Journal of King Saud University – Science 31 (2019) 931–936

Contents lists available at ScienceDirect

Journal of King Saud University – Science

journal homepage: www.sciencedirect.com

Original article

Optimization and production of ascorbic acid by fusant cell of *Aspergillus flavus and Aspergillus tamarii*

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ARTICLE INFO

Article history:
Received 4 October 2017
Accepted 25 April 2018
Available online 26 April 2018

Keywords:
Ascorbic acid
Protoplast fusion
Aspergillus flavus
Aspergillus tamarii
Hybridization

ABSTRACT

Ascorbic acid is an essential vitamin for the optimal growth of most animals including humans. The complexity and expensive nature of ascorbic acid production necessitates the development of a relatively simple and cost-effective method for its production. Hence, this study explored the potential of a somatic hybrid (fusant cell) of *Aspergillus flavus* and *Aspergillus tamarii* for enhanced production of ascorbic acid. Optimization studies of the ascorbic acid fermentation processes were performed at pH range of 4–8, temperature range of 30–45 °C and agitation speed range 60–160 rpm for 96 h of fermentation. Quantification of the ascorbic acid produced was done titrimetrically. There was significant difference ($P < 0.05$) in the ascorbic acid production of the fusant cell and the parental cells. The ascorbic acid produced by the somatic hybrid was 8.85 g/L compared to its parental strains (3.92 g/L and 4.57 g/L). However, pH, temperature and agitation speed had no significant effects ($P > 0.05$) on ascorbic acid production. The study revealed that maximal ascorbic acid production of 9.95 g/L was obtained at pH 5.0, temperature 40 °C and agitation speed of 100 rpm at 96 h of fermentation. This study shows the potential of fusant cell for enhanced ascorbic acid production.

1. Introduction

Ascorbic acid (vitamin C or L-ascorbic acid) is an essential dietary factor which must be present in the human diet to prevent scurvy and improve immune status (Food Standards Agency., 2007). Ascorbate is required for a range of essential metabolic reactions in all animals and plants (Li and Schellhorn, 2007). Although, it is synthesized internally by almost all organisms, human beings are the most well-known exception. According to Higdon (Higdon, 2006), ascorbic acid is used as a nutritional supplement, colour fixing agent, flavouring and preservative agent in meats and other foods. Additional usefulness of ascorbic acid include utilization as anti-oxidant in bread dough and as a reducing agent in analytical chemistry. Among several methodologies for ascorbic acid production, one possible approach to ascorbic acid production is its production from microorganisms. This has been reported in some fungi such as *Cryptococcus dimennae* (Kumar, 2000). Microorganisms can be easily grown on an industrial scale with recent evidence that L- ascorbic acid production is cost effective and highly demanding (Hancock et al., 2000).

Protoplast fusion technique has a great potential for strain improvement in industrially useful fungi for ascorbic acid production. Protoplasts are widely used in biotechnological transformation of organisms because they fuse readily (Davey et al., 2005). In addition, protoplasts of different microbial cells or species can be fused even if they are not closely linked taxonomically. With the help of protoplasts fusion, genetic information is transferred and recombinant features constructed (Murlidhar and Panda, 2000). Industrially, important cells such as yeasts have cell walls which make them difficult to fuse with cells of different species hence impeding the production of good hybrids through genetic manipulation (Ahmed and Barkly, 2006). This problem is overcome by the adoption of protoplast fusion approach. The protoplast fusion of *Saccharomyces cerevisiae* has been carried out by Chinenye et al. (2015). However, reports on the application of

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.jksus.2018.04.032
1018-3647/

fusant cells for the production of ascorbic acid are scanty. Hence the aim of this study is to carry out the protoplast fusion of *Aspergillus flavus* and *Aspergillus tamarii* for enhanced ascorbic acid production.

2. Materials and methods

2.1. Fungal source

Pure strains of *Aspergillus flavus* and *Aspergillus tamarii* were obtained from the Culture Collection Center of the Federal University of Agriculture, Abeokuta, Nigeria. The strains were sub cultured on Sabouraud Dextrose Agar to revive the cultures.

2.2. Protoplast fusion

Ascorbic acid-producing strains of Aspergillus identified using acid indicating medium according to the method of Roland et al. (1986) were grown aerobically to early stationary phase in 250 ml flasks containing 50 ml glucose (10%), yeast extract (0.3%) and ammonium sulphate (0.5%). Cells were harvested by centrifuging 5 ml cultures at 500 rpm for 5 min. The cells were subsequently washed three times with sterile distilled water and suspended in 5 ml sterile 0.1 M potassium phosphate buffer (pH 7.5) 0.5 M sorbitol and later digested for 1 h at 30 °C with 6 $mgml^{-1}$ of lysing enzymes. The suspension was incubated at 30 °C for one hour with occasional shaking. It was periodically examined under microscope for formation of protoplasts. Protoplasts were harvested by centrifugation at 1000 rpm for 10 min. Thereafter, protoplasts were washed three times with protoplasting buffer and 0.08 M sorbitol.

Fusion of the protoplasts was carried out to produce improved recombinants according to the method described by Nwachukwu et al. (2008).

2.3. Phylogenetic characterization of parent and fusant cells

The genomic DNA of *Aspergillus flavus*, *A. tamarii* and the fusant cell were extracted using the ZR Fungal/Bacterial DNA extraction kit (Zymo Research, CA, USA) by following the manufacturer's instruction. The Internally transcribed spacer (ITS 1-5.8S and ITS 2.0) were amplified using primers ITS1 and ITS4. (White et al., 1990). PCR was performed in a thermocycler (C100 ^{TM} Bio-Rad Laboratories, CA, USA) by using the same components and conditions as described by Oladipo et al. (2016). PCR amplicons were sequenced and aligned on the UNITE ITS database.

2.4. Genomic pairwise alignments

Nucleotide sequences of parent cells (*Aspergillus flavus* and *Aspergillus tamarii*) and their fusant cell were aligned using the Emboss needle nucleotide pairwise alignment software to determine the level of genetic diversity of the fusant cell to the parent cells (Huang and Miller, 1991).

2.5. Production and quantification of ascorbic acid under optimum conditions by parent and fusant strains of Aspergillus spp

Parent and fusant strains of *Aspergillus* spp. were cultured on the brewery waste medium (brewery waste, D-glucose, L-galactose, yeast extract, peptone and monosodium glutamate) and the effect of the various optimized condition was determined by the response surface plot. Ascorbic acid production was monitored at 24 h interval for 7 days. Quantitative assay of ascorbic acid was done by using the titrimetric method of the Association of Vitamin Chemist (Association of Vitamin Chemists, 2010).

2.6. Optimization of ascorbic acid production by parent and fusant strains of Aspergillus flavus and Aspergillus tamarii

Spores of *Aspergillus flavus*, *Aspergillus tamarii* and their fusant (2×10^9 spore/ml) were inoculated on brewery waste medium (0.6% brewery spent grain, 2% D-glucose, 0.3% L-galactose, 0.3% yeast extract, 0.5% peptone and 0.2% monosodium glutamate) in a 250 ml Erlenmeyer flask under optimum conditions for 96 h.

2.6.1. Effect of temperature on ascorbic acid production

The effect of temperature on the quantity of ascorbic acid produced was studied at different temperatures (30, 35, 40 and 45 °C). The ascorbic acid produced was quantified at 96 h of fermentation.

2.6.2. Effect of pH on ascorbic acid production

The effect of pH on ascorbic acid production was studied at pH range 4.0–8.0 (pH 4.0, 5.0, 6.0, 7.0 and 8.0) and incubated at 40 °C. The ascorbic acid produced was quantified at 96 h of fermentation.

2.6.3. Effect of different agitation speeds on ascorbic acid production

Effect of agitation speed on the quantity of ascorbic acid formed was studied at different agitation speeds (60, 80, 100, 120, 140, and 160 revolution per minute). This was carried out at pH 5 and 40 °C. The ascorbic acid produced was quantified at 96 h of fermentation.

2.6.4. Effect of inhibitors on ascorbic acid production

The effect of inhibitor Ethylenediaminetetraacetic Acid (EDTA) on ascorbic acid production was determined according to the methods of Prakash et al. (2011). EDTA is a known inhibitor of the ascorbic acid production pathway. EDTA was added to the fermentation medium at different concentrations (0.5 g/ml, 1.0 g/ml, 1.5 g/ml, 2.0 g/ml, 2.5 g/ml, 3.0 g/ml, 3.5 g/ml and 4.0 g/ml) and incubated pH 5, 40 °C and 100 rpm. The ascorbic acid produced was quantified at 96 h of fermentation.

2.7. Data analysis

Mean and standard deviation of the duplicated data (N = 2) were analyzed while the significance of the effects of optimization parameters such as pH, temperature range, agitation speed, EDTA and protoplast fusion of *Aspergillus flavus* and *Aspergillus tamarii* on ascorbic acid production were determined by using a one-way ANOVA at 95% confidence interval (0.05 level of significance).

3. Results and discussion

3.1. Protoplast fusion of Aspergillus flavus and A. Tamarii

Protoplast fusion of *A. flavus* and *A. tamarii* resulted in three regenerated recombinants designated as FI, F2 and F3 Table 1). Only F3 showed significant enhancement ($P < 0.05$) of its ascorbic acid production (8.85 g/L) compared to that of parental strains with 3.92 g/L and 4.57 g/L Table 1. This showed a significant increase of 126% and 94% ascorbic acid production over the parental strain of *A. flavus* and *A. tamarii*. This correlates with the

Table 1
Ascorbic acid production by parent and fusant cells of *Aspergillus flavus* and *Aspergillus tamarii*.

Fusant	Ascorbic acid concentration (g/L)	T-test	P value
F1	4.86 ± 1.26		
F2	5.48 ± 2.07	1.94	0.034
F3	8.85 ± 2.35		

findings of Nwachukwu et al. (2008), that protoplast fusion enhanced the production ability of recombinants over the parent strains. Out of the three regenerated recombinant moulds screened, only one (F3), seemed to possess desired recombination genes for increased ascorbic acid production. This could be as a result of duplication/mutation of the genes responsible for ascorbic acid production.

3.2. Genomic pairwise alignments

Protoplast fusion of ascorbic acid producing strain of *Aspergillus flavus* and *Aspergillus tamarii* resulted in the production of a fusant cell that share similar genomic characteristics with the parent cells. However, high level diversity in genomic constituent of the fusant cell with *A. flavus* was observed but highly similar in genomic nucleotide with *A. tamarii* with 93.7% similarity Table 2). Numerous mutational changes were also observed in pairwise alignment of the fusant cell nucleotides and individual parent cell Figs. 1–2). Mutational nucleotide deletion, insertions and various substitutions observed in the fusant cell could enhance high production of ascorbic acid and increase its industrial large scale production for various applications.

3.3. Production of ascorbic acid under optimum conditions by parent and fusant strains of Aspergillus flavus and Aspergillus tamarii

Studies on the fermentation of the selected medium with the parent and fusant strains of *Aspergillus* spp showed that ascorbic acid production peaked at 96 h of fermentation. The highest ascorbic acid production of 9.95 g/L was obtained by Fusant F3 while the least ascorbic acid production of 6.25 g/L was given by *Aspergillus flavus*. The yield of ascorbic acid reduced with increase in fermentation time for the fusant and parent cells. Fig. 3). However, at 120 h the yield of ascorbic acid by *Aspergillus flavus* was 0 g/L. This shows that ascorbic acid has been completely degraded in the fermentation medium. There was no significant difference ($P > 0.05$) in the ascorbic acid produced by the fusant and parent cell between 0 and 168 h of fermentation.

3.4. Effect of pH on ascorbic acid production

Investigations on the effect of pH on ascorbic acid production showed that maximum ascorbic acid was produced at pH 5.0 by the fusant and parent cell Fig. 4). There was no significant difference ($P > 0.05$) in the ascorbic acid produced by the fusant and parent cell. The highest ascorbic acid production of 9.15 g/L was produced by the Fusant (F3). However, ascorbic acid production reduced drastically to 4.25 g/L as the pH of the medium increased to pH 8 Fig. 4. This correlates with a similar work carried out by Shindia et al. (2006), who reported the suitability of pH range of 5–6 for organic acid production. Hence, the pH of the culture medium directly influences the growth of microorganisms and the biochemical processes they perform (Leandro et al., 2015; Sindhu et al., 2009). The finding of the present study is also in line with that of Chaurasia et al. (Chaurasia et al., 2014) who reported an optimum pH of 5.0 in their work on organic acid production by a fungus (*Sclerotium rolfsii*).

Table 2
Genetic diversity between *Aspergillus flavus*, *Aspergillus tamarii* and their fusant cell.

Genetic variables	*A. flavus*/Fusant	*A. tamarii*/Fusant
Length	584	588
Identity (%)	82.9	93.4
Similarity (%)	87.8	93.7

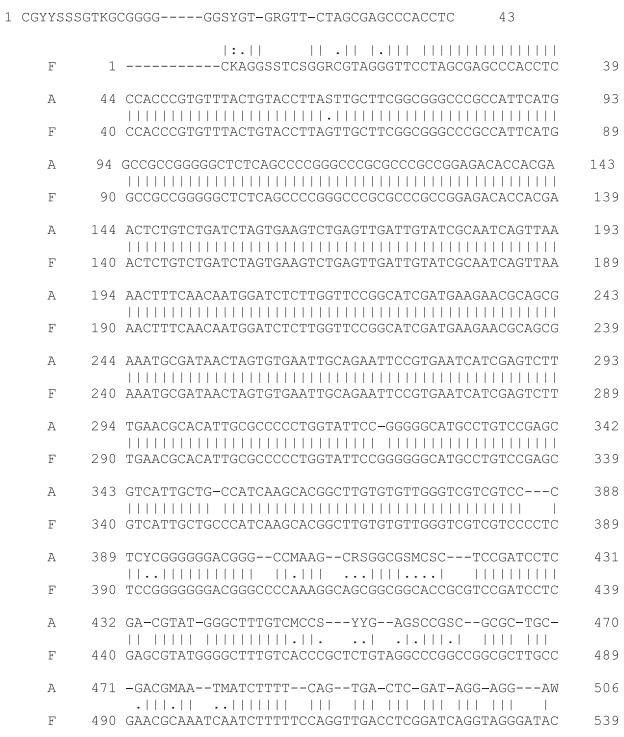

Fig. 1. Pairwise alignment of *Aspergillus flavus* (A) and fusant (F) nucleotides.

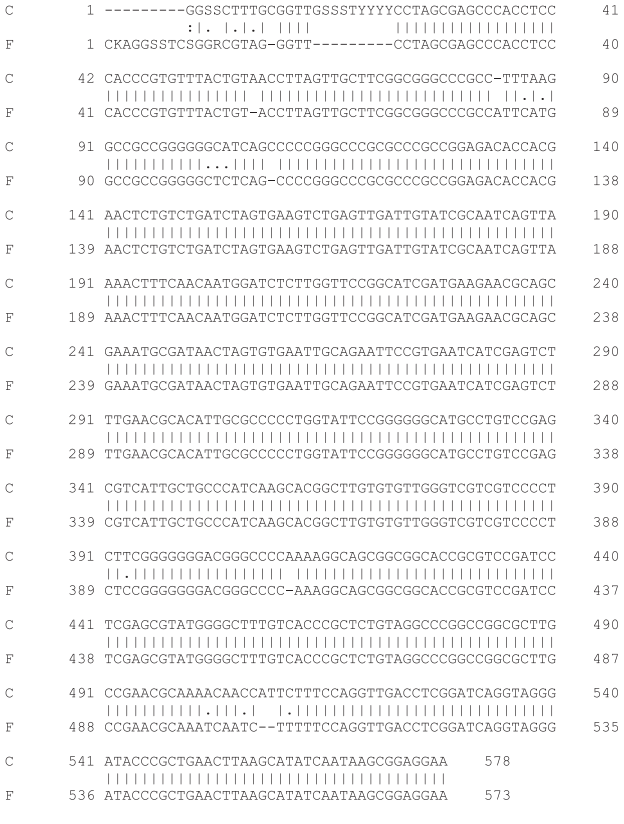

Fig. 2. Pairwise alignment of *Aspergillus tamarii* (C) and fusant (F) nucleotides.

3.5. Effect of temperature on ascorbic acid production

The effect of temperature on ascorbic acid production showed that optimum ascorbic acid yield of 9.80 g/L was produced by the fusant cell at 40 °C Fig. 5). There was no significant difference in

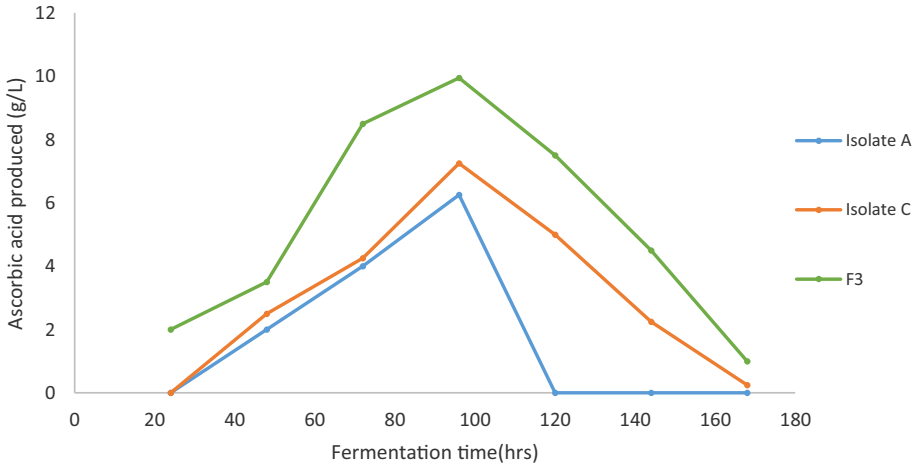

Fig. 3. Production of ascorbic acid by parent and fusant strain (F3) of *Aspergillus flavus* (A) and *Aspergillus tamarii (C).* F = 1.032, *P* = 0.413.

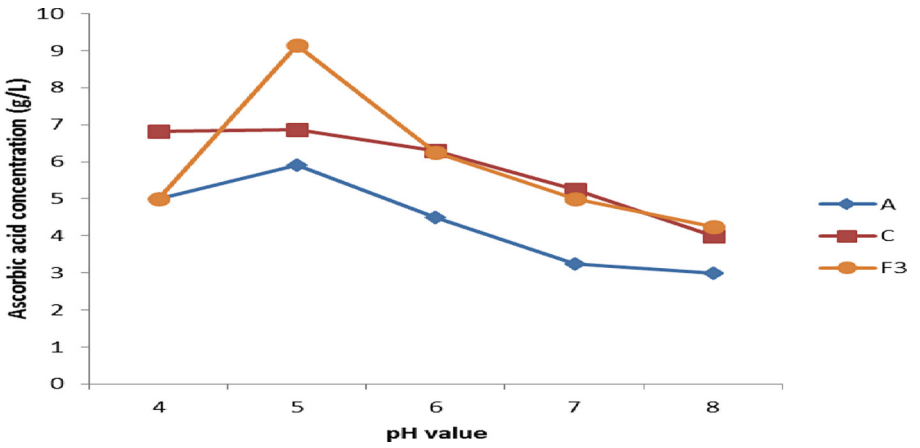

Fig. 4. Effect of pH on ascorbic acid production by parent and fusant strain (F3) of *Aspergillus flavus* (A) and *Aspergillus tamarii* (C). F = 1.812, *P* = 0.205.

the ascorbic acid yields by the fusant and parent cells at different temperatures (*P* > *0.05*). There was a decrease in ascorbic acid production at higher temperature. This correlates with the findings of Kaleel et al. (Kaleem et al., 2016) who reported a decrease of ascorbic acid production at temperature above 50 °C. This might be due to loss of activity as the temperature increases.

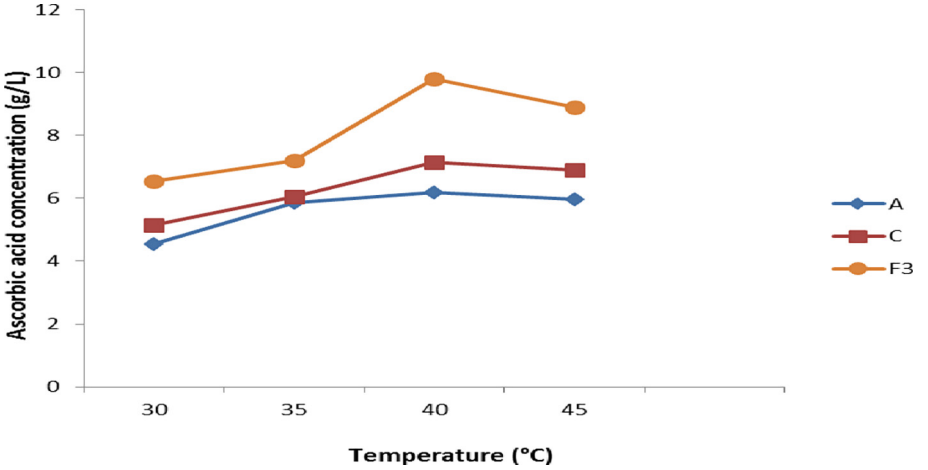

Fig. 5. Effect of temperature on ascorbic acid production by parent and fusant strain (F3) of *Aspergillus flavus* (A) and *Aspergillus tamarii* (C). F = 1.212, *P* = 0.512.

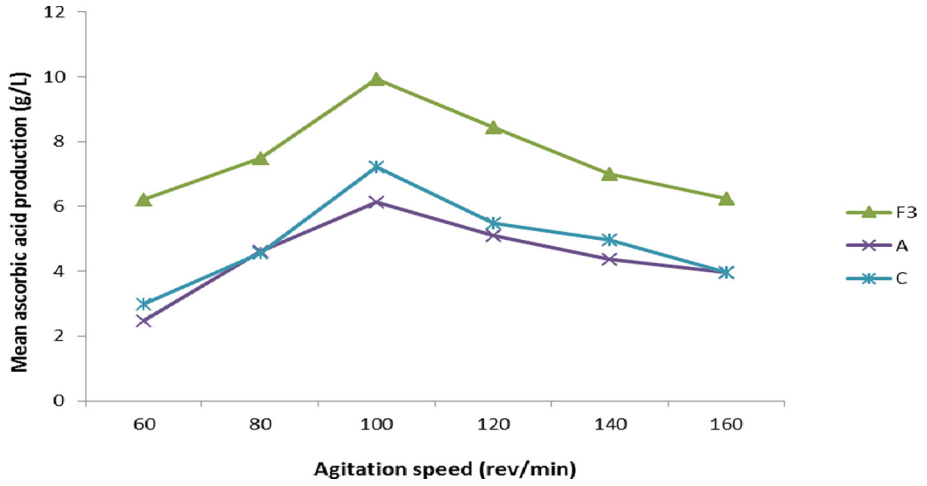

Fig. 6. Effect of agitation speed on ascorbic acid production by parent and fusant strain (F3) of *Aspergillus flavus* (A) and *Aspergillus tamarii* (C). F = 0.736, *P* = 0.564.

3.6. Effect of agitation speeds on ascorbic acid production

The effect of different agitation speed on the quantity of ascorbic acid formed by parent and fusant strains of *Aspergillus* spp is presented in Fig. 6. Optimum ascorbic acid yield of 9.92 g/L produced at an agitation speed of 100 rpm by the Fusant. There was no significant difference in the ascorbic acid yield at different agitation speed (*P* > *0.05*). Further increase in agitation speed resulted in decrease in ascorbic acid production with the least value of 4.37 g/L given by *Aspergillus flavus* at 140 rpm. Decrease in the ascorbic acid production at higher agitation speeds might be due to the harmful effect of the shear forces on the fungi as a result of the agitation intensity. However, at lower agitation speeds less amount of ascorbic acid produced might be due to improper distribution and transportation of oxygen and nutrients which affects the growth of the fungi (Ikram-ul-Haq et al., 2002; Jimenez et al., 2005). Different agitation speeds seemed to provide different distribution and transportation of air and nutrients to the cells (Pena et al., 2008).

3.7. Effect of inhibitors on ascorbic acid production

Studies on the effect of EDTA on ascorbic acid production by *Aspergillus* spp. showed that increase in the concentration of the inhibitor resulted in decrease in ascorbic acid. At an initial concentration of 0.5 g/ml of EDTA, the ascorbic acid yield was 9.85 g/L. There was no significant difference in ascorbic acid yield by the parent and fusant cell at different concentrations of EDTA (*P* > *0.05*). However, increase in the concentration of EDTA to 4 g/ml resulted in the inability of all isolates to produce ascorbic acid. Thus, enzymes responsible for ascorbic acid production were inhibited from concentration of 4 g/ml (Fig. 7). EDTA is known to chelate Ca^{2+} which is required as a cofactor for α-amylase. The denial of Ca^{2+} to the α-amylase makes the active sites of the enzyme less catalytically efficient, hence low activity (Igbokwe et al., 2013).

4. Conclusion

In conclusion, protoplast fusion of *Aspergillus flavus* and *A. tamarii* resulted in a fusant cell with increased ascorbic acid production of 126% and 94% over the parental strain of *A. flavus* and *A. tamarii*. The optimum conditions under which ascorbic acid can be produced by wild and fusant strains of *A. flavus* and *A. tamarii* was established at 40 °C, pH 5 and agitation speed of 100 revolution per minute. This study showed that protoplast fusion of fungal isolates resulted in an increased ascorbic acid production which is of importance to the pharmaceutical industry.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

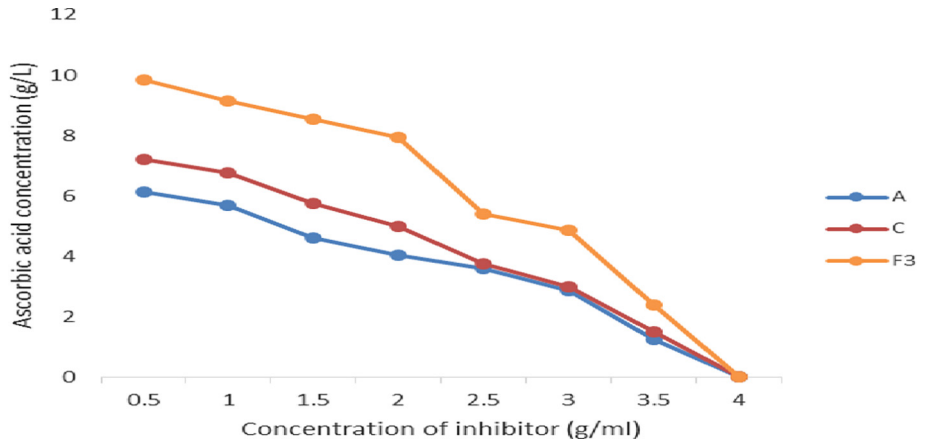

Fig. 7. Effect of inhibitor (EDTA) on ascorbic acid production by parent and fusant strain (F3) of *Aspergillus flavus* (A) and *Aspergillus tamarii* (C). F = 0.436, *P* = 0.657.

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