiologic culture (16) Identification of Lactobacillus species by microbiologic culture frequently entails submission of multiple samples because of variability in the number of organisms being shed. Because detection of specific regions of the bacterial genome by the PCR is more sensitive than microbiologic culture, only a single sample might be needed for detection of Lactobacillus species by the PCR. Compared to methods of gene amplification that do not utilize thermostable DNA polymerases, the PCR is simple and rapid and increases the specificity, yield, sensitivity, and length of targets that can be amplified (17).

CONCLUSIONS

Nanotechnology offers important new tools expected to have a great impact on many areas in medical technology. It is expected to accelerate scientific as well as economic activities in medical research and development. Nanotechnology has the potential to make significant contributions to disease detection, diagnosis, therapy, and prevention. In the present study we have reported a simple biological extracellular, easy, low cost, non toxic economical and ecofriendly approach for synthesizing titanium nanoparticles by using Lactobacillus, which provides extraordinary opportunities to improve materials and medical devices. The titanium nanoparticles formed were characterized by UV-Vis spectra, SEM and EDS studies. Our further studies will be focused on various medical applications of titanium nano particles.

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