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Extending Shelf Life of Pasteurized Milk via Chitosan Nanoparticles

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Abstract

The current study aimed to extend the shelf life of pasteurized milk through addition of chitosan nanoparticles (CNP), which were obtained from chitosan by ionotropic gelation and sonication methods. Physicochemical characteristics of CNP were characterized by fourier transform-infrared spectroscopy (FTIR), zetasizer and transmission electron microscopy (TEM). FTIR spectra analysis of CNP shows a distinctive band at 1150 cm^{-1} due to stretching vibration of phosphate groups. Size of the freshly prepared CNP ranged from 90 to100 nm but after two weeks of storage at room temperature the size increased to 120 -130 nm. By using TEM, freshly prepared nanoparticles appeared as spherical in shape with a little fragmentation of polymer chain and swelling but still translucent after 2 weeks of storage. Synthesized CNP at a concentration of 0.3% w/v has the ability to inhibit growth of vegetative cells of *B.cereus* in pasteurized milk stored at 5°C and extend the shelf life of pasteurized milk from two weeks to 30 days. The addition of 0.3% w/v CNP had no effects on the physicochemical properties, such as rancidity, pH and color, of pasteurized milk.

Keywords: Chitosan, Nanoparticles, Psychrotrophic Bacteria, Pasteurized milk, *Bacillus cereus.*

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INTRODUCTION

Nanotechnology is a promising technology that has proven its significance in various areas such as medicine, agriculture and environment, with a wide range of applications. Nanotechnology involves manipulation of material size scale ranging from 1 to 100 nm. The nanostructured materials have superior properties due to increasing the surface area to volume ratios compared to the same material in bulk[1]. Nanomaterials are synthesized by many techniques, including thermal decomposition, co-precipitation, microemulsion, chemical vapor deposition and laser pyrolysis. However, most these techniques are complicated processes with dilemmas of being inefficient in energy utilization, expensive, toxic, and consequently their potential environmental impact. Therefore, there is a growing demand to implement green and sustainable methods for nanoparticles synthesizing[2-4].

In current study, we mainly focus on the aspects of functionality and applicability of chitosan in food science by using chitosan nanoparticles as a preservative material. Chitosan is a linear polysaccharide that's commercially produced by deacetylation of chitin[5]. It has been widely used in different applications because of its favorable properties such as solubility, fat binding capacity, water binding capacity, biocompatibility, non-toxicity, biodegrability, selective permeability, reactivity of the deacetylated amino groups and antimicrobial activity. These properties are affected by the method of preparation and origin species[6,7]. Antimicrobial activity of chitosan is related to its positive charge and by interacting with negative charge of bacterial membranes. This electrostatic interaction leading to cell membrane lysis presenting as a reasonable cause of chitosan antimicrobial activity[8,9]. Chitosan also can interact with essential microbial nutrients leading to disruption of microbial growth and eventually death[10]. The physico-chemical properties of chitosan such as increasing its solubility in water, antimicrobial activity and other sensory properties have been improved at the nano-size[11].

Raw milk is transported in refrigerated tanks at <7°C and contamination may occur due to the growth of psychotropic organism, particularly *Pseudomonas spp*, *Bacillius spp* and *Clostridium spp.*[12]. Milk spoilage could occur at any stage from farm to consumers even when milk is treated by heat (pasteurization technique). The storage of milk in refrigerators encourages the growth of Psychrotrophic bacteria, which are able to grow below 7°C and form vegetative cells that resist the pasteurization process. Additionally, pasteurization induces sporulation process of some species like Gram-positive *Bacillus cereus*[13]. Psychrotrophic bacteria have the ability to form hydrolytic enzymes such as lipase, proteases and phospholipases. These enzymes are resistance to high temperature and cause milk spoilage by changing the physicochemical and sensory properties of milk leading to economic loss. The hydrolysis of casein by proteases enzymes leads to milk coagulation and undesirable changes in liquid milk and dairy products flavor, such as bitterness, metallic taste and rancidity[14]. The objective of the current study was to examine chitosan nanoparticles effects, particularly chitosan effects on extending the shelf life of pasteurized milk.

MATERIALS AND METHODS

Preparation of Chitosan Nanoparticles

Chitosan was purchased from Chemical Point UG Company (Germany), degree of deacetylation was ≥75%, molecular weight 200 KDa. Nanochitosan solution was prepared by ionotropic gelation method by dissolving 100 mg of chitosan in 1% v/v acetic acid solution; the reaction mixture was stirred at room temperature until the solution became clear. The mixture was adjusted to pH 6.5 by addition of 0.1 molar sodium hydroxide solution. At room temperature, 10 ml of tripolyphosphate aqueous solution with a concentration of 0.80 mg/ml was added dropwise under magnetic stirrer at 750 rpm in a Pyrex glass flask. Then, the solution was sonicated at 80% amplitude for 10 min by SB-5200 DTD Ultrasonic Cleaner, at room temperature[15]. The solution containing chitosan nanoparticles was filtered by nylon syringe 0.22µm mesh and then was freeze-dried for subsequent analysis.

Characterization of CNP

The structural feature of the prepared chitosan nanoparticles was studied by fourier transforms infrared spectroscopy (FTIR), 2 mg of the freeze-dried product was added to 100 mg of KBr powder, the mixture was pressed under 8 bars for 60 sec to form pellet shapes, which

were placed in FTIR for analysis using wave number range of 600 - 4000 cm^{-1} using a Sell FT-IR Spectrometer Model FTIR-600. To study the morphological characteristics of chitosan nanoparticles, transmission electron microscope (TEM) (Jeol JEM-1400, Japan) was used to investigate CNP morphology. A suspension of freeze-dried nanochitosan with distilled water was prepared and sonicated for 3 min. A drop of this suspension was left to dry on a copper grid at room temperature; the sample was stained with tungsten phosphoric acid. CNP size was measured by zetasizer (Malvern Instruments Ltd., UK).

Physico-chemical and sensory properties of milk after addition of CNP

Rancidity, Color measurements and pH

Rancidity of pasteurized milk after addition of CNP was investigated by measuring the reduction of surface tension by (Sigma 703D Du-Nouy-Ring tensiometer). Color measurement was done by (CM-2600d spectrophotometer, Konica Minolta). pH was measured by using (HI-2210-02 Bench Top pH Meter with pH electrode and °C probe, HANNA). All experiments were done at room temperature in triplicates and best result was taken for different concentrations of CNP (0.0 - 0.1 - 0.2 and 0.3% w/v) during 1, 15 and 30 days of storage at 5°C. Low concentrations of CNP were applied to pasteurized milk, previous study reported that using high concentrations of polysaccharides could interact with milk proteins causing coagulation[16].

Detection antibacterial activity of CNP by measuring the diameter of inhibition zone on MHA

Thirty samples of raw milk were collected from the Collage of Veterinary's farm, University of Baghdad. *Bacillus cereus* was isolated and differentiated from other *Bacillus spp*. by chromogenic agar (CHROMagar, France). 0.2 ml of each samples were inoculated at chromogenic agar and incubated 48 hr at 30°C. Colonies from sample with highest count of *B.cereus* were transferred from chromogenic agar into a test tube that contained sterile nutrient broth and incubated for 24 hr at 37°C. After incubation, 400 µL of bacterial suspension of 10^6 CFU/ml was transferred by micropipette into Muller-Hinton agar (MHA) and spreading by sterile spreader then petri dish was incubated for 24hr at 37°C. Four wells with 6mm diameter were punched on MHA by a cork borer. These wells were sealed by adding drops of molten MHA. Fifty µL of CNP solutions with different concentrations (0.1 - 0.2 - 0.3%) w/v were poured into the wells. Petri dishes were kept at 4°C for 3 hr to ensure complete diffusion of CNP. After that, the dishes were incubated at 37°C for 24 hr. The diameter of the inhibition zone was measured[17].

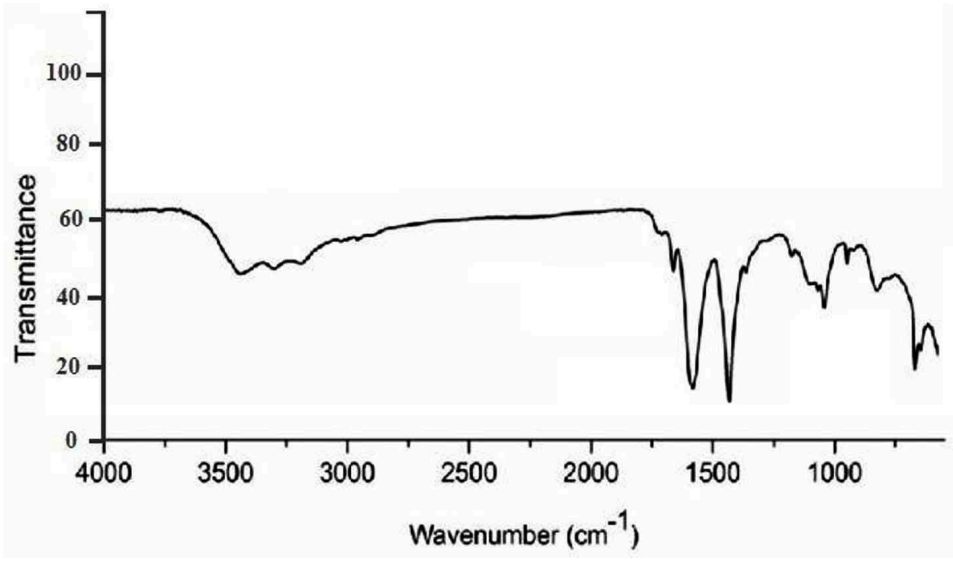

Fig. 1. Characterizations of chitosan nanoparticles (CNP) by sell FT-IR spectrometer 600.

Addition of chitosan nanoparticles to raw milk

Chitosan nanoparticles were added at different concentrations (0.0- 0.1- 0.2 - 0.3 %) w/v to 15 ml test tubes containing raw milk sample which was previously diagnosed with highest count of *B. cereus*. The treatments were done in triplicates. After addition of chitosan nanoparticles, the raw milk was pasteurized at 72°C for 15 sec. Then, these samples were stored at 5°C for 30 days. During storage period, the microbiological test (culturing on chromogenic agar) was done to investigate the growth of *B.cereus* at day 1, 15 and 30 of storage.

RESULTS AND DISCUSSION

Characterization of CNP

In fig.1, FTIR spectra of chitosan nanoparticles shows a distinctive band at 3440 cm^{-1}, which was attributed to stretching vibration of $–NH_2$ and –OH groups that is give an indication on enhancing hydrogen bonding of molecules. Bands at 1650 cm^{-1},1590 cm^{-1} and 1315 cm^{-1} were caused by the absorption of Amide I ($CONH_2$), $–NH_2$ bending and Amide III respectively. CH_2 wagging gave an absorption band at 1410 cm^{-1} another distinctive band at 1150 cm^{-1} due to P=O stretching vibration from phosphate groups which was not obtained in FTIR of classic chitosan as previous studies mentioned[18-20].

TEM micrograph shown in fig. 2 was observed immediately after formulation and upon two weeks of storage at room temperature. The freshly ultrasonicated chitosan nanoparticles showed a spherical shape with a little fragmentation of polymer chain, zetasizer gives average size diameter range of (90nm-100nm). After two weeks of storage, the nanoparticles became larger due to

Table 1. The mean of inhibition zones diameter (mm) of three concentrations of chitosan nanoparticles against *B. cereus*.

Concentration of CNP (w/v)	Zone of inhibition (mm)
0.1 %	6
0.2 %	10
0.3 %	15

Table 2. *Bacillus cereus* count (CFU/mL) of pasteurized milk with chitosan nanoparticals (CNP) at different concentrations (0.1, 0.2 and 0.3% w/v) and without CNP during (1, 15 and 30) days of storage at 5°C.

Storage period	*B. cereus* count (CFU/ml) Concentration of CNP (day) sample (% W/V)			
	0%	0.10%	0.20%	0.30%
1	1×10^{-1}	1×10^{-1}	1×10^{-1}	1×10^{-1}
15	6×10^{-7}	5×10^{-1}	3×10^{-1}	2×10^{-1}
30	$>10^{-8}$	3×10^{-1}	2×10^{-1}	<10

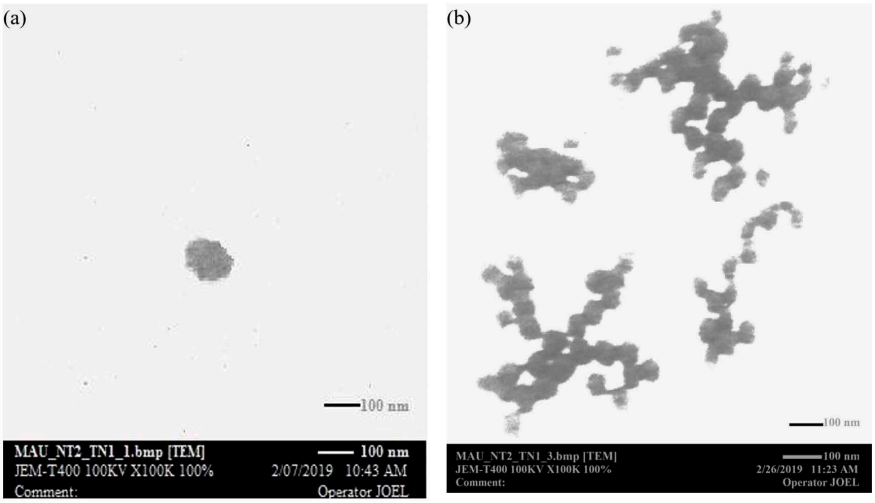

Fig. 2. TEM photographs of chitosan nanoparticles (a) freshly prepared with size diameter range of 90nm -100nm; (b) two weeks of storage with size diameter range of 120nm -130nm.

swelling by osmosis with an average size of about (120nm-130nm). These swelling nanoparticles fragmented and became as feeble branches. The mixture had a translucent appearance upon storage because the radius of nanoparticles was not increasing to the size which causing turbidity.

Detection antibacterial activity of CNP by measuring the diameter of inhibition zone on MHA

Four out of thirty raw milk samples were diagnosed with *B.cereus* which appeared as blue with white halo colonies on chromogenic agar. Sample with highest count (1x10^6 CFU/ml) of *B.cereus* was selected to detect the diameter of inhibition zone by CNP. Table 1 shows the inhibitory activity of chitosan nanoparticles against *B.cereus*. Results showed that at the concentration 0.1% w/v, the diameter of inhibition zone was 6 mm but when the concentration have been increased to 0.2% w/v inhibition zone diameter was also increased to 10 mm, with a concentration of 0.3% w/v the inhibition zone diameter was increased to 15mm. These results indicate that the inhibitory activity of CNP against microbes increases as its concentration increases according to previous studies[21].

Addition of chitosan nanoparticles to raw milk

Results showed that *B.cereus* counts of raw milk after pasteurization with and without CNP which stored at 5°C (table 2). During the first day of storage, *B. cereus* count for pasteurized milk with different concentrations of CNP (0.1-0.2- 0.3%) w/v was comparable as *B.cereus* count of pasteurized milk without CNP (1x10 CFU/ml). At day 15 of storage, the count of *Bacillus cereus* was increased to (5x10, 3x10 and 2x10) CFU/ml for 0.1, 0.2 and 0.3% w/v, respectively. Due to the growth of *B.cereus* spores but these values did not effect on biological quality of milk as compared with *B.cereus* count of pasteurized milk without CNP which was 6 x10^7 that is indicate heat treatment alone was not enough to kill and prevent the growth of vegetative cells of psychrotrophic bacteria, pasteurized milk without CNP after 15 days of storage was deteriorated by *B. cereus* and became dangerous for human consummation. After 30 days of storage*, B. cereus* count for pasteurized milk with CNP was (3x10, 2x10, <10) CFU/ml for the mentioned concentrations respectively as compared with *B.cereus* count of untreated pasteurized milk which was >10^8 this value effects markedly on biological quality of milk leading to its spoilage and considered high even when compared with acceptable limits of *B.cereus* in raw milk as mentioned in previous studies[22-24]. The best inhibitory outcome was obtained with 0.3% of chitosan nanoparticles. The obtained results indicated that the addition of chitosan nanoparticles to milk prevents microbial growth of vegetative cells, enhances microbiological quality, prevents spoilage and increasing its storage life to a maximum 30 days because chitosan is a reusable material and do not loss its antimicrobial activity during storage period according to previous study[25]

Table 3. Changes in rancidity of pasteurized milk with chitosan nanoparticals (CNP) at different concentrations (0.1, 0.2 and 0.3% w/v) and without CNP during (1, 15 and 30) days of storage at 5°C.

Storage period (day)	Concentration of CNP sample (% W/V)			
	0%	0.1%	0.2%	0.3%
1	50.00	50.00	50.07	50.10
15	45.00	49.02	50.04	50.50
30	40.00	49.01	50.03	50.40

*All surface tension values measurement was given by (dynes /cm^2) at room temperature

Table 4. Changes in color of pasteurized milk with chitosan nanoparticals (CNP) at different concentrations (0.1, 0.2 and 0.3% w/v) and without CNP during (1, 15 and 30) days of storage at 5°C

Storage period	Concentration of sample (% W/V)	L*	a*	b*
1 d	0	79.96	- 3.12	4.81
	0.1	79.46	-3.25	5.20
	0.2	79.86	-3.27	5.29
	0.3	78.70	-3.30	5.38
15 d	0	80.88	-3.04	5.84
	0.1	80.52	-3.21	5.37
	0.2	80.11	-3.13	5.42
	0.3	79.91	-3.10	5.45
30 d	0	79.88	-2.80	6.06
	0.1	78.51	-3.15	5.41
	0.2	77.58	-3.07	5.46
	0.3	76.86	-3.05	5.49

as compared with free milk treated by heat alone which has a maximum storage life of two weeks. The inhibitory activity of chitosan nanoparticles increases as its concentration increase.

Physico-chemical and sensory properties of milk after addition of CNP

Rancidity, Color measurements and pH

The rancidity of milk is measured for two reasons, first to detect the influence of CNP addition to milk on rancidity and to detect the activity of resistant *B.cereus*, rancidity caused by lipolytic activity which leading to free fatty acids liberation; the resulting free fatty acids reduce pasteurized milk surface tension. Rancidity was measured at room temperature in order to activate the lipolytic enzymes of *B.cereus*[26]. Surface tension values of pasteurized milk without CNP addition were 50.00 dynes /cm^2, 45.00 dynes /cm^2 and 40.00 dynes/cm^2 after day 1, 15, 30 of storage respectively. Reduction in surface tension of untreated pasteurized milk was caused by lipolytic enzymes of resistant *B.cereus* leading to rancidity. Surface tension of pasteurized milk with addition of CNP was measured at different concentrations (0.1- 0.2- 0.3% w/v), the results in table 3 show that, at first day of storage as the concentration of CNP increases the surface tension will also increase but with addition of 0.1% w/v of CNP there was a little reduction in surface tension from 50 to 49.02 and 49.01 dynes /cm^2 at day 15 and 30 of storage respectively because this concentration of CNP was not enough to prevent microbial enzymes activity and growth as compared with 0.3% w/v which gives the best results during all tested days of storage, the results indicate that when concentration of CNP increases the surface tension will also increase due to the prevention of microbial growth and subsequently lipolytic activity[27] another reason is that CNP has fat binding effect as proved by[28] that subsequently leading to increase surface tension.

Results in (table 4) showed color measurement of pasteurized milk after addition of CNP, L* values of all samples was not markedly changed during storage period however a concentration of 0.3% w/v had a considerable decreasing effect on L* value at day 30 of storage. a* and b* values of control pasteurized milk samples at day 1 and 30 was increased from -3.12 to -2.80 and from 4.81 to 6.06, respectively. The color of control samples at day 30 of storage was yellowish as b* values increase to 6.06. From these results chitosan nanoparticles at these tested concentrations did not affect markedly on milk color during its storage period.

The results from fig.3 showed that pasteurized milk stored at 5°C without addition of chitosan nanoparticles, pH levels were (6.75-6.58) at first and 15th day of storage respectively. At 30th day of storage pH value was declined to lower than 6.52 which may indicate the growth of microbes. By adding 0.1%w/v of chitosan nanoparticles, pH values were (6.72, 6.67 and 6.63) at day 1, 15, 30 of storage respectively. As CNP concentration

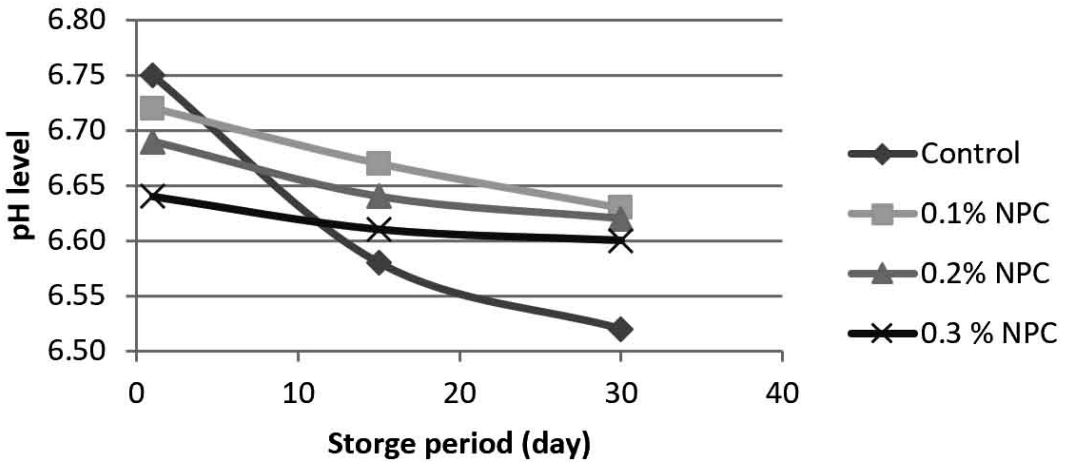

Fig. 3. Changes in pH of pasteurized milk with chitosan nanoparticals (CNP) at different concentrations (0.1, 0.2 and 0.3% w/v) and without CNP during (1, 15 and 30) days of storage at 5°C.

increased to 0.2% w/v, pH values were (6.69, 6.64, and 6.62) at day 1, 15, 30 of storage respectively. When CNP concentration increased to 0.3% w/v the pH values were declines to (6.64, 6.61 and 6.60) at day 1, 15 and 30 respectively as compared with pH values of untreated pasteurized milk. These results indicate that the addition of chitosan nanoparticles to pasteurized milk at these tested concentrations leading to a little reduction of pH values with each increase in CNP concentration but this reduction did not effect on pasteurized milk physical properties and quality because these values still with acceptable limits of pasteurized milk pH which indicates that the addition of chitosan nanoparticles did not effect on the quality of milk.

CONCLUSION

In the present study, synthesized chitosan nanoparticles have antibacterial activity by preventing the growth of vegetative cells of *B. cereus* which responsible on milk spoilage after pasteurization. Addition of CNP at 0.3% w/v has no effects on the physicochemical properties of pasteurized milk during its storage period under 5°C. However, a reduction in pH occurred and this reduction within expectable limit of pasteurized milk. Addition of CNP is considered as a method for reducing economic losses and health hazards which happen by milk spoilage.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

None.

AUTHOR'S CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

DATA AVAILABILITY

All datasets that are generated or analyzed during this study are included in the manuscript and/or in the supplementary files.

ETHICS STATEMENT

All experimental protocols were approved under the Al-Qasim Green University, College of Food Science, Department of Dairy Science and all experiments were carried out in accordance with approved guidelines.

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