Study of the sugarcane (*Sachharum* spp.) antimicrobial activity against the fungi *Aspergillus* sp. and *Fusarium* sp.

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Preservatives and synthetic pesticides are commonly used to increase the shelf life of food, however, the uncontrolled use of these substances, especially antibiotics, have shown at times to be either inefficient or more harmful to humans. Therefore, there have been more and more studies on how natural substances are able to inhibit pathogenic or spoilage microorganisms. Given the need of obtaining new antimicrobial substances that may replace the conventional ones, arises the current work which aims to analyze the antimicrobial activity of the pasteurized sugarcane juice against *Aspergillus* sp. and *Fusarium* sp. The inhibition analysis consists of verifying the value of PGI (Percent Growth Inhibition): the percentage of the colony growth inhibited compared to the control sample. By observing the measures, it concludes that the sugarcane juice has antimicrobial activity against the tested fungi. The juice diluted with water at 90% during 48 hours of action, a PGI of 84% was obtained against *Aspergillus* sp. The dilution of 70% during 48 hours, a 41% PGI was obtained against *Fusarium* sp.

Keywords: Antimicrobial peptides; defensins; Fitopathogenic fungi

1. Introduction

The usage of various conservation methods used by the food industry aims to increase the shelf life of foods which are naturally susceptible to alterations. These changes can be produced by many agents, whether physical (light and heat), chemical (oxygen and water) or biological (microorganisms and enzymes) that may be the cause diseases or simply the rejection by the consumer. Through some techniques, it´s possible to delay these deteriorations, maintaining the nutrients and sensory characteristics of a given food, some examples of these techniques are: heat (pasteurization, sterilization and whitening), cold (refrigeration and freezing), reduction of the water content (drying or dehydration), microbial action that transforms and ensures better conservation (special fermentation), the use of package which serves as barrier against the factors responsible for the deterioration (active packaging, vacuum and inert gases), irradiation, and the use of chemicals that prevent or delay these changes (additives and chemical preservatives) [1, 9, 24].

Recently, many studies turned to combat spoilage microorganisms, especially phytopathogenic fungi, given that these cause various injuries to economically important plants (annually, it´s estimated that over 25-50% of the harvest of fruits and vegetables are destroyed by the action of fungi) and also to humans, given the incidence of aflatoxins, important class of allergens that presently have gained prominence due to its high carcinogenic power [9,17].

Preservatives and synthetic pesticides are commonly used for many years to prevent fungal growth, but with the constant use of these substances, especially antibiotics, they have shown sometimes to be inefficient or ever-growing aggressive to humans and the environment. Thus, there are increasing studies on natural substances that may inhibit spoilage or pathogenic microorganisms such as the stud of eugenol, garlic´s allicin, cinnamon´s cinnamic aldehyde, mustard´s allyl isothiocyanate, oregano´s thymol and the egg white´s lysozyme. It has been long since its known that natural antimicrobial effect of some foods, condiments and spices, which are used since ancient times as preservation method [3,7,11].

In order to obtain new antimicrobials able to replace conventional synthetic ones, arises the idea of this work, which aims the studying of the antimicrobial capacity of the pasteurized sugarcane broth against *Aspergillus sp*. and *Fusarium sp*., two kinds of spoilage and pathogenic fungi common on the food industry.

2. Literature Review

It is known that plants do not present specific immunity (antigens, antibodies, immunological memory, etc.) and they are dependent on the non-specific immunity. For some time, many studies have been made in order to apply the defense system of a resistant plant to another non-resistant plant susceptible to a particular pathogen. Other research aim to extend protection to processed foods, reducing the use of synthetic antimicrobials. Specifically, one of the variety of research branches include the use of secondary metabolites of plants that exhibit antibacterial and/or antifungal properties, as in the case of various natural chemical substances, such as: phytoalexins, defense proteins, alkaloids, xanthones, lactones, steroids, terpenoids, and phenolic compounds such as lignans, flavonoids, coumarins and quinones [2,4,23].

The sugar cane´s juice is a viscous, foamy, opaque, greenish-yellow colored liquid, pH between acid and neutral and complex chemical composition and also very variable, but that basically consists of water, solutes and dispersed colloidal systems, including sugars, nitrogenous materials, salts, pectins, gums, lipids, pigments, minerals, vitamins and

organic acids, a range of compounds that may act in synergism, mentioning in particular phenolic compounds of high antioxidant power, for example: flavones as apigenin, luteolin and derivatives of tricine, in addition to the phenolic hydroxycinnamic acid, caffeic and sinapic, among others [2,5,13,23].

The choice for sugar cane on this work was given by recent studies suggesting that the β-defensins are the main responsible for its antimicrobial capacity. In fact, the defensins are a very specific group of peptides generally characterized by cationic character, low molecular weight (between 5 and 7 kDa), its widely different tissues distribution and it´s produced by different groups of living beings, important features to determine its action mechanism. It is interesting to notice that these antimicrobial peptides are extensively reported in the literature in many other living groups other than plants, as in bacteria, fungi, shellfishes, insects, arthropods, amphibians, fishes, reptiles, birds and mammals (including humans), therefore, by this means, been considered primitive elements of the immunological system of all living beings [2,5,15,21].

The advantages of the applicability of such substances include, for example, the fact that most of them are considered innocuous by the World Health Organization; many do not confer odor or color, may be easily hydrolyzed on digestion and exhibit relatively high thermal stability to pH variations and the action of certain proteases. Furthermore, they are source of researches for new pesticides with low environmental impact and less aggressive therapeutic agents to humans and the environment; They can also be applied in genetically modified plants, and act in synergy with other antimicrobial agents [3,4,7].

It´s found on the literature many descriptions of the action mechanism that antimicrobial peptides may have when interacting with a microorganism, given that not all of them are fully understood, but almost everyone agrees that action is directly related and initially with the outer lipid layer of microorganisms. The peptides tend to a random structure when in an aqueous environment and a specific one when found on a biological membrane. Regardless, the surface charge of the peptides is generally positive, while the surface membrane of the microorganism is usually negatively charged compounds. Given that first electrostatic attraction between positive and negative charges there is the formation of peptide specific structure, an amphipathic structure, in other words, with a hydrophilic face and the other hydrophobic, which enable itself to insert into the lipid bilayer of the microorganism [2,3, 5.10]. When the peptide integrates into the lipid layer (which depends both on the peptide´s charge and the charge of the micro-organism surface layer), it forms small pores on the cell surface. This, by itself, could lead to the cell´s death by changing its selective permeability, otherwise, the lipid layer could disintegrate and solubilize in the form of micelles. There are cases where the layer fails to disintegrate completely, occurring the passage of peptides into the cell, where they can attack any intracellular organelle [5,15,17].

In the case of fungi, the main binding sites for defensins are sphingolipids and glicosilceramidas. Specifically it has been shown that in fungi the action of some vegetable peptides cause alterations on the gradient of Ca2+ and K+ ions (essential for the growth of hyphae), and morphological changes in hyphae, leaving branched, swollen and less elongated. Furthermore, defensins also stimulate the formation of intracellular oxygen-reactive species, core clutter and blistering of the plasma wall, which can lead to cell´s death [2,3,5,11,5].

3. Methodology

3.1 Isolation and identification of fungi

In order to obtain pure yeast colonies for inhibition analyzes it was performed isolation and replating of these microorganisms, following the recommendations of Moraes; Paes & Holanda (2010), with modifications, where papaya samples (*Carica papaya*) and hamburger-type bread were left in different containers, exposed to sunlight at room temperature until they present clear signs of microbial contamination by fungi. Next, it was taken 25 grams of parts that showed more signs of contamination, and then separately homogenized in a blender with 225 mL of sterile saline 0.85%. From those samples it was prepared three serial dilutions on a ratio of 1:10, for each one, for every single contaminated food, that were seeded on SDA (Sabouraud Dextrose Agar). The surface, before being sown in Petri dishes of 8.5 cm on diameter, was acidified with sterile solution of tartaric acid 10% up to pH 3.5 according to the manufacturer's instructions. Aliquots of 0.1 ml of each dilution were homogenized in the solidified surface of SDA with Drigalsky handles and then inverted and stored at 28°C until they showed microbial growth, approximately three days. For the identification of these fungi obtained from papaya and hamburger-type bread, it was proceeded with microcultive analysis according Ribeiro & Soares (2002), with modifications proposed by the identification key from Taniwaki & Silva, (2001): Scraps of solidified SDA 4 cm^2 were placed on a glass slide, which is placed on top of a blade holder. The set was placed in an empty petri dish with only a little distilled water at the bottom for imparting moisture to fungus growth. The side walls of the solidified environment were inoculated, with disposable inoculation loop, the colony of isolated fungus, and this was placed a cover slip. The plate was closed and placed at 28°C for 3 days. After the incubation period and fungal growth, the coverslips were removed from the microculture and observed under a microscope. Photographs were taken in several objective and these were compared with various mycology´s catalogs till the visual identification of the species tested.

3.2 Preparation of sugarcane juice

The analyses were developed in the Laboratory of Microbiology, Faculdade de Tecnologia Termomecanica: The samples of sugarcane (*Saccharum* spp) were provided by the Centro Educacional da Fundação Salvador Arena, in São Bernardo do Campo, SP (Brazil). The maturation stage was checked visually, of which were chosen the more mature culms and suitable for extraction. After selection, the culms were treated according recommendations of Oliveira (2012) with adaptations: the odd parts were removed such as soil, leaves and roots and appropriate stalks were cut into cylinders of about 60cm wide, rinsed with running water and brushed manually. After washing, they were cut longitudinally immersed in a 150 ppm chlorine solution for 15 minutes, then rinsed and drained. The broth was obtained by mechanical pressing in common machine previously sanitized. After extraction, the juice was immediately filtered and pasteurized for 25 minutes at 70°C. The inhibition analysis was performed according to Hillen (2012), with adjustments. Different plates with solidified SDA environment were added to 150µL of the diluted sugarcane juice 50, 60, 70, 80, 90 and 100%. The solution was homogenized by the surface of the solidified environment with Drigalsky handles. After homogenization, with the aid of a flamed stick stoppers, it was perforated to three holes in each plate, 5mm on diameter. With disposable inoculation handles, the inside walls of each hole were inoculated with a purview of microorganisms, since each of them received only one isolated microbial genus. Fungi *Aspergillus* sp. and *Fusarium* sp. used were at least 4, and at most, 7 days old, been initially isolated from the hamburger bread and papaya (*Carica papaya*), respectively, and identified by microculture.

For purposes of control, a plate was prepared, inoculated and drilled in the same manner without receiving any addition of broth. Once properly inoculated plates were inverted and stored in an oven at 28°C and observed at 24h and 48h. At these intervals the diameters of the colonies around each well were measured: The measures were taken based upon the larger diameter of each colony formed around each well and then a second and third diametrically opposite to the first measures were also taken. With these measures it was obtained the mean and standard deviation. To verify if there was a significant difference between the measurements, it was used the statistical treatment of Dunnet, test at 5% significance level.

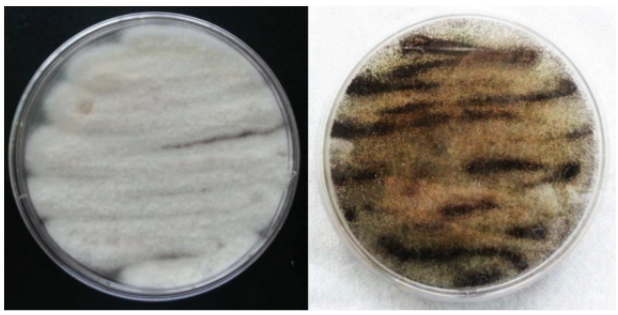
The principle of inhibition analysis consists on checking how much the fungal colony can grow from where it was inoculated to be inhibited by the antimicrobial agent which was disposed on the surface of the plate. The PGI (Percent Growth Inhibition) is, in percentage terms, how much a colony had their growth inhibited compared to control, as shown in equation 1.

$$PGI = \left(\frac{Growth_{control} - Growth_{Sample}}{Growth_{Control}}\right) x100$$

Equation 1 Percent Growth Inhibition Ratio (Source: Hillen et al, 2012).

4. Results and Conclusions

The papaya (*Carica papaya*) and the hamburger bread showed colony formation after 2 weeks of exposure to the environment. The mycelium of fungi is responsible for the characteristic appearance of the colonies and in the case of papaya (*Carica papaya*) is presented as several small diameter cylindrical spots, low and white, with dry appearance and rough texture. On the other hand, the hamburger bread stains display themselves as a cylindrical shape, greenish, soft, dry appearance, which were initially spaced apart but subsequently spread out thru the entire surface of food. The culture used for the growth and isolation of fungi was the PDA acidified with 10% tartaric acid, in order to avoid bacterial contamination. After the serial dilution analysis, it was withdrawn a heave of a single isolated and pure colony to avoid entrainment of more than one species, from this it was done a new SDA plate by smear technique, being this step sufficient to isolate the fungus. The colonies of fungi isolated on plates are shown in Figure 1:



(A) (B)

Fig. 1 Macromorphology of fungal colonies after isolated and purified, respectively for the papaya and hamburger bread. (Source: Authors).

The principle of the microculture is to facilitate fungal growth, such that the fruiting bodies (which have a very characteristic morphology for the most common genera) grow more in a dispersed form on the coverslip, facilitation its view on the microscope [12].

The isolated microorganism of bread had fast-growing colonies by taking the entire surface of the plate. Initially the colonies were white and then with black beads, with velvety appearance. After four days the colonies were black, with its reverse dark-green with clear edges. Observation under a microscope showed branched and septate hyphae with conidiophores unbranched, ending in a dark globular end. The comparison, both of the macromorphology as of the micromorphology, with images and descriptions of textbooks and handouts indicated as a result the gender *Aspergillus* sp., as outlined in Figure 2.

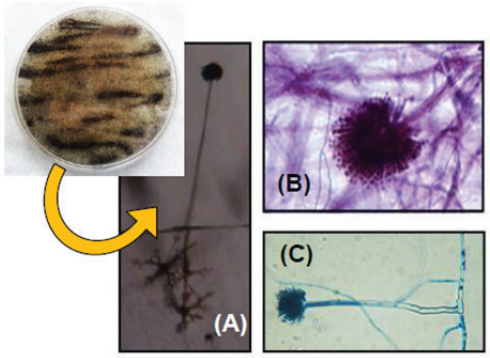


Fig. 2 Comparison between (A) the morphology of Aspergillus sp. found by the authors (400X magnification) with pictures (B) and (C) other authors (Source: (A) of the Authors; (B) Oliveira, 2012; (C) Adapted from Araújo, 2009).

This genus comprises over 100 species, of which the most common examples are *A. niger*, *A. oryzae*, A. *flavus, A. parasiticus, soyae* and *A. terreus*; colonies can be found in yellow, green and black colors on a wide variety of foods. They are mostly saprophytic (they parasitize even dead host vegetables) or opportunistic pathogens capable of growing with low water activity, high temperatures and high concentrations of sugar, dominating warm and/or dry environments. Most species are thermophilic, being more frequent in tropical than in temperate climates, some others have even industrial applications such as production of citric acid, gluconic acid and gallic acid; and enzymes such as lipases, pectinase, invertase, glucoamylase[1,20,24]. The species commonly attack seeds and other grains, such as soybeans, and cereal, peanut butter, cocoa and coffee, during storage, but are also present in the field, where some of them can infect crops and produce aflatoxins, ochratoxin A and Sterigmatocystin, important mycotoxins. Other examples of foods include easily damaged peaches, citrus fruits, figs, edible oils, bacon and corn [19,18].

The second microorganism also showed rapid growth with white-velvety colonies, dry and soft which spread thru the whole plate and its reverse been pink with clear edges. The observation under a microscope showed septate hyphae, a little branched, many macroconidias sickle-shaped, multiseptate and clear. The comparison of both macromorphology as the micromorphology, with images and descriptions of textbooks and handouts indicated as a result the *Fusarium* sp., as shown in Figure 03.

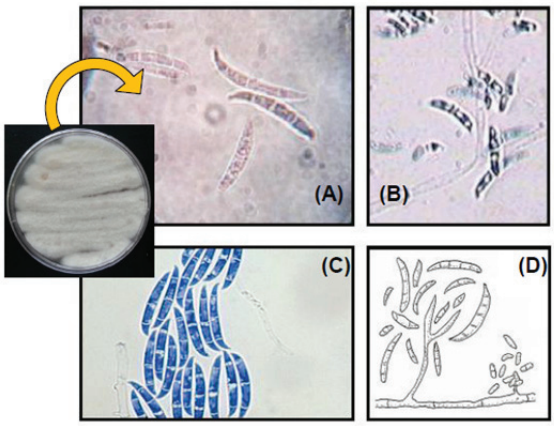


Fig. 3 Comparison between (A) the morphology of *Fusarium* sp. found by the authors (100X magnification) with (B), (C) and (D) found by other authors (Source: (A) of the Authors; (B) Oliveira, 2012; (C) and (D) Adapted from Araújo, 2009).

Fusarium sp. comprises a very common genus of filamentous ascomycete fungus, with species associated with disease in many plants such as citrus, cotton, corn, peanut, barley, wheat and coffee, causing major losses in the agricultural industry. Most species have rapid growth and can be found in soils worldwide. Some species can cause

disease in humans but rarely exhibit toxicity via feeding, although certain species produce mycotoxins such as zearalenone, fumonisins and trichothecenes [1,24,10].

Currently it's very difficult to identify the species of this genus, given the wide genetic variability of this group, among which we can mention, for example, *Fusarium oxysporum; F. solani; F. moniliforme; F. graminearum; F. brasiliensis; F. verticillioides; F. dlaminii, F. culmorum* and *F. decemcellulare*. Usually the colonies have white mycelium of aspect, bright or not, with its reverse color as pink, violet, red, yellow, gray and brown, depending on the species and growing conditions [10,11,24]. Find below the tabulated results of PGI analysis for *Aspergillus* sp. (Table 01) and *Fusarium* sp. (Table 02):

Table 1 Mean and standard deviation of diameters (mm) and PGI of inhibition zones caused by pasteurized Sugarcane broth solutions in different concentrations in relation to *Aspergillus* sp, seen in 24 and 48 hours.

	24h	PGI_{24h}	48h	PGI_{48h}
Control	47 ± 2^a	-	85^a	-
50%	20 ± 2^b	57,4%	16 ± 1^b	81,2%
60%	18 ± 2^b	61,7%	15 ± 2^b	82,4%
70%	17 ± 2^b	63,8%	16 ± 1^b	81,2%
80%	20 ± 4^b	57,4%	$18 \pm 0,6^b$	78,8%
90%	14 ± 1^b	70,2%	14 ± 2^b	83,5%
100%	16 ± 1^b	66,0%	$16 \pm 0,8^b$	81,2%

Values with the same letters in the same column indicate that there is no significant difference between the data compared to the control, 5%. (Source: Authors).

It's observed that within 24 hours there is a considerable inhibition of the growth of *Aspergillus* sp., however there is no statistical difference between the concentrations tested, in other words, the 50% solution is as effective as the pure juice itself.

The PGI_{48h} measures are even greater than 24 h, indicating that this is the optimum time for maximum inhibition, but also there is no significant difference between the concentrations tested.

Table 2 Mean and standard deviation of diameters (mm) and PGI of halos of inhibition caused by pasteurized Sugarcane broth solutions in different concentrations in relation to *Fusarium* sp, seen in 24 and 48 hours.

	24h	PGI_{24h}	48h	PGI_{48h}
Controle	*	-	$28 \pm 0,5^a$	-
50%	*	-	18 ± 2^b	35,74%
60%	*	-	20 ± 2^b	28,53%
70%	*	-	16 ± 2^b	41,14%
80%	*	-	22 ± 2^b	21,92%
90%	*	-	18 ± 3^b	34,53%
100%	*	-	19 ± 2^b	32,73%

* No measurements. - Not applicable. Values with the same letters in the same column indicate that there is no significant difference between the data compared to the control, 5%. (Source: Authors).

In 24 hours the *Fusarium* sp. did not present any growth, precluding its inhibition analysis calculations, however at 48h it was observed an inhibition of its growth, but to a lesser extent compared to the fungus *Aspergillus* sp. On the growth of *Fusarium* sp., there were no statistically significant differences between the concentrations tested, showing that the 50% solution is as effective as 100%.

From the studied theoretical framework and practical experiments made, it can be concluded that in fact the broth of sugarcane, when pasteurized at 70°C for 25 minutes has antimicrobial ability against the fungi *Aspergillus* sp. and *Fusarium* sp., since the lowest PGI *Aspergillus* sp. occurs at concentrations of 50% and 80% at 24h (57.4% inhibition) and higher in 90% concentration in 48h (84% inhibition); for *Fusarium* sp. the PGI was the lowest at 60% broth over 48h (28.53%), and most at 70% broth, also at 48h (41.14%). It should be noticed that the chemical responsible for the inhibition, not presented on its purified form but in the own juice, cannot ensure adequate homogenization, thus modifying the inhibition, carrying more or less antimicrobial between a plate or other, for example. Another factor that interferes with the concentration is the condensed vapor that sometimes falls over the culture and dilutes the substances there present. Additionally, in the integral extract there may be other chemicals, which could interfere directly with results of the analysis.

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