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Strain improvement of *Aspergillus flavus* for enhanced ascorbic acid production by physical and chemical mutagenesis

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Abstract

The demand for ascorbic acid in the food and pharmaceutical industry led to the search for hyper ascorbic acid producing strains by physical and chemical mutagenesis. Spores of *Aspergillus flavus* were subjected to Ultraviolet (UV) radiation (240 nm) and Ethidium Bromide (EB) (25, 50, 75 and 100 µg/ml) to develop hyper-producing mutants. The selected mutants were cultured in a liquid fermentation medium containing Brewery Spent Grain (0.6 % w/v) at pH range 4 - 8, temperature range 30 - 45 °C, agitation speed range 60 - 160 rpm for 96 h. Ascorbic acid produced was quantified by titration techniques and with High Performance Liquid Chromatography (HPLC). The UV and EB mutant strains of *A. flavus* gave increased ascorbic acid yields of 6.99 g/L and 7.28 g/L respectively when compared to the parental strain with ascorbic acid yield of 3.92 g/L. Optimum ascorbic acid yields were produced at 40 °C, pH 5.0 and 100 rpm at 96 h of fermentation. This study shows the potential of strain improvement for enhanced ascorbic acid production.

Keywords Ascorbic acid; Ethidium bromide; Ultraviolet radiation; *Aspergillus flavus; Mutagenesis*

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Introduction

Ascorbic acid is an essential nutrient for a large number of higher primate species, a small number of other mammalian species, a few species of birds and some fish (Saalu et al., 2009). This organic acid whose deficiency causes scurvy in humans is also required for a range of essential metabolic reactions in all animals and plants (Food Standards Agency, 2007). Ascorbic acid acts as food additive (Higdon, 2006), and antioxidant which protects the body against oxidative stress (Padayatty et al., 2003). The production of increased quantities of ascorbic acid by improved strain of microorganisms has been reported (Banjo et al., 2018).

Strain improvement is a veritable tool used in the screening of better isolates among mutagen-treated microbial population. Moreover, it is used in the screening of hyper-producing mutants for enhanced industrial metabolites production. Strain improvements of microorganisms by chemical and physical mutagens have been carried out on *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* (Sauer et al., 2004); *Gluconobacter frateurii* and *Acetobacter aceti*. (Beuzelin-Olivier et al., 2012), for increased ascorbic acid production. Improved ascorbic acid yield by genetically altered microorganism means an increase of at least 5 % ascorbic acid yield compared to a cell which is not genetically altered. (Beuzelin-Olivier et al., 2012).

Mutagens are strain improvement tools employed in the screening of microorganisms of industrial importance (Suribabu et al., 2014). Metabolites of industrial importance are produced in low concentrations by the parent cell of microorganisms but this may be increased by alteration of the genome of the microorganism (Suribabu et al., 2014). There are several reports on strain improvement of microorganisms for the production of enzymes and other secondary metabolites; however there is insufficient information on the strain improvement of *Aspergillus flavus* for increased ascorbic acid production. Hence the objective of this study is the strain improvement of *Aspergillus flavus* by mutagenesis using Ultraviolet ray and Ethidium bromide for enhanced ascorbic acid production which is of great importance in the pharmaceutical industry.

Materials and Methods

Source of fungi for ascorbic acid production

Strain of *Aspergillus flavus* was 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.

Sources of substrates used for the cultivation of Aspergillus flavus

Brewery waste was obtained from Sona Breweries, Ota, Ogun State. Cassava starch flour was obtained from Local markets in Abeokuta, Ogun State.

Production of mutant strains by exposure of Aspergillus flavus to ultraviolet ray

Ultraviolet ray mutagenesis of the fungal isolates was carried out by a modified method of Irfan et al. (2011). Spore suspension was prepared in serial dilution method from five days old culture slant. One ml of 10^6 dilutions was poured in petri dish and placed under UV lamp (240 nm) for a specified time intervals (10, 20, 30, 40 and 50 minutes). The irradiated spores were cultured on SDA plates and incubated at 30 C for five to seven days until sporulation of fungal culture. The ascorbic acid produced by the mutagenized colonies was quantified and the best producing strain selected for further study.

Production of mutant strains by treatment of Aspergillus flavus with Ethidium Bromide

A stock solution of Ethidium bromide was prepared in which 1.0 mg Ethidium bromide (EB) was added per mL to deionized distilled water. Dilutions of 25 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml were prepared from the stock solution. One ml of Ethidium Bromide was added separately to 9 ml of Vogel's medium containing fresh fungal spore. The treated spores were then harvested by washing with sterile saline solution after specified time intervals (30, 60, 90 and 120 minutes) after which the harvested spores was spread on agar plates for colony formation.

Production and quantification of ascorbic acid under optimum conditions by parent and mutant strains of Aspergillus flavus

Parent and mutant strains of *Aspergillus flavus* 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 (Banjo et al., 2018) 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 (2010). Quantitative assay of Ascorbic acid was carried out by titration using the method of Association of Vitamin Chemists (2010). Equal weights (200-300 g) of the sample was blended with 6 % metaphosphoric acid to yield a homogenous slurry.10 g of this slurry was weighed into a 100ml volumetric flask and dilute to 100ml with 3 % metaphosphoric acid and filter the diluted sample. 10ml of the filtrate was pipetted into a flask and titrated immediately with the standardized solution of 2, 6-Dichlorophenol indophenol to a faint pink end point which persisted for 15 seconds.The quantity of ascorbic acid in samples was calculated as shown below

$$\mathbf{B \times A/10}$$

Where A= Titre values from standard ascorbic acid

B= Titre values from ascorbic acid in samples

Optimization of ascorbic acid production by parent and mutant strains of Aspergillus flavus

Spores of *Aspergillus flavus* and their mutant

strains (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 hours. The ascorbic acid produced was quantified according the methods of Association of vitamin chemist (2010).

Effect of pH on ascorbic acid production by parent and mutant strains of Aspergillus flavus

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 after 96 h of fermentation according the methods of Association of vitamin chemist (2010).

Effect of temperature on ascorbic acid production by parent and mutant strains of A. flavus

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 after 96h of fermentation according the methods of Association of vitamin chemist (2010).

Effect of 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 after 96h of fermentation according the methods of Association of vitamin chemist (2010).

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.

Results and Discussions

Physical mutagenesis of Aspergillus flavus by exposure to ultraviolet ray

Studies on the effect of ultraviolet ray on ascorbic acid production by the *Aspergillus flavus* showed that the maximum yield of 6.99 g/L was produced by *A. flavus* at 10 minutes of exposure (Table 1). This mutant strain was selected for further studies and designated as A10. Mutagenesis of *A. flavus* by Ultraviolet ray at an exposure time of 10 seconds resulted in an increased ascorbic acid yield of 6.99 g/L compared to a yield of 3.92 g/L from the parental strain of *A. flavus*. This showed an increase of 78% ascorbic acid production by the mutant strain of *A. flavus*, over that of the parent cell. Increased secondary metabolite production is brought about by excitation of the electrons and subsequent formation of extra bonds in DNA molecules by UV light. However, ascorbic acid production decreased to 3.50 g/L at exposure time of 20 minutes and ascorbic acid was not produced above this time (Table 1). This agrees with the findings of Khanam et al. (2013), who reported that fungal growth rate decreased with increase in the time of exposure to UV rays thus leading to reduced and complete loss of metabolite producing ability by the fungus.

Table 1: Effect of exposure time of Ultraviolet radiation on ascorbic acid production by Aspergillus flavus

Exposure Time (Minutes)	Ascorbic acid concentration (g/L)
10	6.99
20	3.50
30	0
40	0
50	0

Chemical mutagenesis by Ethidium Bromide treatment

The effect of Ethidium bromide on ascorbic acid production by *Aspergillus flavus* showed that ascorbic acid yield varied with different concentrations of Ethidium bromide (Fig. 1). The highest ascorbic acid yield was produced by *Aspergillus flavus* at 50 µg/ml of Ethidium bromide for 120 minutes. The mutant strain was designated as AB120 and selected for further studies. Mutagenesis of *A. flavus* with Ethidium bromide gave the highest ascorbic acid yield of 7.28 g/L at 50 µg/ml for 120 minutes (Fig. 1). This showed an increase of 80 % ascorbic acid production over the parent strain. This result agrees with the work of Khanam and Prasuna (2014), who reported that Ethidium bromide increased enzyme production. Similarly, Roland et al. (1990) reported the mutagenesis of *Candida Norvegensis* for increased ascorbic acid production. Ethidium bromide acts as a mutagen because it intercalates double stranded DNA and deforms the DNA. This could result in frame shift mutations of the genes responsible for ascorbic acid production leading to increased ascorbic acid yield.

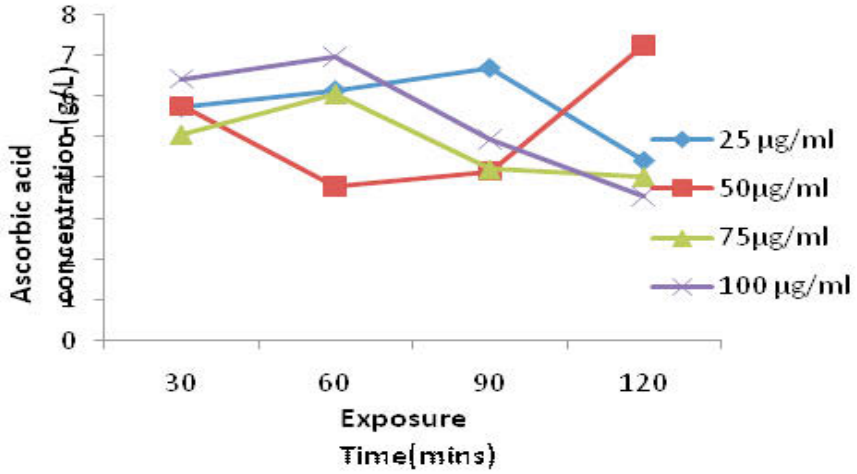

Fig. 1: Effect of exposure time of Ethidium bromide on ascorbic acid production by Aspergillus flavus

Effect of pH on ascorbic acid production

Investigations on the effect of pH on ascorbic acid production showed that optimum ascorbic acid yields of 5.95 g/L, 7.21 g/L and 8.05 g/L were produced by *Aspergillus flavus*, UV mutant strain (A10) and EB mutant strain (AB120) respectively at pH 5.0 (Fig. 2). However, ascorbic acid yield reduced drastically to 3 g/L, 4 g/L and 4.5 g/L by *Aspergillus flavus*, A10 and AB120 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 Chaurasia et al. (2014), who reported the suitability of pH 5 for organic acid production, by a fungus (*Sclerotium rolfsii*). Hence, the pH of the culture medium directly influences the growth of microorganisms and the biochemical processes they perform (Leandro et al., 2015).

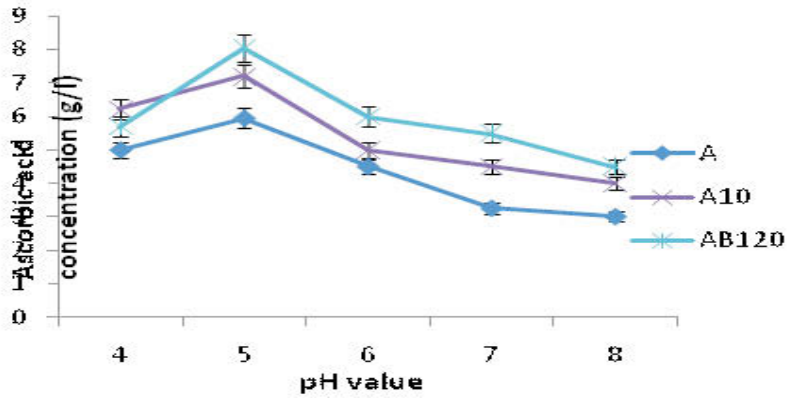

Fig. 2: Effect of pH on ascorbic acid production by Aspergillus flavus (A), EB mutant strain (AB120) and UV mutant strain (A10).

Effect of temperature on ascorbic acid production

The effect of temperature on ascorbic acid production showed that optimum ascorbic acid yields of 6.05 g/L, 8.18 g/L and 8.35 g/L were produced at 40°C by *Aspergillus flavus*, A10 and AB120 respectively. There was a decrease in ascorbic acid production at higher temperature as shown in Fig. 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.

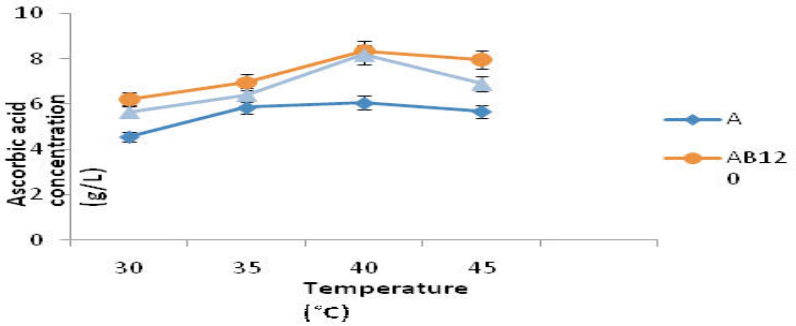

Fig. 3: Effect of temperature on ascorbic acid production by Aspergillus flavus (A), EB mutant strain (AB120) and UV mutant strain (A10).

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 4 revealed that optimum ascorbic acid yields of 6.25 g/L, 8.5 g/L and 8.7 g/L were produced at an agitation speed of 100 revolution per minute by Aspergillus flavus, A10 and AB120 respectively. Further increase in agitation speed resulted in reduction in ascorbic acid yield by the parent and mutant strains. 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).

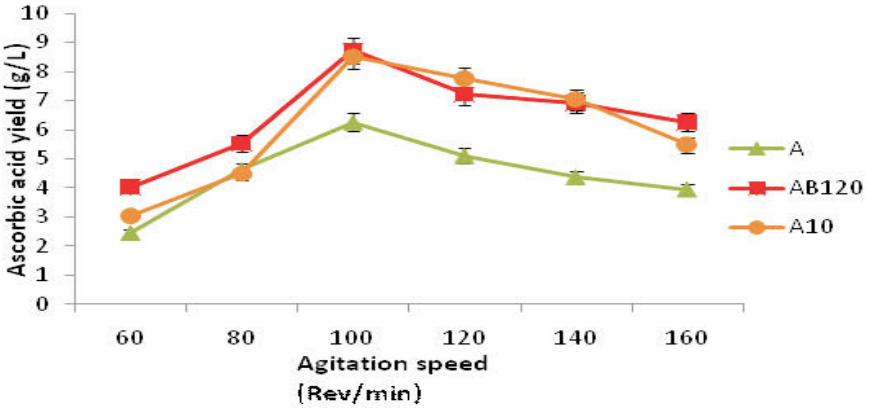

Fig. 4: Effect of agitation speed on ascorbic acid production by Aspergillus flavus (A), EB mutant strain (AB120) and UV mutant strain (A10).

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 its mutant strains. It showed the ascorbic acid yields by *A. flavus*, A10 and AB120 to be 6.248 g/L, 8.462 g/L and 8.658 g/L respectively. The extracts from the fermentation medium of the mutant strain, AB120 produced only one peak while A10 and *A. flavus* showed two peaks (Peaks 1 and 2). 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.

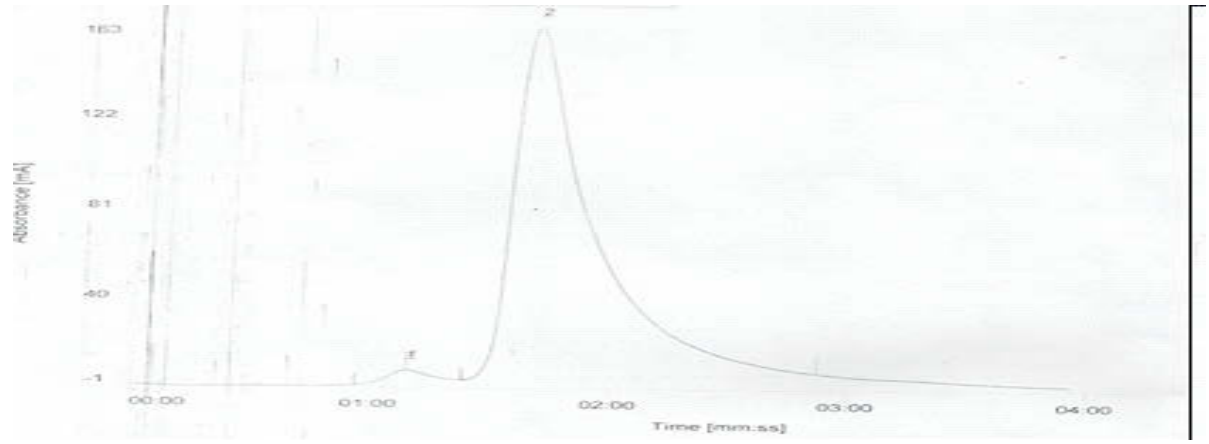

Fig. 5 High Performance Liquid Chromatography of *Aspergillus flavus*

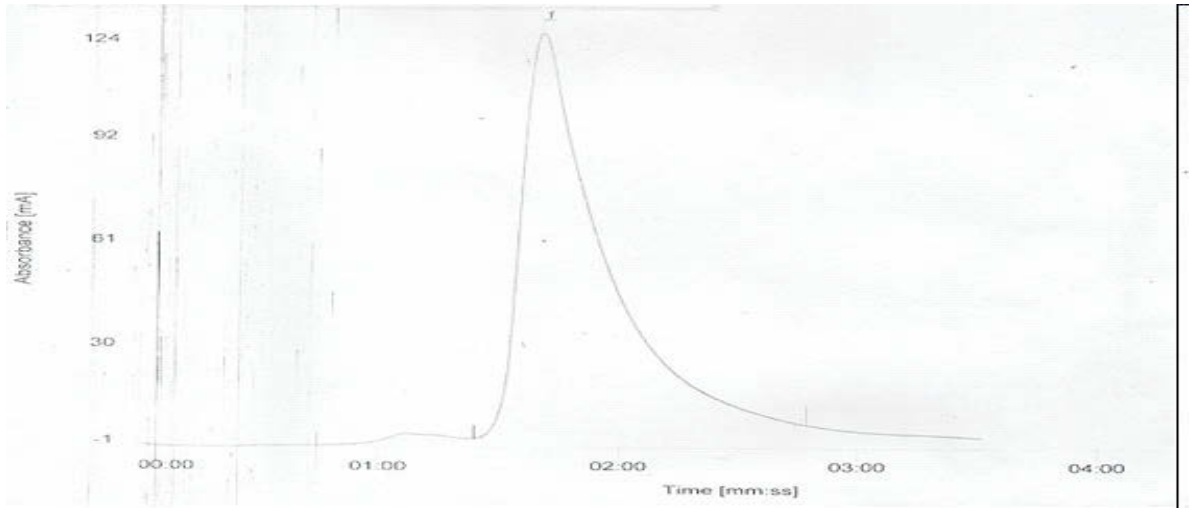

Fig. 6: High Performance Liquid Chromatography of *Aspergillus* strain AB120

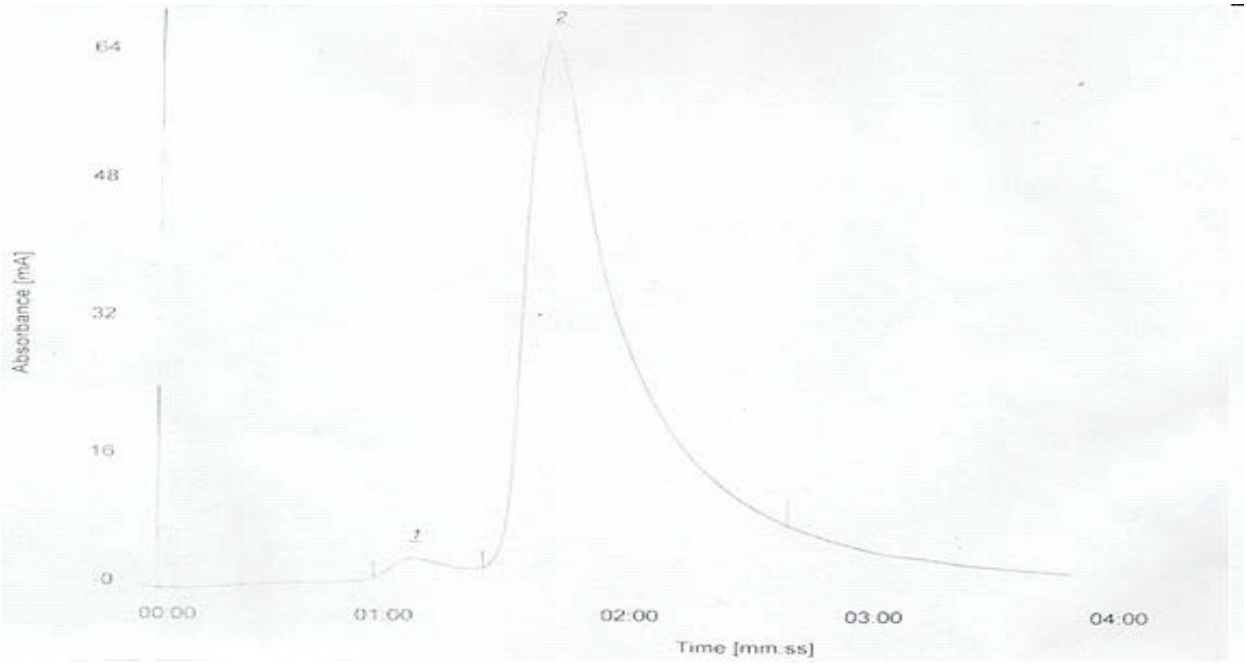

Fig. 7: High Performance Liquid Chromatography of *Aspergillus* strain A10

In conclusion, Strain improvement of *Aspergillus flavus* resulted in an increased ascorbic acid yields of 6.99 g/L and 7.28 g/L by the UV and EB treated mutant strains of *A. flavus* respectively at 40 °C, pH 5.0 and 100 rpm for 96 hours. The UV and EB treated mutant strains of *A. flavus* resulted in a significant increase of 78% and 80% ascorbic acid production over the parental strains. This shows the potential of strain improvement for increased ascorbic acid production which is of importance to the food and pharmaceutical industries.

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