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CHARACTERIZATION OF CHITOSAN NANOPARTICLES ADDED WITH ESSENTIAL OILS. *In vitro* EFFECT ON *Pectobacterium carotovorum*

CARACTERIZACIÓN DE NANOPARTÍCULAS DE QUITOSANO ADICIONADAS CON ACEITES ESENCIALES. EFECTO *in vitro* EN *Pectobacterium carotovorum*

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Abstract

Chitosan is a biomacromolecule with antibacterial activity against a broad spectrum of bacteria, while essential oils are known to possess antimicrobial activity. In this work, lime and thyme essential oils were encapsulated in chitosan nanoparticles by nanoprecipitation method. The success of the encapsulation was confirmed by Scanning Electron Microscopy (SEM), Energy Dispersive Spectroscopy (EDS), Fourier Transform Infrared Spectroscopy (FTIR) and Z Potential. The obtained nanoparticles exhibited a regular distribution and spherical shape with size range of 117-250 nm. The antibacterial activity of the chitosan nanoparticles and chitosan nanoparticles added with thyme essential oil presented a significant inhibitory effect on the growth of *Pectobacterium carotovorum*.

Keywords: phytopathogenic bacteria, thyme, lime, antimicrobials, polymer.

Resumen

El quitosano es una biomacromolécula con actividad antibacterial contra un amplio espectro de bacterias, mientras que los aceites esenciales son reconocidos por poseer actividad antimicrobiana. En este trabajo, aceites esenciales de limón y tomillo se encapsularon en nanopartículas de quitosano, mediante el método de nanoprecipitación. El éxito de la encapsulación se confirmó mediante Microscopía Electrónica de Barrido (SEM), Espectroscopia de Energía Dispersa (EDS), Espectroscopia Infrarroja de Transformada de Fourier (FTIR) y Potencial Z. Las nanopartículas que se obtuvieron presentaron una distribución regular y forma esférica con un tamaño promedio de 117-250 nm. La actividad antibacteriana de las nanopartículas de quitosano y nanopartículas de quitosano adicionadas con aceites esenciales de tomillo tuvieron un efecto inhibitorio significativo en el desarrollo de *Pectobacterium carotovorum*.

Palabras clave: bacterias patógenas, tomillo, limón, antimicrobianos, polímero.

1 Introduction

Chitosan is a biologically compatible biomacromolecule widely studied, which is typically derived from chitin (2-acetamido-2-deoxy-β-1, 4-D-glucan), the main component of the exoskeleton of insects and crustaceans. Chitosan is biodegradable, nontoxic (Carlson, 2008; Salazar-Leyva *et al*., 2014), and it has a broad spectra of activity and a significant effect in the control of phytopathogenic fungi and Gram-positive and Gram-negative bacteria (Kaur *et al*., 2012; Martinez-Camacho *et al*., 2010; Cota-Arriola *et al*., 2013).

Due to the chemical properties of chitosan, it may be combined with essential oils, which are complex mixtures of various aromatic and volatile

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oily liquids, products of plants secondary metabolism. These are typically formed in specialised cells or groups of cells, found in leaves, stems, bark and/or fruits. Essential oils extracted from plants are rich sources of biologically active compounds, such as terpenoids, esters, aldehydes, and phenolic compounds, conferring medicinal, antibacterial and antifungal properties being an alternative to the conventional system of pesticides (Oussalah, 2007; Bakkali *et al*., 2008; Sánchez-Rangel *et al*., 2014).

Within the wide variety of essential oils, thyme and lime have gained acceptance due to its abundance, low cost and antimicrobial properties (Ramos-García *et al*., 2012; Çetin *et al*., 2011). Moreover, they are considered GRAS products (generally recognized as safe). However, despite its important properties, essential oils are volatile compounds that evaporate easily and/or decomposed due to direct exposure to heat, light or oxygen (Hosseini *et al*., 2013).

In order to overcome the sensitivity and improve the stability of these bioactive compounds, nanoparticles development arises (materials whose dimensions lie in the order of nanometers) (Santacruz-Vázquez *et al*., 2013). Nanoparticles are known to be more reactive and therefore are more efficient in their antimicrobial activity (Sanpui *et al*., 2008; Chen *et al*., 2009) due to the larger contact surface with the microbial membrane and the consequent agglomeration on the surface of the cell wall (Radzig *et al*., 2013).

There are several methods for obtaining nanoparticles, among them, the nanoprecipitation method (or solvent displacement), which has many advantages. It is quick, easy to perform, and the whole procedure may be carried out in one step. Besides, the formation of nanoparticles is instantaneous (Bilati *et al*., 2005; Luque-Alcaraz *et al*., 2012).

On the other hand, agricultural products are susceptible to pathogens attack, especially some genera of bacteria agents which cause various diseases like *Erwinia carotovora subsp. carotovora*, recently classified as *Pectobacterium carotovorum*, which is a phytopathogenic bacteria member of the enterobacteriaceae family, characterized by having straight bacilli of 0.7-1.5μ, it is a Gram-negative, facultative anaerobe, and producer of pectinolytic enzymes, which favor the disease commonly named 'soft rot' (Toth *et al*., 2003; Smadja *et al*., 2004). This disease occurs most often, among others in fleshy vegetables with soft tissues, such as potatoes, carrots, radishes, onions, cucumbers, squash, eggplant, peppers, cabbage and tomato (Toth *et al*., 2003; Agrios, 2005; Hibar *et al*., 2007; Zhao *et al*., 2013). This disease can occur in the field and during transport, mainly in storage, resulting in considerable economic losses (Agrios, 2005; Bhat *et al*., 2012).

In general, control of diseases caused by bacteria, depends primarily on the use of synthetic bactericides. However, the increase in public concern about food safety and the environment, in addition to the acquired resistance has led to the cancellation of some chemical bactericides. Therefore, research has been focused on the development of alternative methods of disease control, implementing the use of natural compounds such as chitosan and essential oils (Sánchez-González *et al*., 2010, 2011; Du *et al*. 2009; Ramos-García *et al*., 2012).

The objectives of this study were then to characterize chitosan nanoparticles alone and chitosan nanoparticles added with lime and thyme essential oils by the nanoprecipitation method, and to evaluate their effect on the growth of *P. carotovorum* by two *in vitro* methods.

2 Background

The antibacterial activity of chitosan has been reported for Gram-negative bacteria including among others *Escherichia coli*, *Pseudomonas aeruginosa, Salmonella typhimurium, Pseudomonas fluorescens* and *Vibrio parahaemolyticus*. Previous studies about the antimicrobial activity of chitosan in bacteria such as *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*, *S. typhimurium*, *Listeria monocytogenes*, and fungi such as *Aspergillus niger*, *Alternaria alternata* and *Colletotrichum gloeosporioides*, indicate that application of chitosan nanoparticle coatings could be an effective antimicrobial method against these pathogens (Qi *et al*., 2004; Martínez-Camacho *et al*., 2010; Ali *et al*., 2011; Cota-Arriola *et al*., 2013; Sanpui *et al*., 2008; Shi *et al*., 2006; Chen *et al*., 2012).

Natural compounds as essential oils have been studied as an alternative to reduce the use of chemicals. In addition, these substances can be incorporated in the chitosan, to enhance its antimicrobial potential. For example, it has been reported that lime essential oil has significant antimicrobial effect against phytopathogenic fungus including *C. gloeosporioides* and *Rhizopus stolonifer* (Ronquillo, 2007; Ramos-García *et al*., 2012), whereas the thyme essential oil has been used for the control of bacteria such as *Bacillus subtilis*,

P. aeruginosa, *Acinetobacter faecalis*, *Enterobacter cloacae*, *S. typhimurium*, *E. coli*, *Campylobacter* sp., *Listeria* sp. and *Yersinia* sp. (Çetin *et al*., 2011; Sheeladevi and Ramanathan, 2012). The combination of chitosan added with essential oils such as tea Tree (*Melaleuca alternifolia*) and lime (*Citrus* sp.) have been evaluated on pathogenic bacteria such as *L. monocytogenes*, *E. coli* and *S. aureus* and on fungi such as *Penicillium italicum* (Sánchez-González *et al*., 2010, 2011).

The production of fruit and vegetables is one of the most important sectors in Mexican agriculture; however, they have the disadvantage of being perishable and therefore susceptible to be infected with microorganisms that accelerate ripening. A large number of bacterial pathogens have been involved in the decay of fresh fruits and vegetables (CDC, 2010) that is the case of soft rot caused by *P. carotovorum*, which has been reported as a phytopathogen which causes destructive diseases in pre and postharvest of many vegetables worldwide. In recent years, the incidence of damage caused by *P. caratovorum* in fruits like tomatoes has been reported.

3 Methodology

3.1 Materials

Medium molecular weight pure chitosan (degree of deacetylation 75-85%) was purchased from Sigma Aldrich (St. Louis, MO,USA), JT Baker methanol and acetic acid was supplied by Fermont Chemicals Inc. Monterey (Colonia Mirador Monterrey N.L.). Essential oils (white thyme and Mexican lime 91 061 91031) were purchased from Essential Oils-esencefleur (Lake Victoria No. 52. Col. Granada, Mex. D.F.)

3.2 Chitosan nanoparticles preparation with essential oil

In order to obtain the nanoparticles using the nanoprecipitación method, the methodology proposed by Luque-Alcaraz *et al*. (2012) was used. Chitosan (0.05% w/v) was dissolved in acetic acid (2% v/v) to obtain the solvent phase. Ten mL of this phase was added to the non-solvent phase (essential oils with methanol) composed of 20% lime and 5% of thyme (40 mL) essential oils using a peristaltic pump (Bio-Rad, EP-1 Econo Pump) under moderate magnetic stirring. Once the nanoparticles were formed, the obtained solutions were placed in a rotary evaporator at 40°C with a speed of 50 rpm (Rotary Evaporator RE 300, BM 500 Water Bath, Yamato CF 300) and stored under refrigeration (4°C) for further analysis.

3.3 Nanoparticles morphology

The morphology of the nanoparticles was analyzed by scanning electron microscopy (SEM) using a JEOL electron microscope JSM-6390 LV. A drop of each sample was placed on a glass plate and dried at room temperature. Then were coated with gold-palladium metal for 60 s at high vacuum and examined. Also, energy dispersive spectroscopy (EDS) was determined.

3.4 Fourier Transform Infrared Spectroscopy

Infrared spectroscopy analysis of pure chitosan, chitosan nanoparticles and chitosan nanoparticles added with lime and thye essential oils was performed in a spectral range of 650-4000 cm^{-1} using a Shimadzu FTIR spectrophotometer, Affinity model, equipped with an ATR module of zinc selenide. For measurement, a drop of sample was placed in the center of the spectrophotometer.

3.5 Z Potential

To determine the potential Z a Zetasizer Nano-ZS90 (Malvern Instruments) was used. Three mL of each sample were placed in a quartz cell and analyzed.

3.6 Determination of antibacterial activity

3.6.1 Pectobacterium carotovorum activation

The strain of *P. carotovorum* was donated by Dr. Sergio Aranda from the Bacteriology Laboratory of the Graduate School at Montecillo, Texcoco. The strain was stored in a cryogenic mixture (glycerol/ Svelty skim milk, Nestlé) at -70°C. For activation, the bacteria were seeded in tubes containing 3 mL of brain heart infusion broth and incubated for 24 h at 30°C. To confirm the purity of the strain, a seeding groove on tryptone soy agar plates was performed, once the purity was verified, a culture replanting was performed in inclined test tubes adding tryptone soy agar and incubated at 30°C for 24 h (Lara-Cortez, 2006).

3.6.2. Determination of Minimum Inhibitory Concentration (MIC) of chitosan and essential oils in P. carotovorum using the agar plate method

First, the MIC of each of the compounds (chitosan, thyme and lime essential oils) that inhibited the growth of the bacterial strain was determined. Six replicates of each treatment were done. This was carried out by the agar plate technique, which consisted in tryptone soy agar (10 mL) in Petri dishes. The medium was allowed to solidify and then a 20 mL bacterial suspension previously added to the semisolid agar (10 mL) was incorporated. Once solidified, the agar was punctured with a tunneler (5 mm in diameter) making 6 wells or cavities on each Petri dish. Subsequently, for each small well, 20 μL of the solution with the compound were placed. Finally, the boxes were sealed and incubated for 24 h at 30°C. After that time, the zones of inhibition (lighter areas around the well) that indicated death or bacterial inhibition were measured (Lara-Cortez, 2006).

3.6.3. Evaluation of the effect of the nanoparticles in vitro

The bactericidal effect of the prepared nanoparticles was determined by two methods: agar plate and broth dilution. Four treatments with six replicates were tested: 1. chitosan nanoparticles (chitosan 0.05%), 2. chitosan nanoparticles added with lime essential oil (0.05% chitosan, 20% lime), 3. chitosan nanoparticles added with thyme essential oil (0.05% chitosan, 5% thyme), and 4. control.

3.6.3.1. Broth dilution method

For this technique, tryptone soy broth was placed (5 mL) in test tubes and a bacterial suspension (20 μL) and the nanoparticles tested (20 μL) were incorporated. They were shaken and incubated for 24 h at 30°C. Subsequently, to determine the concentration of cells and absorbance spectrophotometer (Spectronic Instruments, Genesys 5) was used. Finally, one mL of this solution was placed in Petri dishes with tryptone soya agar to determine the colony forming units (CFU) of bacteria.

3.7 Statistical analyses

A completely randomized design was used for statistical analyses. One-way analysis of variance with a significance level of P <0.05 was applied. Similarly, when significant differences were found, a comparison of means was performed using a Tukey's multiple comparison tests. A confidence interval of 95% was employed. The analysis was performed using a SigmaStat 3.5 program.

4 Results

Characterization of chitosan nanoparticles with essential oils

4.1 Nanoparticles morphology

As aforementioned, the particle morphology was observed by SEM. The micrographs of the chitosan and chitosan added with oils show a spherical shape with an average size of 117 to 250 nm. In Figure 1 (a) chitosan nanoparticles are observed. The EDS results confirmed the presence of carbon with an atomic weight of 72.63%, 23.18% of oxygen, 3.20% of nitrogen and other elements such as Na, Cl, K and Ca in minor proportions. In Figure 1 (b) the image of the chitosan nanoparticles added with lime essential oil having spherical morphology and 250 nm to 1 micron in size is observed. The EDS also confirmed the presence of carbon with an atomic weight of 55.83%, 33.21% of oxygen, 1.73% of nitrogen and other elements in minor proportions as Na, Al, Si, K, Ca, Cu and Zn. On the other hand, in Figure 1 (c) chitosan nanoparticles with added thyme essential oil presented average sizes from 117 to 226 nm are seen. The EDS confirmed the presence of carbon with an atomic weight of 37.20%, 39.06% of oxygen, 14.59% of nitrogen and other elements such as Si, Ca and Zn.

4.2 Fourier Transform Infrared Spectroscopy of the nanoparticles

Figure 2 shows the infrared spectra of pure chitosan; chitosan nanoparticles; chitosan added with lime essential oil, and chitosan added with thyme essential oil. The infrared spectrum of pure chitosan showed absorption bands located at 1377 cm^{-1} (-CH deformation), 1470 cm^{-1} (amide I) and at 2967 cm^{-1} (-CH stretching). The infrared spectrum of the chitosan nanoparticles showed absorption bands at 1017 cm^{-1} (C-O-C stretching), at 1375 cm^{-1} for -CH deformation (previously identified for pure chitosan), and a new peak located at 1644 cm^{-1} for $-NH_2$ bending. In the infrared spectra of chitosan nanoparticles with added lime and thyme essential oils, showed the characteristic peaks at 910 cm^{-1}

and 935 cm^{-1} corresponding to the formation of the pyranoside ring were identified. The band located at 3354 cm^{-1} indicated the formation of hydrogen bonds with the formation to the -OH and -NH groups, which were not observed in the spectrum of pure chitosan.

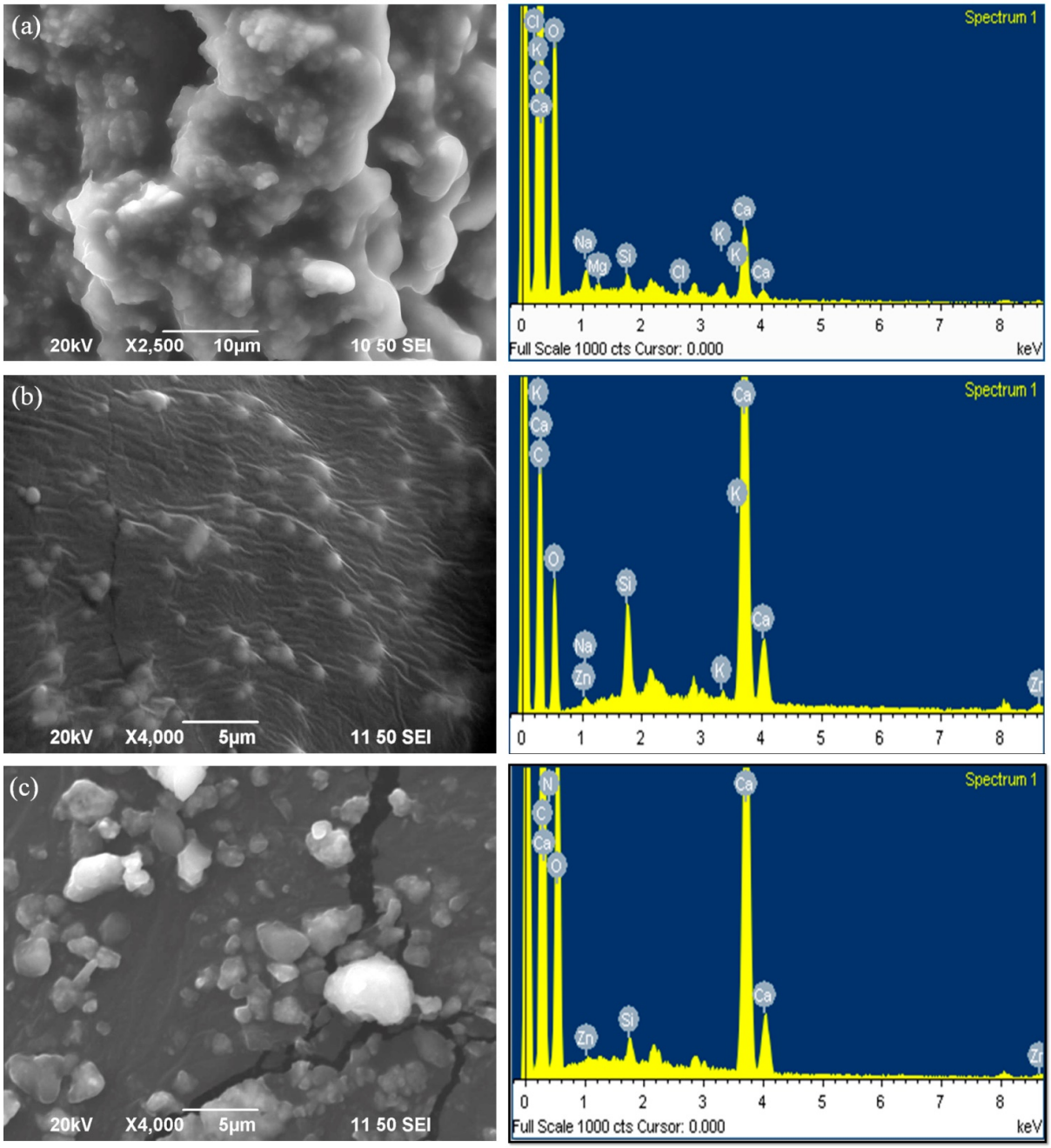

Fig. 1. (Left) Micrographs of chitosan nanoparticles (a), chitosan nanoparticles added with lime essential oil (b) and chitosan nanoparticles added with thyme essential oil (c). (Right) EDS analysis.

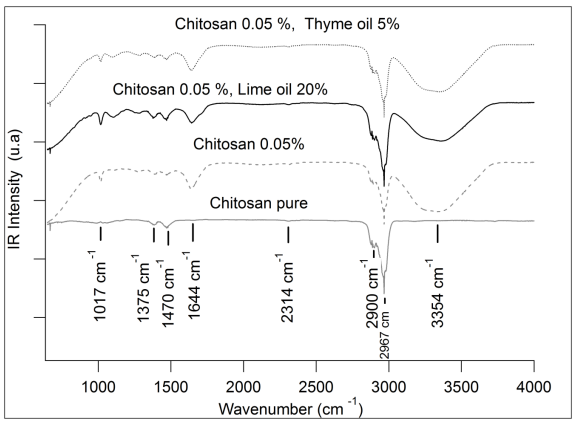

Fig. 2. Infrared spectra of pure chitosan, chitosan nanoparticles and chitosan added with lime and thyme essential oils.

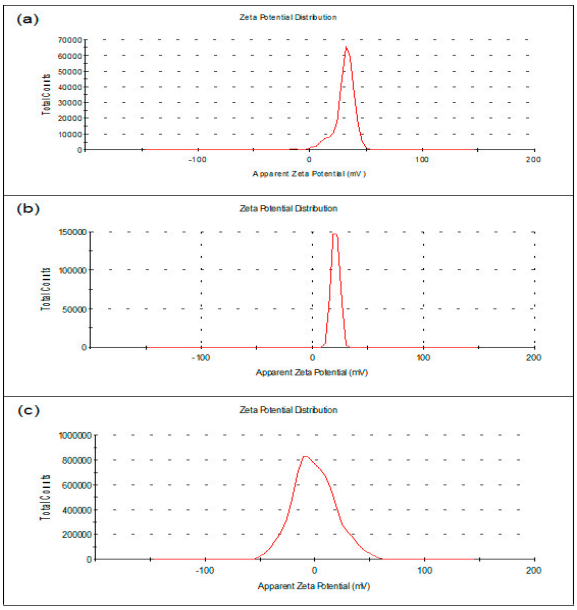

Fig. 3. Z Potential. Chitosan nanoparticles a), chitosan nanoparticles added with lime essential oil b), chitosan nanoparticles added with thyme essential oil c).

4.3 Z Potential

To determine the stability of the nanoparticles, the Z potential was measured. The chitosan nanoparticles prepared using the nanoprecipitation method had a +20.2 mV Z potential and the chitosan nanoparticles added with lime essential oil presented a potential of +10 mV, while the chitosan nanoparticles added with thyme essential oil had a Z potential of +27 mV (Figure 3).

4.4 Determination of Minimum Inhibitory Concentration (MIC) of chitosan and essential oils in P. carotovorum

Pectobacterium carotovorum treated with chitosan showed growth inhibition at a concentration of 0.5%. In the lime essential oil the inhibition was observed at a concentration of 11.2%, whereas for the thyme essential oil, *P. carotovorum* presented inhibition at the concentration of 2.8% (Table 1).

4.5 Evaluation of the effect of the nanoparticles in vitro

4.5.1. Agar plate method

In vitro evaluations of nanoparticles on *P. carotovorum* (Table 2), showed inhibition values significantly different (P <0.05) among the applied treatments. The highest inhibition of the bacteria was for the treatment of the chitosan nanoparticles added with thyme essential oil in which the corresponding halo value was of 24 mm in diameter, compared with the control, in which inhibition halo was not present.

4.5.2. Broth dilution method

In the evaluation of the nanoparticles by the broth dilution method, the chitosan nanoparticles showed an average absorbance of 0.227, therefore, the number of CFU (countless colonies) could not be determined. For the chitosan nanoparticles with added lime essential oil, an absorbance of 0.132 with CFU 450 was obtained, while in the chitosan nanoparticles added with thyme essential oil, the absorbance was 0.028 with 240 CFU. As can be seen, significant differences (P <0.05) of these samples compared with the control (Table 2) were found.

5 Discussion

The micrographs of the chitosan nanoparticles and chitosan nanoparticles added with thyme essential oil were similar in size. However, an increase in size was observed in the chitosan nanoparticles added with lime essential oil, with sizes in the range of microns. In previous studies conducted by Hosseini *et al.* (2013) and Keawchaoon *et al.* (2011) an increase in size of the nanoparticles due to the addition of essential oils was reported. In these studies the values corresponded to a range of 309-716 nm.

Table 1. Minimum inhibitory concentration (MIC) of chitosan and essential oils. *In vitro* monitoring of *Pectobacterium carotovorum*

Concentration (%)	Inhibition halo (mm)		
	Chitosan	Lime essential oil	Thyme essential oil
0.5	1.8 ± 2.8	ND	ND
0.7	ND	NI	NI
1.0	5.2 ± 0.9	ND	ND
1.4	ND	NI	NI
1.5	3.9 ± 3.1	ND	ND
2.0	6.3 ± 0.5	ND	ND
2.8	ND	NI	8.5 ± 1.7
3.0	7.0 ± 1.4	ND	ND
5.6	ND	NI	11.6 ± 2.0
11.2	ND	7.8 ± 3.4	10.9 ± 2.5
100	ND	17.0 ±7.3	28.1 ± 7.9

Data represented as mean ± standard deviation
NI = No inhibition, ND = Not determined

Table 2. *In vitro* evaluation of the nanoparticles on *Pectobacterium carotovorum* development, by agar plate and broth dilution methods.

Treatments	Inhibition halo (mm)	Absorbance	CFU
Chitosan nanoparticles	15.0 ± 0.2^{b}	0.227 ± 0.0^{b}	Countless
Chitosan nanoparticles added with lime essential oil	13.0 ± 0.1^{c}	0.132 ± 0.0^{c}	450
Chitosan nanoparticles added with thyme essential oil	24.0 ± 0.1^{a}	0.028 ± 0.0^{d}	240
Control	0 ± 0.0^{d}	0.929 ± 0.0^{a}	Countless

Data represented as mean ± standard deviation. Different letters in the same column indicate statistical significance (Tukey, P <0.05).

Comparing the infrared spectra of the pure chitosan with the chitosan nanoparticles and chitosan nanoparticles with added essential oils, a new peak appeared at 1644 cm^{-1} for -NH_2 bending due to the complex formation *via* electrostatic interaction between NH^{3+} groups of chitosan and phosphoric groups of TPP (P-O and P=O) within the nanoparticles (Hosseini *et al*., 2013; Yoksan *et al*., 2010). The broad band at 3354 cm^{-1} is related to -OH, and -NH groups of chitosan (Maza *et al*., 2007), lime (Thummanoon *et al*., 2012) and thyme (Pensel *et al*., 2014) essential oils.

Other factors involved in particle size may be the concentrations used and the type of chitosan used during the preparation method. For higher concentrations, the probability of aggregation of particles is increased, yielding low Z potential indicating an incipient instability of the particles due to a weak electrostatic interactions, causing the aggregation between them (Calvo *et al*., 1997; Qi *et al*., 2004; Xu *et al*., 2003; Velasco-Rodríguez *et al*., 2012).

Ali *et al*. (2011) mentioned the relationship between particle size and Z potential. The higher the Z potential, the smaller particle size, and viceversa. This is in agreement with our results where this relationship was observed for the chitosan nanoparticles added with lime essential oil, which had the highest size and the less Z potential. Also, Yien *et al*. (2012) referred that the size and the Z potential is one of the most important properties affecting antimicrobial activity. Nanoparticles with different size or Z potential should have different mechanisms of inhibition against microorganisms, and this factor contributes to the interaction that occurs between the positive charges present on the chitosan polymer chain, with the negative charges on the membranes of the microbial cells. However, there may be other factors involved in the antibacterial activity, such as pH and the components added to the nanoparticles, as is the case

of the chitosan nanoparticles added with silver and metal ions such as silver nitrate, copper sulfate, zinc sulfate, manganese sulfate and iron sulfate which have been shown to have inhibitory effect against bacteria such as *S. aureus*, *E. coli*, *Klebsiella pneumoniae* and *Salmonella* sp, among others (Shi *et al*., 2006; Sanpui *et al*., 2008; Du *et al*., 2009). However, until date, there are few studies related to the evaluation of chitosan nanoparticles added with essential oils for the control of plant phytopathogenic bacteria. In our case, there was a significant decreased of bacterial development when grown in a medium with chitosan nanoparticles and nanoparticles added with thyme essential oil, demonstrating that polymer chitosan nanoparticles added with essential oils have a high antimicrobial potential.

Conclusions

The chitosan nanoparticles with essential oils had an average size of 117-250 nm. The infrared spectrum showed the presence of characteristic compounds of chitosan, with an interaction between the chitosan and essential oils to form the nanoparticles. Z potential showed unstable solutions due to weak electrostatic interaction causing the agglomeration among particles. Bacterial efficiency of chitosan nanoparticles added with thyme essential oil in controlling *P. carotovorum* was better than chitosan alone and those added with lime essential oil.

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Nomenclature

w/v	weight /volume
v/v	volume/volume
mL	mililliters
μL	microliters
rpm	Revolutions Per Minute
nm	nanometers
cm^{-1}	wavelength
mV	milivolts
h	hours
SEM	Scanning Electron Microscopy
EDS	Energy Dispersive Spectroscopy
CFU	Colony Forming Units
MIC	Minimun Inhibitory Concentration
FTIR	Fourier Transform Infrared Spectroscopy
IR	Infrared Spectroscopy

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