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Amylase production by *Aspergillus flavus* immobilized in polysaccharide beads of *Adansonia digitata*.

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ABSTRACT: A novel matrix for the immobilization of amylase produced by *Aspergillus flavus* was exploited in this study. Spores of *A. flavus* were immobilized on *Adansonia digitata* matrix cross-linked with glutaraldehyde (2.5 %) and the effects of gel concentration (9 – 13 %), spore load (100 – 500 mg), bead size (2 - 7 mm) and bead number (2 - 10) on amylase activity were determined. Optimum amylase activity of 280 U/ml was obtained under batch fermentation at 10 % gel concentration, 280 mg spore load with 9 beads of 3.0 mm bead size at 96 h of fermentation compared to 190 U/ml by the free cells. The immobilized *A. flavus* retained amylase activity of 250 U/ml after four repeated cycle and also exhibited increased activities over the free cells. This study shows the potential of *Adansonia digitata* as a novel matrix for increased amylase production.

Keywords Amylase; Immobilization; *Adansonia digitata*; *Aspergillus flavus.*

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Introduction

Recent discoveries on the potentials of using microorganisms as biotechnological source of industrially relevant enzymes have led to increased search for microorganisms with enzymes of industrial relevance (1). Amylases are hydrolytic enzymes employed in the processing industries for the hydrolysis of starch into simpler sugar constituents (2). This group of enzymes has a wide application in the brewing, textile, detergent and pharmaceutical industries. In recent times, the application of α-amylase in the field of laundry and dish washing detergents is on the increase (3).

Several attempts have been made to optimize the culture conditions of different strains of fungi because the amylase of fungal origin was found to be more stable than the bacterial enzymes on a commercial scale (4). Moreover, many fungi had been found to be good sources of amylolytic enzymes. Studies have been reported on the production of amylase by *Rhizopus sp*. and *Aspergillus niger* (5). Also, a high amylolytic activity in biomass production has been reported by Ikenebomeh and Chikwendu (6).

Microbial cells and enzymes immobilized by entrapment in polymeric matrices are currently receiving attention as industrial biocatalysts. 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 (7).The most frequently used matrices include agar, agarose, alginate, carrageenan and polyacrylamide (8). Some of these matrices are expensive and also have weak mechanical strength (9). However, in Nigeria, there are some

underutilized natural polymers such as *Adansonia digitata* with hydrocolloid properties comparable with conventional entrapment agents.

Adansonia digitata belongs to the family Bombacaceae and is predominant in the semi-arid and arid zones of Africa. The seed is underutilized, non-toxic, cheap, readily available and biodegradable key economic species used daily in the diet of rural communities in West Africa with various important medicinal and food uses (10). Whole cell and enzyme immobilization with *Afzelia africana*, *Detarium microcarpum* and *Irvingia gabonensis* had been reported (11-13). However, there are no known reports on immobilization of amylase with *A. digitata*, hence, the need for this study. The aim of this study therefore is to use a novel matrix, *Adansonia digitata* for the immobilization of amylase produced by *Aspergillus flavus.*

Materials and Methods

Fungal source

Pure strains of *Aspergillus flavus* 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.

Pretreatment of *Adansonia digitata* seeds.

Dehulling of A. digitata 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.

Milling of A. digitata seeds

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.

Removal of Lipids from A. digitata seeds

The powder sample of *A. digitata* was defatted as described by Shivani *et al*. (14) using a soxhlet apparatus. The extraction was carried out using hexane as the solvent for 6 h. The defatted sample of the *A. digitata* was removed and oven dried at 105 °C for 1 hour. The difference between the initial and final weight of the sample was taken as the lipid content of the sample as shown below;

Percentage of lipid = $W_1 - W_2 / W_1 = W_3 / W_1 \times 100/1$ %

Where W_1 = Initial weight of the sample
W_2 = Final weight of the sample
W_3 = Difference between the initial and final weight

Screening of amylase positive molds

This was carried out by using the method of Kareem *et al*. (15). The plate medium is made up of nutrient agar containing 1 % Starch (Starch-Agar). The medium was autoclaved at 121°C for 15mins at pH 4.5. The plates were inoculated with pure culture of *Aspergillus flavus* and incubated at 30° C for 72 h. The plates were flooded with grams iodine solution to confirm the formation of clear zones around the mould.

Amylase production

This was carried out using the medium of Osho *et al*. (2001). The production medium was adjusted to pH 4.5 before autoclaving at 121°C for 15 min. Spores of *Aspergillus flavus* (2×10^9 spore/ml) were inoculated on the sterilized medium and incubated at 30 °C for 120 h.

Determination of amylase activity

Crude amylase extract (0.5 ml) from *Aspergillus flavus* was added to 1.5 ml of the 2% gelatinized starch and incubated at 65°C for 10 min. The reaction was stopped with 0.5 ml of NaOH and HCl. The Solution was made up to 10ml out of which 0.5 ml of aliquot was added to 0.5 ml of iodine solution. This was also made up to 10 ml and the absorbance was measured at 470 nm colorimetrically using the method of Harahito *et al*. (16)

Immobilization of *Aspergillus flavus* and *Aspergillus tamarii* on *A. digitata* matrix

This was carried out as described by Kareem *et al*. (13). Defatted powder of *A. digitata* was cross-linked using glutaraldehyde (2.5 %) v/v. Spores of *Aspergillus flavus* were mixed with 100 mls of cross-linked *A. digitata* 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.

Properties of free and immobilized amylase produced

Effect of fermentation time on amylase production.

The effect incubation time on amylase production was studied by assaying for the amount of amylase produced at 24, 48, 72, 96 and 120 h of incubation respectively as previously described.

Effect of temperature on amylase production

The effect of temperature on the quantity of amylase produced was studied at different temperatures (30, 35, 40 and 45 °C). The amylase activity was determined as previously described.

Effect of pH on amylase production

The effect of pH on amylase 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 the optimum temperature. The amylase activity was determined as previously described.

Optimization of Immobilization of *Aspergillus flavus* on *A. digitata* matrix

Effect of gel concentration on amylase production by immobilized cells

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

Effect of spore load on amylase production by immobilized cells

The effect of spore load on amylase production was studied at optimum gel concentration by weighing spores in the range 100 – 500 mg. The spores were mixed with defatted powder activated with 2.5 % (v/v) glutaraldehyde solution. Beads were formed and the amylase activity determined at 96 h of fermentation.

Effect of bead size on amylase production by immobilized cells

At optimum gel concentration and spore load, the effect of bead sizes on amylase 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 amylase activity determined at 96 h of fermentation.

Effect of number of beads on amylase production by immobilized cells.

The effect of the number of beads on amylase production was investigated by varying the number of beads in the range 2–10. This was carried out at optimum gel concentration and bead sizes. The amylase activity was determined at 96 h of fermentation.

Effect of reusability on amylase production by immobilized cells.

The effect of reusability of the immobilized cells of *Aspergillus flavus* on amylase production was investigated. At the end of each batch, the beads were washed in phosphate buffer (pH 7.0),

dried, and used for the next batch. The amylase activity at the end of each batch was determined at 96 h of fermentation.

Comparative yield of amylase produced by free and immobilized cells of *Aspergillus flavus* in *Adansonia digitata* matrix

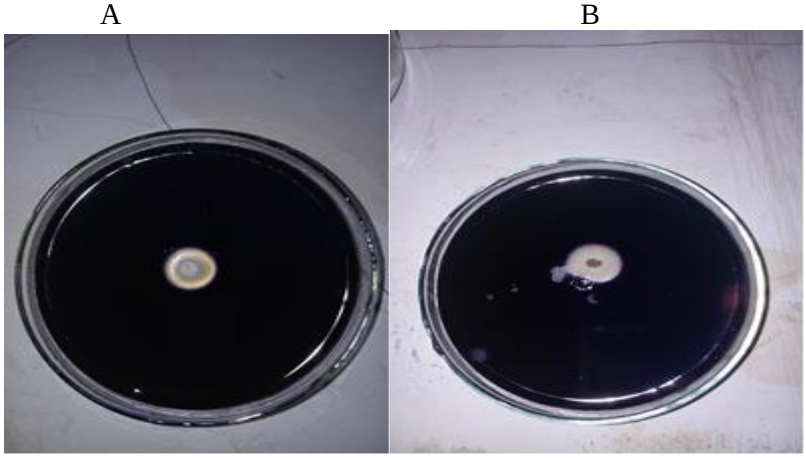
A comparative study of amylase produced by the free and immobilized cells of *Aspergillus flavus* in *A. digitata* was carried out. The amylase activity by the free and immobilized cells was determined and compared at 96 h of fermentation.

Results and Discussion

Screening of amylase positive molds

The zone of clearance exhibited around colonies of the *Aspergillus flavus* (A) showed their amylolytic potentials. The non-amylase producing mould (B) gave no zone of clearance around its colony because amylase was not produced (Plate 1). The clear zones around colony of the mould on Starch-Agar showed that it is an amylase producer able to degrade the starch in the medium around. This showed the mould to be an amylolytic microorganism. The zone of clearance increases as a function of increased amylase production by the mould.

A B



A = Amylase positive mould B= Non-amylase producing mould

Plate 1: Amylase and Non-amylase producing moulds

Effect of incubation time on amylase production by *Aspergillus flavus*

Studies on the effect of incubation time on amylase production showed that amylase yield peaked at 96 h of fermentation. Amylase yields of 170 U/ml and 180 U/ml were produced by the free and immobilized cells of *Aspergillus flavus* respectively. Thus, 96 h was adopted as the optimum incubation time for further studies. Amylase production reduced with increase in incubation time for the free and immobilized cells (Table 1). This is in agreement with the works of Osho *et al*. (2001) who reported an optimum incubation time of 96 h for amylase production by *Aspergillus oryzae* immobilized in gelatin matrix. Fadahunsi and Garuba (17) reported that the decrease in amylase production beyond the optimum incubation time could be attributed to catabolite repression caused by glucose released into the medium. It could also be as a result of the secretion of proteolytic proteins which are known to cause the denaturation of proteins (18).

Effect of pH on amylase production

The effect of pH on amylase production showed that optimum amylase yields of 170 U/ml and 175 U/ml were produced at pH 6 and 4 by the free and immobilized cells of *A. flavus* respectively.

Thereafter, an increase in pH led to a decrease amylase production (Fig. 1). This variation in amylase production as pH of the medium varies could be as a result of the various morphological changes induced in microbes due to the changing pH. The optimum pH of the free amylase was pH 6, while that of immobilized enzyme tends towards acidic medium with optimum pH 4 (Figure 1). This may be a result of the micro environmental pH of the polysaccharide hydrogel support. This result correlates with the findings of Fadahunsi and Garuba (17) who reported an optimum pH of 6 for amylase production by *Aspergillus flavus.* Similarly, Sasi *et al.* (19) reported that the maximum amylase production by a strain of *Aspergillus* spp was at pH 6.

Table 1: Effects of incubation time on amylase production by *Aspergillus flavus.*

Isolates	**Fermentation time (Hours)**									
	12	**24**	**36**	**48**	**60**	**72**	**84**	**96**	**108**	**120**
Free cells (U/ml)	**0**	**40**	**60**	**90**	**100**	**120**	**150**	**170**	**140**	**100**
Immobilized cells (U/ml)	**0**	**60**	**90**	**110**	**125**	**140**	**165**	**180**	**160**	**110**

Effect of temperature on amylase production

The effect of temperature on amylase production showed that optimal amylase activities of 180 U/ml and 190 U/ml were produced by the free and immobilized cells of *Aspergillus flavus* respectively at 40 ºC (Fig. 2). However, as the temperature was increased to 40 ºC, amylase activities by the free and immobilized cells reduced to 140 U/ml and 160 U/ml respectively. Reduced amylase activity at higher temperature above the optimum may be as a result of the inactivation of amylase enzyme or suppression of cell viability. However, low amylase activity observed at low temperature values may be as a result of reduction in the metabolic activities of the microorganism and consequently, the amylase production (20)

Effect of Gel concentration on amylase production

Optimum amylase production of 240 U/ml was achieved with gel concentration of 12 % as shown in Figure 3. Further increase in gel concentration resulted in decrease in amylase production. 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 amylase production. Whereas at lower gel concentration, the beads were unstable and fragile which resulted in poor immobilization and reduced amylase production. This may be due to larger pore size of the beads and consequently leakage of enzyme from the beads will increase (21).

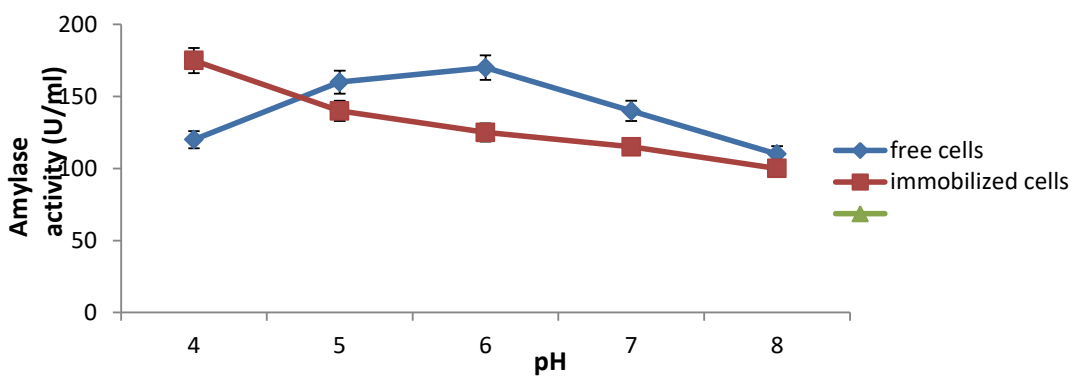


Fig 1: Effect of pH on amylase production by free cells of *Aspergillus flavus*

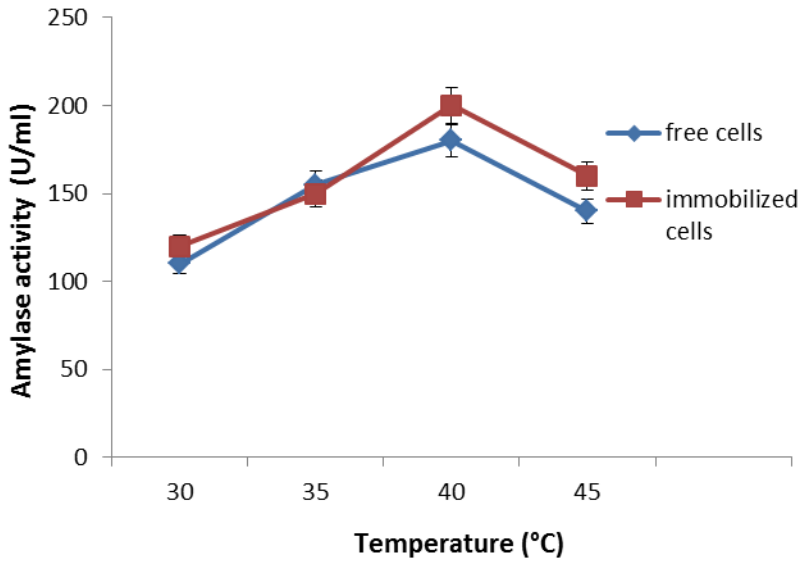


Fig 2: Effect of temperature on amylase production by free cells of *Aspergillus flavus*

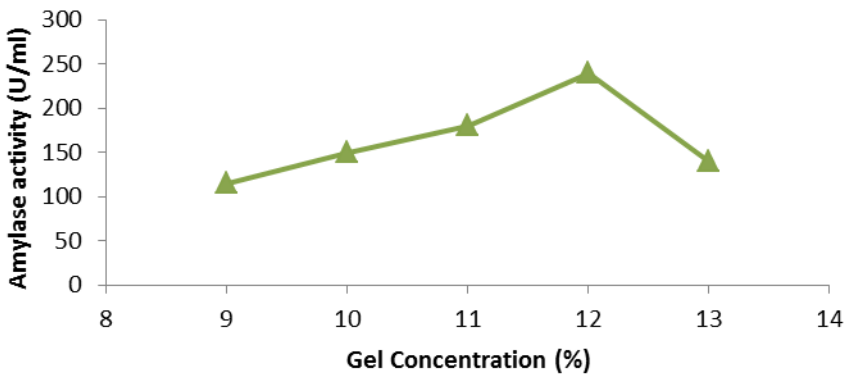


Fig 3: Effect of gel concentration on amylase production by *Aspergillus flavus*

Effect of spore load on amylase production by immobilized cells

The influence of spore load on amylase production was investigated and presented in figure 4. Amylase activity of 250 U/ml was produced at optimum spore load of 300 mg. However, higher concentration of spores did not lead to increased amylase production. At spore load of 500 mg, amylase activity reduced to 170 U/ml. It has been reported that the mechanical strength of gel particles decreases with increasing cell loading and may be accountable for this decrease in amylase production (22).

Effect of Bead Size on ascorbic acid production

The significance of bead size in amylase production was also studied as shown in Figure 5. Optimum amylase activity of 260 U/ml was produced at bead size of 3 mm. An Increase in the bead size to 7.0 mm resulted in amylase activity of 90 U/ml. This is due to the fact that at lower bead size, the gel matrix is thinner, which makes the fungal mycelia in the gel cavities more accessible to substrate than at higher bead size (8).

Effect of Number of Beads on amylase production

The effect of the number of beads on amylase production showed that amylase production increased with increase in the number of beads (Fig. 5). Optimum amylase activity of 280 U/ml was achieved with 9 beads. However, further increase in bead number did not result into increased

amylase activity. Amylase activity became asymptotic above the optimum number of beads. This agrees with Kareem *et al*. (12) who reported that as reaction rate was maximum at 15 beads, further increase in bead number will achieve the same rate.

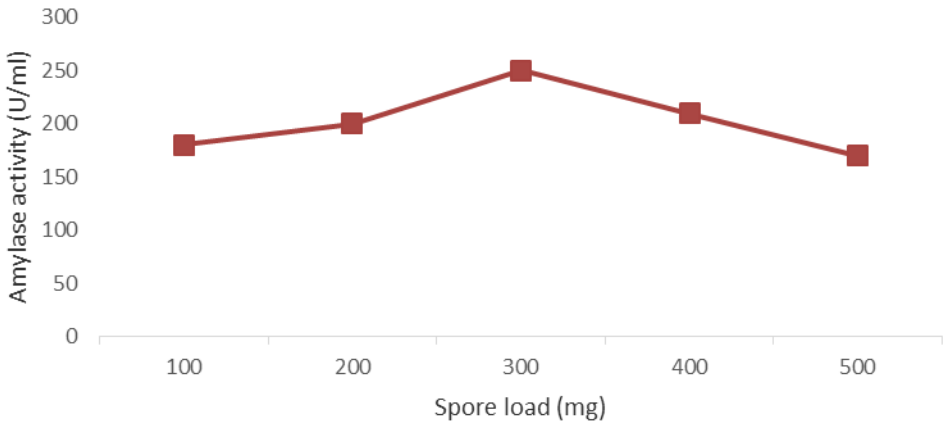


Fig 4: Effect of spore load on amylase production by *Aspergillus flavus*

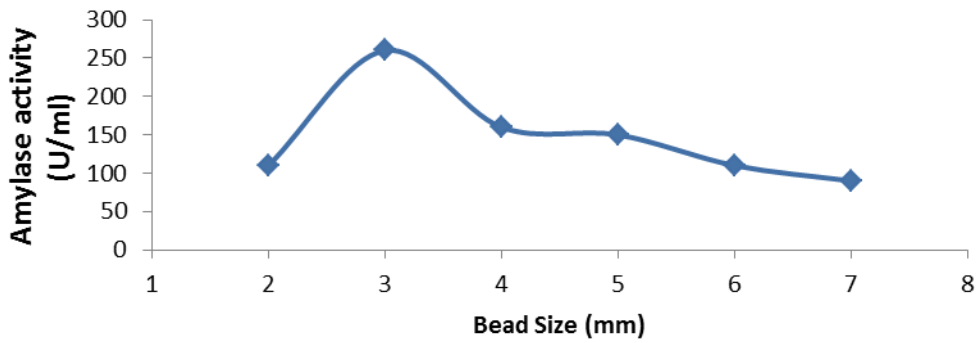


Fig 5: Effect of bead size on amylase production by *Aspergillus flavus*

Effect of Beads Reusability on amylase production

One of the main advantages of immobilization is the ease of separation and reusability. The effect of reusability of beads on amylase activity showed that 250 U/ml of enzyme activity was produced after 4 repeated uses (Fig. 7). However, there was a decline in amylase activity with further use of the immobilized cells. The decrease in enzyme activity could be due to the leakage of enzyme from the beads, occurring due to the weakening of the binding strength between the beads and the enzyme (23). Similarly, Osho *et al.* (8) reported that the low yield of amylase in sequential batch fermentations could be due to low stability of the gel beads.

Comparative amylase production by free and immobilized cells of *Aspergillus flavus* in *Adansonia digitata* matrix

Comparative studies on amylase production by the free and immobilized cells of *Aspergillus flavus* revealed that immobilized cells of *A. flavus* gave an improved amylase activity of 280 U/ml while the free cells gave a lower activity of 190 U/ml (Fig. 8). 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 (24).

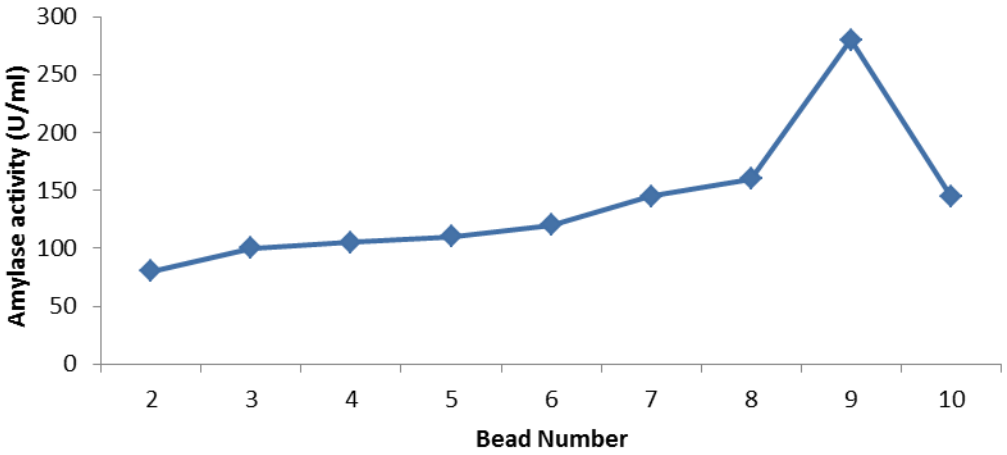


Fig 6: Effect of bead number on amylase production by *Aspergillus flavus*

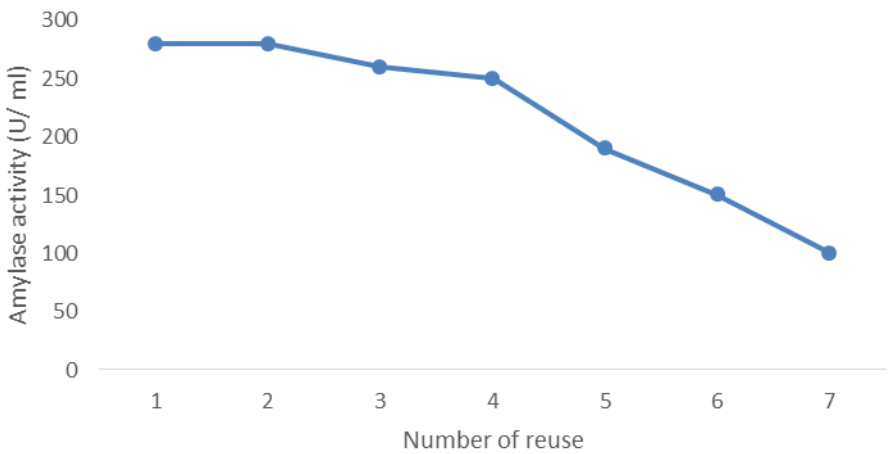


Fig 7: Effect of bead reusability on amylase production by *Aspergillus flavus*

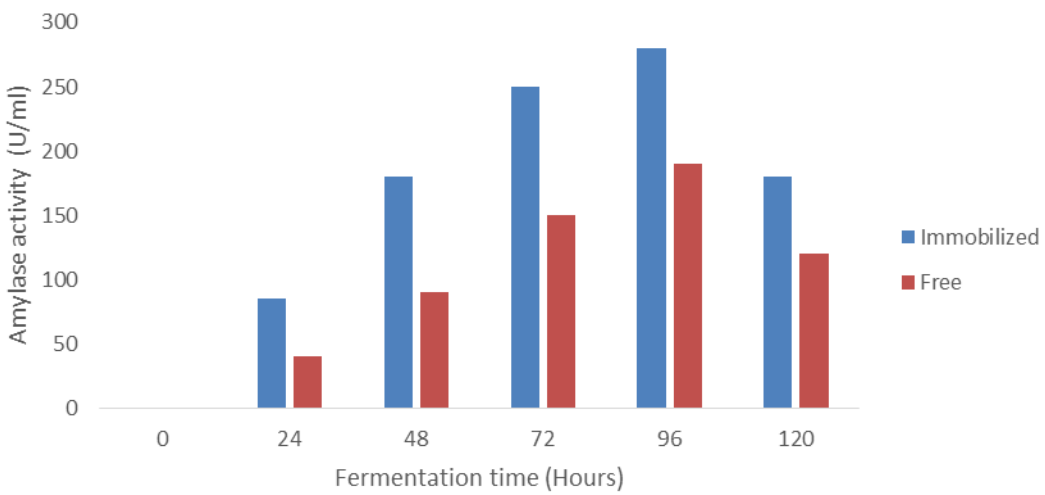


Fig. 8: Comparative amylase production by free and immobilized cells of *Aspergillus flavus* in *Adansonia digitata* matrix

Conclusion

The present study showed that immobilized cells of *Aspergillus flavus* produced a higher yield of amylase compared to the free cells. Furthermore, the immobilized *A. flavus* retained 250 U/ml of enzyme activity after 4 repeated uses. This shows the potential of *Adansonia digitata* as matrix for enhanced amylase production.

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