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Synthesis of Silver Nanorods from Food Industrial Waste and Their Application in Improving the Keeping Quality of Milk

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Supporting Information

ABSTRACT: A novel method for the synthesis of silver nanorods is reported, in which industrial milk waste was utilized, that were then used to extend the stability of milk. During the synthesis, the size of the silver nanorods were affected by pH and temperature. Silver nanorods were formed at alkaline pH in room temperature, whereas nanoparticles were formed in lower pH at elevated temperature. The obtained nanostructures were characterized by UV−visible spectrophotometer, energy dispersive X-ray analysis (EDAX), and transmission electron microscope (TEM). These silver nanorods were used to control coliform and standard plate count (SPC) in milk. This was confirmed by an increase in 4 to 5 folds of methylene blue reduction time as compared to the control. The Hom inactivation model was proposed to express microbial inactivation in milk. The cytotoxic effect of silver nanorods shows that they have been nontoxic to humans even at higher concentration.

1. INTRODUCTION

In recent years nanotechnology has been rising as a rapidly growing field with abundant application in science and technology. The active surface area of AgNPs would be significantly large when compared to their compounds, which exhibits remarkable abnormal physiochemical properties and biological activities when exposed to cells of tissues.[1−5] The medicinal properties of silver nanoparticles and their utilization of silver as a drinking agent are not new. Silver compounds act as an effective antimicrobial agent for both aerobic and anaerobic bacteria by precipitating microbial cellular protein and also by blocking the microbial respiratory chain system.[6−8] With advancement of nanotechnology, the interest in the use of the antibacterial potency of silver nanoparticles has been rekindled. The antimicrobial mechanism of AgNPs is similar to that of silver compound. However, because of the larger surface area to volume ratio, AgNPs may have far better efficiency.[4]

Nanosize silver has a large surface area for better contact with the micro-organism. The nanoparticles get attached to the cell membrane and react with amino acids containing sulfur that successfully affects bacterial cell viability.[9] AgNPs can also attack the respiratory chain in the mitochondria of micro-organisms and result in their death.[6] A recent study reveals that the antimicrobial activity of AgNPs is solely due to Ag^+ release even at lower concentrations (released or adsorbed on AgNP coatings).[10]

One dimensional metal nanostructures such as wires, rods, and tubes have attracted substantial interest in research because of their unique applications.[11] Silver nanostructures synthesized through chemical methods have adverse chemicals present on the surface of the nanomaterials, which are highly reactive and hazardous.[12] Hence, a green synthesis method employing algae, plants and fungi extracts are used for nanomaterial synthesis.[13] Recently harmless green chemicals like glucose, maltose, lactose, galactose, etc., are also used.[14]

Generally two approaches were employed for metal nanorods synthesis, either by hard template or soft template methods.[15,16] Silver nanorods have been synthesized by using polymers, wet chemicals, sono-chemical, surfactant assistant method, etc.[17,18] When compared to AgNPs, silver nanorods are particularly interesting because of their higher electrical and thermal conductivity and exhibit higher antimicrobial property.

In this paper a seedless, cost-effective method was proposed to synthesize silver nanorods by using industrial waste materials. In earlier methods reducing agent, capping agent, and surfactants were used for synthesis. Here the liquid waste which contains lactose and a small quauntity of whey acts as both reducing and capping agent. The only affecting parameters were pH and temperature, which decide both the particle size and shape. The industrial waste material can be reused and the product also will be useful for some other industrial purpose.[19,20]

Paneer is a high protein cheese food from South Asian countries, which is produced by curdling of milk. The milk is heated to 70−90 °C and acidified using lemon juice or food grade acids like viniger or citric acid, etc. The coagulated solids settle and the liquid part is separated as waste. At present there is no literature evidence for the synthesis of silver nanorods from these wastes and its application in the keeping quality of

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milk. SPC, coliform counts, and methylene blue reduction test (MBRT) were conducted to assess the microbial quality of raw milk. The inactivation kinetics of the Hom model was used to compare the microbial inactivation between control and experiment.

2. MATERIALS AND METHODS

The milk waste and cow milk samples were collected from Aavin Dairy (Chennai, India). NaOH and HCl were received from SRL, India. Deionized water and micrometer filters were purchased from Fischer Scientific, India. $AgNO_3$ was obtained from Merck, India. Methylene blue thiocyanate tablets, buffered peptone water, standard plate count agar, and violet red bile agar were purchased from Merck, India.

2.1. Synthesis of Silver Nanorods. The wastewater drained from the milk industry while making paneer was collected. It was then filtered through 0.4 μm filter to separate fine suspended particles. Silver nitrate solution (0.001M) was prepared and dissolved in 90 mL of deionized water. The pH of the filtrate was adjusted by using NaOH. In the course of adjusting pH, the colloidal particles formed were removed by centrifuging at 6000 rpm for 10 min. The supernatant was then collected and stored in an airtight container below 5 °C.

The pH of supernatant solution was adjusted to acidic condition and maintained at room temperature. To 120 mL of 1 mM silver nitrate 10 mL of the above solution was added. Samples were collected at different time intervals and analyzed. The nanorods were removed by centrifuging at 15 000 rpm for 20 min. The settled nanorods were redispersed by adding deionized water and centrifuged again as mentioned earlier in the procedure.The same procedures were repeated two times to obtain silver nanorods.

2.2. Instrumentation. The results were analyzed using UV−vis spectrometer. All absorbance measurement were carried out on a shimadzu UV−vis spectrophotometer. (UV-1800, Japan). Milk industrial waste was used as a blank solution. The samples were analyzed without further dispersion. The sample was characterized using XRD with an X'Pert Pro X-ray diffractometer with Cu Kα radiation (λ = 1.54178 Å). The size and morphology of silver nanorods were performed on a high resolution TEM with an accelerated voltage of 250 KV equipped with SAED pattern (HITACHI-TEM 2010, Japan). Quantitative analyses of elemental composition were investigated by EDAX (Noran System Six, Thermo Electron Corporation, Japan).

2.3. Cytotoxicity Assay. Cytotoxicity assays are essential for determining the responses of human normal cells. Cytotoxic effects of synthesized silver nanorods on skin epithelial, normal human fibroblasts, and breast carcinoma cells were studied. A 1 mL stock concentration of the silver nanorods solution was diluted with DMEM medium to obtain a working concentration. The cytotoxicity assays were carried out using 100 μL of cell suspension containing 10^4 cells. These live cells were counted and distributed in a 96-well ELISA plate with 100 μL of culture medium. The plate was incubated for 24 h to 48 h at 37 °C in 5% CO_2 atmosphere to allow the cells to attach to the bottom of the well. This forms the monolayer of seed cells, which serve as cell control. After 24 h from seeding of cells, fresh medium containing 100 to 1000 μL concentration of the silver nanorods were inoculated to all the wells except control. The microtiter plate was incubated for 48 h at 37 °C in 5% CO_2 enriched atmosphere, seeded at different densities with (positive control) and without (negative control). Eight wells (duplicates) for each test concentration against the above said three cells were studied. The cell population was then determined by optical microscopy at 24 and 48 h.

2.4. MBRT Test. The hygienic status of the raw milk samples was determined using MBRT. One milliliter aliquots of methylene blue solution was transferred to labeled and sterilized screw-cap test tubes containing 10 mL of milk samples with different concentrations of silver nanorods. The tube was tightly capped and gently inverted to mix the solution. Each tube was incubated at 37 °C and examined at 10 min time intervals until the color of solution disappeared. The MBRT can thus be used to evaluate the growth curve and the viability equivalent to plate count.[21]

2.5. SPC and Coliform. Milk samples with different concentrations of silver nanorods were investigated through SPC and a coliform count method for finding the microbial load. Samples were taken at different time intervals, and different dilutions were made using buffered peptone water; 10-fold dilutions were prepared ranging from 10^{-2} to 10^{-7}. SPC agar was used to enumerate the total count of mesophlic aerobic bacteria, and coliform numbers were determined using violet red bile agar plates incubated at 37 °C for 48 h. The above-mentioned experiments were repeated again at different time intervals for microbial inactivation kinetics.

3. RESULTS AND DISCUSSION

3.1. Preparation and Characterization of Nanorods. Color change occurred from white to yellow by mixing the silver nitrate solution with milk waste. The solution has a lower pH at room temperature, and the colorless solution changes to yellow with increasing time interval. The color changes from light yellow to dark yellow on increasing the pH from 4 to 8 at 60 °C. Both resultant samples were tested using UV−visible light and TEM analysis. Reactions under both parameters give different effects on nanomaterial synthesis.

3.2. UV−Visible Absorption Spectra. By increasing the pH to 6, 7, 8, and 10 nanorod formation is predominant. At lower pH, double absorption spectra are seen between 400 nm and 600 nm which are attributed to the transverse and longitudinal plasmon bands indicating the formation of nanorods and some nanoparticles (Figure 1). At lower pH,

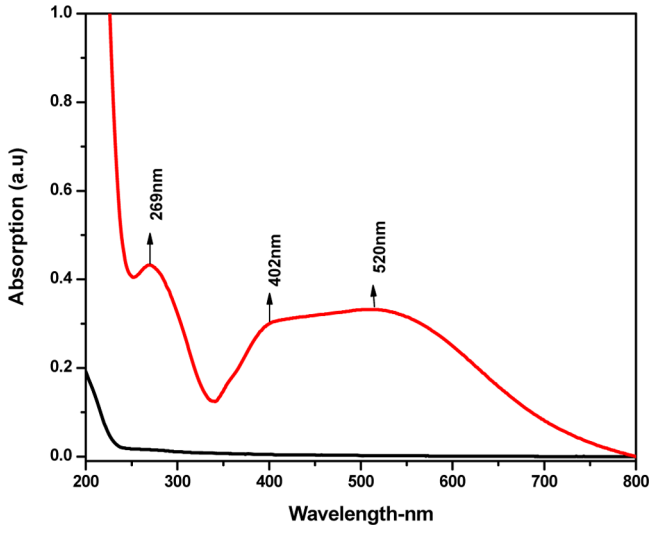

Figure 1. UV spectrum of silver nanorods at lower pH.

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double absorption spectra are seen at 402 and 520 nm which are attributed to the transverse and longitudinal plasmon bands indicating the formation of nanorods and some nanoparticles.[22] From Figures 1 and 2, the adsorption peak at 269 nm is attributed to lactose and with increasing pH the rod formation increases and only a single broad peak is seen.[23,24]

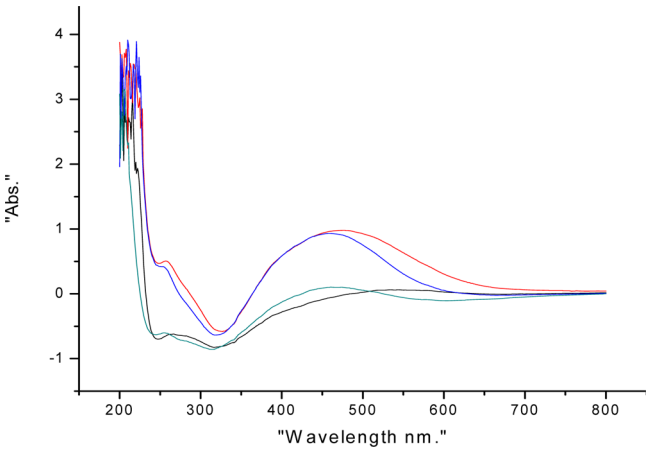

Figure 2. UV−vis spectrum for silver nanorods at different pH (6−10).

At pH 8, with a constant temperature of 60 °C and an increasing time interval from 15 to 120 min, the spectrum gives a sharp peak at 420 nm because of the formation of fabricated non-agglomerated particles (Figure 3). The heating of colloids converts the shape of the silver nanorods to spheres.[25] However with increasing pH, the reduction of silver increases and rod formation also increases due to interparticle surface attraction. At a controlled temperature, uniform sized non-agglomerated nanoparticles were obtained because of decreasing interparticle surface energy.[26] The source materials have a lower pH, but for synthesis of nanorods a higher pH is required. The size of the nanorod varied based on the amount of reacting NaOH concentration, and the materials were stable. At room temperature, microorganisms are present in the milk.[27] The longitudinal surface plasmon band shifts to longer wavelengths with an increase in rod formation.[28]

At lower pH, the reduction is low so the lower numbers of nanoparticles were in the solution. Fewer numbers of nanoparticles joined together to form nanorods and some free nanoparticles were also present at lower pH. Waste milk contains lactose which gets degraded with increasing temperature forming acids. The pH decreased with increasing temperature; hence, the reduction of Ag^+ was also decreased. Because of low pH and high temperature nonagglomerated nanoparticles were formed. Upon an increase in time, a greater number of particles get reduced thus giving a sharp peak at 420 nm in the spectrum. But after 90 min suddenly the sharp peak recedes. This shows that the silver nanoparticles were unstable over 120 min of heating.

3.3. XRD, TEM, and EDAX Analysis. Silver nanorod diffraction peaks correspond to (111), (200), (220), and (311) at 38.1, 44.6, 64.4, and 77.3 (JCPDS 87-0720). The XRD pattern reveals the face-centered cubic structure of a Ag rod with an (111) strong peak.[29] TEM and SAED images have been employed to investigate the morphology of the resultant products and mechanism of formation of silver nanorods. The nonagglomerated silver nanoparticles present in the solution express the mechanism of particles joined together by surface attraction; here pH was adjusted to get the silver nanorod for uniform size (Figure 4a,b). EDAX spectra showed that the nanoparticles were composed of silver. No other peak for any other element has been found, which confirms again that the grown nanoparticles are silver.

3.4. Mechanism for Silver Nanorods Formation. A new approach is proposed for synthesis of silver nanorods from milk industrial waste without the use of a specific surfactant or any other seed growth method. The waste whey contains 6 to 7% solids in which 70% of lactose, 0.9% protein, and traces of water-soluble vitamins, minerals and fats are present. Lactose present in waste acts as both reducing and stabilizing agent. Silver nanorods were synthesized without the use of surfactants and only by changing pH. In the initial stage of the addition of

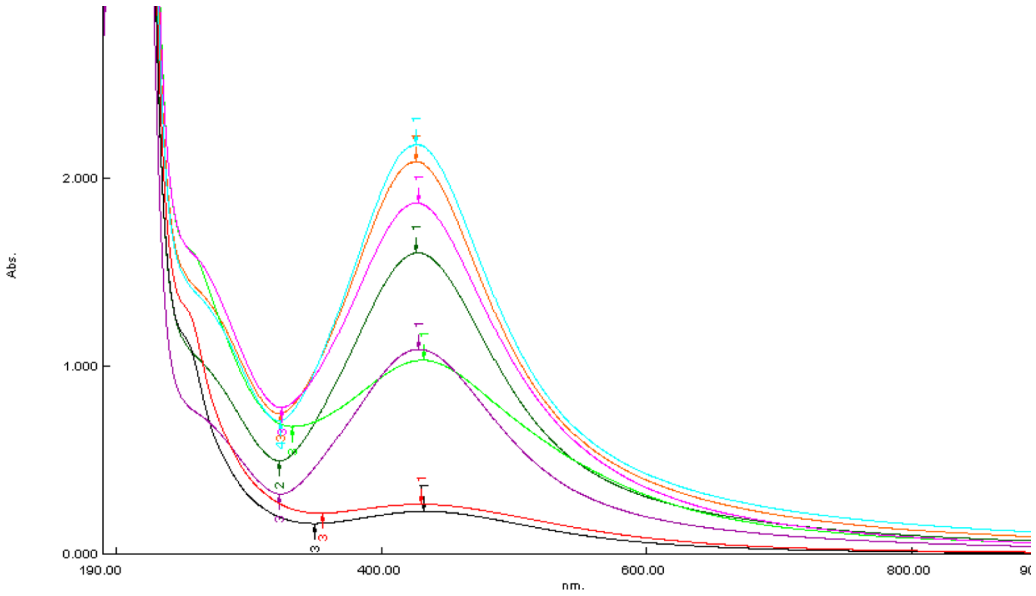

Figure 3. UV−vis spectrum for silver nanorods at 60 °C in different time intervals.

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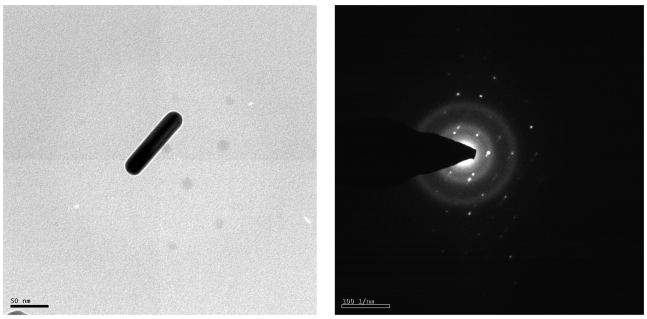

(a) Nano-rods (b) SAED pattern

Figure 4. TEM images.

NaOH, slow reduction occurs; further with increasing time, the reduction of silver might occur on the surface of existing colloids. The quantity of added alkaline ion would decide how much silver could be produced.[30] A greater number of silver nanoparticles were formed with increasing pH. The growth of rodlike crystallites usually results from different surface energies of the respective lattice planes. During growth, the formation of chainlike aggregates and nanorods was observed.[31] The mechanism of formation of these silver chainlike nanostructures was explained. It was confirmed that initially Ag nanoparticles were formed. With an increase in pH they self-assembled and fused with each other to yield nanorods. Free anomeric carbon (reducing end) present in the lactose reduces silver ion. The reduction of silver nitrate to silver nanoparticles and release of H^+ ion was in the ratio of 2:3 which is expressed in the following equation.

$$2Ag^+ + C_{12}H_{22}O_{11} + H_2O \xrightarrow{NaOH} 2Ag + C_{12}H_{21}O_{12}^- + 3H^+ \quad (1)$$

3.5. Temperature Effect. Waste-containing lactose was taken at pH 8 with 60 °C constant temperature. At elevated temperature lactose degradation starts and acids form. With the decrease in NaOH concentration, the reaction rate decreased and formation of nanorod aggregation also decreased. Non-agglomerated silver nanoparticles are formed with increasing time interval, Here temperature controls the size of silver nanoparticles via degradation of lactose.

3.6. Cytotoxicity Analysis of Silver Nanorods. The *in vitro* cytotoxic effects of silver nanorods were screened against three normal human cell lines. The morphology of the cells were inspected day by day (from 24 to 48 h) and observed for microscopically detectable alterations. The viability of the cells were analyzed through standard MTT assay. The absorbance value obtained for silver nanorod treated cells with respect to the control was determined. The sample does not show any direct response in the relationship with cytotoxicity analysis in human cell lines. Silver nanoparticles synthesized from dairy waste do not show any inhibition in cytotoxic analyses at 24 and 48 h, at maximum concentration of 800 μL concentration. There was a slight variation in absorbance at higher concentrations of 1000 μL at 48 h. The nanoparticles were unable to inhibit the cell line's growth.

3.7. MBRT, SPC, and Coliform Observed Results. Methylene blue reduction test reveals that the stability of milk increased from 4 to 5 times than that of the normal milk when silver nanoparticles were added (Table 1). Different

Table 1. Methylene Blue Reduction Test

	MBRT (min)						
concn	control (0)	0.25	0.5	0.75	1.0	1.25	1.5
sample A	45	45	110	110	195	195	195
sample B	60	60	90	90	230	230	230
sample C	20	20	45	45	105	105	105

dilutions were made from 10^{-1} to 10^{-8} 10-fold. From the dilution experimental values, the better results were selected for the three samples on 10^{-4} cfu mL^{-1} for coliform and 10^{-5} cfu mL^{-1} for SPC. Standard plate count values show that the total bacterial count controlled more than the coliform count for all the three samples. On the basis of the time interval of MBRT, colony count values also changed for the same samples. The MBRT time increases with a decrease in multiplication of microbial count because of the silver nanorods concentration and vice versa.

The initial concentration of coliform was 2 300 000 cfu mL^{-1}, and after adding silver nanorods, the coliform count decreases to 700 000 cfu mL^{-1} in 5 min and increased to 1 400 000 cfu mL^{-1} at 45 min, whereas in control the coliform increases from 2 400 000 cfu mL^{-1} to 3 200 000 cfu mL^{-1} at 45 min. SPC initial concentration was 20 000 000 cfu mL^{-1}, after 5 min the values decreases to 2 900 000 cfu mL^{-1} and reaches 9 000 000 cfu mL^{-1} in 45 min. The control SPC value increases from 22 000 000 in 5 min to 34 000 000 cfu mL^{-1} at 45 min.

Under aerobic conditions the silver nanorods react with oxygen to form silver oxide, which further reacts with H^+ and gets converted to Ag^+. Now this Ag^+ reacts with the microorganisms present in the milk resulting in inhibiton of oxygen intake capacity which leads to cell lysis. Equations 2 and 3 explain the mechanism for the formation of Ag^+ from silver nanorods.

$$4Ag_o + O_2 \rightarrow 2AgO \quad (2)$$

$$2Ag_2O + 4H^+ \rightarrow 4Ag^+ + 2H_2O \quad (3)$$

3.8. Inactivation Kinetics Using Hom Model. The Hom model kinetics was used to identify the inactivation of the microbial population. It is represented in eq 4.

$$\log N/N_0 = -kC^nT^m \quad (4)$$

where N is the number of microbial population at inactivation time and N_0 is the initial number of microbial population; k is the reaction constant, C is the concentration, T is the time in min, and n and m are variables.

The model results were shown in the Table 2, experimental and control values are different and have opposite log values. It shows that the control was not fit for the inactivation. Hom inactivation kinetics gives a different sign of the R^2 value for the control and experiment because there is no inactivation in the control, instead microbial counts increase with the increase in

Table 2. Inactivation Kinetic Constants for Coliform and SPC Using the Hom Model

kinetic constants	k	m	n	R^2
experiment coliform	0.4621	0.6209	0.6386	0.9308
control coliform	0.1499	1.4047	−10.92	−4.855
experimental SPC	1.374	0.9969	0.6145	0.925
control SPC	2.079	0.7964	−10.77	−5.554

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time interval. A graphical representation of the kinetics was plotted using MATLAB 7.8.0.347, and the results are shown in Table 3. The experimental plot for coliform and SPC were drawn using the Hom model curve. It was found that the control plot for coliform and SPC did not match with the Hom model curve.

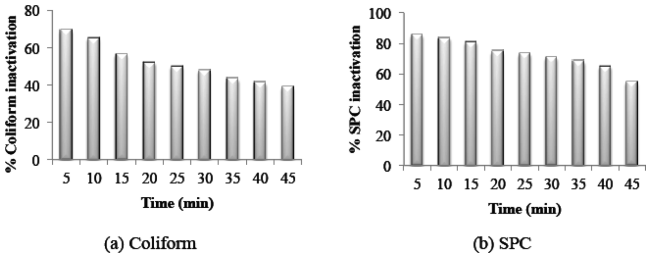
Table 3. Inactivation Kinetics Experiment at Time Intervals from 5 to 45 min for Control and Sample

time (min)	coliform (experimental) log N/N_0	coliform (control) log N/N_0	SPC (experimental) log N/N_0	SPC (control) log N/N_0
5	−0.51	0.018	−0.838	0.041
10	−0.48	0.036	−0.782	0.060
15	−0.36	0.069	−0.721	0.096
20	−0.32	0.077	−0.602	0.146
25	−0.30	0.100	−0.570	0.161
30	−0.28	0.115	−0.537	0.161
35	−0.24	0.129	−0.501	0.176
40	−0.23	0.129	−0.455	0.204
45	−0.21	0.143	−0.479	0.230

3.9. Microbial Inactivation Percentage Calculation. The microbial inactivation percentage can be calculated as

$$(B_C - B_T)100/B_C \quad (5)$$

where B_C is the initial control microbial concentration and B_T is the microbial concentration in time. The charts in Figure 5



(a) Coliform (b) SPC

Figure 5. Inactivation percentage versus time.

panels a and b explain the percentage of inactivation of coliform and SPC. After 5 min from an intitial concentration, 69.56% of coliform and 85.5% of SPC were reduced by the addition of silver nanoparticles. When the time increased the reduction of microbial percentage decreased. With the gradual decrease in concentration of the silver nanoparticles, the microorganisms multiplication gradually increased due to reaction with microbes.

4. CONCLUSION

In this study, industrial milk liquid waste was utilized for synthesis of nanorods. It was observed that pH and temperature are the main parameters that affect the particle size and shape. The produced nanorods were analyzed by both UV−visible spectrophotometer and high resolution TEM and were applied to increase the keeping quality of fresh milk. It was found that the keeping quality of raw milk was improved by inhibiting the microorganisms growth, and the raw milk was stable for a longer time after the addition of silver nanorods. These results were confirmed by cytotoxicity MBRT, SPC, and coliform count. Thus nanorods from milk waste were used to increase the shelf-life of raw milk without sacrificing the physical, chemical, and nutritive values of the milk.

■ ASSOCIATED CONTENT

Supporting Information

Mechanism for the formation of silver nanorods (Figure S1); structure of lactose with the free anomeric carbon (reducing end) (Figure S2); methylene blue reduction time (test) at 37 °C for silver nanorods-added milk samples and control which shows the changes in color (Figure S3); coliform and SPC counts for different samples with dilutions showing the reduction of microbial counts (Figure S4); inactivation Hom model for experimental coliform log reduction versus time showing inactivation and fit to the model (Figure S5); inactivation Hom model for control coliform log reduction versus time showing no inactivation and no fit to the model (Figure S6); inactivation Hom model for experimental SPC log reduction versus time showing inactivation and fit to the model (Figure S7); inactivation Hom model for control SPC log reduction versus time showing no inactivation and no fit to the model (Figure S8). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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