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### Biosynthesis of silver nanoparticles using native *Acetobacter* and *Pediococcus* strains

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#### ABSTRACT

Among metal nanoparticles, silver due to its high electrical and thermal conductivity and being profoundly anti-bacterial and inexpensive has more importance in comparison with other metals such as gold and platinum. Many acidophilic bacteria can be effective in regenerating many of the nanoparticles due to their metabolism. Experimental samples were collected from Faraman Dairy Factory in Rasht industrial town in May 2019. After isolation and molecular identification to investigate the possibility of biosynthesis of silver nanoparticles, isolates were cultured in liquid medium including Nutrient Broth (NB), Luria-Bertani (LB) and De Man, Rogosa and Sharpe (MRS) to compare their yield. After incubation time for 24 hours, the silver nitrate salt was added separately to supernatant and material that dissolves in liquid. Characteristics of silver nanoparticles determined using Transmission Electron Microscopy, XRD and ultraviolet spectrophotometer (UV-Vis) were investigated. The results gained from the study displayed that the isolated *Acetobacter* and *Pediococcus* bacterial strain could produce silver nanoparticles in LB medium. It was also found that the *Acetobacter* and *Pediococcus* bacteria could not produce silver nanoparticles in NB and MRS culture media. The study showed that the *Acetobacter* bacteria in the supernatant phase and the precipitate phase of LB culture medium could produce silver nanoparticles. It was also found that the *Acetobacter* bacteria could produce silver nanoparticles with sizes of 50 nm and 30 nm. Also, the study showed that the *Pediococcus* bacteria just in the supernatant phase of LB culture medium could produce silver nanoparticles. It was also found that the *Pediococcus* bacteria could produce silver nanoparticles with sizes of 100 nm and 50 nm.

#### 1. Introduction

Metal nanoparticles are used in various fields of science and technology (Shipway et al., 2000). Meanwhile, silver nanoparticles (AgNPs) are much considered for good conductivity, chemical stability, catalytic, photonic and optoelectronic properties (Hussain and Pal, 2008; Sadeghi, Mohammadzadeh, et al., 2015). In the field of research related to the

development of AgNPs production, the reuse of silver as a powerful bactericidal material has flourished (Mohtashami et al., 2013). Silver salt and colloid formulation have caused it to be used in the treatment of burns, chronic ulcers, infections, acute epididymis, inflammation of the tonsils, and prevention of eye diseases in infants (Sintubin et al., 2009).

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Additionally, different chemical methods have been proposed for the synthesis of nanoparticles. They include microemulsion (Richard et al., 2017), microwave wave synthesis (Seku et al., 2018), template methods (Liu et al., 2013), chemical regeneration methods (Kataria, and Garg, 2018), electrochemical reduction (Virginia Roldán et al., 2013), irradiation reduction (Chen et al., 2007), chemical reduction in aqueous solutions (Leopold and Lendl, 2003) and biochemical methods (Ashraf Sabri et al., 2016).

Additionally, there are biological methods used for the synthesis of silver nanoparticles, the use of microorganisms (Gaidhani et al., 2013; Singh et al., 2013; Fang et al., 2019) and herbs (Ghosh et al., 2012; Salunkhe et al., 2014; Ganesan et al., 2019). Microbes have recently considered the biosynthesis of nanoparticles as an appropriate substitute for mass production of nanoparticles. "Green nanomaterial" is used to identify such type of nanoparticle which presents the target of many researchers in nanotechnology several factors such as the chemical composition of final nanomaterials, their particle size, high monodispersity and the ability to the high production is emphasized in the development of experimental protocols on the rapid and reliable production of green nanomaterials (Welman and Maddox, 2003).

In the recent years, silver has been identified as a toxic substance for many bacterial cells (Durán et al., 2005). But other types of bacteria not only resist against silver, but they can also collect about 25% of the silver cell's living space in their walls. This combination of different mechanisms arises from external emission systems, changes in solubility and dissociation rates by changing the state of metal ion regeneration, the formation of extracellular complexes and metal deposition, and the lack of specific metal transduction in the system. This method has been used to extract silver from its mining tin. The *Pseudomonas stutzeri* AG259 is capable of retrieving the 46-35 nanometer nanoparticles in it is a wall from a sulfide environment. This bacterium can also produce larger sized powders in a more isolated environment and in nitrate conditions. Nanoparticles with sizes less than 200 nm, with appropriate size distribution, shape and morphology of intercellular space in bacteria can be achieved (Mandal et al., 2005).

Bacteria that do not tolerate high concentrations of metal ions can also be used as beneficial organisms. *Lactobacillus*, which is found in milk fat, produces silver and gold nanoparticles well among its cells. Lactic acid bacteria in whey can also produce nano-alloy powders from these two metals (Ahmad et al., 2003).

Moreover, antimicrobial and anticancer properties and some other properties silver nanoparticles have been identified since a few years ago, and for this reason, the synthesis of these nanoparticles is of great importance. Methods biodegradable synthesis of silver nanoparticles, the importance of it is used in treatment raises a person. Obtaining knowledge of synthesis of nanostructures using eco-friendly methods and so-called "green" methods it is important (Sadeghi and Rostami et al., 2015). Recently, biosynthetic methods of microorganisms like bacteria and fungi, or from plant extracts the title is a very simple and acceptable alternative to chemical synthesis the complexity of some of the nanostructures (Fayaz et al., 2010; Philip et al., 2010; Sadeghi and Gholamhoseinpour 2015). Biosynthesis has many advantages, such as lower costs, environmental compatibility life and production are easy to scale. Insight, biosynthesis requires the use of high temperature and pressure as well as compounds Chemical is not toxic (Khan et al., 2012; Kaviya et al., 2012).

Lactic acid bacteria have been utilized in the industry for food and food fermentation from the past to today (Dysvik et al., 2019). Lactic acid is a principal metabolite produced by acidic bacteria that have many applications in the industry, including as a preservative, food flavoring, textile and pharmaceutical industries, as well as the production of lactate esters, propylene glycol, propylene oxide, acrylic acid, etc (Varadarajan and Miller, 1999; Åkerberg, and Zacchi, 2000). The LAB is capable of producing various antimicrobial compounds such as ethanol, formic acid, acetone, hydrogen peroxide, diestyle and bacteriocins (Oliveira et al., 2009).

Acidophilic bacteria have a special strategy to maintain the pH of the cell that enables it to survive in acidic environments. For this purpose, they have developed gradients in the membrane. Also, acidophilia contains cytoplasmic buffering molecules that contain amino acids such as

lysine, histidine, arginine, and the like that are able to capture protons (Zychlinsky and Matin, 1983). Other buffering molecules include dihydrogen phosphate and potassium ions (Spijkerman et al., 2007). Interestingly, all acidophiles that can grow in severe acidic environments are heterotrophic.

## 2. Materials and Methods

### 2.1. Sample collection and isolation of bacteria

At first, samples were collected from effluent of food factories in May 2019. After the first transfer of samples to the laboratory, the amount of 100 µl of sediment was transferred to the MRS broth for initial condensation. The medium was further placed in an incubator at 25°C for 24 hours. Some of the microbial suspension was transferred to MRS agar medium, after incubation time and observation of turbidity by the sterile loop. Further, in order to isolate the aerobic strains, the culture medium was incubated at 25°C for 24 hours. Also, to isolate the anaerobic bacteria, the plates were inserted into the anaerobic jar in the same circumstances as mentioned above.

After observing the colony and purifying them, the microbial suspension was prepared in the phosphate buffered saline. Subsequently, for the test of acid resistance determination, 10 µl of the suspension was transferred to tubes containing MRS broth medium, which was adjusted with lactic acid at pH 3 to 7. The specimens were then placed in an incubator for 24-48 h at 25°C. The specimens grown in acidic conditions were selected for molecular identification (Sadeghi et al., 2010).

### 2.2. Molecular identification

For the purpose of molecular investigation, DNA extraction was performed on the samples collected from the BHI medium cultures using an extraction kit (Sina Clone-Iran). PCR amplification was carried out using a pair of specific primers made by TAG Copenhagen Company (Denmark). Each reaction was performed in a total volume of 25 µl containing 13 µl of molecular biology-grade water (Takara-Japan), 2.5 µl of 10× PCR buffer (Takara-Japan), 1 µl of 10 pmol of 1392R (5'-GGT TAC CTT GTT ACG ACT T-3'), 1 µl of 10 pmol of 27f (AGA GTT TGA TCC TGG CTC A)

primers, 1 µl of 10 mM dNTPs (Takara-Japan), 0.5 µl of smart taq DNA polymerase (Takara-Japan), 1 µl of 50 mM MgCl<sub>2</sub> (Takara-Japan) and 5 µl of DNA template. The negative control tube contained the same PCR reagents as above but had 5 µl of water substituted for the DNA template.

PCR amplification conditions on a thermocycler (Biorad-Germany) were as follows: 95°C for 5 minutes, followed by 35 cycles of 95°C for 60 seconds, 60°C for 60 seconds and 72°C for 75 seconds, with a final extension at 72°C for 10 minutes (Dong et al., 2011). An aliquot of all PCR products was run on a 1.5% (w/v) agarose gels with a 100-1500 bp DNA ladder (Fermentas-Russia) and electrophoresed at 75 V for 40 min. The bands were visualized using safe staining and photographed by a UV-transilluminator (UV doc, England). PCR samples were sent to the South Korean Macrogen Corporation for sequencing.

### 2.3. Nanoparticle synthesis

After isolating and identifying the acidophilic strain, in order to investigate the production of silver nanoparticles, 100 µl of the microbial suspension was inoculated into liquid medium NB, LB, and MRS, and incubated for 48 hours at 25°C.

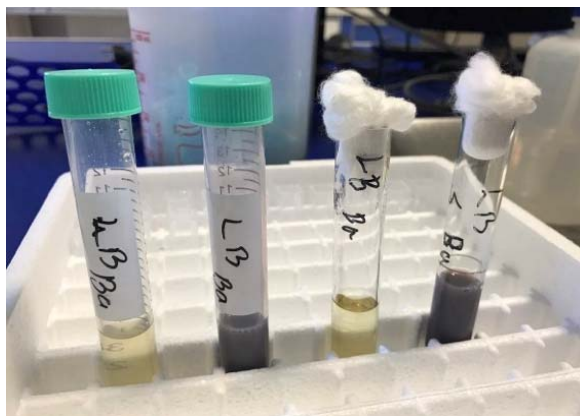
Subsequently, the selected samples were centrifuged for 10 minutes at 6000 rpm to remove the precipitate from the supernatant phase after growing and observing turbidity. Then, the amount of silver nitrate 50 Mgr (AgNO<sub>3</sub>) was added to the supernatant phase and precipitate phase, as well as to the base culture media without bacterial growth as the control, silver nitrate was added. After about 2-3 hours, the contents of the tube containing the colored silver envelope were mirrored.

Further, the optical absorption of the sample at 450 nm was investigated with a spectrophotometer. Sample light absorption was also investigated after 24 hours. Finally, in order to measure the size of the nanoparticles produced, the sample was sent to the Khajeh Nasir Tusi University of aerospace for imaging with Transmission Electron Microscope (TEM).

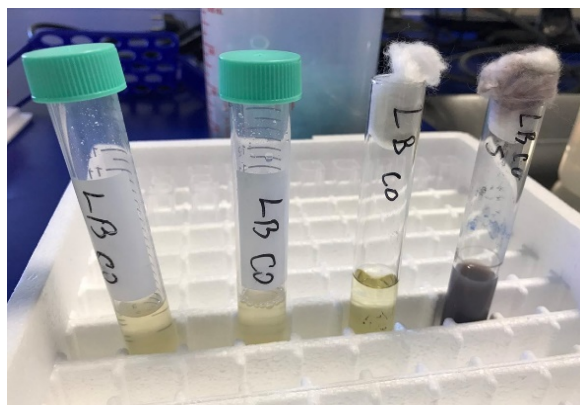
### 3. Results

The result of cultivating a sample of the effluent in MRS agar was the observation of a white colony of 2 mm and 3 mm in size. The results inferred that the isolated bacterium belonged to the *Acetobacter* and the *Pediococcus* genera that has the ability to grow in the MRS Agar environment and acidic conditions.

Additionally, the results showed that after adding silver nitrate to the supernatant solution and precipitating the NB, LB and MRS culture media containing the *Acetobacter* and the *Pediococcus*, the color change were observed only in the LB culture medium, which indicates the production of silver nanoparticles (Figure 1-2).



**Figure 1.** A) The supernatant phase of LB medium with *Acetobacter* bacteria and silver nitrate. B) The control tube containing the supernatant phase of LB medium with *Acetobacter* bacteria without silver nitrate. C) The precipitate phase of LB medium with *Acetobacter* and silver nitrate. D) The control containing the precipitate phase of the LB medium with the silver nitrate-free bacterium *Acetobacter*. The A and C tubes to which silver nitrate was added were darkened, indicating the production of silver nanoparticles.



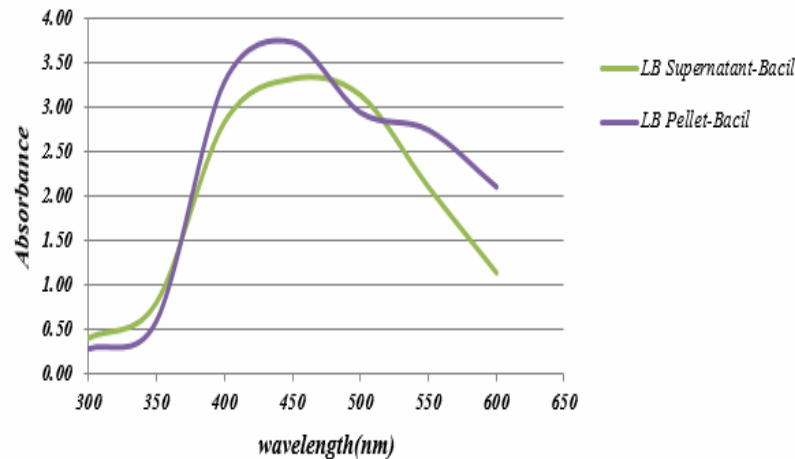
**Figure 2.** A) The supernatant phase of LB medium with *Pediococcus* bacteria and silver nitrate. B) The control tube containing the LB culture medium with *Pediococcus* bacteria without silver nitrate. C) The precipitate phase of LB culture medium with *Pediococcus* bacteria and silver nitrate. D) The precipitate phase of the LB medium with the bacterium *Pediococcus* without silver nitrate. The A tube to which silver nitrate was added were darkened, indicating silver nitrate production, but the C tube was transparent, indicating that it did not produce silver nanoparticles.

The results of the absorption spectrum are based on the wavelength of the spectrophotometer after 24 hours for the LB medium with an *Acetobacter* and the *Pediococcus* in two conditions: the supernatant phase and the precipitate phase (Sediment) are shown at 450 nm wave has the highest absorption (Figure 3-4).

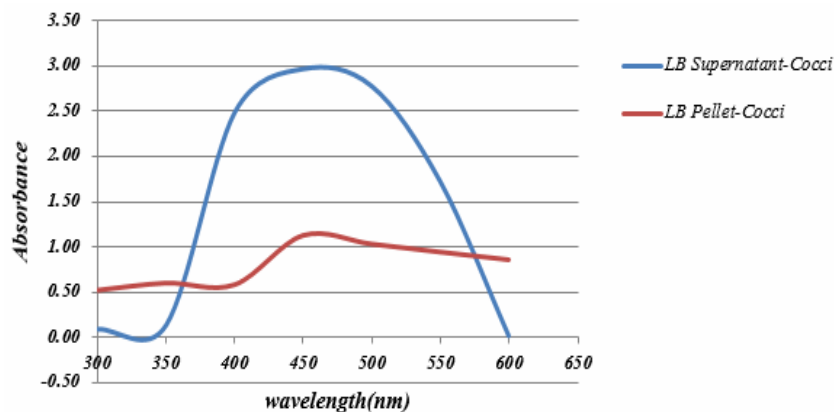
The results of the measurement of silver nanoparticles by the Transmission electron microscopy (TEM) are shown in the (Figure 5-

6). Silver nanoparticles with 100 nm and 30 nm are spherical in LB medium. Cloudy halo observed in images around nanoparticles is a bacterial tissue that is not seen in other nanoparticle synthesis methods.

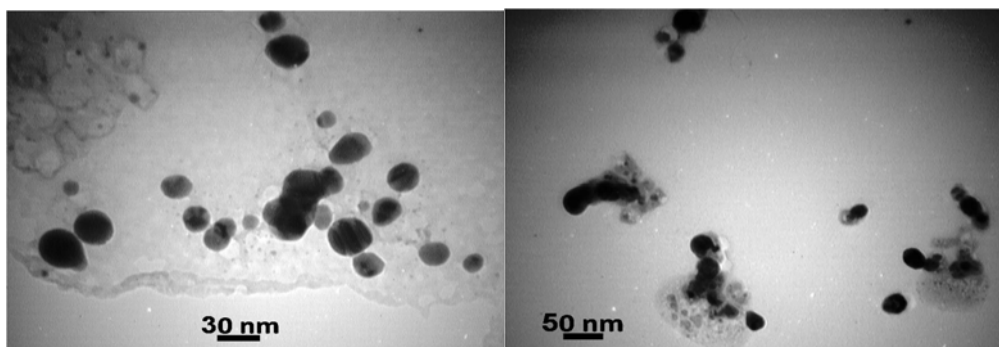
The XRD results from the *Acetobacter* and the *Pediococcus* are shown in the (Figure 7). In this method, X-ray diffraction is used to examine the properties of the sample. In the form of peaks of silver nanoparticles produced in the silver nanoparticle production intervals.



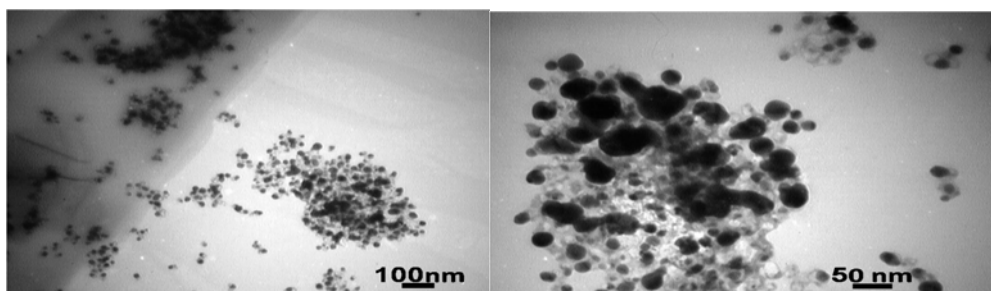
**Figure 3.** The absorption spectrum is based on the wavelength of the spectrophotometer after 24 hours for the LB medium with an *Acetobacter* bacteria in two conditions: the supernatant phase and the precipitate phase (Sediment).



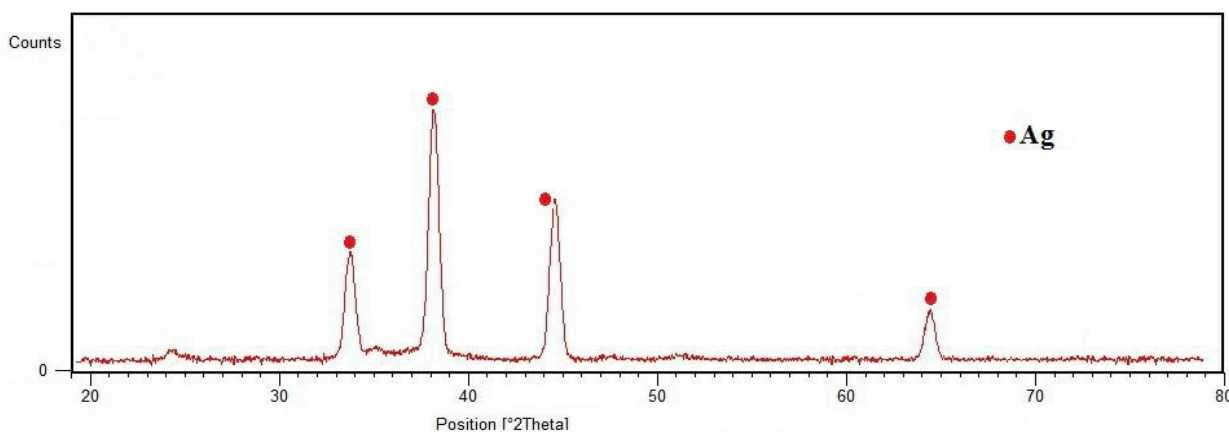
**Figure 4.** The absorption spectrum is based on the wavelength of the spectrophotometer after 24 hours for the LB medium with *Pediococcus* bacteria in two conditions: the supernatant phase and the precipitate phase (Sediment).



**Figure 5.** Nanoparticles produced by the *Acetobacter*.



**Figure 6.** Nanoparticles produced by the *Pediococcus*.



**Figure 7.** XRD pattern of silver nanoparticles using *Acetobacter* and *Pediococcus*.

#### 4. Discussion

Amongst nanoparticle, silver nanoparticles, because of its high electrical and thermal conductivity, silver has an antibacterial property, it is inexpensive in comparison with other noble metals such as gold and platinum, it is unique in optical properties, and it has the highest reflectivity in the entire IR region. The 400 nm

wavelength is of great importance. The synthesis of silver nanoparticles by bacteria involves the synthesis of extracellular cells, intracellular synthesis, and synthesis using bacterial-derived compounds. Extracellular synthesis of nanoparticles occurs outside of the bacterial cell. The nanoparticles have various shapes, cells are cultured, supernatants are grown, and the aqueous extract of the cell is synthesized.

Extracellular synthesis methods have advantages compared to intracellular type, the most important of which are the possibility of easy recovery of the nanoparticles from the solution. Recovery of these compounds is usually made in the form of a pellet precipitate that can be dispersed again in a suitable solvent, by the high speed of the centrifuge solution containing the nanoparticles.

Ashengroph in June 2013, a study on the extracellular synthesis of silver nanoparticles was performed by *Ralstonia* bacteria isolated from Sarcheshme copper mine. In this study, eight strains of bacteria with high tolerance to toxic silver ions were isolated from the copper and gold minerals using morphological observations and biochemical diagnostic tests. The results obtained from ocular observations, UV-vis spectrophotometry and images obtained from SEM electron microscopy and X-ray diffraction (XRD) showed that supernatant of *Ralstonia* sp. SM8 is capable of regenerating silver ions into extracellular silver nanoparticles. The silver nanoparticles produced were spherical, and their size was in the range of 20 to 50 nm. Based on the results of this study, the supernatant of *Ralstonia* sp. SM8, the rapid and extracellular production of silver nanoparticles, without the need for complex extraction processes, can be done. Whereas, in the present study, silver nanoparticles produced were spherical, and their size was in the range of 100 to 30 nm, they were also produced by the green synthesis method (Ashengroph, 2013).

In 2007, Shahverdi *et al.* conducted a study to quickly synthesize silver nanoparticles using supernatant cultures of gram negative bacteria of *Klebsiella pneumoniae*, *Escherichia coli*, and Enterobacteriaceae family. The results showed silver nanoparticles formed from silver ion within 5 minutes. Additionally, the results of the research indicate that the supernatant of acidophilic bacteria similar to Enterobacteriaceae can produce silver nanoparticles. Whilst, in the present study, acidophilic bacteria *Pedicococcus* and *Acetobacter* were studied and after 2 to 3 hours they were evaluated for the production of silver nanoparticles (Shahverdi *et al.*, 2007)

Korbekandi *et al.* (2012) conducted a study to optimize the biological synthesis of silver nanoparticles using *Lactobacillus casei*. In the culture of AgNO<sub>3</sub>, it was used as an enzyme

inducer and from glucose as an electron donor. As biomass increased, nanoparticles also increased. The results showed that *Lactobacillus* is capable of producing silver nanoparticles. In this study, *Lactobacillus casei* was studied and glucose was used as electron donor. In the present study, however, *Pedicococcus* and *Acetobacter* bacteria were studied and glucose was not used as electron donor (Korbekandi *et al.*, 2012).

In 2015, Kushwaha *et al.* conducted a study on the isolation and identification of *E. coli* for silver nanoparticle synthesis, particle properties, and antibacterial activity study. Urine samples were used for this study and grown in EMB agar medium and *E. coli* isolated. In this study, EMB agar medium was used and *E. coli* was investigated. While, in the present study, cultures of MRS broth, LB, NB and MRS agar were used and *Pedicococcus* and *Acetobacter* bacteria were studied (Kushwaha *et al.*, 2015).

Aguilar *et al.* (2018) conducted a study on the synthesis of silver nanoparticles (AgNPs) using natural extracts is a process that has already been studied due to their excellent antimicrobial activity. In this study, the AgNPs were prepared to use the extract of the sugar industry waste, sugar cane bagasse, as a reducing and capping agent, by a soxhlet extraction system. It was observed that varying the pH of the reaction medium it was possible to avoid the formation of AgCl and in addition, the pH has an important role in controlling the particle size and dispersion. In the present study, however, Nanoparticles size was in the range of 100 to 30 nm in various pH (Aguilar *et al.*, 2018).

In 2018, Tandeep *et al.*, conducted a study on the variable morphology of the silver nanoparticles (AgNPs) synthesized by using *Pleurotus florida* mycelia extracted as a bioreductant at two different reaction conditions (shaking and static), was reported. The formed AgNPs were characterized for the specific SPR (Surface Plasmon Resonance) peaks position around 400 to 450 nm at different time intervals by UV-vis spectroscopy. Under shaking conditions silver nanoparticles took least time for synthesis. Whereas, in the present study, just it was used static situation and the formed AgNPs were peaks position around 450 nm at 24 hours' intervals by UV-vis spectroscopy (Tandeep *et al.*, 2018).

## Conclusion

The study showed that the *Acetobacter* bacteria in the supernatant phase and the precipitate phase of LB culture medium could produce silver nanoparticles. It was also found that the *Acetobacter* bacteria could produce silver nanoparticles with sizes of 50 nm and 30 nm. Also, the study showed that the *Pediococcus* bacteria just in the supernatant phase of LB culture medium could produce silver nanoparticles. It was also found that the *Pediococcus* bacteria could produce silver nanoparticles with sizes of 100 nm and 50 nm.

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