Inactivation of E. coli and B. subtilis spores in ozonized cassava starch
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Abstract
In the present study, the efficacy of ozone inactivation of B. subtilis spores and E. coli in cassava starch was evaluated. Cassava starch with 18 and 30% moisture content was processed with ozone at concentrations of 40-118 ppm and exposure times of 15-120 minutes. The processing at 113 ppm/120 minutes (maximum exposure level to ozone evaluated) at 18% of moisture content did not cause significant reduction of B. subtilis spores and caused the reduction of only 2 decimal of E. coli. On the other hand, when the ozonation process was carried out for 120 minutes at 30% of moisture content, 3.6 decimal reduction of B. subtilis was achieved at 40 ppm of ozone and total B. subtilis load reduction (>5 log cycles) was observed at 118 ppm of ozone. Similarly, total E. coli load reduction (>7 log cycles) was achieved at 40 ppm of ozone exposure for 60 minutes. Therefore, the results indicate that the ozone efficacy against microorganisms in cassava starch was mainly dependent on the sample moisture content and to ozone concentration and exposure time. Moreover, it was observed that ozone is a promising technology to reduce microbial counts in dried food.

Keywords: ozone; food processing; non-thermal technologies.

1 Introduction
Today’s consumers have demanded processed products very similar to fresh food (TRIBST; SANT’ANA; DE MASSAGUER, 2009). For such reason, the development of non-thermal methods for food processing has been extensively studied – e.g. high-hydrostatic pressure, irradiation, UV radiation, pulsed electric field, pulsed light, ultrasound, and high-pressure homogenization, and ozone (DIELS; WUYTACK; MICHIELS, 2003; TRIBST; SANT’ANA; DE MASSAGUER, 2009; KLOCKOW; KEENER, 2009).

It is expected that products processed by such technologies maintain the nutrients and sensory characteristics better than thermal process. Among these technologies, ozone stands out for its antimicrobial properties and for being totally degradable in oxygen with no waste/ toxic products (TIWARI et al., 2008, 2010); thus it is generally recognized as safe (GRAS) for food applications (GUZEL-SEYDIM; GREENE; SEYDIM, 2004; D’HILLON et al., 2009; TIWARI et al., 2010). An additional advantage is the lower cost of ozone equipment (GUZEL-SEYDIM; GREENE; SEYDIM, 2004).

The ozone is a high oxidative compound with a broad antimicrobial spectrum; it is able to inactivate bacterial vegetative cells and spores, yeast, molds, and viruses and to kill insects in stored grains and degrade mycotoxins (TIWARI et al., 2010). Ozone inactivates microorganisms by the progressive oxidation of vital cellular components. The microbial surface is the primary target of ozonation with the oxidation of the polyunsaturated fatty acids and consequent loss of selective permeability and cell disruption. Additionally, ozone causes the oxidation of sulfhydryl groups and amino acids of enzymes, peptides, and proteins including nucleic acids and vital enzymes (KHADRE; YOUSEF; KIM, 2001; GUZEL-SEYDIM; GREENE; SEYDIM, 2004). In general, Gram negative bacteria are more sensitive to ozone treatment than Gram positive ones (KHADRE; YOUSEF; KIM, 2001). However, several other factors affect the ozone efficacy including the strain of the microorganism, age of the culture, density of the treated population, and presence of ozone-demanding medium components and method of applying ozone (KHADRE; YOUSEF; KIM, 2001).

In spores, ozone degrades the coat layer components thus exposing the cortex and the core to oxidation by ozone (KHADRE; YOUSEF; KIM, 2001). The molecular ozone and the reactive by-products of ozone decomposition (free radicals: OH, O2 , and HO2) have antimicrobial activity. Due to its high efficacy, low concentration and short contact time are normally enough to achieve the desired microbial inactivation.

Ozone has been previously used in food industry to inactivate microorganisms in packages and stainless steel surfaces (KHADRE; YOUSEF, 2001), for microbial decontamination of poultry carcasses, on chilled water treatment and recycling (GUZEL-SEYDIM; GREENE; SEYDIM, 2004; KHADRE; YOUSEF, 2001), for fish shelf-life extension (PASTORIZA et al., 2008), for fruit juice processing (TIWARI et al., 2009a, b), for sanitation of fruit and vegetables (ACHEN; YOUSEF, 2001; BIALKA; DEMIRCI, 2007; NAJAFI; KHODAPARAST, 2009; KIM; YOUSEF; CHISM, 1999; KOSEKI; ISOBE, 2006), and...
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for decontamination of dry products as seeds, grains, and pepper (TIWARI et al., 2010; AKBAS; OZDEMIR, 2006; EMER; AKBAS; OZDEMIR, 2008; IBANOGLU, 2001, 2002; KETTERINGHAM et al., 2006; ZHAO; CRANSTON, 1995).

In addition to ozone antimicrobial potential, this gas can also be used to change physicochemical characteristics of corn, sago, and tapioca starch (CHAN; BHAT; KARIM, 2009) and wheat flour (LI et al., 2012) by oxidation improving their functional performance and presenting better results than those obtained by the traditional oxidation process (using chlorine).

Cassava starch is an important raw material in the food industry due its absence of "cereal flavor", typically observed in corn starch, its viscoamylographic standard during cooking (DEMIATE et al., 2005), its pasta low temperature, and its syneresis low susceptibility (SILVA et al., 2003).

Considering that cassava starch is extracted from plant root, it is potentially contaminated with many microorganisms, mainly bacterial spores. Therefore, some processes are required to guarantee the microbial stability of cassava starch before its use as raw material in the food industry. Due to low temperature gelation, the thermal process application for microbial inactivation of cassava starch is not feasible (CEREDA; EMER; AKBAS; OZDEMIR, 2008; IBANOGLU, 2001, 2002, 2004) and ozonized air (Model UV-100, Eco Sensors, Inc., Santa Clara, USA), Ozonized air was injected in a stainless steel powder mixer of 25 L through two air inlet devices located in the mixer lid. The spinning blades promoted an efficient contact between starch and ozonized air.

2.4 Ozone treatment of cassava starch inoculated with E. coli

1 kg of cassava starch (initial moisture of 11%) was inoculated with 100 mL of E. coli suspension reaching a final count of 10^6-10^7 CFU·g^{-1} and moisture of 18% (maximum limit of starch moisture allowed by the Brazilian law). For assays using cassava starch moisture at 30% (moisture of starch immediately after cassava centrifugation carried out in the starch industry just before the drying processing), the samples were previously humidified with sterile water, followed by inoculation with 100 mL of E. coli suspension. After inoculation, cassava starch rested for one hour before ozonation to enable the adherence of microorganism to starch.

The starch of 18% moisture content was processed at ozone concentration of 40 and 113 mg·L^{-1} for 30, 60, 90, and 120 minutes. The starch of 30% moisture content was processed at ozone concentration of 40 mg·L^{-1} for 30, 60, 90, and 120 minutes or at ozone concentration of 113 mg·L^{-1} for 15, 30, 45, and 60 minutes. The 113 and 118 mg·L^{-1} were the higher ozone levels reached in the equipment for the samples at 18 and 30% of moisture. This small variation in the maximum ozone concentration can be attributed to few oscillations in the oxygen concentration and ozone generated in the equipment.

Survivor counts of E. coli were performed on nutrient agar just after the ozonation process. The control samples were obtained by exposing previously inoculated cassava starch to air (without ozone) under the same conditions used for the ozonized samples. Initial count was determined by starch plating immediately after inoculation. The samples were incubated at
37 °C for 24 h. The Number of Decimal Reductions (NDR) of each process was determined according to Equation 1.

\[
\text{NDR} = \log(\text{initial count}) - \log(\text{survivor’s count})
\]  

(1)

### 2.5 Ozone treatment of cassava starch inoculated with B. subtilis

1 kg of cassava starch was inoculated with B. subtilis spores suspension at the same conditions described for E. coli. B. subtilis final count was \(10^7\) CFU·g\(^{-1}\), and cassava starch moisture was 18 and 30%. The inoculated sample was allowed to rest for 1 hour for the adherence of microorganism to starch, and the starch of 18% moisture content was then ozonized at ozone concentration of 118 mg·L\(^{-1}\) for 30, 60, 90, and 120 minutes, and starch of 30% moisture content was processed at 40 and 118 mg·L\(^{-1}\) for 30, 60, 90, and 90 minutes.

The B. subtilis enumeration was carried out after a heat shock (70 °C/20 minutes) for spore activation. The samples were plated on nutrient agar and incubated at 30 °C for 48 hours. The control samples were obtained by exposing previously inoculated cassava starch to air (without ozone) under the same conditions used for the ozonized samples. Initial count was determined by starch plating immediately after inoculation. The Number of Decimal Reductions (NDR) of each process was determined by Equation 1.

### 2.6 Statistical analysis

The analysis of variance (ANOVA) was performed to compare the effects of the different treatments, and the Tukey test was used to determine their differences at a 5% confidence level. Statistical analyses were carried out using STATISTICA 5.0 software (StatSoft, Inc., Tulsa, Okla., U.S.A). All of the tested conditions were evaluated in triplicate. The results were presented as mean ± standard deviation.

### 3 Results and discussion

#### 3.1 E. coli inactivation

The E. coli inactivation on ozonized 18% moisture cassava starch is shown in Figure 1. Statistical analysis indicated that 30, 60, and 90 minutes of contact time with 40 mg·L\(^{-1}\) ozone caused about one decimal reduction, with no differences between them (p > 0.05). On the other hand, 120 minutes of contact at this same concentration caused about 2 decimal reduction, and it was different from other conditions evaluated (p < 0.05).

The results of cassava starch ozonized at the concentration of 113 mg·L\(^{-1}\) showed 1-2 decimal reduction, with no significant differences between different ozone contact times. Considering the control sample results (<0.5 NDR), it was observed that ozone was able to reduce E. coli counts in 18% moisture cassava starch, but these reductions (<2 decimal reductions) were not enough to guarantee the cassava starch safety when used as food raw material. Moreover, no significant differences were observed between E. coli inactivation in the samples ozonized at 40 and 113 mg·L\(^{-1}\), which probably indicates ozone saturation at concentration of 40 mg·L\(^{-1}\).

The effect of starch humidification on the ozone efficacy was evaluated through ozonation process carried out on previously humidified starch at 30% moisture (moisture of the starch after centrifugation). The E. coli inactivation at this condition is shown in Table 1.

The control treatment (carried out with no ozone injection) showed 1.3-1.6 decimal reductions, indicating that at 30% moisture, the microorganism was more sensitive in non-ideal growth media or its recovery after the process was reduced. Comparing the results of control and ozonized samples, on the other hand, it was observed that ozonization was able to inactivate the microorganism.

The results showed that increases in ozone concentration and exposure time improve E. coli inactivation during the process. Processes applying ozone concentration at 40 mg·L\(^{-1}\) for 60 minutes or 118 mg·L\(^{-1}\) for 30 minutes are enough to guarantee an adequate level of E. coli inactivation assuring the safety of foods with this starch as raw material.

E. coli inactivation by ozone has been previously studied by several authors in different products including water, fruits, vegetables, meat, and dry products (MUTHUKUMARAPPAN; HALAWEISH; NAIDU, 2000; ZHAO; CRANSTON, 1995; KHADRE; YOUSEF; KIM, 2001; AKBAS; OZDEMIR, 2006, 2008b; EMER; AKBAS; OZDEMIR, 2008). These studies were carried out using different equipment and strains of E. coli, but, in general, it was observed that higher inactivation occurred in water or products with high moisture (MUTHUKUMARAPPAN; HALAWEISH; NAIDU, 2000). The use of 0.35 ppm of ozone resulted in 5 decimal reductions of E. coli in water, and the use of 18 ppm for 50 minutes resulted in total load inactivation of E. coli O157:H7

![Figure 1. Inactivation of E. coli in ozonized 18% moisture cassava starch. Vertical bars represent the standard deviation of each value.](image)

### Table 1. Inactivation of E. coli in ozonized 30% moisture cassava starch.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>NRD at different ozone concentration</th>
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<tr>
<td></td>
<td>0 mg·L(^{-1})</td>
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<tr>
<td>15</td>
<td>-</td>
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<tr>
<td>30</td>
<td>1.26</td>
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<tr>
<td>45</td>
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<tr>
<td>60</td>
<td>1.45</td>
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<td>90</td>
<td>1.51</td>
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<tr>
<td>120</td>
<td>1.64</td>
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*Results of control sample. These values are the NDR average of two processes.*
in several foods (MUTHUKUMARAPPAN; HALAWEISH; NAIDU, 2000). The concentration of 1.0 ppm of ozone applied for 360 minutes resulted in 3.5 decimal reduction in dried figs (AKBAS; OZDEMIR, 2008b). For pistachio (AKBAS; OZDEMIR, 2006), red pepper (AKBAS; OZDEMIR, 2008b), and black pepper (EMER; AKBAS; OZDEMIR, 2008), similar inactivation level (about 2 decimal reductions) was obtained after ozonation for 120–360 minutes.

Therefore, the presence of water in foods is very important to reach high microbial inactivation level by ozone, as observed in the present study. Similarly, previous results showed that microbial inactivation by ozone on black pepper was improved at high moisture content (ZHAO; CRANSTON, 1995). It is known that ozone is a powerful oxidative compound that reacts with microorganisms and organic materials (KHADRE; YOUSEF; KIM, 2001; MUTHUKUMARAPPAN; HALAWEISH; NAIDU, 2000). Thus, the increase in the organic material content may have affected the ozone efficacy.

Additionally, the ozone gas is rapidly decomposed in oxygen (MUTHUKUMARAPPAN; HALAWEISH; NAIDU, 2000). It is possible that ozone partial dissolution in the food free water results in the increase in the antimicrobial half-life, which can become more effective. Another possible effect is that moisture can improve the gas–product contact area or promote water free-radical formation that was effective for microbial inactivation in addition to the ozone effects.

Comparing the results obtained for E. coli inactivation in the cassava starch and other dry products (AKBAS; OZDEMIR, 2006, 2008b; EMER; AKBAS; OZDEMIR, 2008), it was observed that, for the other products, lower ozone concentration of 0.5–1 mg L\(^{-1}\) was required to reach the same level of inactivation (i.e., 40 times smaller than that required for cassava starch). Additionally, to the differences in the moisture content and water activity of these foods and cassava starch, the differences between the results obtained can be explained by different ozonized air flow and the amount of product ozonized, which interfere in the ozone available amount and also on ozone mass/product mass ratio. Moreover, the E. coli adherence in cassava starch for one hour before ozonation and the powder small dimension with rough surface can also affect ozone activity.

### 3.2 B. subtilis inactivation

Considering the results obtained for E. coli inactivation in ozonized 18% moisture cassava starch and the expected higher ozone resistance of B. subtilis, its inactivation in 18% moisture cassava starch was evaluated only at the ozone concentration of 118 mg L\(^{-1}\) (which was the maximum ozone concentration reached in the ozonation equipment). No B. subtilis spore reduction was observed in cassava starch of 18% moisture content, even after ozonation at 118 mg L\(^{-1}\) concentration for 120 minutes (data not shown). This indicates that, at this cassava starch moisture, ozone was not effective for B. subtilis inactivation.

Comparing the results obtained for B. subtilis and E. coli inactivation (113 mg L\(^{-1}\) ozone concentration), it was confirmed that Bacillus spores are more resistant to ozone than E. coli, as previously described by Akbas and Ozdemir (2008a, b) and Muthukumarappan, Halaweish and Naidu (2000). This can be explained by the high resistance of the spore coat and outer membrane to the oxidative mechanisms of the ozone (AKBAS; OZDEMIR, 2008a, b; MUTHUKUMARAPPAN; HALAWEISH; NAIDU, 2000), compared with oxidation of vegetative cell envelope and membrane.

The effect of 30% moisture starch on the ozone efficacy against B. subtilis was also evaluated, and the results are shown in Figure 2. The results showed that the control processes caused no significant reduction on B. subtilis spores. On the other hand, ozone promoted B. subtilis spores inactivation at the concentration of 40 and 118 mg L\(^{-1}\), and it was higher at higher ozone concentration (p < 0.05). In addition, increased contact time up to 60 and 120 minutes caused a significant increase in spore inactivation at ozone concentration of 40 and 118 mg L\(^{-1}\), respectively. Again, with the increase in the starch moisture, a significant increase in the microbial inactivation was observed.

Data on B. subtilis spores in water also demonstrated higher level of spore inactivation, with 3 log reductions after processing with 0.7 ppm of ozone, indicating that the water content is important for spore inactivation.

It is interesting to highlight that by increasing ozone concentration by three times (40 mg L\(^{-1}\) to 118 mg L\(^{-1}\)) it was able to reduce 50% of process time aiming to achieve the same level of B. subtilis spores inactivation, which can be a possible solution when low time processing is required.

Previous data on ozone treatment of wheat and wheat flour showed low level of inactivation of total aerobic microorganism and yeast and moulds (<1 decimal reduction) using ozonated water at concentrations up to 16.5 ppm (IBANOGLU, 2001, 2002; LI et al., 2012). These results corroborate the findings that food with low water content possible slows ozone reaction speed and therefore reduces the disinfection ability of the ozone gas.

### 4 Conclusion

The ozonation process can be effective for E. coli and B. subtilis inactivation in cassava starch, and it is highly dependent on starch moisture. At low starch moisture (18%), the process was not enough to guarantee microbial inactivation; however, at 30% moisture starch, it was possible to reach >5 decimal reductions, ensuring safe products. E. coli were less resistant to ozone treatment, and it was inactivated after 30 minutes treatment with 118 ppm of ozone. On the other hand, 90 minutes are required to reach an appropriated inactivation of B. subtilis. Therefore, the application of ozone at these conditions makes cassava starch a safe product to be used as a raw material in the food industry.
Acknowledgments

The authors would like to thank the São Paulo Research Foundation (FAPESP) for financial support to the project #2008/10942-5, the Brazilian National Research Council (CNPq) for the E.O.C Amorim fellowship, and the Corn Products Brasil for the cassava starch donation.

References


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