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Original Article

Nitrogen-fixing *Cyanothece* sp. as a mixotroph and silver nanoparticle synthesizer: a multitasking exceptional cyanobacterium

Cyanothece sp. fixadora de nitrogênio como um sintetizador de nanopartículas mixotrófico e prata: uma cianobactéria excepcional multitarefa

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Abstract

To investigate the best carbon source for mixotrophic growth of *Cyanothece* sp. This cyanobacterium is also used as a source of biogenic silver nanoparticles. The study also investigates the antimicrobial activity of nanoparticles (both gold and silver) biosynthesized by this cyanobacterium. Those particles are tested solely and in combination as antimicrobial agents against two farm bacteria commonly found in Al Ahsa. Soil extract was prepared from pesticide-free soil whereas dextrose was prepared in 6 mM concentration and the cyanobacterial culture cell density was determined after two weeks. Cultures of *Cyanothece* sp. were incubated with silver nitrate until turning brown. The external solution of culture was analyzed using chemical analyses including UV- visible and FTIR to confirm nano silver formation. Previously-biosynthesized nano gold particles by *Cyanothece* sp. were used solely and in combination with newly biosynthesized nano silver particles for antimicrobial bioassay against the two farm bacterial pathogens. The best mixotrophic cyanobacterial growth was obtained for soil extract followed by dextrose which were significantly different from control. The synthesis of nanoparticles using this cyanobacterium was confirmed using UV-visible light spectrophotometry which detected the characteristic surface plasmon resonance peak in the range of 410-450 nm and the FTIR spectroscopy which showed the characteristic silver nanoparticles peak at 3.297 cm^{-1} which overlaps with -OH- in addition to the other functional groups associated with nano silver particles detected at 2,927, 1,631 and 1,383 cm^{-1}. Silver nanoparticles showed the strong antimicrobial effect against both pathogens *Staphylococcus aureus* (MRSA) and *Staphylococcus warneri* with inhibition zone diameter 1.3 cm for both followed by the combination of silver and gold nanoparticles. Soil extract is a natural medium rich in all types of organic and inorganic nutrients which enhance algal mixotrophic growth. Biosynthesized silver nanoparticles showed the strongest antibacterial action against both pathogens most likely due to its ease of penetration, interaction with cellular components, generation of reactive oxygen species and induction of oxidative stress leading to bacterial death. **Conclusion**, Mass culturing of cyanobacteria by using soil extract is both economic and eco-friendly. In addition, the green biosynthesis of silver nanoparticles and their use in biocontrol of farm pathogens serve the purposes of environmental sustainability and food security.

Keywords: antimicrobial, biocontrol, cyanobacteria, *Cyanothece* sp., environmental sustainability, food security, mixotrophy, silver nanoparticles.

Resumo

Investigar a melhor fonte de carbono para o crescimento mixotrófico de *Cyanothec*e sp. Esta cianobactéria também é utilizada como fonte de nanopartículas de prata biogênicas. O estudo também investiga a atividade antimicrobiana de nanopartículas (ouro e prata) biossintetizadas por essa cianobactéria. Essas partículas são testadas isoladamente e em combinação como agentes antimicrobianos contra duas bactérias agrícolas comumente encontradas em Al Ahsa. O extrato do solo foi preparado a partir de solo livre de pesticidas, enquanto a dextrose foi preparada na concentração de 6 mM e a densidade celular da cultura de cianobactérias foi determinada após duas semanas. Culturas de Cyanothece sp. foram incubadas com nitrato de prata até ficarem marrons. A solução externa de cultura foi analisada usando análises químicas incluindo UV-visível e FTIR para confirmar a formação de nano prata. Nano partículas de ouro previamente biossintetizadas por Cyanothece sp. foram usados apenas e em combinação com nano partículas de prata recém-biossintetizadas para bioensaio antimicrobiano contra os dois patógenos bacterianos da fazenda. O melhor crescimento de cianobactérias mixotrópicas foi obtido para o extrato de solo seguido pela

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dextrose que foram significativamente diferentes do controle. A síntese de nanopartículas usando esta cianobactéria foi confirmada por espectrofotometria de luz UV-visível que detectou o pico característico de ressonância plasmônica de superfície na faixa de 410-450 nm e a espectroscopia FTIR que mostrou as nanopartículas de prata características - pico em 3.297 cm-1 que se sobrepõe com -OH- além dos outros grupos funcionais associados a nanopartículas de prata detectadas em 2.927, 1.631 e 1.383 cm−1. As nanopartículas de prata mostraram forte efeito antimicrobiano contra ambos os patógenos (Staphylococcus aureus (MRSA) e Staphylococcus warneri com diâmetro de zona de inibição de 1,3 cm para ambos, seguido pela combinação de nanopartículas de prata e ouro. O extrato do solo é um meio natural rico em todos os tipos de nutrientes orgânicos e inorgânicos que favorecem o crescimento mixotrófico de algas. As nanopartículas de prata biossintetizadas mostraram a ação antibacteriana mais forte contra ambos os patógenos, provavelmente devido à sua facilidade de penetração, interação com componentes celulares, geração de espécies reativas de oxigênio e indução de estresse oxidativo levando à morte bacteriana. Conclusão, o cultivo em massa de cianobactérias usando extrato do solo é econômico e ecologicamente correto. Além disso, a biossíntese verde de nanopartículas de prata e seu uso no biocontrole de patógenos agrícolas atendem aos propósitos de sustentabilidade ambiental e segurança alimentar.

Palavras-chave: antimicrobiano, biocontrole, cianobactéria, *Cyanothece* sp., sustentabilidade ambiental, segurança alimentar, mixotrofia, nanopartículas de prata.

1. Introduction

Cyanothece sp. is a nitrogen-fixing unicellular cyanobacterium that is capable of performing photosynthesis and nitrogen fixation in a diurnal cycle controlled by circadian rhythm. Many reports demonstrated its fantastic metabolic activities and physiological attributes including gold nanoparticle biosynthesis and plant biofertilization (Younis et al., 2019; El Semary et al., 2020). In order to exploit this cyanobacterium further and to grow it at minimal cost, we tested two natural different carbon sources including dextrose (glucose) derived from corn and soil extract as carbon sources. The purpose was to obtain maximum growth from the mixotrophy of this alga by growing it on natural cost- effective sources and to use the resulting biomass for abundant nanoparticle synthesis. With regard to silver nanoparticles' antimicrobial abilities, El Dayel et al. (2020) showed that nano silver biosynthesized by *Chlorella vulgaris* had antimicrobial effect against multi-drug resistant *Staphylococcus aureus* (MRSA) isolated from clinical sample, which is a potent pathogen that can be deadly. Similarly, Younis et al. (2019) showed the powerful antimicrobial effect of silver nanoparticle biosynthesized by *Phormidium* sp. against multi-drug resistant *Staphylococcus aureus* (MRSA). Similarly, El-Semary et al. (2021) showed the strong antimicrobial activity of silver nanoparticles from a filamentous cyanobacterium. Cyanobacteria grow rapidly in relatively inexpensive growth media. Some cyanobacteria can even grow mixotrophically benefiting from autotrophic and heterotrophic modes of nutrition at the same time) (Feng et al., 2010). Enriched culturing of *Cyanothece* sp.by natural carbon compounds would allow mass nanoparticles production. Some cyanobacterial genera: *Anabaena, Calothrix, and Leptolyngbya* were reported to produce intracellular gold and silver nanoparticles, which are released into the culture medium and stabilized by cyanobacterial exopolysaccharides/peptides. However, the size of the recovered particles and yield depend on the cyanobacteria genus (Brayner et al., 2007). With regard to *Cyanothece* sp., it was previously shown to have a great gold nanoparticle biosynthetic potential (Younis et al., 2019). This finding inspired the current study in which the biosynthesis of silver nanoparticles by the same *Cyanothece* sp. is investigated. The antimicrobial action of these biogenic nanoparticles, both silver and gold individually or in combination, is going to be investigated on dates' pathogenic bacterial strain, *Staphylococcus warneri* isolated from Al-Ahsa Governorate, Eastern Province, Kingdom of Saudi Arabia. Dates is an economic plant in that region and pathogenic bacteria cause yield damage and economic losses. As the harvesting of dates may involve skin cuts and wounds, the antimicrobial bioassay will also be performed against multidrug-resistant *Staphylococcus aereus* which is highly pathogenic bacterium with a wide range of hosts (Kozajda et al., 2019) and can also be found in animal manures. The objective of the study is to investigate the natural carbon source that enhances mixotrophy for maximized algal production. Moreover, we aim to capitalize on *Cyanothece* sp. exceptional activities and to exploit its nanoparticles' biosynthetic ability in the biocontrol of two pathogenic farm bacteria namely: Dates' pathogen *Staphylococcus warneri* and multi-drug resistant (MRSA-)*Staphylococcus aureus* usually found in the animal manures.

2. Materials and Methods

2.1. The different carbon sources and growth estimation of cyanobacterial cells

The coccoid cyanobacterial monospecific culture was previously collected, isolated and purified (Younis et al., 2019). Mid-logarithmic cultures were used in the biosynthesis of nanoparticles. BG11 medium was supplemented with 50 ml of 6 mM Dextrose (Sigma). Soil extract was prepared by weighing 100 gm of herbicide-free soil after sieving and removing gravels. This amount of soil was added to 1000 ml water, stirred thoroughly and left to stand for an hour. The soil solution was filtered by multiple layers of cheese cloth then the filtrate was autoclaved and 50 ml were added to BG11 to grow the cyanobacterium. Each treatment was done in triplicates. The growth of the cyanobacterium was monitored for two weeks and the final growth was estimated by direct cell counts Petroff-Huaser cell (El Semary, 2010). Initial inoculum contained 4x 10^2 cells/mL Statistical analysis of the results was carried out using One-way ANOVA, Minitab package version 13, Minitab incorporation.

2.2. The pathogenic bacteria

The pathogenic bacterial samples; MRSA-*Staphylococcus aureus* and *Staphylococcus warneri* were provided by courtesy of Dr Munirah Aldayel, Biological Sciences Department, College of Science, King Faisal University, Al Ahsa, KSA.

2.3. Gold nanoparticles biosynthesis

Gold nanoparticles were prepared and identified as previously described in Younis et al. (2019).

2.4. Silver nanoparticles biosynthesis

Silver nitrate (Sigma–Aldrich, USA) was prepared at 10 mM concentration and kept in dark bottle. Cells of *Cyanothece*sp. were washed with distilled water and centrifuged. The cells were incubated with silver nitrate solution for two weeks. The external solution gradually turned brown and got darker in time and the formed silver nanoparticles in the unfiltered external solution were subjected to the following analyses.

2.5. UV–visible spectroscopic (UV–vis) analysis

The UV–vis spectrophotometer (Genesys10S UV–visible double beam spectrophotometer) was used. Scanned spectrum was recorded for the wavelength range of 200 nm to 600 nm.

2.6. FTIR analysis

Characterization of silver nanoparticles was performed on unfiltered external solution of cultures containing nanosilver particles using Fourier-Transform infrared spectrometer (FTIR, Agilent Cory 630, Agilent Technologies, USA).

2.7. Antibacterial bioassay

Dates' pathogenic bacterial strain Staphylococcus warneri strain and the multidrug-resistant MRSA-Staphyococcus aureus strain were used in the antimicrobial assays using nanosilver. They are both Gram positive bacteria. Antimicrobial bioassay was performed according to Loo et al. (2018).

The freshly-sub cultured pathogenic bacteria were evenly spread on the Mueller-Hinton agar (Merck, Germany). Sterile paper disks were loaded with 30 µL of negative control including silver nitrate solution (1 mM) whereas chloramphenicol was used as positive control at 5% concentration. Disks containing 30 µL *Cyanothece*-biosynthesized AgNPs were used (Younis et al., 2019). Sterilized-paper disks were saturated with 30 µLof silver nanoparticles solution at concentration of 100 µg/ml. Disks were dried and placed on the surface of the inoculated medium. The plates were incubated at 37 °C. The zones of inhibition were measured after 24 h. The antimicrobial bioassay was further repeated but this time with an equimolar combination of nanogold nanoparticles and silver nanoparticles prepared in this investigation and treatments involving nanogold alone. Nanogold particles were prepared according to Younis et al. (2019).

3. Results and Discussion

With regard to the effect of different carbon sources on growth of *Cyanothece* sp., the highest cyanobacterial growth was obtained for soil extract followed by dextrose (Table 1). Dextrose is a monosaccharide sugar derived from corn or wheat and is chemically identical to glucose. The rationale behind using dextrose is that it can be derived from natural sources and their residual waste can be used to grow cyanobacteria at minimal cost. The cyanobacterial Maximum growth (Table 1) was obtained by using soil extract where it showed significant difference from control and dextrose treatment. The extract is cost-free, easy to prepare and full of nutrients whether organic or inorganic. Indeed, old algal collections were kept growing in the past using soil extracts. Although its exact chemical composition is not defined, nevertheless, it is efficient and economic. Silver nanoparticles formation was indicated by the change of the extracellular solution which turned brown from the start of incubation and became darker with time. T he biosynthesized silver nanoparticles showed a strong specific surface plasmon resonance peak which was obtained within the range of 413-450 nm (Figure S1). This is in accordance with the range reported by Yasin et al. (2013). In addition, FTIR confirmed the biosynthesis of silver nanoparticles. The FTIR spectrum (Figure 1) showed the characteristic silver nanoparticles associated functional groups peaks at 2,927, 1,631, 1,383 cm^{-1} (Devaraj et al., 2013) and 3297cm^{-1} (Aldayel et al., 2020) which are present in the unfiltered external solution. The biological synthesis of nanoparticles has many advantages as it is eco-friendly, cost-effective and can be easily optimized for mass production. Some cyanobacterial species have been reported for their potential for bio-reduction of Au^{3+} to Au^0 and subsequent formation of gold nanoparticles (Lengke et al., 2006). With regard to inorganic ions of silver and gold, there is a suggested enzymatic reduction that converts metallic ions to form to nano-neutral particles. This possibly proceeds through carrying electrons by quinones to be used by enzymes such as NADH-dependent reductases to reduce metal ions (Lengke et al., 2006). The suggested mechanism of gold bioaccumulation by cyanobacteria involves the precipitation of nanoparticles of amorphous gold (I)-sulfide at the sulfated-polysaccharide sheath (Younis et al., 2019) of *Cyanothece* sp. and/or at the

Table 1. Effect of presence of two different carbon sources on growth of *Cyanothece* sp.

Carbon source	Cyanobacterial count cell/mL
Dextrose	$8x\ 10^{8*}$
Soil extract	$16x\ 10^{8*}$
Control	$4x\ 10^{6}$

*Significant difference is denoted by the sign (*).

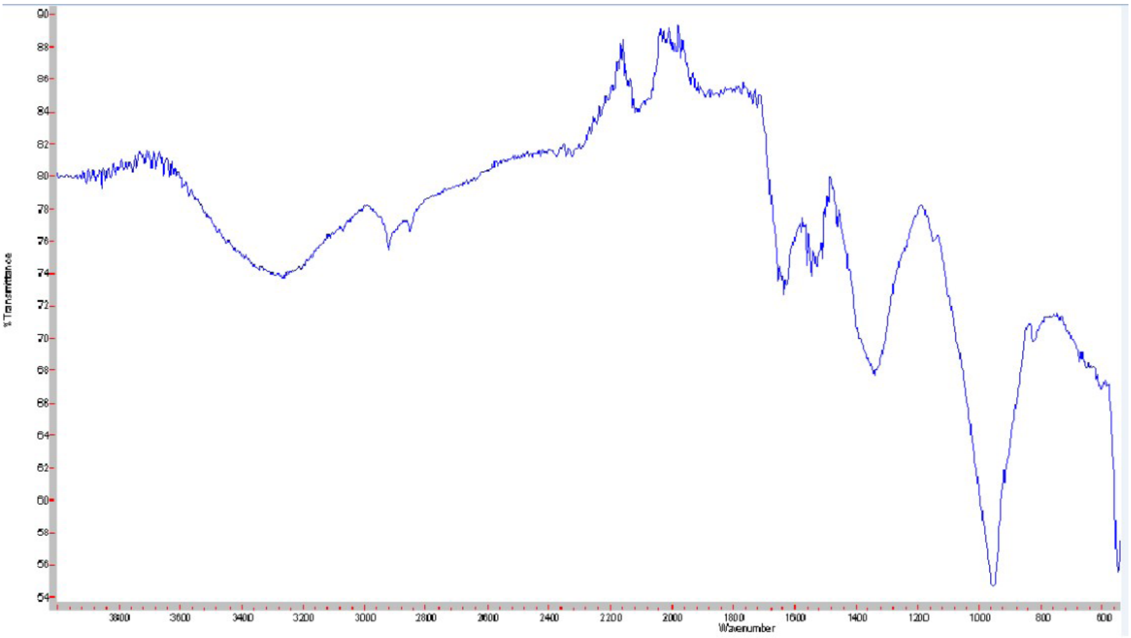

Figure 1. FTIR spectrum of biogenic silver nanoparticles from unfiltered external solution of *Cyanothece* sp.

cell wall, and finally depositing metallic gold in the form nanoparticles (Lengke et al., 2006). With regard to silver nanoparticles antimicrobial action, Khorrami et al. (2018) attributed the adherence of silver ions to the cell wall and cytoplasmic membrane to the electrostatic attraction to sulfur proteins, thereby increasing the permeability of the cytoplasmic membrane and eventually leading to disruption of the bacterial envelope. After the uptake of silver nanoparticles, respiratory enzymes can be inactivated and reactive oxygen species can be generated leading to oxidative stress in addition, to the interruption in adenosine triphosphate production (Durán et al., 2016).

3.1. The antibacterial bioassay

Silver nanoparticles were effective against all three tested pathogenic bacteria but being most effective against gram positive bacteria showing inhibition zone of average diamter 1.3 cm (Table 1). To further potentiate their antimicrobial effect, both silver and gold colloidal nano-solutions were used in combination in the antimicrobial bioassay for both pathogenic strains of *Staphylococcus warneri* and MRSA-*Staphylococcus aureus*. The results showed that thre treatment including nanosilver only had the highest antimicrobial activity against the two bacterial pathogens (Table2) whereas the nanogold action was somewhat lowest. The combined mixture showed antimicrobial action of moderate degree in both (Table 2, Figure 2).

Table 2. The antimicrobial bioassay of nanosilver and nanogold particles, biosynthesized by *Cyanothece* sp.

Bacterial species	Treatment	Average Inhibition zone (cm) ± SD
Staphylococcus warnei (dates pathogenic bacterium)	Nano-Silver	1.3± 0.1
	Nanno-Gold	1.0± 0.1
	Nano Silver+Gold	1.1± 0.1
MRSA-*Staphylococcus aereus*	Nano-Silver	1.3± 0.1
	Nanno-Gold	1.1± 0.2
	Nano Silver+Gold	1.2± 0.2

Inorganic nanoparticles such as gold and silver are increasingly used in many biological applications due to their unique features such as ease of application, functionality and biocompatibility (Xu et al., 2006). *Cyanothece* sp. is a unicellular prokaryotic cyanobacterium. It has the ability to both perform photosynthesis and fix nitrogen in an alternating day/night cycle. Another important derivative of carbohydrates secreted by *Cyanothece* sp. is the sulphated polysaccharides. The sulphated polysaccharides have negatively charged hydroxyl group and a sulfate group (Liberman et al., 2020). Many active functional groups are present on sulphated polysaccharides which serve as reducing entities for the reduction of silver ions to neutral nano silver particles (López-Miranda et al., 2012). Similarly, gold ions can be reduced in the same manner to nanogold particles (Younis et al., 2019). Sulphated polysaccharides serve dual functions as they are both active synthesizers of nanoparticles and efficient agents for capping and stabilizing those nanoparticles (Jeon et al., 2021). Nano silver particles were shown to possess the

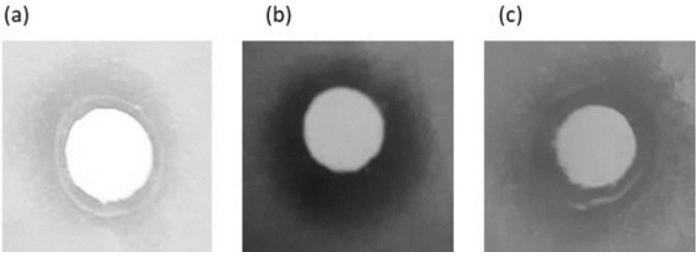

Figure 2. Disc inhibition zone against MRSA *Staphylococcus aureus* using (a) gold nanoparticles, (b) silver nanoparticles, and (c) both silver nanoparticles and gold nanoparticles.

highest antibacterial action which may be attributed to the damage they cause to bacterial cell wall as well as to plasma membrane (Hamida et al., 2020a). Indeed, our study on the two pathogenic Gram-positive organisms; *Staphylococcus aureus* (MRSA) and *Staphylococcus warneri* showed that both silver and gold biogenic particles were effective as antimicrobial agents. However, silver nanoparticles showed the highest antimicrobial effect as compared to effect of nanogold particles or the combination between nanogold and nano silver particles. Previously silver nanoparticles were reported to induce the generation of reactive oxygen that damaged the bacterial cell wall and cell membrane, thereby disrupting respiration and permeability (Hamida et al., 2020a). The results demonstrated for the first time the relationship between biogenic silver nanoparticles and genes related to the bacterial secretion system and showed that silver nanoparticles caused downregulation of those enzymes. Moreover, silver ions can inhibit the synthesis of proteins (Hamida et al., 2020c). In the present study, the efficacy of the silver nanoparticles against *Staphylococcus warneri* and MRSA-*Staphylococcus aureus* indicates that applicability of these particles in agriculture as antibacterial agents. Overall, silver nanoparticles (AgNps) were proved to be highly effective against different microbial pathogens which is in accordance with what Hamida et al. (2020c) reported. They possess excellent antimicrobial property due to their extremely large surface area which provides better contact with microorganisms and allow nanoparticles to exert their toxic effect. Nanoparticles bio-synthesized by microorganisms tend to be stabilized by peptides which prevent aggregation (Seshadri et al., 2012) which only adds to the advantage of using cyanobacteria as a green platform for nanoparticles synthesis. Indeed, the silver nanoparticles developed in the unfiltered external solution of the cyanobacterial cultures showed indications of many functional groups including peptides as indicated from the functional group bands detected in the FTIR spectroscopy. With regard to the gold nanoparticles, it is suggested that they owe their antimicrobial activity in part to their electrical and optical properties (Rabiee et al., 2022). the antimicrobial superiority of silver nanoparticles over that of gold nanoparticles is mainly attributable to release of silver ions from those nanoparticles unlike gold nanoparticles. These silver ions interact with phosphorus-containing sulfur molecules in membrane proteins, and with DNA and proteins thereby inhibiting cell division. Moreover, the interaction with the thiol group leads to the generation of reactive oxygen species, oxidative stress, inhibition of respiratory enzymes, and apoptosis of cells whereas gold nanoparticles do not seem to undergo same interactions (Rabiee et al., 2022). One of the Bindhu and Umadevi (2014) showed similar results and recommended using silver nanoparticles for antibacterial applications. Another important consideration is that the broad-spectrum antimicrobial nature of silver nanoparticles and the ability to combat even the multi-drug resistant bacterial strains (Aldayel et al., 2020) make silver nanoparticles good candidate for antimicrobial formulations and applications. Concomitantly, Surwade et al. (2019) reported the strong antibacterial action of silver nanoparticles against MRSA and its possible combination with antibiotics for even more potent effect. Recently, El Semary and Bakir (2022) showed that silver nanoparticles of different sizes had broad-spectrum antimicrobial effect and the smallest particles had the most potent antimicrobial effect. All of these considerations support the use of AgNPs as biocontrol agent against bacterial pathogens.

4. Conclusion

The soil extract offers a rich, cost-free, easy to prepare medium for mixotrophy and *Cyanothece* sp. is an exceptional nitrogen-fixing mixotroph and nano silver synthesizer as well. This can be further exploited for the economic and ecofriendly production of antibacterial nanoparticles.

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Supplementary Material

Supplementary material accompanies this paper.

Figure S1. UV-visible spectrum showing plasmon resonance between 410-450 nm characteristic of nanosilver in the unfiltered external solution of the Cyanothece sp. culture.

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