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Synthesis and Characterization of Nisin Loaded TPP Cross Linked Chitosan Nanoparticles

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Abstract: ***The goal of this study was to develop a biocompatible nanoparticle for prolonged action of approved antimicrobial peptide (bacteriocins) against food borne pathogens. Chitosan tripolyphosphate nanoparticles were developed as carriers of well studied bacteriocin nisin. The nisin tagged nanoparticles were developed by well studied Ionic cross linking method. Zeta-sizer, FTIR spectra, and X-ray powder diffraction were employed for studying the physiochemical properties of nanoparticles. Encapsulation efficiency was found to be 95-99 %. The nisin loaded nanoparticles exhibited better antimicrobial activity (16 mm) compared to free Nisin (10 mm). The result of this study suggests the encapsulation of nisin into chitosan tripolyphosphate nanoparticles could be an efficient process for enhancing the potential of bacteriocins in food products.***
Keywords: Nisin, Chitosan, nanoparticle, drug delivery, Listeria monocytogenes

I. INTRODUCTION

Nisin, as well as many other antimicrobial compounds, is effective in inhibiting pathogenic bacteria in food and other nutrient containing systems. Nisin belongs to the lantibiotic group of class-I bacteriocins that are generally synthesized by Lactococcus lactis. Reduction of bacterial contamination by nisin treatments has been reported in various studies [1, 2]. Nisin either single or in combinations with other antimicrobials have found to be effective as a hurdle for pathogen survival [3]. Listeria monocytogenes is a gram-positive food borne pathogen grows widely in food based environments, even at refrigerated temperatures [4]. Nisin is a positively charged antibiotic peptide that is able to bind to negatively charged cytoplasmic membranes [5-7]. Nisin has been approved as a food preservative and is effective in suppressing Gram-positive bacteria such as L. monocytogenes and has been approved as a food additive by WHO as they are treated as Generally regarded as Safe organisms [8]. The antimicrobial action of nisin is through the formation of pores in the cell membranes of pathogen and is now widely used in various food applications [9-14]. Liposome encapsulated nisin has been developed and their efficacy was tested in milk fermentation and the ripening of cheddar cheese [15, 16]. The stability of Nisin in liposome and micro particles of calcium has also been studied by several authours [17-20]. Cheese treated with liposome tagged nisin revealed excellent sensory character and good flavour during storage [16]. Works done earlier on liposomes, alginate films loaded with nisin showed disadvantages like decreased antibacterial activity of nisin possibly due to interaction between the protein and polymer systems. Ionic cross linking amid phosphate groups of tripolyphosphate and the amine groups of chitosan lead to formation of poly ionic complexing of chitosan tripoly phosphate. The benefits of chitosan tripolyphosphate nanoparticles and their ability to act as a vector to deliver drug were evaluated; the properties of Chitosan Tripolyphosphate nanoparticles such as dimensions, shape, zeta potential and encapsulation efficacy of Nisin, and release profile of Nisin from the nanoparticles were determined and also antibacterial effects of nisin stacked nanoparticles on controlling L. monocytogenes was analyzed. These results may help us for better understanding of nano carriers for delivery of bacteriocins to food and pave the way for their use in food industry.

II. MATERIALS & METHODS

A. Preparation of Chitosan Tripolyphosphate Nanoparticles

Chitosan nanoparticles were prepared according to the literature based on the ionic gelation of chitosan with TPP anions. CS–TPP nanoparticles were synthesized using ionotropic gelation between positively charged amino groups of chitosan and negatively charged TPP, as reported previously by Krauland and Alonso [21]. Briefly, chitosan (0.1%), TPP (0.1%) and Nisin 10 mg/ml were prepared and filtered with 0.22-µm filter. TPP solution was added dropwise to the chitosan solution with stirring at room temperature for 15 minutes to obtain chitosan nano particle. The nano particles formed spontaneously were concentrated by

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centrifugation at 9000g for 15 minute in a glycerol bed. The centrifuged CS–TPP nanoparticles were resuspended in100 ml purified water.

B. Package of Nisin in chitosan Tripolyphosphate Nano Particles

Package of nisin in chitosan tripolyphosphate were carried out by a method followed by Zohri et al. [22] with few modifications. 300 µl of Nisin (10 mg/ml) was supplemented drop by drop to 10 ml solution of chitosan and stirred for 5 min. Further, 3 ml of tripoly phosphate solution was added drop wise to resulting chitosan solution and was continuously agitated for further 15 min. Nisin packed nanoparticles which formed spontaneously were concentrated by centrifugation at 9000g and 4^{o}C for 10 min.

C. Nanoparticles Characterization

1) *Scanning Electron Microscope:* Morphology of nanoparticles was determined using scanning electron microscopy (Hitachi, Model: S-3400N). A 5-10 µL of nanoparticle suspension was dropped on clean glass slide and the slide was placed on a aluminium sample holder and dried completely in a dessicator and examined by scanning electron microscope.

D. Zeta sizer:

To find the size of the nanoparticles in aqueous dispersions zeta sizer 3000HS (Malvern Instruments, UK) was employed. Zeta Sizer measurements of chitosan nanoparticles, chitosan nanoparticles loaded with nisin were done.

E. Fourier Transform Infrared Analysis

IR analysis for the lyophillized powders of chitosan tripolyphosphate nanoparticles and nisin packed chitosan tripolyphosphate nanoparticles was done. Both the test samples were blended using moist potassium bromide (Qauligens) and ground into fine powder and were scanned over a region of 400–4,000cm^{-1} at 4 mm per sec. IR peaks for chitosan TPP nanoparticles, nisin, and chitosan TPP nanoparticles loaded nisin were analyzed.

F. X-Ray Powder Diffractometry

Powder X-ray diffraction patterns (XRPD) were taken of the samples in question with a Bruker D8-Advance X-ray diffractometer (Germany), over the range of 2–75. The experimental parameters were set as: current, 20mA; voltage, 40kV; angular speed,48/min. XRPD patterns of chitosan tripolyphosphate nanoparticles, chitosan nanoparticles loaded with nisin were recorded.

G. *Differential Scanning Calorimetry*

DSC measurements were done on a on a Q600 SDT and Q20 Differential scanning calorimeter at a heating rate of 10^{0}C/min. Samples (2mg) was heated in closed aluminum cells at the temperature range of 30–360^{o}C with continuous nitrogen flow of 40mL/min. Thermal analysis of chitosan-tripoly phosphate loaded nanoparticles with standard bacteriocin nisin, chitosan TPP nanoparticles were carried out [22, 23].

H. Deduction of loading Proficiency of Nanoparticles

Centrifugation of the dispersion containing nisin-loaded nanoparticles was carried out using a 100-kDa molecular weight cut off ultrafilter (AmiconUltra -100K) at 9000g; 25^{o}Cfor 10 min. Concentration of the protein in the outer tube was estimated using Bradford method. The experiment was repeated three times and the loading potential or efficiency was calculated by the below equation. [22]

$LE = Nisin_{total} - Nisin_{supernatant} / Nisin_{total}$

I. Release profile of nisin from Chitosan Nanoparticles

Dialysis of nisin loaded nanoparticle dispersions was carried out to evaluate the release profile of nisin, for this a dialysis membrane of size 12 kDa was used. For this experiment, a 5mL of 1mg/mL solution phosphate-buffered saline (pH 7.4) was transferred to dialysis tube, the tube was positioned into a 100-mL glass cylinder comprising release media, and agitated with the magnetic stir bar. Nisin will diffuse into the outer solution (release media) from the pores of dialysis tube. Release media was assessed at regular intervals of 0, 15, 30, 60, 120, 180, 240, 300, 360, 420, 480 and 520 min by taking out a small amount of sample.

III. RESULTS & DISCUSSION

In this analysis, ionic cross linking method was used to synthesize nisin tagged nanoparticle and the prepared material was characterized employing physiochemical test and the anti-bacterial efficiency of the synthesized material was compared with the

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free nisin. SEM Image shows the presence of formation of nanoparticles. Smooth and Spherical shape of chitosan nano particles (Fig.1A) and chitosan nanoparticles loaded with nisin were seen (Fig.1B). A narrow size range distribution of 40–250 nm (not shown) of chitosan nanoparticles were noted. Zeta sizer data revealed a mean Z-average diameter of 157±1 nm (mean±S.E.M.), a number average size of 84±8 nm, and a polydispersity index (PDI) of 0.815±0.02 whereas nisin loaded chitosan nanoparticles had a mean Z- average number of 160±2 nm (Fig. 2 and 3). DSC thermograms provided little more information on encapsulation of nisin into chitosan Tripolyphosphate nanoparticles, the endothermic peak found in thermogram of chitosan tripolyphosphate nanoparticles (Fig.4A) shifts from 100.75 ^{o}C to 91.9 ^{o}C in thermogram of nisin loaded nanoparticles (Fig.4B) which indicates the binding of bacteriocin in to the back bone of chitosan tripoly phosphate nano particle. The shift in endothermic peaks can be considered as a proof of interaction between nisin and chitosan Tripolyphosphate polymers.

FTIR evaluation was done to examine chemical interactions. In FTIR spectrum of pure chitosan (not shown) a band at 3450 cm^{-1} corresponds to the combined peaks of the NH2 and OH group stretching and a band at 1650 cm^{-1} is attributed to the CO-NH2 group. The peak at 3450 cm^{-1} of pure Chitosan shifts to 3423 cm^{-1} (Fig.5A) in the synthesized chitosan nanoparticles indicating the formation of hydrogen bond due to nano particle formation. The peak was found to be sharper in the chitosan nanoparticles, The signal intensities of (CONH2) band at 1650 cm^{-1} and (NH2) band at 1421 cm^{-1} (Fig. 5A) decreases in chitosan nanoparticle but was found to be close in pure chitosan (not shown).

Two new absorption bands at 1643 cm^{-1} and 1417 cm^{-1} appear (Fig. 5A), which may be due to the cross linking of ammonium groups with Tripolyphosphate. It is well known that negatively charged phosphate group of Tripolyphosphate network with the positively charged amino group of chitosan and this interaction results in the formation of ionic complex. Formation of ionic complex was prominently confirmed by shifts in the absorption bands of carboxylic and amino clutches and amide bonds in the fourier transform infra-red spectra. N–H and O–H broadening rhythms for the nisin-loaded chitosan tripolyphosphate nanoparticles exhibited high intensity peaks at 3517 cm (Fig. 5B) compared to the chitosan tripolyphosphate nanoparticles which is devoid of nisin. Deviation in spectra may be due to the encapsulation of bacteriocin nisin into the nanoparticles. Similar trends were observed by Zohri etal [22]. Changes in the FTIR diagram (Fig. 5B) are indication of encapsulation of nisin into chitosan nanoparticles X-ray diffractograms of pure Nisin powder show sharp peaks at a diffraction angle of 2ɵ = 27.3346, 31.6832, 45.4244, 53.8857, 56.4640, 66.2419 that denotes the crystalline property of nisin (Fig. 6 A). Two sharp peaks in diffractograms of native chitosan at 2ɵ= 17.7914, 20.128, denotes the crystalline property of chitosan (Fig.6 B). However this crystalline property was missing in the diffractogram recorded for nisin loaded chitosan nanoparticle (Fig. 7B), The results of XRD confirms tagging of Nisin and the absence of crystalline material after encapsulation, nisin-loaded chitosan- TPP nanoparticles exist in the amorphous state.

The results obtained for loading efficiency and prolonged release of nisin from chitosan tripolyphosphate nanoparticles were similar to that of Zohri et al [22].

Loading efficiency of nisin was achieved in between 90–95% which was determined using the equation mentioned. Nisin which possess a positive charge imparts positive charge to nanoparticles up on encapsulation, it is one of the prime factors influencing good physical stability, higher loading capacity and release.

Release profile of nisin shows a maximum release of 80% during the time period of 180-240 minute, while a noteworthy release at other time intervals. The initial fast release of protein Nisin from chitosan tripolyphosphate nanoparticles is due to the hydrophilic nature of chitosan, which results in its swelling and loosening of chitosan nanoparticles structure allowing nisin molecules encapsulated to diffuse across the particle structure. This release was seen up to 240 min and there is a total release of nisin between 420-480 min of incubation.

There was a decline in the nisin release rate with the increase in the incubation time suggesting that nisin molecules were trapped inside newly formed swollen chitosan polymer chain structure [24]. The synthesized nisin tagged nanoparticles exhibited better anti listerial activity due to the synergistic action of chitosan in antibacterial strength.

The nisin loaded nanoparticles activity showed more inhibition zone (16 mm) than that of free Nisin (10 mm). One of the reasons for this higher antibacterial activity is also the organized discharge of nisin by the nanoparticles and also shielding nisin from being degraded during initial phase [22]. Brandelli et al [25] prepared phopshotidyl choline nano vesicles loaded with nisin and they were less stable compared to nisin loaded chitosan tripolyphosphate nanoparticles.

Nisin loaded chitosan tripolyphosphate nanoparticles showed similar results with Pectin/PLA films incorporated Nisin [26], in the inhibition of pathogenic Listeria monocytogenes, but a small quantity of nisin is required in preparing nisin loaded chitosan nanoparticles for better antibacterial activity. Encapsulation of nisin in nano molecules is a potent tool to enhance the stability of nisin and to have prolonged inhibitory action in protecting the quality of food products [16, 22, 27].

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1V. CONCLUSION

The results were encouraging, inclusion of nisin into chitosan tripolyphosphate nanoparticles may potentially improve the delivery of nisin to foods. Moreover the use of hydrophilic food grade polymer chitosan provides stability to the nano particulate systems. A great deal of work has to be done in order to exploit the nanoparticle targeted drug delivery systems for delivery of bacteriocins.

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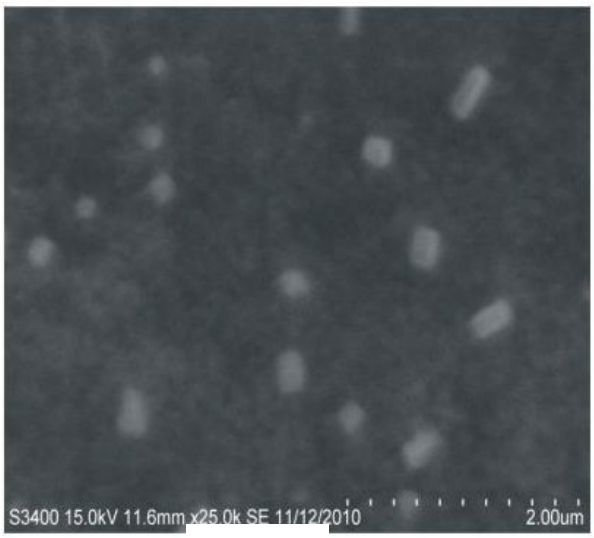
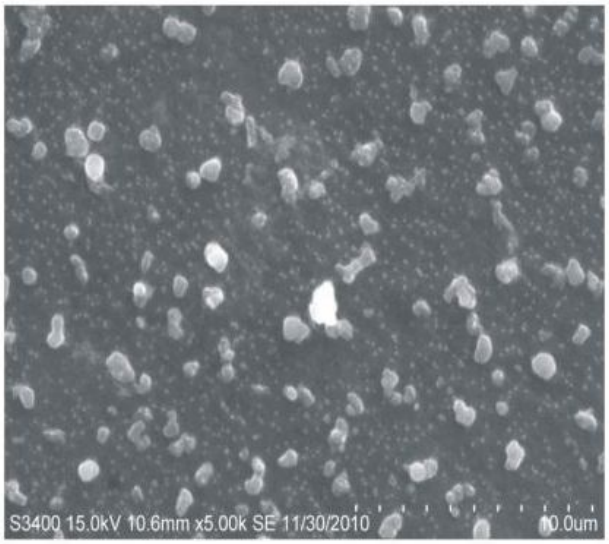
Figure 1. Scanning electron microscope image of Chitosan Tripolyphosphate Nanoparticles (A) and Nisin loaded Chitosan Nan

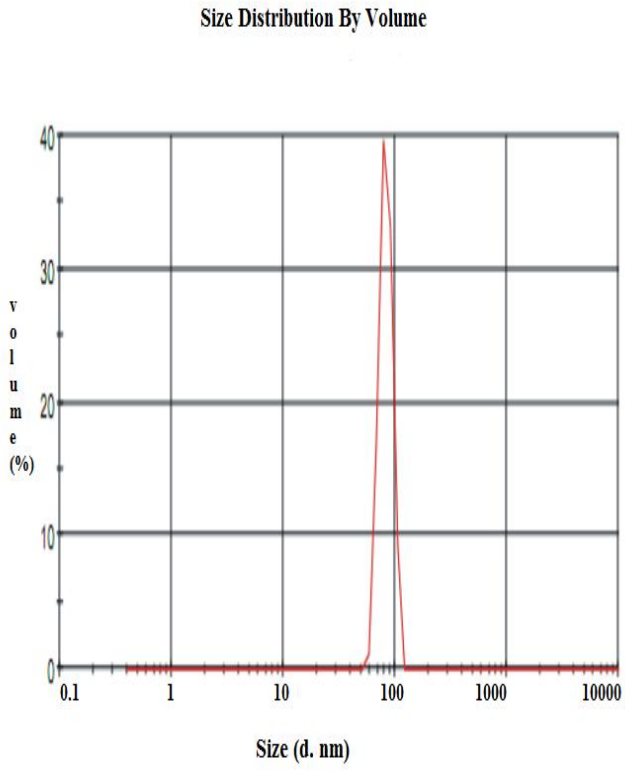


A



particle (B). B

Figure 2. Image showing size distribution by volume of Chitosan Tripolyphosphate Nanoparticles (A) and Nisin loaded Chitosan Tripolyphosphate Nanoparticles (B) in liquid dispersion medium



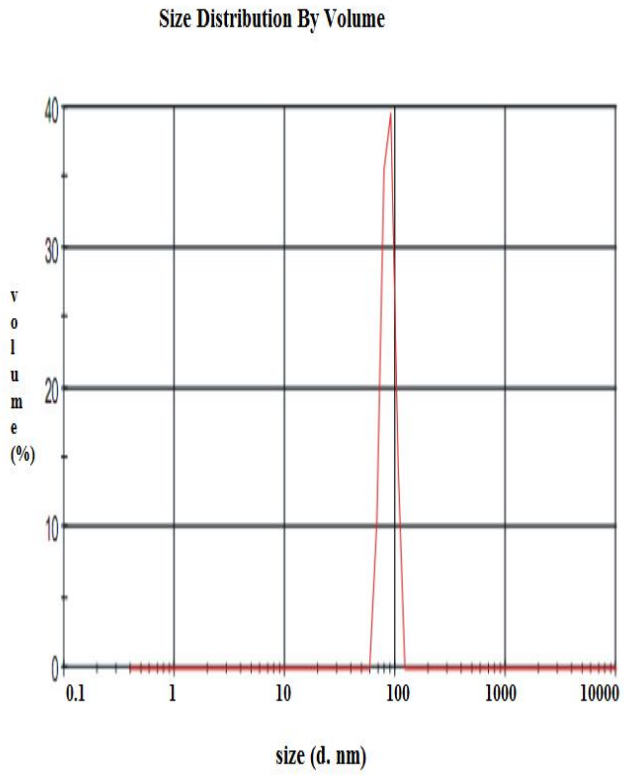


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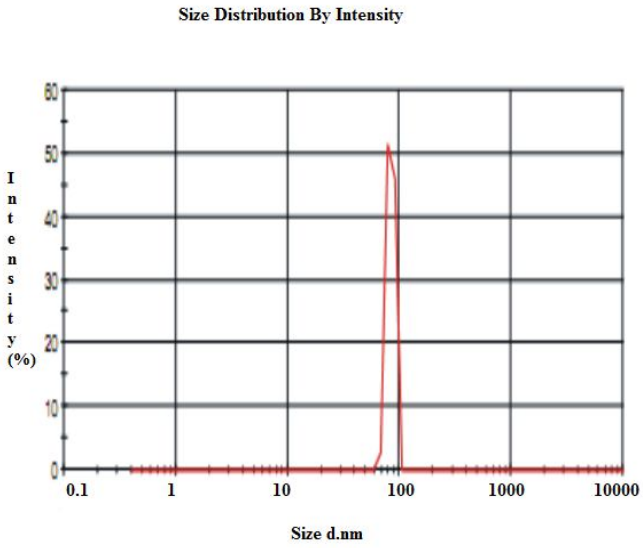
Figure 3. Image showing Size distribution by intensity of Chitosan Nanoparticles (A) and Nisin loaded Chitosan Tripolyphosphate Nanoparticles (B) in liquid dispersion mediu



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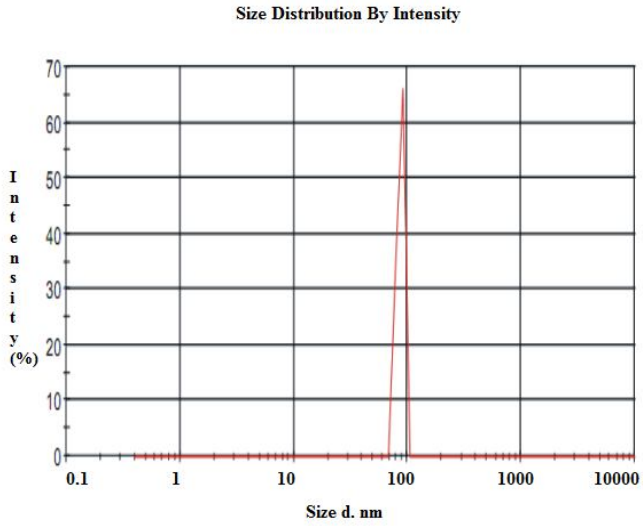
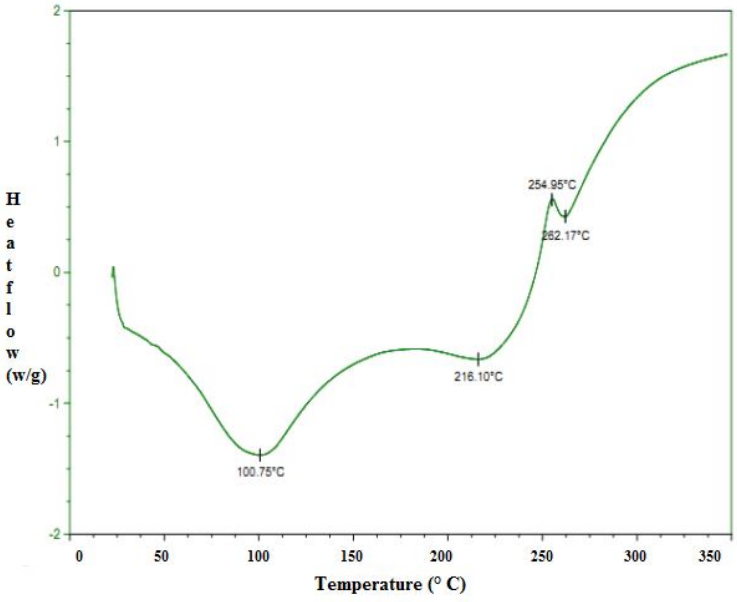


B

Figure 4. DSC Image showing the Chitosan Tripolyphosphate Nanoparticles (A) and Nisin loaded Chitosan nanoparticles (B)



A

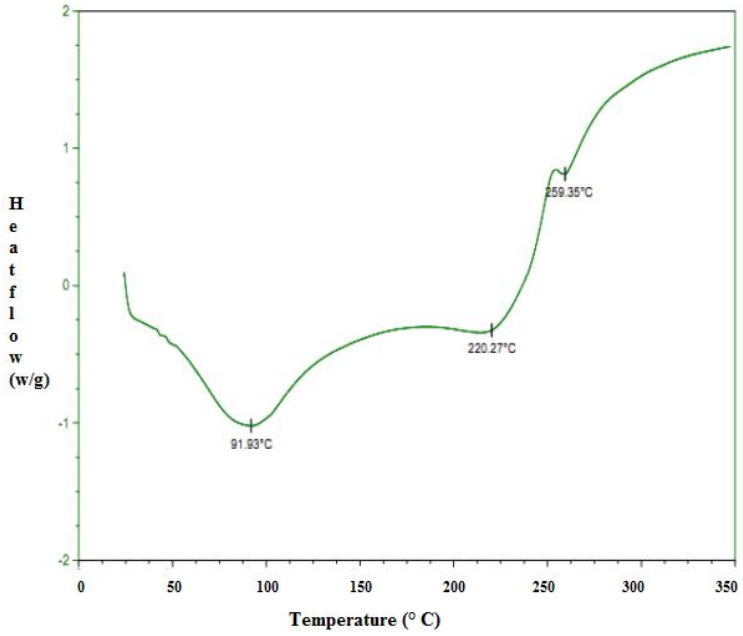


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Figure 5. FTIR spectrum of Chitosan Tripolyphosphate nanoparticles (A) and Nisin loaded Chitosan nanoparticles (B)

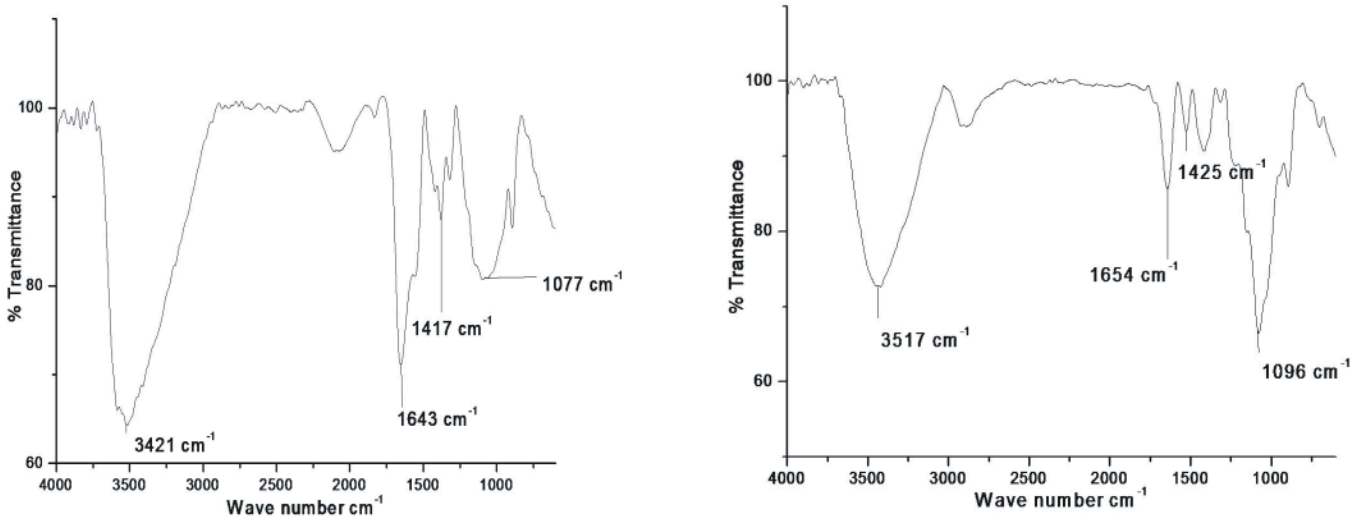

Figure 6. Image showing Powder XRPD analysis of pure nisin (A) and pure chitosan (B)

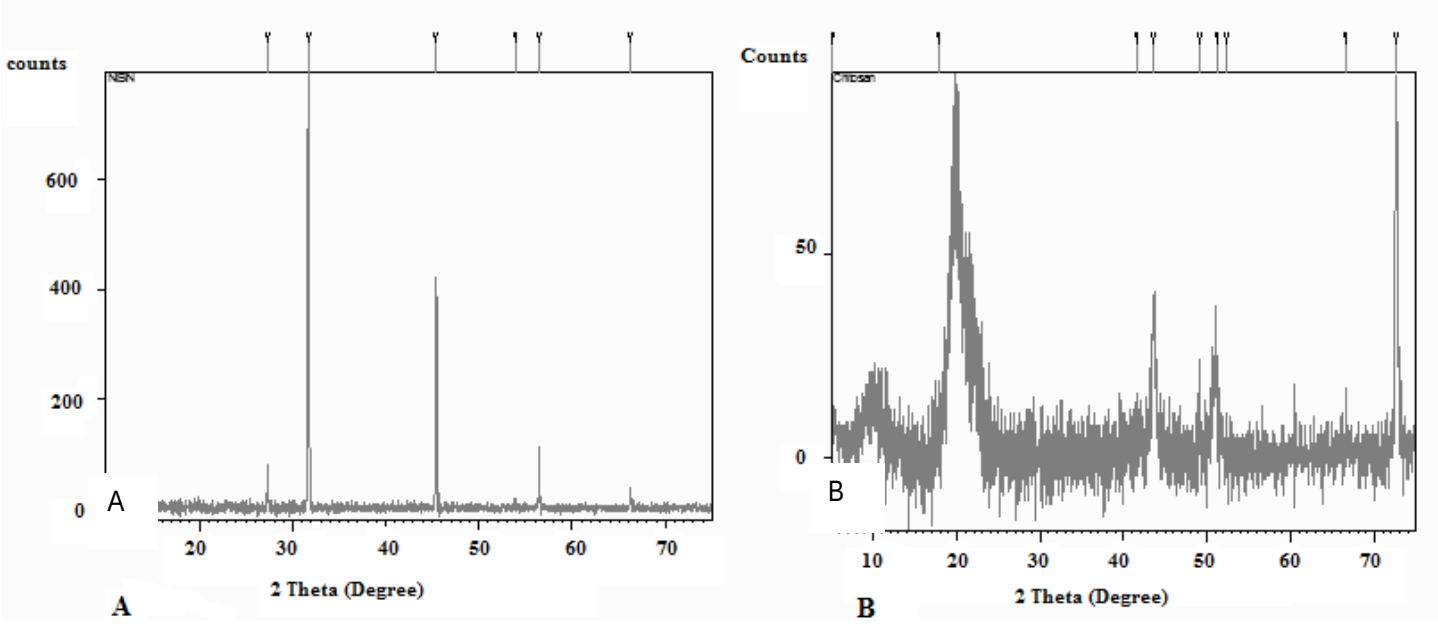

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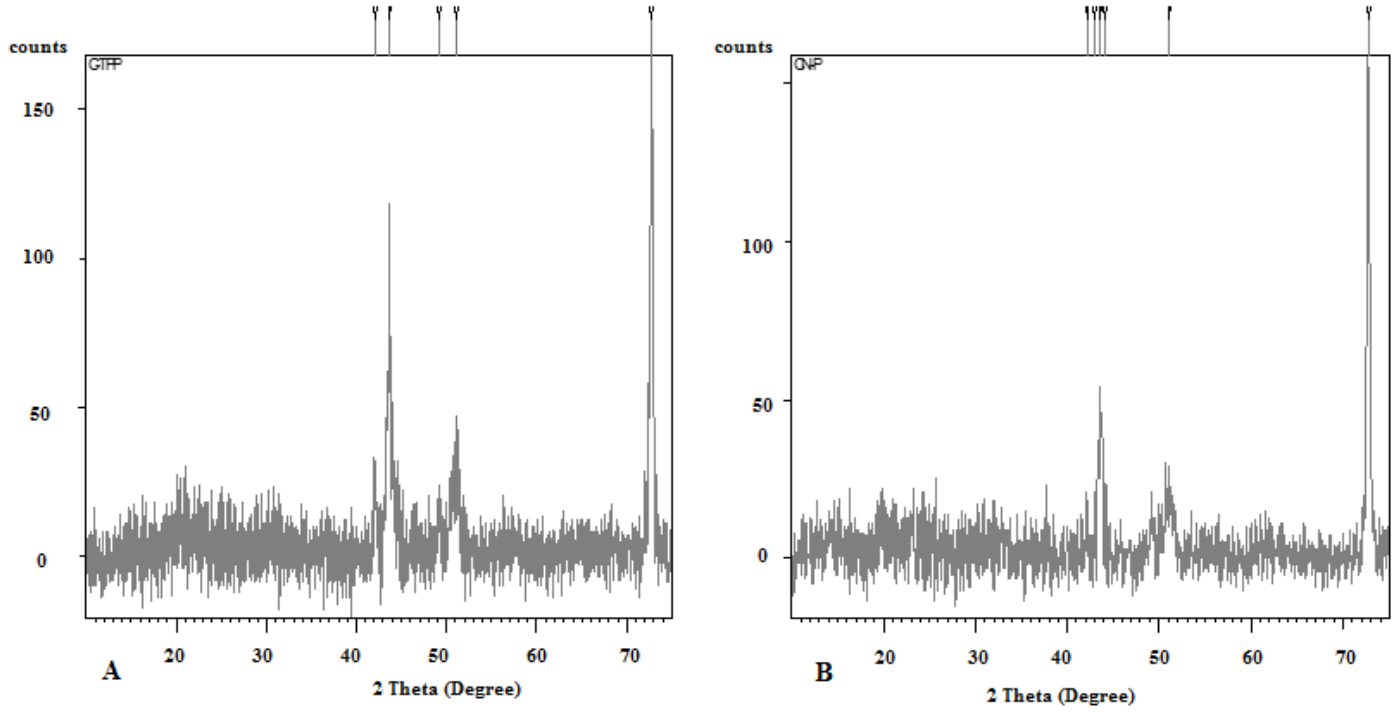
Figure 7. Image showing Powder XRPD analysis of chitosan tripolyphosphate nanoparticles (A) and nisin loaded chitosan nanoparticles (B).



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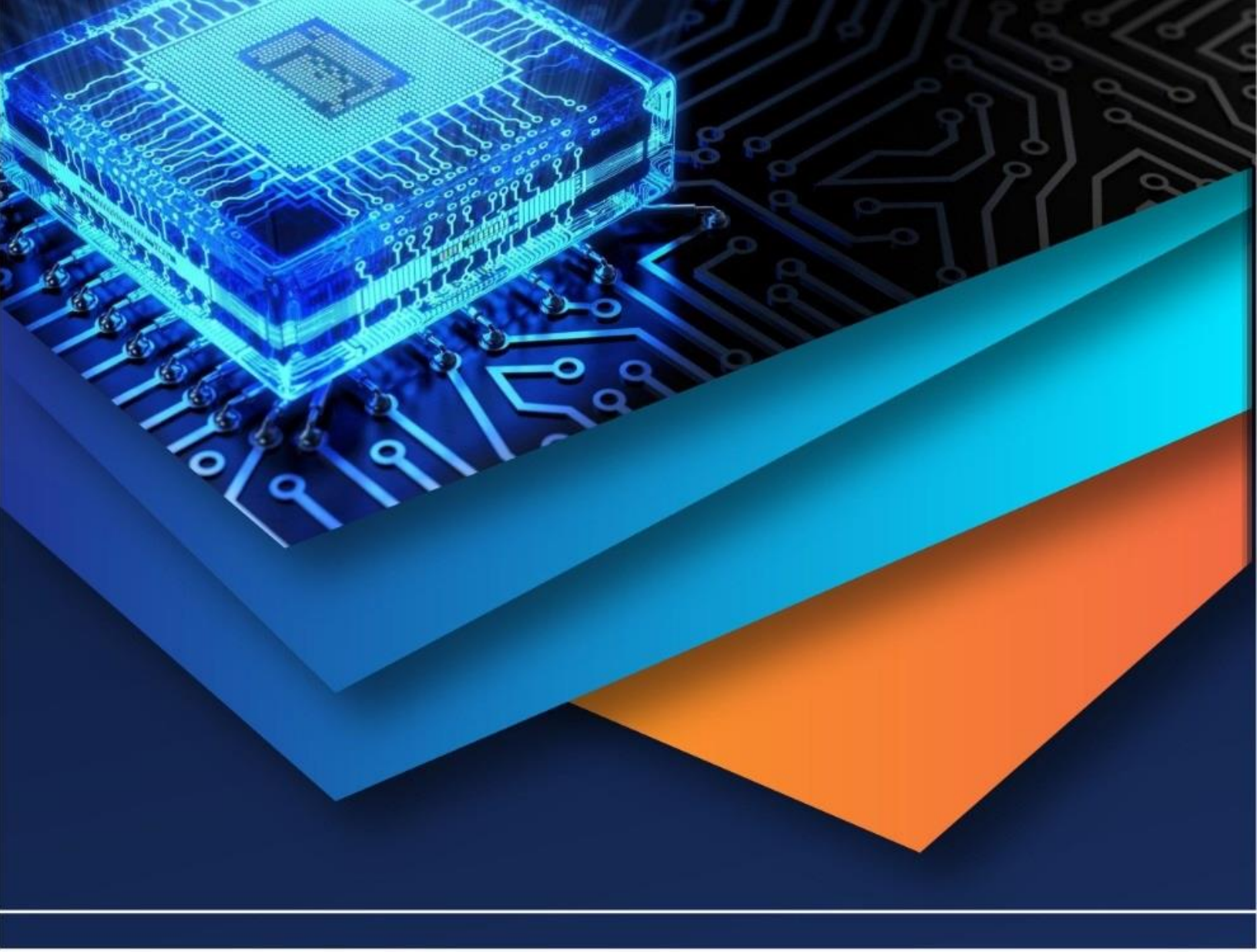

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