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Simultaneous Production of Glucose and Ascorbic Acid from Cassava Starch by Mixed Culture of *Aspergillus Flavus* and *Aspergillus Tamarii*

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Abstract: The study evaluated simultaneous production of glucose and ascorbic acid from cassava starch by fungal amylases of *Aspergillus flavus* and *Aspergillus tamarii* grown on rice bran solid medium at 30°C for 72 h. Partially purified amylase of *Aspergillus flavus* (A), *Aspergillus tamarii* (C), and partially purified amylase extracted from the mixed cultures of *A. flavus* and *A. tamarii* (B), hydrolyzed cooked cassava starch to glucose optimally at 70°C, pH 6.0 with 90%, 70% and 85% conversion efficiency respectively. On the contrary, bioconversion of cassava starch by enzyme extracts of the three cultures A, B, and C to ascorbic acid occurred mainly at 65°C, pH 4.5. Enzyme extracts of A and C converted cooked cassava starch to ascorbic acid in a two-step reaction at 65°C, pH 4.5 after 6 h of fermentation to yield 15 mg/ml and 25 mg/ml ascorbic acid respectively. The synergy between *Aspergillus flavus* and *Aspergillus tamarii* in the mixed culture resulted in an increased ascorbic acid production of 43 mg/ml at 6 h. Glucose production was inversely proportional to ascorbic acid production indicating a direct conversion of glucose to ascorbic acid by enzyme extracts of *Aspergillus flavus*, *Aspergillus tamarii* and mixed cultures of *A. flavus* and *A. tamarii*. This shows that enzyme of the mixed cultures contain enzyme complex that can be useful in the direct conversion of cassava starch to ascorbic acid.

Keywords: Amylase; Ascorbic acid; Cassava Starch; Glucose; Mixed cultures.

Introduction

Ascorbic acid also known as Vitamin C is a dietary factor which must be present in the human diet to prevent scurvy; as a nutritional supplement, color fixing agent, flavoring and preservative in meats and other foods, antioxidant in bread dough, abscission of citrus fruits in harvesting and as a reducing agent in analytical chemistry (Higdon, 2006). Additionally, for use as a dietary supplement, ascorbic acid can be isolated from natural sources or synthesized chemically by the oxidation of L-sorbose a variation of the Reichstein process (Porro and Sauer, 2003).

The first chemical synthesis of ascorbic acid from L-xylosone was achieved in 1933 (Reichstein *et al.*, 1933). The process of converting glucose to ascorbic acid on a commercial scale is done by the Reichstein process. It includes seven steps, one of which is fermentation. Although, this is the classical method of Vitamin C production in industry, it requires the use of hazardous chemicals and high energy steps (Hancock and Viola, 2002). However, another possible approach for the synthesis of ascorbic acid is the production of L-ascorbic acid from microorganisms. 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 *S. 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 (Huh, 1998; Hancock *et al.* 2000). In yeasts (*Candida* and *Saccharomyces* species), the production of erythroascorbic acid has been reported (Huh *et al.*, 1998). In such yeasts, a physiological pathway has been proposed proceeding from D-glucose via D-arabinose and D-arabinono-1, 4-lactone to erythroascorbic acid (Kim *et al.*, 1996). Mixed cultures of microorganisms have been employed in the production of 2-keto-L-gulonic acid (2-KLGA) a key intermediate of ascorbic acid (Xu *et al.*, 2004). Mixed cultures of different microorganisms such as *Pseudomonas striata*, *Gluconobacteroxydans* and *Corynebacterium* sp. were used for the direct production of 2-KLGA from D-gluconate (Zinsheng *et al.*, 1981).

Recent attempts to reduce the complex steps and improve the overall yields involve the bioconversion of L-galactonic acid to ascorbic acid by *Candida* yeast. However, all these technological modifications could not improve on the efficiency.

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Abundance of by-product in the medium, difficulty of substrate and the complexity of the operation renders the process commercially unviable. Therefore the discovery of a two-step, cost effective and relatively simple method for the production of ascorbic acid is the objective of this study. Hence, this study reports the development of a new method for the production of ascorbic acid directly from cassava starch using mixed fungal cultures.

Materials and Methods

Amylase production by monocultures and mixed cultures using Solid State Fermentation

This was carried out by using the method of Akpan and Adelaja (2004). The medium is made up of Rice bran, Soybean flour and Starch formulated in the ratio 10:3:1 w/w respectively. The mixture in a petri dish was moistened with distilled water to 55% moisture content and sterilized at 121°C for 15 min. The sterilized medium was inoculated with 1 ml of spore inoculums in the following combination; 1:1(a), 1:2(b), 1:3(c), 1:4(d), 1:5(e), 2:1(f), 3:1(g), 4:1(h), 5:1(i) of the two moulds. These were incubated at 30°C for 72 h.

Crude Enzyme recovery from mouldy bran

The crude enzyme was recovered from the moldy bran using 0.1M Acetate buffer pH 4.5 for the mixed culture and pH 6.0 for the monocultures. The moldy bran was weighed and dissolved in the acetate buffer in the ratio 1:10 after which it was stored at 4°C for 2h with intermittent shaking. The supernatant which was decanted and filtered was used as the crude enzyme source. The enzyme activity was determined using the method of Harahito *et al.* (1992). One unit of enzyme activity was defined as the amount of enzyme which liberated from soluble starch 1 µmol of maltose per minute under assay conditions

Hydrolysis of starch by mixed and monocultures of *Aspergillus spp*

Enzyme extracts from the single and mixed culture were used in the hydrolysis of various starches (cassava, corn, potato, rice, millet). 0.5 ml of the crude enzyme extracts of the monocultures and mixed cultures of *Aspergillus flavus* and *A. tamarii* were

separation of the products from 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.* (1992).

Ascorbic acid and glucose production by mixed and monocultures of *Aspergillus spp*

Cassava starch was hydrolyzed by monoculture and mixed cultures of moulds of the selected combinations designated as 1:1(a), 1:5(e), and 2:1 (f) of *Aspergillus flavus* to *A. tamarii*. The ascorbic acid hydrolysate was quantified using the method of Association of Vitamin Chemists (1966), while the glucose concentration in the hydrolysate was measured in percentage by the use of a refractometer.

Determination of ascorbic acid in cassava starch hydrolysate

This was carried out using the method of Association of Vitamin Chemists (1966). 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 100 ml with 3% metaphosphoric acid and filter the diluted sample. 10 ml of the filtrate was pipetted into a flask and titrated immediately with the standardized solution of 2, 6-Dichlorophenol indophenols to a faint pink end point which persists for 15secs.

RESULTS

Amylase production by mixed and monocultures of *Aspergillus spp* using Solid State Fermentation

The amylase activity of monocultures and mixed cultures of *Aspergillus flavus* and *Aspergillus tamarii* was determined and presented in figure 1. The mixed cultures of *Aspergillus flavus* and *Aspergillus tamarii* in the ratio 1:1 (a), 1:5 (e) and 5:1 (f) produced high amylase activities of 65%, 70% and 62% respectively when compared to the monocultures which produced lower amylase activities of 50% for *A. flavus* and 55% for *A. tamarii* (Banjo *et al.*, 2014).

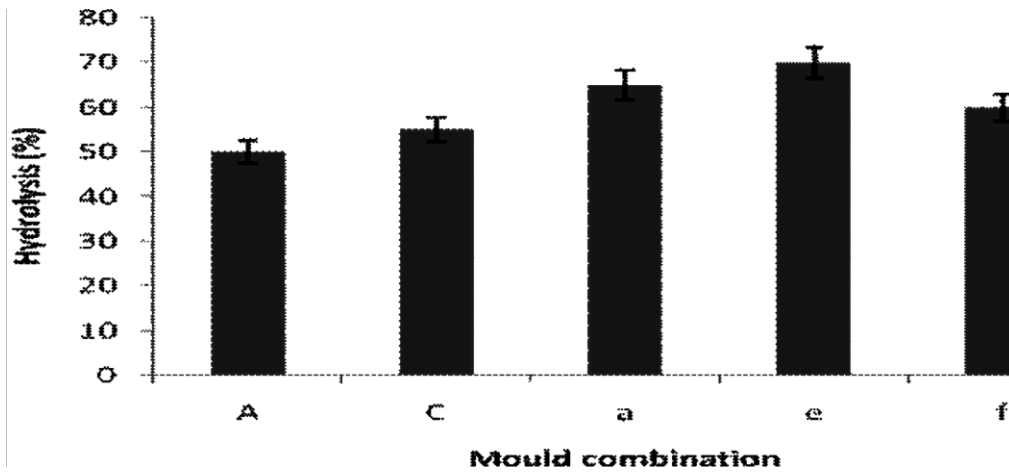


Figure 1: Amylase production by mixed and monocultures of *Aspergillus flavus* and *Aspergillus tamarii*
Hydrolysis of starch by mixed and monocultures of *Aspergillus* spp

The result indicated that all the enzyme extracts were able to hydrolyze the different starches (Cassava, Corn, Millet, Rice and Potato) to different extents. Cassava starch has the highest hydrolysis while the least hydrolysis was recorded by rice starch (Fig.2)

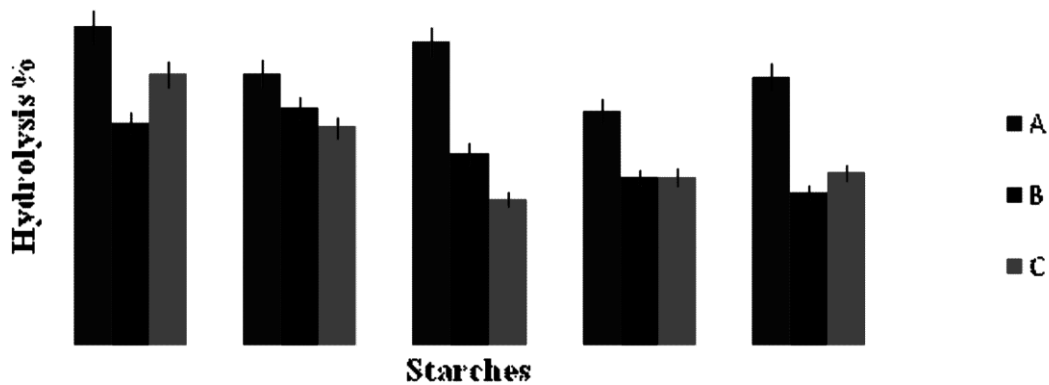


Figure 2: Hydrolysis of starch by monocultures (A, C) and mixed cultures (B) of *Aspergillus flavus* and *Aspergillus tamarii*

Ascorbic acid and glucose production by mixed and monocultures of *Aspergillus* spp

Studies on glucose and ascorbic acid production by enzyme extracts of the mixed and monocultures from cassava starch was investigated (figs. 3-5). The mixed cultures produced the highest

ascorbic acid yield of 43 mg/ml while *A. flavus* produced the least ascorbic acid yield of 15 mg/ml. Also, the mixed cultures produced the lowest glucose concentration of 46% while *A. flavus* produced the highest glucose concentration of 88%.

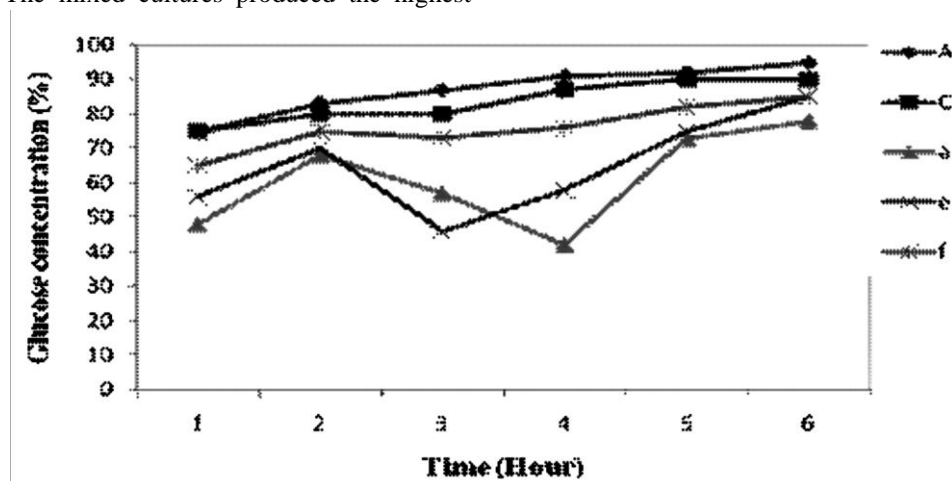


Figure 3: Glucose production from cassava starch by monoculture of *Aspergillus flavus*(A) and *A. tamarii*(C) and its mixed cultures in ratios 1:1 (a) 1:5, (e) and 2:1 (f)

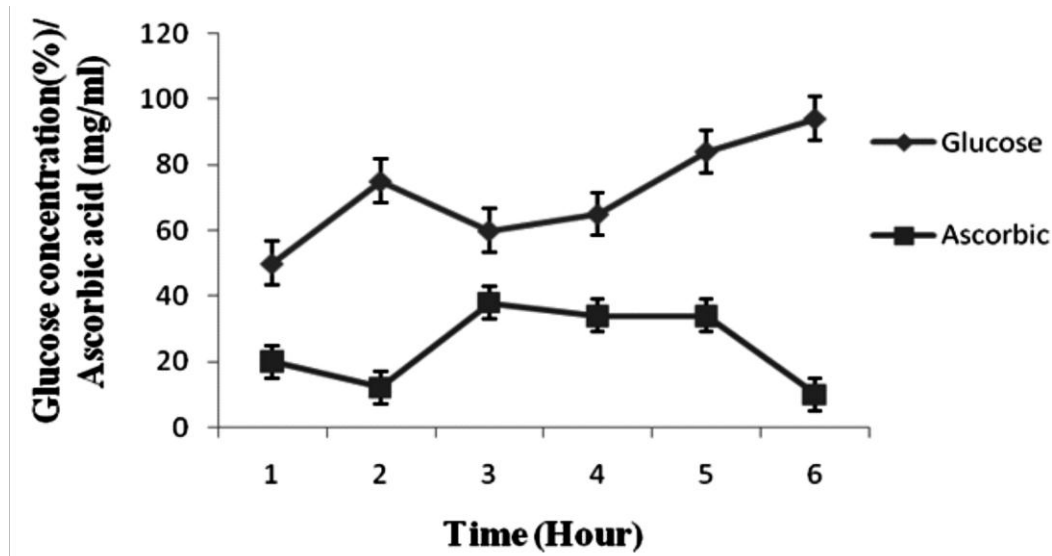


Figure 4: Glucose and Ascorbic acid production from cassava starch by mixed culture of *Aspergillus* spp

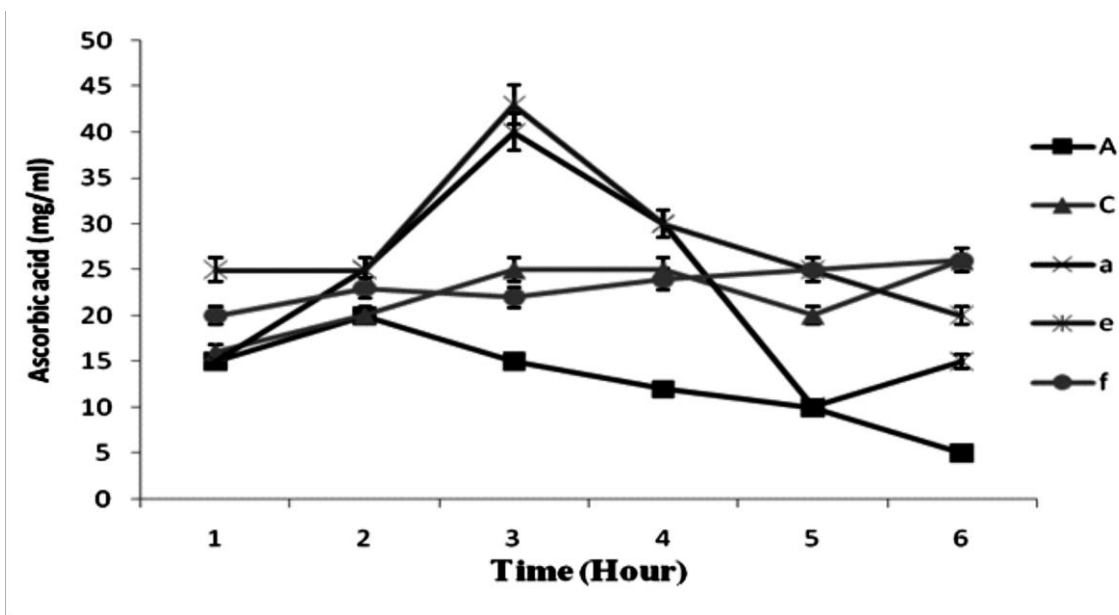


Figure 5: Ascorbic acid production from cassava starch by monoculture of *Aspergillus flavus*(A) and *A. tamarii*(C) and its mixed cultures in ratios 1:1 (a) 1:5, (e) and 2:1 (f)

DISCUSSION

The mixed cultures produced the highest amylase activity because the association of microbial population of mycelial fungi allows an even higher enzyme production of hemicellulases and cellulases which by acting synergistically effectively degrade vegetable refuse. This has also resulted in an increased amylase production (Abate *et al*, 1999). This result agrees with the findings of Abate *et al* (1999) who also reported a 2.5 fold increase in amylases produced by mixed cultures of *Bacillus* and *Zymomonasmobilis*. Therefore mixed cultures of *Aspergillus flavus* and

Aspergillustamarii in the ratio 1:1 (a), 1:5 (e) and 2:1 (f) with high amylase activities were selected for ascorbic acid production (Fig. 1)

Maximal enzyme activity was recorded with cassava starch as the substrate (Fig. 2) Hence the production of glucose syrup from cassava starch was exploited using the amylase extracts from the single and mixed cultures. The hydrolysate containing enzymes of the mono cultures produced a higher concentration of glucose compared to that of the mixed culture. Ascorbic acid was also detected in the

hydrolysates with enzyme from the mixed and monocultures combinations.

The hydrolysis of cassava Starch at 65°C, pH 4.5 and 6.0 by the mixed and monocultures led to the production of ascorbic acid after 6 h of starch hydrolysis (fig. 5). The qualitative determination of glucose concentration after 6 h hydrolysis of the starch indicated that glucose was more in the monoculture than in the mixed culture with *A. flavus* and *A. tamarii* producing 14 mg/dl of glucose each and the mixed culture 5.6 mg/dl of glucose. The possibility of more of the glucose of the mixed culture being converted to ascorbic acid is suspected to be responsible for the low level of glucose in the mixed culture. This correlates with the findings of Huh et al (1994) who reported the production of erythroascorbic acid from D-glucose by some species of *Candida* and *Saccharomyces*. The quantitative determination of ascorbic acid produced by different combinations of the moulds, *A. flavus* and *A. tamarii* was carried out. The synergy between the monocultures in the mixed cultures resulted in an increased ascorbic acid production of 43 mg/ml, while that of the monocultures, *A. flavus* and *A. tamarii* were 15 mg/ml and 25 mg/ml respectively at 6 h of hydrolysis. Whereas the mixed culture produced the lowest glucose concentration of 46%, *A. flavus* produced 88% and *A. tamarii* 80%. (Figs 3-4). Hence, an inverse relationship exists between ascorbic acid and glucose concentration. This relationship brought about by the conversion of glucose to ascorbic acid at 6 h of hydrolysis suggests the presence of the enzyme L-gluconoxidase.

Conclusion

The synergistic effect of mixed cultures of *A. flavus* and *A. tamarii* was exploited in this study which led to increased ascorbic acid production. This two-step bioconversion of starch to ascorbic acid unlike the conventional method which involves five steps could be of great contribution economically because of its cost-effectiveness and because of the dietary importance of ascorbic acid as a supplement in the health sector.

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