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Evaluation of antifungal activity of chitosan coating on cut apples by image analysis technique

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Abstract
Image analysis techniques have often been chosen as a fundamental tool for evaluating the surface and/or structure of organic and inorganic materials over a broad range of magnification. In this work, a simple image capture system comprising a commercial desktop scanner combined with free image analysis software was used to evaluate the efficiency of antifungal activity of commercial medium molecular weight chitosan as edible coating on the evolution of fruit decay by fungi infestation (predominantly *Penicillinum* sp. and *Alternaria* sp.) on cut apple surfaces. The images were acquired twice daily and binary transformed for quantitative analysis. The inhibitory effect of chitosan coating in the mycelial fungi growth in on apple cut surface was confirmed.
Keywords: Chitosan, Edible coating, Antifungal activity, Minimally processed fruit.

INTRODUCTION

Protective treatment of minimally processed and post harvest products entails a variety of different techniques, including controlled cold room environment, ozone washing, biocidal action by irradiation and additional protective procedures such as packaging and coatings. Recent emerging technology provides the ability to apply biobased materials on freshly cut surfaces, offering an alternative method of controlling and extending the quality and shelf life during storage. Chitosan has been suggested as a potential material for edible coatings processing, mainly concerning its non-toxic nature, biocidal activity and gas barrier properties. It is widely known that chitosan has excellent antimicrobial activity against bacteria, viruses and fungi and have the ability to induce the expression of a variety of genes involved in plant defense responses.[1] The antifungal property of chitosan has been observed for a broad range of concentrations upon several spoilage yeasts.[2] Chitosan, as edible coatings have been evaluated, for example, on carrots, on mangos, on strawberries and on apples.[3] When deposited on fruit cut surfaces, chitosan forms a high transparent film, allowing a statistical and comparative quantification of fungi spreading against time storage. In this study, image analysis technique was used in order to follow the fungi area evolution on coated and non-coated processed fruits.

MATERIALS AND METHODS

Chitosan coating and sample contamination

Commercial medium molecular weight chitosan of shrimp (60% or more deacetylated units, from Sigma) was dissolved in 1% acetic acid in deionized water with constant stirring for 2 hours. Solutions of 2.0 g/L chitosan were prepared at a pH approximately 4.0. Supermarket apples, cv. Gala (*Malus domestica*) were first sliced into two halves and then displayed separately in two groups of 20 samples each. The first group underwent chitosan coating by direct dipped into the solution. Excess gel was allowed to drain off and the coating was then formed by drying at room temperature.

Groups of 20 coated and 20 non-coated apple slices were put into a controlled temperature chamber (25 ± 0.5°C) where petri dishes containing non-classified cultures of fungi (predominantly *Penicillium* sp. and *Alternaria* sp.) were equidistantly allocated amongst the samples, so as to allow spontaneous inoculation of the fruit slices by ambient contamination.

The fungus was originally isolated from a decayed apple and the cultures prepared by growing on potato dextrose agar (PDA) at pH 5.5, according to Zhang & Han.[4]

Image capture and analysis

Qualitative and quantitative analysis were performed using a commercial scanner (HP ScanJet 4C) for image capture. All cut surfaces were individually scanned twice a day. Images were 250% enlarged from original and 512 by 512 pixels, each with a grayness (brightness) level ranging from 0 to 255. Images were recorded to allow monitoring of the samples, providing a visual history and track alterations on the surface by assessing the percentage of blackness due to the fungus spreading. The acquired images were directly imported into the image processing software and a threshold applied, i.e., binary transformed to remove debris and quantification was performed considering a two dimensional growth. We adopted the grayness level of 130 for all captured images. The infected area was then isolated and automatically estimated by pixel counting and numerically compared to each precedent data. The analysis program used was the free software Image Tool v.3 from UTHSCSA.[5]

RESULT AND DISCUSSION

Fungal evaluation

Figure 1 illustrates the two dimensional digital image analysis procedure of a non-coated sample surface after 6 days exposure. Setting a threshold enables a selection of ranges of pixel values in grayscale images that distinguish the objects under consideration from the background, thus allowing assessment of the infected area.

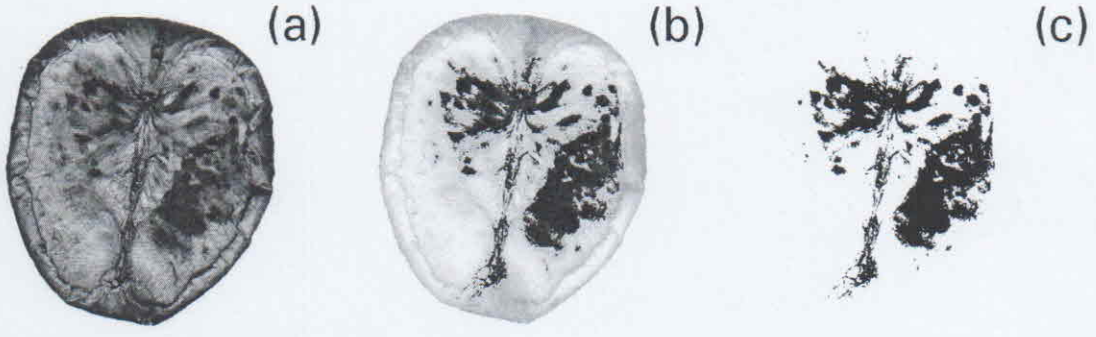

Figure 1. An example of image processing and infected area measurement. The image of a fungi-contaminated non-coated sample is digitally recorded (a), and the fungal pattern proliferation identified (b), and the correspondent area (c), after a threshold is removed for percentage fraction measurement (27.25 % of the area infected).

Fungi are filamentous microorganisms that grow as tubular cells extended by a vesicle based process of apical growth. The typical colonies are characterized by pellet morphology which is highly entangled, dense masses of hyphae. According to Cox et al.,[6] the general structure of fungal pellets is a dense core showing gradual steps to a hairy external region. Such morphology allows an easy visual approach of microbial progressive spreading.

Numerically, as expected, the non-protected faces entail higher marked fungal growth and proliferation with time, as can be seen when comparing the evolution data as plotted in Figure 2. Infected samples were considered as those having at least 10% of the total scanned area identified as covered by fungus pattern. After 10 days of image acquisition, it was apparent that 90% non-protected samples and 40% chitosan-coated fruits slices were infected.

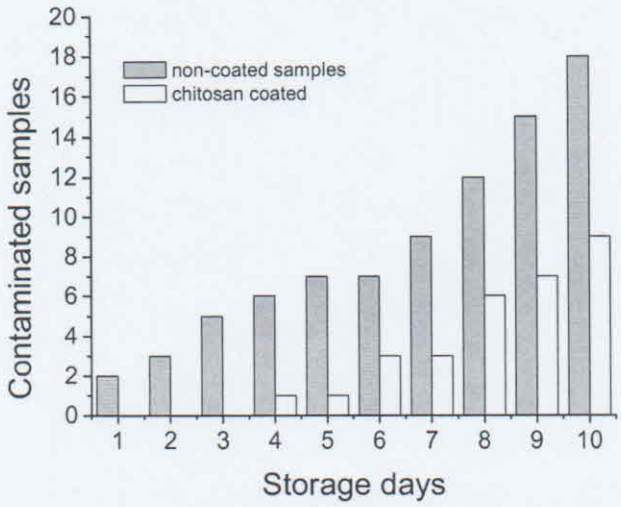

Figure 2. Evolution of the number of infected samples during 10 days of storage, over a total of 20 samples (chitosan coated and non-coated), according to the analysis of scanned surfaces.

The software was used to count pixels in the acquired images corresponding to the isolated area. A simple comparison of the evolution area in the same set of samples over a period reveals the kinetic tendency of the fungal growth. Figure 3 summarizes the percentage of infected area. The dissemination is, as expected faster for non-protected surfaces, with a reduced rate of proliferation on chitosan-coated surfaces, mainly in the first 1-4 days of exposure to fungi.
The profile of the curves clearly approach to typical standard growth kinetics found in fungi, i.e., comprising lag, exponential, and stationary phases in accordance with the literature (see for comparison the kinetics presented by Viniegra-Gonzalez, *et al.*[7] and Olsson,[8] and the numerically not far different from those reported with conventional chemical analysis.[9]

Antifungal chitosan activity
Similarly to bacteria, the chitosan activity against fungus is assumed to be fungistatic (hinders the growth of fungus but does not imply whether or not fungi are killed), rather than fungicidal (kills the live fungus or some fraction therein) with a potential to communicate regulatory changes in both the host and fungus.

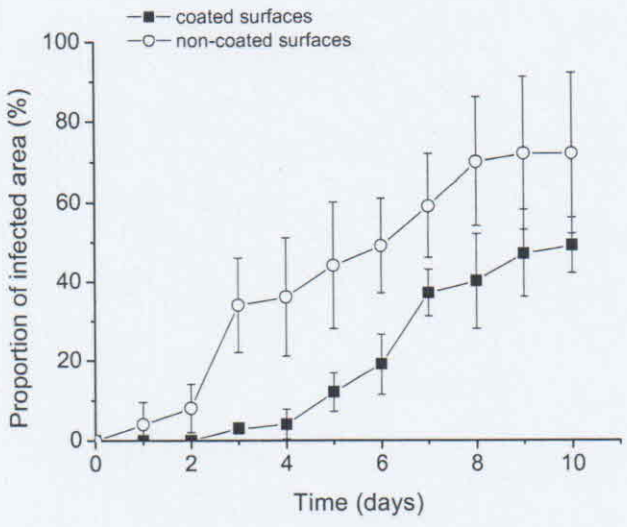

Figure 3. Proportion of infected area for sliced surfaces versus exposure time, as measured by image analysis. The error bar represents the standard deviation on 20 image acquisitions in each condition.

Generally chitosan has been reported as being very effective in inhibiting spore germination, germ tube elongation and radical growth.[10] The antifungic mechanism of chitosan involves cell wall morphogenesis with chitosan molecules interfering directly with fungal growth, similarly to the effects observed in bacteria cells.[11] The most accepted model for explaining antimicrobial activity is related to the polycationic nature of the polysaccharide that can interact with anionic sites in proteins. Such interaction is mediated by the electrostatic forces between the protonated NH_2 groups in chitosan and the negative residues at cell surfaces. Such interaction interferes with fungal cell wall membranes causing alterations in the permeability, promoting internal osmotic imbalances.[12] Microscopic observation reported that chitosan oligomers diffuse inside hyphae interfering on the enzymes activity responsible for the fungus growth. The intensity of degradation action of chitosan on fungal cell walls is also dependant upon the concentration, degree of acetylation and local pH[11].

CONCLUSIONS

From the results of the present work, the chitosan was confirmed a successful material in protecting sliced apples against fungus contamination. When directed applied on cut surfaces (with no additives) chitosan has the potential to form invisible coatings with inhibitory activity on fungi development. Such activity can be easily traced by means of image observation. Adequate application, however, requires additional knowledge of the factors that determine chitosan performance including the effects of pH, temperature, concentration, strain-specificity, etc. Anyway, chitosan may be considered as a good source for preservation of cut fruits in substitution to conventional antifungal agents. Evidently, the storage life of coated samples may be much prolonged by the conjugated use of refrigeration.

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