The effect of copper (II) ion on the production of citric acid by *Aspergillus niger* using corncobs.

Abstract

Citric acid production using agro-waste as cheap carbon source helps in waste management and reduction in cost of production. The study was aimed at optimizing citric acid production from corncobs as substrate amended with copper ions. Corncobs were collected, air dried, milled and pretreated with acid hydrolysis. The pretreated corncob media were inoculated with 10^6 spores/ml of *Aspergillus niger* and fermented for 10 d at 28 ± 2 °C. Effect of corncob concentrations of 5 – 25 % w/v on citric acid production was examined. The concentration with highest yield of citric acid was optimized using copper ions concentration of 0 – 0.4 g/l. All through the fermentation processes samples analyzed were citric acid produced, fungal biomass, and changes in pH. The supplemented corncob medium had the highest citric acid production and fungal biomass after 8 d of fermentation. Studies on corncob concentrations showed that 25 %w/v of corncobs had the highest citric acid production and fungal biomass. The highest citric acid production and fungal biomass of 2.94±0.05 and 3.11±0.04 g/l respectively were observed in the medium amended with 0.3 g/l of copper ions. Statistically, the values were significant different from other concentrations of copper ions ($p < 0.05$). Corncob was observed to be a good substrate for citric acid production by *A. niger* and amendment of substrate with copper ions at lower concentration can help to optimize the production of citric acid.

Keywords: Corncobs, citric acid, copper ions,, *Aspergillus niger*

1. INTRODUCTION

The bioconversion of cellulosic waste by enzymatic hydrolysis to useful products has great potential with the possibility of using lignocellulosic biomass from the waste as an inexpensive raw material for the production of important products such as citric acid is an attractive goal [1].

Citric acid (2 hydroxyl -1, 2, 3 – propane tricarboxylic acid) is a nearly universal intermediate and an important commercial product of metabolism and its traces are found in virtually all plants and animal tissue [2]. This weak acid is one of the most sought after products which have a never ending demand in the global market [3]. Citric acid is used commercially in industries such beverages, food, cosmetics and pharmaceutical thus, requires an effective production process. It has many applications in food, pharmaceutical and cosmetic industries as an acidulant, flavor enhancer, preservative, antioxidant, emulsifier and chelating agent [3,4]. It global production rate is about 1.8 Mt per annum. To meet the industrial demands low cost medium is required for the production of citric acid [3]. Microbiological processes are more preferred for commercially citric acid production to chemical processes [5]. Studies have shown that citric acid can be produce from four species of *Aspergillus* such *Aspergillus niger*, *A, phoenicis, A. luchuensis* and *A. awamori*. However, the fungus *Aspergillus niger* is more preferable due to the ease in handling, ability to utilize a variety of substrate and to have high production of citric acid at low pH [4,6].

The increased demand of citric acid by industries coupled with the search for an efficient raw material from agricultural waste residue for its production is of great concern [4]. Substrate selective for citric acid production depends on several parameters mostly, related with cost and availability. These substrates are rich in organic matter as nutrients for the microbial growth and metabolism. The metabolic process of the organism resulted in production of useful products such as citric acid. A typical example of nutrient source is corn cobs termed as waste that is cheap and available. The abundance of corn in Nigeria has made the residues to pose serious environmental threat [7]. Biotechnological application of corn cobs as substrate for microbial fermentation not only form a low cost substrate but also help in solving related problems of waste disposal [8].

The use of corn cobs as a substrate for the production of citric acid by *A. niger* have been reported by several authors [2,4,8,9]. Optimization process for citric acid production is media supplementation. The supplementation of the fermentation media with a nitrogen source is important for citric acid biosynthesis [2,4]. Trace metal ions such as zinc, magnesium, copper and manganese have a significant influence on citric acid accumulation by *Aspergillus niger* [10,11,12]. The level of copper is quite critical and the content added to the fermentation media should be controlled carefully [12]. When the copper ion level is in the right proportion together with operational parameter, the medium will allow high production of citric acid. Copper ion is known to have chelating effect and also been used as an antagonist to iron [13,14]. Furthermore, copper ion addition also provide the advantage of selectively inhibits Penicillia growth as a contaminant [15]. Sporulation of fungi is also effected by Cu^{2+} concentration in the medium [16]. The aim of this study was at optimizing citric acid production from corn cob as a substrate amended with copper ions.

2. MATERIALS AND METHODS

2.1. Sample Collection and Preparation of Fermentation Medium

Corn cob were obtained from waste areas in markets in Benin city, Edo state. Corncobs were sun dried for 5 d and ground to powder using a blender. Dilute hydrolysis of 10 g of corncob powder was carried out using 0.1 N HCl. The hydrolysis reaction was quenched by adding 1.0 M NaOH. Then the pH will be adjusted to 5.5 by addition of 0.1 N NaOH and the hydrolysis was autoclaved at 121 ^{o}C for 20 mins. After acid hydrolysis the reaction mixture was filtered using a thin cloth and the filtrate was collected for citric acid production [2].

2.2. Isolation and Inoculum Preparation of *Aspergillus niger*

An isolate of *Aspergillus niger* from an onion left at room temperature to undergo spoilage was used for the experimental study. The fungal isolate was identified based on cultural and microscopy characterization following standard methods and maintained on potato dextrose agar (PDA) slant and stored at 4 ^{o}C. Inoculum was prepared from a subculture of *Aspergillus niger* on PDA plates and incubated for 5 d. After the inoculation period, a solution of 1 % tween 80 was prepared and 10 ml of the solution was poured each time onto the cultured agar plate and the spores was gently scrapped using sterile glass rod. The number of spores was counted using haemocytometer and inoculum size of 10^6 spores/ml was used to inoculate all the media [17,18].

2.3. Fermentation process

Submerged fermentation was carried out at temperature of 28 ± 2 ^{o}C on an orbital shaker at a speed of 120 rpm for 10 d using the corncob media. The media were supplemented with ammonium sulphate and trace elements. The constitution of the supplemented medium is as follows: $(NH_4)_2SO_4$ – 2.0 g/l, KH_2PO_4 – 1.0 g/l, $MgSO_4.7H_2O$ – 0.5 g/l and $ZnSO_4.7H_2O$ – 0.1

g/l added to varied concentration (5 – 25 g/l) of corn cobs. The pH was adjusted to 5.5 using either 0.1N HCL or 0.1N NaOH after testing initial pH level [19].

The Substrate concentration was varied to investigate the best concentration: conical flask of 250ml with 100ml of distilled water is supplemented with 5, 10, 15, 20 and 25 g of the best substrate to obtain a substrate concentration of 5, 10, 15, 20 and 25 % w/v respectively.

Using the best substrate concentration, the effect of different concentration of copper (II) ions (0, 0.1, 0.2, 0.3 and 0.4 g/l) was investigated in the medium to obtain the concentration of copper (II) ion with the highest yield of citric acid. The copper ion used is $CuSO_4 . 5H_2O$.

All flask containing prepared medium was sterilized at 121°C for 15 mins, cooled and inoculated with 1ml (10^6 spores/ml) from the homogenate *Aspergillus niger* culture aseptically transferred into each medium [18]. Parameters analyzed at every 2 d interval were citric acid concentration, amylase content, fungal biomass and pH.

2.4. Analytical methods of the fermented media

Determination of fungal biomass, citric acid produced and changes in pH of the fermented corncob media were carried out after 2 d interval for 10 d. Fungal biomass was determined after the medium was pasteurized at 65 °C for 30 min in a water bath. Filtration process was carried out to collect the fungal mycelia using pre-weighed Whatman No 1 filter paper and washed twice with 50 mL sterile distilled water. The fungal biomass and filter paper were dried at 90 °C in a Genlab hot air oven (YIA 110 model, England) to a constant weight. [18]. Citric acid produced was determined titrimetrically by using 0.1N NaOH and phenolphthalein as indicator and calculated as g/l according to the formula:

$$\text{Citric acid (g/l)} = \frac{\text{Normality x Vol of NaOH x Molar mass of citric acid}}{\text{Volume of sample}}$$

Where molar mass of citric acid is 196 [20].

Changes in pH were determined using use of pH meter (3305 Jenway, England) [21].

2.5. Statistical Analysis

Data obtained in this study were expressed as the mean and standard error of triplicates analysis. Statistical analysis was performed using Analysis of variance (ANOVA) (Spss version 23). The means were compared using Duncan's multiple range test (MRT) and $P < 0.05$ were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Effect of substrate concentration

The isolate fungus from the onion was identified using cultural, morphological and microscopically as *Aspergillus niger*. The result revealed increase in pH from day 0 to day 4 after with decrease in pH recorded in all media from day 6 till the end of the study seen in Table 1. The highest pH at the end of the study was recorded in 5 % w/v of supplemented corncob media (6.28±0.38) while the least was recorded in 25 % w/v corncob supplemented media (5.88±0.18).

During fermentation, pH of the medium is important in citric acid accumulation. Behera *et al*. {14] reported that at pH lower than 5 favour accumulation of citric acid and reduced the possibility of other microorganisms contaminating the medium, thus making recovery of product easier [22]. The pH of the media throughout fermentation period of 10 d increased till day 4 and reduced from then till the end of the fermentation with lowest pH seen in 25 %w/v with value of 5.88 ± 0.18. This could be as a result of the presence of a weak organic acid (citric acid) and is in agreement with Haq *et al*. [23] investigation, who reported that the optimal production of citric acid by *Aspergillus niger* GCMC-7 was at pH 5.4, and it also aligns with Ali *et al*. [24] reports, that fungi strains seem to thrive between acidic medium ranging 3 – 6.

Table 1: pH value of the varying substrate concentration of corncob media

Substrate Concentration (%)	Fermentation Period (D)					
	0	2	4	6	8	10
5	5.50±0.00	5.87±0.18	6.48±0.39	6.41±0.40	6.33±0.38	6.28±0.38
10	5.50±0.00	5.85±0.17	6.43±0.41	6.38±0.05	6.27±0.37	6.21±0.22
15	5.50±0.00	5.73±0.05	6.36±0.05	6.23±0.23	6.15±0.59	6.11±0.03
20	5.50±0.00	5.69±0.00	6.31±0.38	6.26±0.38	6.09±0.08	5.98±0.12
25	5.50±0.00	5.66±0.35	6.28±0.38	6.19±0.22	6.03±0.08	5.88±0.18

Key: *Values are the mean and standard error of triplicate;

The varied concentration of corn cob in the medium was found to support growth of *A. niger* (Figure 1). The result revealed increase in fungal biomass from day 0 to day 8 after which there was a decrease at day 10. The highest fungal biomass was recorded in 25 % w/v of corn cob supplemented media (2.05±0.06 g/L) after day 8 of fermentation while the least was recorded in 5 %w/v of corncob supplemented media (1.74±0.07 g/L). There was significant difference ($p< 0.05$) between the fungal biomass of the varying substrate concentration throughout the period of fermentation.

Currently, great attention has been on the use of agro-waste as substrate for industrial production of metabolic products such as organic acid [25]. For the ideal substrate to be selected for organic acid production, cost and availability must be put into consideration. Corn cob as a potential substrate for citric acid production by *A. niger* have been studied [4,8,9]. The media supplementation was carried out to provide the fungi with basic nutrient required for growth which agrees to the findings of Adudu *et al*. [4] and Shetty [2] that fermentation media for citric acid production should consist of nutrient necessary for fungal growth. Corn cob was converted into fermentable sugars through acid hydrolysis. From the investigation, appreciable fungal biomass was observed in varied corn cob concentration. The concentration of 25 %w/v gave the highest biomass, indicating the amount of corn cob needed to support more of *A. niger* growth.

According to Shetty [2] biomass is a fundamental parameter in the characterization of microbial growth and the production of citric acid paralleled the consumption sugar which is also seen in my work. An increase in fugal biomass throughout the fermentation was observed with the highest fungal biomass seen in 25 %w/v CCS media on day 8. This may be a result of an increase in sugar or carbon source which could mean more sugar is utilized that also translates to increase in microbial growth. The findings of Dashen *et al*. [26] revealed that there was a steady increase in mycelial weight and Citric acid yield with a proportional decrease in residual sugars.

Citric acid concentration increased as fermentation progressed up to day 8 and declined at day 10 as shown in Figure 2. The highest concentration of citric acid day 8 was recorded in 25 % w/v of corncob supplemented media (1.04±0.06 g/L) while the least was recorded in 5% w/v of corncob supplemented media (0.36±0.03 g/L).

In the corn cob media fermentation, increase in concentration increased citric acid production. Corn cob concentration of 25 %w/v resulted in the highest production of citric acid indicating that the sugar content was high enough for *A. niger* growth and to accumulate more citric acid than other concentration [27]. At this appreciable concentration of corn cob, nutrient released was able to increase the activity of enzyme involve in citric acid production [27,28]. Maximum citric acid production was observed after 8 d of fermentation and decreased after 10 d. This agreed with the report of Anastassiadis *et al*. [11] that fermentation duration of 8 d favours the accumulation of citric acid by *A. niger*. The findings supported the report of Dutta *et al*. [22], who stated that citric acid accumulation increased as fermentation period increases but decreased after a longer period. Decrease in citric acid accumulation may be as a result of the fungi age, nutrient depletion and inhibitory effect of high citric acid concentration [18].

An increase in the citric acid concentration throughout the fermentation period was noticed, with the highest citric acid yield seen in 25 %w/v CCS media on day 8, which could be as a result of an increase in substrate concentration [8]. The findings stated corncobs concentration increased from 6 - 24 %, increased the yield of citric acid by *Aspergillus niger* The findings of Addo *et al*. [9] stated that large increase of the sugar concentration resulted in a considerable increase in the amounts of citric acid produced.

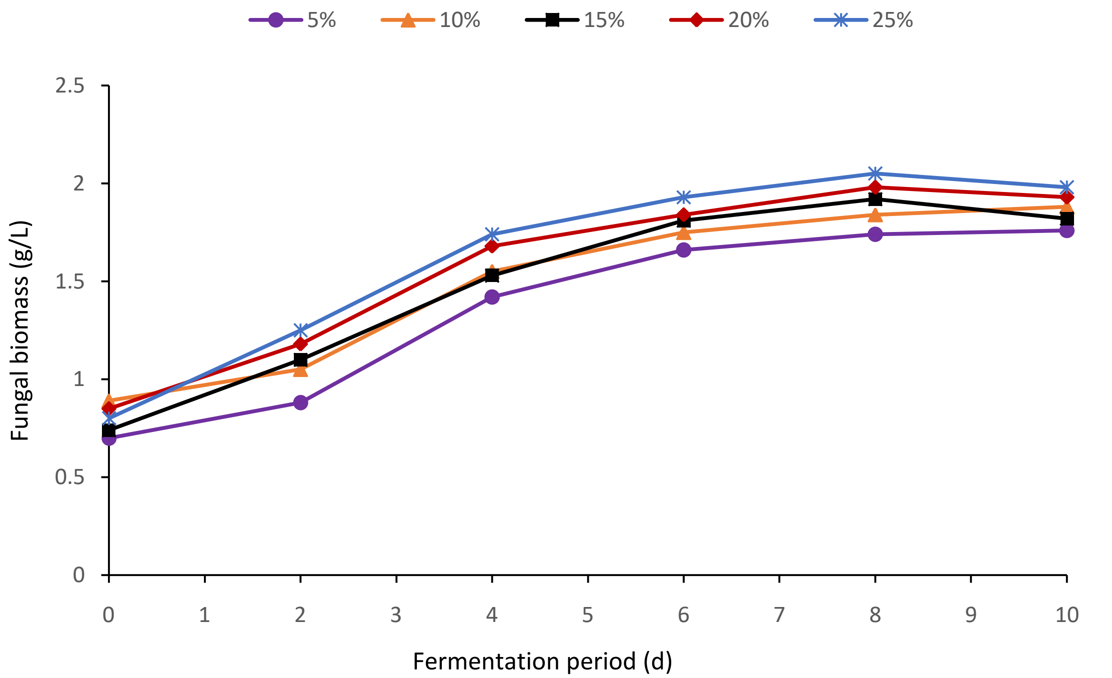

Figure 1: Fungal biomass of *A. niger* inoculated into varying substrate concentration corncob media

Key: 5 % w/v Corncob supplemented medium; 10 % w/v Corncob supplemented medium; 15 % w/v Corncob supplemented medium; 20 % w/v Corncob supplemented medium; 25 % w/v Corncob supplemented medium.

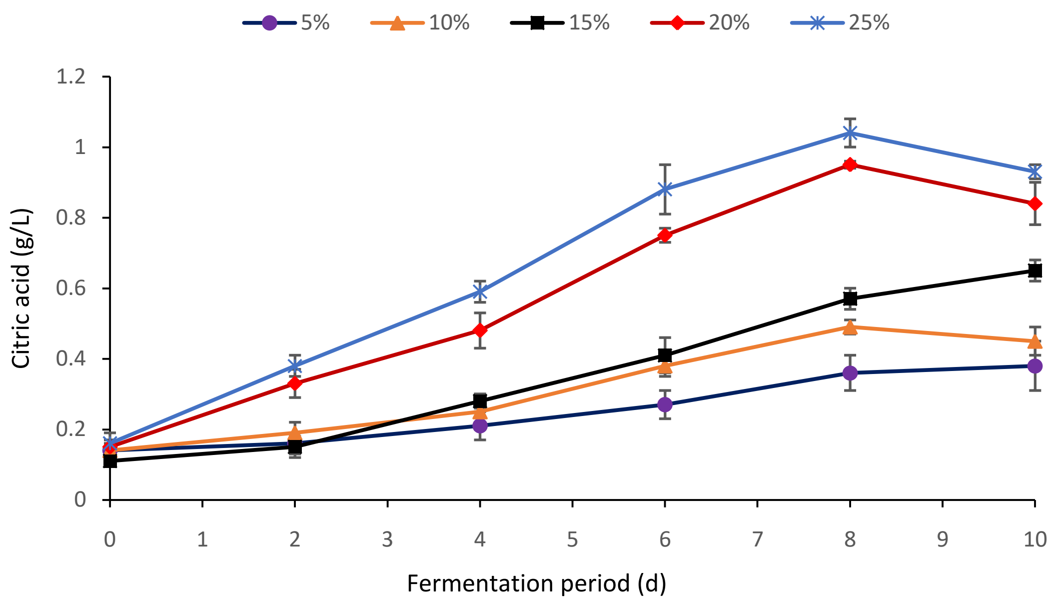

Figure 2: Citric acid production of *A. niger* inoculated in varying substrate concentration of corncob media

Key: 5 % w/v Corncob supplemented medium; 10 % w/v Corncob supplemented medium; 15 % w/v Corncob supplemented medium; 20 % w/v Corncob supplemented medium; 25 % w/v Corncob supplemented medium.

3.2. Effect copper ion concentrations on citric acid production

The effect of media supplemented with varied copper ion concentrations on *A. niger* growth and citric acid production was investigated using 25 % w/v Corncob medium, which previously showed the highest citric acid yield. In this study, varied copper ions concentrations (0 – 0.4 g/l) were investigated. Dried *A. niger* biomass cropped from the varied copper ions concentrations is presented in Figure 3. In the study, medium supplemented with 0.3 g/l of copper ion recorded the highest biomass yield of 3.11±0.04 g/L while the least 2.05±0.06 g/L was recorded in medium supplemented without copper ion (0 g/l). Statistically, medium supplemented with 0.3 g/l of copper ion was significantly different from other media ($p < 0.05$). The amount of citric acid produced when different concentration was copper ions was used is shown in Figure 4. The highest citric acid accumulated was in the medium supplemented with 0.3 g/l of copper ion and the least was medium supplemented with 0 g/l of copper ion with values of 2.94±0.04 and 1.04±0.06 g/L respectively.

789Changes in pH values with the different concentrations of copper ion in the medium is shown in Table 2, The highest and lowest final pH recorded after 8 d of fermentation were 5.5 ± 0.21 and 4.8 ± 0.07 for media supplemented with 0.3 and 0 g/l of copper ion respectively.

There have been studies that copper ions enhanced the production of citric acid and also played an important role in reducing the deleterious effects of iron on citric acid production [16]. Also, copper addition is an excellent technique for achieving highest yields of productivities and constitutes the most efficient modern technology for citric acid production. Tsekova and Todorova [29] reported that generally the growth of *Aspergillus* species is less affected by the presence of Cu^{2+} than *Rhizopus* species, with the most resistant strain to the copper ions inhibition being *Aspergillus niger*. Yigitoglu [30] also found that copper ions play an important role in minimizing the deleterious effects of iron on citric acid production.

In this study at 0.3 g/l copper ions in 25 %w/v Corn cob media produced the highest yield of citric acid. The presence of copper ions in the media, at a lower concentration, is very important to enhance fungal cellular physiology during fermentation for citric acid production [31]. Copper ion in the medium helps to eliminate the toxic effect of iron II ions [14] thus, 0.3 g/l of copper ion favours the production of citric acid. Karren and Rahman [32] reported high accumulation of citric acid when medium was supplemented with 1 %w/w of copper ion. Cayford *et al*. [33] reported that at a concentration of 0.5 g/l of copper ions using *Aspergillus niger*, citric acid accumulated the highest. In the presence of copper (II) ions there was an increase in fungal biomass and citric acid accumulation in the 0.3 g/l copper ions CCS media.

Table 2: pH of varying copper (II) ions concentrations supplemented in corncob media

Copper ion Concentration (g/l)	**Fermentation Period (D)**					
	0	2	4	6	8	10
0	5.50±0.00	5.66±0.35	6.28±0.38	6.19±0.22	6.03±0.08	5.88±0.18
0.1	5.50±0.00	5.63±0.14	6.25±0.38	6.17±0.58	5.94±0.13	5.32±0.06
0.2	5.50±0.00	5.61±0.39	6.21±0.22	6.17±0.23	5.83±0.19	5.74±0.14

0.3	5.50±0.00	5.59±0.14	6.11±0.03	6.13±0.02	5.42±0.02	4.85±0.02
0.4	5.50±0.00	5.60±0.39	6.19±0.58	6.15±0.59	5.56±0.09	5.15±0.05

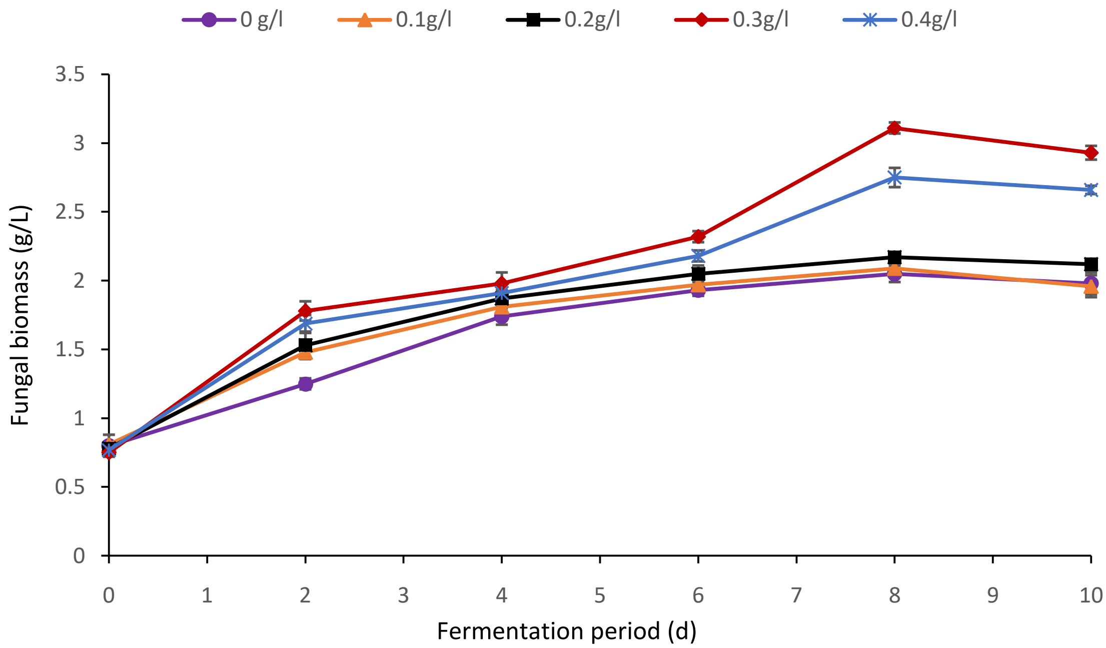

Figure 3: Fungal biomass of *Aspergillus niger* inoculated into varying copper (II) ions concentrations supplemented in corncob media

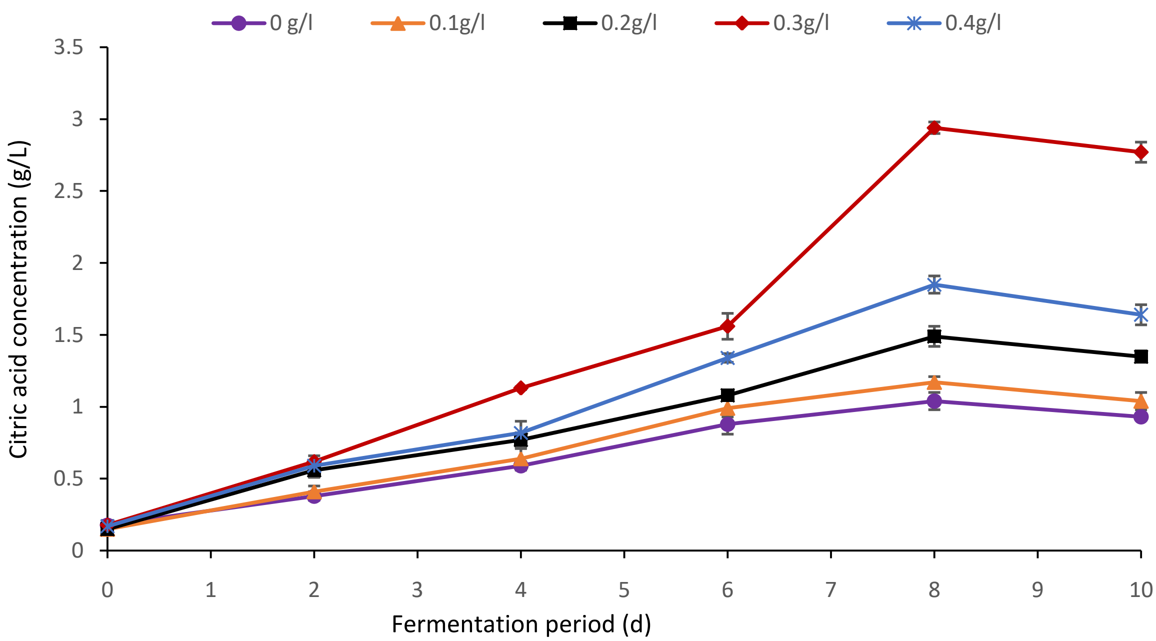

Figure 4: Citric acid yield by *Aspergillus niger* inoculated into varying copper (II) ions concentrations supplemented in corncob media

4. CONCLUSION

Citric acid is commercially produced from various sources by *Aspergillus niger*. An increase in the demand of citric acid has led to finding cheaper fermentable substrates and more ways to improve the yield of citric acid. This study revealed that agro waste can be processed for use as a substrate in the industrial production of citric acid. Hence, sourcing the amylolytic fungus locally and utilizing it for the local production of citric acid that is in high demand for many industrial and biotechnological processes, will go a long way to boosting the local industries as well as the economy. Also, the addition of copper ions at lower concentrations can help to improve the yield of citric acid. However, further studies on manipulating the genes of the fungal isolate for efficient citric acid production should be considered.

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