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Full Length Research Paper

Production of citric acid by *Aspergillus niger* isolated from coffee environments in Tanzania

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Abstract

A study was carried out to determine the suitability of coffee pulp residue (CPR) for production of citric acid using fungus Aspergillus niger isolated from coffee processing environment in Kilimanjaro, Tanzania. The components of the CPR were analysed and the filamentous fungi isolated using standard methods. The fungal isolates were identified by amplification and sequencing of the ITS 1- 5.8-ITS 4 region of ribosomal DNA. Growth parameters were optimized against pH, nitrogen supplement, methanol and potassium ferrocyanide. The citric acid was determined using the Megazyme kit and measured spectrophotometrically. The CPR contained appreciably high levels of carbohydrate (7.5% w/v), nitrogen content (0.05%), and trace elements. Nine out of 12 fungal isolates produced citric acid at varying levels (0.70 to 6.64 g/l) out of which 5 were identified as Aspergillus niger, 1 as Fusarium sp. and 3 belonged to other Aspergillus species. Isolate X1 which produced highest amount of citric acid was used for optimization studies. Optimum pH 6.0 increased citric acid production from 6.64 mg/ml to 7.12 mg/ml. Addition of ammonium nitrate declined citric acid production. Methanol supplementation improved citric acid production from 6.6 g/l to 35.5 g/l whereas addition of small amounts of Potassium ferrocyanide further enhanced citric acid concentration up to 43.6 mg/l, indicating that these compounds play an important role the citric acid production. In conclusion, the overall citric acid production from CPR by the locally isolated fungus Aspergillus niger was greatly enhanced up to 7 fold through optimization of growth conditions.

Key words: *Coffee pulp residues, Citric acid, Aspergillus niger, optimization*

Introduction

Citric acid is a commercially important specialty chemical with several applications in pharmaceuticals, food and beverage industries (Soccol *et al*., 2006; Yalcin *et al*., 2010). It is one of the products with highest production rate worldwide where 1,000,000 tonnes are produced annually (John *et al.,* 2012). The acid occurs as a natural component of most citrus fruits and its production was first done by crystallization from lemon juice (Papagianni, 2007). Commercially citric acid is produced by submerged state fermentation. However, in recent years considerable interest has been shown in solid state production of citric acid by fungal strains mainly from the species *Aspergillus niger* (Kumar and Jain, 2007). Less expensive substrates such as agro-industrial residue and by-products such as orange and pineapple waste, cassava peels, coffee husk, rice and wheat bran among others, have been used elsewhere in the world as substrate to produce citric acid (Soccol *et al.*, 2003, Sukesh 2013). Novel and cheap agricultural wastes such as banana peels have also been utilized and have shown their ability to produce citric acid (Kareem and Rahman, 2013).

Coffee is one of the most important agricultural commodity crops in the world. However only 9.5% of the fresh weight of the coffee cherries is used as coffee bean and 90.5% is left as residue (Murty and Manonmani, 2008). Coffee pulp residues (CPR) are amongst the waste generated in large quantities during coffee cherry processing. CPR is rich in proteins (22%) and fermentable sugars (44%) which make it an ideal substrate for microbial processes for the production of value-added products (Pandey *et al.,* 2000). However the presence of ant-nutritional factors (tannins, caffeine and polyphenols) in (CPR) has limited its utilization. CPR finds limited application as fertilizers, livestock feed and compost as these utilize only a fraction of available waste (Soccol and Vandenberghe, 2003).

Underutilization of CPRin many countries has caused a disposal burden. In Tanzania, approximately 50,000 tons of coffee beans are produced annually giving about 143,259 metric tons of industrial wastes and effluents (Tanzania Coffee Board, 2010; Hamadi *et al*., 2014).Coffee production in Tanzania is a significant aspect of the economy as it is Tanzania's largest export crop . However the use of coffee pulp residue as a biotechnological resource is yet to be explored despite its tremendous production and accumulation in the coffee processing sites. This study therefore focused on exploring bioconversion potential of CPR generated in Tanzania into citric acid using locally isolated fungi from coffee pulp residues.

Materials and Methods

Sample collection

Coffee pulp residues were collected from the coffee factory located at Lyamungo estate in Kilimanjaro, Tanzania. The factory processes Coffee Arabica type and the process involves wet method in which coffee pulp residues are produced as waste during removal of the pulp. The collected pulp was preserved in a cool box containing ice cubes and transported to Molecular Biology and Biotechnology (MBB) laboratory at the University of Dar es Salaam for analysis and further studies.

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Analysis of Coffee Pulp Waste Components.

At MBB laboratory, the CPR was placed at 4^0C in a refrigerator (Panasonic, Model NR-B301M, Panasonic UK) and thereafter and thereafter characterized by analyzing the following parameters; pH, carbohydrates and mineral content. These parameters are essential for growth of microorganisms and bioconversion of waste products into useful compounds (Yigitolu 1992; Kareem *et al*., 2009 and Max *et al.,* 2010). pH was measured by using a standardized pH meter (Hanna-270671).Total carbohydrates was determined by phenol-sulphuric acid method as previously described by Dubois *et al.* (1956). The method involved a reaction between standard glucose samples with phenol sulphuric acid to obtain a standard series of mean absorbance value using spectrophotometer (Jenway-Genova model 2384, Bibby scientific UK). Nitrogen content was processed by Kjeldhal method of nitrogen determination as described by (Egan *et al*., 1981) and analyzed together with trace metals (Iron, Copper, Zinc, Magnesium and Manganese) using Atomic Absorption Spectrophotometer (UNICAM, Model 939, Solaar system UK) as described by Allen (1989), at a commercial facility, Southern and Eastern African Mineral Centre (SEAMIC).

Isolation of filamentous fungi from coffee pulp residue,

For the purpose of this study, isolation targeted filamentous fungi as they are associated with production of high level of citric acid (Alagarsamy and Nallusamy, 2010 and Majumder*et al*., 2010). 1ml of coffee pulp residue was inoculated in a plate using spread plate method on; malt extract agar (Van der Walt and Yarrow, 1984)) and Czapeckdox agar culture media. The plates were then incubated at 30ºC for 5 to 7 days where the growing colonies of filamentous fungi were marked and sub-cultured on the same media to obtain pure cultures. Pure isolates of filamentous fungi in agar slants were stored at -20^0C (West Point Model WBES 238.X WestPoint UK) until further use.

Screening of isolates for CA production

Pure cultures of filamentous fungi were then tested for citric acid production ability using coffee pulp residue as a medium. Spores of fungi were prepared by cell count method (counting chamber) where (1 ml from (1.0 X 10^6 cell/ml) was inoculated in 100 ml of the medium in a conical flask. Surface culture fermentation was carried out for 28 days. Samples for analysis of citric acid were collected at the interval of 7 days stored in the freezer (-20^0C) (West Point Model WBES 238.X WestPoint UK) and finally analyzed.

Determination of citric acid content

Citric acid produced by fungal isolates during screening and optimization studies was analyzed by using the Megazyme kit (K-CITR 07/11, Megazyme International Ireland Ltd) as described by Mato*et al*. (1998). The procedure involves enzymatic reactions where the citric acid is broken down by citrate lyase. The citric acid produced during fermentation is quantified by finding the consumption of NADH which is measured by the decrease in absorbance at 340 nm (Jenway-Genova model 2384, Bibby scientific UK). Polyvinyl pyrollidone (PVP) is incorporated into the megazyme kit to prevent inhibition caused by anti-nutritional factors found in the coffee pulp residues. Calculations for the produced citric acid were done by using megazyme formula from the manufacturer as follows,

$$c = \frac{V * MW * \Delta A}{\varepsilon * d * v} (mg / mL)$$

Where;

V = final volume of the sample (ml)
MW = molecular weight of citric acid g/mol
ΔA = Change in absorbance
$\mathcal{E}$ = extinction coefficient of NADH at 340 nm
d = light path (cm)
v = sample volume (ml)

Optimization of initial pH.

Production of citric acid depends strongly on the pH of the substrate as it is one of the factors that affect the growth of the fungi that is used in the fermentation process (Darouney *et al.,* 2009; Max *et al.,* 2010 and Nwoba *et al.,* 2012). In the current study, optimization of parameters was done using the best CA producing fungal isolate identified during screening in Section 2.4 above. Conical flasks containing 100 ml of CPR were prepared by measuring pH and sugar concentration. The effect of pH was studied by setting the CPR to pH range varying from pH 4.5 to 6.5. The CPR media was sterilized at 121^0C for 15 minutes using autoclave (Wiseclave®, WACS-2060, DAIHATIN SCIENTIFIC, KOREA). One ml (1.0 x 10^6 cell/ ml) containing spore of a fungal isolate which showed the highest ability in citric acid production during screening was inoculated in the CPR and fermentation was done by surface culture method at

room temperature (28 – 31^{0}C). Samples were withdrawn aseptically from the fermentation flask and analyzed for production of citric acid on the 7th, 14th, 21st and 28th day.The optimized pH value was included in the subsequent optimization studies.

Optimization of nitrogen

Nitrogen is an important mineral for making up of the cell proteins and is also involved in the cell metabolism, thus, it is important to optimize its effect in fermentation (Javed *et al*., 2011, Kareem *et al*., 2010). Different concentrations of ammonium nitrate were used as a source of nitrogen added at a rate of 0.1, 0.2, 0.3, 0.4, 0.5 % (wt/v) in the coffee pulp. The supplemented media was sterilized in an autoclave (Wiseclave®, WACS-2060, DAIHATIN SCIENTIFIC, KOREA) and inoculated with best citric acid producing fungal isolate as described in section 2.6. Control was prepared in the same manner using optimum pH obtained from section 2.6, but without addition of nitrogen source.

Optimization of methanol

The effect of methanol in citric acid production has been associated with different factors one of them being the ability of it to increase the cell permeability thus accelerating the excretion citric acid to the medium (Max *et al*., 2010). Its optimization for citric acid production was assessed by addition of 1, 2, 3, and 4 percents of methanol in the coffee pulp residue. Fermentation media and inoculation of filamentous fungi X1 was done as described in section 2.7**.** Optimum pH obtained from section 2.6 was used but without supplementation with ammonium nitrate.

Optimization of Potassium Ferrocyanide.

The effect of metal complexing agent was assessed by addition of Potassium ferrocyanide to the CPR in the following concentrations;50 ppm, 100 ppm, 150 ppm, 200 ppm. Optimal pH from section 2.6 and methanol from section 2.8 were used, where the medium was sterilized and inoculated with the isolate producing highest amount of citric acid.

Identification of fungal isolates

The locally isolated filamentous fungi which showed the ability to produce citric acid from CPR were identified by studying theITS 1-5.8-ITS 4 region of the rDNA. Amplification involved the use of the following sets of primers which are mainly used for fungi identification; ITS 1-sequence: 5'_TCCGTAGGTGAACCTGCGG forward primer; and ITS 4-sequence: 5'_ TCCTCCGCTTATTGATATGC reverse primer as previously reported by White *et al.* (1990). The PCR products were sequenced using the Big Dye chain termination method employing an ABI 3730 Genetic analyzer, done in a commercial facility at Inqaba Biotechnical Industries, South Africa. Each sample was sequenced twice usingeither forward or reverse primer. BLAST which is an online tool found at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) was used to find the region of similarities between the partial sequences of the filamentous fungi found from CPR and those found in the Genbank (Altschul*et al*., 1997).

Results and Discussion

Characteristics of coffee pulp

Coffee pulp waste from Lyamungo coffee estate and factory used throughout in this study contained the following characteristics; total carbohydrates 7.5% and nitrogen content was 0.05%. Trace element analysis gave the following results in mg/kg: Manganese 1.7; Magnesium 135.8; Iron 44.3; Zinc 5.3 and Copper 4.1. These values are indeed very similar to those reported by Hamadi *et al.* (2014) who characterized coffee pulp waste from the same source for other studies. Other studies on coffee pulp residues have reported even lower values of total carbohydrates ranging from 0.3-3 %, yet producing reasonable amounts of useful organic products (Hamadi *et al*., 2014; Kefale*et al*., 2012 and Navia P. *et al.* 2011). Except where specified, coffee pulp residue has been used in this research as the sole source of food for growth of the studied fungi.

Screening for Citric acid production.

A total of 12 filamentous fungal isolates were found to be native of coffee pulp residue. The targeted fungal strain *Aspergillus niger* belongs to the filamentous fungi and was thus easier to be identified from others. During screening for citric acid production from the CPR, 9 filamentous fungal isolates among the 12 produced citric acid at different concentrations (Figure 1). The highest amount of citric acid was produced by isolate X1 (6.64 mg/ml), followed by X3 (5.7 mg/ml) and isolate 1 (5.3 mg/ml) after 14 days of incubation. The citric acid values obtained in this study were higher than some reported results of citric acid production from other agricultural products like undersized semolina, which produced lower values (0.052, 0.055, 0.068 g/l) even after optimization of pH, methanol and nitrogen respectively (Ermen and Osman 2004). Fungal isolate X1, which produced the highest citric acid concentration in this research was thus used for optimization studies to enhance its production.

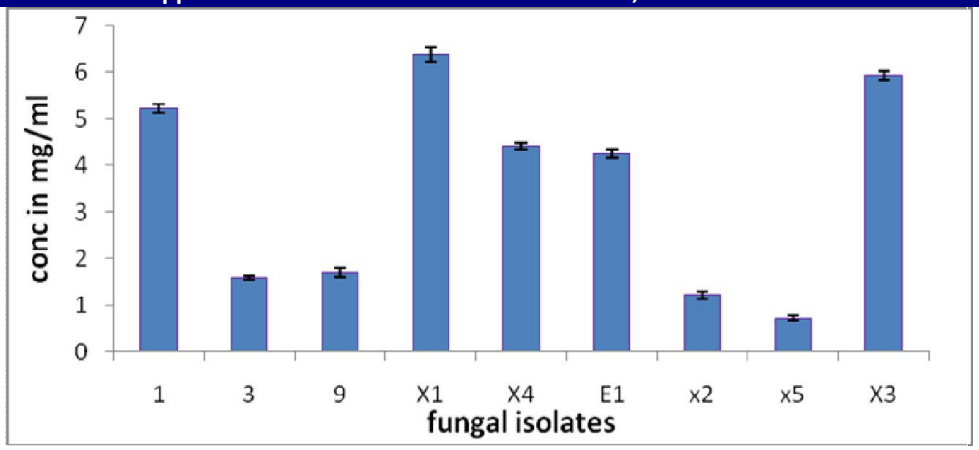

Figure1. Screening of isolate for citric acid production after 14 days of incubation.
(Error bars indicate standard error of the mean of the triplicates)

Identification of fungal isolates

Genomic DNA was extracted from the nine fungal isolates that had the ability to produce citric acid. PCR amplification using the ITS 1 and 4 primers gave DNA regions with sizes ranging from 580 to 600 bp, within the expected range reported for fungi of 450 to 700bp (Bellemain *et al.,* 2010 ; Beeck *et al.,* 2014). Sequencing of the PCR products gave different sizes of DNA sequences with nucleotides ranging from 503 to 562 bp. Results from the BLAST showed that the isolated fungi had 99 to 100% similarities to those found in the database (Table 1).

Table 1: Percentage similarity of the isolated filamentous fungi to fungi in the database after blasting.

Isolate from coffee environments	Strain that is similar too	Accession number	Maximum Identity in percentage
1	*Aspergillusniger*	KF691796	99
E1	*Aspergillusniger*	KJ 534379	99
X4	*Aspergillusniger*	JN688869	99
X1	*Aspergillusniger*	HQ891869	99
3	*Aspergillusfumigatus*	GU566217	99
9	*Aspergillustamarii*	HQ340111	99
X5	*Fusarium sp.*	GU973619	100
X3	*Aspergillusniger*	HQ285563	99
X2	*Aspergillusfumigatus*	HQ285569	99

All isolates which produced highest amounts of citric acid X1, X3, 1, X4 and E1 were identified as *Aspergillus niger* as they resembled by 99% with the *Aspergillus niger* strains from the database. In principle, a strain is considered to be similar to that on the database if the similarity index is above 97% (Kurtzman and Robdnett1998). Reports show that *Aspergilllus niger* is the leading strain in the production of citric acid from different sources (Lotfy *et al*., 2007; Femi Ola *et al*., 2009; Afifi, 2011), the fact supported by results of the current study. Moreover, isolate 1, X3 and X1 resembled *A. niger* strain that was previously reported as a potential microbe responsible for fermentation of different substrates from different countries in the production of valuable biotechnological product (Zhang *et al.,* 2011; Yang and Lee 2010;Ang*et al.,*2013). Isolate X2 and 3 had 99%similarity with *Aspergillus fumigatus* strains where isolate 9 resembled *Aspergillus tamarii* by 99% while isolate X5 resembled *Fussarium* sp by 100%.

Effect of pH on citric acid production

Results on pH optimization for growth of isolate X1 show an increase in citric acid production with increase in initial pH from 4.5 to 6.0 (Figure 2), with the optimum citric acid production at pH 6.0. Maximum citric acid of 7.12 mg/ml was reached on the 14th day of incubation which was 15.5% increase compared to the control at pH 5.5 (before optimization) which produced 6.64 mg/ml. This increase may be attributed to the fact that germinating spores require a pH greater than 5 where as citric acid production phase which occurred at pH below 2.The germinating spores absorb nitrogen source and causes the release of hydrogen ions which in turn lowers the pH of medium. This may explain the observed decrease in citric acid production with increase in incubation period and subsequent lowering of pH in the growth medium. Nevertheless, lower pH values have also been reported in some studies to be optimal for production of citric acid. Femi-Ola and Atere (2013) optimized citric acid production from brewers spent grain and found initial pH of 4.5 to be optimal using *Aspergillus niger* and *Saccharomyces sereviceae* strains. These findings imply that there is a need to establish optimal pH for every fungus or strain that is used in citric acid production as previously suggested by Hess *et al*. (2000).

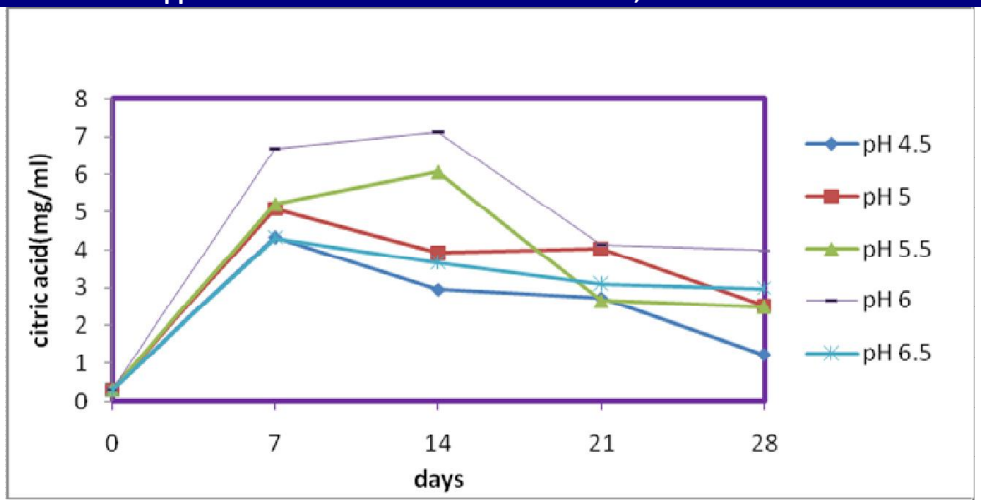

Figure 2. Effect of pH on citric acid production by isolate X1 grown Coffee pulp residues.
(Error bars indicate standard error of the mean of the triplicates)

Effect of nitrogen supplementation on citric acid production

The trend of citric acid production when different amounts of ammonium nitrate were added is presented in (Figure 3).It was generally observed that addition of ammonium nitrate in the CPR reduced the amount of citric acid production from 6.06 mg/ml (control) to about 3.5 mg/ml (by 42%). Although the CPR used in this research was characterized to have high sugar concentration suitable for citric acid accumulation, the production of citric acid decreased due to increase in the concentration of ammonium salts. Results obtained in this study could be explained by the fact that the amount of nitrogen (0.05%) contained in the coffee pulp residue was more than sufficient for metabolic activities of the *Aspergillus* strain, and thus any increase in nitrogen source would enhance the rate of fungal growth by supporting spore formation instead of citric acid production. Literature shows that excessive nitrogen promote higher growth and consequently diverts the source of carbon towards energy and biomass production which restrict the production of citric acid (Vandenberghe *et al*., 1999; Kim *et al*., 2006). Soccol *et al*., 2006 suggested that for citric acid fermentation nitrogen should range between 0.1-0.4 g/L, where as CPR used in this study had 0.5 g/l. Our findings concur with literature that supplementation of ammonium salts is required only on highly pure media rather than on complex substrates, suggesting that CPR could be used without addition of nitrogen (Angumeenal and Venkappayya 2013; Wang *et al*., 2013).

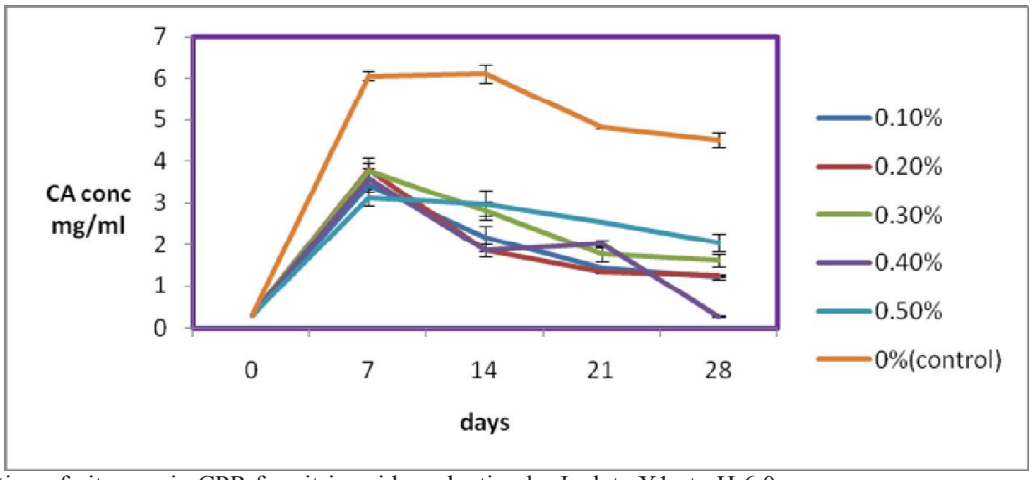

Figure 3. Optimization of nitrogen in CPR for citric acid production by Isolate X1 at pH 6.0
(Error bars indicate standard error of the mean of the triplicates)

Effect of Methanol on citric acid production

Results on the effect of methanol on the production of citric acid are presented in (Figure 4). Addition of methanol at different concentrations produced considerable increase in citric acid after incubation for 7 days as compared to control. Maximum citric acid yield occurred at 3% methanol, producing 35.53 mg/ml. This percentage increase in citric acid production was about 83% compared to control which produced only 4.2 mg/ml. It was also observed that at all methanol concentrations, the maximum citric acid production was reached after 7 days of incubation except for the control, which took 14 days.

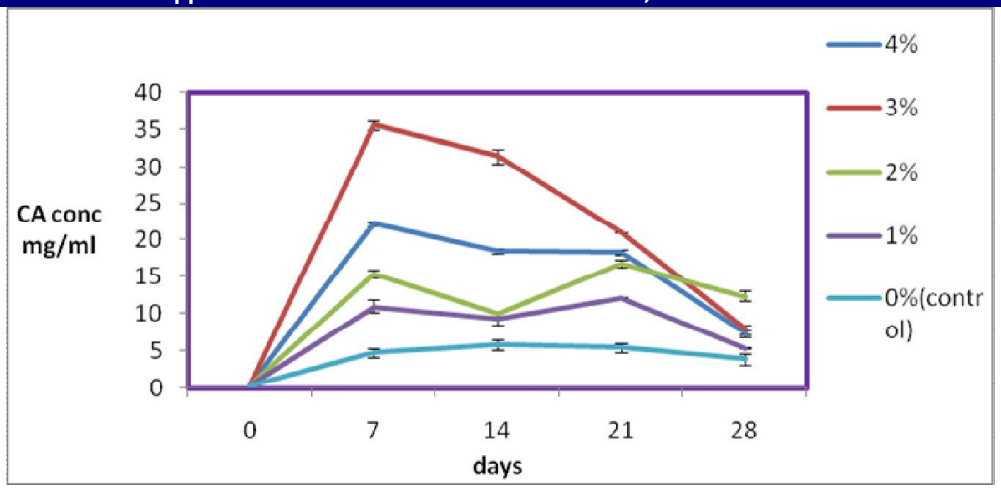

Figure 4. Optimization of methanol on citric acid production from coffee pulp residues.
(Error bars indicate standard error of the mean of the triplicates)

The effect of methanol on citric acid production may be related to growth and spore formation of the studied fungal isolate. The production of citric acid is suggested to work better when there is little or no sporulation during fermentation (Moyer, 1953). Results showed that addition of methanol delayed the formation of spores in the fermentation medium during the first 10 days of incubation, a period characterized by high citric acid production. As seen in (Figure 5), spore formation was delayed on flasks supplemented with methanol compared to un-supplemented one, which was characterized by black spores. This study confirms previous observations by other researchers who found out that addition of alcohols stimulate citric acid by affecting growth and sporulation, by acting on cell membrane permeability and also changing lipid composition of cell wall (Ingram and Buttke, 1984; Soccol *et al*., 2006).

Figure 5. Growth of *Aspergillus niger* in media with and without methanol. The first three from the leftwere supplemented with methanol and one on extreme right was not supplemented with methanol.

3.5Effect of Potassium Ferrocyanide on citric acid production

Results as presented in (Figure 6) show that addition of 50 ppm potassium ferrocyanide in the fermentation medium produced highest citric acid production (43.6 mg/ml) compared to the control (31.23 mg/ml), where as higher levels of ferrocyanide either produced little effect (100 and 150 ppm) or resulted in decreased citric acid production (200 ppm). The fact that the lowest concentration of ferrocyanide used (50 ppm) produced highest citric acid is an indication that smaller amounts of this chelating material (Potassium ferrocyanide) are needed to enhance citric acid production. In this study an optimal value of potassium ferrocyanide as 50 ppm was established. However, further studies involving lower concentrations than 50 ppm are needed. Ferrocyanide plays an important role in controlling the amount of trace metals in complex medium by forming precipitates and thus reducing their amount in fermentation medium (Max et al., 2010)). Literature shows that complex substrates such as coffee pulp residues contain trace metals which are required in optimal amount for citric acid production (Yigitoglu, 1992). Indeed, the amount of Iron found in the fermentation medium used in this study was far much higher (45.52 mg/kg) than 1 mg/kg suggested in literature (Mirminachi *et al.,* 2002).

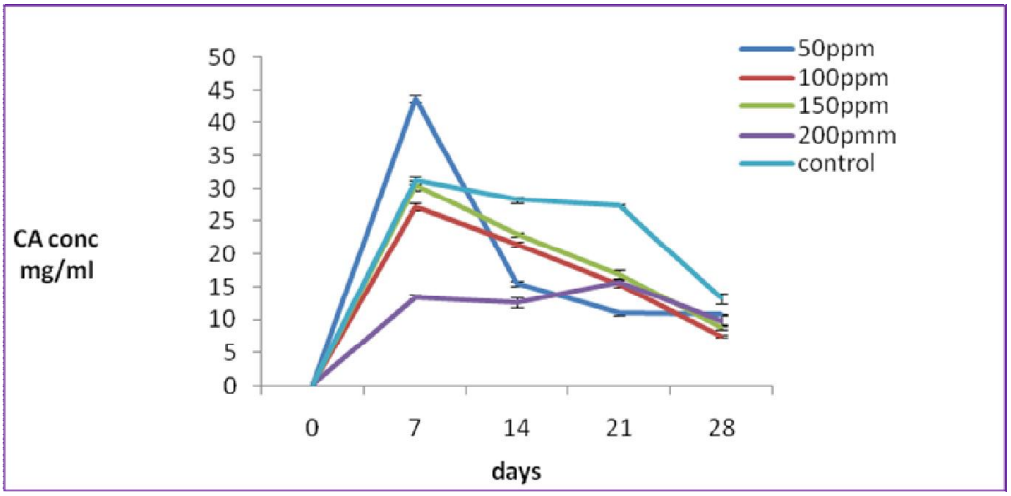

Figure 6. Effect of potassium ferrocyanide on citric acid fermentation.
(Error bars indicate standard error of the mean of the triplicates)

On the other hand, copper at an optimum level (40 mg/kg) is suggested to reduce the deleterious effect of iron in the production of citric acid and also antagonizes the effect of manganese in the fermentation medium (Najafpour, 2007). In the present study, the amount of Copper in the fermentation medium was found to be 4.02 mg/kg which is far less than the optimal amount suggested, as such it could not play a role of neutralizing the effects of manganese and iron. It is thus postulated that the addition of potassium ferrocyanide played a role of regulating levels of other trace elements by suppressing the toxic effect of some ions during citric acid fermentation (Hauka *et al*., 2005), resulting into increased citric acid production. The values obtained for citric acid production in the current study after supplementation with potassium ferrocyanide and methanol are comparable to those reported in literature when industrial strains NRRL2270, ATCC942 and CBS733.88 were used, yielding citric acid from carrot waste, beet mollases and sugarcane bagasse at 29m/ml, 35mg/ml and 21.24 mg/ml respectively (Soccol *et al*. 2006, Pandey and Nigam 2009).

Conclusion

This study reports for the first time isolation of filamentous fungi identified as *Aspergillus niger* capable of producing high levels of citric acid from coffee pulp residues in Tanzania. Citric acid production performance of the fungi was greatly enhanced up to7 folds through optimization of pH, supplementation with nitrogen, methanol and potassium ferrocyanide. Citric acid levels attained are comparable with those used for industrial production.This study thus opens an avenue for turning this otherwise underutilized bio-resource, into array of useful products through bioconversion. However, its practicability for up-scaling requires a different evaluation in future research endeavors.

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