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Nisin-loaded bacterial cellulose: evaluation of its antimicrobial activity stability

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Abstract

Nisin is a 3.4 kDa antimicrobial peptide, produced by *Lactococcus lactis* (ATCC 11454). This bacteriocin can inhibit spores gemination and gram-positive bacteria development and has gained visibility in therapeutic use. The bacterial cellulose (CB) has been considered an ideal material and with high quality applied in food and medical-pharmaceutical inputs. Because of all this benefits, it is important to know the system proceeding of CB with nisin. Therefore, it was realize nisin release profile analysis of CBs was performed; analysis of the minimum inhibitory concentration (MIC) of nisin against *Escherichia coli* ATCC 25922, *Pseudomonas aeuroginosa* ATCC 9721 and *Staphylococus aureus* ATCC 10390; antimicrobial stability test, for 100 days at different temperatures of 4º, 25º and 37 ° C against microorganisms: *S. aureus* e *L. sakei*. The results show that nisin is released by the CB in 4 hours of contact with medium and the MIC of nisin is 78 µg/mL for *S. aureus,* doesn't have gram-negative inhibition. It had stability until 100 days against *L. sakei* and 60 days for *S. aureus*. The system proved to be efficient and CB potentiated the antimicrobial action of nisin, acting as a selective barrier for other compounds present in the standard solution, serving as protection of the peptide at different temperatures. The CB loading system can be an ideal antimicrobial stability system for nisin.

Keywords: Bacterial cellulose, Nisin, Antimicrobial activity, Stability.

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INTRODUCTION

Antimicrobial peptides are synthesized by several organisms. Nisin is a lantibiotic, part of bacteriocins subfamily among peptides produced by microorganisms [1]. This biomolecule has 34 aminoacids and is secreted by gram-negative bacteria as *Lactococcus* [2–5]. Antimicrobial property of nisin is attributed to pore formation in cell membrane of microorganisms, with specific binding to the lipidic precursor of the cell wall, attached to the membrane [2, 6].

Nisin was considered safe by World Health Organization (WHO) and Food and Drug Administration (FDA), being used initially as a food additive [7, 8]. However, its antimicrobial action awakens potential applications for clinical use, whether in topical or systemic therapies, due to its broad spectrum against gram-positive bacterial and low probability of developing microbial resistance [8, 9].

Nisin's application extends to several medical areas, being used in relation to mastitis, oral and gastrointestinal diseases, among [9–11]. One of the fields studied is its immobilization in solid matrices that could control its release, as for example membranes of bacterial cellulose (CB) [12].

CB is a polysaccharide extracellularly excreted by several microorganisms, such as *Agrobacterium*, *Rhizobium*, *Escherichia*, *Sarcina* e *Acetobacter* [13–15]. Among them *Gluconacetobacter xylinus*, a non-pathogenic gram-negative *Acetobacter*, can produce significant amounts of cellulose [16]. CB is a linear glucose polymer, formed by a matrix of nanofibers, which give it porous characteristics in a three-dimensional network structure. Although it resembles vegetable cellulose, bacterial cellulose presents a high degree of purity, crystallinity, tensile strength and high water absorption [17].

Due to its biocompatibility and non-toxicity for biological systems, CB application has been directed to medical devices and tissue engineering [18]. Due to its structural proximity to the extracellular matrix, an ideal dermal substitute is proposed, capable of inducing the direction of cells for tissue repair in tissue reconstruction [19, 20]. Moreover its absorption characteristic facilitates exudates removal from wounds and its morphological shape helps in protecting the barrier, until epithelial healing is achieved [21, 22].

Immobilization of biomolecules in CB has also been studied, either by increasing its antibacterial or enzymatic, and may providing a control release system [23–25]. Although previous studies show the antimicrobial action on nisin incorporated in CB and its antioxidant capacity [26], it is necessary to investigate the stability of this system. Therefore, nisin stability after its incorporation into bacterial cellulose membranes was evaluated, aiming its future application in the development of a wound healing dressing.

MATERIAL AND METHODS

Materials

Standard nisin and bicinchoninic acid kit were purchased from Sigma-Aldrich (Sao Paulo, Brazil). All other reagents were of analytical grade.

CB Production and Purification

CBs was produced by *Gluconacetobacter xylinus* ATCC 53582, using 24-well plates with *Hestrin & Schramm* medium. Each well was filled with 1 mL and incubated at 30ºC in static condition for seven days. After formed, membranes were immersed in a 2% SDS solution under stirring overnight, washed in running water and

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bleaching process was carried out with NaOH for one hour until reaching 60 ºC. The CBs were washed to remove NaOH and sterilized at 121 °C for 15 minutes [27].

Total Proteins Quantification

Total protein concentration was determined using bicinchoninic acid method. Bovine serum albumin (BSA) in different concentrations of 0.1 to 1 mg/mL was used as standard. Absorbance reading was performed on 96-well microplates with a wavelength of 562 nm, by spectroscopy (Inifinite M200 PRO, RCHISTO, Austria). Each analysis was performed in triplicate, and the mean of these absorbance values was used to determine protein concentrations.

Nisin Standard Curve

The standard solution that was used in all tests for the calibration of the procedure, had 2.5% nisin in 1 g of the product, corresponding to an initial activity of 10^6 AU/mL. The nisin solution used had 0.25 mg/mL. For the construction of the standard curve, the microorganism bioindicator of the antimicrobial activity of nisin it was *Lactobacillus sakei* ATCC 15521 [26, 28].

Nisin Loading in CB

The CBs membranes were disposed in 24-well plates and 1 mL of nisin solution (250 µg/mL) was added in each well. The plate was placed on shaker (NT 715, NovaTecnica) at 30 ºC and 100 rpm for 4 h. After the membranes were dried at room temperature in sterile gauze.

Release of Nisin from N-CB

Nisin-loaded CB membranes (N-CB) were disposed in triplicate in 24-well plate with 1 mL of PBS pH 7 solution, as well as membranes without the incorporation of nisin (CB), which were used as controls. The plates were placed on shaker at 25 ºC and 100 rpm. Between 2h to 36h, 1 mL of the samples were collected, and total protein concentration and antimicrobial activity were quantified.

Microbiology Analyses

Microorganisms

Escherichia coli ATCC 25922, *Pseudomonas aeuroginosa* ATCC 9721 (both representing gram-negatives) e *Staphylococus aureus* ATCC 10390 (representing gram-positive) were used. *Tryptone Soya Broth* (TSB) was used as culture medium. After growth, microorganisms were counted respecting the range of 30 to 300 colonies and selecting those with 10^6 UFC/mL to carry out antimicrobial activity tests.

Minimum Inhibitory Concentration (MIC)

MIC method [23, 29] was adapted was adapted in 96-well microplates and performed in triplicate. The plate was stored in oven at 37 ºC overnight. After this, 5 µL of each well were collected and dripped into Petri dishes containing Tryptone Soya Agar (TSA) for activity analysis. The plates were kept in oven at 37ºC overnight.

Agar Diffusion and Stability Test

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N-CB were stored in Petri dishes at different temperatures: i) 4 °C, ii) 25 °C and iii) 37 °C, and evaluated using the agar diffusion methodology with *L. sakei* and *S. aureus* microorganisms in different time periods from 0 to 100 days, adapted from methodology [30].

RESULTS AND DISCUSSION

Nisin Standard Curve

Zone of inhibition were formed between 10.87 to 36.30 mm, depending of solution concentrations from 0.25 ug/mL (10^1 AU/mL) until 2500 µg/mL (10^5 AU/mL). This result shows that microorganism was sensitive even when exposed to low nisin concentration, and it is important to establish the relationship between the degree of solubility and pH of nisin in relation to the method used [31]. As it is a soluble peptide with low molecular weight, this facilitates its diffusion and complements its antimicrobial activity [32, 33].

Release of Nisin from N-CB

Nisin release from N-CB membranes was evaluated for 36 hours (Figure 1). It was possible to observe a detachment of 580 µg/mL of nisin in the first 2 hours, reaching a peak of 620 µg/mL after 4 h, and a plateau in 600 µg/mL. This result shows that nisin was absorbed by CB membranes, and after 4 hours maximum release was reached and maintained, which may be used as a strategy to minimize the time of reapplications.

Figure 1. Nisin release profile from bacterial cellulose membranes in PBS pH 7.

Retention and consecutive release are reported in several studies with molecules and drugs, mainly with potential antimicrobials, where they try to maintain this release in a controlled manner [34]. CB provides ideal retention, with structural and functional protein stabilization, due to its nanofiber structure [24]. Cellulose membranes have also been shown to be efficient for retaining and releasing bromelain, maintaining the enzyme activity of the enzyme and providing antimicrobial activity to the final product [23]. In the literature, it is suggested a potential use of cellulose hydrogel for application and release molecules, where this release can be controlled through ultrasonic treatments [35]. Previous study with cellulose microcrystals concluded that low molecular weight molecules and drugs were faster released when compared with molecules with high molecular weight [36].

Minimum Inhibitory Concentration (MIC)

Nisin was effective to inhibit *S. aureus* growth, with MIC of 78 µg/mL. N-CB absorbed a nisin concentration 8 times higher than MIC, which would already warrant antimicrobial activity. In the literature, the concentration of nisin with EDTA (20 mM) is described at 125 µg/mL, at pH varying between acid and base, necessary to inhibit the growth of gram-positive microorganisms [37]. Our study showed a lower MIC, however, without the use of EDTA gram-negative microorganisms, as *E. coli* and *P. aeuruginosa*, were not inhibited even in high nisin concentration (250 µg/mL). For this reason, the stability work was carried out only with *S. aureus* and *L. sakei*, the latter being used as a bioindicator of nisin antibacterial action.

Modugno et al. (2018) [38] demonstrates that nisin antimicrobial activity is favoured around neutral pH, which our analyses were performed on. However, peptide activity can vary depending on microorganisms' type,

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as already observed in our studies. Nisin acts by interrupting transglicosylation in gram positive, causing pores formation in cell membrane and inhibits cell wall formation. On the other hand, gram-negatives bacteria have a high resistance to nisin molecule, once their outer membrane is thicker and acts preventing access of antimicrobial peptide [38, 39]. Thus, it is necessary to unite the antimicrobial activity of nisin with EDTA, optimizing it, against gram-negatives, where EDTA will bind metal ions to lipopolysaccharide layers of bacterial membrane [37, 40].

Agar diffusion and stability test

Agar diffusion of N-CB after 7 days of storage at room temperature is shown in Figure 2.

Fig. 2 Agar diffusion in plates with CB with nisin against of microorganism *L. sakei* (a) and *S. aureus* (b)

Against *L.sakei*, N-CB maintained the stability during the 100 days in 4, 25 and 37 ºC temperatures (Figure 3). After 3 days of storage, there was an increase in nisin released by N-CB, which resulted in an increase of 1 AU/mL. For the last period (100 days), there was a non-significant decrease in AU/mL in all temperatures. It is possible that N-CB stability exceed the study period.

Initial nisin solution presented 5 $\log_{10}$ AU/mL, and after 24 h in contact with CB, the N-CB system showed an increase of 1 $\log_{10}$ AU/mL in antimicrobial activity. This fact corroborates the observation that CB may function as a filter, separating active biomolecule from other molecules in the solution and, consequently, potentiates its antimicrobial action. The same behaviour of CB membrane was reported during bromelain release of bromelain [23]. The process of nisin purification described in the literature showed that nisin activity can be reduced by the presence of fat clusters, salts concentration and aggregates of soluble proteins in the medium, and that after purification its antimicrobial activity was increased [41].

Figure 3. Nisin antimicrobial activity stability against *L. sakei* ATCC 15521 in N-CB membranes after storage at 4, 25 and 37 ºC.

Antimicrobial activity against *S. aureus* was stable for 60 days at 4 ºC and 25 ºC, with a decrease of approximately 1.5 log representing up to 38 times less of nisin released, however, maintaining its antimicrobial activity. At 37 ºC, the stability reached a period of 21 days, against the microorganism, obtaining a decay of 2 log, thus continuing its effectiveness.

Figure 4. Nisin antimicrobial activity stability against *S. aureus* ATCC 10390 in N-CB membranes after storage at 4, 25 and 37 ºC.

Staphylococci are bacteria that live on epithelial surface of humans and animals, being responsible for food poisoning and infections. Mainly referring to bacterial resistance, nisin has shown itself convenient for not presenting problematic growth of bacterial resistance when applied [7, 42]. *S. aureus* also appears among the main microorganisms responsible for secondary infections in burns [43,44]. Another study confers the antimicrobial capacity against the microorganism *S. aureus*, with samples supplemented with nisin, making possible a significant

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decrease of viable cells in a period of 1 day and even inhibiting biofilm formation [45]. Through the tests of nisin encapsulation in nanofiber polymer, they were able to observe that it provided stability to nisin for a period of up to seven days, however, not in a longer period, where the inhibition zone was inferior to our studies [42].

CONCLUSION

CB membranes significant incorporate nisin from a solution, and also showed a sustained release of this biomolecule for 36 hours. In addition, CB acted as a selective barrier for other compounds present in initial nisin solution, potentiating its antimicrobial action. After nisin incorporation, N-CB system was stable after storage in different conditions and can be considered an ideal system for nisin delivery. N-CB system has potential to be used as dressing in patients with skin lesions, in which nisin can be released preventing infection caused by gram-positive bacteria, and CB may act providing a physical barrier protecting wounded area helping cell matrix regeneration.

Conflict of interest: The authors declare that there are no conflicts of interest.

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