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Research Article

EFFECTS OF SILVER NANOPARTICLES ON STORAGE STABILITY OF RAW MILK

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ABSTRACT

Silver nanoparticles show anti-microbial and antibacterial effect as well as used for milk-spoiling microbes. In this work, cultures of milk spoilage microbes were isolated and the silver nanoparticles were synthesized using sodium borohydride. The milk-spoiling microbes, *Trichosporon asahii* and *Lactobacillus casei* were isolated from the spoiled milk. The antibacterial effect of the silver nanoparticles was investigated for both of these microorganisms. The kinetic growth studies as well as the test of zone of inhibition and colony counting showed the successful synthesis of the nanoparticles and their influence on the microbial activity i.e. their inhibitory effects. Moreover, tests were performed for the impregnation of nanoparticles on an aluminum surface. The microbial studies were carried out with silver nanoparticles impregnated on the surface of the aluminum foil. This will provide a stable packaging system for milk storage.

Keywords: Ag nanoparticles, Anti-microbial effect, Impregnation, Aluminum foil

INTRODUCTION

Nanotechnology emerges from the physical, chemical, biological and engineering sciences, where, novel techniques for the synthesis of nanoparticles are employed for the development of eco-friendly and sustainable methods [1]. The antibacterial effect of silver is well known for 3,000 years. Nanoparticles have been used as colloids of gold, silver and copper to embellish calligraphy and in stained glass windows in medieval European churches and used a silver coin to keep the milk safe from the spoilage. In the last few years, the industry has started to use silver and its antibacterial effects in a lot of products like non-smelling socks or other cloths and it has shown better wound-healing capacity [2].

The antibacterial mechanism of silver is related to its interaction with sulfur and phosphorus, most notably thiol groups (S–H) present in cysteine and other compounds. Interaction of ionic silver (which can be released from nAg) with thiol groups and formation of S–Ag or disulfide bonds can damage bacterial proteins, interrupt the electron transport chain and dimerize DNA. That means that enzymes are inhibited, oxidative stress is induced and the defense molecules are depleted. Similarly, the antiviral properties of silver ions involve interactions with viral DNA and thiol groups in proteins [3]. Most of the antibiotics block only certain mechanisms like that of streptomycin which blocks 70-S-ribosomes. The only way that a microbe is safe from the effects of silver, is that the microorganism build some sulphurous molecules which react with the silver as protecting molecules [4].

In recent years, Drug resistance to human pathogenic bacteria has been commonly reported from all over the world [5]. The wide use of antibiotics in the treatment of bacterial infections has led to the emergence and spread of resistant strains which would result in the formation of new antimicrobials. In recent scenario, much attention has been paid to metal nanoparticles which exhibit novel chemical and physical properties owing to their extremely small size and high surface area to volume ratio [6]. The toxicity of silver nanoparticles to bacteria is greatly influenced by nanoparticles particle size and shape. The silver nanoparticle synthesis and their uptake in bacteria have been reported for spheres and rods up to 80 nm. The toxicity mechanisms of Ag+ ions that dissolve from nAg are well understood, but the extent to which direct contact between bacteria and silver nanoparticles causes toxicity remains unclear. Bactericidal action of silver ions also increases with increasing temperature and pH, which are two important factors with respect to the toxicity of silver nanoparticles. The high specific surface area of silver nanoparticles is responsible for the higher amount and better delivery of silver ions than that in bulk silver [7].

The lower thresholds for silver ion (Ag+) toxicity lie between 0.01 and 0.1 mg/L. The World Health Organization established that 0.1 mg/L of silver in drinking water can be tolerated without risk to human health. In humans, silver ions cannot cross the blood–brain barrier, and they are regulated by blood metallothioneins that bind with metal-thiolate-cluster structures for transport, storage and detoxification. The microbes produce milk acid i.e. lactic acid. This inhibits the growth of microbes, but it is also the reason for the milk-spoilage [8].

Some of the milk spoiling microbes is pathogens but there are some other microorganisms which are important for the human's health like the probiotic bacteria. Often lactic acid bacterias (LABs) are called probiotics. Fungi like yeasts and other bacterial species can also be probiotics. Probiotics is Greek word with the meaning "for the life" (pro bios). On the other hand, there are milk microorganisms which are dangerous for the human health like *Streptococcus sp* [9].

MATERIALS AND METHODS

Preparation of Ag nanoparticles

Nanosilver particles were synthesized by using Creighton's method by using sodium borohydride mixed with silver nitrate [10, 11]. Beaker was kept in a box of ice cubes for preparation of 100 ml of 2 mM sodium borohydride solution. 7.56 mg of sodium borohydride (M=37.83 g/mol) was mixed with 100 ml of distilled water. To stabilize the synthesized silver nanoparticles, 1% of tri-sodium citrate (1g of 100 ml distilled water) was used. The synthesis was carried out in a dark reaction space. 2 ml of the 1% tri-sodium citrate was added under stirring. After 5 min incubation, 10 ml of 1 mM silver nitrate was added drop wise. The first indication for a positive result at the synthesis is the formation of yellow colored synthesis solution. The second indication is the creation of silver nanoparticles typical peek around 390 to 420 nm in UV–spectrogram.

Bacterial cultures and evaluation of antibacterial activities

To study the anti-microbial experimentations, raw spoiled milk was taken and serially diluted. All materials need for experiment is sterilized by autoclaving MSR-Agar, which is a special nutrient agar for *Lactobacillus sp* used as a main source for culturing and incubated in 37 °C. The staining and biochemical tests were done to know the morphology of isolated colonies. The target species identified was *Lactobacillus casei* and *Trichosporon asahii*. The density of bacterial and yeast cells in the liquid cultures were estimated by optical density (OD) measurements at the range of 200 to 800 nm wavelength. The zone of inhibition was studied with different concentrations and the kinetic growth study was done with and without silver nanoparticles.

RESULTS

Determination of the microorganisms

Staining

The Gram staining showed that one culture consists of Gram-positive long thin Bacilli which were mostly separated, though sometimes found in pairs. The other one showed violet colored big oval organisms. To rationalize the second culture, let us assume that this is a fungi-culture. The second culture on staining with methylene blue showed some structures similar to hyphae. This proves that the assumption for the second culture being a fungi/yeast-culture holds good.

Sugar fermentation test

The sugar fermentation test showed that the Durham's tubes which contained gas bubbles had the sugar metabolizing microorganisms. The positive and negative result is represented by the following Table 1.

Table 1: Sugar fermentation test for sample 1 and 2

Microbs	Arabinose	Cellulose	Cellubiose	Dextrose	Fructose	Galactose	Lactose	Maltose	Mannitol	Raffinose	Sucrose	Xylose
1	-	-	+	+	+	+	+	+	+	-	+	+
2	+	-	+	+	+	+	+	+	+	+	+	+

This test lets us infer that the first sample is *Lactobacillus casei* and the second sample contains *Trichosporon asahii.* For these inferences, the databases like mycobank and certain articles describing the reaction of bacterias with those sugars were used. To confirm these inferences, other tests were performed.

Growth at different pH-value

There was microbial growth found with the probability of having *Trichosporon asahii* in the media with the pH of 2.0 or 9.5. Furthermore, this microorganism grew very well in the pH of 6.0. The *Lactobacillus spp.* grew well in the media with the pH value of 6.0. In the alkaloid media, no growth could be observed, but in the acid media some cell-growth was found. *Lactobacillus spp.* is well known for their survival at low pH. They have the ability to produce lactic-acids; with this production, they decrease the pH of the nutrient media and so they are adapted to survive in low pH. On the other hand, the *Trichosporon spp.* is adapted to both high and low pH values.

Catalase test

In the catalase test, the reaction of *Trichosporon asahii* with hydrogen peroxide produced effervescence in the form of bubbles, but not in the case of the *Lactobacillus* culture. Hence, this test shows that *Trichosporon asahii* is catalase positive, but on the other hand the *Lactobacillus spp.* is catalase negative.

Oxidase test

In this experiment, the Oxidase test disk colored after 1 min in the case of *Lactobacillus casei* culture; but for the *Trichosporon asahii* culture, the testing disk changed the color directly after coming in contact with the microorganism solution. The inference is that the *Lactobacillus casei* is oxidase negative and the *Trichosporon asahii* is oxidase positive.

Mobility test

Both microorganisms can't be observed with any kind of mobility in the hanging drop experiment. This infers that they don't have flagella or any other locomotory organelle for any type of movement. They move around using the dynamics in the fluid.

Micro-organism isolated from raw milk that cause spoilage

Trichosporon asahii occur in tropical places. The milk which was used for the attempts was produced in India, which is a tropical country, and hence a warm place. This microbe is pathogenic for the human body and can be infectious to people with low immunity. It is an opportunistic organism and this *Lactobacillus sp.* is well known as the lactic acid bacteria (LAB). They have the ability to produce lactic-acid by the fermentation of lactose. This microorganism is nonpathogenic, *Lactobacillus casei* keeps *Samonella sp.* at bay and saves the human body from diarrhoea.

Quantification of silver nanoparticles

The measurement of the amount of silver nanoparticles in the synthesis solution and the solution with the immobilized nanoparticles was done directly after the synthesis with the help of a UV–spectrophotometer. Both solutions showed two peaks around 210 and 393 nm respectively. The second peak implied the successful synthesis and represents the presence of silver nanoparticle in the solution, since, the range should be between 390 to 400 nm. The pH value of the solution with only silver nanoparticles was 8.75, and for the solution in which the nanoparticles were synthesized in the presence of aluminum foil has a pH value of 8.84 on the first day.

Zone of inhibition

This experiment [12] showed the antibacterial effect of silver nanoparticles for the milk spoiling microbes and also for the spoiled milk sample. Silver nanoparticles have an antibacterial effect and the zone of inhibition was observed. The following table shows the size of zone of inhibition for the different samples used.

Table 2: Size of diameter of zone of inhibition for silver nanoparticles (in cm)

	Trichosporon asahii		Lactobacillus casei		Spoiled milk	
	1st plate	2nd plate	1st plate	2nd plate	1st plate	2nd plate
100 µL	1.4 cm	1.4 cm	0.5 cm	0.5 cm	0.0 cm	0.0 cm
150 µL	1.5 cm	1.5 cm	0.5 cm	0.4 cm	0.1 cm	0.0 cm
200 µL	1.6 cm	1.5 cm	0.5 cm	0.5 cm	0.2 cm	0.1 cm

The result showed that the antibacterial effect of the silver nanoparticles is better for the *Trichosporon asahii* than that of the *Lactobacillus casei*. Serially diluted sample of the spoiled milk was used. The sulfur is present in the milk proteins as well as in the agar media (MRS agar), these proteins being smaller and free; while, the microbes also have sulfur present on their surface as well as in their intracellular proteins, but those proteins are not free. The nanoparticles bind easily with sulfur molecules present on the free and smaller proteins.

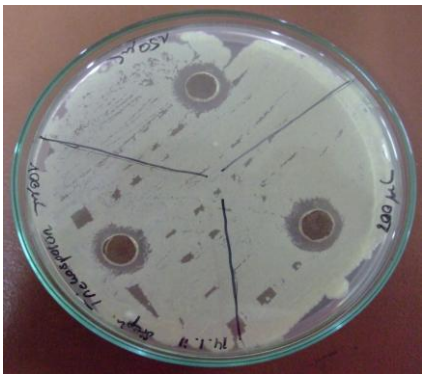

Fig. 1: It shows the zone of inhibition with silver nanoparticles for *Trichosporon asahi*

Growth kinetics

In this study, the reaction of the milk-spoiling microbes with silver nanoparticles (free and impregnated) as well as with the impregnation material was observed. For both microorganisms, it was found that the solution with only microorganisms have the highest growth. The solution with the free silver nanoparticles showed the lowest growth of microbes or the highest inhibition, followed by the immobilized nanoparticles.

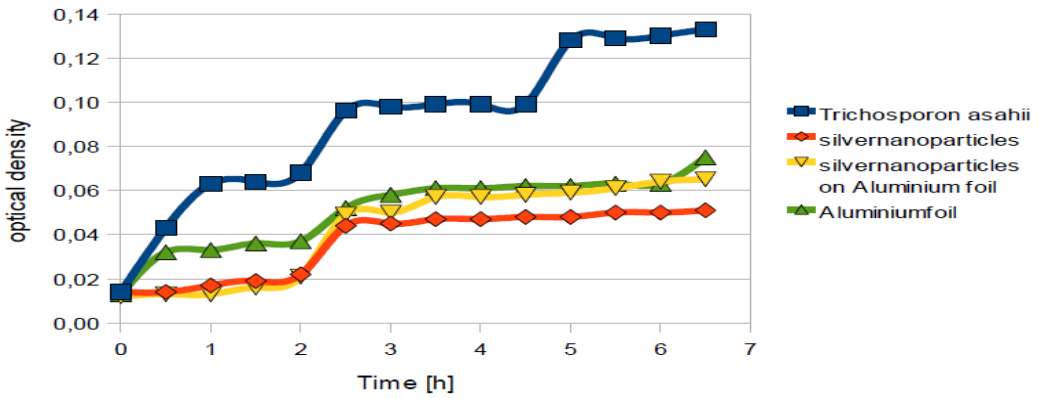

Fig. 2: It shows the kinetic studies with *Trichosporon asahii*

The graph for the plain silver nanoparticles shows a lesser growth and more inhibition when compared to the aluminum foil. When the aluminum foil with nAg and the silver nanoparticle culture's growth activities are compared with that of the pure microorganism culture, it could be observed that the antibacterial effect of silver and aluminum is very high.

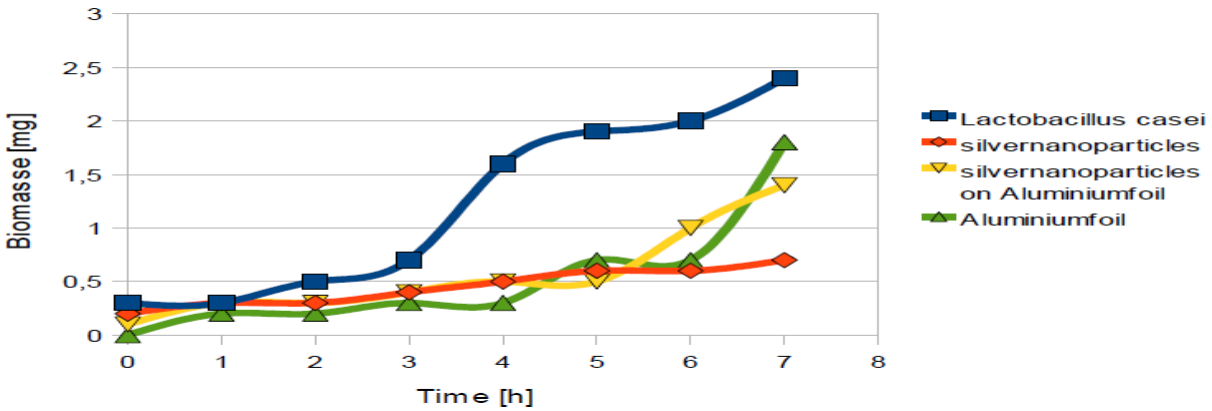

Fig. 3: It shows the kinetic studies for the antibacterial effect of nAg for *Lactobacillus casei*

Correlation of the biomass of *Lactobacillus casei* as a function of the time for the different antibacterial agents added to the culture media. The inhibition by silver nanoparticles and aluminum were found to be similar. In the beginning, the antibacterial effect of the aluminum foil is higher than the inhibition by silver nanoparticles. A possible explanation can be given that the silver nanoparticles might react with some sulphurous components of the MRS-Agar, so that the antibacterial effect of aluminum seems to be higher. The aluminum foil delays the log-phase; hence, the stationary phase is achieved later on.

CONCLUSION

The silver nanoparticles were successfully synthesized via Creighton's method. These silver nanoparticles showed a good stability over the time-period of 25 days. The spoiled milk was confirmed with the presence of *Lactobacillus casei* and *Trichosporon asahii.* The experiments on the antibacterial effects of silver nanoparticles showed a better inhibition of *Trichosporon spp.* than that of *Lactobacillus spp*. From the results of the kinetic studies, it can be said that there is only a less amount of impregnation of silver nanoparticles happened on the aluminum foil. This showed that the difference of the antibacterial effect between impregnated aluminum foil and pure aluminum foil is not very significant. Furthermore, it could also prove the well known antibacterial effect of aluminum.

A really important factor which must be investigated is the toxicity of nanoparticles in the humans; since, more and more of the silver products are being used in our day-to-day life. In this case, a different nanoparticle which also has antibacterial and anti-microbial effect as well as no reaction with raw milk can be searched for. Thus, from this study, the silver nanoparticles impregnated on the aluminum surface prevent the milk from spoilage and provides a stable package systems for the milk and milk products.

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