# PRODUCTION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF CHITOSAN NANOPARTICLES AGAINST LACTIC ACID BACTERIA

Bianca Pereira da Silva[1], Bruna Casagrande Amorin-da-Silva[1,2*], Dânia E. Christofoletti Mazzeo[3], Kelly R. Francisco[4], Tatiana Santana Ribeiro[4] & Sandra R. Ceccato-Antonini[1]

*[1] Dept. Tecnologia Agroindustrial e Socio-Economia Rural, CCA – UFSCar – Campus de Araras – SP, Brazil,*
*[2] Post-Graduation on Biological Sciences, UNESP – Campus de Rio Claro – SP - Brazil,*
*[3] Dept Produção e Biotecnologia Vegetal e Animal, CCA – UFSCar – Campus de Araras – SP, Brazil*
*[4] Dept. Ciências da Natureza, Matemática e Educação, CCA - UFSCar – Campus de Araras – SP, Brazil*
** Corresponding author's email address:* brunacamorin@hotmail.com

## ABSTRACT

Chitosan is a polysaccharide derived from chitin, found naturally in the cell walls of fungi or from the deacetylation of chitin derived from the exoskeleton of insects and crustaceans. Among the various properties of chitosan, antimicrobial and antioxidant activities stand out. Chitosan, in the form of nanoparticles, due to its reduced size, has the ability to allow access to certain compounds more efficiently, promote improved interactions and offer a larger contact area. This work aimed to test two protocols for the production of chitosan nanoparticles, as well as to verify their antimicrobial activity on *Limosilactobacillus fermentum*, one of the most important contaminating bacteria in the ethanolic fermentation process. The particle samples were subjected to Scanning Electron Microscopy, Zeta Potential and Dynamic Light Scattering analysis for characterization. Protocol II produced particles with sizes ranged from 437 to 529 nm, stable and with less tendency to agglomerate than protocol I. At a concentration of 1000 µg/mL, chitosan nanoparticles reduced the number of *L. fermentum* in approximately one log cycle. The results open the perspective of studying these nanoparticles as antimicrobial in the context of the bioethanol industry, replacing sulfuric acid in the cell treatment step between fermentation cycles.

**Keywords:** *Lactobacillus*. Antibacterial activity. Chitosan.

## 1 INTRODUCTION

Chitosan is a biopolymer obtained from the deacetylation of chitin, a polysaccharide present in the cell wall of Zygomycete fungi and in the exoskeleton of crustaceans, which is extensively used in a variety of sectors, including the food, medical, pharmaceutical, textile and agronomic, due to its antimicrobial, antioxidant, biodegradability, film and sphere formation properties, and biocompatibility with various materials.[1,2]. Chitosan, when transformed into polymeric nanoparticles, due to its reduced size, allows access to certain compounds more efficiently, promotes improved interactions, offers a larger contact area, regulates the release rate and optimizes the dosage of the applied substances.[3].

Ethanol production in Brazil has traditionally used a combination of sugarcane juice and molasses, under non-aseptic conditions, which allows the entry of bacteria and wild yeasts. The main problem is the presence of lactic acid bacteria, such as *Limosilactobacillus fermentum*, which results in a decreased fermentative yield.[4,5]. Treating cell mass with sulfuric acid (aqueous solution pH 2.0-2.5) between the fermentation cycles is the most common practice in the industry, but it is urgent to look for cheaper and more sustainable alternatives. In this work, we propose the production, characterization and evaluation of the antibacterial activity of chitosan nanoparticles, with a view to their potential use in the bioethanol industry. Two nanoparticle production protocols are tested. The chitosan used is obtained in a two-step process where chitin is obtained from shrimp wastes via lactic fermentation, and chitin is deacetylated via chemical method. The chitosan obtained by our research group (*molasses chitosan*), without purification, has been studied for a series of applications[6], among them, the production of micro and nanoparticles.

## 2 MATERIAL & METHODS

The production of chitosan (molasses chitosan) was carried out as described by Tanganini et al.[6]. For the production of nanoparticles, two protocols were tested. Protocol I followed the methods described by Calvo et al.[7] and Fan et al.[8] with some modifications. Chitosan was dissolved in an aqueous acetic acid solution (0.2 mg/mL) to form a solution of 0.5 mg chitosan/mL. The chitosan solution was stirred at room temperature for 24 h on a magnetic stirrer. Sodium tripolyphosphate (TPP) was dissolved in ultrapure water at a concentration of 0.5 mg/mL. A volume of 10 mL of chitosan solution was placed in a water bath preheated to 60◦C for 10 min, and then placed on a magnetic stirrer in an ice bath. A volume of 3 mL of ice-cold TPP solution was quickly added to the chitosan solution. The reaction was carried out for 10 min and the resulting suspension was dried at 30◦C.

Protocol II was carried out using the ionic gelation method.[9]. An amount of 0.5 g of chitosan was dissolved in 10 ml of 0.5% acetic acid, stirring for 24 h. In the final step, 0.1 g of sodium tripolyphosphate was dissolved in 10 ml of distilled water and added to the chitosan solution, dripping slowly. The final solution was stirred for 30 min and centrifuged at 1000 rpm for 20 min and the resulting suspension dried at 30◦C.[10].

The nanoparticle samples were submitted to Scanning Electron Microscopy (SEM) with the ThermoScientific Prisma E equipment operating at 20 kV, using secondary electron imaging and energy dispersive X-Ray (EDX) mode. The colloidal stability of the dispersions was evaluated by Zeta Potential (ZP) and the size distribution of the samples was analyzed by Dynamic Light Scattering (DLS) using the Zetasizer® Nanoseries model NanoZS90 equipment (Malvern Instruments Inc.).

For the antimicrobial assay, only nanoparticles obtained through protocol II were used. The *L. fermentum* strain (CCT0559/ATCC9338) was incubated overnight in de Man-Rogosa-Sharpe (MRS) medium at 35°C. The tests were conducted under non-proliferative conditions; for this, the cells were subjected to centrifugation, washed three times with Phosphate-Buffered-Saline (PBS) buffer and suspended in saline solution (0.85% NaCl). A volume of 1 mL was pipetted into Falcon tubes containing 9 mL of saline solution with 0, 100, or 1000 µg/mL of nanoparticles. Then, the tubes were incubated with shaking at 150 rpm for 3 h at 35°C. After this period, serial dilution and plating onto MRS medium were performed, with incubation at 35°C for 48 h to determine the number of colony-forming units (CFU/mL). The data were transformed to log of CFU/mL.

## 3 RESULTS & DISCUSSION

The chitosan particles obtained through protocols I and II were visualized by SEM (Figure 1). In protocol I (Figure 1A, 1B) it was not possible to measure the size of the nanoparticles formed. In protocol II (Figure 1C, 1D), particles with size ranged from 488 to 1038 nm were observed. Nanoparticles are defined as solid particles with a size between 1–1000 nm[11], so that chitosan nanoparticles were obtained using Protocol II.

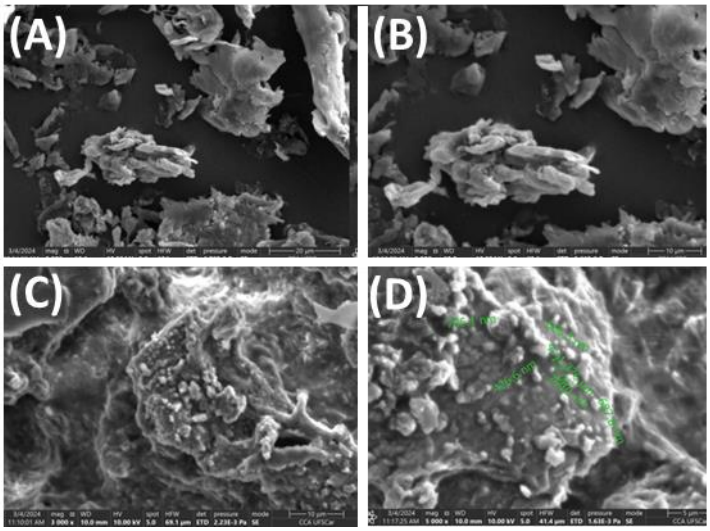


**Figure 1** SEM images of chitosan particles obtained using protocols I (A, 2000x; B, 3000x) and II (C, 3000x; D, 5000x).

The ZP values obtained for the particles in protocols I and II were -10.8 mV and -25.2 mV, respectively, indicating that the particles produced from protocol II are more stable and with less tendency to form agglomerates.[12] . The sizes of the particles obtained from the DLS data using protocol II were between 437 and 529 nm, sizes compatible with those observed in the SEM images (Figure 1C, 1D). However, it was not possible to determine these values for the particles produced from protocol I, possibly due to their large size not covered by the DLS technique. The ZP and DLS results confirmed the production of chitosan nanoparticles by protocol II.

The antimicrobial assay (Figure 2) showed a reduction in the number of bacteria by approximately 1 log cycle at the concentration of 1000 µg/mL (equivalent to 1 g/L) of chitosan nanoparticles in relation to the control (without nanoparticles). Similar test with molasses chitosan microspheres (average size of 1530 µm) demonstrated no antibacterial effect at the concentration of 10 g/L.[6,13]. This suggests that by reducing particle size to the nano scale, the antimicrobial efficiency is increased. However, the efficiency of chitosan nanoparticles at the concentrations tested is much lower when compared to the efficiency of sulfuric acid (aqueous solution pH 2.0-2.5) against *L. fermentum*.[5]. Higher concentrations of nanoparticles and the effect on ethanolic fermentation yeast, *Saccharomyces cerevisiae*, will be tested with a view to the possible use of chitosan nanoparticles as an antimicrobial in the context of the bioethanol industry.

## 4 CONCLUSION

After evaluating two protocols to produce chitosan nanoparticles, stable and less likely to clump nanoparticles (437 a 529 nm size) were obtained from unpurified molasses chitosan. At a concentration of 1000 µg/mL of chitosan nanoparticles, a reduction in approximately one log cycle of *L. fermentum* was obtained in non-proliferative conditions. Since this bacterium is one of the major bacterial contaminant in ethanolic fermentation, this result may open an avenue to evaluate these particles as antimicrobial in the bioethanol industry.

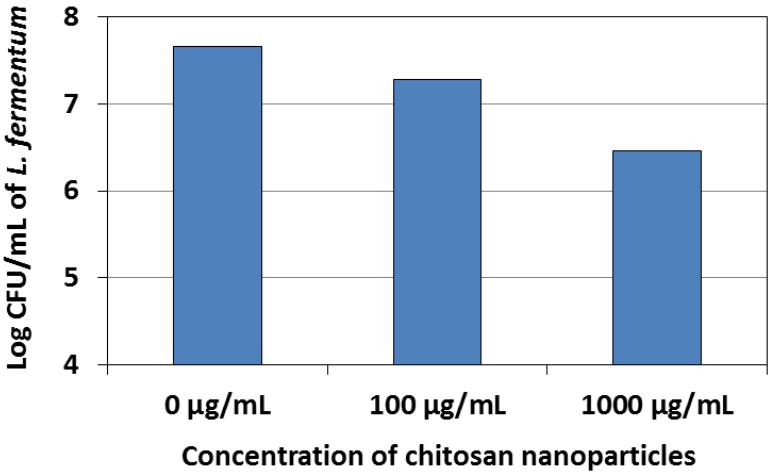


**Figure 2** Effect of chitosan nanoparticles on the number of *L. fermentum* after 3 h of treatment, in non-proliferative conditions, at 35ºC, 150 rpm.

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