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EFFECT OF CITRIC ACID AT DIFFERENT pH ON THE SURVIVAL OF *Escherichia coli*

Mahe, A.[1*], Sabiu, B.[2], Adam, A. A.[1] Abdullahi, U. Z.[1] and Ado, B. A. [1]
[1]Biochemistry Department, Federal University Dutse
[2]Microbiology and Biotechnology Department, Federal University Dutse
*Corresponding author: maheasmau@gmail.com. Tel: +2348036010772

ABSTRACT
Microorganisms are included among the major spoilers of food, they achieve this by using the nutrients present in food material. Susceptibility of microorganisms to the most currently used preservatives has been decreasing. Organic acids have been considered as valuable food preservatives. This study aimed to isolate, identify and determine the effect of citric acid at different pH levels on the survival of E. coli. The E. coli was isolated and a pure culture was obtained after series of sub-culturing on Eosine Methylene Blue agar. The biochemical tests known as IMViC were performed to confirm the presence of the organism. The organism was also identified using polymerase chain reaction (PCR) in which the DNA was extracted, amplified and viewed by gel electrophoresis. The organism was then inoculated in nutrient broth containing citric acid at pH levels of 3.0, 4.5 and 6.0 in different test tubes. Negative controls were included. Results were analyzed using one way ANOVA to compare the means obtained. Results obtained was positive for indole and methyl red tests but negative for Voges Proskauer and citrate tests which confirmed the organism. After 24 hours of inoculation, the results of spectrophotometry showed that at pH level of 3.0, the absorbance was lower than the results obtained at pH level of 4.5 and 6.0. This is an indication of higher reduction in the count of the organism at pH of 3.0. There was significant difference between the control and the test groups ($p<0.05$) but the difference obtained between the test groups were not significant ($p>0.05$). Results from this study showed that citric acid could not eliminate the whole organism but was effective in inhibiting the growth of the organism dependent on pH level. This indicates that a pH dependent citric acid can be used as a good preservative.
Keywords: Escherichia coli, Citric Acid, pH, Isolation, Identification

INTRODUCTION

Many researches have been carried out to test the activity of organic acids on the survival of pathogenic microorganisms on food materials. This is owing to the fact that microorganisms are implicated among the major spoilers of food, they grow on food utilizing the nutrients that are present in those food materials and in the process release toxins or waste metabolic products on such foods leading to food spoilage (Kantor *et al.*, 1997). Food spoilage is a metabolic process that makes foods to be unacceptable or undesirable for human consumption because of changes in sensory characteristics (Rawat, 2015).

Susceptibility of microorganisms to the most currently used preservatives has been decreasing (Rawat, 2015).Organic acids used as additives can act as buffers to regulate acidity, thus can function as preservatives. Low molecular weight organic acids like citric acid, acetic acid, lactic acid are produced by microorganisms through the fermentation pathway (Singh *et al.*, 2017).

Citric acid, an organic acid that is commonly found in powdered form. Fruits which are sour in taste contains high amounts of citric acid e.g orange, blackberries, lemon, raspberries, mangoes and pineapples (Van De Walle, 2019; Penniston *et al.,* 2008). Citric acid is biodegradable and it is a non-pollutant in the sense that it is ecofriendly (Singh *et al.*, 2017). Industrial-scale production of citric acid first began in 1890 based on the Italian citrus fruit industry, where the juice was treated with hydrated lime (calcium hydroxide) to precipitate calcium citrate, which was isolated and converted back to the acid using diluted sulfuric acid (Verhoff,2014).

Citrate is an intermediate in the tricarboxilic acid cycle (TCA cycle or Krebs cycle), a central metabolic pathway for animals, bacteria and plants. Citrate synthase catalyzes the condensation of oxaloacetate with acetyl CoA to

form citrate. Citrate then acts as the substrate for aconitase and is converted into aconitic acid. The cycle ends with regeneration of oxaloacetate. Some bacteria (primarily *E. coli*) can produce and consume citrate internally as part of their TCA cycle (Stryer, 2012).

Citric acid being among the stronger edible acids is used as preservative and flavoring agent in food and beverages. It also possess numerous industrial applications (Verhoff,2014).The efficacy of organic acids as antimicrobials vary widely depending on pH, concentration, and the concentration of the non-dissociated form (Beth *et al.*, 2004). Microorganisms grow in beef and its products primarily at the surface, numerous methods were used to delay spoilage by using an organic acid (citric acid) spray or dips as antimicrobial agents (Siragusa and Dickson, 1992). The mechanism of action of organic acids mainly citric acid is that the undissociated acid penetrate the cell of microorganisms through diffusion, then dissociates and acidify the cell interior, this interferes with cellular metabolism and or decrease in biological activity due to the changes in pH of the cell's environment (Cherrington *et al.*, 1991).

E. coli is a gram-negative, rod-like bacterium that is mostly found in the lower intestine of warm-blooded organisms (endotherms). Most *E. coli* strains are harmless, but pathogenic varieties such as *E. coli* O157:H7 caused serious food poisoning, meningitis, urinary tract infections, septic shock (Vogt and Dippold, 2005).They are from the family that have the ability to ferment glucose or lactose (Feng and Weagant, 2009). Pathogenic *E. coli* produce toxins and other virulence factors that can damage the host cells. These pathogenic traits are encoded by genes within the pathogens (Mobley *et al.*, 2004). *E. coli* is an indicator for fecal contamination in foods and drinking water. It is considered as an indicator in food safety and hygiene (Ekici and Dümen, 2019).This study is to identify *E. coli* and determine the effectiveness of citric acid at different pH levels on *E. coli* in order to have better alternatives for food preservation.

MATERIALS AND METHODS

ISOLATION OF TEST ORGANISM

All materials for the isolation and culturing of the organism were obtained from Microbiology Laboratory, Federal University Dutse. The inoculum used as test microorganism was obtained from microbiology laboratory. Using a differential media known as Eosine Methylene blue agar (EMB agar), two plates of the culture were prepared. To prepare two plates, 1.1 gram of the powdered agar was dissolved in 35ml of distilled water (according to manufacturer's specification) and was shaken to dissolve completely. The media was then autoclaved at 121ºc for 15 minutes. After cooling, the media was dispensed into sterile petri dishes and then allowed to solidify. The organism was streaked on the media aseptically after solidification. It was then incubated at 37ºC for 24 hours.

BIOCHEMICAL TESTS

The biochemical tests carried out was IMViC test which comprises of four main reactions; Indole test, Methyl Red test, VogesProskauer test and Citrate utilization test. IMViC reactions are employed in the identification of members of family enterobacteriaceae in which *E. coli* is among.

Indole test

Some bacteria can produce indole from amino acid tryptophan using the enzyme typtophanase. Production of indole is detected using Ehrlich's reagent or Kovac's reagent. Indole reacts with the aldehyde in the reagent to give a red color (Bachoon *et al.*, 2008). An alcoholic layer concentrates the red color as a ring at the top. Bacterium to be tested was inoculated in peptone water, which contains amino acid tryptophan and incubated overnight at 37ºC. Following incubation few drops of Kovac's reagent were added.

Methyl Red (MR) test

Methyl Red is a pH indicator, which remains red in color at a pH of 4.4 or less. The bacterium to be tested was inoculated into glucose phosphate broth, which contains glucose and a phosphate buffer and incubated at 37ºC for 48 hours. Over the 48 hours the mixed-acid producing organism must produce sufficient acid to overcome the phosphate buffer and remain acid. The pH of the medium was tested by the addition of 5 drops of MR reagent (Koneman, 2016).

VogesProskauer (VP) test

In this test two reagents, 40% KOH and alpha-naphthol are added to test broth after incubation and exposed to atmospheric oxygen. If acetoin is present, it is oxidized in the presence of air and KOH to diacetyl. Diacetyl then reacts with guanidine components of peptone, in the presence of alpha- naphthol to produce red color (Bachoon *et al.*, 2008). Role of alpha-naphthol is that of a catalyst and a color intensifier. Bacterium to be tested was inoculated into glucose phosphate broth and incubated for 48 hours. 0.6 ml of alpha-naphthol was added to the test broth and shaken. 0.2 ml of 40% KOH was added to the broth and shaken.

The tube was allowed to stand for 15 minutes. While MR test is useful in detecting mixed acid producers, VP test detects butylene glycol producers. Acetyl-methyl carbinol (acetoin) is an intermediate in the production of butylene glycol.

Citrate utilization test

This test detects the ability of an organism to utilize citrate as the sole source of carbon and energy. Bacteria are inoculated on a medium containing sodium citrate and a pH indicator bromothymol blue. The medium also contains inorganic ammonium salts, which is utilized as sole source of nitrogen. Utilization of citrate involves the enzyme citritase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and CO_2. Production of Na_2CO_3 as well as NH_3 from utilization of sodium citrate and ammonium salt respectively results in alkaline pH. This results in change of medium's color from green to blue (Mac, 1980). Bacterial colonies were picked up from the culture media using a straight wire and inoculated into slope of Simmon's citrate agar and incubated overnight at 37^{o}C. If the organism has the ability to utilize citrate, the medium changes its color from green to blue.

MOLECULAR BASED IDENTIFICATION OF *E. coli*

Polymerase chain reaction was used to further identify the test organism. It involves stages from DNA extraction, PCR and gel electrophoresis.

Genomic DNA extraction

DNA extraction protocol was followed as described by Mohammed *et al.* (2011). From the broth sample, 1.5ml of the organism was added to an Eppendorf tube and then centrifuged at 10,000rpm for 5 minutes. The supernatant was discarded and the pellets were washed twice with sterile water. After that, 200µl of sterile water was added to the pellets and the mixture was vortexed to homogenize and boiled in a dry bath at 100ºC for 10 minutes. This was followed by vortexing and centrifugation at 12,000rpm for 5 minutes. The supernatant containing the DNA were then transferred into another tube and stored at -20ºC. The concentration and purity of the DNA was estimated using a Nanodrop spectrophotometer.

Polymerase Chain Reaction (PCR)

The following reagents were added to the PCR tube; 1.5µl of taqbufferA, 0.75µl of $MgCl_2$, 0.12µl of $dNTP_s$, 0.51µl of Bac1492 forward primer, 0.51µl of Bac1494 reverse primer, 0.12µl of kappataq, 10.49µl of distilled deionized H_2O and 1.0µl of genomic DNA which made total volume to 15µl. the sample containing the reagents were loaded on the PCR machine and the cycling conditions set was 95ºC for five minutes which was for denaturation followed by a repeated cycle for 35 times in which the temperature was set to 94ºC for 60 seconds, 60ºC for 90 seconds which enables annealing of primers and 72ºC for 90 seconds for elongation or extension. The final temperature was 72ºC for 10 minutes.

Gel Electrophoresis

First, 1.5% of the gel was prepared by dissolving 1.5g of the agarose in 100ml of Tris-acetate-EDTA (TAE). The solution was then heated in a microwave oven so as to dissolve the agarose, it was allowed to cool. After cooling, 4µl of ethidium bromide was added to the gel. The mixture was poured into a cartridge with a comb to make wells and then allowed to solidify. After solidification, the PCR products were loaded into the wells and then a voltage of 340V was applied for 30 minutes. After the separation, the gel containing the separated DNA was slided into an electrophoretic chamber and then viewed. A DNA ladder (Hyper ladder 1 kb) was used to determine the number of base pairing.

EXPERIMENTAL PROTOCOL

The method employed to determine the effect of the acidity of citric acid was as described by Albashan (2009) through spectroscopy in which the isolated pure culture of the organism was inoculated in nutrient broth and the citric acid was added to the culture broth, 2 test tubes at each of the following pH levels, 3.0, 4.5 and 6.0. There were 2 test tubes serving as negative control (no addition of citric acid). The tubes were then incubated for 24 hours after which the absorbance was taken using a spectrophotometer at wavelength of 540nm.

Statistical Analysis

Values are expressed as mean **±** standard deviation (SD). Data were analyzed by ANOVA after performing normality test using the SPSS statistical package (Version 20).

RESULTS AND DISCUSSION

Colony characteristics of *E. coli* shows colonies with a dark center and a metallic green sheen as shown in figure 1.

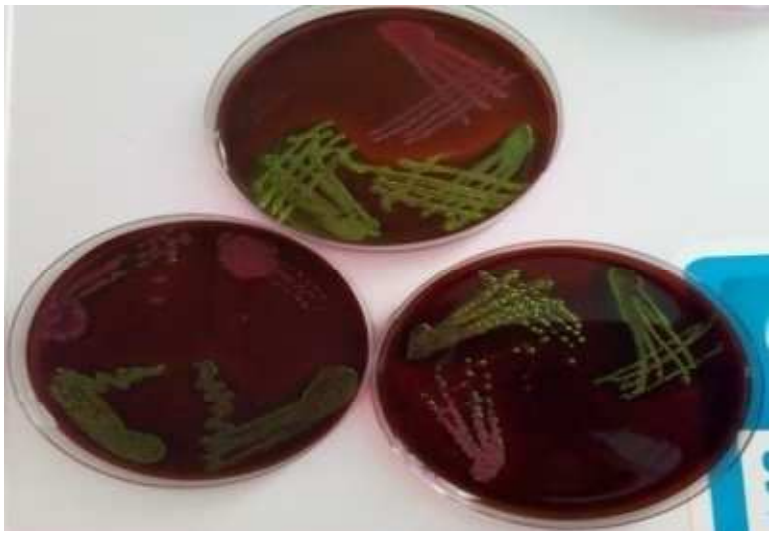
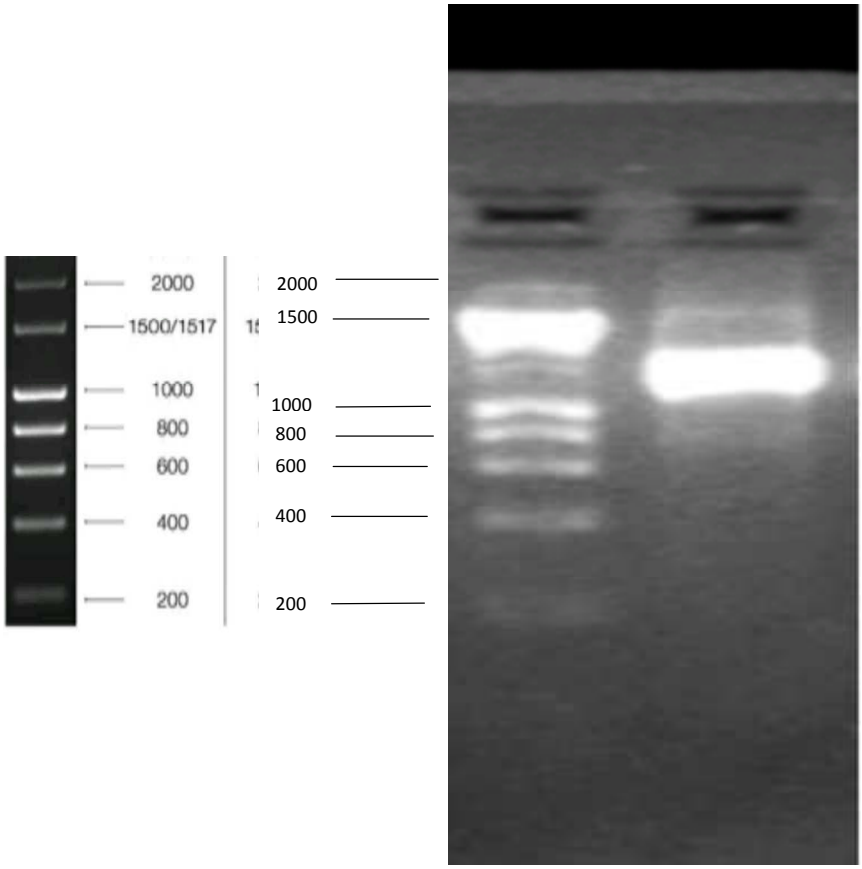


Figure 1: Colony appearance of *E. coli* on Eosine Methylene Blue agar

Table 1: Biochemical reactions of the organism.

Indole	Methyl red	Vogesproskauer	Citrate utilization
Positive	Positive	Negative	Negative



(a) (b)

Figure 2: (a) hyper ladder 1kb (b) Electrophoresed band product of PCR.

The results of the spectrophotometry that was performed 24 hours after culturing of the organism at various pH levels of 1ml citric acid solution added to culture broth is shown in table 2 below.

Table 2: Mean absorbance at each specific pH.

pH (citric acid)	Growth (OD* at 540nm) of *E.coli*
3.0	1.140
4.5	1.285
6.0	1.340
7.0	1.660

*OD, read as absorbance.

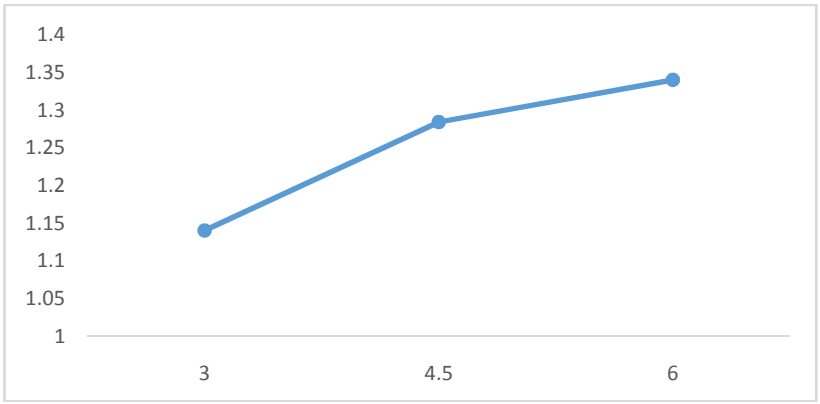


Figure 3: Absorbance of the organism at specific pH level

Table 3: Statistical analysis using one way ANOVA

Groups	Control	Group 1 (pH 3.0)	Group 2 (pH 4.5)	Group 3 (pH 6.0)	F value	P-value
Absorbance	1.66 ± 0.21^{abc}	1.14 ± 0.03^{a}	1.29 ± 0.10^{b}	1.34 ± 0.12^{c}	10.956	0.001

Rows taking the same superscripts are significantly different ($p<0.05$).

DISCUSSION

E. coli formed a green metallic sheen as a result of fermentation of the lactose present in the agar which then produces acid that reacts with the dyes, the metachromatic properties of dyes (Xavier *et al.*, 2016).

Table 1 shows the various biochemical reactions carried out, the result shows that the organism was able to produce indole from tryptophan using the enzyme typtophanase encoded by the gene tnaA (Mingxi *et al*, 2010) and this was detected through the addition of kovac's reagent. For methyl red test, it shows the organism was able to produce and maintain stable acid end products from glucose fermentation in the glucose broth which made the methyl red indicator remain red because the acid pH level was not above 4.4. The vogesproskauer test indicated that, the organism was not able to utilize the butylene glycol pathway thereby not producing acetoin. For the citrate utilization test, it also shows that the organism was not able to utilize citrate as the sole carbon source because it lacks a citrate transporter (Dustin *et al.*, 2016), therefore, this caused the inability of the medium to change its color from green to blue.

From the results of the agarose gel electrophoresis in figure2, the band obtained when compared with hyper ladder 1kb which is a DNA ladder with numbers of bands ranging from 200 base pairs to 10,037 base pairs showed the amplified DNA band was lying slightly above 1000. The result agrees with Mohammed *et al.* (2011) experiment that had a total of 1,183 base pairs. According to Ponce De Leon *et al.* (1993), the extent of inhibitory activity of this acid coincided with their degree of undissociation (pH dependant) because at lower pH of 3, the absorbance was lesser compared to that of 4.5 and 6. According to fig.3, at pH level of 4.5 and 6, the level of absorbance increases which is linked to the decreased level of acidity. The absorbance difference between 3 and 4.5 was higher than the absorbance difference between 4.5 and 6.0. Study of Albashan (2009) obtained an absorbance of 0.713 when the pH of the broth was adjusted to 3.0 while in this study, a higher absorbance was recorded at same pH. The effect shows higher reduction in the count of the organism. Data obtained were subjected to normality test and found to be evenly distributed. One way ANOVA was used to compare the means. There was significant

difference between the control and each of the test groups ($p<0.05$). While, no significant difference ($p>0.05$) was observed between groups as the values were slightly different.

CONCLUSION

Based on the present study, it was discovered that, the use of citric acids is efficient and has some usefulness in controlling microbial growth in foods. It would help reduce the amount of food spoilage, increase the shelf life of food and reduces the use of chemical preservatives. Citric acid can be in the evaluation and integration of new processing/preservation treatments.

Conflict of Interest

The authors declare that there is no conflict of interest with regards to this manuscript.

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