Original Article

Synthesis and Antioxidative Study of Silver Nanoparticles Using *Lactobacillus* Species Isolated from Breast Milk

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Abstract

Background: Synthesis of silver (Ag) nanoparticles (NPs) from lactic acid bacteria is an alternative and eco-friendly way than many chemical and physical methods.

Objectives: This study aims to determine and understand if there is an antioxidant property of nanoparticle synthesized from Lactobacillus isolated from breast milk

Materials and Methods: The isolated *Lactobacillus* strains were confirmed by a series of biochemical tests and were used for in vitro silver nanoparticle biosynthesis from silver nitrate solution. The AgNPs formation was characterized by UV-visible spectroscopy.

Results: The antioxidant property of AgNPsprepared from isolating *Lactobacillus* from the breast milk was studied by DPPH and FRAP assay. These nanoparticles noted increased inhibition (98%) of DPPH at 5 µg concentration of AgNPs and reducing property of FRAP.

Conclusions: Series of experiments were performed to isolate and confirm the increased antioxidant properties in breast milk.

Key words : Silver nanoparticles, *Lactobacillus*, breast milk, antioxidant activity

Introduction

Human breast milk is an integral part of the supplement for survival and also for the proper development of infants. This is not only essential for the supply of nutrients but also is crucial to transport essential microflora that is initiated in breast milk. Even though there are a large amount and number of nutrients that are indigestible by infants they play a vital role in the initiation of microflora that is indigenous.[1-3] Breast milk is a major spring of probiotic bacteria or commensal and mutualistic to the infant gut, including lactic acid bacteria and Bifidobacteria. In recent years, more than 200 different species have been found in human milk. The rich source of these bacteria that are mutualistic or commensal or probiotic also include *streptococci* and *staphylococci*.[3-5] It is a researched fact that during the gestation period the antioxidant capacity is low and hence the oxidative stress is exhibited during the neonatal stage, and it is also established that human breast milk has got a superior antioxidant capability than other forms of milk.[5]

Lactic acid bacteria (LAB) like the *Lactococcus* or *Lactobacillus* or *Pediococcus* and also the strains or species of these, are majority for the mass production also for food preservation or even as probiotics for human or animal consumption. LAB plays a significant role in balancing the flora of the intestine, can boost immunity, reported to reduce cholesterol, and many specific strains are shown to have anti-aging properties as well as possess antioxidant properties.[6] A gastrointestinal tract that is a significant player for interfacing the host with the environment is believed to obtain benefit from LAB that is mostly enteric flora.[7]

Recent trends have shown an increase in the number of research and publication related to preparation of silver nanoparticles (AgNPs) from bacteria, plants, active components, etc.[8-10] This trend can be attributed to the fact that synthesis of nanoparticle using bacteria by biological methods could be a reliable and eco-friendly compared to many harsh physical and chemical methods. The LAB strains provide a great source of means to

synthesize silver nanoparticles.[11,12] Antibacterial activity of AgNPs against pathogenic bacteria and to combat antibiotic-resistant strains is now well recognized as an alternative to ionic silver.[13,14] Research findings of lactic acid bacteria isolated from different sources of milk indicate the antioxidant activity of LAB from milk.[6,15,16] But the antioxidative nature of AgNPs that are prepared using *Lactobacillus* strains isolated from human breast milk and the mechanisms involved in Ag NPs biosynthesis by *Lactobacilli* are not elucidated yet.

The aim of this research topic is to determine and understand the antioxidant property of silver nanoparticles prepared by isolating lactobacillus species present in the breast milk. Series of experiments were performed to isolate and understand the antioxidant properties of silver nanoparticles prepared using bacteria from breast milk.

Materials and Methods

Method of expressing breast milk:

The women providing her breast milk as a sample, washed her hands thoroughly with soap and warm water, and dried with a clean towel. The women then gently wiped the nipples and then breast, from the nipple out, with a clean, damp wash cloth and mild soap. Then the milk was expressed into a sterile Eppendorf tube and refrigerated within 30 min of pumping until use.

Isolation of Bacterial strains and AgNPssynthesis

1 mL of the sample of LAB was serially diluted transferred to 10^{-2} dilution tube and repeated to make it till 10^{-6}, respectively. Spread plate method using 10^{-4}, 10^{-5} and 10^{-6} dilutions were considered for spread plating. 0.1 mL of aliquot was transferred on to MRS agar medium and placedat 37°C for 24 h. After incubation, the bacterial sample underwent centrifugation at 1500 g for 10 min and washed several times with sterile deionized water. Then, 1 mM $AgNO_3$ was added to the bacterial sample for the synthesis of AgNPs, spectra were recorded.

Characterization of silver NPs

The reduction of pure Ag^+ ions was monitored by measuring the UV-Vis spectrum by diluting a small amount of the coated silver nanoparticles along with the bacterial capping agents into distilled water. Analysis of LAB coated nanoparticles was done by using UV-Vis spectrophotometer at the range of 200 – 550 nm.

In vitro Antioxidant Assay:

The antioxidant activity was examined for the prepared silver nanoparticle solutions. To assess the free radical scavenging activity, in this study, we used DPPH and FRAP, which is reliable for determining the free radical scavenging activity of the LAB components capped silver nanoparticles, and the results were compared with the standard antioxidants.

DPPH radical scavenging activity

DPPH radical scavenging activity was measured with the help of the spectrophotometric method.[13] Here the reaction mixture i.e. the prepared silver nanoparticles, was incubated at 25 °C for 5 min, after which absorbance was measured at 517 nm. Control: The DPPH with corresponding solvents (without sample) Test: The methanol with the respective test sample. Standard Curve: 0.1 g of L-ascorbic acid were dissolved in oxalic acid (0.05 M) solution freshly prepared and made up the volume to 100 mL. The different amounts of stock solution were made with oxalic acid making concentrations of 20, 40, 60, 80, and 100 mg/100 mL, respectively. The percentage inhibition of DPPH radical scavenging activity of the sample was calculated as

% Inhibition of DPPH radical = (Control-Test)/ Control * 100

20 min after incubation, the absorbance was recorded at 517 nm and the percentage of inhibition was computed by using the formula. The experiment was performed three times.

FRAP assay:

FRAP assay was carried out to understand the reducing power and the antioxidant activity if any for the silver nanoparticles coated with components of LAB isolated from breast milk. Here, for blank: 2mL FRAP reagent + 1 mL of water was added. For Samples: 100 µL sample + 900 µL water + 2 mL FRAP reagent was added. The tubes are mixed and placed for 30 min in the dark. Then the optical density was measured at 593 nm. The equation Y= mX+C is considered for calculating X= Unknown concentration and expressed as µM.

Results

Breast Milk collection and serial dilution:

Isolation of *Lactobacillus* is from breast milk was considered essential to study their capability of formatting coated nanoparticlesand various procedures were adopted and carried out for isolating *Lactobacillus* from breast milk (Figure 1a). The sample was serially diluted and the spread plate was performed and incubated (Figure b). After incubation, gram staining was performed to visualize *Lactobacillus*. Quadrant streaking was done to obtain complete rods without any cocci.

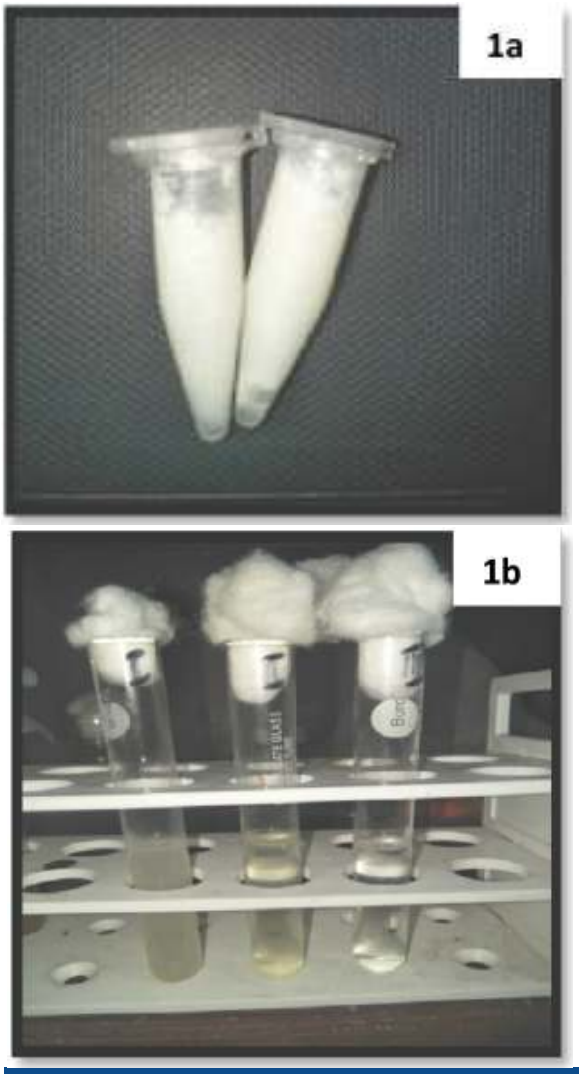

Figure 1a and 1b: Breast milk sample and serial dilution of the sample

Isolation of lactobacillus sp., from breast milk:

From the collected breast milk sample *Lactobacillus* sp., was isolated by streaking on nutrient agar plate and the growth was observed. The Figure 2a shows the mixed culture, and then based on staining techniques the pure culture of *Lactobacillus* sp., was isolated (Figure 2b).

Identification oflactobacillus sp. gram staining

The results obtained after gram staining indicated and confirmed that the organism was found to be *Lactobacillus* species by their morphology (Figure 3). Gram-positive rod shaped facultative anaerobic bacteria was observed.

Synthesis of silver nanoparticles:

The *Lactobacillus* species was inoculated with silver nitrate and incubated in dark for 72 h for the synthesis of silver nanoparticles. The change in the

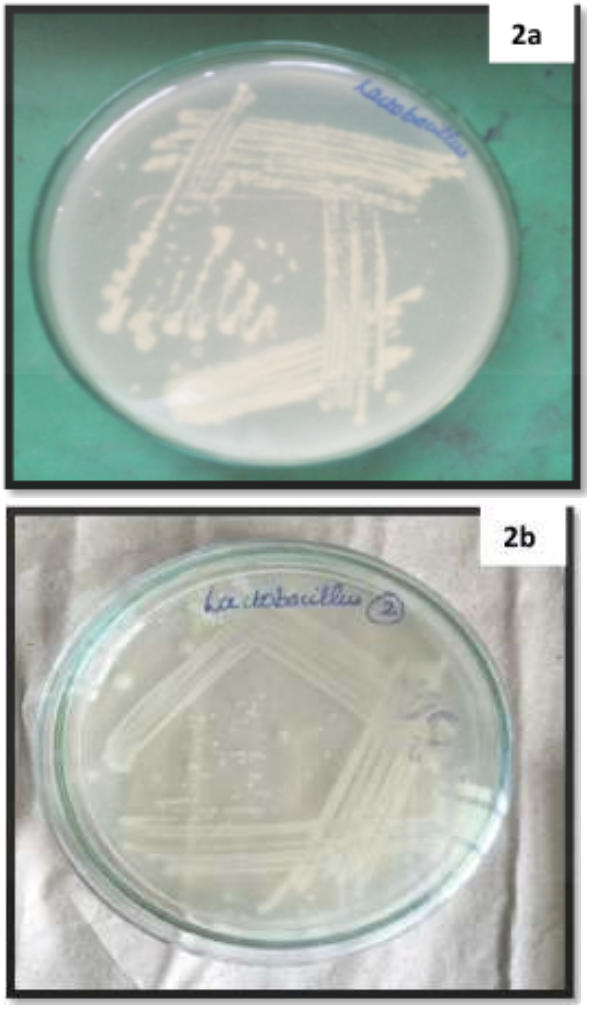

Figure 2a and 2b: Mixed culture and pure culture of LAB isolated from breast milk

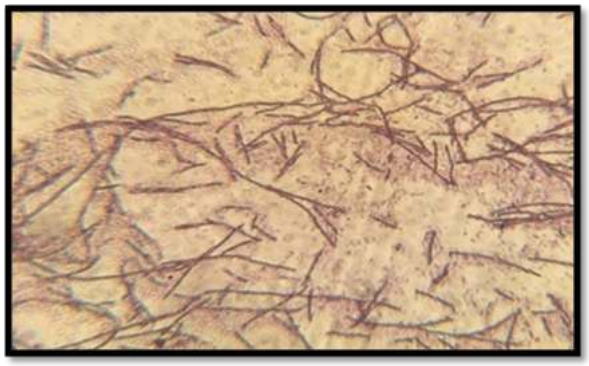

Figure 3: Identification oflactobacillus sp. gram staining

colour was observed indicating that the formation of silver nanoparticle was showed in Figure 4

UV- VIS absorption spectra

The UV- VIS absorption spectra of *Lactobacillus* species coated silver nanoparticles was observed and a strong peak obtained in the UV region (Figure 5)

Figure 4: Synthesis of silver nanoparticle

DPPH assay:

Figure 6 indicates theresults of DPPH assay. The results show elevated percentage of inhibition of DPPH which gets increased with increasing amount of oxalic acid (Figure 6). From this we found that nanoparticles that exhibited more inhibition(98% in 5g/ml) due to electron donated and accepted by DPPH.

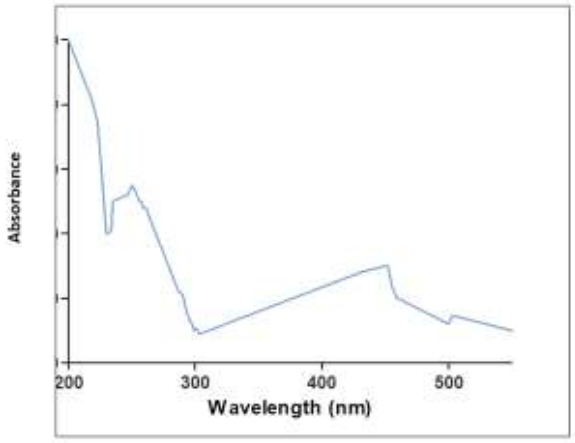

Figure 5: Spectral studies of LAB coatedsilver nanoparticlesUV-Vis

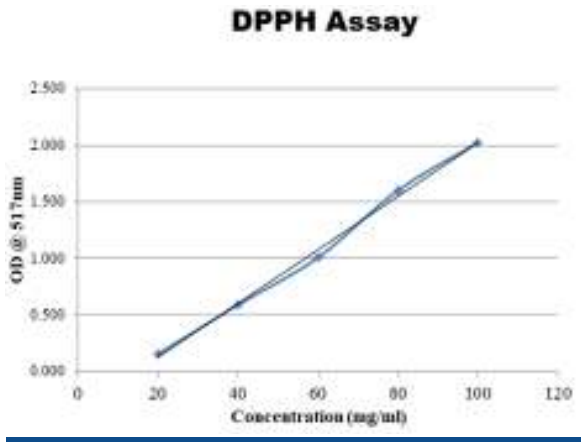

Figure 6: DPPH Assay

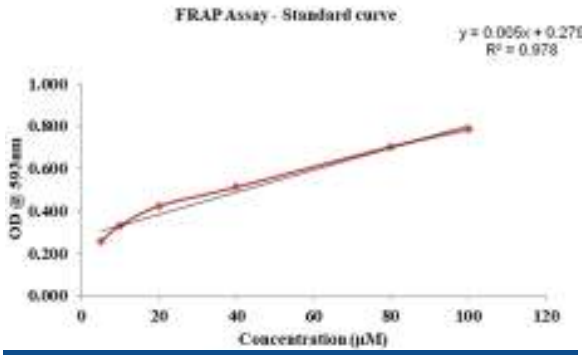

Figure 7: FRAP Assay

FRAP Assay

Antioxidant activity of breast milk *Lactobacillus* coated nanoparticles was studied by FRAP assay (Figure7 Standard Plot). Coated samples exhibited superior ferric reducing antioxidant power compared to that of vitamin C. Values indicated that the ferric reducing antioxidant potential of coated nanoparticles by *Lactobacillus* species was about 2-fold higher compared to the standard.

Discussion

LAB strains can reduce silver ions into silver nanoparticles. Synthesis of nanoparticles takes place by the interaction of silver ions and the organic compounds in the cell. Silver nanoparticles produced earlier indicated an average size of 11.2 nm which was by *Lactobacillus fermentum*. Smaller size nanoparticles can easily go inside the cell whereas large size particles are distributed outside of the cells. Increase in the pH increases the production of nanoparticles.[14]

Here in this study of our work we employed the potential of *Lactobacillus* species isolated form breast milk to synthesize AgNPs. The appearance of brown color in solution is a clear indication of the formation of silver nanoparticles in the reaction mixture due to the reduction of Ag^+ ions to Ag by the presence of reducing agents in the cultural supernatants which is a component of LAB. Similar observations were indicated earlier. The exact reaction mechanism leading to the formation of silver nanoparticles by the components of the LAB from breast milk is yet to be elucidated. It has been observed in several earlier studies that extract from microorganisms may act both as reducing and capping agents in AgNPs synthesis.[17]

Our research on the results of DPPH assay shows the elevated percentage of inhibition of DPPH which gets increased as the concentration of the silver nanoparticles increased. The inhibition of DPPH to 98% was observed at 5mg/mL of the solution. Even though this is a preliminary result, these results are

promising and further purification of silver nanoparticles coated with LAB can improve the inhibition of DPPH at a lower concentration. It can be understood that the inhibition potential of LAB coated AgNPs could be attributed by preventing the production of free radicals rather than the scavenging of free radicals.[18]

So as to understand whether LAB coated silver nanoparticles played a role in preventing free radical production or the LAB or cell-free supernatant, separate studies were carried out. For the same dilution of LAB nanoparticles used LAB only indicated 17% inhibition whereas cell free supernatant indicated 10.3 % as compared to 98% of LAB coated AgNPs. It can be deciphered that LAB components, along with AgNPs plays a vital role in preventing free radical production.

Antioxidant activity of *Lactobacillus* in breast milk was also studied by FRAP assay. Bacterial sample exhibited superior ferric, reducing antioxidant power compared to that of standard. LAB coated AgNPs indicated higher antioxidant activity.

Conclusion

Silver nanoparticles is shown to exhibit excellent antioxidant properties due to theunique physical and chemical properties of the silver. Though various studies report the efficacy of silver nanoparticles produced by bacteria, but the mechanism of action is not fully understood. Synthesis of nanoparticles via physical and chemical methods are considered expensive and conventional one and it exerts a certain toxic effect. To overcome these problems, the biological synthesis of silver nanoparticles is the preferred option. Our studies indicated LAB from breast milk can trigger the antioxidant potential of silver nanoparticles

Conflict of Interest

The authors declare no conflict of interest.

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