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Full Length Research Paper

Perspectives in the hurdle techniques in the preservation of a non alcoholic beverage, zobo

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The effect of some commonly used chemical preservatives (acetic acid and sodium benzoate), natural plant extracts (clove, garlic, ginger and lime) and pasteurization on the elongation of shelf-life of zobo beverage were investigated by determining total counts and characterization of microorganisms before and during storage at ambient temperature for 14 days. Five bacterial species, namely *Staphylococcus aureus, Micrococcus leteus, Micrococcus rosues, Bacillus subtilis* and *Enterococcus faecalis* were predominant isolates. Two fungal species, namely, *Saccharomyces cerevisiae* and *Rhizopus stolonifer* were also isolated. *Bacillus subtilis* and *Saccharomyces cerevisiae* remained the predominant organisms throughout the storage. The role played by some of the isolates in food spoilage, deterioration and intoxication had been discussed. The microbial population increased for two days as the organisms utilize the nutrients present in the sample. There was a drastic reduction in microbial load as the effects of the preservative became evident, except for the control sample. Antimicrobial activities of the preservatives revealed that samples with chemical preservatives were most effective against bacteria and fungi species, followed by the natural plant extracts samples. Pasteurization confers temporary preservation as its effect was short lived. The phytochemical and antimicrobial activities of the plants used had been reported. Of all the samples, only those treated with chemical preservatives remained organoleptically attractive after preservation for 14 days. The overall reduction in the microbial population could be a concerted effort of the preservatives and exhaustion of nutrients in the zobo drink. The study recommends the use of chemical and natural preservatives to enhance the keeping quality of zobo beverage which conforms to good manufacturing practices globally.

Keywords: Hurdle, techniques, zobo, beverage.

INTRODUCTION

Zobo, a non-alcoholic beverage popularly consumed in northern Nigeria (Aliyu, 2000; Ogiehor and Nwafor, 2004), is produced from the dried calyces of the rosell plant *Hibiscus sabdariffa* by boiling and filtration. It is gaining wide acceptance, being consumed by several millions of people from different socio-economic classes and background. Zobo drink has been shown to be good source of natural carbohydrate, protein and vitamin C which constitutes the major reason for consuming soft drink and fruit juice (Ogiehor and Nwafor, 2004).

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In spite of the increasing popularity of Zobo beverage, one of its limitations for large scale production is that it deteriorates rapidly. The drink contains microorganisms which can cause food spoilage (Omemu *et al*., 2006). Several groups of microorganisms (*Bacillus, Streptococcus, Staphylococcus, Leuconostoc, Lactobacillus, Aspergillus, Penicillium, Geotrichum, Fusarium and Alternaria*) have been associated with zobo beverage during storage (Akinyosoye and Akinyele, 2000; Ogiehor and Nwafor, 2004). Some health implications of these spoilage microorganisms include: food poisoning, food intoxication and sometimes death in severe cases. At present, the production process is neither standardized nor mechanized. The proliferation of the associated micro-organisms potentiates spoilage and the short shelf life

of 1-4 days (Ogiehor and Nwafor, 2004) associated with this relish beverage. Considering the increasing acceptance, socio-economic potentials and ready sources of vitamin C, the need to enhance and extend the shelf life by an effective means becomes imperative.

It is possible to improve the shelf life zobo beverage by slowing down the fermentation process, thus inhibiting discoloration of the zobo drink and possibly permitting large-scale production and preservation for a longer period with maximum retention of nutritive values. Ilondu and Iloh (2007), Akpomedeya and Ejechi (1998) Ogiehor *et al*. (1998), Akinyosoye and Akinyele (2000) have demonstrated different methods of zobo preservation.

The deleterious role of *Aspergillus niger* and *Penicillium citrinum* in low pH and high sugar content foods have been reported (Efiuwevwere and Akoma, 1997; Prescott *et al*., 2002). The use of chemical preservatives at low allowable concentrations to control the growth of microorganisms in beverages is desirable and gaining research interest worldwide (Efiuwevwere and Akoma, 1997).

The effect of plant and spice extracts and salt of sodium on the growth of *Aspergillus* spp. and related fungi are well documented (Kreb *et al*., 1983) but not enough literature is available on zobo drink.

This study is aimed at developing basic data and indices for the production and handling of zobo drink and possibly controlling the microbial activities associated with zobo drink while maintaining the nutritive and economic quality, thus enhancing the shelf life of the beverage.

MATERIALS AND METHODS

Collection and preparation

Matured dried reddish-purple petals of *Hibiscus sabdariffa* used was obtained from the Imo State botanical garden and identified by a plant taxonomist and horticulturist. Zobo drink was prepared in the laboratory by the methods of Ogiehor and Nwafor (2004) and Adenikpekun (1998).

Preparation of preservatives

Four natural plant materials (cloves, garlic, ginger and lime) and two chemical preservatives (acetic acid and sodium benzoate) were used in this study. Physical method (pasteurization) was also employed. Sample without any preservatives served as the control.

Plants materials and chemical preservatives were prepared in 2ml w/v and 2ml v/v concentration respectively. Pasteurization was maintained at 72^0C for 5 seconds (Perry and Staley, 1997).

Inoculation of zobo beverage with preservatives

Ninety eight milliliters (98ml) of freshly prepared zobo was dispensed into 100ml previously sterilized Uniscope conical flask. Two milliliters (2ml) of the preservatives (natural and chemical) were added into the flask. One set of the sample was pasteurized in a water bath. All the samples were kept on an electrically controlled shaker at ambient temperature.

Microbiological analysis of samples

One milliliter of each sample was transferred into sterile 9ml peptone water and diluted decimally. Aliquot portions (0.1ml) of the 6th and 7th dilutions were inoculated onto freshly prepared potato dextrose agar and nutrient agar respectively. The plates were spread evenly and incubated at ambient temperature for 24h (Cheesbrough, 2002). This procedure was repeated concurrently for one week and subsequently after one week, covering a total of 336 hours. Figure 1.

Enumeration and characterisation of bacteria and fungi

Total bacteria count was done using Gallenkemp colony counter. Fungal count was done with the aid of a hand lens. The total numbers of viable microorganisms (colonies) developed were enumerated and expressed as colony forming unit per milliliters (cfu/ml) by the methods of Harrigan and McCance (1990).

The characterization and identification of bacteria and fungal isolate were based on the colonial morphology, staining reaction (gram staining) and standard biochemical tests, as described by Kregervan (1984), Sneath *et al.* (1986), Claus (1991), Harrigan and McCance (1990) Barnett and Hunter (1987), Fawole and Oshe (2002) and Cheesbrough (2002).

Yeasts were identified using the yeast identification program of physico-chemical analysis and their ability to ferment certain fermentable sugars as well as their ability to grow aerobically with various compounds each as sole source of carbon and nitrogen at room temperature ($29^{\pm}1^{o}$C) on laboratory tables (Barnett *et al*., 1994).

RESULTS

Tables 1 and 2 shows respectively the total counts and characteristics of bacteria and fungi isolated from freshly prepared zobo drink. Four species of bacteria and five species of fungi were isolated from the untreated sample (Tables 1 and 2). Total bacteria and fungi counts of preserved and control samples are recorded in Tables 3 and 4 respectively. Total population

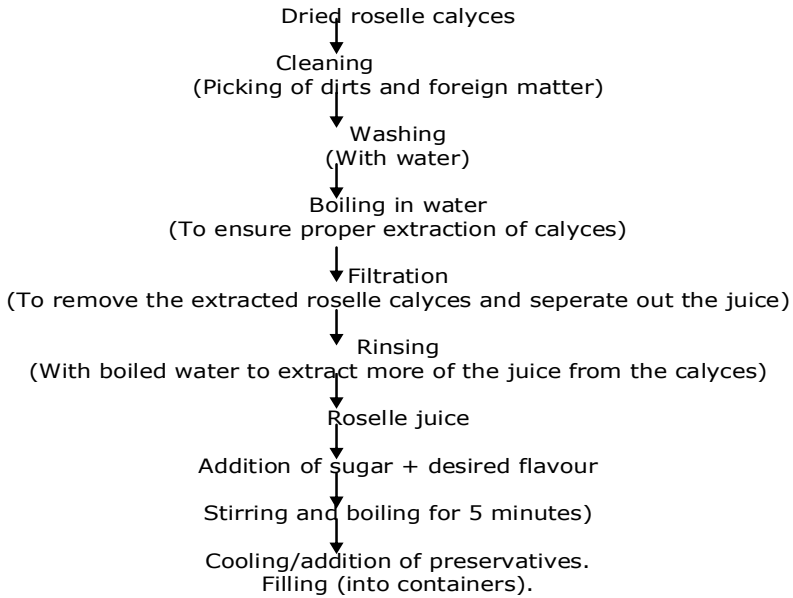

Figure 1. Flow chart for the production of Zobo beverage

Table 1. Total colony count and colonial characteristics of heterotrophic bacteria isolated from sample before treatment

Colony code	total count	colonial characteristics	microscopic morphology	Cat	Oxi	Coag	In	MR	VP	Cit	Mot	G	S	MN	L	F	Identity of isolate
Zb1		smooth circular small Yellow colonies	+S	+	-	-	-			+	-	-	-	-	-	-	*Micrococcus luteus*
Zb2		smooth circular small golden yellow colonies	+S	+	-	+	-			-	-	+	+	+	+	+	*Staphylococcus aureus*
Zb3		flat irregular shaped cream dry colonies	+R	+	-	-	-			+	+	+	+	+	+	+	*Bacillus subtilis*
Zb4	5.7×10^{8}	small circular shiny cream colonies	+S	-	-	-	-			+	-	+	+	+	+	+	*Enterococcus faecalis*

Cat, catalase; Oxi, oxidase; Coag, coagulase; In, indole; MR, methyl red; VP, Voges Proskaeur; Cit, citrate;Mot, motility; G, glucose; S, sucrose; L, lactose; MN, mannitol; F, fructose

Table 2. Total heterotrophic counts and characteristics of fungi isolated from sample before treatment

Colonial characteristics	Microscopic characteristics	dentity of isolates	Counts Cfu/ml
Dirty green powdery colonies	septate hyphae. Conidia attached to sterigma	*Penicillium caseicolum*	
Small black spores on short white Hyphae	septate hyphae. Conidia arranged like mob-head	*Aspergillus flavus*	
Creamy butyrous circular dull and dry colonies	gram positive ellipsoidal budding cells	*Saccharomyces cerevisiae* var *ellipsoideus*	
Small creamy white circular and shiny colonies	gram positive oval and spherical budding cells	*Saccharomyces cerevisiae*	
Tall white filamentous hyphae covering entire culture plate	non- septate hyphae. Spores enclosed in a sporangium	*Rhizopus stolonifer*	
Short dirty white mycelia	non- septate hyphae. Sporangiophore septate	*Mucor* sp	
Fine white cotton wool like hyphae slightly raised	septate hyphae. Conidia arranged like sickle cell	*Fusarium* sp	2.01×10^{9}

Table 3. Total bacteria counts on preserved zobo samples and Total bacteria counts (cfu/ml)

Duration of preservation (hours)	A	B	C	D	E	F	G	H
24	1.7×10^{10}	2.9×10^{9}	NG	2.01×10^{10}	TNTC	NG	5.1×10^{10}	2.3×10^{8}
48	2.5×10^{10}	2.75×10^{10}	2.31×10^{10}	2.76×10^{10}	2.30×10^{10}	1.60×10^{10}	TNTC	TNTC
72	2.60×10^{10}	1.92×10^{10}	1.76×10^{10}	4.64×10^{10}	1.89×10^{10}	TNTC	2.26×10^{10}	2.24×10^{10}
96	1.5×10^{9}	2.03×10^{10}	TNTC	5.2×10^{9}	TNTC	3.0×10^{8}	1.1×10^{9}	4.1×10^{9}
120	2.12×10^{10}	8.0×10^{8}	TNTC	9.4×10^{9}	1.0×10^{10}	1.8×10^{8}	1.7×10^{10}	2.0×10^{9}
144	1.60×10^{10}	2.90×10^{10}	1.62×10^{10}	5.2×10^{9}	9.9×10^{9}	6.6×10^{9}	1.9×10^{9}	2.4×10^{9}
168	7.5×10^{9}	4.6×10^{9}	3.55×10^{10}	8.6×10^{9}	6.6×10^{9}	4.1×10^{9}	NG	2.2×10^{9}
336	2.0×10^{8}	2.31×10^{9}	3.0×10^{9}	2.14×10^{9}	3.0×10^{9}	1.0×10^{8}	NG	1.6×10^{9}

NG, no growth; TNTC, too numerous to count;A,garlic;B,ginger; C,pasteurisation;D,control; E,clove;F,acetic acid;G,sodium benzoate; H,lime

Table 4. Total fungi counts on preserved zobo samples and Total fungi counts (cfu/ml)

Duration of preservation (Days)	A	B	C	D	E	F	G	H
24	NG	NG	6.0×10^{7}	4.9×10^{9}	NG	NG	NG	1.6×10^{8}
48	1.0×10^{7}	3.4×10^{8}	1.7×10^{8}	9.7×10^{8}	4.0×10^{7}	TNTC	8.0×10^{17}	TNTC
72	8.0×10^{7}	3.55×10^{9}	1.4×10^{8}	2.08×10^{9}	1.2×10^{8}	1.5×10^{8}	2.0×10^{7}	1.7×10^{7}
96	NG	3.8×10^{9}	1.0×10^{9}	2.08×10^{9}	1.0×10^{9}	3.0×10^{7}	8.0×10^{7}	2.0×10^{9}
120	TNTC	1.60×10^{10}	TNTC	2.44×10^{9}	8.0×10^{9}	NG	1.50×10^{10}	TNTC
144	4.7×10^{8}	1.77×10^{9}	2.53×10^{9}	3.68×10^{9}	3.0×10^{8}	1.8×10^{8}	7.0×10^{7}	TNTC
168	4.0×10^{8}	4.76×10^{9}	1.48×10^{9}	TNTC	1.8×10^{8}	4.5×10^{8}	TNTC	TNTC
336	4.0×10^{8}	2.17×10^{9}	1.8×10^{8}	TNTC	1.0×10^{8}	4.0×10^{7}	8.0×10^{7}	TNTC

Table 5. Distribution of bacteria isolated from preserved samples

Bacterial isolates	Garlic	Ginger	Pasteurization	control	Clove	Acetic acid	Sodium benzoate	Lime
M. luteus	+	++	+	+++	++	+	+	+
M. roseus	+	+	+	+++	+	-	-	+
B. subtilis	+	++	+++	+++	++	+	+	+
S. aureus	+	+	+	+++	+	-	+	-
Ent. faecalis	+	+	+	+++	+	-	-	+

-, no growth; +, scanty growth; ++, moderate growth; +++, copious growth

Table 6. Distribution of fungi isolated from preserved samples

Fungal isolates	Garlic	Ginger	Pasteurization	control	Clove	Acetic acid	Sodium benzoate	Lime
R. stolonifer	-	+	+	+++	++	++	-	++
A. flavus	-	+	+	++	++	++	-	++
F. poae	-		+	++	-	-	-	-
Mucor sp	+	+	+	++	+	++	+	+
Sacch. cerevisiae	++	++	++	++	++	+	+	+
Sacch. ellipsoideus	++	++	++	++	++	+	+	+
P. caseicolum	-	+	+	++	+	++	-	++

counts increased with days of fermentation, but decreased gradually except the control sample. The distribution of the bacteria and fungi across the treated and control samples is shown in Tables 5 and 6 respectively. Samples preserved with acetic acid, sodium benzoate, lime and garlic recorded low/limited presence of bacteria, whereas samples preserved with garlic and sodium benzoate recorded low presence of fungi. *Bacillus* and *Saccharomyces* species dominated in both the preserved and control samples.

DISCUSSION

The zobo juice produced from the calyces of *Hibiscus sabdariffa* was very acidic with low pH values as was reported by Frazier and Westhoff (1986). The high acidity of the juice could account for the low numbers and few types of organisms isolated from the fresh preparation (Tables 1-2). There was a gradual increase in the population asthe organisms utilize nutrients from the zobo beverage and some of the preservatives (Tables 3 and 4). The effect of the different preservative on the microbial growth was noticed as the population decreased during storage (Tables 3 and 4).

Zobo drink when left for two or three days at room temperature turns sour. This may result from fermentation due to microbial action. This fermentation process has been found to lead to loss of taste and nutritional value, increased rate of browning and offensive odour, and perhaps presence of cloudy materials at the bottom of the container (Adenipekun, 1998).

Results obtained showed that zobo drink, raw or preserved supports the growth and proliferation of a wide variety of microorganisms. Some of the isolates have been found to be associated with food spoilage and intoxication (Stainer *et al*., 1987; Prescott *et al*., 1999) and portends health risk to consumers. Omemu *et al*. (2005) suggested that the presence of microorganisms in zobo drink produced with a boiling method is indicative of post-production contamination during the addition of sugar and other additive(s). *Bacillus subtilis*, a spore former can withstand adverse effects of the preservative(s). Spores are extraordinarily resistant to environmental stress such as heat, ultraviolet radiation, chemical disinfectant and desiccation (Prescott *et al*., 1999; Prescott *et al*., 2002).

Though the count of viable microorganisms was too high to be acceptable in a drink, the count was generally low compared with count of microflora in related food materials (Frazier and Westhoff, 1986; Prescott *et al*., 2002). From the result, it can be deduced that the chemical and natural preservative used were effective against the microorganisms. This is shown by the reduction in bacterial count over time, which contrasted in the case of the sample without preservative. Investigation of the antimicrobial activity of the preservatives revealed that sodium benzoate was the most effective on both fungal and bacterial species. This was evident as sample preserved with sodium benzoate had the lowest bacterial count. Kreb *et al*. (1983) had reported on the antifungal action of sodium benzoate.

Pasteurization as a form of preservation of zobo beverage slowed down the microbial growth in the sample since it is not intended to kill all the organisms in the zobo beverage but aimed

to reduce the number of viable pathogens which are likely to cause diseases (Grant, 2002).

Preservative have been used to store food substances and they act by inhibiting, retarding or arresting the growth of microorganisms or of any such deterioration resulting from their presence or of masking the evidence of any such deterioration (Ihekoronye and Ngoddy, 1995; Fawole and Oshe, 2002). To be in accordance with good manufacturing practice (GMP), the use of preservative should not adversely affect the nutritive value of foods or should not permit the growth of food poisoning organisms while suppressing the growth of others that would make spoilage evident (Ihekoronye and Ngoddy, 1995).

Sodium benzoate maintained the sweet taste and red colour characteristic of the zobo beverage throughout the period of the storage, and had more keeping quality than the other preservative methods. Acetic acid preserved sample turned dark red, bacame sour but retained the strong pungent smell characteristic of acetic acid. The shelf-life can thus be extended by the use of preservatives or storage at conditions that will not favour bacterial multiplication (Pelczar *et al*., 2002). Unpreserved samples, however, exhibited the highest bacterial and fungal count as the storage lasted and consequently, had the poorest keeping quality when compared to those with preservatives.

Most of the microbial isolates are natural inhabitants of soil, water and vegetations. *Bacillus* and *Saccharomyces* species are predominant is the samples analyzed suggesting their ubiquity and ability to survive in extreme conditions including low nutrient availability (Tables 5 and 6).

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