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Citric acid Production from Agricultural Wastes using *Aspergillus niger* Isolated from some Locations within Kaduna Metropolis, Nigeria

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ABSTRACT: This investigation was carried out to assess citric acid production by *Aspergillus* species isolated in some locations within Kaduna metropolis Nigeria using standard methods. Fungal isolates identified as *Aspergillus niger* were obtained from samples collected from four (4) different locations. Isolates were subjected to substrate preference test using local substrates such as wheat straw, rice straw and potato peel powder. Wheat straw supported the highest growth value of 41%. Screening of *A. niger* isolates for the production of citric acid showed that the isolate from Kawo (Kw) gave the highest yield of citric acid (0.38g/100ml) on basal screening media. Optimization of pH and temperature were carried out and the optimum temperature and pH for citric acid production by isolate Kw was 30^{O}C (0.65g/100ml) and pH 6.0. The wheat straw treated with 5 N HCl produced a high yield of citric acid with a value of 25.60g/kg while the untreated wheat straw produced a yield of 13.3g/kg. Molecular characterization to confirm the identity of the fungal isolates was carried out by the amplification and sequencing of the 5.8S gene of the ribosomal RNA and the two intergenic spacers ITS1 and ITS2 of the strains. By comparison (BLAST) of *Aspergillus niger* isolate to reference sequence in the gene bank, a sequence similarity of 99% to 100% of other *Aspergillus niger* strain was obtained. Various methods of strain improvement techniques could be adopted to increase citric acid production by the *A. niger* isolates obtained from this study.

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Biotechnology has been key in exploiting the diversity and/or metabolic activities of different microorganisms in fermenting wide range of agricultural wastes to produce several useful products such as crude enzymes (Saha and Santra, 2014) electrical energy using microbial fuel cell (MFC) (Aderiye *et al*., 2018); chemicals like biogas, bioethanol (Muradin and Foltynowicz, 2014) and organic acids (like citric acid) (Muharani *et al*., 2014; Dienye *et al*., 2018). Citric acid (2-hydroxy-1, 2, 3-tricarboxylic acid) is in high demand in different industries as food, chemical, beverage, cosmetic and pharmaceutical for stabilization, flavour enhancement, emulsification, preservation, anti-oxidation, acidulation, as synergistic agent and plasticizer (Soccol *et al*., 2006; Maharani *et al*., 2014). This acid is the most important organic acid produced by fermentation and it is designated by FDA as GRAS (Generally Recognized as Safe) (Yalcin *et al*., 2009). Citric acid can be produced chemically and synthetically from acetone or glycerol (Haq *et al*., 2004); microbial fermentation has however received tremendous attention recently perhaps, because it is applicable for large scale production of the acid (Bhattacharjee and Baruah, 2015). Among different microorganisms including bacteria and yeast reported

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as capable of producing citric acid, fungi are commonly used in commercial production of the organic acid. However, some potentials like easy to handle, ability to produce higher acid per unit time and ability to utilize cheap raw biomass (Schuster *et al*., 2002; Kareem and Rahman, 2011), has made *Aspergillus*. *niger* most preferred organism in the production of citric acid (Lotfy *et al*., 2007; Maharani *et al*., 2014; Dienye *et al*., 2018). *Aspergillus niger* has been used in the industrial preparation of citric acid (Max *et al*., 2010). *A. niger* grows aerobically on organic matter therefore it can be found almost everywhere in environments that contains soil. Also, it is found in waste, decaying plant material and compost in outdoor environments. The thermotolerant abilities of *A. niger* enable growth over a wide temperature range from 6 to 47ºC with a preferred optimum temperature at 35-37ºC (Schuster *et a*l., 2002.). Using agricultural waste for producing valuable products like citric acid in recent time has not only increased the sustenance of the acid for industrial application, but also enhanced control and management of environmental pollution and littering (Dienye *et al*., 2018). Cheap agricultural wastes like rice straw, wheat straw and potato peel powder are among substrates that are reported promising at cost reduction in citric acid production (Haq *et al*., 2004; Ali *et al*., 2012; Femi-Ola and Atere, 2013; Meenakshi and Kumaresan, 2014). The present study hence aimed at assessing citric acid production potentials in *Aspergillus niger* isolated from samples obtained from different locations within Kaduna metropolis, Nigeria.

MATERIALS AND METHODS

Sample collection: Soil samples were collected from groundnut and cowpea farms in Kawo (KW), and Mando (MD) respectively; and dumpsite in Kaduna State University (KSU), while spoilt fruits were obtained from Kaduna Central Market (CM). Samples were collected in clean nylon bags and taken to the Laboratory of the Department of Biological Sciences, Nigerian Defence Academy, Kaduna, Kaduna State, Nigeria. For the substrate preference, wheat straw and rice straw were collected from farmlands in Kaduna, while potato peel powder was obtained from Kaduna Central market.

Isolation of samples: Five (5) grams of each soil sample were suspended in 90 ml of sterile distilled water. Then 0.1ml of the resulting mixture was spread on the surface of solid potato dextrose agar (PDA) plates using a bentglass rod and incubated at room temperature (29±3°C) for 5 days observing daily for fungal growth. The fungal colonies formed were further sub cultured on fresh potato dextrose agar to obtain pure cultures. The pure cultures were maintained on potato dextrose agar slants and stored in a refrigerator at 4^0C for further use.

Morphological Identification of Aspergillus niger: The fungal pure cultures were macroscopically observed for characteristics such as colony diameter and exudates at day 4 and day 7 of incubation. The fungal isolates were mounted on grease-free glass slide, then stained with lacto-phenol cotton blue and observed for nature of hyphae and conidia under microscope (at Mag. X40) after which photographs were taken. The fungal species were distinctly identified following the Manual about the Genus Aspergilli (Raper and Fennell, 1965; Domsch *et al*., 1980). The characterized *A. niger* isolates were then used in the subsequent tests.

Substrate preference test: The method of Prashant *et al*. (2003) was adopted to assay for the substrate preference. Ten (10) grams of natural substrate (rice straw, wheat straw and potato peel powder) was taken in a strainer and soaked in distilled water for 3 min. The strainer was then removed, and the excess water was drained for the same duration. This procedure was repeated thrice to remove dust and ensure sufficient soaking. The substrate was transferred into conical flasks of 250 ml capacity and sterilized at 121 °C for 20 minutes. Ten (10) ml of sterile water was aseptically added to adjust final moisture content. *Aspergillus niger* isolates were inoculated into flask by adding 1 ml to each flask respectively. Inoculated flasks were kept at room temperatures for 5 days. On the third day flasks were shaken gently to ensure uniform distribution of mycelia over the solid particles. Beakers filled with water were placed in the incubator to keep the air inside the incubator humid.

Spore Extraction and Quantification of Conidia: Fifty (50) ml of sterile water was added to the slants and flasks, respectively. To harvest the spores on the natural substrates, the flasks were vigorously shaken for 15 min, and then filtered with filter paper. The solution which was brown in colour was kept in the refrigerator for 24 hours. Quantification of conidia was done at 560nm using a spectrophotometer.

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Screening for citric acid producing strain among the Aspergillus niger: The *Aspergillus niger* isolates were screened quantitatively for the production of citric acid by inoculating 1 ml of 5 days old spores suspension in sterile liquid basal medium containing; soluble starch 10 gm/L, $(NH_4)_2SO_4$ 2.2 gm/L, K_2HPO_4 1 gm/L, $MgSO_4.7H_2O$ 0.05 gm/L, $CaCl_2$ 0.05 gm/L; with pH adjusted to 6.0. Fermentation was carried out in 250 ml Erlenmeyer flasks containing 100 ml of fermentation medium at room temperature. The

concentration of citric acid in the fermentation medium was estimated titrimetrically (AOAC, 1995) at regular intervals of 12 hours for 72 hours.

Optimization of fermentation medium: Optimization of the culture conditions taking two different parameters, Temperature and pH, for citric acid production using *Aspergillus niger* isolate was done. The citric acid production was estimated after 72 hours of incubation of the inoculated medium. Different temperature ranges were selected, 25°C, 30°C, 35°C and 40°C and a range of pH starting from 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 was selected for citric acid production by *A niger*. The concentration of citric acid in the fermentation medium was estimated titrimetrically (AOAC, 1995).

Fermentation of wheat straw using A. niger
Untreated wheat straw: Wheat straw was washed, air dried and then dried in a hot air oven at 70 °C for about 2-3 hours. The substrate was then grounded to about 1-2 mm size. Slurry of untreated wheat straw was prepared with distilled water at 1:10 (w/v).

Treated wheat straw: Grounded wheat straw was pretreated using acid treatment by slurring in 5 N HCl at a solid liquid ratio of 1:10 (w/v), and incubated in water bath at 100 ºC for 1 hour. After cooling, the substrate was washed with distilled water and dried in an oven at 100ºC.

Fermentation: Fermentation Experiments were conducted in 250 ml flasks, each containing 4 g of crude wheat straw (untreated and treated), and moistened with the appropriate amount of distilled water in order to contain 65 % (w/v) moisture. Substrate was supplemented with sugar cane molasses to contain 14 % (w/w) initial sugar (the water content of molasses was considered in moisture adjustment). The initial pH of the substrate was adjusted to 6.0 with 2 N NaOH and autoclaved at 121ºC for 15 min, the flasks were cooled to ambient temperature and inoculated with 1 ml of spore suspension. The flasks were incubated at 30 ºC in an incubator for 5 days. The medium was then filtered and the filtrate was used to determine citric acid concentration titrimetrically (AOAC, 1995; Khosravi *et al.*, 2008).

Genomic DNA isolation: Fungal genomic DNA was extracted by phenol/chloroform method using molecular DNA Laboratory manual guide Sambrook *et al*., (1999). The DNA concentration and purity was checked using a spectrophotometer.

PCR amplification: Amplification of ITS rDNA and 5.8S rDNA of the fungal isolates were carried out according to method described by Gene *et al*. (1996) using a Perkin Elmer 2400 thermal cycler. The primer pairs ITS5 (3′GGAAGTAAAAGTCGTAACAAGG5′) and ITS4 (3′TCCTCCGCTTATTGATATGC 5′) used were described by White *et al*. (1990). The amplification process consisted of a pre-denaturation step at 95^0C for 5 minutes, followed by 35 cycles of denaturation at 94^0C for 40 seconds, annealing at 52^0C for 40 seconds and extension at 72^0C for 40 seconds, and a final extension of 5 minutes at 72^0C.

Gel Electrophoresis: The amplified DNA was run on 1.5% agarose gel. DNA bands were visualized and photographed using UV transilluminator (gel documentation system). To ascertain the amplification of the target gene, a 100-bp DNA ladder (Gibco BRL) was loaded into the first lane of the agarose gel and the amplicon size compared with the DNA ladder.

PCR Product Purification and Sequencing: PCR products were purified following the protocols of the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA, USA). The purified DNA was eluted from spin columns with 30 µl of nuclease free H_2O and DNA concentrations were determined with a spectrophotometer. Samples were prepared in sequencing tubes with 1 µl of either the ITS-4 or ITS-5 primer added to 3 µl (300 ng) of DNA template and brought up to a final volume of 12 µl with nuclease free H_2O. The samples were then sequenced. Sequences obtained were then blasted against reference sequences in the NCBI database to confirm species identification.

RESULTS AND DISCUSSION

Morphological identification of Aspergillus niger: Early growth of isolates on PDA and SDA showed white mycelia which gradually turned to velvety black coloured spores upon maturation, the conidial head showed black colouration. Microscopic examination revealed distinct conidiophore which ended with a swollen vesicle bearing flask-shaped biseriate phialides as shown in Table 1, Figure 1.

Table 1: Microscopic characteristic of *Aspergillus niger*

Features	Matured	Young
conidiophore	Larger	Smaller
Hyphae	Sepetate brown transparent growing on substratum	Sepetate blue-black transparent growing on substratum
Head	Dark brown globular head on hyphae	Black globular head on hyphae

Screening of isolates from different locations for citric acid production: Figure 2 presents the amount of citric

acid produced by the different isolates of *Aspergillus niger* recorded at regular intervals of 12 hours over a span of 72 hours. The experiment was performed in duplicate. Maximum mean value of 0.38 g/100ml was obtained at 72 hours for isolate from location KW, 0.27g/100ml was obtained from isolate CM, a mean value of 0.24g/100ml and 0.225g/100ml was obtained from isolates KSU and MD respectively. Since isolate KW gave the highest mean value of citric acid of all the isolates, *Aspergillus niger* KW was used for optimisation studies.

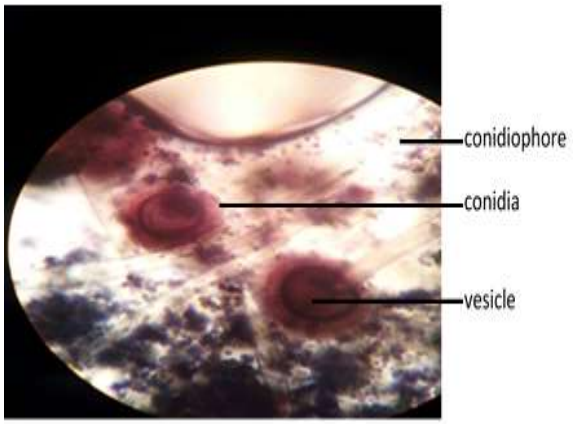

Fig 1: Matured *Aspergillus niger* as viewed under the microscope (Magnification X40)

Effect of pH on citric acid production: The pH of the medium affects the performance of submerged fermentation. In this study, initial pH of 6.0 was optimal for citric acid production as shown in Figure 3. At pH 5.5 the value of citric acid obtained was 0.26g/100ml, at pH 6.0. value was 0.4gm/100ml, at pH 6.5 value was 0.27g/100ml. Further increase to pH 8.0 was associated with a marked decrease in citric acid yield of 0.09g/100ml.

Effect of incubation temperature on Citric acid production: The effect of temperature on citric acid production is shown in Figure 4. In this study a pH of 6.0 was used at different temperatures, the mean value of citric acid production at a temperature of 25°C was 0.4 g/100ml, at 30°C the mean value was 0.65g/100ml, at temperature of 35°C and 40°C the values obtained were 0.12g/100ml and 0.06 gm/100ml respectively. So the optimum temperature for citric acid production in this study was 30^{O}C.

Substrate Preference: Result of *Aspergillus niger* isolates from location; KSU, CM, KW And MD is presented in Table 2. The highest percentage growth yield of 41% was obtained when wheat straw was used as a substrate. This was followed by potato peel powder with 32% yield and rice straw with 26%.

Wheat straw as a substrate for citric acid production: Citric acid obtained from untreated and pretreated wheat straw is shown in Figure 5. For the untreated and the pretreated wheat straw, the amount of citric acid produced was highest in Day 5 of fermentation. Untreated wheat straw had a maximum value of 13.30g/kg of dry weight at day 5, followed by 12.20g/kg on day 4. For pretreated wheat straw the maximum value of 25.6g/kg was obtained followed by 20.00g/kg at day 4.

Table 2: *Aspergillus niger* isolates from different locations grown on natural substrate

Substrate	Location				
	KW	MD	CM	KSU	% growth of isolates
Wheat straw	16.05	14.02	16.02	13.30	41
Rice straw	9.02	10.05	9.00	9.05	26
Potato peel powder	13.03	12.00	11.04	11.04	32

Key: CM-Central market, KSU- Kaduna State University, MD-Mando and KW-kawo

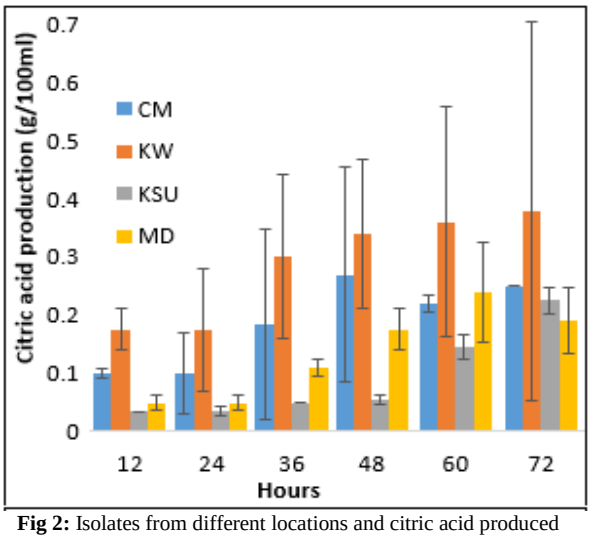

Fig 2: Isolates from different locations and citric acid produced

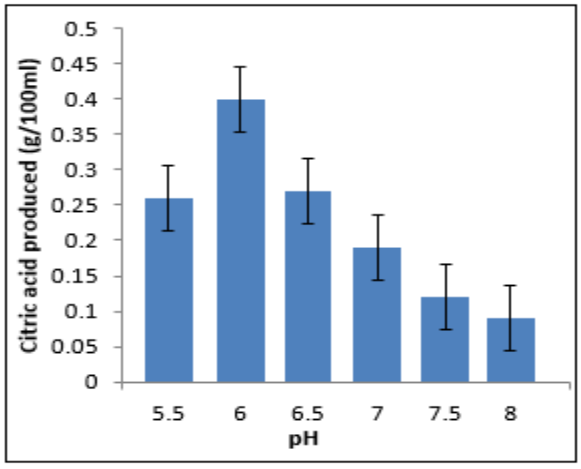

Fig 3: Citric acid production by *A. niger* Kw isolate at different pH

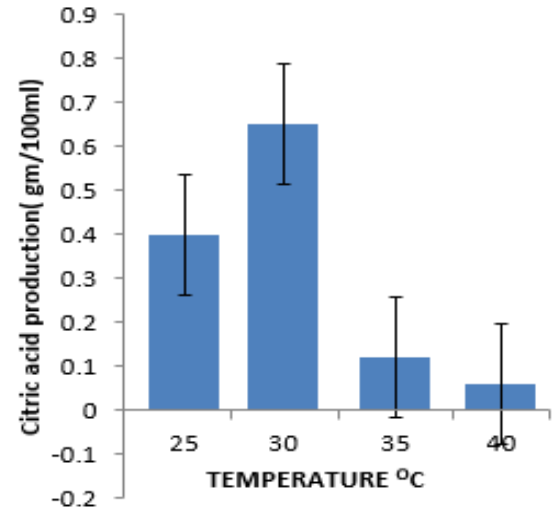

Fig 4: Citric acid production by *A.niger* Kw isolate at different temperatures

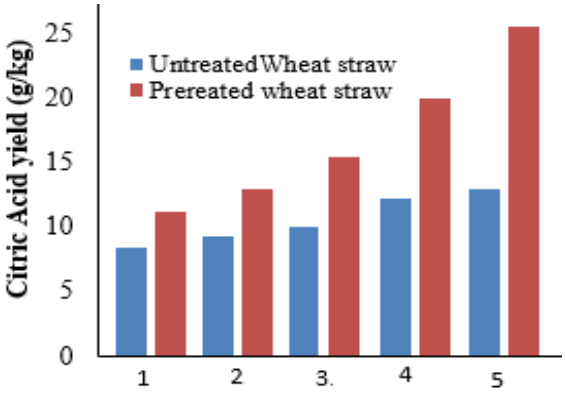

Fig 5: Wheat straw as a substrate for the production of citric acid by *A. niger* Kw isolate

Polymerase chain reaction (PCR) Amplification of isolates from different locations: A. niger DNA were amplified and resolved on agarose gel to detect the isolates with the expected band size of 500bp. Amplicons obtained from all the isolates corresponded to the expected band size.

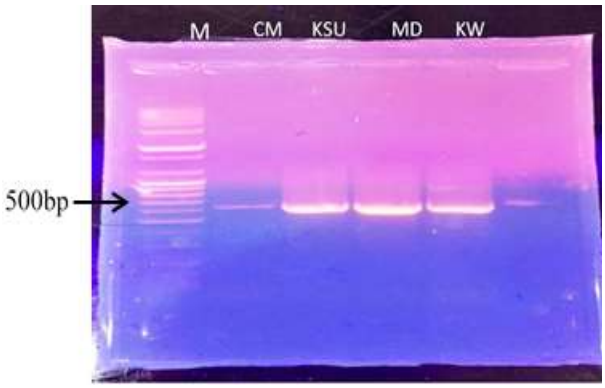

Fig 6: Agarose gel electrophoresis of PCR amplification of ITS region of *Aspergillus niger* isolates from different locations

Sequence similarity of ITS-5.8s –ITS 2: In order to further study isolate KW which gave the highest yield of citric acid, the PCR product for Kw from Figure 6 above was sequenced, then using the sequence, a BLASTN search was performed against the NCBI database. Table 3 shows the sequence similarity of the ITS 1-5.8s- ITS 2 gene of *Aspergillus niger* KW to some of the *A. niger* sequences in the NCBI database.

Fungal isolates obtained from soil samples of cowpea and groundnut farm, spoilt fruits and dump site of different locations in Kaduna metropolis were identified based on their cultural and morphological characteristics. The identity of the KW isolate (which produced appreciable amounts of citric acid) was further confirmed by molecular characterization through the amplification and sequencing of the 5.8SrRNA gene and the two intergenic spacers (ITS1-5.8-ITS2). The DNA that encodes the 5.8S gene of the ribosomal RNA and the two intergenic spacers ITS1 and ITS2 of the two proposed type strains of the *Aspergillus niger* aggregate (*A. niger and Aspergillus tubingensis*) has earlier been sequenced by Accensi *et al*. (1999). The nucleotide sequence of *Aspergillus niger* KW obtained in this study is homologous to referenced sequences in the NCBI database with the accession numbers; KX363462.1, KY962978.1, KY698415.1, KY657577.1, KP670427.1, KP748369.1, KT002562.1, KP329617.1, KP329655.1, and KT315445.1. Isolation of *A. niger* from dump site is in line with the work of several researchers that reported the isolation of this species of fungi from different dump sites in Nigeria (Obire *et al*., 2002; Williams and Hakam, 2016; Evangeline *et al*., 2017). Obire *et al*. (2002) further reported the frequency of the genus *Aspergillus* in soil sample of dumpsite as second highest with 25.3% after *Saccharomyces* with (42.8%). Natural substrate preference of *Aspergillus niger* isolates revealed that wheat straw gave the highest growth value of 41% at OD_{540} of 16.05. The 540nm wavelength used in this study has been reported by Llop *et al*., (2000), Dannaoui *et al*., (1999) and Shiradiyi (2011).The natural substrate preference agrees with a work reported by Prashant *et al*., (2003). Screening of *Aspergillus niger* for the production of citric acid showed that isolate from Kawo gave the highest yield of citric acid (0.38g/100ml). The value of citric acid obtained in this study is slightly higher than that from the work reported by Ipsita and Baruah (2015) in which 8 cultures of *Aspergillus* spp were screened for citric acid production. Of these cultures, *Aspergillus niger* S-6 produced 0.33 g/100 ml of citric acid. Futhermore, the pH optimum of 6.0 for citric acid production obtained in this work was also reported by Ipsita and Baruah (2015).

Table 3: Identification of fungi isolates based on sequence analysis of the ITS region of *Aspergillus niger* KW

Isolate code	Morphological identification	Gene bank(BLAST) Result	E-value	Identity	Query cover	Accession
KW	*Aspergillus niger*	*Aspergillus niger* isolate 6029	0.0%	100%	100%	KX363462.1
		Aspergillus niger strain X-5	0.0%	100%	100%	KY962978.1
		Aspergillus niger strain UPMZ01	0.0%	100%	100%	KY698415.1
		Aspergillus niger strain SS4	0.0%	100%	100%	KY657577.1
		Aspergillus niger strain An0314M	0.0%	100%	100%	KP670427.1
		Aspergillus niger strain TA01-24	0.0%	100%	100%	KP748369.1
		Aspergillus niger isolate K7	0.0%	100%	99%	KT002562.1
		Aspergillus niger strain DTO:129-E9	0.0%	100%	100%	KP329617.1
		Aspergillus niger strain DTO:132-A5	0.0%	100%	100%	KP329655.1
		Aspergillus niger strain DTO 132-C7	0.0%	100%	100%	KP329662.1
		Aspergillus niger strain DTO:133-E8		100%		

Shetty (2015) also assessed the production of citric by *A. niger* using medium containing molasses and reported a yield of 10.4 mg/ml at pH 2.5. Optimization of temperature carried out in this study indicated that 30ºC was more suitable for *A. niger* KW for citric acid production as it showed a significantly high yield of 0.65g/100ml. This report agrees with the work of Kareem *et al*. (2010), where citric acid production was optimum at a temperature of 30ºC. Hang and Woodams (1986) had emphasized the effect of temperature of a fermentation medium on the production of citric acid by solid state fermentation of Agricultural wastes. It is however in contrast to the report of Helen *et al*. (2014) that indicated 55ºC as the optimal temperature for Citric acid production by *Aspergillus niger* using *Parkia biglobosa* fruit pulp, the sharp difference in temperature could be attributed to the substrate used. Natural substrate for citric acid production was examined and wheat straw was used as a substrate, the use of wheat straw as a substrate was selected based on the high percentage of fungi growth obtained for the substrate preference assay in this study. The 14% (w/w) sugarcane molasses added to the substrate for fermentation was as a result of the relatively low sugar content of wheat straw (about 4%) in contrast to the initial sugar concentration of 14-22% reported by Rohr *et al*. (1998) as optimum for industrial fermentations. The variation in the initial sugar requirement however depends on the species and/or strain, but Fatemi and Shojaosadati (1999) had reported the importance of this variable on citric acid production using *A. niger*. Untreated wheat straw produced a maximum yield of 13.00g/kg on day 5 of fermentation while wheat straw treated with 5 N HCl produced a maximum value of 25.60g/kg on day 5 of fermentation. The treated wheat straw was found to produce more citric acid than the untreated, this could be attributed to the fact that pretreatment is needed to convert the agro-industrial residues into a more metabolizable form to increase its utilization by microorganisms (Khosravi *et al*., (2008). Similar observation was noted by Khosravi *et al*., (2008) where data from investigations carried out on citric acid production from pretreated and untreated wheat straw showed that the concentration of citric acid was increased when 5N HCl-treated wheat straw was used. Our data on citric acid production using wheat straw as substrate correlates with the works of other researchers that investigated the use of several other agricultural waste such as apple pomace, cassava bagasse, coffee husk, pineapple waste, sugar beet cosset, kiwi fruit peel, African star apple peel, etc. as suitable and cost reducing substrates for citric acid production by solid state fermentation (Hang and Woodams, 1986; Khare *et al*., 1995; Pandey *et al*., 2001; Haq *et al*., 2004; Kareem *et al*., 2010; 2013; Maharani *et* al., 2014; Dienye *et al*., 2018).

Conclusion: The data obtained from this study suggest that the use of wheat straw as substrate for citric acid production by *A. niger* Kw could represent an efficient method for minimizing the wheat straw disposal problems and concomitantly producing a commercially valuable organic acid that is less expensive. The results also showed that strains of *A. niger* capable of producing reasonable amounts of citric acid are present in the soil of farms and dumpsite in some locations within Kaduna metropolis. These fungal isolates could be exploited for the production of citric acid from low cost agri-industrial wastes/residues like wheat straw and molasses.

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