Biosynthesis of Ascorbic Acid by *Aspergillus Flavus* and *Aspergillus Tamarii* Immobilized in *Afzelia Africana* Matrix.

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Abstract

A novel matrix for the immobilization of ascorbic acid produced by *Aspergillus flavus* and *Aspergillus tamarii* was reported. Spores of *A. flavus* and *A. tamarii* were immobilized on *Afzelia africana* matrix cross-linked with glutaraldehyde (2.5%) and the effects of *Afzelia africana* gel concentration (9–13%), spore load (100–500 mg/100 ml), bead size (2–7 mm) and bead number (2–10) on ascorbic acid yield were determined. The immobilized fungi were cultured in a liquid fermentation medium containing BSG (0.6% w/v) for ascorbic acid production for 144 h. The ascorbic acid produced was quantified titrimetrically. The statistical analysis of the effects of gel concentration, spore load and bead size on ascorbic acid production showed no significant difference at $p>0.05$. However, there was significant difference in the effect of bead number on ascorbic acid production at $p<0.05$. Ascorbic acid yield of 8.5 g/L and 7.5 g/L was produced by *Aspergillus tamarii* and *Aspergillus flavus* respectively using 9 beads at 96 h of fermentation. The immobilized *Aspergillus tamarii* and *Aspergillus flavus* retained activities of 72% and 70% respectively after five repeated cycle and also exhibited increased activities over the free cells. This study shows the potential of *Afzelia africana* as a novel matrix for enhanced ascorbic acid production.

Keywords: Ascorbic acid, Immobilization, *Afzelia Africana*, Matrix, *Aspergillus flavus*

1. Introduction

Ascorbic acid, vitamin C or L-ascorbate which is white to light-yellow in appearance is a water soluble sugar acid with antioxidant properties. The L-enantiomer of ascorbic acid is also known as vitamin C (Lupulescu, 1993). Ascorbic acid which is made internally by almost all organisms except human being presence of ascorbate is required for a range of essential metabolic reactions in all animals and plants (Higdon, 1996). Ascorbic acid is a dietary factor which must be present in the human diet to prevent scurvy (Li and Schellhorn, 2007). It is an antioxidant which protects the body against oxidative stress and is a cofactor in several vital enzymatic reactions (Brett, 2007).

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Microbial production of L-ascorbic acid has been established. Ascorbic acid precursors and end products are known to occur in a catalogue of microorganisms including recombinants. It has been shown that wild type of *Saccharomyces cerevisiae* cells accumulated intracellularly L-ascorbic acid when incubated with L-galactose, L-galactono-1, 4-Lactone, or L-gulono-1, 4-Lactone (Hancock *et al*., 2000). Microorganisms can be easily grown on an industrial scale. Although the production of L- ascorbic acid from microorganisms has been reported in the past, recent evidence proves that L-ascorbic analogues and not L-ascorbic acid are found (Hancock *et al*., 2000). In yeasts (*Candida and Saccharomyces* species), the production of erythroascorbic acid has been reported (Huh *et al.,* 1998).

The immobilization of enzyme on insoluble supports has been a topic of active research in industrial enzyme technology and is essential for their application to individual processes (Jaiswal and Prakash, 2011). For industrial application, the immobilized form of enzyme offers several advantages, including repeated use of the enzyme, ease of product separation, improvement of enzyme stability, and continuous operation in packed-bed reactors (Abdel-Naby *et al.,* 1999). Different polysaccharide hydrogel beads have been used as support for lipase entrapment immobilization. This includes agarose beads, alginate beads (Betigeri and Neau, 2002), chitosan beads (Alsarra *et al.,* 2004), k-carrageenan beads (Jegannathan *et al*., 2009) and nanogel beads (Sawada and Akiyoshi, 2010). Some of these matrices are expensive (Park and Chang, 2000). However, in Nigeria, some natural polymers such as *Afzelia africana* which is cheap, readily available and rich in carbohydrate (Which may contribute to its ability to form gel) has comparable hydrocolloid properties to conventional entrapment agents (Ejikeme *et al*., 2009).

Afzelia africana plants are largely cultivated in the Savannah, fringing forest and the drier parts of the forest regions of Africa. In Nigeria, *A. africana* is referred to as *kawo, APA, akpalata* and *gayoki* by the Hausa, Yoruba, Igbo and Fulani speaking people respectively. The tree is a widespread species with a broad rather open crown and massive branches (most readily recognized by the conspicuous hard blackish fruits), up to 30.5 m high and a girth up to 3 m. The seeds have waxy orange cup-like structure at their base and are used in Nigeria generally as soup thickening ingredient in much the same way as melon and *Irvingia gabonensis* seeds.

Whole cell and enzyme immobilization with *Detarium microcarpum* and *Irvingia gabonensis* had been reported (Kareem *et al*., 2012, 2014). *Afzelia africana* popularly called African oak is most widely distributed species in Africa. The plant is used in local medicine for general pain relief and digestive problems. Most legumes including *Afzelia africana* are underutilized except as soup thickener (Adebayo and Ojo, 2013). The use of *A. africana* in

whole cell immobilization has never been reported. Hence, this study investigated the potentials of *A. africana* in immobilization of *Aspergillus flavus* and *Aspergillus tamarii* and their subsequent usage 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, Nigeria. Microbiological media used in these experiments were: Saboraud Dextrose Agar, Potato Dextrose Broth (BDH Chemicals, UK). The Chemicals used are 2, 6 Dichlorophenol Indophenol dye (Sigma Chemical Ltd, U.S.A) Glacial acetic acid, Glutaraldehyde and Metaphosphoric acid were all analar grade obtained from BDH chemicals, UK. Glucose, Galactose and Lactose were obtained from Surechem Products, U.K. Others include ethanol and methanol, (BDH chemicals, UK). The major equipment used include Autoclave (Prestige medical series 2100, England), Hot box oven, incubator (Gallenkamp, size 1 England) and Weighing balance (Mettler Toledo PB 3002, Switzerland).

2.2 Pretreatment of *Afzelia africana* seeds.

The seeds of *A. africana* were removed from the coats mechanically by the use of sharp objects. The seeds were later washed with water and sun dried. The sun dried seeds were grounded with laboratory mortar and pestle after which it was milled into powdery form using a local milling machine. This was sieved through 1.0 mm sieve to obtain a fine powder which was kept in an airtight container until further use.

The powder sample of *A. africana* was defatted as described by Shivani *et al*. (2011) using a soxhlet apparatus. The extraction was carried out using hexane as the solvent for 6 h. The defatted sample of the *A. africana* was removed and oven dried at 105°C for 1 h. The difference between the initial and final weight of the sample was taken as the lipid content of the sample as shown below;

$$\text{Percentage of lipid} = W_1 - W_2 / W_1 \times 100/1\ \%$$

Where W_1 = Initial weight of the sample

W_2 = Final weight of the sample

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) at 40°C and pH 5. Ascorbic acid production was monitored at 12 h interval for

7 days. Quantitative assay of Ascorbic acid accumulated in the medium was carried out using the method of Association of Vitamin Chemists (1996).

2.4 Immobilization of *Aspergillus* spp and their mutant strains on *A. africana* matrix

This was carried out as described by Kareem *et al.* (2014). Defatted powder of *Afzelia africana* was cross-linked using glutaraldehyde (2.5%) v/v. Spores of *Aspergillus flavus* and *Aspergillus tamarii* ($2x10^9$ spore/ml) were mixed with 100 ml of cross-linked *Afzelia africana slurry* at 35°C under vigorous stirring. The slurry was made into spherical beads by dropping through a syringe into ethanolic formaldehyde for 24 h.

2.5 Optimization of Immobilization of *Aspergillus spp* and their mutant strains on *A. africana* matrix

2.5.1 Effect of *Afzelia africana* gel concentration on ascorbic acid production by Immobilized cells

The effect of gel concentration on ascorbic acid production was investigated. Various gel concentrations (9, 10, 11, 12 and 13%) were used and the ascorbic acid produced determined at 96 h of fermentation.

2.5.2 Effect of Spore Load on ascorbic acid production by immobilized cells

The effect of spore load on ascorbic acid production was studied at optimum gel concentration by weighing spores in the range 100–500 mg/100 ml. The spores were mixed with defatted powder activated with 2.5% (v/v) glutaraldehyde solution. The ascorbic acid produced was quantified at 96 h of fermentation.

2.5.3 Effect of Bead Size on ascorbic acid production by immobilized cells

At optimum gel concentration and spore load, the effect of bead sizes on ascorbic acid production was studied by varying the bead sizes in the range 2–7 mm. This was achieved by dropping gel through laboratory dropper of various diameter sizes and ascorbic acid produced determined at 96 h of fermentation.

2.5.4 Effect of Number of Beads on ascorbic acid production by immobilized cells.

The effect of the number of beads on ascorbic acid production was investigated by varying the number of beads in the range 2–10/100 ml. This was carried out at optimum gel concentration, spore load and bead sizes. The ascorbic acid produced was determined at 96 h of fermentation.

2.5.5 Effect of stabilizing agents on ascorbic acid production by immobilized cells.

At optimum gel concentration, spore load, bead size and bead numbers, stabilizing agents (ethanol and formaldehyde) were varied at different ratios (90:10, 70:30, 60:40, 50:50, 10:90, 40:60 and 30:70% v/v) of ethanol and formaldehyde respectively. The ascorbic acid produced was later quantified at 96 h of fermentation

2.5.6 Effect of Reusability on ascorbic acid production by immobilized cells.

The effect of reusability of the immobilized cells of *Aspergillus spp* on ascorbic acid production was investigated. At the end of each batch, the beads were washed in phosphate buffer (pH 7.0), dried at 40°C for 3 h and stored in a desiccator (to prevent rehydration) for the next batch fermentation. The ascorbic acid produced at the end of each batch was determined at 96 h of fermentation.

2.6 Cell leakage

Cell leakages from the gel matrix into the fermentation medium were determined with the use of haemocytometer. One milliliter of the fermentation medium was dropped on the haemocytometer and placed under the microscope. The number of spores were evaluated in Spores/ml.

2.7 Comparative yield of ascorbic acid by free and immobilized cells of Aspergillus spp in *Afzelia africana* matrix

A comparative study of ascorbic acid produced by the free and immobilized cells of *Aspergillus spp* in *A. africana* was carried out. Spores ($2x10^9$ spore/ml) of the free and immobilized cells of *Aspergillus flavus* and *Aspergillus tamarii* were inoculated on the brewery waste medium in a 250 ml flask. The ascorbic acid produced by the free and immobilized cells were determined and compared at 96 h of fermentation.

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 gel concentration, spore load, bead size and bead numbers on ascorbic acid yield were determined using ANOVA at 95% confidence interval with p value<0.05. Significance of **c**omparative yield of ascorbic acid by free and immobilized cells of *Aspergillus* spp in *Afzelia africana* matrix was performed using Pair T-test taking p<0.05.

3. Results and Discussion

3.1 Production of ascorbic acid by *Aspergillus flavus* and *A. 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 yield of 7.25 g/L and 6.25 g/L was produced by *Aspergillus tamarii* and *Aspergillus flavus* at 96 h of fermentation. Thus, 96h was adopted as the optimum fermentation time for the immobilization studies. The yield of ascorbic acid reduced with increase in fermentation time for the two isolates (Figure 1). However, at 108 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 is in correlation with the report of Ajibola *et al.* (2009) that ascorbic acid is destroyed by oxidation especially at temperature above 0°C.

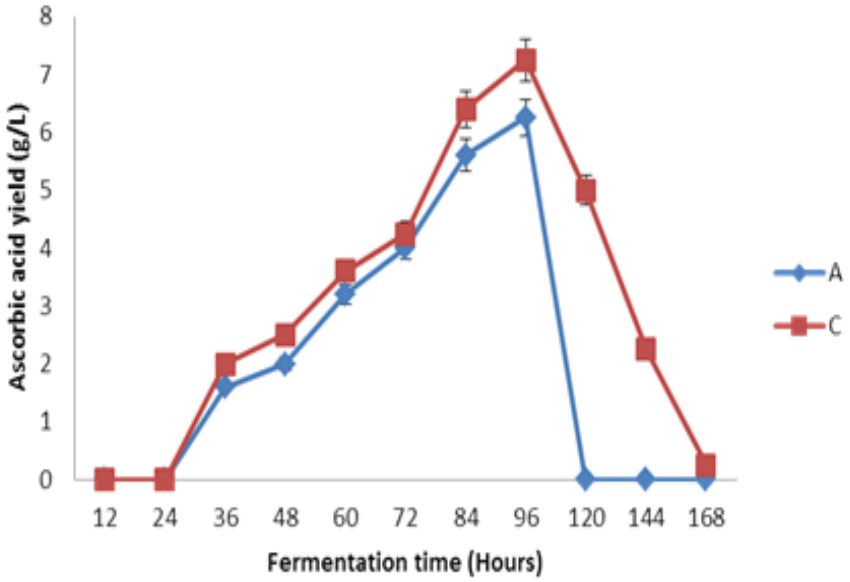


Figure 1 Production of ascorbic acid by *Aspergillus flavus* (A) and *Aspergillus tamarii* (C)

3.2 Effect of *Afzelia africana* gel concentration on ascorbic acid production

The Effect of gel concentration on ascorbic acid yield by *Aspergillus tamarii* and *Aspergillus flavus* showed no significant difference (p>0.05). Optimum ascorbic acid yield was achieved with gel concentration of 10% as shown in Figure 2. Ascorbic acid yield of 7.10 g/L and 7.0 g/L was produced by *Aspergillus tamarii* and *Aspergillus flavus* respectively. Further increase in gel concentration resulted in decrease in ascorbic acid yield. Increase in gel concentration reduces the pore size of the bead which interfered with the entry of substrate into the bead, thus leading to decreased ascorbic acid production. Whereas at lower gel concentration (9%), the beads were unstable and fragile which led to poor immobilization. This may be due to larger pore size of the beads and consequently leakage of cells from the beads will increase (Talekar and Chavare, 2012).

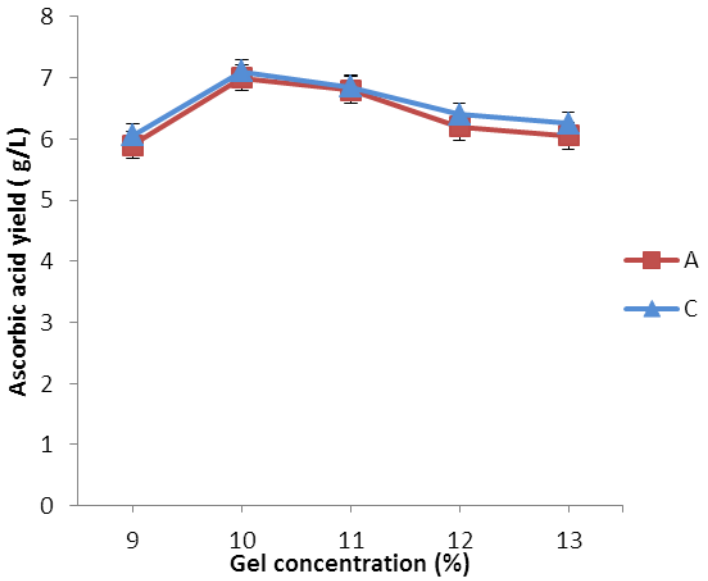


Figure 2 Effect of *Afzelia africana* gel concentration on ascorbic acid production by immobilized *Aspergillus flavus* (A) and *Aspergillus tamarii* (C) (F=0.233, P=0.642).

3.3 Effect of Spore Load on ascorbic acid production

The effect of spore load on ascorbic acid production was not significant as maximum ascorbic acid yield of 7.5 g/L and 8.5 g/L was produced by *Aspergillus flavus* and *Aspergillus tamarii* respectively with a spore load of 300 mg (p>0.05) (Figure 3). However, increase in spore load above 300 mg resulted in decreased ascorbic acid yield. This may be attributed to a decrease in mechanical strength of gel particles as cell loading increases (Dong *et al.,* 2006)

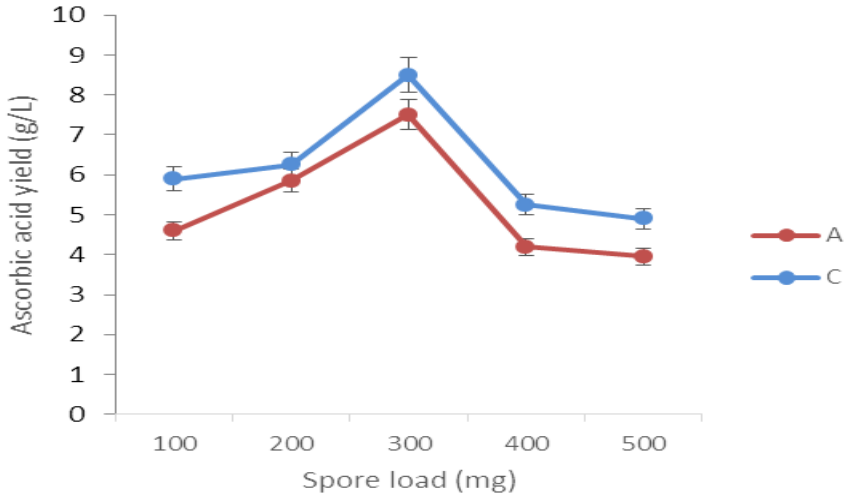


Figure 3 Effect of spore load on ascorbic acid production by immobilized *Aspergillus flavus* (A) and *Aspergillus tamarii* (C) (F=1.065, P=0.332).

3.4 Effect of Bead Size on ascorbic acid production

The Effect of bead size on ascorbic acid yield by *Aspergillus tamarii* and *Aspergillus flavus* showed no significant difference at $p>0.05$. Increase in bead size resulted in increased ascorbic acid with optimum ascorbic acid yield of 8.10 g/L by *A. tamarii* and 7.05 g/L by *A. flavus* at a bead size of 3.0 mm (Figure 4). An Increase in the bead size to 7.0 mm resulted in an ascorbic acid yield of 6.35 g/L and 5.20 g/L by *A. tamarii* and *A. flavus* respectively. This is due to the fact that at lower bead size, the surface area of the bead is increased, the gel matrix is thinner, which makes the fungal mycelia in the gel cavities more accessible to substrate than at higher bead size (Ahmad and Sardar, 2015; Kareem *et al.*, 2012).

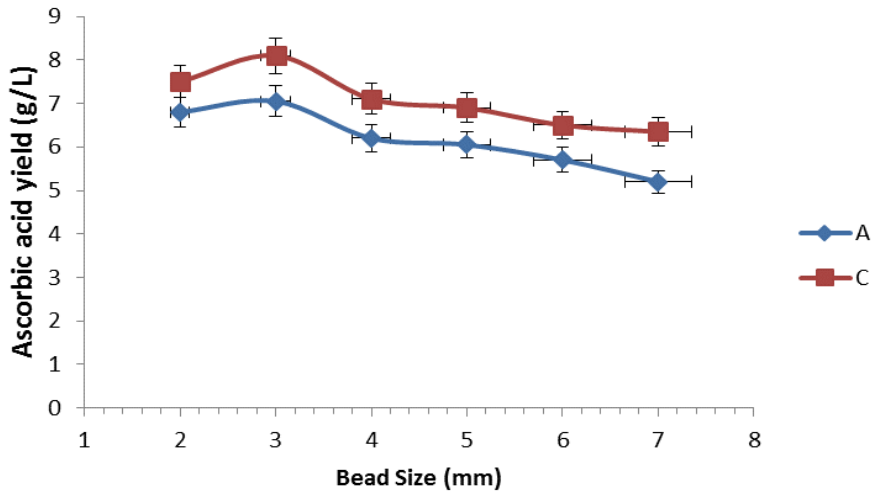


Figure 4 Effect of bead size in ascorbic acid production by immobilized *Aspergillus flavus* (A) and *Aspergillus tamarii* (C) (F=1.212, P=0.303)

3.5 Effect of Number of Beads on ascorbic acid production

The effect of the number of beads on ascorbic acid production by the *Aspergillus* spp showed a significant difference at $p<0.05$. Increase in the number of beads resulted in an increased ascorbic acid production (Figure 5). Optimum ascorbic acid yield of 8.15 g/L produced by *Aspergillus tamarii* was achieved with 9 beads. Whereas, *Aspergillus flavus* gave a reduced ascorbic acid yield of 7.25 g/L. However, further increase in bead number did not result into increased ascorbic acid production with further increase in bead number, activity became asymptotic. This could be attributed to the fact that, when the number of beads increases, the nutrient/bead ratio decreases, which may become limiting (Beshay, 2003)

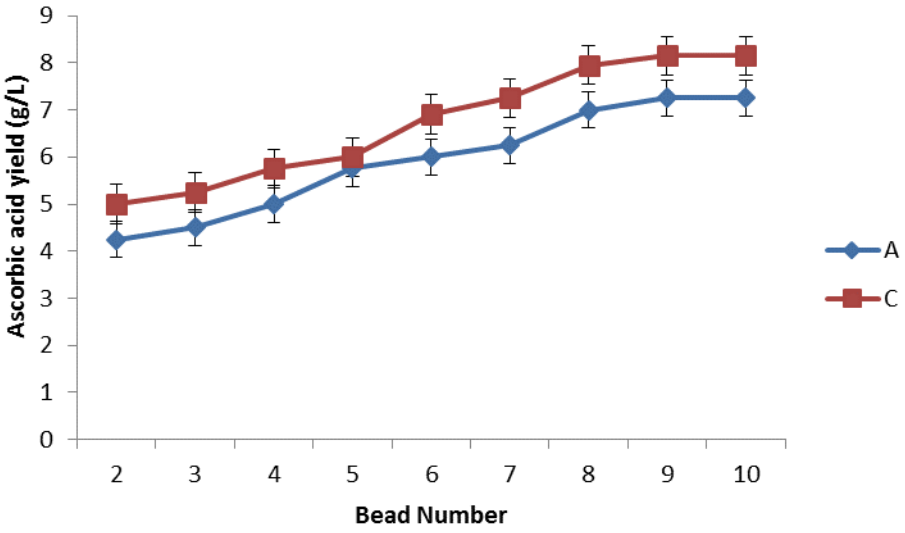
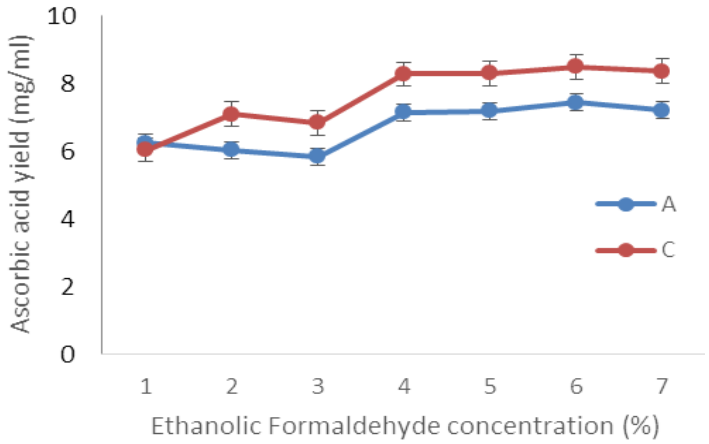


Figure 5 Effect of bead number on ascorbic acid production by immobilized *Aspergillus flavus* (A) and *Aspergillus tamarii* (C) (F=7.008, P=0.029)

3.6 Effect of stabilizing agents on ascorbic acid production

The effect of various concentrations of stabilizing agents (ethanol and formaldehyde) on ascorbic acid production showed that 8.5 g/L and 7.45 g/L of ascorbic acid was produced by *Aspergillus tamarii* and *Aspergillus flavus* respectively at 40:60 v/v of ethanol and formaldehyde (Figure 6). There was no significant difference in the effect of the stabilizing agents on the ascorbic acid yields at $p>0.005$. Formaldehyde enhances stability of the carbohydrate matrix and eventually overcomes the tendency to agglomerate or form a gel in aqueous solutions (Kareem *et al.,* 2012).



1=90:10, 2=70:30, 3=60:40, 4=50:50, 5=10:90, 6=40:60 and 7=30:70 of Ethanol:Formaldahyde

Figure 6 Effect of stabilizing agents (Ethanol: Formaldehyde) on ascorbic acid production by immobilized *Aspergillus flavus* (A) and *Aspergillus tamarii* (C) (F=1.363, P=0.277).

3.7 Effect of Beads Reusability on ascorbic acid production

One of the main advantages of immobilization is the ease of separation and reusability. The effect of reusability of beads on ascorbic acid production was not significantly different (p<0.05) with 72% and 70% relative activities retained by *Aspergillus tamarii* and *Aspergillus flavus* respectively after 5 repeated uses (Table 1). However, there was a decline in ascorbic acid yield with further use of the immobilized cells due to cell leakage. This was also reported by Elnashar (2010). The loss of activity upon reuse could be due to weakening in the strength of binding between the matrix and enzyme on repeated use and hence the enzyme might leach out from the matrix, therefore resulting in loss of activity (Jaiswal and Prakash, 2011).

Table 1 Effect of bead reusability on ascorbic acid production by immobilized *Aspergillus flavus* (A) and *Aspergillus tamarii* (C)

Bead Reuse	*Aspergillus flavus*	*Aspergillus tamarii*
1	100	100
2	90	90
3	85	87
4	80	82
5	70	72
6	65	65
7	50	50

Note: F=2.619, P=0.157 (p<0.05)

3.8 Cell leakage efficiency of *Afzelia africana*

Cell leakage efficiency of *Afzelia africana* matrix for the immobilization of *Aspergillus flavus* and *Aspergillus tamarii* was investigated (Table 2). The cell leakage value of 24 spores/ml was given by *Aspergillus flavus* while that of *Aspergillus tamarii* was 22 spores/ml at the end of the 7th cycle of batch fermentation. There was no significant difference in the leaked cells on ascorbic acid production (p<0.05). There was a sharp decline in ascorbic acid production after each cycle of fermentation. This might be due to increase in the cell leakage hence, there is an inverse relationship between cell leakage and ascorbic acid production.

Table 2 Cell leakage efficiency of *Afzelia africana* matrix for immobilization of *Aspergillus flavus* (A) and *Aspergillus tamarii* (C)

Cell leakage Spores/ ml	*Aspergillus flavus*	*Aspergillus tamarii*
1	0	0
2	4	4
3	7	6
4	10	8
5	15	12
6	16	16
7	24	22

Note: F=2.911, P=0.139 (p<0.05)

3.9 Comparative yield of ascorbic acid by free and immobilized cells of *Aspergillus* spp in *Afzelia africana* matrix

Comparative studies on ascorbic acid yield by free and immobilized cells of *Aspergillus* spp revealed that immobilized cells of *Aspergillus tamarii* and *Aspergillus flavus* gave an improved ascorbic acid yield of 8.5 g/L and 7.5 g/L while the free cells gave a lower yield of 7.25 g/L and 6.25 g/L respectively. There was a significant difference in the ascorbic acid yield of the immobilized and free cells (p<0.05). When free cells are compared with immobilized cells, the productivity obtained in the latter is considerably higher, due to high cell density and immobilization-induced cellular or genetic modifications (Ivanova *et al.,* 2011). Bayraktar and Mehmetoglu (2000) reported that immobilized cells offer several advantages over free cells such as decreased medium viscosity and enhanced oxygen and nutrient transfer, higher productivity, operational stability and decreased contamination of the product by free cells.

4. Conclusion

The present study showed that immobilized cells of *Aspergillus flavus* and *Aspergillus tamarii* produced a higher yield of ascorbic acid compared to the free cells. Furthermore, the immobilized *Aspergillus tamarii* and *Aspergillus flavus* retained activities of 72% and 70% respectively after 5 repeated uses compared to 60% reported in literature for Calcium alginate. This shows the potential of *Afzelia africana* in enhanced ascorbic acid production.

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