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Comparative Study on Antifungal Activity of Some Nanoencapsulated Natamycin Systems

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ABSTRACT

Food grade microorganisms can form different substances that are inhibitory to other microorganisms. Natamycin is a naturally occurring antifungal agent produced during fermentation by the bacterium *Streptococcus natalensis*. It is a save antifungal preservative and its sensitivity in vitro in most cases below 20ppm. Nanoencapsulation has been applied to a number of food biopreservatives.

The aim of this study is to determine if the nanoencapsulation system could enhance of its activity. Our results indicated that it is active against yeasts and moulds tested at low concentrations used (4-6ppm). Inhibition was variable and significantly influenced by strain of the organism. Growth of yeast and mould couldn't be detected in cheese treated with 6ppm natamycin (free or encapsulated) after 30 days of storage. Natamycin has strong antifungal activity and can extend cheese shelf life during storage period in all treatments.

Key words: Nanoencapsulation- Natamycin- Antifungal Activity- Cheese - yeast and mould .

Introduction

Microbial contamination is one of the major cause of food spoilage. Antimicrobial agents can be applied to food for controlling microbial growth. Natamycin is commonly is used in many countries as food additive to prevent the growth of moulds and yeast on food products (Jay *et al.,* 2005 and Stark, 2003).

Natamycin can be used in a variety of ways such as mixed into brine (aqueous suspension) (Eissa *et al.,* 2014) or incorporation into the bio-based packaging material (Chinnan & Cha, 2004).
Natamycin when incorporated or applied on food products, it didn't affect the food quality (taste, color, and odor). Repentantly, application by direct incorporation of natamycin in food products such as yoghurt and beverage in some countries have been approved by the Food and Drug Administration (FDA).

Natamycin have a selectivity action towards fungi without activity against bacteria. It possesses broad spectrum activity and its effect at low doses (below 3-10ppm). Thomas & Delves-Broughton (2001) recorded that the concentration of natamycin for yoghurt preservation to be in range of 5-10ppm. The poor aqueous solubility of natamycin limits its availability in molecular state for antimicrobial activity and also its diffusion rate to the site of antifungal action. Nano-encapsulation system could provide higher availability and improved antifungal efficiency of natamycin (Bouaoud *et al.,* 2016).
A limited number of attempts have been reported so far with such nano-carriers for the encapsulation of natamycin and were all focused on application antifungal treatment.

The main goal of this research was to determine if the incorporation of natamycin molecule within nano-encapsulation systems could provide benefits for antimicrobial efficiency.

Materials and Methods

Pathogenic strains:

Aspergillus flavus 3357 and *Saccharomyces cerevisiae* Y-2223 were provided by the Northern Regional Research Laboratory Illinois, USA (NRRL). *Aspergillus niger*, *Penicillium roqueforti* J5 were

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obtained from Department of Microbiology, Swedish University of Agricultural Sciences, *Candida albicans* were provided by the Institute of Applied Microbiology, University of Tokyo, Japan.

Preparations of natamycin-loaded whey protein nanoparticles:

Whey protein nanoparticles prepared by the method described by Hassan *et al.* (2014). Whey protein nanoparticles prepared at final concentration 1%. Natamycin suspension (10mg/ml) was added drop-wise to 1% aqueous solution of whey protein nanoparticles and stirred for 1h at 1500rpm.

Preparation of natamycin-loaded alginate nanoparticles:

Sodium alginate was prepared by dissolving 2% of sodium alginate in deionized water. Then, it was filtered with syringe filter prior to use of natamycin solution (10mg/ml) was added drop-wise to aqueous solutions of sodium alginate (2%) and stirred for 1h (Zohri *et al.,* 2010).

Nanoparticles characterization:

Measurement of particle size and zeta potential:

The size and zeta potential of the particle in the prepared CPP containing particles were determined using Zeta sizer var. 704 instrument (Malvern Instruments, Malvern, UK). Samples was diluted with ultrapure MQ water before measurement its light scattering for a laser beam (633nm) at an angle of 173 at 25°C over time intervals. The changes in laser beam scattering versus time was used to determine the particle size distribution. The mean particle diameter (the scattering intensity-weighted mean diameter, Z-average) and polydispersity index (PDI) were calculated from the particle size distribution. Narrowness of the particle size distribution. With values of ≤0.1 indication a very narrow distribution (Saberi *et al.,* 2013). Zeta potential was measured in the same sample by electrophoresis and results were expressed as Mv.

The effect of natamycin and nanoparticles on pathogenic fungi was determined as the following:
First step:

Different fungi and yeast strains were activated individually and grown in potato dextrose broth medium, after that, natamycin added at 6, 4 and 2ppm to fortified potato dextrose agar media individually in different flasks. Then 1ml of different pathogenic fungi and yeast were added to different flasks, after that pouring 10ml of different flasks containing different concentration of natamycin in petri dishes and incubated for 3 days at 25°C.

Second step: Fortified potato dextrose agar media individually in different flasks with 0.6, 4.00 and 2.00ppm microencapsulated natamycin as above.

Manufacturing of contaminated soft cheese:

Fresh buffalo's milk was standardized to 5% fat, pasteurized at 70°C for 10min, cooled and adjusted to 37°C, then calcium chloride and sodium chloride were added in 0.02% and 2% (w/v) contents, respectively. Then, the rennet was added and the coagulation was performed after 3h according to Fahmi and Sharara (1950). The resulting cheese was contaminated with a cocktail of two pathogenic stains (*A. flaus* and *A. niger*) obtained by mixing the same population (~ 10^4cfu/ml) of the different strains of each microorganism. The inoculated cheese was divided into three portions, the first portion sever as control and stored under refrigeration at 7°C for 30 days, the second portion stored under refrigeration at 7°C for 30 days in whey containing 6ppm and third portion stored under refrigeration at 7°C for 30 days in whey containing 4ppm.The cheese samples were analyzed microbiologically at 0, 15 and 30 days of storage.

Results and Discussion

I- Size and zeta potential of nanoparticles:

From the results gained and summarized in Table 1, the main size of nanoparticles is 12.46±1.577 in case of alginate loaded nanoparticles and 4.288±0.7602 in case of whey protein nanoparticles. The zeta potential of nanoparticles was about -57.7±637 for alginate and -43.39±483 for whey protein treatment which could result in good stability of the nanoparticles during the manipulation and storage (Zohri *et al.,* 2010).

Table 1: Size and zeta potential of nanoparticles

	Alginate	WPI
Particle size (nm)	1.577 ±12.46	0.7602 ±4.288
Zeta potential (mV)	6.37±-57.7	4.83 ±-43.79

II- Effect of different natamycin concentrations on some moulds and yeasts

A- Free natamycin:

It is clear from Table 2 that naturally occurring fungal resistance to natamycin at low concentrations (2-4ppm) for some tested organisms is present. On the other hand, in the presence of 6ppm natamycin in growth media as tested organisms could not be detected. .

In this respect, Ray and Bullerman (1982) recorded that, inhibition was highly variable but significantly influenced by the strain of the organism. *A. flavus* had highly tolerance to natamycin as shown in previous results.

Table 2: Effect of natamycin concentrations on moulds and yeasts

Strains	Control	2ppm	4ppm	6ppm
A. niger	+++	N.D	N.D	N.D
A. flavus	+++	++	++	N.D
P. roqueforti	+++	N.D	N.D	N.D
Sac. cerevisiae	+++	++	++	N.D
Candidia albicans	+++	N.D	N.D	N.D

N.D: no growth detected ++: found growth (10^2 log cfu/ml) +++: found growth (10^5 log cfu/ml)

It is clear that, natamycin has minimal inhibitory concentration of less than 4ppm for some yeast and mould and not less than 6ppm from food borne fungi tested.

In this concern, Stark (2003) stated that a minimal inhibitory concentration for *A. flavus* ranging from 10 to 20ppm, while this concentration was <5ppm for *A. niger*. Finally, Thomas & Delves-Broughton (2001) stated that natamycin can be added directly to yoghurt mix at concentration ranging from 6 to 10ppm but when added to fruit juice, the concentration became 2.5-10ppm. Lastly, Abdel hameed (2016) stated that natamycin has strong antifungal activity and can extend cheese shelf-life during storage period.

B- Microencapsulated natamycin concentrations

Nanoencapsulation systems could indeed provide higher availability and improved efficiency of natamycin nanoparticles. Nanoparticles can be easier incorporated into food. It is clear form Tables 3 and 4 that sodium alginate nanoencapsulated natamycin at concentration ≥4ppm was more efficient to inhibit the tested organisms. In the same time, some effects were observed at concentration 4ppm when whey protein nanoencapsulated natamycin was applied. All tested organisms could be inhibited at concentration 6ppm of whey protein encapsulated natamycin.

Table 3: Effect of nano-natamycin concentrations (encapsulated with sodium alginate) on moulds and yeasts

Strains	Control	4ppm	6ppm
A. niger	+++	N.D	N.D
A. flavus	+++	N.D	N.D
P. roqueforti.	+++	N.D	N.D
Sac. cerevisiae	+++	N.D	N.D
Candidia albicans	+++	N.D	N.D

N.D: no growth detected +++: found growth (10^5 log cfu/ml)

Table 4: Effect of different nano-natamycin concentrations (encapsulated with whey protein) on moulds and yeasts

Strains	Control	4ppm	6ppm
A. niger	+++	++	N.D
A. flavus	+++	N.D	N.D
P. roqueforti.	+++	N.D	N.D
Sac. cerevisiae	+++	N.D	N.D
Candidia albicans	+++	N.D	N.D

N.D: no growth detected ++: found growth (10^2 log cfu/ml) +++: found growth (10^5 log cfu/ml)

These preliminary results confirm that, interesting properties can be generated via the encapsulation of natamycin inside nanocarriers.
This phenomena could be attributed to the fact that some oligosaccharides such as carrageenans or alginate containing functional groups show biological activity as antibacterial, antiviral as well as antioxidant activity (Cieśla, 2017). It is worthy to mention that, Gunasekaren et al. (2007) recorded that whey protein isolate is a suitable wall material for encapsulation and can release properties can be altered by addition of sodium alginate.

Lastly, Fuciños *et al.* (2012) stated that the antibacterial efficiency of natamycin was improved when encapsulated in nanohydorgels due to the protection that the hydrogel gives against environmental degradation whilst also lowering the presence of preservatives in food bulk.

III- Inhibitory effect of natamycin free and microencapsulated on mould growth in Egyptian soft cheese:

It is clear form Table 5 that, addition of 6ppm natamycin to whey cheese (free or encapsulated) resulted in some decrease of fungal count after 15 days of storage at 8-10°C compared with control. The organism could not be detected after 30 days of storage in both treated samples, while, counts recorded ~log 5 in natamycin free samples, these results nearly similar to Eissa *et al.* (2014), El-Diasty *et al.* (2009) and Var *et al.* (2004). They mentioned that no growth of mould was detected in yoghurt samples after 30 days of storage. Abdel hameed (2016) concluded that natamycin has strong antifungal activity and can extend cheese shelf-life during storage period.

Table 5: Counts of mould in contaminated soft cheese samples during storage period

Samples	Storage period (days)				
	Zero	7	15	21	30
C	$30x10^4$	$36x10^4$	$38x10^4$	$35x10^4$	$33x10^4$
T1	$28x10^4$	$14x10^4$	$21x10^2$	14x10	nil
T2	$31x10^4$	$10x10^4$	$9x10^2$	11x10	nil

Soft cheese contaminated by *A. flavus* and *A. niger*
C: control T1: soft cheese containing 6ppm free-natamycin.
T2: soft cheese containing 6ppm microencapulated (alginate).

Ombarak & Shelaby (2017) found that, in cheese samples treated with 5ppm natamycin, mould growth inhibition percentage compared with control group at the same storage time were 75.4, 95.5, 97.8 and 98.4 after the 1st, 2nd, 3rd and 4th week of refrigerated storage. No clear differences could be observed between the activity of free and encapsulated natamycin.

From the above results it could be concluded that the solubility of natamycin in water at neutral pH level is 30ppm, therefore, for many applications or treatments of natamycin, suspensions is quite effective. The level of the dissolved active natamycin is high enough to prevent the growth of fungi.

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