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Full Length Research Paper

Microbiological Status of Mixed Fruit Juice Preserved with different Concentrations of Sodium Benzoate.

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Abstract
A mixed fruit juice produced from pineapple (40%), orange (30%), paw-paw (20%) and guava (10%) was treated with different concentrations (0.0%, 0.04%, 0.06%, 0.08%) of sodium benzoate as preservative. The four samples were pasteurized and stored for three months. They were microbiologically analyzed as fresh sample (not stored), after one month and three months storage. Isolates were characterized colonially, microscopically and biochemically. The result of the microbial analysis showed that the sample preserved with 0.04% sodium benzoate and control (0.0%) had higher microbial load of 9.8 X 10^6 (Bacterial) and 8.0 X 10^6 (fungal) and 3.5 X 10^6, 5.6 X 10^6 respectively, after three months of storage. The microorganisms identified in them were Lactobacillus fermentum, Staphylococcus aureus and Penicillum species. All the samples preserved with 0.06% and 0.08% sodium benzoate had microbial load within the acceptable range of 1.0 X 10^6 cfu/ml. Lactobacillus fermentum, Saccharomyces cerevisiae and Penicillum species were identified in the sample preserved with 0.06% sodium benzoate after three months of storage. Lactobacillus fermentum and Saccharomyces cerevisiae were the only microorganisms identified in all the samples preserved with 0.08% sodium benzoate. The organisms have not been associated with any food-borne disease. From the above results, 0.08% sodium benzoate could preserve the mixed fruit juice for the storage period of three months on the shelf.

Keywords: *Microbiological status, Mixed fruit juice, Sodium benzoate, Preservative*

Introduction

A mixed fruit juice is the non-fermented, though fermentable liquid extract of more than one fruit (Bates et al, 1997).It is usually expressed by mechanical extraction process and could be preserved exclusively by chemical and physical means (Woodroof and Luh, 2000). Fruit juices are rich in vitamins and minerals which are mostly anti-oxidants. The anti-oxidant components of fruit juices have been associated with some beneficial long term health effects such as, decreasing the risk of cancer and heart disease (Boyer and Liu, 2004). The liquid nature of the juice and availability of many tropical fruits offer a blending opportunity of many mixing options thereby creating variety in fruit products. Nigeria is blessed with so many tropical fruits such as pineapple, orange, paw-paw, sour-sop, guava etc and with its large population of about

150 million, Nigeria has the largest market in the sub-Saharan Africa (Akinwale, et al, 2001). This, not withstanding, Nigeria remains a major importer of food and agricultural products including fruit juice concentrates and other food drinks which merely qualify as fruit nectars (Gain Report, 2009).

Moreso, some food poisoning cases have been attributed to consumption of fruit juices (Ashurst, 1995). Ordinarily, fruit juices processed under hygienic condition could enhance consumer's health because of its nutrient composition. In absence of good manufacturing practice, the fruit juices become favorable medium for microbial growth and associated food borne pathogens (Tsiga, et al, 2008).

Several factors encourage or inhibit the growth of micro-organisms in juices. Some of these factors are water activity (aw), low pH, hygienic practice, storage condition and concentration of preservative (Jay, 1987). In some processed fruit juices, bacteria are the most diversified micro-organism causing its spoilage. The lactic acid bacteria such as Acetobacter and Acetomonas are reported to be the frequent spoilers of fruit juices (Fraizer and Westhoff, 1998).

Also, most fruit juices are acidic enough and have sufficient sugar to favour the growth of yeast. Moulds generally are considered to be the least important group of micro-organisms causing spoilage in fruit juices with the exception of Penicillum and Aspergillus spore forming organisms (Parish and Haggin, 1998). Some food borne diseases have been associated with the consumption of fruit products which were probably not hygienically processed or not adequately preserved. This research is therefore intended to analyze and identify the micro-organisms in mixed fruit juice prepared from pineapple, orange, paw-paw and guava with different concentration of preservative and stored for three months.

Materials and Methods

Collection of Materials

The fruits (pineapple, orange, paw-paw and guava) used for the mixed fruit juice production were purchased from Eke Ukwu main market in Owerri Municipal, Imo State.

Chemical Reagents

The chemical reagents of analytical grades used for the various analyses were obtained from the microbiological lab of Federal University of Technology, Owerri.

Method of Production of Mixed Fruit Juice

The different fruits were separately washed, peeled, cut, deseeded and fed into an already cleaned screw press. The juice of each fruit was pressed out separately, collected and filtered through a muslin cloth. The different juices obtained were blended in the proportion (pineapple 40%, orange 30%, paw-paw 20%, and guava 10%) according to the formula mostly accepted by the production guidance panel. The mixed fruit juice was preserved with different concentrations of sodium benzoate. The preserved mixed juice samples were filled into sterilized plastic containers and pasteurized at 80^{O}C for 10 minutes. They were then covered hot and cooled.

Generation of Samples

Different trials were done to produce some mixed juice formulations. These trial samples were presented to a twenty-man production guidance panel (PGP) to assess the mixed juice samples based on the quality parameters of colour, flavor and overall acceptability. Considering the comments of the PGP and the rating of each presented trial sample on a 7-point hedonic scale (where 7 stood for very acceptable and 1 stood for very rejectable).

The formula for the sample rated most acceptable by the PGP was used in the production of final experimental samples for this study. A trial of 10 liters of fresh mixed fruit juice was produced with this formula. Two and a half litres of this was poured into each four transparent bottles. To the juice contents of these bottles were added 0.0%, 0.04%, 0.06%, or 0.08% of sodium benzoate as preservative. These formed the four treatment samples. Each stock was filled into sterilized 300ml plastic transparent white bottles and pasteurized at 80^{O}C for 10 minutes. They were then corked hot and cooled in cold water. Each sample was left on the shelf and microbiologically analyzed when freshly prepared and after storage periods of one month and three months forming a total of twelve samples.

Microbiological analysis of samples

Microbiological analysis included enumeration and identification of microbes in the samples. The fruit juice samples were cultured using the spread plate method described by Uriah (2004) as the best for bacterial enumeration of food samples. The plates were incubated at 37^{O}C for 24 – 48 hours before observation for total viable count on nutrient Agar and total coliform on MacConkey Agar. The potato Dextrose Agar plates for yeast and moulds were incubated for 5 days. These plates were maintained at required temperature and time.

Total bacterial counts were done with Gallenkamp digital colony counter. The mean number of colonies counted was expressed as Colony Forming Units (CFU)/ml. Subculture was carried out to obtain pure isolates and discrete colonies. Identification of organisms was done based on morphological, biochemical and cultural characteristics. Bacterial isolates were analyzed for Gram character and their motility and various biochemical tests were performed by inoculating small portion of well-isolated colony into a series of media such as sugar fermentation, catalase test, coagulase test, citrate test, oxidase and indole tests (Uriah, 2004). Fungi isolates were characterized on the basis of pigmentation, sporulation, mycelia arrangement and microscopically (Abbey, 2007). The identities of the isolates were confirmed with reference to standard bacteriological and mycological manuals (Barnett and Hunter, 1987).

Identification of Fungi

The method described by Barnett et al, (1995) was used. A drop of 0.05% methylene blue was placed on a clean grease-free glass slide with the aid of a Pasteur pipette. The fungi cells from the PDA medium were picked with a sterilized teasing needle and placed on the slide with methylene blue. This was teased with the needle to release the spores. The preparation was covered with a cover slip and examined under low-power objective lens (X10 and X40)

Identification of Bacteria

Various tests were done on the bacteria isolates picked from the nutrient Agar in order to identify and clarify their physiology and morphological nature such as gram staining reactions and motility test. Biochemical tests such as catalase and indole tests were carried out and the various isolates were assigned to their probable genera using Bergey's manual determinative bacteriology (Buchanan and Gibbons, 1974). This was done by comparing characteristics of the isolates with those of the known species.

Gram Staining

In gram staining for morphological characteristics, smears of isolates were made on a clean grease-free slide which was allowed to air-dry and heat fixed by passing the slide over a gentle flame three times. The fixed slide was flooded with crystal violet dye for about

one minute and washed under running tap water. The slide was then flooded with Lugol's iodine solution. It was decolorized rapidly with alcohol and washed with water. It was then counter stained with safranin for 30 seconds, washed with water, blot dried and examined under the microscopic using oil immersion (X 100) objective.

Catalase Test

One drop of hydrogen peroxide was placed on a glass slide with Pasteur pipette. A colony of the cells was aseptically collected with a sterile glass rod and placed on the slide; it was well mixed and observed for bubbles of gas on the slide. (Evolution of bubbles of gas shows catalase positive which indicates the presence of an enzyme that catalysis the release of oxygen from hydrogen peroxide).

Coagulase Test

One drop of distilled water was placed on a clean slide and an isolated colony was picked with a sterile wire loop and placed on the slide. A drop of plasma was mixed with the bacteria suspension and examined for visible clumping within 10 seconds. Clumping indicates that the organism possesses the enzymes coagulase, which coagulates plasma.

Motility Test

A ring was made on the central part of a slide using a plaster seal. Then a drop of an overnight broth culture was placed on the cover slip and the man slide was placed on the cover slip ensuring that the dropped culture did not come in contact with the slide. The slide was inverted, so it was viewed through the cover slip under the microscope (X 10 and X 40). When the bacteria are motile, there will be vertical or downward movement of organisms.

Citrate Utilization Test

Simmons citrate agar was prepared by dissolving 3gm of the powder in 100ml of distilled water (by water bath heating) and distributed into 5ml-volume bijon bottles, sterilized at $121^{O}C$ and 15psl for 15 minutes. The agar slant was inoculated with the test organism and incubated at $37^{O}C$ for 24 hours. It was observed for colour change in agar (to blue). Any colour change indicates that the organism utilizes citrate as the sole carbon source.

Indole Test

This was done to test the ability of the organisms to convert tryptophan amino acid to indole. A 5ml portion of overnight peptone water-both culture of the isolates was introduced into a test tube. Then 0.5ml of kovac's reagent was added. A pink or red colour indicates indole positive.

Oxidase Test

Two (2) drops of oxidase reagents (1% aqueous tetra-p-phenyl-nedianuene solution) was dropped on a filter paper using Pasteur pipette. The isolated colony was picked with a sterile wire loop, which was also used for the streaking of the isolated organism. It was then examined after few seconds. A positive oxidase reaction turns the filter paper to dark purple within 10 seconds while negative result retains the original paper colour.

Carbohydrate Fermentation Test

Some 1% sugar solutions (glucose, sucrose, lactose) were prepared using peptone water in some test tubes containing inverted Durban tubes. These were inoculated with the isolates and incubated at $37^{O}C$ for 24 hours. The contents were examined for acid and gas production as indication of the presence of sugar fermenting bacteria.

Results and Discussion

The impact of preservative concentration on the total microbial counts of the mixed fruit juice samples.

The total microbial counts for the freshly prepared samples (with preservative) ranged from 1.2×10^3 to 2.9×10^3 cfu/ml (table 1). These variations in values could not be explained besides equipment/handling contamination, though all the fresh samples had very acceptable level of microbial load. The samples with 0.04% sodium benzoate, had higher rate of microbial growth, with bacterial count increasing from 2.9×10^3 to 2.6×10^4 at one month storage period and to 3.5×10^5 at 3 months storage period as compared to the samples preserved with 0.06% whose bacterial count increased from 1.6×10^3 to 6.0×10^4 and 0.08% sodium benzoate whose bacterial count was relatively stable, increasing from 1.2×10^3 to 3.8×10^3 at one month storage period and to 4.8×10^3 at 3 months storage period. It was observed that the 0.08% level of sodium benzoate effectively checked fungal growth for the storage period studied. These samples maintained the total fungal load of 1.0×10^1 CFU/ml up to 3 months storage period. Thus, this preservative level should be recommended for the preservation of this product for its effectiveness in controlling both total bacterial and fungal growth in the product. This view was held because the maximum total microbial count recommended for single strength and mixed juice samples is 1.0×10^5 Cfu/ml, (Hatcher et al, 1992). The control sample which had no added preservative (sodium benzoate) had the highest bacterial load among all the samples and at periods of storage. It increased from 3.5×10^3 in the freshly produced mixed juice to 7.5×10^5 at one month storage to 9.8×10^6 at 3 months storage. This showed that the freshly produced mixed juice can be taken without preservative within few hours of production, but becomes unsafe when stored since the bacterial load are above the acceptable microbial load level (1.0×10^5 cfu/ml) in fruit juices.The bacteria identified in the freshly produced mixed fruit juice samples for all levels of treatment (preservative) were *lactobacillus specie* (table 2). *Staphylococcus aureus* was identified in the

control and 0.04% benzoate treated samples stored for 1 and 3 months. While lactobacillus is a common bacteria used in lactic acid fermentation, the incidence of *Staphylococcus Aurues* in these other samples is considered a safety risk since it had been associated with food borne illnesses (Peng et al, 2001).Penicillum, a food spoilage organism was also identified in the samples preserved with 0.4% and0.6% sodium benzoate. This suggests that the fruit juice product for storage, required up to 0.08% sodium benzoate (as preservative) to be shelf-stable and wholesome. The non-identification of any coliform or pathogens in the mixed juices preserved with 0.08% sodium benzoate was not surprising since; Hatcher et al (1992) reported that the pH of most fruit juices (< 4.0) is usually too low for the growth of pathogenic bacteria. *Saccharomyces cerevisiae* was identified in all the fruit juice samples (table 3). This confirmed a report by Ray (1996) that varieties of *Saccharomyces cerevisiae* were the most common yeasts in fermented foods and beverages based on fruit and vegetables. The fungi identified in the stored control samples were *Saccharomyces cerevisiae, Penicillum,* and *Candida* species. The isolation of *Saccharomyces cerevisiae, Penicillum, Rhodotorula* and *candida* species in fruit juices was reported by Mathews and Monthville (2000). They stated that *candida* was responsible for spoilage of apple under study. This could also be responsible for the off flavor observed in the stored control samples of the mixed fruit juice.

Table 1: The total microbial count for mixed juice samples

Samples	Fresh (not stored) (Cfu/ml)		1 Month (Cfu/ml)		3 Months (Cfu/ml)	
	Bacteria	Fungal	Bacterial	Fungal	Bacterial	Fungal
Mixed juice with 0.0% sodium benzoate	3.5×10^3	4.0×10^1	7.5×10^5	7.6×10^2	9.8×10^6	8.0×10^2
Mixed juice with 0.4% sodium benzoate	2.9×10^3	3.1×10^1	2.6×10^4	4.2×10^2	3.5×10^5	5.6×10^2
Mixed juice with 0.6% sodium benzoate	1.6×10^3	2.0×10^1	4.2×10^3	3.5×10^1	6.0×10^4	4.8×10^1
Mixed juice with 0.8% sodium benzoate	1.2×10^3	1.0×10^1	3.8×10^3	1.0×10^1	4.8×10^3	1.0×10^1

Table 2: Bacteria identified in the mixed juice samples

Samples	Fresh	1 Month	3 Months
Mixed juice with 0.0% sodium benzoate	*Lactobacillus specie*	*Lactobacillus fermentum Staphylococcus Aurues*	*Lactobacillus fermentum Staphylococcus Aurues*
Mixed juice with 0.04% sodium benzoate	*Lactobacillus specie*	*Lactobacillus fermentum Staphylococcus Aurues*	*Lactobacillus fermentum Staphylococcus Aurues*
Mixed juice with 0.6% sodium benzoate	*Lactobacillus specie*	*Lactobacillus specie*	*Lactobacillus specie*
Mixed juice with 0.8% sodium benzoate	*Lactobacillus specie*	*Lactobacillus specie*	*Lactobacillus specie*

Table 3: Fungi identified in the mixed juice samples

Samples	Fresh	1 Month	3 Months
Mixed juice with 0.0% sodium benzoate	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae Penicillum Candida specie*	*Saccharomyces cerevisiae Penicillum Candida specie*
Mixed juice with 0.04% sodium benzoate	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae Penicillum*	*Saccharomyces cerevisiae Penicillum*
Mixed juice with 0.6% sodium benzoate	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae Penicillum specie*	*Saccharomyces cerevisiae Penicillum specie*
Mixed juice with 0.8% sodium benzoate	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*

Table 4: Fungal morphology on potato dextrose agar (pda) of mixed juice

Samples/Source	Colour	Microscopic Observation	Fungal Isolates
Fresh juice with 0.04% and 0.06% sodium benzoate	Creamy smooth colonies on PDA	Spherical ascospores with rounded ends formed	***Saccharomyces spp.***
1 month storage	White cotton-like colonies on PDA	Branched conidiosphere with bush like conidial head seen	***Penicillium spp.***
	Creamy smooth colonies on PDA	Spherical ascospores with rounded ends formed	***Saccharomyces spp.***
3 months storage	White cotton-like colonies on PDA	Branched conidiosphere with bush like conidial head seen	***Penicillium spp.***
	Creamy smooth colonies on PDA	Spherical ascospores with rounded ends formed	***Saccharomyces spp.***
Juice with 0.08% sodium benzoate	**Creamy smooth colonies on PDA**	**Spherical ascospores with rounded ends formed**	***Saccharomyces spp.***

Table 5: Physiological and biochemical properties of bacterial isolates from mixed juice preserved with sodium benzoate

Sample/Source	Colonial morphology	Gram reaction	Motility	Catalase	Coagulase	Citrate	Oxidase	Indole	Glucose	Sucrose	Lactose	Gas	Probable organisms
Control and 0.04%	Milky,bunched colonies on NA	+ve cocci in clusters	-ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	***Staphylococcus***
Fresh juice with 0.06% and 0.08%	White rough colonies on NA	+ve Rods in chains	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	***Lactobacillus fermentum***
1 month juice	White rough colonies on NA	+ve Rods in chains	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	***Lactobacillus fermentum***
3 months juice	**White rough colonies on NA**	**+ve Rods in chains**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**+ve**	**+ve**	**+ve**	**+ve**	***Lactobacillus fermentum***

Conclusion

The results of this study revealed that the concentration of preservative affects the nature and microbial load of micro-organisms found in fruit juices during storage. The bacterial and fungal load of mixed fruit juice preserved with 0.04% sodium benzoate increased from $2.9X10^3$ and $3.1X10^1$ in fresh sample to $3.5X10^5$ and $5.6X10^2$ after three months of storage respectively. The micro-organisms identified were *Lactobacillus fermentum, Saccharomyces cerevisiae* and *Penicillum species*. The bacterial and fungal load in samples preserved with 0.08% sodium benzoate were $1.2X10^3$ and $1.0X10^1$ in fresh sample and $4.8X10^3$ and $1.0X10^1$ after three months of storage respectively. The organisms identified in this sample after three months storage were *Lactobacillus fermentum* and *Saccharomyces cerevisiae*, which have not been associated with any food borne disease. The concentration level of 0.08% sodium benzoate is considered adequate for preservation of fruit juices for the storage period of three months.

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