# American Journal of Food Technology

ISSN 1557-4571

Academic Journals Inc.

www.academicjournals.com

American Journal of Food Technology 9 (4): 200-208, 2014
ISSN 1557-4571 / DOI: 10.3923/ajft.2014.200.208


# Comparison on Two Methods of Preparation of Zobo Drink on the Survival of *Bacillus* species

[1]S.F. Mohammed and [2]B.B. Ismail
[1]Department of Food Technology, School of Science and Technology, Fedral Polytechinc, PMB 1012, Kaura, Namoda, Zamfara State, Nigeria
[2]Department of Food Science and Technology, Bayero University Kano, Faculty of Agriculture, PMB 3011, Kano State, Nigeria

*Corresponding Author: S.F. Mohammed, Department of Food Technology, School of Science and Technology, Fedral Polytechinc, PMB 1012, Kaura, Namoda, Zamfara State, Nigeria Tel: +234903474404, +447438619690*

## ABSTRACT

The present study was aimed at gaining a comparison of the microflora of Zobo Drink (ZD) prepared by two different methods. Samples of ZD produced by boiling and steeping methods were evaluated on Plate Count Agar and Bacillus Agar at 30°C (±1°C) for 48 h (±1 h) to ascertain their levels of microflora and *Bacillus* spp. The study emphasises on the production of ZD by boiling method over the steeping method and provides guidance for developing a suitable preparation method to control organisms of food safety and quality concern.

**Key words:** Boiling method, steeping method, zobo drink, *Bacillus* agar, survival, *Bacillus* spp.

## INTRODUCTION

Zobo Drink (ZD) is a traditional soft drink produced by either steeping or boiling the calyx of the sorrel plant (*Hibiscus sabdariffa* var. *sabdariffa*) in potable water and usually sweetened with sugar and served chilled to consumers (Muhammad and Umar, 2007; Alobo *et al*., 2009). But harsh conditions, such as boiling and refrigeration temperatures, can initiate and support the production of spores by *Bacillus* species (Moeller *et al*., 2008). Sorrel calyces are products of an herbaceous plant species from the family *Malvaceae*, widely grown in the North-Eastern (Adamawa, Katsina, Kaduna, Kebbi, Sokoto and Zamfara states) and middle-belt regions (Benue, Kogi and Nasarrawa states) of Nigeria. The red and purple varieties are used for the production of ZD (Morton, 1987; Oboh and Elusiyan, 2004; Mady, 2010). The preparation of ZD varies from one community to another (Foline *et al*., 2011). The rural community produce ZD by steeping the calyx over-night for about 3-7 h, while the urban community produce ZD by boiling the calyx for 25-35 min (Mohammed, 2013).

Furthermore, *Bacillus subtilis* and *Bacillus stearothermophilus* have been isolated from commercial soft drink (Sakuraoka and Madsen, 2001). Also, *Bacillus* species were isolated from synthetic fruit drinks (Lawley *et al*., 2012). A study by Burnett and Beuchat (2000) reported that unpasteurised juices were implicated in foodborne outbreaks in USA which was later linked to contaminated processing equipment used for production. Unfortunately, recent studies have shown that *Bacillus* spp. (*Bacillus cereus* and *Bacillus subtilis*) were detected in ZD and this could signify a source of food poisoning for consumers because they are known as potential pathogenic organisms (Omemu *et al*., 2006; Nwachukwu *et al*., 2007; Nwafo and Ikenebomeh, 2009; Braide *et al*., 2012; Fernandez-No *et al*., 2013). Furthermore, it has been established that *Bacillus cereus* and

*Bacillus subtilis* produce spores and are potential food poisoning agents causing emetic and diarrhoeal infection (Lawley *et al*., 2012; Marsden *et al*., 2012; Fernandez-No *et al*., 2013). Therefore, a comparison of the production of ZD from the two common methods (steeping and boiling) was undertaken to assess the impact of both methods in ensuring ZD safety for human consumption as its production in Nigeria is been done without regulation (Ojo, 2011). Hence, this study aimed at evaluating the significance of preparation approaches on the survival of *Bacillus* species during production of ZD by the two methods.

Nevertheless, building on the studies of (Doughari *et al*., 2007: Adebayo-Tayo and Samuel, 2009; Braide *et al*., 2012) it is timely to identify *Bacillus* species in ZD using a differential medium. This approach is designed to ensure isolation of pure culture from ZD and enable easy identification and characterization of the *Bacillus* spp. and closely related genera in ZD produced by boiling and steeping methods. This is because it has been well established that *Bacillus* species are closely related and they are difficult to distinguish from one another on nutrient media such as Plate Count Agar (PCA) and Nutrient Agar (NA) and by biochemical and microscopic tests (Ouoba *et al*., 2008; Tallent *et al*., 2012).

Nonetheless, *Bacillus* species and other closely related species are playing an important role in beverage drinks. Yet, their significance is poorly understood and the extent to which they may be responsible for outbreaks of food associated illness is most likely understated and under-investigated in Nigeria. This could be attributed to difficulties with the identification of these spore-forming pathogens and closely related genera and confusion over their taxonomy (Chang and Chen, 2003).

The focus of this study is investigation on the survival of *Bacillus* species from samples of ZD prepared by two methods using Bacillus Agar for ease of presumptive isolation and identification of *Bacillus* spp. This is to respond to the research question on whether steeping or boiling method can reduce and/or eliminate the cells population of microbial contaminants and *Bacillus* species in ZD.

## MATERIALS AND METHODS

**Source of raw materials:** Sample of raw calyx of sorrel (purple variety) was transported in a plastic container from Nigeria to the United Kingdom in August 2013. The granulated sugar, (Whitworths granulated sugar, 13235F31, UK) used as the sweetener for ZD production, was purchased from Iceland Super market, Gillingham, United Kingdom (UK). The de-ionised water used for the extraction of zobo juice was sourced from the Microbiology laboratory, NRI, University of Greenwich, UK (Mohammed, 2013).

**Experimental designs:** The effect of two preparation methods on the survival of *Bacillus* species in ZD was studied. Two treatments (steeping and boiling) were employed in the production of ZD and microbial evaluation (duplicates) was conducted on the samples of ZD prepared. To conduct further basic biochemical analysis, cultures of ZD were grown on Nutrient Agar. In this study, microbiological examination was conducted using pour plate and spread plate methods. Results were presented in Figures and Tables.

### Preparation and microbiological evaluation of samples of ZD

**Preparations of samples of zobo drink:** Sorrel calyx (15 g) and sugar (35 g) were weighed into cleaned media bottles and conical flasks (500 mL capacity). The first set was steeped (for 0, 3, 5 and 7 h) at room temperature (18°C), while the second set was boiled at 100°C (for 0, 10, 15 and 20 min), respectively in deionised water (300 mL). The samples of ZD were then

stored immediately after production at 4°C for 30 min. (Bolade *et al.,* 2009; Mohammed, 2013). Each Sample was aseptically measured (30 mL) into a sterile media bottle containing MRD (270 mL) for microbiological analysis (Mohammed, 2013).

**pH determination:** The pH levels of samples of ZD were determined using a digital pH meter (Jenway 3510, UK). The probe was adjusted by dipping into 7 and 4 buffer solutions, respectively before placing into the prepared samples of ZD and readings were taken (Omemu *et al.*, 2006; Mohammed, 2013). The pH values of both samples were recorded after taken average mean from three observed readings (Foline *et al.*, 2011; Mohammed, 2013).

**Enumeration of aerobic bacteria:** The $10^{-1}$ diluted samples were homogenized and a dilution series prepared. 1 mL of each dilution was aseptically inoculated into a petri dish into which about 25 mL of Plate Count Agar (PCA) (CM 0325M, Oxoid) was added. The contents were mixed and allowed to solidify. After drying, the petri dishes (duplicates) were dried and incubated at 30°C (±1°C) for 48 h (±1 h) for enumeration and identification of aerobic bacteria. Plates with 30-300 colonies were selected for counting using an automatic colony counter (Gallenka□p, England). Also, control experiments were carried out as described (Roberts and Greenwood, 2003; Da Silva *et al.*, 2013). Results obtained were recorded in CFU $g^{-1}$.

**Enumeration, isolation and biochemical evaluation of *Bacillus* species:** All samples previously mixed with 270 mL of MRD were homogenized and diluted aseptically as described earlier. An aliquot of each dilution (0.1 mL) was inoculated onto Bacillus Agar (92325HiChrome™Agar, Fluka). The petri dishes (duplicates) were incubated at 30°C (±1°C) for 48 h (±1 h) for enumeration and identification of presumptive *Bacillus* spp. Colonies were selected for counting as described. From plates with visible growth colonies were randomly picked and carried out as described. After checking purity, isolates were examined as described. From plates with visible growth colonies were randomly picked from each plate using a Harrison Disc (Harrigan and McCance, 1976). The selected colonies (10) were recovered, purified on Nutrient Agar (NA) (CM 0003, Oxoid) by streaking and incubated at 30°C (±1°C) for 18 h (±1 h). After checking for purity, isolates were examined for Catalase production and Oxidase and Gram reactions (Hucker's method) and observed under a microscope (Nikon, 2303841, Japan) (Roberts and Greenwood, 2003; Ouoba *et al.*, 2008; Da Silva *et al.*, 2013).

**Statistical analysis of data:** Results are presented as duplicates for 2 treatments for Steeped Zobo Drink (SZD) and Boiled Zobo Drink (BZD). Data obtained after determination of APC and PBSC were converted to $\log_{10}$CFU $mL^{-1}$ using FEPTU 557 $\log_{10}$ conversion table prior to analysis. t-Test (two-tail) was used for statistical analysis of all the data ($p>0.05$). The statistical analysis was performed using Microsoft Excel 2010 (Mohammed, 2013).

## RESULTS AND DISCUSSION

**pH value and microorganisms associated with samples of ZD:** The aim of this part of the study was to compare preparation methods (boiling and steeping) employed in the production of ZD and their impact on the survival of bacteria. In this study, the pH value was also determined. The results indicated that irrespective of preparation methods, there was no significant difference in the pH values of the samples ($p>0.05$) as presented in Table 1. This suggests that the methods of preparation did not have much effect on the pH of ZD. The pH of the ZD produced in this study suggests that ZD could be classified as a highly acidic soft drink. Although, a substance is said

Table 1: pH values for samples of zobo drink produced at varying times by steeping and boiling methods

| | SZD (h) | | | | BZB (min) | | | |
|---|---|---|---|---|---|---|---|---|
| Sample code | 0 | 3 | 5 | 7 | 0 | 10 | 15 | 20 |
| pH (± 0.10) | 2.9 | 2.9 | 2.9 | 2.9 | 2.9 | 3.0 | 3.0 | 2.9 |

pH values represent average mean from three observed readings. SZD: Steeped zobo drink, BZD: Boiled zobo drink

Table 2: APC and PBSC of ZD samples on PCA and BA expressed in CFU $mL^{-1}$

| Sample code (time) | APC | PBSC |
|---|---|---|
| **SZD (h)** | | |
| 0 | $2.2\times10^3$ | $4.5\times10^4$ |
| 3 | $3.0\times10^3$ | $4.0\times 10^4$ |
| 5 | $3.0\times10^3$ | $4.2\times10^4$ |
| 7 | $2.7\times10^3$ | $3.2\times10^4$ |
| **BZB (min)** | | |
| 0 | $3.1\times10^3$ | $3.7\times10^4$ |
| 10 | $<1.0\times10^1$ | <1 |
| 15 | $<1.0\times10^1$ | <1 |
| 20 | $<1.0\times10^1$ | <1 |

h: Hour, min: Minute, APC: Aerobic plate count, PBSC: Presumptive *Bacillus* species count. Results presented are counts of duplicates. No visible growth of microbial cells in a petri dish represents <10 counts = $1.0\times10^1$ and <1

to be highly acidic especially if the substance dissolves in water and releases hydrogen ions. The concentration of released hydrogen ions determines the level of acidity of a substance (McGlynn, 1992). Pathogenic microorganisms are not expected to survive in foods with pH below 4.5 (Roberts and Greenwood, 2003) but *Bacillus* species were isolated from high acid food (Canned Tomato juice) and synthetic fruit drinks (Lawley *et al*., 2012). The pH value of the ZD produced in this study was slightly higher than the values reported by other studies between 2.60 and 2.78 by Omemu *et al*. (2006) and 2.50 and 2.53 by Foline *et al*. (2011).

**APC and PBSC for samples of ZD:** Aerobic Plate Count (APC) and Presumptive *Bacillus* Species Count (PBSC) from samples of SZD and BZD were determined in order to understand the survival of microorganisms in ZD after steeping and boiling preparation methods. The APC and PBSC results indicated that SZD samples had higher bacterial counts than BZD samples (Table 2).

Table 2 presents the APC and PBSC results for samples of ZD produced by steeping and boiling methods. Samples produced from steeping method showed higher counts of APC and PBSC than the samples produced from boiling method. In this study, it was observed that microbial cells were not detected from samples of ZD produced by boiling method during 10, 15 and 20 min, respectively.

Figure 1 and 2 showed that the Aerobic Colonies Count (APC) and presumptive Bacillus Count (PBSC) stayed more or less constant over the steeping period. Boiling for 10, 15 and 20 min resulted in no detectable growth on either Nutrient Agar and on the Bacillus Agar (Fig. 1). But, in contrast, the 0 min BZD shows PBSC. Study has reported that the vegetative cells of *Bacillus cereus* are heat sensitive but spores can survive harsh heat treatment because they are fairly heat resistant (Lawley *et al*., 2012). The spores can withstand the temperature of about 85 to 95°C for 1 to 36 min (Lawley *et al*., 2012). The results from this study suggest that *Bacillus* spores were destroyed within 10 min of boiling.

There was no significant difference ($p<0.05$) with respect to APC and PBSC between the samples of SZD from 0, 3, 5 and 7 h. But, there was significant difference ($p<0.05$)

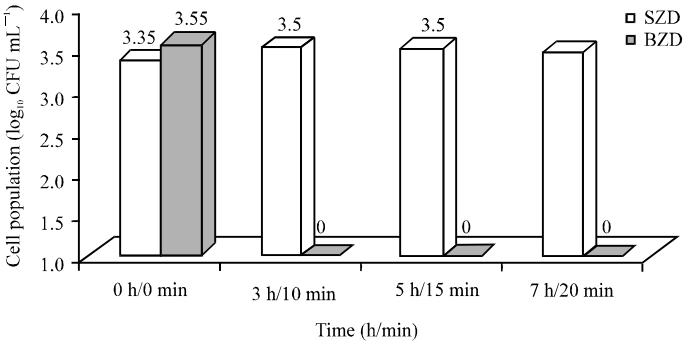


Fig. 1: Aerobic plate count for microorganisms during steeping and boiling preparation methods for the production of ZD at room temperature for 0, 3, 5 and 7 h and at 100°C for 0, 10, 15 and 20 min

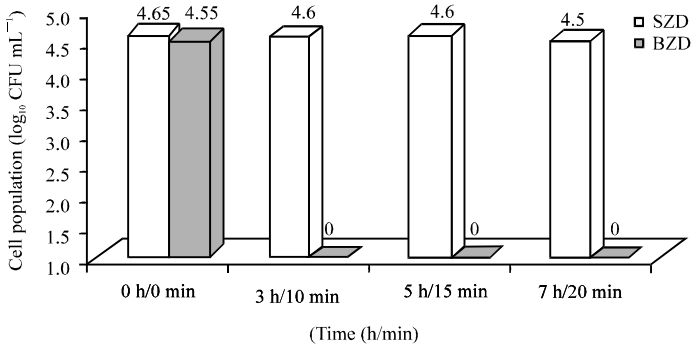


Fig. 2: Presumptive *Bacillus* species count during steeping and boiling preparation methods for the production of ZD at room temperature for 0, 3, 5 and 7 h and at 100°C for 0, 10, 15 and 20 min

with respect to APC and PBSC between the time 0 sample of BZD and those taken after 0, 10, 15 and 20 min boiling (Fig. 2).

The APC found in this study differed from those reported by Omemu *et al.* (2006) (4.15 $\log_{10}$ CFU $mL^{-1}$) but are more in agreement with the result (2.6 $\log_{10}$ CFU $mL^{-1}$) reported by Nwachukwu *et al.* (2007). The presumptive identification of the colonies (Table 2) agreed with several studies reporting the presence of *Bacillus* spp. in ZD produced and sold in Nigeria (Omemu *et al.*, 2006; Nwachukwu *et al.*, 2007; Braide *et al.*, 2012).

Table 3 presents a description of colonies grown on Bacillus Agar from samples of ZD, as well as other results obtained from the pure isolates and results of pure isolates grown on Nutrient Agar.

It has been reported that thermal processing, such as boiling of acid foods, is designed to destroy vegetative cells of microorganisms capable of growing in food products during storage. Although, spores may survive boiling and toxin will not be produced at pH of 4.6 or less. Many bacterial spores will survive thermal processing at low pH levels but typically do not germinate and grow in acid foods that have been thermally processed (Stannard, 1997). Furthermore, low pH is probably the single most important factor in the preservation of soft drinks that enhances the effect of thermal processes, such as pasteurization and also acts as an additional hurdle to the growth of micro-organisms of food safety concern (Stratford *et al.*, 2000). This is because, boiling has destructive effects on microorganisms leading to the reduction and/or destruction of pathogens

Table 3: Cultural characteristics of *Bacillus* spp. observed from samples of ZD on BA and NA

| Sample code | Colour of colony | Presumptive *Bacillus* spp.[1] | Gram | Catalase | Oxidase | Cell morphology[2] |
|---|---|---|---|---|---|---|
| **SZD (h)** | | | | | | |
| 0 | Yellow mucoid colonies | *B.megatarium* | + | + | - | Rod-shape alone, in pairs and in chain |
| | Light blue, large, flat colonies with blue centre | *B.cereus* | + | + | - | Rod-shape alone, in pairs and in chain |
| 3 | Pink, small, raised colonies | *B.coagulans* | + | + | - | Rod-shape alone, in pairs and in chain |
| | Yellow mucoid colonies | *B. megatarium* | + | + | - | Rod-shape alone, in pairs and in chain |
| | Light blue, large, flat colonies with blue centre | *B. cereus* | + | + | - | Rod-shape alone, in pairs and in chain |
| | Green | *B. subtils* | + | + | - | Rod-shape alone, in pairs and in chain |
| | Yellow colonies | *Staphylococcus aureus* | + | + | - | Cocci, alone, in pairs and in chain |
| 5 | Pink, small, raised colonies | *B. coagulans* | + | + | - | Rod-shape alone, in pairs and in chain |
| | Yellow mucoid colonies | *B. megatarium* | + | + | - | Rod-shaped |
| | Light blue, large, flat colonies with blue centre | *B. cereus* | + | + | - | Rod-shape alone, in pairs and in chain |
| | Yellow colonies | *Enterococcus faecalis* | + | + | - | Cocci, alone, in pairs and in chain |
| | Yellow colonies | *Staphylococcusaureus* | + | + | - | Cocci, alone, in pairs and in chain |
| 7 | Yellow, mucoid colonies | *B. megatarium* | + | + | - | Rod-shape, alone, in pairs and in chain |
| | Light blue, large, flat colonies with blue centre | *B. cereus* | + | + | - | Rod-shape, alone, in pairs and in chain |
| | Yellow colonies | *Enterococcus faecalis* | + | + | - | Cocci, alone, in pairs and in chain |
| | Yellow colonies | *Staphylococcus aureus* | + | + | - | Cocci, alone, in pairs and in chain |
| **BZD (min)** | | | | | | |
| 0 | Pink, small, raised colonies | *B. coagulans* | + | + | - | Rod-shape, alone, in pairs and in chain |
| | Yellow mucoid colonies | *B. megatarium* | + | + | - | Rod-shape, alone, in pairs and in chain |
| | Green | *B. subtilis* | + | + | - | Rod-shape, alone, in pairs and in chain |
| | Yellow colonies | *Enterococcus faecalis* | + | + | - | Cocci, alone, in pairs and in chain |
| | Yellow colonies | *Staphylococcusaureus* | + | + | - | Cocci, alone, in pairs and in chain |
| 10 | NVG | NVG | ND | ND | ND | ND |
| 15 | NVG | NVG | ND | ND | ND | ND |
| 20 | NVG | NVG | ND | ND | ND | ND |

h: Hour; m: Minutes, ND: Not determined, NVG: No Visible Growth. [1]These species were observed on Bacillus Agar Culture. [2]These shapes of bacterial species were observed under the microscope from NA culture. (Source: Fluka Analytical, 2013)

(Jay, 2000). Heating and cooling are channel of heat transport contributing to the destruction of vegetative microbial cells but the combination of time and temperature will determine the fatal effect of heat treatment on food (Nout, 2001).

This study suggests that boiling can be employed to produce safe ZD for human consumption because the levels of APC and PBSC in the samples of BZD are within the limits of APC (10 to $<10^2$ CFU $mL^{-1}$) and PBSC ($<10^3$ CFU $mL^{-1}$) for ready-to-drink soft drink (FDA, 2013).

Toxin production is not going to be an issue due to low pH of ZD. This is because *Bacillus* species cannot produce enterotoxins at low pH values (2 to 4.3) (Lawley *et al*., 2012). Also, an intoxication dose of 8 μg $kg^{-1}$ body weight of spores of *Bacillus* species has been suggested (Paananen *et al*., 2002). But large doses ($10^5$-$10^8$ CFU $g^{-1}$) of viable cells of *Bacillus* spp. (*Bacillus cereus* and *Bacillus subtilis*) are required before toxin (cereulide) becomes detectable in the food (HPA, 2009). Emetic toxin in foods implicated in an outbreak in Japan ranged from 0.01-1.28 μg $g^{-1}$ (Agata *et al*., 2002). Although, it has been opined that *Bacillus cereus* can cause food poisoning at low dosage ($10^3$ CFU $g^{-1}$) but high levels (= $10^5$ CFU $g^{-1}$) are necessary to produce enough toxin to cause illness (McIntyre *et al*., 2008; Lawley *et al*., 2012; FDA, 2013).

## CONCLUSION

This study employed Plate Count Agar and Bacillus Agar in the enumeration and presumptive identification of *Bacillus* species associated with Zobo Drink (ZD) samples produced by steeping and boiling methods. This study also presumptively confirms the presence of species of *Bacillus* associated with the ZD as reported by several studies (Omemu *et al*., 2006; Doughari *et al*., 2007; Braide *et al*., 2012).

The findings of this study indicate that boiling method will be a better preparation approach for the production of safe ZD over the steeping method because results for PBSC were less than the samples from steeping methods. Therefore, production of safe ZD is possible by boiling sorrel calyx in potable water and chilling before consumption because microbial growth was not observed in the samples of ZD produced at 100°C for 10-20 min boiling times and refrigerated at 4°C for 30 min. Also, if ZD is produced and consumed on the same day this will give little or no chance for microbial growth in the drink that might cause foodborne problems. ZD produced by the steeping method in rural communities is also safe providing that is prepared and consumed on the same day. Investigation into the reformulation of ZD would need to be produced commercially because of possibility of the presence of spores from pathogenic *Bacillus* species.

In summary, the management of identified hazards in ZD production using a HACCP plan will contribute to food safety in Nigeria. The detection of the foodborne pathogenic bacteria in steeped ZD samples could represent an unacceptable risk to health regardless of number of bacterial cells present. Even though *Bacillus* species levels detected in this study are within the acceptable limits that might not cause food poisoning to consumers but they should not be found in ready-to-drink soft drink that has been adequately prepared (HPA, 2009; FDA, 2013).

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