THE INHIBITORY EFFECT OF CHITOSAN ON NATURAL MICROFLORA AND INOCULATED CHALLENGE SPOILAGE YEASTS IN LABORATORY OZONATED TROPICAL FRUIT JUICES

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Abstract: The major problem of fresh tropical juices is their limited shelf life. Food grade chitosan was investigated for its ability to inactivate both inherent microflora and challenge spoilage yeasts in three tropical fruit juices from orange, pineapple and water melon. Juices were prepared using Binatone juice extractor fitted with a special filter. The natural flora were isolated and enumerated following standard microbiological techniques. The juice was ozonated using an ozone gas generator with a concentration of 3.33 mg/min. Chitosan concentrations of 1000, 1500 and 2000 µg/ml were biocidal on bacteria with number reduced by > 0.5-1.27 $\log_{10}$CFU/ml immediately after addition to orange, pineapple, watermelon, and mixed fruits juices. At a concentration of 2000 µg/ml, non-detectable levels of *Saccharomyces cerevisiae*, *Candida pulcherrima* and *Pichia fermentans* were obtained in all juice samples stored at 28±2^{0}C showing the fungicidal nature at this concentration of chitosan. Concentrations of ≥1500 µg/ml were considered fungicidal in tropical fruits juices. The effect thus demonstrated chitosan can delay the spoilage of tropical fruits juices by both bacteria and yeasts at chill or ambient storage temperatures.

Keywords: Ozonation, chitosan, fruit juices, yeasts, inactivaion

Introduction

Consumers in the twenty-first (21st) century are increasingly and consistently demanding the highest quality for available fruit juice products at the fairest price with corresponding expectations that such quality will be maintained at a high level during the period between purchase and consumption. These expectations are a consequence not only of the primary requirement that the fruit juice should remain safe, but also of the need to minimize unwanted changes in sensory quality. Consumers will accept any new technology that extends shelf life, if they perceive that the process does not diminish product quality and if a long shelf life does not deteriorate both the quality and convenience of the product (Eskin and Robinson, 2001).

Food preservatives are employed principally to prevent the spoilage of foods during storage and throughout distribution, retailing and use by the consumer. An important current trend in food preservation is towards the use of procedures that deliver food products that are less "heavily" preserved, higher in quality, perceived as being more "natural", contain less additives, and are nutritionally healthier (Beales and Smith, 2004). Some new and "emerging" techniques which act by inactivating microbes include the application of high hydrostatic pressure, pulsed electric field, ultrasonication, high pressure carbondioxide and naturally occurring antimicrobials which are being explored for use as food preservatives (Lee and Kang, 2004).

There is considerable interest in the possible use of naturally occurring antimicrobial compounds, in combination with already existing preservative mechanisms. Combinations may produce products with increased shelf-life and reduced chemical preservatives. The requirement to manufacture food products with lower levels of conventional preservatives is a strong driving force in today's consumer market. Natural alternatives have the potential to be used to replace some or all of the conventional preservatives in perishable and ambient stable products. Novel processing and packaging technologies can also contribute to the microbiological safety and stability of fruit juice products, thus reducing the reliance on conventional chemical preservatives. Future developments will see more use of naturally derived antimicrobial systems for food preservation (Beales and Smith, 2004).

Chitosan is a natural polysaccharide which can be obtained by the deacetylation of chitin from crustacean shells (No and Meyers, 1989). Chitin can be transformed into chitosan that has free amino groups by removing acetyl groups (CH_3-CO) from chitin molecules. Thus chitosan is the deacetylated form of chitin in which the acetamide groups (CH_3CO-NH) in chitin are substituted into amino groups (-NH_2) in chitosan (Kurita, 1998). Hsu *et al*. (2002) reported that chitosan is insoluble in water, alkali and organic solvents, but soluble in most diluted acids with pH less than 6. When chitosan is dissolved in an acid solution, it becomes a cationic polymer due to the protonation of free amino groups on the C-2 position of pyranose ring. The cationic properties of chitosan in acidic solutions give it the ability to interact readily with negatively charged molecules such as lipids and cholesterol. In this respect, chitin and chitosan have attained increasing commercial interest as suitable resource materials due to their excellent properties of biocompatibility, biodegradability, absorption, ability to form films and to chelate metal ions (Li *et al*., 1992). In conventional beverage processing fruit juices are heated to inactivate all non-spore-forming microorganisms. Any remaining bacteria spores are generally unable to germinate because of the acidic nature (pH <4.6) of the juices (Splittoesser *et al*., 1994). However, heat treatment causes the loss of vitamins and possible changes in flavour of the juices. Many consumers regard heated-treated, shelf stable products as low in quality and are demanding minimally processed fruit juices. Furthermore, increasing consumer pressure for the exclusion of many chemically synthesized preservatives or chemical additives from foods and beverages has challenged food scientists to find alternative and "more natural" methods of preserving food (Dillon and Board, 1994; Naidu, 2000). An area that holds promise but yet to be explored is the use of saccharidic natural substances notably deacetylated chitin or chitosan which when combined with a number of milder preservation techniques may achieve an enhanced level of product safety and stability.

Materials and Methods

Source of fungi used in experimentation

The yeasts used were *Candida pulcherrima, Pichia fermentans* and *Saccharomyces cerevisiae,* isolated from deteriorating fruit (orange, pineapple and watermelon) samples.

Source of fruits

Tropical fruits used in the experiment: pineapple and orange were obtained from Ehor fruit market, Edo State, Nigeria. Watermelon was obtained from Uselu market, Benin City, Nigeria. The fruits were transported in clean black polyethene bags to the experimental postgraduate laboratory of the

Department of Microbiology, University of Benin, Benin City for processing into fruit juice.

Preparation of fruit juices

Freshly harvested fruits of pineapple, orange and watermelon were sorted, washed, peeled and diced into cubes using a sharp sterile stainless steel knife. The diced or chopped pieces were fed into a sterilized Binatone juice extractor (Model JE-500, Binatone, England). The device was fitted with a special filter which enabled the fruit juice to be obtained directly without further filtration and homogenization. The resultant pulp from the fruit extraction was discarded. The juice obtained was immediately received in sterile bottles and corked after filling using manually operated bottle corking machine. Samples were subjected to microbiological and physicochemical analysis. Fresh juice samples were either refrigerated at 4^0C or kept at 28 ± 2^0C until needed for use. Packaging materials used for storage include PET plastic bottles and polythene sachets (Omogbai, 2012).

Microbiological analysis of tropical fruit juices

Four fruit juices: pineapple juice, orange juice and water melon juice were analyzed microbiologically for bacteria and fungi using standard methods.

Isolation and enumeration of microflora of juice samples

Fruit juice (100 ml) was dispensed into 200 ml containers (in duplicate for each treatment) and stored at 4^0C and 28 ± 2^0C. Duplicate samples (10 ml) were taken periodically from each container for microbiological analysis. It was daily for 10 days for inherent microflora but hourly for 72 h for yeasts.

The pour plate method was used for the isolation and enumeration of bacteria in the fruit juice samples. Ten-fold serial (1:10) dilutions of homogenized suspension were prepared in sterile peptone water –a maximal recovery diluent. Viable numbers were determined by pour-plating 1.0 ml on nutrient agar for total counts, deMan Rogosa Sharpe agar (MRSA) for lactic acid bacteria and ethanol bicarbonate agar (EBA) for acetic acid bacteria. MRSA was supplemented with 0.001% w/v actidione (cycloheximide) in order not to suppress overgrowth of lactic acid bacteria by yeasts. Nutrient agar and MRSA plates were incubated at 37^0C for 24 h. While EBA plates were incubated at 28^0C for 3 days. Violet red bile glucose agar was used for the enumeration of *Enterobacteriaceae* after incubation at 37^0C for 24 h. Yeasts and moulds were enumerated by spread-plating (0.1 ml) on Sabouraud dextrose agar incubated at 28 ± 2^0C for 3 – 5 days. Microbial counts were taken after incubation, calculated and expressed as colony forming units per milliliter (CFU/ml) of the juice samples tested (Omogbai and Ikenebomeh, 2016).

Ozonation of tropical fruit juices

The modified method of Hunt and Marinas (1997) was employed in ozonation of fruit juice. Ozone gas was generated using an ozone generator (Model: TS-III, Tianjin Bioengineering Co., Ltd China) in a 500 ml Erlenmeyer flask. An ozone concentration of 3.33 mg/min was applied. Ozone concentration was recorded using an ozone analyzer (built-in ozone module in the device). Prior to ozone treatment, a measured 10-20 µl sterile antifoaming agent was added to the juice to prevent excess foam formation. The juice samples were treated for 0 – 20 min with sampling at 2 min intervals. All experiments were carried out in duplicate and replicated at least twice. The efficacy of treatment was determined in terms of reduction of viable counts over time (Kim and Yousef, 2000).

Results and Discussion

The effect of chitosan concentration and storage time on the natural microflora of tropical fruits juices are shown in Tables 1 and 2. Chitosan concentrations of 1000, 1500 and 2000 µg/ml were biocidal on bacteria with number reduced by > 0.5-1.27 $\log_{10}$CFU/ml immediately after addition to orange, pineapple, watermelon, and mixed fruits juices. The numbers reduced to non-detectable level with 2000 µg/ml from the 4^{th} to the 10^{th} day in orange and mixed fruit juices. At a concentration of 1500 µg/ml non-detectable levels were recorded in all fruits juices after 8 days. With a concentration of 1000 µg/ml the bacteria counts in the juices at both 4±1 and 28 ± 2^0C initially killed, later became stable and then the number increased thereafter. In orange juice bacteria numbers decreased from 2.72 $\log_{10}$CFU/ml to 1.50±0.05$\log_{10}$CFU/ml and 1.46±0.02 $\log_{10}$CFU/ml at day 6 for storage at 28±2 and 4 ± 1^0C, respectively. These numbers became stable and then increased from the 8^{th} -10^{th} day to final values of 1.68±0.02 $\log_{10}$CFU/ml and 1.61±0.01 $\log_{10}$CFU/ml, respectively. However the number of bacteria remained below the levels in the control at both storage temperatures in all fruit juices which had reached a little over 8 log CFU/ml (Table 1).

The effect of chitosan concentration and storage time on the survival of inherent fungal population in tropical fruits juices is shown in Table 2. Fungal population were continuously decimated but not totally eliminated from the fruit juices (orange, pineapple, watermelon and mixed fruit juices) with chitosan concentration of 1000 µg/ml at both 4±1 and 28 ± 2^0C. More reduction in viable numbers occurred at 4 ± 1^0C compared to 28 ± 2^0C indicating that chill temperature work better in synergy with chitosan.

Fungal population survived up to 2 days and 1 day with 1500 and 2000 µg/ml, respectively at both 4±1 and 28 ± 2^0C in all fruit juices. Concentrations of ≥1500 µg/ml were considered fungicdal for fungal populations in tropical fruits juices. The effect thus demonstrated showed chitosan can delay the spoilage of tropical fruits juices by both bacteria and yeasts at chill or ambient storage temperatures.

The results of the effect of chitosan on microorganisms playing leading role in the spoilage of tropical fruits juices are shown in Tables 3 – 4. The survival of *Saccharomyces cerevisiae* in ozonated tropical fruit juices treated with chitosan is shown in Table 3. Counts of *Saccharomyces cerevisiae* decreased steadily from a challenge concentration of 7.0±0.02 log CFU/ml in all fruits juices samples treated with chitosan at 4±1 and 28 ± 2^0C. Chitosan concentration of 1000 µg/ml was fungistatic reducing yeast loads by 3.54 log CFU/ml in all samples. At this concentration a non-detectable level was reached in watermelon after 72 h. Chitosan concentration of 1500 µg/ml reduced *Saccharomyces cerevisiae* to non-detectable level in watermelon juices after 24 h. This feat was achieved in other juices after 36 h. At a concentration of 2000 µg/ml, non-detectable levels of *S. cerevisiae* was obtained in all samples except in mixed fruit juice samples stored at 28 ± 2^0C showing the fungicidal nature of this concentration of chitosan against *S. cerevisiae* in foods.

Table 1: Effect of Chitosan (DMPAC) concentration and storage time on inherent bacterial flora in tropical fruits juices stored at 4±1 and 28 ± 2^0C

Storage Time (days)	Chitosan (µg/ml)	Survival bacterial population in tropical fruit juice (Log_{10}CFU/ml)							
		ORANGE		PINEAPPLE		WATERMELON		MIXED FRUIT	
		28^0C	4^0C	28^0C	4^0C	28^0C	4^0C	28^0C	4^0C
0	0	2.72±0.01	2.72±0.01	2.80±0.1	2.80±0.1	2.76±0.1	2.76±0.1	2.85±0.1	2.85±0.1
	1000	1.83±0.02	1.85±0.01	2.53±0.01	2.50±0.01	2.58±0.2	2.55±0.03	2.80±0.1	2.77±0.05
	1500	1.65±0.10	1.60±0.1	2.36±0.02	2.35±0.02	2.25±0.01	2.20±0.01	2.46±0.01	2.43±0.02
	2000	1.48±0.10	1.45±0.01	2.08±0.1	2.08±0.05	2.20±0.02	2.17±0.01	1.68±0.1	1.64±0.01
2	0	4.86±0.5	2.78±0.01	3.98±0.1	2.88±0.01	2.88±0.2	2.76±0.01	4.16±0.3	3.10±0.01
	1000	1.71±0.01	1.68±0.03	2.28±0.02	2.26±0.01	2.17±0.1	2.17±0.1	2.64±0.1	2.60±0.01
	1500	1.43±0.01	1.43±0.02	2.01±0.1	2.01±0.1	1.80±0.01	1.76±0.02	2.28±0.01	2.23±0.02
	2000	1.10±0.02	1.12±0.01	1.63±0.01	1.60±0.01	1.52±0.01	1.50±0.01	1.27±0.1	1.27±0.1
4	0	8.05±0.02	3.40±0.01	7.86±0.4	3.52±0.01	5.04±0.3	2.84±0.01	5.38±0.4	3.57±0.01
	1000	1.64±0.01	1.58±0.02	2.10±0.01	2.06±0.02	2.08±0.01	2.08±0.01	2.34±0.1	2.30±0.05
	1500	1.20±0.01	1.15±0.1	1.41±0.1	1.37±0.01	1.64±0.02	1.60±0.01	1.55±0.01	1.50±0.03
	2000	ND	ND	1.10±0.01	1.10±0.01	1.35±0.01	1.31±0.02	ND	ND
6	0	8.16±0.5	4.71±0.02	8.05±0.5	4.82±0.01	7.76±0.5	3.47±0.01	7.88±.4	4.83±0.02
	1000	1.50±0.01	1.46±0.02	1.52±0.1	1.48±0.01	2.08±0.01	2.08±0.01	1.86±0.1	1.80±0.04
	1500	ND	ND	1.41±0.01	1.39±0.02	1.48±0.1	1.45±0.05	ND	ND
	2000	ND	ND	ND	ND	1.22±0.1	1.22±0.01	ND	ND
8	0	8.30±0.4	6.83±0.01	8.23±0.4	5.42±0.01	8.17±0.6	4.46±0.01	8.14±0.5	6.51±0.02
	1000	1.53±0.01	1.46±0.02	1.65±0.02	1.60 ±0.01	1.56±0.01	1.56±0.01	1.66±0.01	1.63±0.02
	1500	ND	ND	ND	ND	ND	ND	ND	ND
	2000	ND	ND	ND	ND	ND	ND	ND	ND
10	0	8.41±0.5	7.25±0.01	8.28±0.5	6.78±0.01	8.29±0.5	5.82±0.01	8.21±0.3	7.16±0.01
	1000	1.68±0.02	1.61±0.01	1.73±0.01	1.70±0.02	1.30±0.01	1.28±0.02	1.66±0.02	1.56±0.04
	1500	ND	ND	ND	ND	ND	ND	ND	ND
	2000	ND	ND	ND	ND	ND	ND	ND	ND

ND = Not detected, Each value is mean of triplicate determination ±standard deviation

Table 2: Effect Chitosan (DMPAC) concentration and storage time on inherent fungal (yeasts and moulds) flora in tropical fruits juices stored at 4±1 and 28±2^{0}C

STORAGE TIME (DAYS)	CHITOSAN (µg/ml)	SURVIVAL FUNGAL POPULATION IN TROPICAL FRUIT JUICE (Log_{10}CFU/ml)							
		ORANGE		PINEAPPLE		WATERMELON		MIXED FRUIT	
		28^0C	4^0C	28^0C	4^0C	28^0C	4^0C	28^0C	4^0C
0	0	3.40±0.02	3.40±0.02	3.51±0.01	3.51±0.01	3.46±0.02	3.46±0.02	3.6±0.01	3.6±0.01
	1000	2.81±0.01	2.76±0.01	3.02±0.01	2.85±0.02	3.05±0.01	2.87±0.01	2.68±0.01	2.50±0.02
	1500	2.43±0.02	2.38±0.1	2.37±0.02	2.18±0.03	2.06±0.02	1.99±0.00	2.00±0.05	2.00±0.02
	2000	1.65±0.03	1.60±0.02	1.48±0.1	1.36±0.03	1.29±0.01	1.18±0.01	1.03±0.02	1.10±0.01
2	0	4.50±0.01	3.58±0.01	5.60±0.02	3.51±0.01	4.73±0.01	3.58±0.01	5.25±0.02	4.03±0.03
	1000	1.44±0.02	1.40±0.03	2.58±0.01	2.49±0.02	2.77±0.01	2.64±0.02	2.01±0.03	2.34±0.01
	1500	1.06±0.01	1.11±0.01	1.07±0.01	1.05±0.01	1.12±0.03	1.05±0.01	1.19±0.02	1.08±0.01
	2000	ND	ND	ND	ND	ND	ND	ND	ND
4	0	6.86±0.03	3.95±0.02	6.88±0.01	3.63±0.03	6.51±0.3	3.72±0.04	6.78±0.01	5.74±0.02
	1000	1.15±0.01	1.20±0.01	2.04±0.02	1.93±0.02	1.83±0.01	1.83±0.01	1.57±0.02	1.48±0.01
	1500	ND	ND	ND	ND	ND	ND	1.19±0.05	1.07±0.02
	2000	ND	ND	ND	ND	ND	ND	ND	ND
6	0	8.20±0.05	6.58±0.01	8.15±0.3	4.09±0.05	7.17±0.01	4.28±0.10	7.64±0.01	6.09±0.05
	1000	1.15±0.01	1.12±0.02	1.43±0.1	1.38±0.04	2.35±0.01	1.58±0.01	1.14±0.02	1.05±0.03
	1500	ND	ND	ND	ND	ND	ND	ND	ND
	2000	ND	ND	ND	ND	ND	ND	ND	ND
8	0	8.37±0.01	6.76±0.05	8.50±0.01	4.83±0.01	8.00±0.01	5.49±0.03	7.99±0.02	6.33±0.01
	1000	1.15±0.01	1.06±0.10	1.18±0.02	1.06 ±0.02	1.18±0.01	1.07±0.02	1.02±0.01	1.05±0.03
	1500	ND	ND	ND	ND	ND	ND	ND	ND
	2000	ND	ND	ND	ND	ND	ND	ND	ND
10	0	8.37±0.01	8.23±0.01	8.62±0.03	5.12±0.01	8.19±0.10	6.01±0.05	8.17±0.01	7.12±0.1
	1000	1.15±0.01	1.06±0.02	1.06±0.1	1.06±0.02	1.04±0.02	1.07±0.02	1.02±0.01	1.05±0.01
	1500	ND	ND	ND	ND	ND	ND	ND	ND
	2000	ND	ND	ND	ND	ND	ND	ND	ND

ND = Not detected, **Each value is mean of triplicate determination ±standard deviation**

The effect of chitosan on the yeast *Candida pulcherrima* in ozonated tropical fruits juices is illustrated in Table 4. In the control experiments *Candida pulcherrima* continued to grow at both 28±2 and 4±1^{0}C temperatures reaching ≥7.81 log_{10}CFU/ml in all samples. This yeast was however affected by chitosan at 1000 µg/ml more in watermelon, orange and pineapple juices compared to the mixed fruit juices blend. The initial challange population of 7.32 log_{10}CFU/ml was reduced by *ca* 5.07 and 5.17 log_{10}CFU/ml at 28±2 and 4±1^{0}C, respectively after 72h in watermelon juice. Chitosan concentration of 1500 and 2000 µg/ml was more effective against this yeast in fruits juices samples. At a concentration 1500 µg/ml, no detectable levels were found in orange and watermelon juices after 36 h. By 48h chitosan concentration of 1500 and 2000 µg/ml achieved >7 log reduction of *Candida pulcherrima* in all the fruit juices investigated.

The effect of chitosan concentrations on the growth and survival of *Pichia fermentans* in ozonated tropical fruit juices

is shown in Table 5. Chitosan concentration of 1000 µg/ml affected the initial challenge cell concentration of 7.50 log_{10}CFU/ml with reduction by *ca* 6.22, 6.14, 6.34 and 4.81 log_{10}CFU/ml in orange, pineapple, watermelon and mixed fruits juices respectively within 72 h at 28±2^{0}C storage. At 4±1^{0}C the log reductions by 1000µg/ml chitosan in orange, pineapple, watermelon and mixed fruit juice were 6.65, 5.99, 6.47 and 5.39 log_{10}CFU/ml, respectively.

Concentrations of 1500 and 2000 µg/ml were more effective at killing cells of *Pichia fermentans* in fruit juices. Total killing of *Pichia fermentans* was achieved by a concentration of 1500 µg/ml in watermelon at both 4±1 and 28±2^{0}C in 36 h. In orange and pineapple juices gross reduction in yeast numbers occurred with ≥ 5.95Log_{10}CFU/ml at both 4±1 and 28±2^{0}C. The yeast *Pichia fermentans* was totally inhibited by a concentration of 2000 µg/ml in all fruit juices type by 48 h at both 4±1 and 28±2^{0}C.

Table 3: Effect of Chitosan (DMPAC) Concentration and Storage Time on the Growth and Survival of *Saccharomyces cerevisiae* in Ozonated Tropical Fruits Juices Stored at 4±1^{0}C and 28±2^{0}C

STORAGE TIME (H)	CHITOSAN CONCENTRATION (µg/ml)	SURVIVAL POPULATION (Log_{10} CFU/ml) IN TROPICAL FRUITS JUICES							
		ORANGE		PINEAPPLE		WATERMELON		MIXED	FRUIT JUICE
		28^{0}C	4^{0}C	28^{0}C	4^{0}C	28^{0}C	4^{0}C	28^{0}C	4^{0}C
0	0	7.0±0.02	7.0±0.02	7.0±0.02	7.0±0.02	7.0±0.02	7.0±0.02	7.0±0.02	7.0±0.02
	1000	5.62±0.01	5.48±0.02	6.30±0.00	5.57±0.01	5.13±0.03	4.86±0.01	6.65±0.00	6.54±0.03
	1500	3.48±0.05	3.36±0.1	4.45±0.02	4.05±0.00	3.29±0.01	2.84±0.02	5.72±0.01	5.28±0.01
	2000	3.16±0.01	2.95±0.00	3.76±0.01	3.43±0.03	2.85±0.02	2.60±0.00	4.58±0.00	4.02±0.00
12	0	7.28±0.10	7.15±0.03	7.36±0.01	7.25±0.02	7.40±0.02	7.31±0.00	7.31±0.02	7.21±0.00
	1000	3.89±0.02	3.67±0.02	5.72±0.01	4.46±0.02	4.48±0.04	3.66±0.01	6.50±0.02	5.80±0.02
	1500	1.88±0.03	1.64±0.00	2.64±0.03	2.14±0.00	1.75±0.01	1.54±0.02	3.46±0.00	3.14±0.04
	2000	1.42±0.00	1.13±0.00	1.61±0.02	1.07±0.01	1.23±0.04	1.07±0.0	3.06±0.01	2.87±0.00
24	0	7.45±0.05	7.38±0.01	7.64±0.00	7.58±0.01	7.6±0.01	7.50±0.02	7.63±0.03	7.53±0.02
	1000	3.45±0.01	3.30±0.02	5.24±0.02	4.20±0.01	3.27±0.00	2.86±0.03	6.03±0.00	4.86±0.03
	1500	1.56±0.01	1.25±0.00	2.05±0.01	1.95±0.03	ND	ND	2.87±0.02	2.16±0.01
	2000	ND	ND	ND	ND	ND	ND	2.15±0.01	ND
36	0	7.64±0.15	7.56±0.03	7.92±0.00	7.78±0.01	7.9±0.00	7.7±0.01	7.98±0.00	7.58±0.01
	1000	3.10±0.05	2.94±0.01	4.56±0.01	3.71±0.00	2.18±0.01	1.87±0.02	5.47±0.01	4.21±0.00
	1500	ND	ND	1.25±0.00	1.03±0.00	ND	ND	1.51±0.02	1.34±0.02
	2000	ND	ND	ND	ND	ND	ND	ND	ND
48	0	8.52±0.06	8.17±0.02	8.47±0.02	8.36±0.01	8.21±0.01	7.95±0.00	8.16±0.02	7.97±0.00
	1000	2.86±0.01	2.63±0.00	4.15 ± 0.00	3.36±0.02	1.56±0.00	1.31±0.04	4.26±0.02	3.64±0.01
	1500	ND	ND	ND	ND	ND	ND	ND	ND
	2000	ND	ND	ND	ND	ND	ND	ND	ND
60	0	8.68±0.01	8.54±0.06	8.75±0.00	8.58±0.02	8.52±0.02	8.16±0.01	8.43±0.01	8.07±0.02
	1000	2.50±0.00	2.47±0.01	3.46±0.02	3.28±0.02	1.37±0.01	1.16±0.01	3.18±0.03	2.58±0.01
	1500	ND	ND	ND	ND	ND	ND	ND	ND
	2000	ND	ND	ND	ND	ND	ND	ND	ND
72	0	8.85±0.02	8.71±0.02	8.91±0.01	8.66±0.01	8.61±0.00	8.26±0.02	8.47±0.00	8.21±0.00
	1000	2.46±0.01	2.34±0.00	3.46±0.02	3.28±0.02	ND	ND	2.43±0.01	2.27±0.01
	1500	ND	ND	ND	ND	ND	ND	ND	ND
	2000	ND	ND	ND	ND	ND	ND	ND	ND

ND = Not detected, Each value is mean of triplicate determination ±standard deviation

Table 4: Effect of Chitosan (DMPAC) concentration and storage time on the growth and survival of *Candida pulcherrima* in ozonated tropical fruit Juice stored at 4±1 and 28±2^{0}C

STORAGE TIME (H)	CHITOSAN CONCENTRATION µg/ml	SURVIVAL POPULATION (Log CFU/ml) IN TROPICAL FRUITS JUICES							
		ORANGE		PINEAPPLE		WATERMELON		MIXED FRUIT JUICE	
		28^0C	4^0C	28^0C	4^0C	28^0C	4^0C	28^0C	4^0C
0	0	7.32±0.13	7.32±0.13	7.32±0.13	7.32±0.13	7.32±0.13	7.32±0.13	7.32±0.13	7.32±0.13
	1000	6.24±0.00	5.88±0.01	6.37±0.01	6.18±0.02	5.44±0.03	4.61±0.04	7.10±0.00	6.96±0.01
	1500	4.36±0.02	3.95±0.02	4.58±0.00	4.24±0.01	3.48±0.01	3.14±0.01	4.74±0.02	4.18±0.00
	2000	2.39±0.00	2.16±0.03	3.62±0.00	3.45±0.00	2.17±0.00	1.75±0.00	3.95±0.01	3.34±0.02
12	0	7.46±0.05	7.40±0.00	7.52±0.00	7.43±0.02	7.82±0.00	7.63±0.03	7.68±0.01	7.45±0.01
	1000	5.78±0.02	5.56±0.02	5.93±0.02	5.71±0.02	4.36±0.01	3.42±0.02	6.76±0.02	5.88±0.00
	1500	2.48±0.03	2.11±0.04	3.71±0.05	3.01±0.00	1.34±0.00	1.18±0.00	3.15±0.00	2.79±0.00
	2000	1.86±0.02	1.04±0.00	2.05±0.00	1.70±0.02	1.11±0.00	ND	2.09±0.00	1.73±0.02
24	0	7.57±0.00	7.51±0.01	7.64±0.02	7.55±0.01	7.96±0.02	7.71±0.00	7.75±0.00	7.50±0.00
	1000	4.67±0.03	4.53±0.01	5.26±0.06	4.86±0.00	3.65±0.01	3.16±0.04	6.23±0.00	5.46±0.01
	1500	1.31±0.00	1.06±0.00	2.85±0.03	2.53±0.02	ND	ND	2.91±0.02	2.06±0.00
	2000	ND	ND	1.13±0.00	0.96±0.01	ND	ND	1.15±0.00	1.08±0.01
36	0	7.71±0.01	7.68±0.00	7.64±0.00	7.60±0.00	8.14±0.01	7.94±0.01	7.56±0.03	7.50±0.00
	1000	4.21±0.01	3.96±0.01	4.75±0.02	4.31±0.04	3.10±0.03	2.65±0.01	5.73±0.01	5.03±0.05
	1500	ND	ND	1.61±0.00	1.23±0.00	ND	ND	1.72±0.00	1.56±0.01
	2000	ND	ND	ND	ND	ND	ND	1.02±0.00	1.01±0.00
48	0	7.83±0.00	7.75±0.04	7.86±0.01	7.78±0.00	8.21±0.04	8.13±0.01	7.64±0.02	7.61±0.01
	1000	3.83±0.00	3.75±0.04	4.04 ± 0.00	3.94±0.02	2.55±0.00	2.34±0.01	5.16±0.00	4.98±0.00
	1500	ND	ND	ND	ND	ND	ND	ND	ND
	2000	ND	ND	ND	ND	ND	ND	ND	ND
60	0	7.96±0.01	7.90±0.00	7.97±0.02	7.85±0.01	8.30±0.01	8.13±0.01	7.91±0.01	7.75±0.02
	1000	3.68±0.05	3.48±0.01	3.66±0.00	3.48±0.00	2.28±0.03	2.15±0.00	4.81±0.02	4.57±0.01
	1500	ND	ND	ND	ND	ND	ND	ND	ND
	2000	ND	ND	ND	ND	ND	ND	ND	ND
72	0	8.18±0.02	7.98±0.01	8.25±0.00	8.07±0.03	8.34±0.00	8.26±0.02	8.03±0.01	7.81±0.00
	1000	3.56±0.01	3.25±0.05	3.60±0.01	3.40±0.01	2.25±0.03	2.15±0.00	4.26±0.02	4.04±0.02
	1500	ND	ND	ND	ND	ND	ND	ND	ND
	2000	ND	ND	ND	ND	ND	ND	ND	ND

ND = NOT DETECTED

Table 5: Effect Chitosan (DMPAC) Concentration and Storage Time on the Growth and Survival of *Pichia fermentans* in Ozonated Tropical Fruit Juice Stored at 4±1^{0}C and 28±2^{0}C.

STORAGE	CHITOSAN	SURVIVAL POPULATION (Log_{10}CFU/ml) IN TROPICAL FRUITS JUICES

TIME (H)	CONCENTRATION (µg/ml)	ORANGE		PINEAPPLE		WATERMELON		MIXED FRUIT JUICE	
		28^0C	4^0C	28^0C	4^0C	28^0C	4^0C	28^0C	4^0C
0	0	7.50±0.01	7.50±0.01	7.50±0.01	7.50±0.01	7.50±0.01	7.50±0.01	7.50±0.01	7.50±0.01
	1000	5.38±0.00	4.97±0.02	7.01±0.00	6.67±0.01	4.56±0.02	4.38±0.01	7.15±0.00	6.75±0.00
	1500	3.27±0.01	2.89±0.01	5.44±0.00	5.03±0.02	3.11±0.01	2.96±0.03	5.46±0.01	5.18±0.00
	2000	2.51±0.03	2.34±0.00	3.53±0.00	2.78±0.01	2.37±0.02	2.21±0.02	4.55±0.01	4.03±0.02
12	0	7.55±0.00	7.58±0.01	7.60±0.02	7.53±0.00	7.55±0.04	7.50±0.00	7.73±0.02	7.63±0.02
	1000	4.50±0.00	4.36±0.03	6.45±0.02	5.89±0.00	3.97±0.01	3.58±0.02	6.53±0.01	5.18±0.01
	1500	2.65±0.02	2.45±0.01	4.31±0.01	3.95±0.03	2.31±0.02	2.06±0.00	4.18±0.00	3.96±0.03
	2000	1.97±0.00	1.36±0.00	2.45±0.01	2.03±0.01	1.73±0.01	1.18±0.02	2.24±0.00	1.95±0.02
24	0	7.62±0.00	7.71±0.02	7.66±0.01	7.58±0.01	7.74±0.02	7.65±0.01	7.79±0.00	7.50±0.01
	1000	3.38±0.02	3.14±0.01	4.48±0.00	4.01±0.03	3.01±0.02	2.77±0.02	5.37±0.02	3.95±0.04
	1500	1.83±0.00	1.76±0.00	2.86±0.01	2.07±0.01	1.28±0.01	1.08±0.00	3.59±0.00	3.12±0.01
	2000	1.54±0.01	1.06±0.01	1.83±0.00	1.26±0.00	1.33±0.02	ND	1.52±0.01	1.23±0.03
36	0	7.95±0.01	6.94±0.02	7.73±0.00	6.62±0.03	7.81±0.00	7.70±0.00	7.86±0.02	7.75±0.02
	1000	3.06±0.01	2.75±0.00	4.56±0.01	3.63±0.01	2.44±0.01	2.06±0.00	3.76±0.01	3.72±0.00
	1500	1.10	0.89±0.00	1.55±0.02	1.39±0.02	ND	ND	2.53±0.01	2.10±0.00
	2000	0.92±0.00	ND	1.06±0.00	ND	ND	ND	ND	ND
48	0	7.91±0.03	6.94±0.00	7.78±0.01	6.89±0.03	7.89±0.04	7.63±0.01	7.95±0.01	7.83±0.01
	1000	2.43±0.01	2.06±0.00	3.34 ±0.02	2.65±0.02	2.17±0.00	2.06±0.00	3.47±0.00	3.08±0.02
	1500	ND	ND	ND	ND	ND	ND	ND	ND
	2000	ND	ND	ND	ND	ND	ND	ND	ND
60	0	7.96±0.02	7.88±0.01	7.83±0.04	7.78±0.01	7.98±0.01	7.63±0.01	7.98±0.02	7.87±0.01
	1000	1.96	0.85±0.00	2.08±0.00	1.51±0.03	1.57±0.02	1.36±0.02	3.08±0.01	2.53±0.01
	1500	ND	ND	ND	ND	ND	ND	ND	ND
	2000	ND	ND	ND	ND	ND	ND	ND	ND
72	0	7.96	7.90±0.00	7.92±0.00	7.86±0.00	8.05±0.02	7.75±0.02	8.15±0.00	7.94±0.00
	1000	1.28±0.01	0.85±0.00	1.36±0.00	1.51±0.03	1.16±0.00	1.03±0.00	2.69±0.00	2.11±0.02
	1500	ND	ND	ND	ND	ND	ND	ND	ND
	2000	ND	ND	ND	ND	ND	ND	ND	ND

ND = Not detected, **Each value is mean of triplicate determination ±standard deviation**

The efficacy of chitosan in killing or eliminating the inherent microflora in tropical fruit juices is shown to be dependent on concentration, storage time and temperature. A concentration of 1000 µg/ml of chitosan was microbistatic at both ambient and refrigeration temperature. This is possibly due to the organic matter content of the fruit juices and inability to create a complete adverse environment inimical for growth of the organisms. However with increased concentration (≥1500 µg/ml) steady decrease in microbial counts even to non-detectable levels (Tables 1 and 2) were observed.

The decrease in microbial count is evidential of the presence of the microflora in a metabolically hostile environment. Thus chitosan created an unfavorable micro-environment for the organisms through hurdles which they could not overcome. The inability to overcome the hurdles may have arisen from physiological homeostatic stress and metabolic distortion. Further attempts to overcome the adverse conditions may have led to more stress which subsequently led to metabolic exhaustion, gradual death, subsequent decline in population and total non-detection of the organisms in the fruit juices. These findings corroborate earlier reports by Leistner (2000). Furthermore microbial reductions were observed to be more at 4 ± 1^0C compared to 28 ± 2^0C. This is corroborated by Adams and Moss (1995) who reported that the growth of many microorganisms are generally inhibited at low temperatures. Thus chitosan seemed to work in synergy with low temperature for the elimination of the inherent microflora especially yeast which thrived for only two (02) days with the introduction ≥1500 µg/ml chitosan into the fruit juices.

Yeasts have been reported as the primary culprit in fruit juice spoilage. Lima *et al*. (2009) reported *Saccharmyces cerevisiae* as one of the most important yeasts causing spoilage of fruit juices and soft drinks and can be considered as shelf-life indicator. The selective action of chitosan on three species of yeasts occurring naturally in the mixed microflora of the tropical fruit juices shows its potential as an alternative processing technology with likelihood to prevent spoilage and extend the shelf-life of these products. The results (Tables 3 – 5) demonstrate that the presence of 1500 µg/ml chitosan inactivated the yeasts. *Saccharomyces cerevisiae*, *Candida pulcherrima* and *Pichia fermentans* and prevented their regrowth for at least 3 days at both refrigerated (4 ± 1^0C) and room (28 ± 2^0C) storage temperature. However the inactivation was species specific with killing achieved at different times and depending on the organic matter content of the fruit juice. While *Candida pulcherrima* were killed after 12 h in watermelon juice, 24 h was required by *Saccharomyces cerevisiae* and *Pichia fermentans*. In all, yeast numbers were reduced faster in fruit juices with less organic matter content. This is because high concentration of organic matter represses the bioactivity of chitosan.

Conclusion

The use of non-thermal methods for the inactivation of spoilage yeasts in fruit juices as seen in the current study will avoid deleterious effect of heat on flavour and sensory characteristics thus retaining freshness desired by consumers.

Conflict of Interest

The authors affirm that there is no conflict of interest.

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