

Full Length Research Paper

Effect of sodium benzoate on the growth and enzyme activity of *Aspergillus niger* and *Penicillium citrinum* in Zobo drink during storage at $30 \pm 2^\circ\text{C}$

O. E. Nwafor^{1*} and M. J. Ikenebomeh²

¹Department of Microbiology, Delta State University, Abraka, Nigeria.

²University of Benin, Benin City, Nigeria.

Accepted 13 March, 2009

The effect of different concentrations of sodium benzoate on the growth and enzyme elaboration potentials of *Aspergillus niger* and *Penicillium citrinum* in zobo drink packaged in glass bottles were investigated. All the tested concentrations of 0.025, 0.05 and 0.075% (w/v) caused decreases in the counts of *A. niger* and *P. citrinum* at $30 \pm 2^\circ\text{C}$. Concentration of 0.05% (w/v) of the preservative hurdle showed complete inhibition of *A. niger* in the 4th week whereas the same effect was observed in the 5th week on *P. citrinum*. Enzyme activity in the control experiments was significantly higher ($P < 0.05$) than those of treated and fresh Zobo samples.

Key words: Sodium benzoate, enzyme activity, Zobo drink.

INTRODUCTION

Zobo drink is produced from flowers of *Hibiscus sabdariffa*. It is a refreshing drink cherished in many parts of Nigeria and some other African countries. The short shelf-life of about 1 - 4 days is however a major problem faced by producers and consumers of the product (Ogiehor and Nwafor, 2004). The short shelf-life is primarily due to objectionable changes induced by microbial activities as in many other beverages (Efiuvwevwe and Akoma, 1997). To control these microbial activities requires appropriate preservation methods. The use of low temperature which is the method of choice has not proved effective since it is limited by cost of refrigeration and energy.

The deleterious role of *Aspergillus niger* and *Penicillium citrinum* in low pH and high sugar content foods have been reported (Ekundayo, 1984; Ogiehor, 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 (Shelef and Addala, 1994; Ogiehor and Ikenebomeh, 2004). The use of low concentrations of chemical preservatives

either singly or in combinations will remove the risk associated with them (Efiuvwevwe and Isaiah, 1998; Leistner, 2000). Furthermore, the involvement of *A. niger* in the elaboration of the enzyme which caused undesirable changes in the physio-chemical quality of zobo has been reported. The effect of plant and spice extracts and salt of sodium on the growth of *Aspergillus* sp. and related fungi are well documented (Nwafor et al., 1998; Amadi et al., 2007; Idise, 2007) but not enough literature is available on zobo drink. This is in spite of the popularity it is beginning to enjoy and the spoilage experienced by producers and consumers. This study is therefore to determine the effect of sodium benzoate on the growth and enzyme production potentials of *A. niger* and *P. citrinum* following deliberate contamination (challenges test) of zobo drink samples packaged in glass bottles at $30 \pm 2^\circ\text{C}$.

MATERIALS AND METHODS

Preparation Of Zobo drink

Zobo drink was prepared following the recipe in Magi family menu cook book (1996). The processed Zobo drink was divided into three portions and treated with different concentrations, 0.025, 0.05 and 0.075% (w/v), of sodium benzoate (BDH, England). They were thereafter packaged in glass bottles and held at $30 \pm 2^\circ\text{C}$.

*Corresponding author. E-mail: obiomanwafor@yahoo.com.
Tel.: 08035878792.

Inoculation of Zobo drink samples

Stock cultures of *A. niger* and *P. citrinum* isolated from stale Zobo drink during storage and identified using morphological and cultural characteristics (Bounds et al., 1993) were reconstituted according to the method of Harrigan and McCance, 1976. The inoculum size 2.11 and 2.26 log₁₀ cfu/ml for *A. niger* and *P. citrinum* respectively was determined by pour plate method on yeast malt agar (Oxoid) supplemented with chloranphenicol. The Zobo drink samples (20 ml) in 100 ml flasks were inoculated with 1ml of 48 h old broth cultures of *A. niger* and *P. citrinum* in turn, aseptically sealed with hand sealing machine (Super master, England) and kept at 30 ± 2°C for a period of 8 weeks. Microbial growth was monitored at weekly intervals while enzyme activities were assessed at the beginning and at the end of the storage period.

Microbiological analysis of Zobo Drink

Viable counts in the various samples of Zobo drink were determined as described by Vandezant et al. (1997). A 1 ml portion of the sample was aseptically added to 9 ml of 0.10% (w/v) peptone water and allowed to stand for 3 min with occasional stirring. This was serially diluted (1:10) and viable count enumerated by pour plate method on a yeast malt agar (Oxoid) and incubated according to manufacturer's instructions. The colonies that developed were enumerated and expressed as colony forming unit per milliliters (cfu/ml).

Determination of enzyme activity in Zobo drink

Enzyme activity was determined following the method of Odibo et al. (1990). In this, 10 ml of fresh sample, sample treated with 0.05% (w/v) sodium benzoate and challenged, and untreated sample inoculated with the microorganisms (control) were added to 40 ml of 0.10 M phosphate buffer in different 100 ml conical flasks with stirring. They were thereafter filtered through Whatman's No. 1 filter paper. The crude enzyme filtrate was used for enzyme activity determination.

Amylase activity determination

The method described by Plummer (1978) was employed by adding 2 ml of the crude enzyme to 1 ml of 1% (v/v) soluble starch (Hopkin and Williams Ltd). The mixture was incubated at 37°C for 15 min. The optical density (OD) of the solution was read at 600 nm using SP-30 UV spectrophotometer (Pye Unicam, Cambridge). Amylase activity was read off from a standard amylase activity curve. It was assumed that one unit of enzyme activity is the amount of enzyme that will produce a reducing sugar corresponding to 1 mg of glucose from starch in 1 min under the assay conditions.

Protease activity

Protease activity was estimated following the procedure of Lowry et al. (1951) modified by Plummer (1978). Different concentrations (0.20, 0.40, 0.60, 0.80, 1.0 and 2.0 mg/ml) of bovine serum albumen (BSA) were prepared in different test tubes. To 1 ml portion of each was added 1 ml of the crude enzyme solution and incubated at 37°C for 30 min. Thereafter the OD of the preparations was read at 700 nm from SP-30UV spectrophotometer (Pye-Unicam, Cambridge). Protease activity was extrapolated from standard protease activity curve. Protease activity was estimated to be the amount of enzyme that liberates 1 mg of amino acid in 1 min under experimental conditions.

Lipase activity

Lipase activity was determined using 0.1 M phosphate buffer at pH 8.0 and olive oil as the substrate. Fatty acid liberated after incubation at 37°C for 50 min was determined by titration with 0.05 M NaOH using phenolphthalein indicator. One unit of lipase activity is defined as the amount of enzyme capable of releasing 1 mg of oleic acid in 1 min under the assay conditions.

Statistical analysis

The data obtained were subjected to statistical analysis of mean, standard deviation and analysis of variance (ANOVA). The significant value was determined by t-distribution test using appropriate computer software (Ogbeibu, 2005).

RESULTS

The result of the effect of different concentrations of sodium benzoate on the growth of *A. niger* and *P. citrinum* are presented in Figures 1 and 2. The various concentrations inhibited the growth of the test organisms from the first week up to the 6th week and throughout the period of storage. The least concentration 0.025% (w/v) inhibited the growth of *A. niger* in the 5th week whereas the same effect was observed in the 6th week with *P. citrinum*. A similar trend was observed with the highest concentration of 0.075% (w/v), which inhibited the growth of *A. niger* in the 4th week and *P. citrinum* in the 5th week of storage. In the control experiments, there was a steady increase in the population of the organisms up to the 7th week. Thereafter a decrease in population was observed.

Amylase, protease and lipase activities in Zobo drink preserved with 0.05% (w/v) sodium benzoate after storage for 8 weeks is shown in Table 1. The treated and challenged sample showed activities of 3.50 ± 0.02, 2.50 ± 0.01 and 0.60 ± 0.2 µg/ml for amylase, protease and lipase respectively for *A. niger*. For *P. citrinum*, the values obtained were 3.60 ± 0.02, 2.50 ± 0.3 and 1.4 ± 0.3 µg/ml for amylase, protease and lipase respectively. No enzyme activity was observed in the fresh sample. The control experiments showed activities of 18.60 ± 0.01, 8.40 ± 0.2 and 1.50 ± 0.2 µg/ml for amylase, protease and lipase respectively for *A. niger*, and 18.90 ± 0.2, 8.60 ± 0.2 and 1.70 ± 0.2 mg/ml for *P. citrinum*. These values are significantly different ($P < 0.05$) from those of the treated and fresh samples.

DISCUSSION

This study has indicted the efficacy of sodium benzoate as an antimicrobial agent against the growth and survival of *A. niger* and *P. citrinum* in Zobo drink. The decrease in the population of microorganisms may be due to their presence in an unfavourable micro-environment created by sodium benzoate. Sodium benzoate may have created hurdles, which the organisms could not overcome. This

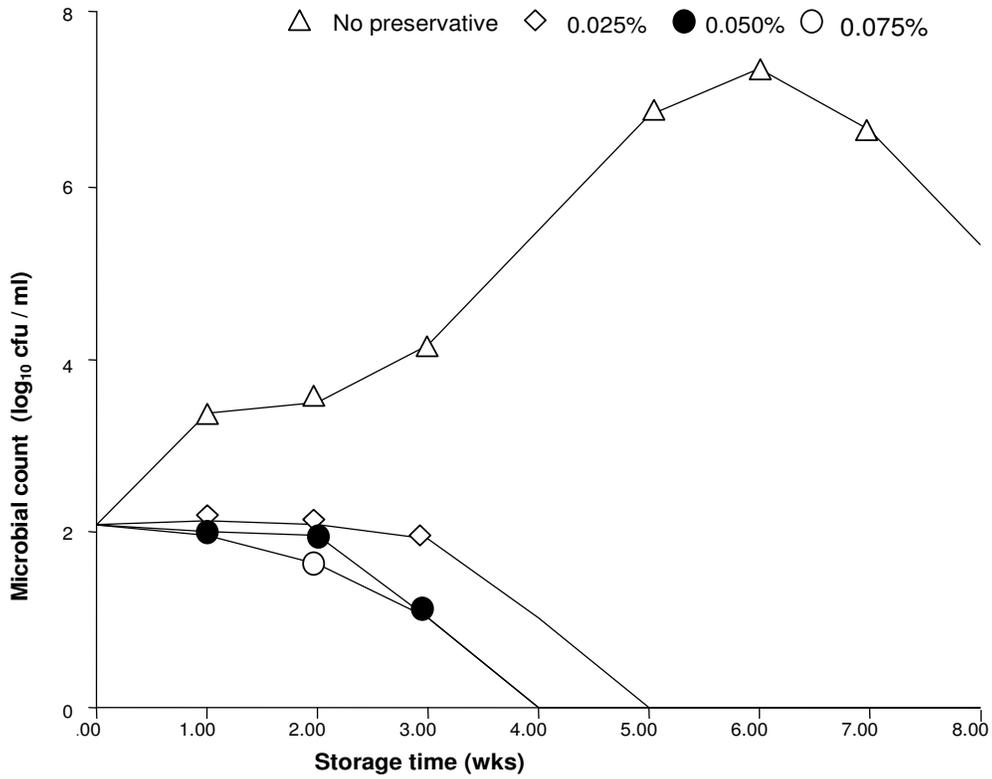


Figure 1. Effect of sodium benzoate on the growth of *A. niger* (\log_{10} cfu/ml) in Zobo drink during storage at $30 \pm 2^\circ\text{C}$

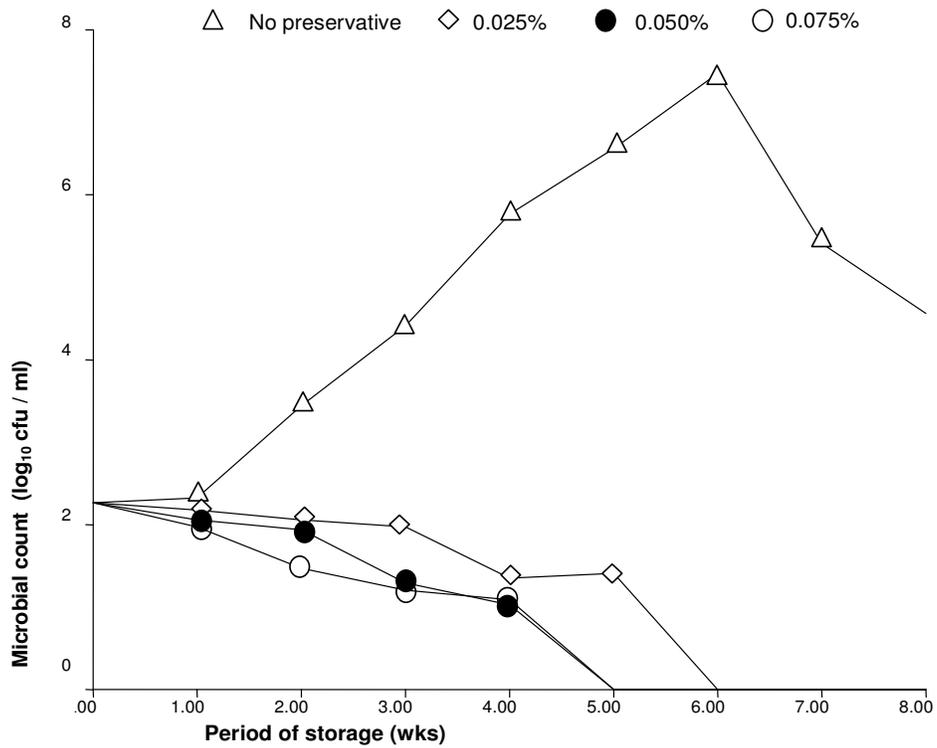


Figure 2. Effect of sodium benzoate on the population of *P. citrinum* (\log_{10} cfu/ml) in Zobo drink during storage at $30 \pm 2^\circ\text{C}$.

Table 1. Enzyme activities ($\mu\text{g/ml}$) of *A. niger* and *P. citrinum* in Zobo drink preserved with 0.05% (w/v) sodium benzoate after 8 weeks storage at $30 \pm 2^\circ\text{C}$.

Zobo drink	<i>A. niger</i>			<i>P. citrinum</i>		
	Amylase	Protease	Lipase	Amylase	Protease	Lipase
Control	18.60 ± 0.01	8.40 ± 0.2	1.50 ± 0.2	18.90 ± 0.02	8.60 ± 0.02	1.70 ± 0.2
Preserved	3.5 ± 0.02	2.30 ± 0.01	0.60 ± 0.2	3.60 ± 0.02	2.50 ± 0.3	1.40 ± 0.3
Fresh	ND	ND	ND	ND	ND	ND

Each value is the mean \pm standard deviation of triplicate determinations; ND- not detected.

may have led to physiological, homeostatic and metabolic distortion. Further attempts to overcome this adverse condition had led to increased stress which in turn brought about metabolic exhaustion, death and subsequent decrease in population observed (Figures 1 and 2). Similar findings have earlier been reported (Bogh-Sorensen, 1994; Ogiehor and Ikenebomeh, 2004). Higher concentrations of sodium benzoate showed greater growth inhibition of *A. niger* and *P. citrinum*. This shows that the effect is concentration dependent. This corroborates some earlier reports (Ogunrinola et al., 1996; Efiuvwevwe and Efi, 1999; Nwafor, 2007).

Steady increase in population observed in the control experiments may be attributed to the absence of preservative hurdles, thus making the micro-environment suitable for the growth and proliferation of the organisms. Decrease in counts observed in the 7th week may be due to depletion in the nutrient content of the medium or competition for limited space as have been previously reported (Ogiehor and Nwafor, 2004).

The significant difference ($P > 0.05$) in enzyme activities between treated and untreated samples is an indication that the alteration of homeostasis of the microorganisms by sodium benzoate may have also resulted in low enzyme activity observed in treated samples. Where the condition is suitable as in untreated inoculated sample, increase in microbial population resulted in higher enzyme activities. This is in line with documented reports of Beuchat (1997).

This work has demonstrated the high antimicrobial effect of sodium benzoate on the growth, survival and enzyme activity of *A. niger* and *P. citrinum* which are common spoilage organisms in Zobo drink. Sodium benzoate at a concentration of 0.05% (w/v) is recommended if a more extended shelf-life of Zobo drink is desired in order to enhance its commercial potential by allowing its sale beyond the locality of its production. However other hurdles or combinations of hurdles can completely eliminate the organisms judging from the result obtained here.

REFERENCES

- Amadi ES, Oyeka CA, Onyeagha RA, Ugbogu OC, Okoli I (2007). Antimicrobial Screening of *Breynia nivosis* and *Ageratum conyzoides* against dental caries organisms. *J. Biological Sci.* 7(2): 354-358.
- Beuchat LR, Rocelle M, Clavero S, Jacqueline CB (1997). Effects of Nisin and Temperature on survival, growth and enterotoxin production characteristics of phytochromic *Bacillus cereus* in Beef Gravy. *Appl. Environ. Microbiol.* 63: 1953-1958.
- Bogh-Sorensen L (1994). Description of hurdles. In: Leistner, L. and Gorris, G. M. (eds). Food preservation by combined processes. Final report, FLAIR Concerted Action No.7. Subgroup B. Eur. pp. 7-24.
- Bounds HC, Boyd FM, Norman JRA (1993). Laboratory exercises in Microbiology. Cambridge University Press, pp. 20-66.
- Efiuvwevwe BJO, Akoma O (1997). The effects of chemical preservatives and pasteurization on the microbial spoilage and shelf-life of kunun-zaki. *J. Food Safety.* 17: 203-213.
- Efiuvwevwe BJO, Efi EU (1999). Antimicrobial effects of butylated hydroxyanisole alone or in combination with potassium sorbate on quality changes in palm oil co-inoculated with *Aspergillus flavus* and *Bacillus* spp. *J. Food Sci. Agric.* 79: 1783-1787.
- Efiuvwevwe BJO, Isaiah AU (1998). Effect of Hygienic handling in combination with potassium sorbate and smoking on the microbial quality and shelf stability of croaker (*Micropogonias furnieri*). *J. Zeitschrift für Lebensmittel und Ernährungsforschung.* 207: 13-17.
- Ekundayo CA (1984). Microbial spoilage of packaged garri in storage. *Microbios letters.* 23: 217-228.
- Harrigan WF, McCance ME (1976). Laboratory methods in food and dairy microbiology, Academic Press, London, p. 416.
- Idise OE (2007). Inhibition of food spoilage organisms with Neem seed oil. *J. Pure Appl. Microbiol.* 1(1): 51-53.
- Leistner L (2000). Basic aspects of food preservation by hurdle technology. *Int. J. Food Microbiol.* 55: 181-186.
- Lowry OH, Nimi JR, Farr AL (1951). Protein measurement with folin-phenol reagent. *J. Biological Chem.* 193: 265-273.
- Magi Family Menu Cookbook (1996). Published by Nestle Foods Nigeria Plc. Ikeja, Lagos, p. 87.
- Nwafor OE (2007). Microbial stability of "Zobo" drink using hurdle technology. Ph.D. Thesis, University of Benin, Benin City, Nigeria. p. 201.
- Nwafor OE, Okoko FJ, Ejechi BO (1998). Activities of Ekimi (*Piptadeniatrum africanum*) extractives against Microsporium species. A preliminary report. *Nig. J. Sci. Environ.* 1(1) 65-68.
- Odibo FJC, Nwanunia E, Osigwe DI (1990). Biochemical changes during fermentation of Telfaria seeds for Ogi production. *World J. Microbiol. Biotechnol.* 6: 425-427.
- Ogbeibu AE (2005). Biostatistics, a practical approach to research and data handling. Mindex Publishing Company Ltd. Benin-City, Nigeria. p. 264.
- Ogiehor IS (2002). Extension of shelf-life of garri by combination of preservative factors. Ph.D. Thesis, University of Benin, Benin City, Nigeria. p. 180.
- Ogiehor IS, Ikenebomeh MJ (2004). Antimicrobial effects of sodium benzoate on the growth, survival and aflatoxin production potential of some species of *Aspergillus niger* in garri during storage. *Pak. J. Nutrition.* 3: 300-303.
- Ogiehor IS, Nwafor OE (2004). Associated microbiological, biochemical and chemical quality changes in zobo beverage produced from *Hibiscus sabbatifa*-Linn. *Nig. Annals Nat. Sci.* 5(2): 1-10.
- Ogunrinola OA, Fung DYA, Jeon IJ (1996). *Escherichia coli* 0157: 117 growth in laboratory media as affected by phenolic antioxidants. *J. Food Sci.* 61: 1017-1078.

Plummer DT (1978). An introduction to practical Biochemistry. (2nd ed.) McGraw-Hill Book Company, England. p. 265.
Shelef LA, Addala L (1994). Inhibition of *Listeria monocytogenes* and other bacteria by sodium diacetate. J. Food Safety. 14: 103-115.

Vandezant C, Splitsoesser DF (1997). Compendium of methods for the microbiological examination of foods. (3rded.). Am. Public Health Association, Washington D. C. p. 596.