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Full Length Article

Antimicrobial activity of silver nanoparticles biosynthesised by *Rhodotorula* sp. strain ATL72

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ABSTRACT

In this study, the pink yeast *Rhodotorula* sp. strain ATL72 was isolated from salt marches near Mediterranean Sea, Egypt. From phylogenetic analysis, the isolated strain ATL72 was closely related to *Rhodotorula bloemfonteinensis* EU075187 by similarity of 40%. The biological synthesis of nanosilver (AgNPs) using the marine pink yeast *Rhodotorula* sp. strain ATL72 was established. The UV–Visible spectral analysis confirmed the synthesis of AgNPs showing a characteristic peak around 400–500 nm. TEM analysis not only confirmed the synthesis of AgNPs but also described the spherical and oval shaped nanoparticle besides size measurements ranged from 8.8 to 21.4 nm. The biosynthesized AgNPs showed a strong antimicrobial activity by causing a complete inhibition of growth for a wide range of Gram positive and Gram negative bacteria as well as fungi with low MIC value. In conclusion, the pink yeast *Rhodotorula* sp., strain ATL72 isolated from Egypt is a promising new biological source for the synthesis of silver nanoparticles having a potent antimicrobial activity against a wide range of pathogenic bacteria and fungi.

1. Introduction

Rhodotorula sp. is unicellular yeast, characterized by a high growth rate. It has a yellow, orange or red color and known as caretogenic yeast or pink yeast due to its production of carotenoids [1]. It is widely distributed and isolated from several sources such as soil, fresh water, flowers, sea water, and food [2].

A lot of studies have been discussed about the importance of genus *Rhodotorula* in the field of biotechnology and its vital applications in the future of industry, which negates the thought that genus *Rhodotorula* is considered as saprophytes [3]. Metal nanoparticles such as silver nanoparticles (AgNPs) possess diverse technological applications due to their particular chemical, optical, electronic and magnetic characteristics that are completely different from the characters of the bulk metals [4]. One of the synthesis techniques of AgNPs is the biological synthesis [5]. It is high yield, low cost, non-toxic and eco-friendly process which can use different biological resources such as plants, bacteria, fungi, yeast, algae and viruses in the synthesis of nanoparticles [6]. Recent studies confirmed the ability of genus *Rhodotorula* such as *Rhodotorula minuta* [7] *and Rhodotorula glutinis* [8] to synthesis metal nanoparticles.

One of the most vital applications of the metal nanoparticles, especially silver nanoparticles, in the field of medicine is using these nanoparticles as antimicrobial agents. The lethal activity of nanoparticles against broad spectrum of Gram-positive bacteria, Gram-negative bacteria and fungi has been approved [9]. This study aimed to add a new biological source for the synthesis of silver nanoparticles besides monitoring the microbial biochemical changes and evaluating the antimicrobial activity of variable silver nanoparticles separated at different centrifugation speeds.

2. Materials and methods

2.1. Isolation and purification of marine pink yeast

Water samples were collected from salt marches near Mediterranean Sea and then diluted via serial dilution method. From the dilutions; 10^{-5}, 10^{-6} and 10^{-7}, 100 μL were inoculated into 50 ml of liquid enriched medium (yeast extract 5 g/l, glucose 10 g/l and 1 L of sea water) [10]. Antibacterial antibiotics were added by the recommended concentrations immediately before the inoculation process to control the bacterial growth. The flasks were incubated on an incubator shaker at 30 °C and 180 rpm. Each week, samples from each dilution were taken to inoculate a plate of agar enriched medium using the compound streaking method till the appearance of pure pink colonies.

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2.2. Molecular identification of the isolated yeast

Total genomic DNA (gDNA) from the strain ATL72was extracted using the Fast DNA® Spin Kit, after that18S rRNA gene was amplified by PCR reaction using the universal forward and reverse primers; 18S rRNA-F:5′-TACCTGGTTGATCCTGCCAG-3′ and 18S rRNA-R: 5′- CCTTCCGCAGGTTCACCTAC-3′ [11]. The sequencing of the PCR product was done using ABI PRISM®BigDyeTM Terminator Cycle Sequencing Kits, followed by basic local alignment search tool (BLAST) and SeqMatch analysis. The quality and quantity of the obtained sequence was examined by Finch TV version 1.4.0. While, assembling the gene contiguous sequences was done by CAP3 software. The 18S rRNA gene sequence of the isolate ATL72 was submitted to the Gene bank under the accession number MG021304 and used for construction of the phylogenetic tree using Sea view software and automated BLAST search for detecting the closest type strain to the isolate ATL72.

2.3. Preparation of AgNPs

Into 100 ml of liquid optimized medium (1 g/l yeast extract, 3.7 g/l malt extract, 35 g/l Nacl, 7.7 g/l fructose and 9 g/l urea), pink yeast were inoculated and incubated in shaking incubator for 72 h at 28 °C with 150 rpm. After that, the liquid culture was centrifuged and the pellet washed extensively three times with sterile distilled water to remove any residues from the media. To have cell free water extract, pellet was added to 100 ml of sterile deionized water in a shaker incubator at 27.5 °C at 150 rpm for overnight and then centrifuged to remove the biomass by decantation-centrifugation. $AgNO_3$ (1 mM) was added to the water extract and kept for 24 h in dark conditions [8]. Control flasks contain deionized water and AgNO3 without cell filtrate. After 24 h the samples showed any change in color were subjected to further analysis.

2.4. UV–Visible spectral analysis of AgNPs

The formation of nanoparticles was monitored using both visual observation of the color and UV–visible spectral analysis (Unicam UV–VIS. Spectrometer UV2, U.S.A). It was noticed that the filtrate was colorless before incubation with silver nitrate then the color turned to dark brown after complete reduction of silver ions. Silver nanoparticles were scanned using spectrophotometer at wavelengths from 200 to 800 nm [12]. The filtrate without silver nitrate was used as a blank sample.

2.5. Transmission electron microscopic (TEM) analysis of AgNPs

For determination of size and the shape of AgNPs, Transmission Electron Microscope (JEOL JEM-2100, U.S.A) was used. The process was performed by coating of AgNPs onto carbon coated grid (Type G 200, 3.05 μμdiameter, TAAP, U.S.A) [13].

2.6. Selected area diffraction pattern (SADP) analysis for AgNPs

SADP is a crystallographic technique accomplished using the transmission electron micrographs and requires a very thin sectioned specimen about 100 nm and about 100–400 KeV as an energy electron volt [14].

2.7. Antimicrobial activity and determination of MIC of AgNPs

AgNPs were centrifuged at three different speeds 4000, 8000 and 14000 rpm to study their activity at each speed. The precipitate of nanoparticles was dried, weighted and suspended in 1 ml of deionized water and subjected to sonication for getting homogenized suspension [15].

Streptococcus sp., *Bacillus* sp., *Staph* sp., *Shigella* sp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* sp. and *Candida* sp. were provided from Genetic Engineering and Biotechnology Unit, Faculty of Science, Mansoura University, Egypt, to study the antimicrobial activity of the biosynthesized AgNPs [16]. These pathogens were activated by culturing 100 μL of each in 10 ml LB medium (10 g/l Pepton, 10 g/l NaCl, 5 g/l Yeast Extract) and incubated at 37 °C for 24 h with shaking at 150 rpm [17]**.** In 10 ml LB broth, 100 μL of AgNPs from each speed (4000, 8000 and 14,000 rpm) and 100 μL of each pathogen were added. The microbial growth was monitored by measuring the optical density (OD) at 600 nm [18].

For MIC determination [19] of the biosynthesized AgNPs, 100 μL AgNPs from different concentrations (50, 25, 10,5,1,0.5 and 0.25 μg/ml) from each speed (4000, 8000 and 14000 rpm) and 100 μL from each test organism (*E. Coli*, *Candida* sp. and *Bacillus* sp.) were applied to 5 ml LB broth medium and incubated at 37 °C with shaking for 24 h. AgNPs with inoculum in broth media were used as a positive control. The results were monitored by measuring the mean OD at 600 nm [20].

2.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)

SDS-PAGE was used to investigate the effect of AgNPs collected at different centrifugation speeds (4000 and 14,000 rpm) on selected pathogens *E. coli*, *Candida* sp. and *Bacillus* sp.. Total proteins from pellets of the treated microorganisms were extracted in 100 mM phosphate buffer at pH 7. For loading protein over the acrylamide gel, 10 μg protein concentrations from each sample were boiled in 2X sample buffer (2.5 ml Tris-HCl pH 6.8, 10 ml Distilled water, 4 ml 10%SDS, 2 ml Glycerol and 1 ml β-mercaptoethanol) for 2 min. Acrylamide gel was prepared in two layers; 4%stacking gel above 12% separating gel [21].The electrophoresis was applied for 2 h at 100 V, then the gel was stained in Commassie Brilliant Blue R250 overnight, followed by destaining on shaker for few hours. Using Gel Analyzer3 Program, the gel was analyzed.

3. Results

3.1. Isolation and purification of marine pink yeast

Under light microscope, the stained cells of the isolated yeast strain were circular in shape and the colonies were smooth, soft and orange in color (Fig. 1).

3.2. Molecular identification

By analysis of the phylogenetic tree, the phylogenetic position of the isolate ATL72 was compared to the closely related species of the genus *Rhodotorula*. Strain ATL72. It was found that, the closely related species is *Rhodotorula bloemfonteinensis* EU075187 with percentage similarity of 40% as shown in Fig. 2.

3.3. Biosynthesis of AgNPs

After incubation of cell free water extract with 1 mM silver nitrate, the color changed from orange into dark brown color which indicates for the formation of nanosilver as shown in Fig. 3.

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