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# Biocompatible chitosan nanoparticles incorporated bacteriocin (CSNps-B) preparation for the controlled release and improved anti-bacterial activity against food borne pathogenic bacteria *Listeria monocytogenes.*

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**ABSTRACT**

Nanotechnology offers higher hopes in food sector by promising longer shelf life, safer packaging, better traceability of food products and healthier food. Because of the distinct properties of nanoparticles,they have a lot of application like food packaging material to improve shelf life,anti-bacterial agent against contamination, food spoilage organisms. Among the different nanoparticles,chitosan nanoparticles have been investigated as a carrier for drug delivery, although have been no attempts to explore the potential of chitosan nanoparticlesas controlled release for food additives..In the present study,chitosan nanoparticles incorporated bacteriocin was synthesized by ionic gelation method. Controlled release of synthesized nanoconjuagte was studied by dialysis bag method and the anti-bacterial activity was studied against *Listeria monocytogenes*. Nano conjugate prepared by ionic gelation method reveals spherical nanospheres with the size range of 90-100nm. The *in vitro* controlled release profiles show 81% release there was a controlled and a steady release of bacteriocin from the nanoconjugate. Anti-bacterial activity against *L.monocytogenes* adopting well diffusion method indicate that all the tested concentration of nano conjugate showed an increased activity.An increase in zone of inhibition was recorded in nano conjugate than free bacteriocin.The present study would suggests the possible utilization CsNps-B as an effective anti-bacterial agent against food spoilage pathogenic bacteria.

**Key words;** chitosan nanoparticles,bacteriocin,nano conjugate,*Listeria monocytogenes*,antibacterial activity

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## INTRODUCTION

Consumers have been consistently concerned about possible adverse health effects from the presence of chemical additives in their foods. As a result, consumers are drawn to natural and fresher foods with no chemical preservatives added. This perception, has stimulated research interest in finding natural but effective preservatives [1]. Bacteriocins, produced by lactic acid bacteria (LAB) may be considered natural preservatives or biopreservatives that fulfill these requirements [2]. Bacteriocins are antibacterial proteins which are produced by bacteria that kill/inhibit the growth of other bacteria. Many lactic acid bacteria (LAB) produce a high diversity of different bacteriocins. They are typically considered to be narrow spectrum antibiotics [3,4].Anti microbial activity of bacteriocin is primarily based on the suitable formulation technique to improve the delivery using micro and nanoencapsulation techniques [5].

Nanoscience and nanotechnology are new frontiers of this century. Their applications to the agriculture and food sector are relatively recent compared with their use in drug delivery and pharmaceuticals [6,7].Current nanotechnology applications in the agro-food production chain are focused on the development of nano-sized food ingredients and additives, delivery systems for bioactive compounds, and innovative food packaging. As a consequence of their small size, NPs show different physical and chemical properties compared to their respective conventional-sized materials, which probably results in different biological interactions. Smart delivery of nutrients, bioseparation of proteins, rapid sampling of biological and chemical contaminants and nanoencapsulation of nutraceuticals are some of the emerging topics of nanotechnology for food and agriculture. Advances in technologies, such as DNA microarrays, microelectromechanical systems and microfluidics, will enable the realization of the potential of nanotechnology for food applications [8].Among the different types of nanoparticles, polymeric nanoparticles have a lot of applications. Polymeric nanoparticles are either nanosized solid particles or capsules which consist of natural or synthetic polymers and to which the drug is attached [9].They are investigated as drug delivery systems for site-specific targeting of tumours and for the transport of drugs across biological barriers, particularly the blood-brain barrier [10] At present the number of companies that work on polymeric nanoparticle drug delivery systems is quite small; only six companies were identified in this study Among the polymeric nanoparticles, chitosan- nanoparticles can be easily integrated into systems relevant for pharmaceutical, biomedical, and biosensor applications. Chitosan (CS) is a polymer of particular interest in this area because it is biodegradable, bioabsorbable,and bactericidal. Due to its polymericcationic characteristics, chitosan nanoparticles may interact with negatively charged molecules and polymers, showing a favorable interactionTherefore, it has attracted considerable interest due to its medicinal properties, such as antifungal, antibacterial, antiprotozoal, anticancer, antiplaque, antitartar, hemostatic, wound healing and potentiates anti-inflammatory response, inhibits the growth of cariogenic bacteria, immunopotentiation, antihypertensive, serum cholesterol lowering, immune enhancer, increases salivary secretion (anti-xerostomial) and helps in the formation of bone substitute materials [11].In the present study,nanoformulation of bacteriocin with chitosan nanoparticles was evaluated against controlled release ,enhanced anti bacterial activity against food borne pathogenic bacteria *Listeria monocytegenes*

## MATERIALS AND METHODS

### Chemicals and Reagent

Bacteriocin (Nisin) was purchased from Duke Thompson's India Pvt ltd. Indore. All the chemicals and reagents (analytical grade) were obtained from Hi media, Mumbai, Ind

### Preparation of chitosan nanoparticles incorporated bacteriocin (CSNps-B)

Ionic gelation method was used to prepare chitosan incorporated nisn. In this method 0.2gram of chitosan suspended in 1% acetic acid in 100ml distilled water was mixed with 10 ml of bacteriocin suspension (mg/ml),the preparation was kept under stirring followed by drop wise addition of sodium tripolyphosphate(TPP) at room temperature for 3hrs. The slurry thus obtained was centrifuged at 10000rpm for 10minute. The collected pellet was lyophilized and used for further studies.

Characterization of nano formulation was carried out by scanning electron microscopy (SEM) for the determination of particles size and morphology (Supra 55-Carl Zeiss, Germany ). Transmission Electron Microscope (TEM) was also used to study particle morphology of the nano drug conjugate. The sample was dispersed in

ethanol and the solution was sonicated for 20mins. Few drops of the solution was dropped on a copper grid at room temperature and the TEM images were recorded using HITACHI H9500 TEM set up at an accelerating voltage of 300kV. Fourrier Transform Infrared Radiation (FTIR) was studied using the dried samples (pelletised with potassium bromide Kbr.

**Entrapment efficiency**

Bacteriocin concentration in the supernatant after the centrifugation of the prepared nanosphere solution was detected using the UV-Vis Spectrophotometer and encapsulation rate is calculated using the formula.

$$\text{Entrapment efficiency (\%)} = \frac{\text{Total bacteriocin-bacteriocin in supernatant}}{\text{Total bacteriocin}} \times 100$$

**In vitro drug release study**

Continuous dialysis bag method was used to investigate in vitro release of bacteriocin from nanoconjugate. CSNp- B was distributed in water and transferred into dialysis bag and dialyzed against physiological saline which was thermostated at 37° C and mechanically stirred at 75rpm. At designated intervals, a portion of the dialysis medium was taken for quantitation of flutamide and the same volume of fresh medium added. The collected dialysis medium was syringe filtered and spectrometrically read at 291nm.

**Anti bacterial activity**

Bacterial strain Listeria monocytegenes was obtained from Microbial Type culture collection (MTCC),Chandigurh,India and the tested bacterial strain was maintained on trypticase soy agar (TSA) slant.

**Inoculum preparation**

Bacterial inocula was prepared from TSA slant.A loopful of culture was taken from the TSA slant and inoculated into 100 ml of sterile tryticase soy broth (TSB) ,incubated under shaking condition (orbital shaker) at 37 C for 16-18 hours.Broth was centrifuged at 10,000 rpm for 10 minutes and the collected cells were washed twice with sterile phosphate buffered saline (PBS).Washed cell suspension was used as source of inoculum.

**Well diffusion assay**

Anti bacterial activity of the tested bacterial strains was studied by well diffusion assay. Inocula of the respective bacterial culture thus prepared was uniformly spread with sterile cotton swabs on sterile Mueller Hinton (MH)Agar Media (Hi-media, India). The wells were made using cork borer and aliquots of free bacteriocin (B) free chitosan nanoparticles (CSNps) and chitosan nanoparticles incorporated bacteriocin (CSNps-B) 25, 50, 75 and 100 µg/ml was loaded into the wells. The plates were incubated at 37ºC for 24 hours.After the incubation period,the plates were observed for zone of inhibition.Three replicates were maintained.

**Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)**

Minimum inhibition concentration (MIC) was determined by a turbidimetric method [12]..Bacterial inocula prepared in tryptic soy broth as earlier was used in this study. In this method, a series of 5 ml of screwcap vial (Borosil) each containing 2mL broth medium was prepared. 2mL of free bacteriocin (B) free chitosan nanoparticles (CSNps) and chitosan nanoparticles incorporated bacteriocin (CSNps-B) suspension with different concentration were prepared and added into the first vial.After mixing, 2 mL of mixture from this vial were transferred to the next vial, and a similar procedure was repeated for the subsequent vials. The bacteria inocula was added to the vial to achieve a final bacterial concentration of 105 cells/mL Inoculated vials were incubated under shaking condition (150 rpm/min ) at 37°C for 20 hours. The MIC was determined as the minimum concentration at which there is no visible change in the turbidity of the medium. The minimum bactericidal concentration (MBC), defined as the lowest concentration of sample that kills 99.9% or more of the initial inoculum, was determined in those test samples after the MIC test showed no growth. The assay was carried out by counting the number of colonies after the bacteria were seeded overnight on agar plates. The MIC and MBC were determined in a similar fashion. Triplicates were maintained in each treatment.

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## RESULS AND DISCUSSION

Nanotechnology has the potential to revolutionize the global food system. Novel agricultural and food safety systems, disease-treatment delivery methods, tools for molecular and cellular biology, sensors for pathogen detection, pesticides, packaging materials, environmental protection, and education of the public and future workforce are examples of the important impact that nanotechnology could have on the science and engineering of agriculture and food systems. The four major areas in food industry that will probably be significantly enhanced by nanotechnology are development of new functional materials; micro- and nanoscale processing; product development; and design of methods and instrumentation for food safety and biosecurity. The potential applications of nanotechnology in the agro-food production chain are claimed to be applicable throughout all phases of food production [13]. In the present study, chitosan nanoparticles incorporated bacteriocin (CSNp-B) was prepared and the anti bacterial activity was studied against major food borne pathogenic bacteria Listeria monocytogenes.

Chitosan nanoparticle incorporated nisin was synthesised by ionic gelation method. The synthesised complex was characterised by Scanning Electron Microscope which revealed the particle size of 90-100nm and the TEM study revealed particles were dispersed equally with electron dense core shell (Figure 1,2).Further characterization was carried out by FTIR.FTIR Spectra for chitosan nanoparticle incorporated nisin-cephalothin drug conjugate shows peaks at 3904.1cm-1 (OH), 3770.2 cm-1 (OH) and 2165.0cm-1 (C≡C), 3433.5 cm-1 (OH), 2928.3cm-1 (CH), 2361.3cm-1 (H–C=O, C≡N), 1737.3cm-1 (C=O), 1406.7cm-1 (C-C), 1127.7cm-1 (C-N), 896.1cm-1 (CH) and 532cm-1 (C-Cl) (Figure 3).

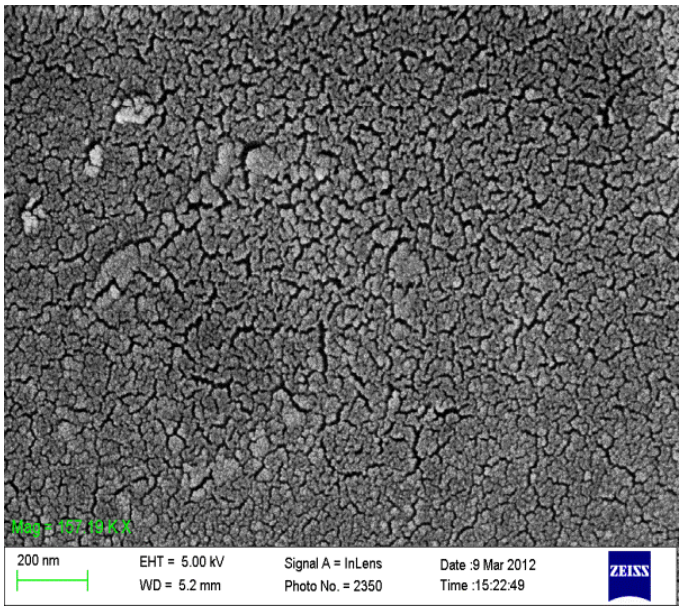


**Figure 1: SEM image of chitosan nanoparticles incorporated bacteriocin**

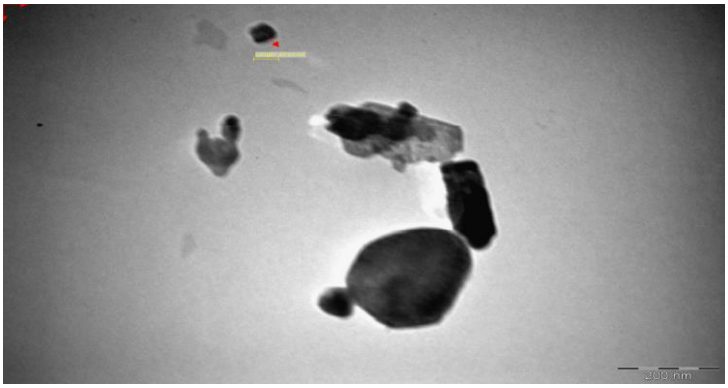

**Figure 2: TEM image of chitosan nanoparticles incorporated bacteriocin**

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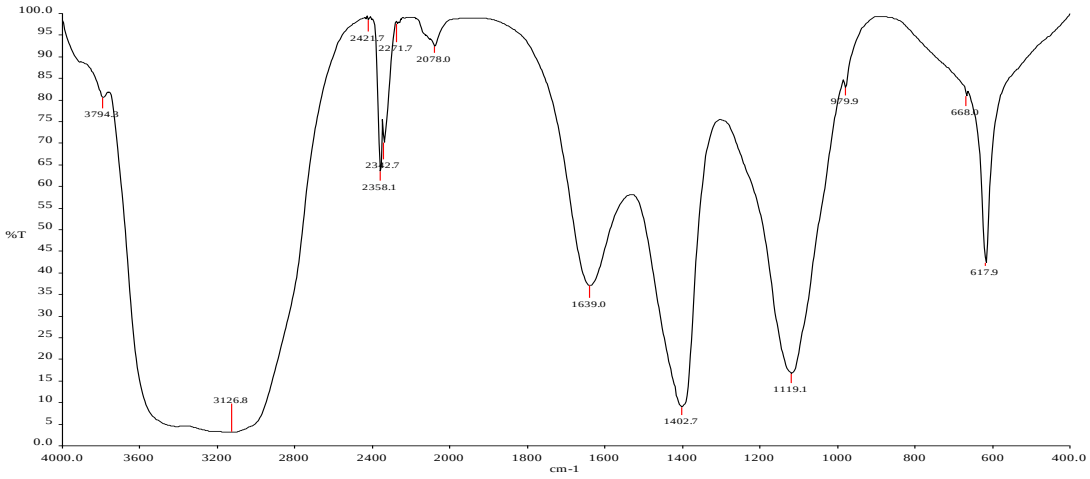


**Figure 3: FTIR spectra of chitosan nanoparticles incorporated bacteriocin**

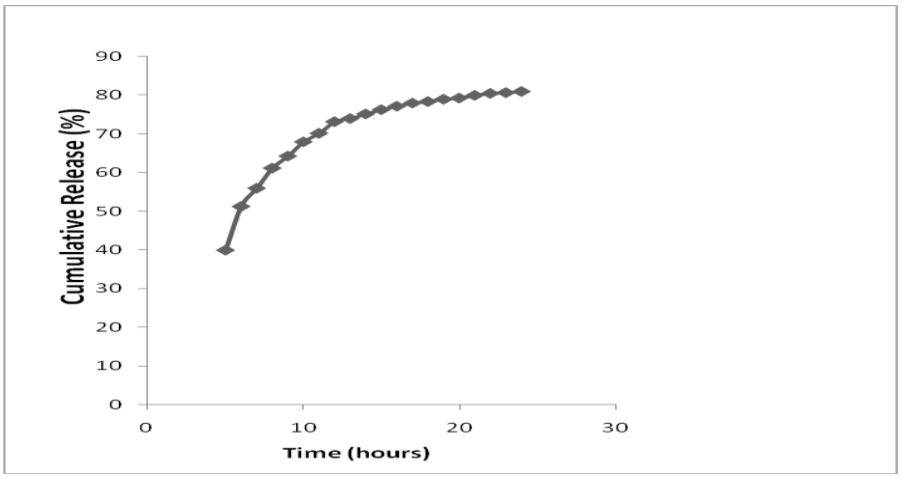


**Figure 4: In vitro bactericin release profile from CSNp-B**

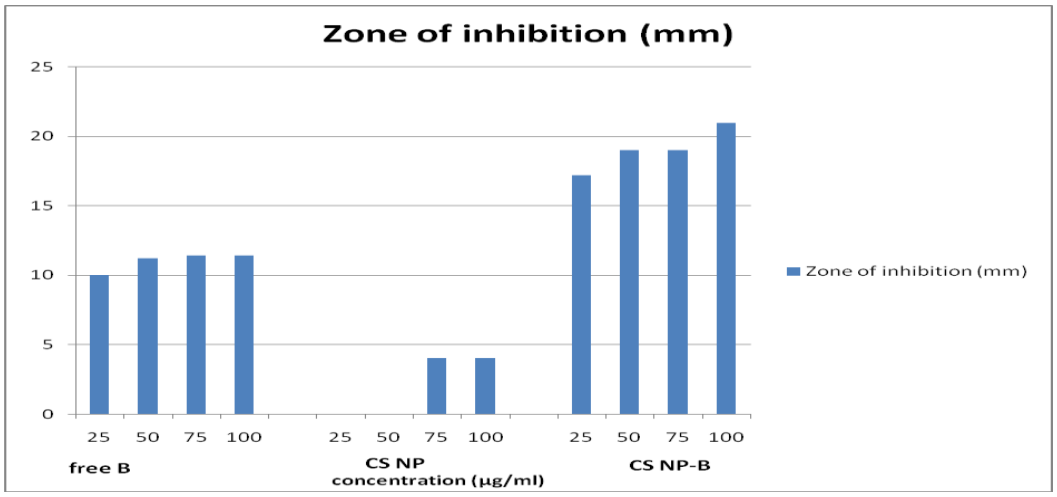


**Figure 5: Zone of inhibition (mm) of free bacteriocin, free chitosan nanoparticles and chitosan nanoparticles incorporated bacteriocin against *L.monocytogenes***

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Entrapment efficiency of the bacteriocin on to the chitosan nanoparticles is found by the spectrophotometric analysis of the bacteriocin-CSNp conjugate suspension. The unbound bacteriocin concentration was found by correlating the absorbance of the supernatant after the centrifugation with the standard absorbance concentration ratio. Entrapment efficiency was in the range of 81 to 83 %.

In vitro drug release was studied by continuous dialysis bag method . The sample was taken at regular intervals and analysed spectrometrically. The release percentage was calculated using the intial drug concentration and the release at specified time. The drug release was calculated for 24 hours. There was a burst release of drug in the early hours and a total release of about 81% was observed. An initial burst of 40% in the first 5 hours can be observed. In the following 6 hours, cumulative release reached 62.0%, in a sustained manner, which provides the possibility to fight continually against target cells, resulting in decreased cell viability. Cumulative release reached almost 81% after 24 hours and showed an almost released ability of the nanoparticle formulation (figure 4). The generally sustained and controlled release profile of facilitates the application of nanoparticles for the delivery.

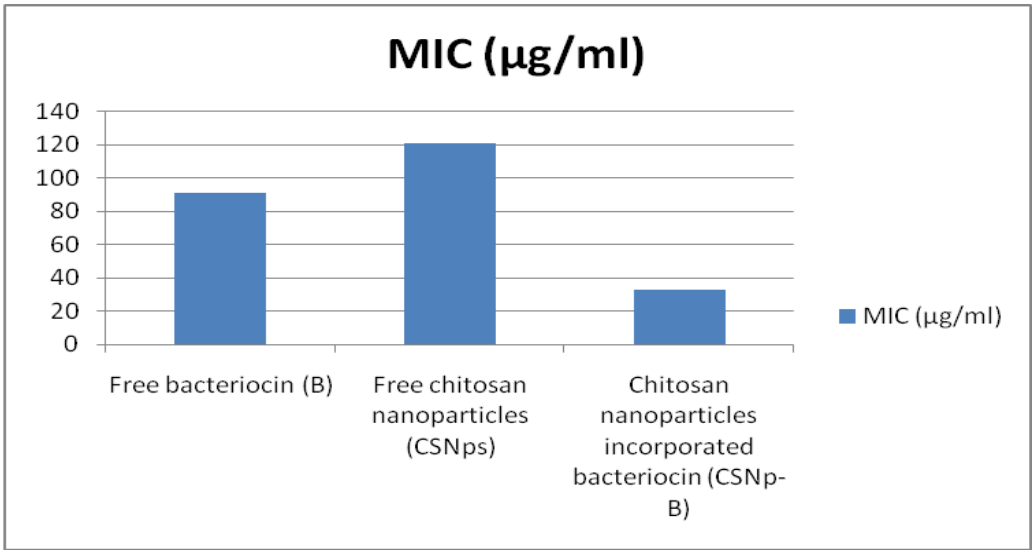


**Figure 6.Minimum inhibitory concentration (MIC) of free bacteriocin, free chitosan nanoparticles and chitosan nanoparticles incorporated bacteriocin against *L.monocytogenes***

Anti-bacterial activity was studied against L.monocytogenes by well diffusion assay and turbidity tube assay.Both assays showed the tested bacteria was susceptible to the nanoformulation.An increase in zone of inhibition was recorded in CSNp-B treatment in all the concentration used (Figure 5).All the tested concentration of free bacteriocin and free chitosan nanoparticles were recorded least zone of inhibition in all the tested concentration. Minimum inhibitory concentration (MIC) of the nanoformulation against the tested bacterial strains was studied by broth dilution method. The MIC values of nanoformulation was found to be 32.5 µg/ml which can be seen that nanoformulation showed high anti bacterial efficacy (Figure 6)., Nanoparticles based on polymeric nanoparticles have the advantages of absorbability, high efficacy and low toxicity.Among the polymeric nanoparticles, chitosan nanoparticles are extensively studied because of the best biocompatibility and bioavailability.Chitosan based nanoparticles and chitosan stabilized various metallic nanoparticles with distinct biological activities have been reported [14,15,16,17]. Further study will helpful to formulate CSNp-B as an effective anti bacterial agent food borne pathogenic microorganisms.

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