Comparative studies on ascorbic acid production by single and mixed cultures of *Aspergillus flavus* and *A. tamarii*

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Abstract

Ascorbic acid is an antioxidant which promotes increased resistance to infection and acts as preservatives in foods. The complexity and expensive nature of its production led to the search for a relatively simple and cheap method of ascorbic acid production. Hence, this study investigated the potentials of mixed cultures of *Aspergillus flavus* and *A. tamarii* for increased ascorbic acid production. Optimization of the fermentation process was carried out at pH (4 – 8), temperature (30 - 45 °C) and agitation speed (60 - 160 rpm) for 96 h. Titration and High Performance Liquid Chromatography techniques were used to determine the quantity of ascorbic acid produced. The highest quantities of ascorbic acid were produced at pH (5.0), temperature (40 °C) and agitation speed of 100 rpm at 96 h of fermentation. The highest quantity of ascorbic acid (9.648 g/L) was produced by the mixed culture compared to acid yield of 6.248 and 7.246 g/L by *A. flavus* and *A. tamarii* respectively. The ability of the mixed cultures of *A. flavus* and *A. tamarii* in hyper-production of ascorbic acid is therefore established in this study.

***Keywords: Aspergillus flavus*, *Aspergillus tamarii*, Ascorbic acid, HPLC, Titration**

1. Introduction

ASCORBIC acid is an important component of the diet which is needed to prevent scurvy. This organic acid which is white to light- yellow crystal or powder also serves as antioxidant in bread dough, colour fixing agent, flavouring and preservatives in foods [1]. In living beings, ascorbic acid is very important in most metabolic processes where they are made internally by almost all organisms except human beings [2]. However, several methodologies have been employed in ascorbic acid production. One of such methodology is its production from microorganisms. Ascorbic acid production has been reported in several microorganisms [3,4]. The conventional method of ascorbic acid production is complex and laborious involving several intermediate steps [5]. However, different modifications employed to improve the efficiency of the ascorbic acid production is energy consuming with much reliance on large quantities of solvents. Therefore, mixed culture of microorganisms which has been utilized in the production of some value added products could be a viable alternative [6].

Mixed culture of microorganisms exhibits more benefits over the single culture which includes; enhanced yield of products (enzymes, food additives, organic acids, antimicrobial agents etc.) and utilization of simple and cheap substrates for fermentation [7]. The production of 2-keto-L-gulonic acid (2-KLGA), a key intermediate of ascorbic acid, by mixed culture of microorganisms has been reported [8, 9]. It has been reported from previous studies that mixed cultures can increase the yield of amino acids and peptides production [10], enhance the levels of organic acids production [11], increase the quantity of volatile compounds [12] in food products produced from fermentation when compared to single cultures [13].

The present methodologies employed in ascorbic acids production is not economical because it is energy consuming and utilizes large quantities of solvents. Therefore, the need to discover a cost effective and simple method for the production of ascorbic acid using mixed cultures of moulds. This study is aimed at investigating the potentials of mixed cultures of *Aspergillus flavus* and *A. tamarii* for enhanced ascorbic acid when compared to the single cultures.

2.0 Materials and Methods

2.1 Fungal source

Pure strains of *Aspergillus flavus* and *Aspergillus tamarii* were obtained from the Culture Collection Centre of Crawford University, Igbesa, Ogun State, Nigeria. Sub culturing of the strains was carried out using Sabouraud Dextrose Agar to revive the cultures.

2.2 Production and quantification of ascorbic acid by single and mixed cultures of *Aspergillus flavus* and *Aspergillus tamarii*

Spores of *A. flavus* and *A. tamarii* (2 x 10^9 spores/ml) were inoculated on brewery waste medium made up of monosodium glutamate, brewery spent grain, peptone, glucose,

galactose and yeast extract in a 250 ml Erlenmeyer flask under optimum conditions for 96 h [14].

2.3 Effect of pH on ascorbic acid production by single and mixed cultures of *Aspergillus flavus* and *Aspergillus tamarii*

The effect of pH on ascorbic acid production was studied by incubating the microorganisms at 30 °C in the brewery waste medium by varying the pH in the range 4.0 – 8.0 (pH 4.0, 5.0, 6.0, 7.0 and 8.0).

2.4 Effect of temperature on ascorbic acid production by single and mixed cultures of *Aspergillus flavus* and *Aspergillus tamarii*

Effect of temperature on ascorbic acid formed was studied by varying the temperature between 30 – 45 °C (30, 35, 45 °C). The fermentation was carried out for 96 hours and the ascorbic acid produced was quantified.

2.5 Effect of inhibitors on ascorbic acid production by single and mixed cultures of *Aspergillus flavus* and *Aspergillus tamarii*

The effect of Ethylene Diamine Tetra Acetic acid on ascorbic acid production was investigated according to the methods of Prakash *et al*. [15]. Different concentrations of the EDTA (0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM, 3.5mM and 4.0 mM) were added to the fermentation medium and the ascorbic acid produced was quantified after 96 hours.

2.6 Effect of different agitation speeds on ascorbic acid production by single and mixed cultures of *Aspergillus flavus* and *Aspergillus tamarii*

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) .The ascorbic acid produced was quantified after 96 h of fermentation.

2.7 High performance liquid chromatography (HPLC)

The quantitative estimation of the ascorbic acid concentration in the extracted samples was determined using High Performance Liquid Chromatography (HPLC) according to the method of El. Gindy *et al*. [16]. The mobile phase consists of Acetonitrile: Water (70:30) with a flow rate of 1ml/min. The concentration of ascorbic acid was calculated based on the area of peak obtained during HPLC analysis.

2.8 Data analysis

Mean and standard deviation of the duplicated data were analyzed while the significance of the effects of optimization parameters such as pH, temperature range, agitation speed and the effects of Ethylene Diamine Tetra Acetic acid were determined using ANOVA at 95 % confidence interval while p value < 0.05.

3.0 Results and Discussion

3.1 Production and quantification of ascorbic acid by single and mixed cultures of *Aspergillus flavus* and *A. tamarii*

Investigation on the fermentation of the brewery spent grain medium with *Aspergillus flavus* and *Aspergillus tamarii* showed optimum ascorbic acid yield at 96 h of fermentation. Ascorbic acid yields of 7.25 g/L, 6.25 g/L and 9.65 g/L were produced by *Aspergillus tamarii, Aspergillus flavus* and their mixed cultures respectively at 96 h of fermentation. The increased ascorbic acid yield by the mixed culture of the two moulds could be attributed to their stable coexistence and interaction which provide various functional factors or materials more effectively than single culture which is as a result of the synergy between the microorganisms involved [17]. The yield of ascorbic acid reduced with increase in fermentation time for the three isolates (Table 1). 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. This complete breakdown of the ascorbic acid observed in the fermentation medium may be due to the increase in the activity of the enzyme ascorbate oxidase which converts ascorbic acid to dehydroascorbic acid [18].

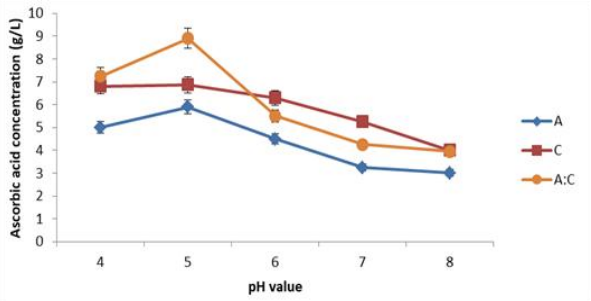
Table 1: Production and quantification of ascorbic acid by single and mixed cultures of *Aspergillus flavus* and *A. tamarii*

Fermentation Time (Hours)	*A. flavus* (A)	*A. tamarii* (C)	Mixed cultures of A and C
12	0	0	0
24	0	0	0.1
36	1.6	2	2.86
48	2	2.5	3.2
60	3.2	3.6	4.6
72	4	4.25	5.65
84	5.6	6.4	7.6
96	6.25*	7.25*	9.65*
120	0	5	7
144	0	2.25	3.75
168	0	0.25	1.25
T-test	2.871	4.027	4.409
P-value	0.017	0.002	0.001

($P<0.05$ is significant, *is optimal temperature with highest production)

3.2 Effect of pH on ascorbic acid production by single and mixed cultures of *Aspergillus flavus* and *A. tamarii*

The the effect of pH on ascorbic acid production showed significant ascorbic acid production at pH 5.0 for the three isolates ($p<0.05$). Optimum ascorbic acid yields of 5.9 g/L, 6.87 g/L and 8.9 g/L were produced by *Aspergillus flavus, Aspergillus tamarii* and their mixed cultures respectively at pH 5.0 (Figure 1). However, there was a drastic reduction in the ascorbic acid yield to 3 g/L and 4 g/L by *Aspergillus flavus* and *Aspergillus tamarii* respectively as the pH of the medium was increased to pH 8, indicating a decrease in ascorbic acid production beyond the optimum pH of 5. The result of this study correlates with a similar finding on organic acid production carried out by Shindia *et al*. [19], in which an optimum pH range of 5-6 was established. Moreover, pH has been reported as one of the critical parameters for fungal growth, enzymatic activities and organic acid production under suitable fermentation conditions [20]. Hence, the pH of the culture medium has direct influence on the growth of microorganisms and the biochemical processes they are involved [21, 22]. Increased organic acid production by the activities of mixed cultures of *Aspergillus ornatus* and *Alternaria alternate* at an optimum pH of 5.0 was also reported by Ambati and Ayyanna [23].

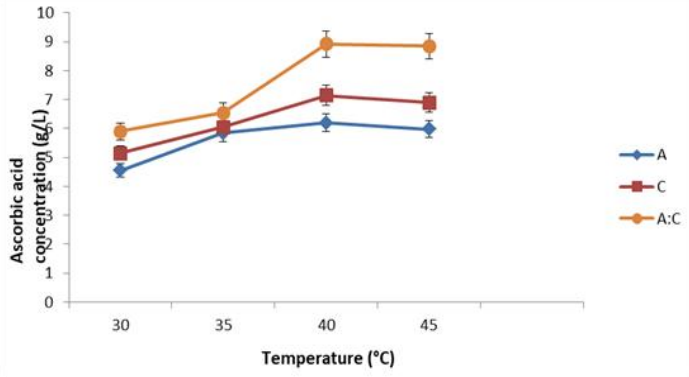


A=*Aspergillus flavus*, C=*Aspergillus tamarii*, A:C=Mixed cultures of *A. flavus* and *A. tamarii*

Fig. 1: Effect of pH on ascorbic acid production by single and mixed cultures of *Aspergillus flavus* and *Aspergillus tamari* (A, p=0.01; C, p=0.001; A:C, p=0.003).

3.3 Effect of temperature on ascorbic acid production by single and mixed cultures of *Aspergillus flavus* and *Aspergillus tamarii*

The effect of temperature on ascorbic acid production showed that optimum ascorbic acid yields of 6.2 g/L and 7.15 g/L and 8.92 g/L were produced by *A. flavus*, *A. tamarii* and their mixed cultures respectively at 40°C. However, there is significant difference in the ascorbic acid produced at different temperatures (p<0.05). There was a decrease in ascorbic acid production at higher temperature as shown in Figure 2. In a related study on another organic acid, Kareem and Rahman [24], reported a decrease in citric acid production at temperatures higher than the optimum temperature. This might be due to accumulation of by-products and eventually, loss of activity as the temperature increases. Moreover, the reduced organic acid production reported at temperature above the optimum could be due to the denaturation of the enzymes present in the microorganisms under study [20].

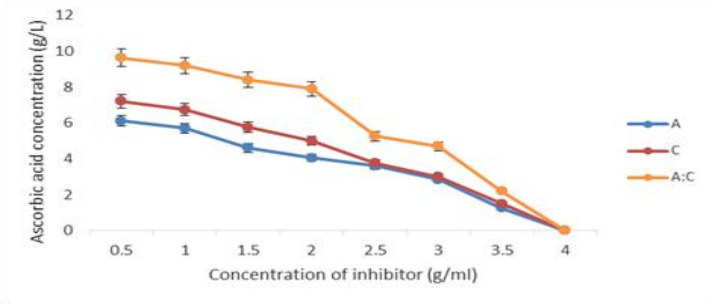


A=*Aspergillus flavus*, C=*Aspergillus tamarii*, A:C=Mixed cultures of *A. flavus* and *A. tamarii*

Fig. 2: Effect of temperature on ascorbic acid production by single and mixed cultures of *Aspergillus flavus* and *Aspergillus tamari* (A, p=0.01; C, p=0.01; A:C, p=0.02).

3.4 Effect of inhibitors on ascorbic acid production by single and mixed cultures of *Aspergillus flavus* and *A. tamarii***

Investigations into the effect of EDTA on ascorbic acid production showed that an initial concentration of 0.5 g/ml of EDTA resulted in maximal ascorbic acid yields of 6.12 g/L, 7.2 g/L and 9.64g/L by *A. flavus* and *A. tamarii* and their mixed cultures respectively. However, an increase in the concentration of the inhibitor to 3.5 g/ml resulted in decreased ascorbic acid production with a yield of 1.25 g/L, 1.5 g/L and 2.2 g/L by *A. flavus*, *A. tamarii* and their mixed cultures respectively. There is significant difference in the ascorbic acid yields at different concentrations of EDTA (p<0.05). Increase in the concentration of EDTA to 4 g/ml resulted in the inability of all the isolates to produce ascorbic acid (Figure 3). Thus, enzymes responsible for ascorbic acid production were inhibited from concentration of 4 g/ml because EDTA is known to chelate Ca^{2+} which is required as a cofactor for enzyme activity. In a related study, Igbokwe *et al*. [25], reported that the low enzymatic activity observed is brought about by the chelation of Ca^{2+} from the enzyme which makes the active sites less catalytically efficient.



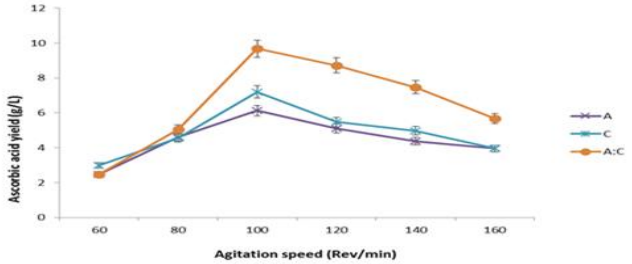
A=*Aspergillus flavus*, C=*Aspergillus tamarii*, A:C=Mixed cultures of *A. flavus* and *A. tamarii*

Fig. 3: Effect of inhibitor (EDTA) on ascorbic acid production by single and mixed cultures of *Aspergillus flavus* and *Aspergillus tamari* (A, p=0.02; C, p=0.03; A:C, p=0.02).

3.5 Effect of different agitation speeds on ascorbic acid production by single and mixed cultures of *Aspergillus flavus* and *A. tamarii***

Investigation on the effect of agitation speed on ascorbic acid production was studied due to its important role in the proper mixing of the components of the fermentation medium. The study as shown in Figure 4 revealed that optimum ascorbic acid yields of 6.13 g/L, 7.21 g/L and 9.65 g/L were produced at an agitation speed of 100 revolution per minute by *Aspergillus flavus Aspergillus tamarii* and their mixed cultures respectively. There is significant difference in the ascorbic acid produced at different agitation speeds (p<0.05). Further increase in agitation speed resulted in reduction in ascorbic acid yield of 3.95 g/L, 3.95 g/L and 5.67g/L by *A. flavus A. tamarii* and their mixed cultures at 160 revolution per minute. The decreased ascorbic acid produced when agitation speed is increased above the optimum agitation speed might be due to the destruction of the fungal mycelium resulting from the shear forces exerted by the increased agitation speed [26]. Also, improper mixing of the constituents of the fermentation medium at reduced agitation speed might be the reason adduced to the reduction in the quantity of ascorbic acid produced [27]. Furthermore, different agitation speeds may be responsible for the distribution of air and nutrients to the microbial cells [28]. It has been reported that the agitation

speed required for a fermentation study will be dependent on the microorganism and what the fermentation medium is made up of [29].



A=*Aspergillus flavus*, C=*Aspergillus tamarii*, A:C=Mixed cultures of *A. flavus* and *A. tamarii*

Fig. 4: Effect of agitation speed on ascorbic acid production by single and mixed cultures of *Aspergillus flavus* and *Aspergillus tamari* (A, p=0.001; C, p=0.001; A:C, p=0.002).

3.6 High performance liquid chromatography (HPLC) of ascorbic acid produced by single and mixed cultures of *Aspergillus flavus* and *A. tamarii*

High performance liquid chromatography was carried out to quantify the ascorbic acid produced by single and mixed cultures of *Aspergillus flavus* and *A. tamarii*. The HPLC result showed that the maximum ascorbic acid yield of 9.648 g/L was produced by the mixed cultures of *A. flavus* and *A. tamarii* and the least ascorbic acid yield of 6.248 g/L by *A. flavus* (Figures 5-7). Two peaks (Peaks 1 and 2) were observed from the HPLC results of the extracts from the fermentation medium of single cultures of *A. flavus* and *A. tamarii*. On the contrary, three peaks were observed from the HPLC results of the extracts from the fermentation medium of the mixed cultures of *A. flavus* and *A. tamarii*. However, Peak 1 of the single culture was identified as ascorbic acid while only peak 3 was identified as ascorbic acid in the mixed cultures. The different elution observed by the three peaks is evident of the fact that there are other compounds present in the extracts which may likely be other analogues of L-ascorbic acid.

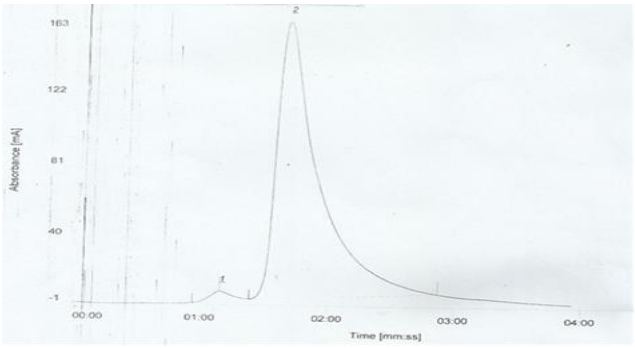


Fig. 5: High Performance Liquid Chromatography of *Aspergillus flavus* (A)

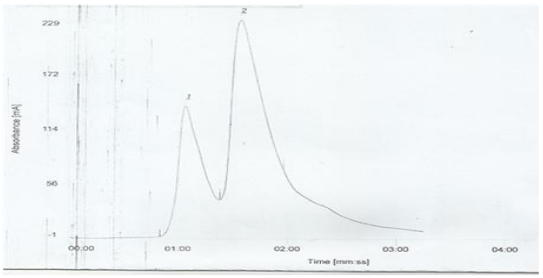


Fig. 6: High Performance Liquid Chromatography of *Aspergillus tamarii* (C)

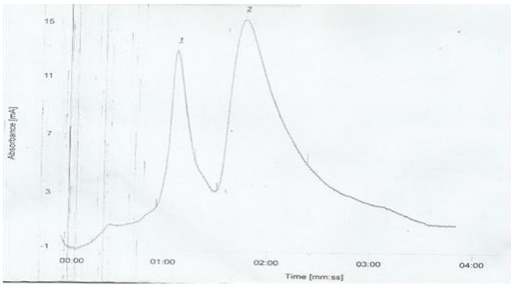


Fig. 7: High Performance Liquid Chromatography of mixed cultures of *Aspergillus flavus* and *Aspergillus tamarii*

Conclusion

In conclusion, mixed cultures of *Aspergillus flavus* and *A. tamarii* resulted in an increased ascorbic acid yield of 9.65 g/L compared to yields of 6.25 g/L and 7.25 g/L by single cultures of A. *flavus* and *A. tamarii* respectively. Optimum ascorbic acid yield was produced at pH 5.0, temperature (40 °C) and agitation speed of 100 rpm at 96 h of fermentation. This study showed that mixed cultures of fungal isolates resulted in an increased ascorbic acid production which is of importance to the pharmaceutical, beverage and food industries.

Conflict of Interest: The authors declare that they have no conflict of interest.

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