Microbial Production of Ascorbic Acid from Brewery Spent Grain (BSG) by *Aspergillus flavus* and *Aspergillus tamarii*

Temitope Banjo [1], Sarafadeen Kareem [2], Temitope Popoola [2] and Oluseyi Akinloye [3]

Abstract

This study evaluated the use of Brewery Spent Grain (BSG) as a novel substrate for the production of ascorbic acid using *Aspergillus flavus* and *Aspergillus tamarii*. Spores of *Aspergillus flavus* and *Aspergillus tamarii* were cultured in a liquid fermentation medium containing BSG (0.6% w/v) for ascorbic acid production. The process was studied at pH 4–8, temperature range 30–45°C, agitation speed range 60–160 rpm for 168 h. Stability studies and the effects of Ethylenediaminetetraacetic Acid (0.5–4.0 g/ml) on ascorbic acid production were studied. Ascorbic acid produced was quantified by titration techniques and with High Performance Liquid Chromatography (HPLC). The statistical analysis of the effects of temperature and agitation speed on ascorbic acid production showed no significant difference at $p<0.05$. However, there was significant difference in the effect of pH on ascorbic acid production at $p<0.05$. Optimum ascorbic acid yields of 7.25 g/L and 6.25 g/L were produced by *A. tamarii* and *A. flavus* respectively at pH 5.0, temperature (40°C) and agitation speed of 100 rpm at 96 h of fermentation. High Performance Liquid Chromatography (HPLC) showed that 6.248 g/L and 7.246 g/L of ascorbic acid were produced by *A. flavus* and *A. tamarii,* respectively. Ascorbic acid production by *A. flavus* and *A. tamarii* was completely inhibited at 4 g/ml of EDTA. This study shows the potential of BSG as a novel substrate for ascorbic acid production.

Keywords: Ascorbic acid, High Performance Liquid Chromatography, Brewery Spent Grain, *Aspergillus flavus*, *Aspergillus tamarii*

1. Introduction

Ascorbic acid also known as Vitamin C is a water-soluble vitamin which plays significant roles in the normal functioning of the human body. The biochemical functions of vitamin C include among others; stimulation of certain enzymes, prevention of scurvy, collagen biosynthesis, hormonal activation, antioxidant, histamine detoxification, formation of nitrosamine, and proline hydroxylation (Walingo, 2005). Also, vitamin C as an antioxidant reduces the incidence of cancer (Lupulescu, 1993).

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Ascorbic acid has been produced from plant, animal and microbial sources. However, this study focused on the use of microorganisms in the production of ascorbic acid. Microorganisms can be easily grown on an industrial scale. The production of erythroascorbic acid has been reported in *Candida* and *Saccharomyces* species (Branduardi *et al.*, 2006). A physiological pathway has been proposed from D- glucose to erythroascorbic acid in these yeasts (Porro and Sauer, 2007).

Significant quantities of wastes are generated by the food industries which results to pollution of the environment (Zvidzai *et al.*, 2007). In the manufacture of beer, which is the fifth most consumed beverage in the world, various residues and by-products are generated (Fillaudeau *et al*., 2006). The most common wastes generated during the manufacture of beer are spent grains, spent hops and surplus yeast (Mussatto, 2009). The brewery spent grain (BSG) constitutes the highest wastes generated (85%) after different separation techniques, leading to the production of beer (Tang *et al*., 2009). It was reported that United Kingdom (UK) generates over 0.5 million tonnes of BSG from the brewing industry (Stojceska *et al*., 2008), while Brazil (the world's fourth largest beer producer) generated about 1.7 million tonnes of BSG in 2002 (Mussatto and Roberto, 2006). Therefore, BSG is a cheap, easily available and ready source of raw material for industrial exploitation (Robertson *et al*., 2010). Several attempts have been made in utilizing brewery spent grain in animal feeds (Szponar *et al.*, 2003), production of value-added compounds (xylitol, lactic acid, etc.) (Bai *et al*., 2008; Mussatto and Roberto, 2008), cultivation of microorganisms (Terrasan *et al*., 2010), or simply used as raw material for extraction of compounds such as sugars, proteins and some acids (Xiros *et al*., 2009). BSG is also applicable in enzymes production and immobilization of various substances (Mussatto, 2009). However, the use of brewery spent grain in the cultivation of *Aspergillus* spp for ascorbic acid production has not been reported. Therefore, this study investigated the use of BSG as substrate in the production of ascorbic acid.

2. Methodology

2.1 Materials

Ascorbic acid producing-strain of *Aspergillus flavus* and *Aspergillus tamarii* were obtained from the Culture Collection Centre of the Federal University of Agriculture, Abeokuta, Ogun State, Nigeria. Brewery Spent Grain (BSG) was obtained from Sona Breweries, Ota, Ogun State Microbiological media used in these experiments were: Saboraud Dextrose Agar, Potato Dextrose Broth from BDH Chemicals, UK. The Chemicals used include; 2, 6 Dichlorophenol Indophenol dye from Sigma Chemical Ltd, U.S.A. Trisodium phosphate, Sodium potassium tartarate, acetic acid, Ethylene Diamine Tetra Acetic acid (EDTA),

Metaphosphoric acid, were all obtained from BDH chemicals, UK. Glucose, galactose, Lactose from Surechem Products, U.K. The major equipment used include Autoclave (Prestige medical series 2100, England), Hot box oven, incubator (Gallenkamp, size 1 England), Weighing balance (Mettler Toledo PB 3002, Switzerland), water bath (Nickel Electro Ltd, England) pH meter (Jenway Ltd England). Waring blender, (Kenwood Ltd, Great Britain)

2.2 Pretreatment of Brewery Spent Grain (BSG)

Samples of BSG collected contained approximately 80% moisture and was dried using hot air oven to approximately 4% moisture at 80°C. The dried BSG were milled using a waring blender for 3 minutes and kept in an airtight container until further use.

2.3 Production and quantification of ascorbic acid by *Aspergillus flavus* and *A. tamarii*

The two moulds were cultured on the brewery waste medium (0.6% brewery waste, 2% D-glucose, 0.3% L-galactose, 0.3% yeast extract, 0.5% peptone and 0.2% monosodium glutamate) as determined by the response surface plot. Ascorbic acid production was monitored at 24 h interval for 168 h. Optimum fermentation time was selected for further studies. Quantitative assay of Ascorbic acid was carried out using the method of Association of Vitamin Chemists (1996). The quantity of ascorbic acid in samples was calculated as shown below

$$B \times A/10$$

A= Titre values from standard ascorbic acid

B= Titre values from ascorbic acid in samples

2.4 Effect of pH on ascorbic acid production

The effect of pH on ascorbic acid production was studied by inoculating the spores of *Aspergillus flavus* and *Aspergillus tamarii* (2×10^9 spore/ml) on brewery waste medium (0.6% brewery waste, 2% glucose, 0.3% galactose, 0.3% yeast extract, 0.5% peptone, 0.2% monosodium glutamate) at 30°C and pH range 4.0–8.0 (pH 4.0, 5.0, 6.0, 7.0 and 8.0) in a 250 ml Erlenmeyer flask at optimum fermentation time.

2.5 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) at 30°C and pH 5. The ascorbic acid produced was quantified at optimum fermentation time.

2.6 Effect of temperature on ascorbic acid production

Effect of temperature on ascorbic acid produced by *Aspergillus flavus* and *Aspergillus tamarii* was studied at 100 rpm, pH 5 and temperature range 30–45°C (30, 35, 45°C). The ascorbic acid produced was quantified at optimum fermentation time.

2.7 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 optimum fermentation time.

2.8 Stability of ascorbic acid

The stability of ascorbic acid produced by *Aspergillus flavus* and *Aspergillus tamarii* under culture conditions was determined according to a modified method of Porro and Sauer (2003) in which the standard solution was reconstituted. The spores of *A. flavus and A. tamarii* were inoculated on a medium containing 2% glucose, 0.3% galactose, 0.3% yeast extract and 0.5% peptone in separate flasks. Ascorbic acid (10 mg) was added to the medium without organism [control (Aw)] and the stability of the ascorbic acid was monitored for six weeks at 40°C.

2.9 Measurement of ascorbic acid by high performance liquid chromatography (HPLC)

The Ascorbic acid concentration in the extracted samples was estimated by High Performance Liquid Chromatography (HPLC) method of El.Gindy *et al*. (2005). The mobile phase was made up 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 and percentage of ascorbic acid formed under different optimized conditions was compared.

2.10 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, agitation speed, stability studies and the effects of Ethylene Diamine Tetra Acetic acid were determined using ANOVA at 95% confidence interval while p value <0.05. Significance of **c**omparative yield of ascorbic acid from brewery spent grain by *Aspergillus flavus* and *Aspergillus tamarii* was determined taking $p<0.05$.

3. Results and Discussion

3.1 Production of ascorbic acid by *Aspergillus flavus* and *Aspergillus tamarii*

Studies on the fermentation of the brewery spent grain medium with *Aspergillus flavus* and *Aspergillus tamarii* showed that ascorbic acid yield peaked at 96 h of fermentation. Ascorbic acid yields of 7.25 g/L and 6.25 g/L were produced by *Aspergillus tamarii* and *Aspergillus flavus* at 96 h of fermentation. Thus, 96 h was adopted as the optimum fermentation time for further studies. The yield of ascorbic acid reduced with increase in fermentation time for the two 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. The loss of ascorbic acid may be a result of the increase in the activity of the enzyme ascorbate oxidase that might have been produced by the fermentation microorganism which strongly depends on the pH of the fermentation environment. The enzymes convert ascorbic acid to dehydroascorbic acid (Adetuyi *et al*., 2008).

Table 1 Production of ascorbic acid from brewery spent grain by *Aspergillus flavus* and *Aspergillus tamarii*

	Fermentation time (Hours)										
Isolates	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**	**120**	**144**	**168**
Aspergillus flavus	0	0	1.6	2	3.2	4	5.6	6.25	0	0	0
Aspergillus tamarii	0	0	2	2.5	3.6	4.25	6.4	7.25	5	2.25	0.25

3.2 Effect of pH on ascorbic acid production

Investigations on the effect of pH on ascorbic acid production showed significant ascorbic acid production at pH 5.0 for both isolates (p<0.05). Optimum ascorbic acid yields of 5.9 g/L and 6.87 g/L were produced by *Aspergillus flavus* and *Aspergillus tamarii* respectively at pH 5.0 (Figure 1). However, ascorbic acid yield reduced drastically 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. 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). Similar result was reported by Chaurasia *et al.* (2014) on organic acid production by a fungus (*Sclerotium rolfsii*) at an optimum pH of 5.0.

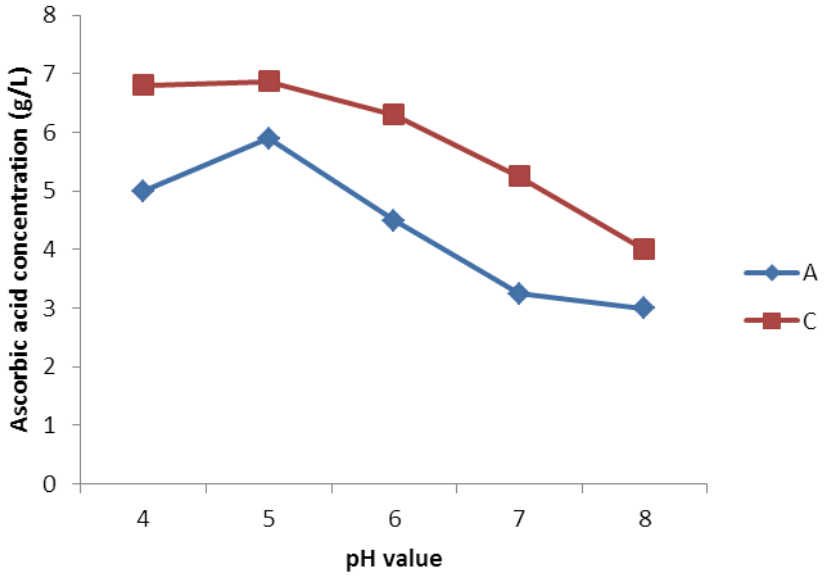

Figure 1 Effect of $_{P}$H on ascorbic acid production by *Aspergillus flavus* (A) and *Aspergillus tamarii* (C) (F=8.041, P=0.025)

3.3 Effect of agitation speeds on ascorbic acid production

A proper agitation speed is important for appropriate air supply and proper mixing of media components, hence the effect of agitation speed on ascorbic acid production was studied. The study as shown in Figure 2 revealed that optimum ascorbic acid yields of 6.13 g/L and 7.21 g/L were produced at an agitation speed of 100 revolution per minute by *Aspergillus flavus* and *Aspergillus tamarii* respectively. There was no 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 by *A. flavus* and *A. tamarii* at 140 revolution per minute. Decrease in the ascorbic acid production at higher agitation speeds might be due to the harmful effect of the shear forces on the fungal mycelium as a result of agitation speed (Techapun *et al*., 2003). At lower agitation speeds, less amount of ascorbic acid produced might be due to improper mixing of the medium (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). The degree of agitation required for a fermentation study will be dependent on the organism and the composition of the fermentation medium (Shyam *et al*., 2009).

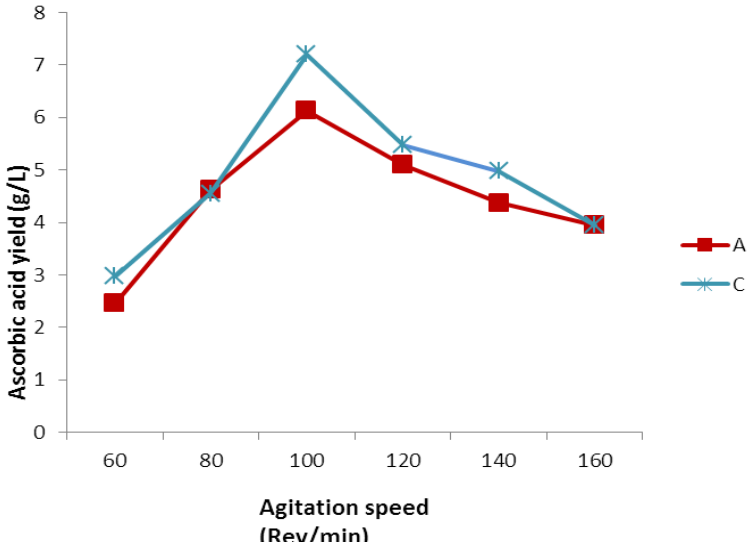

Figure 2 Effect of different agitation speed on ascorbic acid production by *Aspergillus flavus* (A) and *Aspergillus tamarii* (C) (F=3.001, P=0.531)

3.4 Effect of temperature on ascorbic acid production

Optimum ascorbic acid was produced by the two isolates at 40°C. The effect of temperature on ascorbic acid production showed that ascorbic acid yields of 6.2 g/L and 7.15 g/L were produced by *A. flavus* and *A. tamarii* respectively. However, there was no significant difference in the ascorbic acid yields at different temperatures (p<0.05). There was a decrease in ascorbic acid production at higher temperature as shown in Figure 3. In a related study with another organic acid, Kareem and Rahman (2013) reported reduced citric acid production at temperatures above the optimum temperature. This might be due to accumulation of by-products and eventually, loss of activity as the temperature increases.

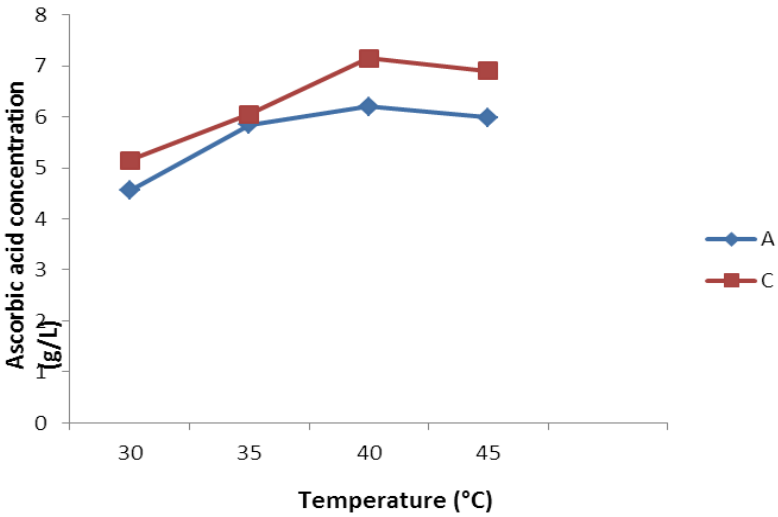

Figure 3 Effect of temperature on ascorbic acid production by *Aspergillus flavus* (A) and *Aspergillus tamarii* (C) (F=2.132, P=0.621)

3.5 Effect of inhibitors on ascorbic acid production

The effect of EDTA on ascorbic acid production showed that at an initial concentration of 0.5 g/ml of EDTA, maximal ascorbic acid yields of 6.12 g/L and 7.2 g/L were produced by the *A. flavus* and *A. tamarii* respectively. At this concentration, ascorbic acid production was not significantly affected when compared to the initial yield of 6.25 g/L and 7.25 g/L by *Aspergillus flavus* and *Aspergillus tamarii* 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 and 1.5 g/L by *A. flavus* and *A. tamarii* respectively. There was no 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 4). Thus, enzymes responsible for ascorbic acid production were inhibited from concentration of 4 g/ml. EDTA is known to chelate Ca^{2+} which is required as a cofactor for enzyme activity. In a similar study, Igbokwe *et al*. (2013) reported that the denial of Ca^{2+} to the enzyme makes the active sites less catalytically efficient, hence low activity.

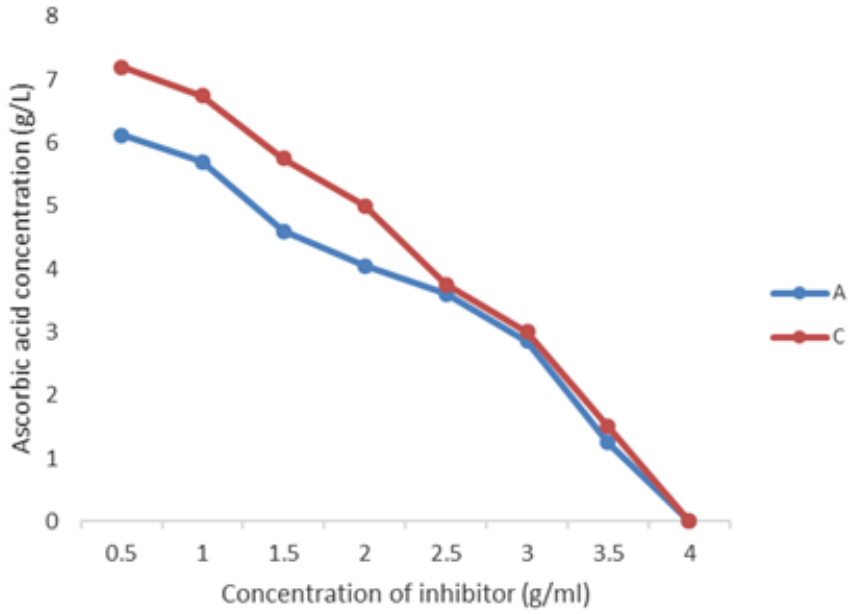

Figure 4 Effect of inhibitor (EDTA) on ascorbic acid production by *Aspergillus flavus* (A) and *Aspergillus tamarii* (C) (F=7.013, P=0.432)

3.6 Stability of ascorbic acid

Ascorbic acid is the least stable of all vitamins and is easily destroyed during processing and storage, hence the need to carry out a stability study of the ascorbic acid produced. The result of the stability studies carried out over a period of six weeks revealed a gradual degradation of ascorbic acid in both the medium without organism (control) labelled Aw and the culture medium. In the first week, ascorbic acid values for the control and culture medium with *Aspergillus flavus* and *Aspergillus tamarii* were 10 g/L, 6.2 g/L and 7.15 g/L respectively. There was no significant difference in the stability of the ascorbic acid over time (p<0.05). However, there was a sharp decline in the ascorbic acid values in the third week with 3.21 g/L, 3.9 g/L and 4.85 g/L yields by the control and culture medium with *A. flavus* and *A. tamarii* respectively. At the end of the six weeks of study, the ascorbic acid in the control has been totally degraded giving a value of 0.25 g/L from an initial value of 10 g/L. This is in agreement with the report of Ajibola *et al.* (2009) that ascorbic acid decreases gradually during storage especially at temperature above 0°C. On the other hand, the culture medium with *A. flavus* and *A. tamarii* still retained ascorbic acid values of 1.15 g/L and 1.62 g/L respectively at the end of six weeks. Although ascorbate was completely degraded within 7 days in a sterile medium with the absence of cells, incubation of ascorbate in culture medium in the presence of growing yeast cells stabilized the ascorbic acid substantially (Bai *et al*., 2008).

3.7 Measurement of ascorbic acid by high performance liquid chromatography (HPLC)

High performance liquid chromatography was carried out to quantify the ascorbic acid produced by *Aspergillus flavus* and *Aspergillus tamarii*. It showed the ascorbic acid yields by *A. flavus* and *A. tamarii* to be 6.248 g/L and 7.258 g/L respectively (Figure 5). The extracts from the fermentation medium of *A. flavus*, *A. tamarii* showed two peaks (Figure 6 and 7). Peak 1 was identified as ascorbic acid. This co-elution observed by the different peaks showed that there are other compounds present in the extracts which may likely be other analogues of L-ascorbic acid.

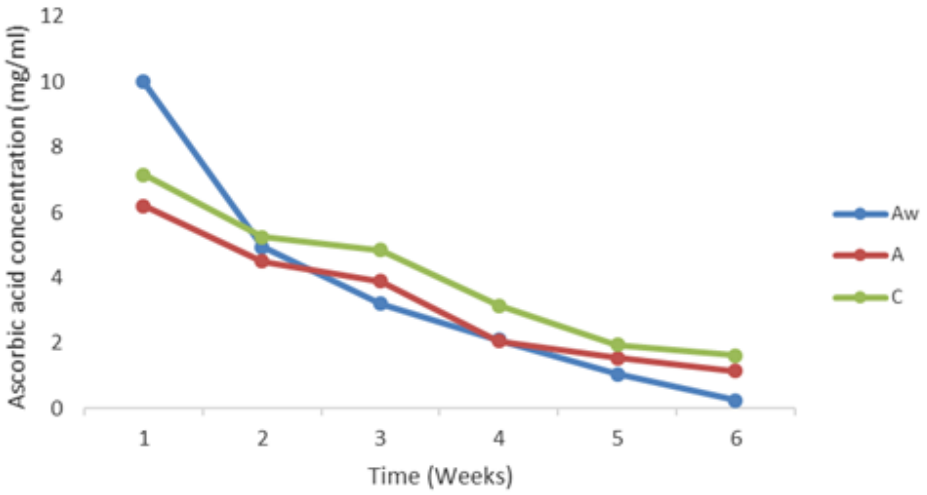

Figure 5 Stability of ascorbic acid produced by *Aspergillus flavus* (A), *Aspergillus tamarii* (C) and the control (Aw) (F=0.432, P=0.532)

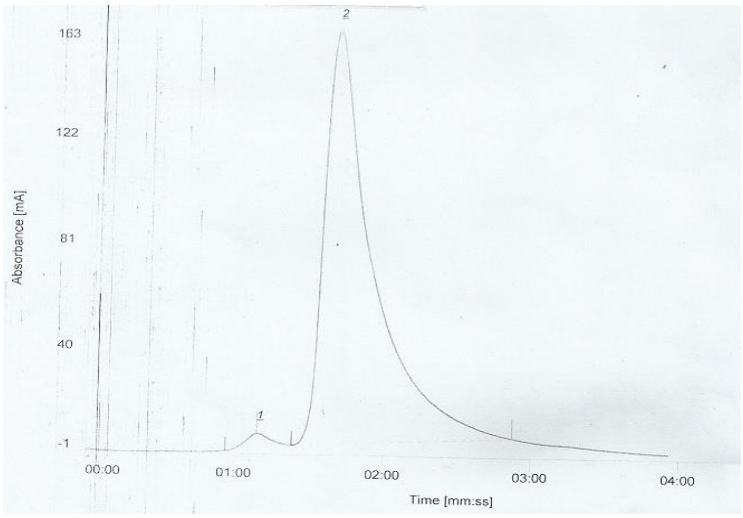

Figure 6 High Performance Liquid Chromatography of *Aspergillus flavus* (A)

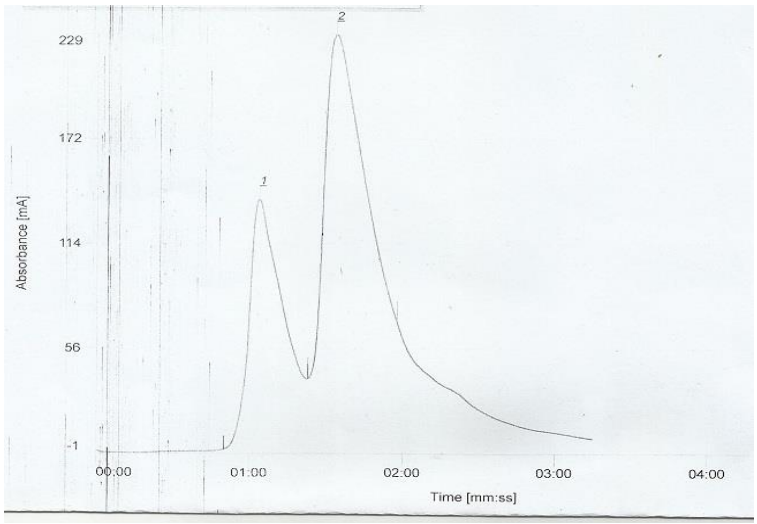

Figure 7 High Performance Liquid Chromatography of *Aspergillus tamarii* (C)

4. Conclusion

The present study showed that optimum ascorbic acid yield of 7.25 g/L and 6.25 g/L were produced by *Aspergillus tamarii* and *Aspergillus flavus* respectively when cultured on a brewery waste medium at pH 5, 40°C and agitation speed of 100 rpm at 96 h of fermentation. This study established the production of ascorbic acid from an industrial waste (BSG), thus turning waste to wealth.

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