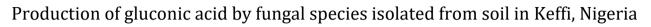


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(Research Article)



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# Abstract

This investigation aimed at production of gluconic acid by fungal species isolated from soil in Keffi. Standard microbiological methods were employed for isolation and identification of the fungal isolates. The yields of gluconic acid produced by the different isolates of the fungi were determined using gas chromatograph and mass Spectrometry. The occurrence of fungi showed that *Rhizopus oryzae* was 100%, *Aspergillus carneus* was 75.0%, *Aspergillus niger* was 75.0% and Aspergillus terreus was 100% while Trichoderma viride was 25.0% and Fusarium moniliforme was 25.0%. The result further demonstrated that three species of the fungal isolates Aspergillus niger, Aspergillus carneus and Fusarium moniliforme were found to produce gluconic acid. Screening for gluconic acid production showed that Aspergillus carneus isolated from locations As1, Cs1 and Cs2, Aspergillus niger isolated from locations Bs1, Bs2 and Cs4 were able to produce gluconic acid. Result of effect of temperature, pH, substrate concentration and fermentation time on production of gluconic acid showed that Aspergillus niger Bs2 produced highest amount of gluconic acid at 28°C, similarly highest amount for gluconic acid produced by Aspergillus carneus As1 was at 28°C, whereas Fusarium moniliforme Bs4 produced highest at 30°C. pH 6.5 was found to the best optima pH for production of both gluconic acid for the fungi studied namely Aspergillus niger Bs2 and Aspergillus carneus As1 and Fusarium moniliforme Bs4 produced highest gluconic acid at pH 5.5. The substrate concentration showed highest production of gluconic acid was produced by Aspergillus niger Bs2 at substrate concentration of 25%. Aspergillus carneus As1 produced highest at substrate concentration of 20% and Fusarium moniliforme Bs4 produced highest at substrate concentration of 20%. The fermentation time showed highest production of gluconic acid by Aspergillus niger Bs2 and Aspergillus carneus As1 was after 144 hours whereas *F. moniliforme* Bs4 produced gluconic acid after 120hrs respectively. The fungi species isolated from soil in keffi revealed great ability in production of gluconic acid.

Keywords: Fungi; Gluconic acid; Temperature; pH; Substrate concentration and fermentation time

# 1. Introduction

Gluconic acid is an important organic acid resulting from the oxidation of D-glucose. The unique property of this acid such as low toxicity, low corrosively and complexing capability with metal ions has enabled it's wide application in the food, pharmaceutical, textile, leather and other industries Shindia, *et al.*[1]]. Thus, the high demand of this organic acid has been on increased for the Pass 20 years and production is growing to more than 60,000 tons every year and still on increase El-Enshasy [2]. Commercially, gluconic acid is produced by three different methods, chemical oxidation of glucose with a hypochlorite solution Kundu and Das [3], electrolytic oxidation of glucose solution containing a known value of bromide, and fermentation process where specific microorganisms are grown in medium containing glucose and other ingredients. The microbial fermentation process offers an attractive technique for the gluconic acid production to alleviate the problems related to chemical production such as the inevitable side reactions and also to further economize the bioprocess.

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A wide group of microorganisms, particularly filamentous fungi have the ability for gluconic acid production Shindia, *et al.* [1]. The production of gluconic acid is mainly done in batch cultivation using several species belonging to the following fungal genera, *Aspergillus, Penicillium, Fusarium, Mucor,* and *Gliocladium* Lee *et al.* [4]. Among the different fungal genera, it has been reported that the accumulation of large amounts of the gluconic acid and its salts are restricted to certain species of *Aspergillus,* especially *A. niger* which considered as the most industrially important gluconic acid producer in fermentation industry Sankpal and Kullkarni [5]. Because of its industrial importance, many investigators have been worked for optimization as well as overproduction of gluconic acid by improving fungal producers Sankpal and Kulkarni [5] El-Enshasy [2]. Also, developing an alternative method in screening techniques to identify and develop possible native fungal strains for commercial exploitation of the process (Spankpal, *et al* [6], despite the abundant availability of commercial gluconic acid. The aim of this research is production of gluconic acid by fungal species isolated from soil in Keffi, Nigeria.

## 2. Material and methods

## 2.1. Sample Collection

Four soil samples were randomly collected (at the top soil) from four different locations of Keffi metropolis location A (high court), location B (Angwan lamnbu), location C (GRA) and location D (Old barracks), using a clean hand trowel and stored using disposable black polythene bags and transported immediately to the Microbiology Laboratory, Nasarawa State University, Keffi for analysis.

### 2.2. Isolation of fungal strains

The isolation of fungal was carried out following a method described by Makut and Ade-Ibijola [7]. One (1) gram of the soil sample was suspension in a test tube containing 9 ml of sterile distilled water to make a soil suspension and tenfold serial dilution was made by transferring one ml of the soil suspension to another test tube containing 9 ml of sterile distilled water. These steps were repeated ten times to obtain a dilution of 10<sup>-6</sup>. From each of the first three test tubes, 0.1 ml of the aliquot was spread on Potato dextrose agar plates and was incubated at 26°C for 4 days.

### 2.3. Identification of fungal species

Identification of fungal species was carried out as described by Makut and Ade-Ibijola [7]. Identification was based on microbiological standard procedure using cultural and morphological characteristics. The cultural characteristics were determined by their appearance on culture plates while the morphological features were determined microscopically using lactophenol cotton blue staining technique. The isolates were identified with reference to the fungi standard chart atlas.

## 2.4. Screening for gluconic acid Producing fungal strains

Screening for gluconic acid Producing fungal strains were carried out as described by El-Enshasy, [8]. The medium used for preparation of calcium carbonate plates was consist of, glucose, 10g; (N H 4\ H PO~, *OA*; MgS04.7Hp, 0.2g; KHl04, 0.2g; CaCOo. *SOg* and agar 20g dissolved in 20ml of double distilled water and autoclaved at 15 lb for 15 min. CaCO, was sterilized separately and added to medium at the time of plating. The medium was inoculated by 10-fold diluted spore suspension following incubation at 30°C for 3days. A clear zone was observed around distinct colonies on the plates indicating the production of calcium gluconate. The fungal producing clear zones were picked up and store for further use

#### 2.5. Preparation of Cassava baggers starch substrates

The substrate was prepared using a method described by Ekeleme, *et al.* [9] with modification corn starch. Cassava baggers was collected and sun dry and grind into powder form using clean grinding machine and sieve. Five hundred gram (500g) powder form was added into 4 liter of distilled water and sieves to form a homogenous mixture and placed at 4°C for 24 h. The starch settled down was separated from liquid and oven dried at 60°C, overnight. A starch solution of 20 g/l was dissolve and autoclaved at 5.0 lbs/ in2 pressure (115°C) for 5 min. To liquefy starch, alpha amylase ( $2.0\mu/ml$ ) was added and heated at 95°C in a water bath for 15 min. For saccharification, amyloglucosidase ( $2.0\mu/ml$ ) was added and heated at 55°C while constant stirring for about 4 h.

## 2.6. Production of gluconic acid

#### 2.6.1. Preparation of inoculum for fermentation

Preparation of inoculum for fermentation was carried out as described by Ekeleme *et al.* [10]. Five (5ml) of 0.3% sterile tween 80 containing in peptone water with some glass beads were transferred into four (4) days' slant culture of fungal species and was shake thoroughly until spores were dislodge and 10<sup>5</sup> of the spores was transferred into peptone broth and incubated at 26°C for 12 hours.

### 2.7. Media formulation and fermentation technique

The batch fermentation was carried out as described by Ekeleme *et al.* [10] with modification, the modification was the use of Cassava baggers Starch hydrolysate. Cassava baggers Starch hydrolysate and nitrogen sources such as: M1 [glucose (20g/L), soya bean cake (2.5 g/L), NH<sub>4</sub>Cl 0.4 g, KH<sub>2</sub>PO<sub>4</sub> 0.1 g, MgSO<sub>4</sub>, 7H<sub>2</sub>O 0.025 g]; and was taken in 250 ml conical flasks. The flasks were plugged with cotton and autoclaved at 15 psi for 15 min. The sterilized flasks were inoculated with 5.0ml of the inoculum under aseptic conditions. Sterilized ferrocyanide (200 ppm free ions concentration) was added to each flask. The flasks were placed in an incubated at different temperature. All the experiments were run parallel in duplicates.

### 2.8. Effect of pH on gluconic acid production

The effect of pH was carried out following a method described by Ekeleme *et al.* [10]. Fifty (200) ml of the different fermentation substrate were transfer into different conical flasks. The pH ranges were adjusted to, 5.5, 6.0 and 6.5 of fermentation media. One (1) N HCl was used in adjusting the pH of the media.

### 2.9. Effect of temperature on gluconic acid production

Effect of temperatures was carried out following a method described by Ekeleme *et al.* [9]. Fifty (50) ml of the different fermentation substrate were transfer into different conical flasks and the fermentation media was incubated at 28°C - 39°C.

### 2.10. Effect of fermentation duration on gluconic acid production

The effect of fermentation duration was carried out as described by Ekeleme *et al.* [9]. Different time interval were monitored during the fermentation of the media after 72 hours to 192hours.

#### 2.11. Estimation of gluconic acid

The gluconic acid produced during fermentation was determined by Gas Chromatography and Mass Spectrometry (GC and MS) Akalin *et al.*, [11] as detailed below;

#### 2.12. Sample preparation

During sample preparation, 7 mL of fermented media was added to 40 mL of buffer-acetonitrile mobile phase (0.5% (w/v) (NH4)2HPO4 (0.038 M) - 0.4% (v/v) acetonitrile (0.049 M), at pH 2.24 with H3PO4), extracted for 1 hour in orbital shaker and centrifuged at 6000 x g for 5 min. The supernatant was collected and filtered once through filter paper Whattman No. 1 and twice through a 0.45  $\mu$ m membrane filter, and then used directly for GC and MS analysis. Duplicate analyses were performed on all samples.

## 3. GC and MS Analysis

Chromatograph equipped with flame-ionization detector. The column used for the separation of solvent was PEG (2.1m x 3.0mm). The operating conditions was mobile phase, aqueous 0.5% (w/v) (NH<sub>4</sub>)2HPO<sub>4</sub> (0.038 M) - 0.2% (v/v) acetonitrile (0.049 M) adjusted to pH 2.24 with H<sub>3</sub>PO<sub>4</sub>; flow rate 0.3 mL min-1; ambient column temperature. The mobile phase was prepared by dissolving analytical-grade (NH<sub>4</sub>)2HPO<sub>4</sub> in distilled deionized water, GC and MS -grade acetonitrile, and H<sub>3</sub>PO<sub>4</sub>. GC and MS -grade reagents was used as standards (Sigma Chemical Co., St. Louis, MO). Solvents were filtered through a 0.45 µm membrane filter and one hundred and twenty degrees centigrade (120°C), Nitrogen gas (30 mL/minutes) was used as carrier gas. The temperatures of injector and detector were 150°C and 200°C respectively. The Peaks were recorded on "SHIMADZU C-R-4\_A, Chromatograph", and was identified by comparison of the retention times with that of standard mixture. The experiment was carried out in duplicate and the means ± standard deviations of the yield of gluconic acid were recorded.

## 3.1. Statistical Analyses

Statistical analyses of data were carried out as described by Si *et al.* [12]. All experiments were conducted in duplicates. Unless otherwise indicated, for each experiment undertaken, the mean levels of variables and the standard deviations (SD) were calculated. Comparisons of variables were made with the One–Way Analysis of Variance (ANOVA). A value of p < 0.05 was considered statistically significant. Statistical analyses were conducted using the software programmer, Statistical Package for Social Sciences (SPSS 21.0; IBM, USA).

## 4. Results and discussion

The cultural morphological characteristics of the fungal isolated from soil in Keffi as showed in Table1.

Table 4.2 shows the percentage occurrence of different fungal species isolated from different soil location in Keffi. The percentage occurrence fungal isolates showed that *Rhizopus oryzae* have 100% occurrence from all the location and 2 were isolated from location A, 1 each were isolated from location B and D respectively. *Aspergillus carneus* have 75.0% occurrence from all the location and 1 was isolated from location A and 2 were isolated from location C. *Aspergillus niger* have 75.0% occurrence from all the location and 2 were isolated from location B and 1 from location C. *Aspergillus niger* have 75.0% occurrence from all the locations and 1 each were isolated from location B and 1 from location C. *Aspergillus terreus* have 100% occurrence from all the locations and 1 each were isolated from all the locations A-D. *Trichoderma viride* have 25.0% occurrences and 1 each was isolated from location B and 1 from location D. Lastly *Fusarium moniliforme* have 25.0% occurrences and 1 each was isolated from location B and 1 from location D respectively.

The screening for the ability of gluconic acid production by the fungal isolates is as given Table 3. The different species of the fungal showed ability of gluconic acid production were *Aspergillus carneus* As1, *Aspergillus carneus*, *Aspergillus carneus*, *Aspergillus carneus* Cs2, *Aspergillus niger* Bs1, *Aspergillus niger* Bs2 and *Aspergillus niger* Cs4 were gluconic acid producers. *Fusarium moniliforme* Bs4 *Fusarium moniliforme* Ds1 also, produced gluconic acid whereas *Rhizopus oryzae*, *Aspergillus terreus* and *Trichoderma viridei* isolates did not have the ability to produced gluconic acid as observed in this study.

### 4.1. Different effects of fermentation parameters on gluconic acid production

The Effect of temperature on gluconic acid production by fungal isolated is as shown in Figure 1. *Aspergillus niger* Bs2 produced high gluconic acid at temperature of 28°C ( $8.04 \mu g/ml$ ) followed by at 30°C ( $7.35 \mu g/ml$ ), at 33°C ( $6.99 \mu g/ml$ ), at 36°C ( $5.82 \mu g/ml$ ) and at 39°C ( $4.75 \mu g/ml$ ). Similarly, *Aspergillus carneus* As1 produced highest gluconic acid at 28°C ( $7.34 \mu g/ml$ ) followed by ( $7.05 \mu g/ml$ ) at 30°C, ( $5.42 \mu g/ml$ ) at 33°C, ( $4.81 \mu g/ml$ ) at 36°C and ( $3.12 \mu g/ml$ ) at 39°C. *Fusarium moniliforme* Bs4 produced high gluconic acid at 30°C ( $5.03 \mu g/ml$ ) followed by at 33°C ( $4.23 \mu g/ml$ ) of gluconic acid, at 36°C ( $4.01 \mu g/ml$ ), at 28°C ( $3.55 \mu g/ml$ ) and at 39°C ( $2.92 \mu g/ml$ ) of gluconic acid respectively.

Characteristics				
Cultural	Morphological	Fungal		
Grow fast with white cotton like at first later becoming brownish grey to blackish-grey	Sporangia are greyish-black, spherical and Sporangiospores are angular to broadly ellipsoidal	Rhizopus oryzae		
Colonies are typically black in colour with white cleistothecia developing within and upon the conidial layer. Reverse is olive and brown	Branched conidiophores with chains of conidia like a brush	Aspergillus carneus		
colonies consist of a compact white or yellow basal felt covered by a dense layer of dark-brown to black	conidial heads are biseriate, large, globose, dark brown, becoming radiate with the phialides borne on metulae	Aspergillus niger		
Colony appear as sand-brown in colour with a yellow to deep dirty brown reverse of plate	Conidiophore stipeses are hyaline and smooth-walled. conidia are conidial heads are biseriate	Aspergillus terreus		
First grow white and downy, later developing yellowish-green to deep green compact tufts, often only in small areas or in concentric ring-like zones on the agar surface	Conidiophores are branched, irregularly verticillate, bearing clusters of divergent, often irregularly bent, flask-shaped phialides	Trichoderma viride		

Table 1 Cultural and Morphological characteristics of Fungal isolated

Figure 2 shows the effect of pH on gluconic acid production by fungal isolates. The various amounts of gluconic acid produced showed that *Aspergillus niger* Bs2 produced highest gluconic acid at pH 6.5 (7.09  $\mu$ g/ml) followed by at pH 6.0 (6.89  $\mu$ g/ml), (5.12  $\mu$ g/ml) at pH 5.5, (4.19  $\mu$ g/ml) at pH 5.0, at pH 4.5 (4.18  $\mu$ g/ml) and at pH 4.0 (3.12  $\mu$ g/ml). Similarly, *Aspergillus carneus* As1 produced highest gluconic acid at pH 6.5 (6.51  $\mu$ g/ml) followed by at pH 6.0 and pH 5.5 (5.43  $\mu$ g/ml), at pH5.0 (4.68  $\mu$ g/ml), at pH4.5 (3.32  $\mu$ g/ml) and at pH 4.0 produced the lowest (3.21  $\mu$ g/ml) respectively. *Fusarium moniliforme* Bs4 produced highest gluconic acid at pH 5.5 (4.03  $\mu$ g/ml) followed by at pH 6.0 (4.00  $\mu$ g/ml), (3.87  $\mu$ g/ml) at pH 6.5, (3.12  $\mu$ g/ml) at pH 5.0, at pH 4.5 (2.88  $\mu$ g/ml) and at pH 4.0 (2.00  $\mu$ g/ml) respectively.

Table 2 Percentage occurrence of fungi species isolated from soil in Keffi

	Locations					
Fungi isolates	No sample	Α	В	С	D	Occurrence (%)
Rhizopus oryzae	4	2	1	0	1	4(100)
Aspergillus carneus	4	1	0	2	0	3(75.0)
Aspergillus niger	4	0	2	1	0	3(75.0)
Aspergillus terreus	4	1	1	1	1	4(100)
Trichoderma viride	4	1	0	0	1	2(50.0)
Fusarium moniliforme	4	0	1	0	1	2(50.0)

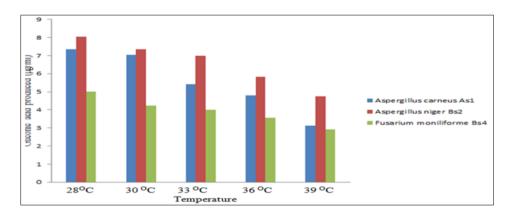


Figure 1 Effect of temperature on gluconic acid production by fungal isolates

Table 3 Screening for gluconic acid producing fungal isolated from soil

Fungal	Sample codes	Gluconic acid producers
Rhizopus oryzae	As2	-
Rhizopus oryzae	As2	-
Rhizopus oryzae	Bs3	-
Rhizopus oryzae	Ds1	-

As1	+
Cs1	+
Cs2	+
Bs1	+
Bs2	+
Cs4	+
As4	-
Bs3	-
Cs3	-
Ds2	-
As4	-
Ds4	-
Bs4	+
Ds1	+
	Cs1 Cs2 Bs1 Bs2 Cs4 As4 Bs3 Cs3 Ds2 As4 Ds4 Bs4

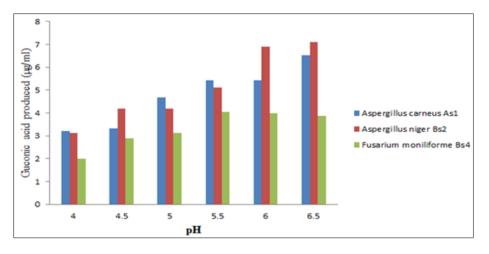


Figure 2 Effect of pH on gluconic acid production by fungal isolates

The effect of substrate concentration on gluconic acid production is as given in Figure 3. The highest gluconic acid produced at different substrate concentration was by *Aspergillus niger* Bs2 at substrate concentration of 25% (6.98  $\mu$ g/ml) followed by at substrate concentration of 20% (5.42  $\mu$ g/ml), 15% substrate concentration (5.09  $\mu$ g/ml), 10% substrate concentration (3.24  $\mu$ g/ml) and the least was at 5% substrate concentration (1.75  $\mu$ g/ml). *Aspergillus carneus* As1 produced highest at substrate concentration of 20% with the yield of 5.51  $\mu$ g/ml followed by 25% substrate concentration with 4.62  $\mu$ g/ml, at 15% substrate concentration it produces 4.01  $\mu$ g/ml. *Tug/ml* and at 5% substrate concentration it produced 1.19  $\mu$ g/ml. *Fusarium moniliforme* Bs4 produced highest at substrate concentration it produced 3.11  $\mu$ g/ml and at 5% substrate concentration it produced 1.19  $\mu$ g/ml followed by 15% substrate concentration with 4.22  $\mu$ g/ml, at 25% substrate concentration it produces 3.03  $\mu$ g/ml, at 10% substrate concentration it produced 2.11  $\mu$ g/ml and at 5% substrate concentration it produces 3.03  $\mu$ g/ml, at 10% substrate concentration it produced 2.11  $\mu$ g/ml and at 5% substrate concentration it produces 1.05  $\mu$ g/ml respectively.

The effect of fermentation time on gluconic acid production by fungal isolates is as given in Figure 4. highest gluconic acid was produced by *Aspergillus niger* Bs2 after 144hrs with 5.02  $\mu$ g/ml followed by after 168hrs with 4.88  $\mu$ g/ml, 120hrs with 4.14  $\mu$ g/ml, 96hrs with 4.01  $\mu$ g/ml, 192hrs with 4.00  $\mu$ g/ml, 72hrs with 3.32  $\mu$ g/ml, 48hrs with 3.18  $\mu$ g/ml and 24hrs with 2.00  $\mu$ g/ml. similarly *Aspergillus carneus* As1 produced highest gluconic acid after 144hrs with 4.50  $\mu$ g/ml followed by after 168hrs with 4.21  $\mu$ g/ml, 120hrs with 4.12  $\mu$ g/ml, 192hrs with 3.75  $\mu$ g/ml, 96hrs with 3.22  $\mu$ g/ml, 72hrs with 2.51  $\mu$ g/ml, 48hrs with 2.49  $\mu$ g/ml and 24hrs with 1.22  $\mu$ g/ml. whereas *Fusarium moniliforme* Bs4

produced gluconic acid after 120hrs with 3.25  $\mu$ g/ml followed by 144hrs with 3.08  $\mu$ g/ml, 168hrs with 2.98  $\mu$ g/ml, 192hrs with 2.77hrs, 96hrs with 2.01  $\mu$ g/ml, 72hrs with 1.97  $\mu$ g/ml, 48hrs with 1.68  $\mu$ g/ml and 24hrs with 1.10  $\mu$ g/ml respectively.

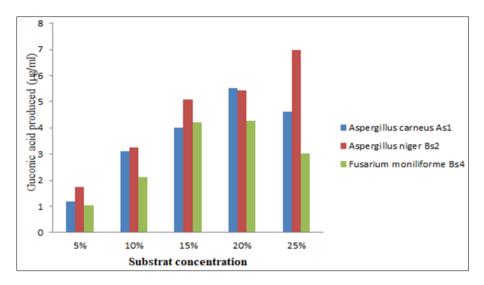


Figure 3 Effect of substrate concentration on gluconic acid production by fungal isolates

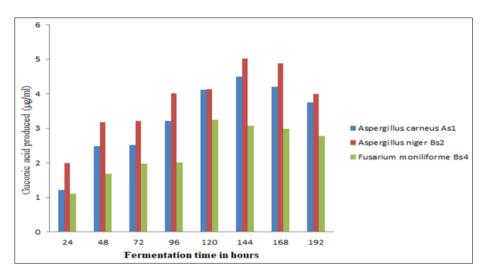


Figure 4 Effect of time on gluconic acid production by fungal isolates

# 5. Discussion

In this study the occurrence of fungal from different location was high which showed a percentage ranging from 25.0% - 75.0% from all the location. This is not as expected because fungi play an important role in breaking down most of both organic and inorganic waste in the environment and is similar to study earlier reported by Nimkar *et al.* [13] and Suganthi, *et al.* [14].

The ability of gluconic acid production in various amount demonstrated by different fungal isolated from soil in different location in this study was in agreement with work reported by Shindia *et al.* [1] on the ability of gluconic acid production by some local fungi which are known to utilized various substrate and release different secondary metabolites.

In this study that highest gluconic acid production was at temperature of 28°C by both *Aspergillus* species (*Aspergillus niger* and *Aspergillus* carneus) while *Fusarium moniliforme* produced highest gluconic acid at temperature of 30°C respectively. This showed that fermentation temperature plays an important role in production of gluconic acid when using different microorganism in bioacid production, which influence the growth and accumulation of the bioacid by the microorganism. This is similar to work early reported by Cheema *et al.* [14] who reported high gluconic acid

production at 28°C by *Aspergillus niger* but in disagreement with work reported by Shindia *et al.* [1] who reported high gluconic acid production by *Fusarium moniliforme* at temperature of 28°C.

The effect of pH is an important parameter in production of organic acid or bioacid using fungal or any other microorganism. In this study high gluconic acid was obtain at pH 6.5 both *Aspergillus* species (*Aspergillus niger* and *Aspergillus carneus*) and pH 6.0 for *Fusarium moniliforme* this is similar to study reported by Dowdells *et al.* [15] and Capuder *et al.* [16]. The realization of gluconic acid production from glucose by fungal species at relatively high pH in the fermentation indicates innovative routes to glucose use for the production of lovastatin and other secondary metabolites without further genetic manipulation of industrial strains, but the production of high concentrations of gluconic acid would disrupt pH regulation in the fermentation and introduce high concentrations of osmotically active solutes especially if the accumulated acid (causing base addition) was later utilized as a carbon source (Dowdells *et al.* [15].

The effect of substrate concentration as observed in this study showed that it is an important parameter in production of gluconic acid. The availability of substrate enhanced the accumulation of this important organic acid.it was observed that at 25% concentration and 20% concentration by the fungal species used in this study which is agreement with work reported by El-Enshasy [8]. Non availability of fermentation substrate will lead in depletion or reused of acid accumulated as source of carbon by the fungal and minimize gluconate formation in secondary product processes, therefore, high concentrations of glucose must be avoided.

## 6. Conclusion

From results obtain from this study the production of gluconic acid by fungal species isolated from soil in different location in Keffi showed the isolation of different fungal from soil namely *Rhizopus oryzae, Aspergillus carneus, Aspergillus niger, Aspergillus terreus, Trichoderma viride* and *Fusarium moniliforme*. These fungal isolates showed varying ability in gluconic acid production base on different fermentation parameter studied and *Aspergillus carneus, Aspergillus* and *Fusarium moniliforme* showed to be a good industrial strain in production of gluconic acid.

## **Compliance with ethical standards**

#### Acknowledgments

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#### Disclosure of conflict of interest

The authors declare that they have no conflict of interest or personal interest.

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