Effect of different sanitizers against *Zygosaccharomyces rouxii*

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Abstract Microbial contamination is a problem in food industry. The effects produced by the metabolic activity of yeasts are diverse. If they are found in high numbers it might affect the organoleptic quality of the product. *Zygosaccharomyces* is the most frequent contaminant genus in sweetened juices that are not properly processed and stored. The aim of this work was to compare the action of five commercial sanitizers against isolates of *Z. rouxii* and to determine appropriate concentrations and time of action for the inactivation. Peracetic acid, monochloramine, iodophor and quaternary ammonium compounds were evaluated on different surfaces (stainless steel, plastic and glass) at room temperature for 30 and 60 s. It was possible to achieve a 99.99 of death Efficiency corresponding to 4 log₁₀ of reduction for a contact time of 60 s for 0.5% (v/v) of peracetic acid, monochloramines and 0.5% (w/v) of iodophors, 1% for compound with a base of 8% (v/v) of benzyl chloride and 7.5% (v/v) of glutaraldehyde and 5% for sodium hypochlorite (55 g/L active compound). Stainless steel was successfully sanitized with all the compounds tested, and then it would be the most appropriate for the food industry because this material is also highly resistant to abrasion, to impact and to different chemical treatments. In view of the great variety of sanitizing products, the selection of the best sanitizer will depend on efficiency, safety and cost.

Keywords *Zygosaccharomyces rouxii* · Fruit juices · Sanitizer · Osmotolerant yeasts

Introduction

Food microbial contamination is a problem in food industry as it gives way to the manifestation of unacceptable foodstuffs for human consumption (Vasconcellos 2004). The use of modern technologies of elaboration using less demanding processing conditions in order to keep flavor, smell and natural color with the purpose of consuming healthier products favors yeast growth in concentrated fruit juices and fruit salads (Shearer et al. 2002; Stratford 2006). The effects produced by the metabolic activity of yeast are varied. If they are found in high numbers it would affect the organoleptic quality of the product due to the production of “off-odor” and discoloration (Renard et al. 2008). The visible signs of alteration are the excess of gas production as result of sugar fermentation, causing the deformation and even the explosion of containers, cans and glass bottles (Barnett et al. 2000; Tournas et al. 2006). *Zygosaccharomyces* is the most frequent contaminant genus in sweetened juices that are not properly processed and stored (Martorell et al. 2007). In order to prevent this contamination, preservatives like sorbic acid or its salts (sorbates) are added.

Nonetheless, the yeasts of the *Zygosaccharomyces* genus can easily grow in high concentrations of sugars and, consequently, they can grow and contaminate juices. A number of volatile derivatives of sorbic acid has been described, and some of them even smell like petroleum, hydrocarbons or plastic paints; the metabolite responsible for such odor has been identified as 1,3 pentadien (Casas et al. 2004).

Zygosaccharomyces rouxii is capable of growing in a wide range of pH values and low aqueous activity particularly caused by a high sugar concentration. Its isolation has been reported from sugar cane and concentrated fruit juice, ginger, sugared strawberries, marmalades, syrups, wine must and honey (Arias et al. 2002; Stratford 2006; Smits and Brul 2005; Tofalo et al. 2009). The high tolerance of *Z. rouxii* to osmotic stress is well known, being able to grow in 15% (w/v) sodium

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chloride or 70% (w/v) sucrose culture medium. The tolerance mechanism is associated to the production of polyols that balance the internal osmotic tolerance. Their presence in juices is a risk for the product stability (Sancho et al. 2002). In order to eliminate them from working surfaces and food processing equipments, cleaning is done using sanitizing solutions in different conditions and contact times since equipments used in the processing are a source of contamination (Rossoni and Gaylard 2000; Taormina and Beuchat 2002; Wirtanen and Salo 2003; Gougouli and Koutsoumanis 2010). Sanitizing solutions (sodium hypochlorite, peracetic acid, iodophors, quaternary ammonium compounds, etc), are used on working surfaces and equipments in food processing plants and/or commercial premises to diminish or inhibit the growth of microorganisms such as bacteria, fungi or viruses (Davidson and Harrison 2002). These products must be applied in adequate concentrations since low doses do not inactivate microorganisms and high ones can cause toxicity in people and negative effects where they are applied (Briñez et al. 2006).

Sodium hypochlorite is considered the most appropriate chlorate derivate for treating most of the surfaces where food is in contact with thanks to its rate of action. Nevertheless, it should always be kept in mind its corrosive capacity, low stability, strong smell and impairment of undesirable flavors to food. Sodium hypochlorite is easy to use but it is toxic as it decomposes in products that could have carcinogenic effects, like trihalomethanes (Block 2001). It is known to be very active in killing most bacteria, fungi, and viruses and it is also known as a strong oxidizing agent (Peng et al. 2002). Sodium hypochlorite is the best example of chlorine compounds used as disinfectant and its germicidal effect is based in the penetration in cells and the oxidation of their enzymes (Lomander et al. 2004). Tough iodophors have many advantages at low temperatures because of their wide action spectrum (bacteria, fungi and viruses), they also present the inconvenient of their relative sensitivity to organic matter, instability linked to temperature as well as the staining they produce on plastics. Iodophors are used to sanitize surfaces and equipment (Russell et al. 2004). The use of monochloramines instead of chlorine diminishes the formation of trihalomethanes; are effective with viruses and fungi as well as for inactivation of the biofilm given they capacity to penetrate it. As monochloramines do not react with organic compounds, it is possible to diminish the chlorinated odor compounds may boost. They are more stable than chlorine solutions, easier to prepare and without ocular or skin risks. Monochloramines have neither corrosive effects nor attack metals, woods, plastics or rubber, that is, they do not present any limitation regarding the nature of the materials to disinfect and it is not necessary to rinse or dry the treated elements (OMS 2003). Peracetic acid is an oxidant compound highly effective over a wide selection of microorganisms. It is also active in the presence of hard waters and an eventual content of organic substance. Peracetic acid has the advantage that is not corrosive

on stainless steel, aluminum and/or plastic material (Alvaro et al. 2009; Kyanko et al. 2010). Quaternary ammonium compounds (QAC) are actively effective to eliminate bacteria, fungi and virus. QAC's activity is developed both in acid and alkaline means. Their properties are practical for cleaning in place (CIP), in equipments, surfaces and piping disinfection. They are both odorless and colorless as well as they present low health risk. The principal matter of importance in these compounds is that their great residual effect lingers well in surfaces as they are not volatiles; QAC neither evaporate nor affect the environment because they are easily biodegradable (Russell et al. 2004). Glutaraldehyde is the most used antimicrobial agent to disinfect plastic material given its effect on bacteria and their spores, fungi and viruses. It is not corrosive, easy to rinse and with a significant low-cost. In view of the great variety of sanitizing products, the selection of the best one will depend on efficiency, safety and cost as well as the corrosive effect over treated surfaces (Wirtanen and Salo 2003).

The objective of this work was to compare the action of five commercial sanitizers against *Z. rouxii* and to determine suitable concentrations and action time for their elimination.

Material and methods

Isolation and identification of yeasts altering of fruit juices

Fifty affected plastic containers of concentrated alcohol free fruit juices (30 of grapefruit juice and 20 of lemon juice) were sampled in 10 stores of Santa Fe, Argentina. Isolations were done by diluting samples in Malt Extract Agar (MEA) medium with chloramphenicol (100 mg/L) to inhibit bacterial development (Beuchat and Cousin 2002). Petri plates were incubated during 5 days at 28°C. Cultures were purified according to Pitt and Hocking (Pitt and Hocking 2009). Isolated yeast colonies were spread on individual Petri plates with MEA and incubated as it was described previously. This procedure was repeated until pure cultures were obtained. Isolates were identified according to Pitt and Hocking (2009). Culture media used for observing the macroscopic characteristics and identification were: Czapek Agar (ACz); MEA, Malt Acetic Agar (MAA); Malt Yeast 50% Glucose Agar (MY50G); and Malt Extract Yeast 10% Salt (sodium chloride) 12% Glucose Agar (MY10-12), cultivating them during 5 days at 28°C; MEA growths were also studied cultivating them for 5 days at 37°C. Obtained strains were then preserved in MEA culture at 4 °C until needed.

Antimicrobial activity

To proceed with the sanitizing treatment, yeast must be at the beginning of the stationary phase as it is here that cell wall is thickened offering resistance to sanitizers (Russell et al. 2004).

With the objective of determining this phase, a growth curve in MEA culture was done. Cell counts were done every 2 h for the first 24 h of culture and then after 48 and 72 h. Plates were incubated during 3 days at 28°C. The experiences were done in triplicate and results correspond to the average of the values obtained. Five isolates, from different samples, were taken at random and cultures were prepared in MEA, incubating them until the beginning of the stationary phase, at 28°C. Two mL of each were pooled. The final concentration was adjusted to 3×10^6 cfu/mL (AOAC 1984). To compare the capacity of the 5 sanitizers, a suspension test was performed (Hollis 1991; PREN 1275. (1994). A 0.1 mL volume of the mixture of yeast isolates were put in contact with 9.9 mL of the sanitizing agent during 30 or 60 s at room temperature. Commercial sodium hypochlorite (55 g/L active compound) at 0.1%, 0.25%, 0.5%, 1%, 2%, 2.5%, 5% and 10%; commercial peracetic acid solution 15% (v/v) of active compound of a balanced mixture of acid peracetic, hydrogen peroxide, acetic acid and water; 0.1%, 0.25%, 0.5% and 1%; monochloramine 17% (v/v) of active compound at 0.1%, 0.25%, 0.5% and 1%; commercial iodophors 2% (w/v) of active compound in 70% ethanol at 0.1%, 0.25%, 0.5% and 1%; compound with a base of 8% (v/v) of benzyl chloride and 7.5% (v/v) of glutaraldehyde (QAC) at 0.1%, 0.25%, 0.5%, 1% and 2%. The effectiveness was determined by the yeast growth capacity on MEA (5 days, 28°C). Each experience was done in triplicate and results correspond to the average values obtained. The results were expressed by death Efficiency (E%) (Bessems 1998); Logarithmic reductions of 1, 2, 3 and 4 Log_{10} are equivalent to the percentages of E% of 90.0; 99.0; 99.90; 99.99% respectively. To consider disinfection as safe to eliminate fungi in food industry, the logarithmic reduction must be at least 4 Log_{10} (99.99% death Efficiency) (Sappers 2001).

Effects of sanitizers on different surfaces

Peracetic acid, monochloramine, iodophor and QAC were evaluated on different surfaces like stainless steel, plastic (hard PVC) and glass, previously sanitized woods with ethanol at 70% (v/v) and dried in a sterilized flow of air during 30 min. To that effect, 0.1 mL of the isolated yeast mixture (10^6 cells) was spread over a $10 \times 10 \text{ cm}^2$ surface of the different materials. It was allowed to dry for 1 h under a sterile flow of air and afterwards the sample was sprayed with the sanitizers under study at a concentration and time selected in the previous essays. Cells were recuperated by swabbing the surfaces with a 3M Quick Swab® (Center Building, USA). Effectiveness of the sanitizing agent was determined as explained before. Samples were processed in triplicates and the results correspond to the average of the values obtained. The results were expressed according to E% defined as: $E\% = [(C_0 - C_f)/C_0] \times 100$, where: C_0 : is the initial concentration of cells and C_f : is the

final concentration of cells after the treatment with the sanitizing.

Results and discussion

Isolation and identification of altering yeasts of fruit juices

All isolates obtained showed the following characteristics: colonies on MEA at 5 days were 2–3 mm diameter, white, circular margins. Cells on MEA were subspheroidal to ellipsoidal, mostly $5\text{--}8 \times 4\text{--}6 \text{ }\mu\text{m}$. Growth on ACz, on MEA at 37°C and no growth on MAA not was observed. Growing in culture medium containing 50% glucose (MY50G) as well as in 10% sodium chloride and 12% glucose (MY10-12) at 28°C was positive, with macroscopic colonies of diameters of 1–2 mm. Isolates were identified according to Pitt and Hocking (2009) as *Z. rouxii*.

Z. rouxii was found as the only contaminant species in all the containers of altered drinks based on concentrated fruit juices. It is a xerophilic organism, able to grow in an aqueous activity below 0.62 in fructose solutions and below 0.65 in sucrose/glycerol. *Z. rouxii* can grow in concentrated orange, apple and grapefruit juices as well as in wine must, balsamic vinegar and honey (Martorell et al. 2007). The isolate was preserved in the Microbiology Laboratory at the Faculty of Chemical Engineering, (U.N.L.), identified as LMFIQ 438.

Growth at 37°C is a typical characteristic that some yeast present in restricted habitats, like certain species of *Candida*, *Kluyveromyces*, *Rhodotorula*, *Debaryomyces* or *Saccharomyces*. *Z. rouxii* does not grow in MEA at 37°C, in ACz or MAA at 28°C. It presents irregular ascus formed by two cells conjugation where 1 to 4 ascospores with soft or finely rough inner walls (Pitt and Hocking 2009).

Antimicrobial activity against *Z. rouxii*

The growth curve showed that early stationary phase was reached between 28 h and 30 h of incubation. The action of peracetic acid and sodium hypochlorite treatments against bacteria, bacteriophages and biofilms has already been studied, yet there are few studies on the action of sanitizers against yeasts (Stopforth et al. 2002; Bore and Langsrud 2005). It was possible to observe a 99.99 of E% corresponding to 4 log_{10} for a contact time of 60 s for 0.5% of peracetic acid, iodophors and monochloramines, 1% of QAC and 5% of sodium hypochlorite (Table 1).

Buschini et al. (2004) determined that sodium hypochlorite and peracetic acid present a genotoxic aspect, with similar effectiveness on *S. cerevisiae*. However, our studies show different results for these compounds, being generally observed higher resistances of *Z. rouxii* to sodium hypochlorite

Table 1 Death efficiency (E %) of five sanitizing agents facing a *Z. rouxii*

Death efficiency (E%)						
Concentration (%)	Time (S)	Peracetic acid	Sodium hypochlorite	QAC	Monochloramines	Iodophors
0.1	30	99.91	99.82	99.90	99.77	99.54
	60	99.93	99.85	99.93	99.96	99.60
0.25	30	99.94	99.84	99.92	99.97	99.69
	60	99.96	99.88	99.94	99.98	99.97
0.5	30	99.98	99.86	99.95	99.98	99.98
	60	99.99	99.89	99.97	99.99	99.99
1	30	99.99	99.88	99.98	99.99	99.99
	60	99.99	99.90	99.99	99.99	99.99
2	30	ND	99.91	99.99	ND	ND
	60	ND	99.93	99.99	ND	ND
2.5	30	ND	99.94	ND	ND	ND
	60	ND	99.95	ND	ND	ND
5	30	ND	99.98	ND	ND	ND
	60	ND	99.99	ND	ND	ND
10	30	ND	99.99	ND	ND	ND
	60	ND	99.99	ND	ND	ND

ND: Not Done

S: Seconds

since concentrations of 5% for 60 s were required to achieve its inactivation, whereas a concentration of 0.5% peracetic acid for the same period of time was sufficient to achieve the same E %. Peracetic acid is less toxic to the environment since it decomposes in acetic acid, hydrogen peroxide, oxygen and water which are not generating toxic mutagenic byproducts (Monarca et al. 2002). This compound has been widely used as a sanitizing agent in different food and beverages industries (Kitis 2004).

Kyanko et al. (2010) studied the action of peracetic acid against *Alternaria alternata*, *Fusarium graminearum*, *Aspergillus ochraceus*, *Aspergillus niger*, *Aspergillus flavus*, *Penicillium roqueforti* and *Penicillium expansum* and determined that a concentration of 0.3% for 30 min was required to inactivate them, while in our case *Z. rouxii* was more sensitive since 60 s at a concentration of 0.5% was enough to achieve an adequate level of inactivation.

There are no reports of the use of iodophors, QAC and monochloramine against yeast. In our experience, a

concentration of 1% for 60 s of these sanitizers was enough for yeast inactivation.

Sanitization of different surfaces

The ability of yeasts off sticking to surfaces such as stainless steel, glass or plastic and form a biofilm, is a problem in the food industry when sanitizing procedures are not sufficient (Moore and Griffith 2002). It can be observed that on the glass surface the E% of 99.99 was obtained with 5% sodium hypochlorite and 0.5% of peracetic acid. On the plastic surface the maximum death Efficiency (e.g., E% 99.99) was obtained with 5% of peracetic acid. Stainless steel was successfully sanitized with all the compounds tested. The wood surface, on the contrary, a 99.99 of E% was not obtained with any of the sanitizing solutions tested. This situation could be because of the porosity that these surfaces present as then it is more difficult for the solution to penetrate it (Table 2).

Table 2 Death efficiency (E %) of sanitizing solutions with different surfaces

Death Efficiency (E%)					
Materials	Monochloramines 0.5%	Peracetic acid 0.5%	Sodium hypochlorite 5%	QAC 1%	Iodophors 0.5%
Wood	99.65	99.85	99.98	99.85	99.94
Glass	99.97	99.99	99.99	99.97	99.98
Plastic (PVC)	99.98	99.99	99.98	99.98	99.98
Stainless Steel	99.99	99.99	99.99	99.99	99.99

Rossoni and Gaylard (2000) compared the action of peracetic acid and sodium hypochlorite with a contact time of 10 min on bacteria adhered to surfaces of stainless steel, and found that sodium hypochlorite was more effective than peracetic acid. In our research, this last one was the most effective sanitizer against this yeast since the required Efficiency (99.99%) was achieved at a lower concentration. Material characteristics and physicochemical properties, hydrophobicity, charge, and also the existence of ridges, bumps, cracks, holes and other irregularities, could influence adhesion. Maukonen et al. (2003) reported that, in general, smooth surfaces are better sanitized than cracked and rough ones. Also, stainless steel surfaces would be more appropriate for the food industry because this material is highly resistant to abrasion, impact and different chemical treatments. Our results are consistent with those previous ones since Efficiency of 99.99% with all tested sanitizers was achieved for this type of surface. Basílico et al. (2004) determined that fungi can colonize and penetrate inside wooden surfaces used in the food industry making it difficult to sanitize with superficial treatments. Our results are consistent with these because the wooden surface was not sanitized effectively by any of the sanitizers tested. This is an area with abundant capillary spaces and poses considerable technical problems when sanitizing. Because of their stability, stainless steel occupies a special place in many fields of food industry, for its advantages this material would be recommended in the first place for surfaces in contact with food, followed if possible by glass (E% of 99.99 for sodium hypochlorite and peracetic acid) since there is a tendency to eradicate its use from the food processing industries because of the physical risks that it displays, thirdly plastic surfaces (hard PVC) (E% of 99.99 for peracetic acid) since these materials play an important role in food industry, for example in cheese molds.

Conclusion

Sanitary treatment is one of the most important components of the control system HACCP. Cleaning and sanitization as a part of everyday operation practice are essential for high hygienic level in food production within the framework of legislation requirements. The count of microorganisms on surfaces in the processing plant varies throughout the day and depends on surface and the moment of the processing stage. Peracetic acid would be effective to avoid *Z. rouxii* contamination and was preferred over the others due to its higher safety. Stainless steel was successfully sanitized with all the compounds tested, and it would be more appropriate for the food industry for its properties. In view of the great variety of sanitizing products, in the beverage processing industry, the selection of the best

sanitizer will depend not only on efficiency but also on safety and cost as well as the corrosive effect over treated surfaces.

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