Catalytic properties of purified alpha amylase from *Aspergillus flavus* cultivated on low-cost agricultural substrate

Propiedades catalíticas de alfa amilasa purificada de *Aspergillus flavus* cultivada en sustrato agrícola de bajo costo

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ABSTRACT

Aspergillus flavus isolated from fermented millet flour produced a crude enzyme, which was purified via ammonium sulphate precipitation and subsequent chromatographic techniques. The biochemical characteristics of the purified amylase were thereafter investigated showing activity in a wide range of pH and temperature, with optimal conditions of pH 6.0 and 50 °C. The enzyme retained even 89% of its activity after 1 h at 50 °C and 2 h at pH 6.0. The purified enzyme was stimulated by Ca^{2+}, Zn^{2+} and Co^{2+}, while Hg^{2+} and EDTA caused mild inhibition of α-amylase activity. The kinetic indices (K_m and V_{max}) and molecular weight of the enzyme were estimated in 1.71 mg mL^{-1}, 2.133 µmol min^{-1} mL^{-1} and 45 kDa respectively. The catalytic properties of α-amylase from *A. flavus* makes it a promising candidate for use in various starch processing industries.

RESUMEN

Aspergillus flavus aislado de harina de mijo fermentada produjo una enzima cruda, que se purificó mediante precipitación con sulfato de amonio y técnicas cromatográficas. Posteriormente se investigaron las características bioquímicas de la amilasa purificada mostrando actividad en un amplio rango de pH y temperatura, con condiciones óptimas de pH 6,0 y 50 °C. La enzima retuvo incluso el 89% de su actividad después de 1 h a 50 °C y 2 h a pH 6,0. La enzima purificada fue estimulada por Ca^{2+}, Zn^{2+} y Co^{2+}, mientras que Hg^{2+} y EDTA causaron una leve inhibición de la actividad de la α-amilasa. Los índices cinéticos (K_m y V_{max}) y peso molecular de la enzima se estimaron en 1,71 mg mL^{-1}, 2,133 µmol min^{-1} mL^{-1} y 45 kDa respectivamente. Las propiedades catalíticas de la α-amilasa de *A. flavus* la convierten en un candidato prometedor para su uso en diversas industrias de procesamiento de almidón.

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The applications of enzymes for various bioproducts formulation for human needs is the driving force behind the evaluation of microorganisms for different extracellular enzymes (Kwatia and Dzogbefia, 2018). The main focus is on starch-degrading enzymes, mainly α-amylase (α-1,4 glucan-glucanohydrolase) which represent more than 60% of the enzyme market due to their usefulness in several industrial processes (Ali *et al.*, 2017). Alpha amylase breaks down the α-1,4 glucosidic bond in starch-containing substrate resulting in monosaccharides such as glucose, short-chain dextrin, and oligosaccharides (Ali *et al.*, 2017).

Being an extracellular enzyme amylase can be extracted from plants, animals, and certain microorganisms. However, more attention has been paid to bacterial and fungal amylases because of their thermostability, ease of cultivation, and higher productivity in industrial applications (Ali *et al.*, 2017, Ahmad *et al.*, 2019). Amylases from fungi are documented to be more commercially consistent than amylases from bacterial sources. They also have a highly accepted GRAS (generally recognized as safe) status. As a result, investigations have been carried out on the process parameters of promising fungal strains (Lim *et al.*, 2020). *Aspergillus* spp. has been reported to possess some unique enzyme machinery with exceptional catalytic properties making them suitable candidates for industrial operations (Karim *et al.*, 2018). For instance, *A. oryzae* serves as a host for heterologous protein production and possesses the capacity to produce large quantities of relevant industrial enzymes that are of great economic value. Also, *Aspergillus niger* highly opposes contamination and is acid-tolerant (Gurung *et al.*, 2013). Solid substrate fermentation (SSF) has been considered a promising technique for the production of enzymes due to its economic and engineering advantages (Razdan and Kocher, 2018). In recent years, the SSF process has been developed and extensively used for the synthesis of bio-products, especially when it involves fungi (Kwatia and Dzogbefia, 2018). SSF cultivated microbes have tight contact with insoluble substrates letting a higher nutrient utilization from them and achieving high enzyme yields during fermentation. Numerous advantages are attributed to SSF these include higher volumetric production, utilization of simple

fermentation media, require less water, generate, fewer effluents, no laborious control of process parameters, bacterial contamination is reduced, and requires less capital involvement for further processing (Ahmad *et al.*, 2019).

With the high demand for novel amylases, getting a cheap, rich, and the readily available suitable substrate is key to meeting the high demand with excellent catalytic efficiency. Hence, cheaper and readily available starchy foods such as cereals (rice, corn, wheat, and millet) and tubers (yam, cassava, potatoes, and cocoyam), could be adopted as an additional use in starch enzymatic breakdown to produce reducing sugars. The enzymatic is inexpensive in terms of energy consumption and the use of a simpler process than the conventional that utilizes pregelatinized starch as substrate (Kwatia and Dzogbefia, 2018).

Purification is an important aspect after enzyme production to obtain a purified component from the crude extract. Without purification, enzyme activities and associated proteins might be difficult to characterize accurately due to the impurities in the crude solution, resulting in unreliable data and information (Lim *et al.*, 2020). Searching for a novel fungal strain with high catalytic properties at a low cost, the present study isolated and cultivated an amylolytic fungus using an economic substrate for amylases production, purification, and characterization. The fungal strain purified amylases demonstrated some biochemical properties making them promising candidates to use in different industries processing starch.

MATERIALS AND METHODS
Collection of samples
Grain samples namely: rice (O*ryza sativa*), wheat (*Triticum aestivum*), corn (*Zea mays*), and millet (*Pennisetum glaucum*) were purchased at Oja-oba market, Akure, Nigeria. Debris and unwanted particles were sorted out from the grains. They were washed, ground into fine particles, packaged in transparent plastic bags, labeled, and transported to the laboratory for further microbial and biochemical analysis.

Fungal isolation and identification
Fungi were isolated from fermented cereals using potato

dextrose agar (PDA) by standard pour plate techniques and incubated at 30 °C. The fungal colonies were sub-cultured to purify the isolates. The isolates were prepared on slides and stained with lactophenol cotton blue to identify their morphology and compare them with a standard fungal atlas (Pitt and Hocking, 2009).

Qualitative screening of isolates for amylase production
Alpha-amylase producing isolates were screened on starch-agar medium (soluble starch 5 g L^{-1}; peptone 5 g L^{-1}; yeast extract 5 g L^{-1}; $MgSO_4 \cdot 7H_2O$ 0.5 g L^{-1}; $FeSO_4 \cdot 7H_2O$ 0.01 g L^{-1}; NaCl 0.01 g L^{-1}; agar 15 g L^{-1} plates. The isolate was inoculated at the center of the plates, at an appropriate temperature and then was stained with Lugol solution. The colonies forming the largest halo zone were selected for further studies (Yalcın and Çorbacı, 2013).

Genetic identification
The genetic identification of the isolates was performed by extracting DNA following the standard technique of Xian *et al.* (2015). Then, using PCR the partial internal transcribed spacer (ITS) region genes were amplified with the corresponding primer sets that target ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC CC GCT TAT TGA TAT GC-3') (Xian *et al.*, 2015). The amplification protocol adopted was: 40 PCR cycles with denaturation at 95 °C for 2 min, annealing at 58 °C for 2 min, and extension at 72 °C for 2 min, with the final extension for 10 min. The sequences of the amplicon were compared with the sequences in the GenBank using a BLASTN search and aligned with related species according to Xian *et al.* (2015).

Fungal inoculum
Spore suspension of the amylase-producing fungal isolates was prepared by scrapping off fungal spores which were dissolved in 40 mL of distilled water until the final volume of 60 mL and vortexed. The SSF production medium was inoculated with 2 mL fungal spore suspension (Kwatia *et al.*, 2017). The mineral salt medium (MSM) contains 0.1 g KH_2PO_4, 0.25 g NaCl, 0.01 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 7H_2O$, and 0.10 g yeast extract in 100 mL of distilled water.

Amylase production in SSF
To choose the best substrate in the SSF for amylase production, commercial starch was substituted with corn, rice, wheat, and millet as selected organic substrates. The organic substrate was used singly; 100% of corn (C); rice (R); wheat (W); millet (M) or in a mixture at different ratios: 1:1 of corn and rice (CR); corn and wheat (CW); corn and millet (CM); rice and wheat (RW); rice and millet (RM); wheat and millet (WM); 1:2:1 corn, rice, and wheat (CRW); rice, corn, and millet (RCM); millet, wheat and rice (MWR); wheat, millet, and corn (WMC); 1:1:1:1 corn, rice, wheat, and millet (CRWM). 4 g of each combination was dissolved in 10 mL of MSM and sterilized at 121 °C for 15 min. At room temperature, the dissolved substrate was inoculated with 2 mL of the fungal spore suspension, thoroughly mixed, and incubated aerobically at 30 °C for 96 h (Kwatia *et al.*, 2017).

Enzyme extraction
The extracellular crude enzyme was extracted from the fermentation medium using 0.05 M citrate phosphate buffer (pH 6.0). 50 mL of the buffer was dispensed into the 4 days old enzyme production medium, homogenized, and kept in a rotary shaker (250 rpm) at 30 °C for 30 min. The resulting mixture was filtrated using a cheesecloth and the filtrate was centrifuged (3600 xg, 15 min at 4 °C). The clear crude extract had crude enzymes for amylolytic activity evaluation (Kwatia and Dzogbefia, 2018). The composite substrate with the highest α-amylase production was considered a potential substrate.

Amylase assay
The amylase activity was determined following the protocol of Ahmed *et al.* (2020). A mixture of 0.5 mL of crude extract and 0.5 mL of soluble starch was incubated at 40 °C in a water bath for 15 min, the reaction was stopped with 1 mL of 3, 5 dinitrosalicylic acid, boiled for 5 min and cooled to measure the reducing sugars and estimate the enzymatic activity according to the method of Miller (1959). The commercial substrate, i.e., soluble starch was prepared by dissolving 1% (w/v) of the substrate in 0.05 M citrate phosphate buffer (pH 6.0). The absorbance was taken at 540 nm against a substrate blank using the UV Spectrophotometer (Axiom 721 vis spectrophotometer). The quantity of reducing sugars released from the enzyme-catalyzed starch was estimated using a glucose standard curve. A unit of

amylase activity was indicated as the amount of enzyme needed to liberate 1 μmol of reducing sugars (maltose/ glucose) per minute under standard assay protocols (Ahmed *et al.*, 2020).

Determination of protein concentration in the crude extract

The protein contents in the crude extract were evaluated by the Bradford method (1976). Different concentrations (0.1-1.0 mg mL^{-1}) of Bovine Serum Albumin were prepared and used as a standard. Finally, the absorbances of the samples were measured at 595 nm.

Crude amylase purification and molecular weight

The crude enzyme was purified by three purification steps: ammonium sulphate precipitation, ion exchange, and gel filtration chromatography (Abdulaal, 2018). After purification, the biochemical characteristics of the purified enzyme, kinetic parameters, and molecular weight were determined.

Ammonium sulphate precipitation

Solid ammonium sulphate was continuously and gently dissolved in crude enzyme solution until 60% of saturation. Allowing the salt precipitation in the mixture at 4 °C for 12 h, the saturated solution was centrifuged for 20 min at 14,000 rpm to obtain the pelletized proteins, which were dissolved in 10 mL of 0.05 M citrate phosphate buffer (pH 6.0). Furthermore, the protein was dialyzed in the same buffer for 96 h (Hassan *et al.*, 2018).

Dialysis

The precipitated protein was aseptically poured into a dialysis bag and dialyzed in the same buffer for 96 h in refrigeration until releasing the sulphate from the solution, replacing the buffer in the bag embedded. After dialysis, the protein content and α-amylase activity were subsequently determined (Hassan *et al.*, 2018).

Ion-exchange chromatography

The dialysate free of salt was loaded on a DEAE Sephadex a-50 column (2.5×40 cm), previously treated with 0.05 M citrate phosphate buffer pH 6.0. The protein was eluted using the same buffer at a flow rate of 2 mL min^{-1}. The unbound inactive protein was eluted from the column, followed by the elution of the bound proteins by a linear salt gradient (0.1–1.0 M NaCl). The protein content eluted was determined by reading the absorbance at 280 nm and the α-amylase activity was assayed following the prescribed protocol. The fractions showing enzyme activities were concentrated with 4 M sucrose solution (Hassan *et al.*, 2018).

Gel filtration

The concentrated enzyme was further purified using a Sephadex G–100 column (2.5×75 cm) previously treated with 0.05 M citrate phosphate buffer pH 6.0. The protein was eluted using the same buffer at a flow rate of 20 mL h^{-1}. The protein content of each fraction was monitored by reading the absorbance at a wavelength of 280 nm and α-amylase activity was assayed as previously described. The pooled fractions were stored at 4 °C and used for biochemical characterization (Hassan *et al.*, 2018).

Biochemical characterization of the purified α-amylase

The biochemical properties of the purified enzyme and kinetic parameters, such as K_m and V_{max}, were studied. The influence of temperature and pH on the activity and stability of purified α-amylase and the effect of metal ions and inhibitory agents on enzyme activity were also determined.

Influence of temperature and pH on the catalytic activity and thermal stability of purified α-amylase

To determine the optimal temperature for α-amylase activity, equal volumes of enzyme and substrate were incubated at different temperatures, from 40 to 90 °C. The thermal stability of the enzyme was evaluated by taking samples every 30 min for 2 h, and the residual activity was evaluated following standard assay protocol (Abdulaal, 2018).

The influence of pH on the catalytic activity of purified α-amylase was determined by incubating at different pH in 50 mM buffer system: glycine-HCl buffer (pH 3.0), sodium acetate buffer (pH 4.0–5.0), phosphate buffer (pH 6.0–7.0), Tris- HCl buffer (pH 8.0–9.0) and glycine-NaOH (pH 10.0–12.0). The stability of the purified enzyme was monitored by incubating in the appropriate buffer solutions for 2 h and taking an aliquot for enzymatic assay every 30 min (Abdulaal, 2018).

Influence of metal ions and inhibitors on the catalytic activity of purified α-amylase

The influence of different concentrations of metal ions and enzyme inhibitors (5 and 10 mM) on the catalytic properties of the enzyme was determined by incubating the mixture enzyme/substrate with the metal ions (Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Hg^{2+}, Na^+, Zn^{2+}, K^+, and K_3Fe (CN) or the inhibitors (EDTA and urea). After incubation, the enzymatic activity was determined following standard assay procedures (Abdulaal, 2018).

Estimation of kinetic parameters of the enzyme

The initial rate of the amylase-substrate reaction was measured using soluble starch (1-10 mg mL^{-1}) as substrate. The kinetic parameters (K_m and V_{max}) of the purified enzyme were estimated from the double reciprocal plot of Lineweaver and Burk (Abdulaal, 2018).

Determination of molecular weight of purified α-amylase

The homogeneity and subunit molecular weight of the purified α-amylase was determined by 10% gel sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a wide range protein molecular weight marker as standards from 11 to 180 kDa and stained with Coomassie brilliant blue (Abdulaal, 2018).

RESULTS AND DISCUSSION

Presumptive identities of fungal isolates from corn, rice, wheat, and millet cereal flour

The isolated fungi from the cereal samples of wheat, rice, corn, and millet were tentatively identified as *Aspergillus niger, A. flavus, Rhizopus stolonifer, Fusarium poae, Penicillium chrysogenum*, and *Cladosporium* sp. From all the samples, *Aspergillus* spp. occurs frequently followed by *Penicillium* spp. The result is similar to that of Hussain *et al.* (2018) who also identified five fungal genera from cereals of wheat, barley, corn, and rice and documented *Aspergillus* as the most predominant. The predominance of *Aspergillus* on fermented cereals could be that they are degraders and common spoilage organisms of carbohydrate-containing (Hussain *et al.*, 2018).

In addition, the genetic identity of the best α-amylase producer resulted in the fungus as *A. flavus* with 97% similarity. The amylase activities of the fungal isolates on different organic substrates are presented in Figure 1.
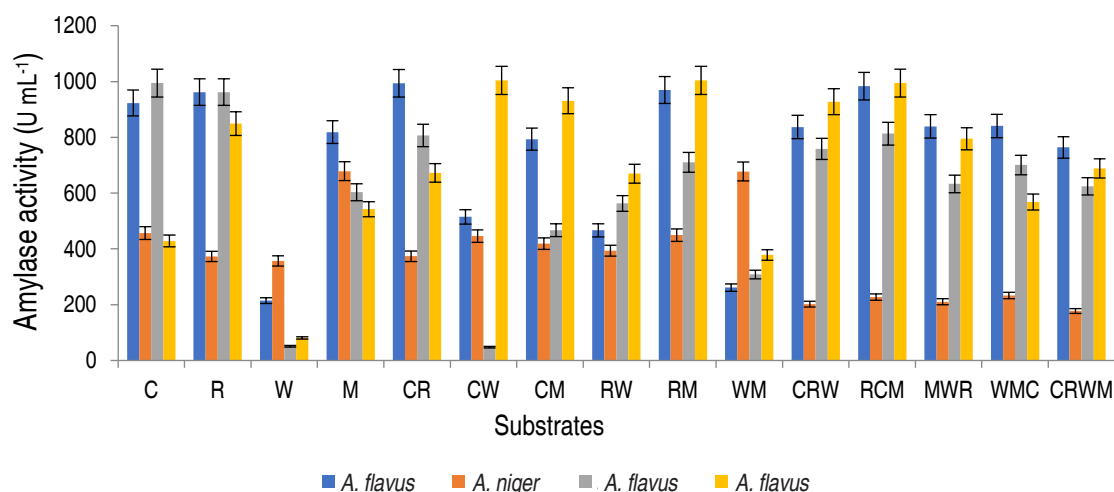

Figure 1. Amylase activity of the amylolytic fungi on different organic substrates viz; 100% corn (C); rice (R); wheat (W); millet (M); 1:1 corn and rice (CR); corn and wheat (CW); corn and millet (CM); rice and wheat (RW); rice and millet (RM); wheat and millet (WM); 1:2:1 corn, rice and wheat (CRW); rice, corn and millet (RCM); millet, wheat and rice (MWR); wheat, millet and corn (WMC); 1:1:1:1 corn, rice, wheat and millet (CRWM).

Aspergillus flavus isolated from millet had the highest amylase activity of 1.004 U mL^{-1} on the combination of rice and millet (RM), *A. flavus* from rice was observed to have the highest amylase activity on rice and millet, with values of 0.814 U mL^{-1}, while *A. niger* from corn had the best activity on millet only. Based on α-amylase activity, *A. flavus* from millet was selected for purification and characterization studies. The production of α-amylase on different substrates from fungal strains using SSF has been studied. Kwatia *et al.* (2017) and Sadhasivam *et al.* (2018) produced amylase from *A. niger* and *A. luchuensis* BS1 by SSF using agro-residues. Abdulaal (2018) and Balakrishnan *et al.* (2021) also separately produced α-amylase from *Trichoderma pseudokoningi* and *A. oryzae* via SSF using orange peels and edible oil cakes, respectively.

In SSF, the selection of a suitable culture medium and a suitable microbial strain are key factors for the production of α-amylases (Razdan and Kocher, 2018). This study revealed appreciable amylase production by *A. flavus* in the composition of rice and millet. This study is in agreement with Ali *et al.* (2017) who documented higher amylase by *A. flavus* on mandarin peels. Oppose to this study, Oshoma *et al.* (2017) found maximum α-amylase yield in *A. niger* using different agricultural wastes via SSF. These variations might be due to the differences in physiological status, and genetic makeup of the organisms leading to varied abilities in utilizing the solid substrates for α-amylase production (Badejo *et al.*, 2021).

Purification summary of amylase
The elution profile of α-amylase from *A. flavus* on the ion exchange column is presented in Figure 2.
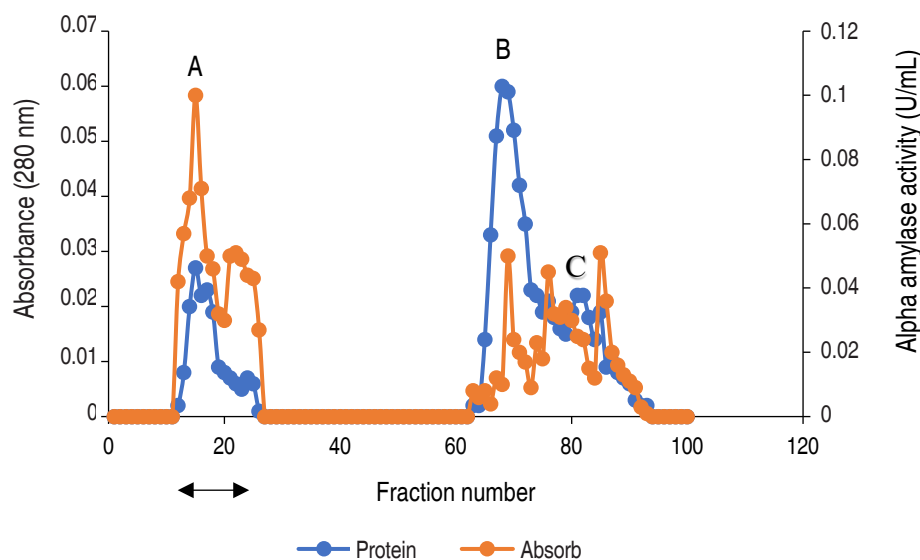

Figure 2. Chromatogram of α-amylase produced by *A. flavus* on ion-exchange column (2.5×40 cm) of DEAE Sephadex.

The profile revealed three (3) activity peaks labeled as A, B, and C. Each peak was pooled together to form the three fractions. The fractions forming peak A had an activity of 0.497 U mL^{-1}, peak B of 0.169 U mL^{-1}, and peak C constitutes the lowest activity of 0.024 U mL^{-1}. The major active peak A was pooled together and was further subjected to purification by gel chromatography. The elution profile of α-amylase from *A. flavus* via gel filtration is shown in Figure 3.

The profile revealed two (2) activity peaks (A and B). Peak B had the highest activity hence, fractions from peak B were pooled together and used for the characterization.
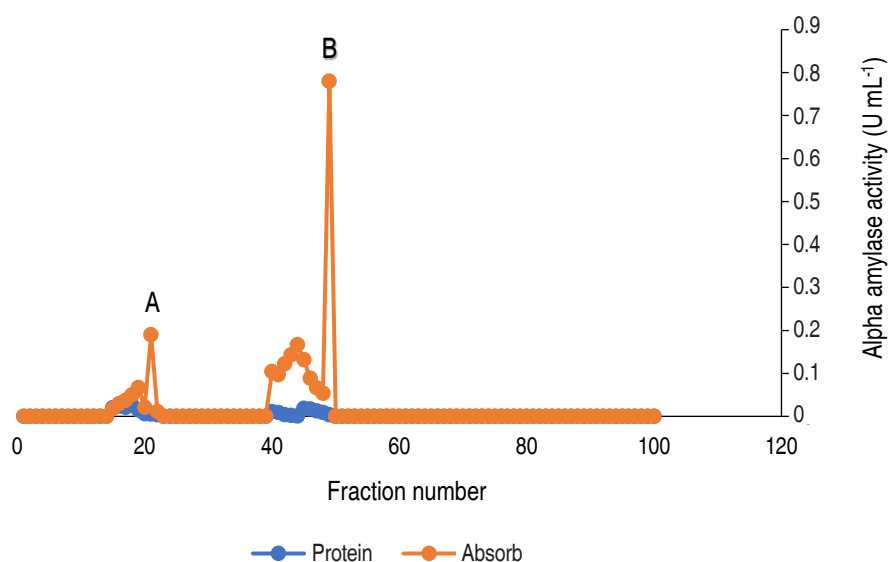
Figure 3. Elution profile of α-amylase via gel filtration with two activity peaks. Pooled fractions were represented with black arrow.

Table 1 has a summary of the processes and the techniques used for the obtaining homogeneity of the enzyme. Alpha amylase from *A. flavus* was purified to homogeneity with a purification yield of 4.3% and a fold of 7.9. Similar to this result, Nithya *et al*. (2017) recorded a purification fold of 7.06, while Hassan *et al*. (2018) and Kwatia *et al*. (2017) reported a lower purification fold of 4.78 and 4.48, respectively. Higher purification folds of 18 and 16.6% were documented by Abdulaal (2018) and

Gbenga *et al*. (2017) for purified α-amylase from different fungal isolates. The differences in the purification yield and fold by different authors is due to the type of resins used and other factors such as buffers and purification conditions (Badejo *et al*., 2021). Fold purification indicates the purification status of the enzyme; an increase in fold purification reveals an increase in the purity of the enzyme subjected to purification when compared with the crude enzyme (Badejo *et al*., 2021).

Table 1. Summary of purification of α-amylase from *A. flavus*.

Steps	Volume (mL)	Protein concentration (mg mL^{-1})	Total protein (mg)	Activity (U)	Total activity (U mL^{-1})	Specific activity (U mL^{-1})	Yield (%)	Purification fold
Crude extract	500	0.259	0.130	0.681	0.341	2.629	100	1
$(NH_4)_2SO_4$	31	0.109	0.003	0.608	0.019	5.529	5.521	2.103
DAEA Sephadex	18	0.066	0.002	0.497	0.009	7.417	2.614	2.820
Sephadex G-100	34	0.021	0.001	0.432	0.015	21.000	4.317	7.987

Physicochemical properties of a purified amylase
Figure 4 shows the catalytic activity of the purified amylase. The highest temperature was considered as 100% and the rest of the temperature was estimated relative to the maximum activity. The relative activity of purified α-amylase increased from approximately 45% at 30 °C and reaches its peak at 50 °C with the value of

100% relative activity, beyond 50 °C, a steady decrease in relative activity was observed, at 90 °C, the activity decreased to 14%.

In this current study, the activity of purified α-amylase reached optimum at 50 °C. Abdulaal (2018) also recorded maximum α-amylase from *Trichoderma*
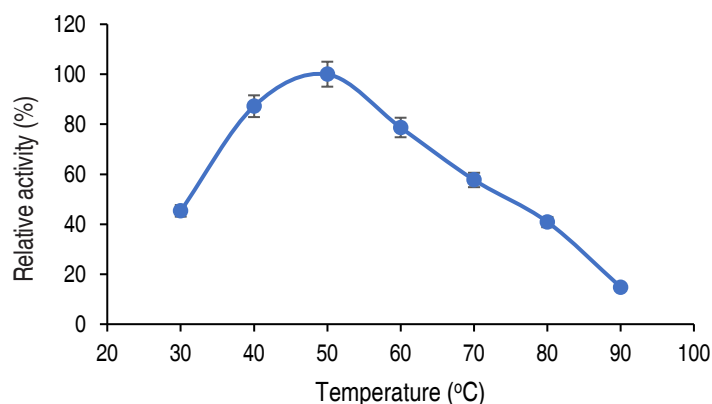
Figure 4. Effect of temperature on the activity of purified α-amylase from *A. flavus.*

sp. at 50 °C. Similarly, El-Sayed *et al.* (2019) also documented optimum amylase activity from *Bacillus mojavensis* at 55 °C. Higher and lower temperatures than those obtained in this study had been recorded from other studies; Hassan *et al.* (2018) showed optimum amylase activity from *E. coli* at 30 °C, while Wang *et al.* (2018) showed that the amylase activity from *Pseudoalteromonas* sp. was 25 °C. Sadhasivam *et al.* (2018), Trabelsi *et al.* (2019) and Wang *et al.* (2018) in separate studies documented optimal activities at 60 °C for purified α-amylase from *A. luchuensis, B. subtilis* and *Thermomyces dupontii* (expressed in *Komagataella*

phaffii, respectively. The decrease in α-amylase activity beyond 50 °C might be connected to the disruption of various bonds in the amylase (Wang *et al.*, 2018). While the variations observed in the optimal temperatures from different studies could be due to the source of the microbial enzyme and differences in the genetic makeup of the organisms (Badejo *et al.*, 2021).

Thermal stability of the purified α-amylase
The infuence of temperature on the stability of the enzyme is shown in Figure 5.
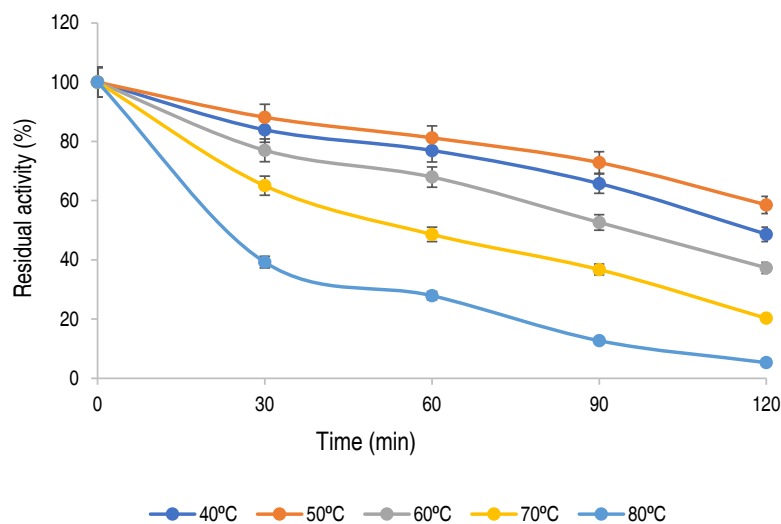The enzyme has a residual activity of approximately 81%

Figure 5. Effect of temperature on the stability of α-amylase from *A. flavus.*

after 1 h at 50 ℃ and 58% of its activity was retained after 2 h, while 76%, 67%, and 48% of residual activities were retained at 40 ℃, 60 ℃, and 70 ℃, respectively after 1 h of incubation. The purified α-amylase from this study was stable and retained 81%, 67% and 48% of its activity at 50 °C, 60 °C and 70 °C for up to 60 min respectively. Karim *et al.* (2018) similarly documented thermo-stable α-amylase from *A. flavus* NSH9 at 50 °C, retaining 87% of its residual activity after 60 min of incubation. The decline in enzyme activity beyond the period of stability could be due to hydrolysis of peptide chain or aggregation, thus causing abnormality in the conformation of the enzyme (Karim *et al.*, 2018).

Influence of pH on purified α-amylase activity
The influence of pH on purified α-amylase is presented in Figure 6.
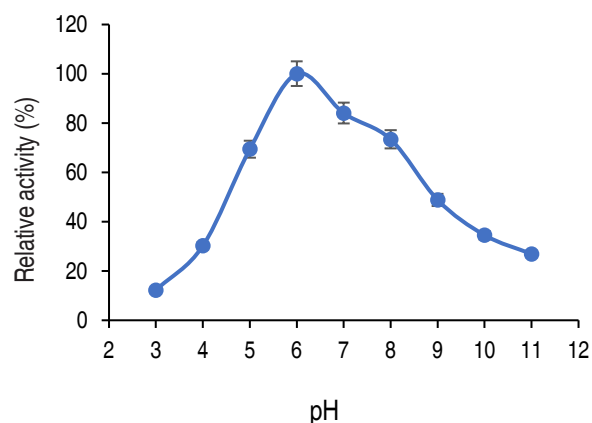

Figure 6. Effect of pH on the activity of α-amylase by *A. flavus*

The relative activity of purified amylase increased from pH 3.0 and reached its optimum at pH 6.0 and then decreased. At pH 8.0 and 9.0, the relative activities were approximately 73% and 48% respectively.

The purified α-amylase from this study was active both in acidic and alkaline regions (5.0 to 8.0), where an optimum pH was recorded at pH 6.0. The purified α-amylase from different fungi have pH optimum ranged from acidic (3.0) to alkaline region (11.0) (Abdulaal, 2018). Different studies have reported various pH optimal for purified α-amylase. Doss and Anad (2012) reported 6.0 as the best pH for optimum α-amylase activity from *A. flavipes* and *A. wentii*. Trabelsi *et al.* (2019) similarly recorded a pH of 6.0 for maximum purified α-amylase activity from different sources. On contrary, a pH of 7.0 was optimum for amylase from *A. nidulans* and *A. versicolor* (Sethi *et al.*, 2016). Hammami *et al.* (2018) and Priyadarshini and Ray (2019) reported an optimum α-amylase from *Bacillus mojavensis* SA and a *Bacillus* sp. at pH 9.0.

The variations in the optimal pH observed in different studies could occur as a result of the microbial origin of the enzyme as well as modification of the genetic make-up of the isolate (Badejo *et al.*, 2021). Also, the activity of the purified α-amylase over a wide range of pH shows the versatility of the α-amylase for several processes. The decline in α-amylase activity at pH 4 and 10 might be due to the denaturation of essential co-factors or dissociation of the enzyme structure (Badejo *et al.*, 2021).

pH stability of the purified α-amylase
The influence of pH on the stability of the purified α-amylase is presented in Figure 7. The purified α-amylase was 100% relatively stable at pH 6.0 for 30 min, and after 2 h of incubation, 68% residual activity was retained. The enzyme retained more than 40% of its residual activity after 90 min at pH 8.0. The stability of this enzyme fulfills the requirement for its application in many industrial operations especially for starch processing.
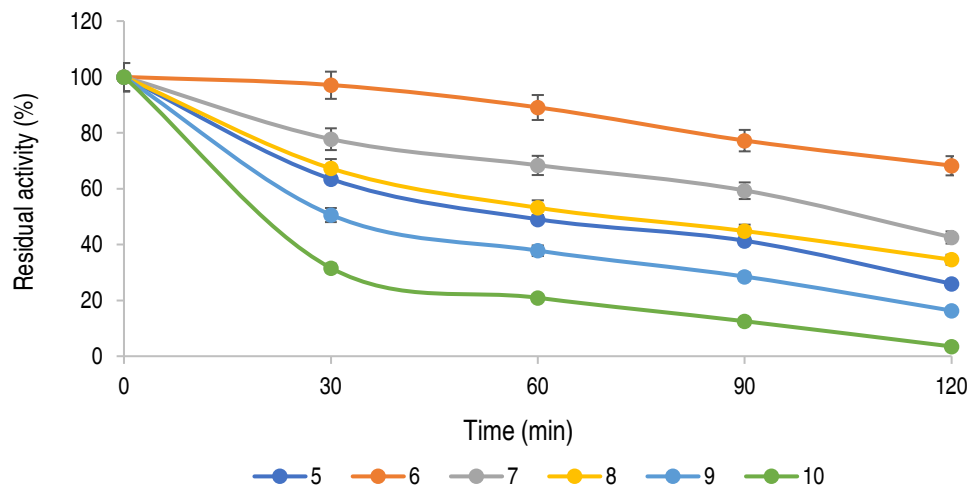
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Figure 7. Effect of pH on the stability of purified α-amylase by *A. flavus.*

Influence of metal ions and inhibitors on α-amylase activity

The effect of different metal ions and enzyme inhibitors on the purified α-amylase from *A. flavus* is presented in Figure 8.

The α-amylase activity was stimulated in the presence of Ni^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, Na^+, Zn^{2+}, Co^{2+} and urea at 5 mM, while Hg^{2+} and EDTA mildly inhibited α-amylase activity at concentration dependent manner.
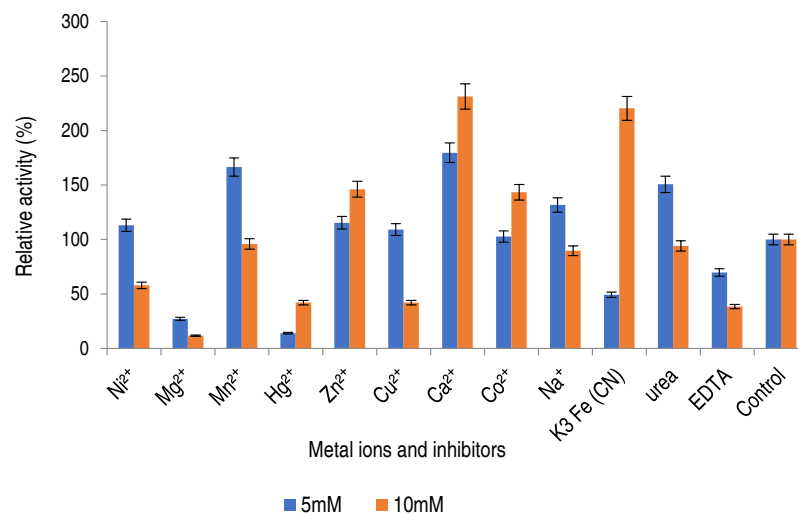

Figure 8 Effect of metal ions and inhibitors.

In this study, the purified enzyme was stimulated by some salts, notably calcium ion. Many of these metal ions are known to enhance enzyme activity, some metal ions act as cofactors increasing the activity of amylase for starch hydrolysis (Lim *et al.*, 2020). From this study, the addition of Ca^{2+}, Co^{2+} and Zn^{2+} positively modulated purified α-amylase at both concentrations of 5 and 10 mM, amylase activity was only activated by Na^+, Cu^{2+}, Ni^{2+} and Mn^{2+} at 5 mM while the enzyme activity was inhibited in the presence of EDTA, and Hg^{2+}. A similar observation was documented by Kwatia and Dzobgefia (2018) and Aladejana *et al.* (2020) when α-amylase from *B. subtilis* was incubated with Ca^{2+}. Also, Bashir *et al.* (2014) documented stimulation of α-amylase from

B. lichiniformis on the addition of Zn^{2+}, Co^{2+}, and Mn^{2+}. Ca^{2+} activated α-amylase suggest that calcium ion might act as an activator and stabilizer of α-amylase and that requirement for Ca^{2+} differs for different α-amylases (Maalej *et al.*, 2021).

In contrary with this study, the inhibitory action of Ca^{2+} at 10 mM against purified amylase was documented by Babu and Satyanarayana (1993). EDTA had an inhibitory effect on the α-amylase activity. Chelating agents such as EDTA are known to inhibit enzymes by either detaching metal ions from the enzyme or by binding to the enzyme to form a ligand (Aladejana *et al.*, 2020).

The findings of this study suggests that the enzyme from *A. flavus* is a metalloenzyme and that the enzyme contains metallic ions which are removed by the chelating agent, forming an active complex with EDTA and consequent loss of activity (Aladejana *et al.*, 2020). In general, variations from different studies occurs because the metal-binding ability of α-amylases, were found to be dependent on the enzyme sources (Maalej *et al.*, 2021).

Kinetic parameters of purified α-amylase
The kinetics parameters, maximum velocity (V_{max}) and Michaelis-Menten constant K_m are showed in Figure 9.
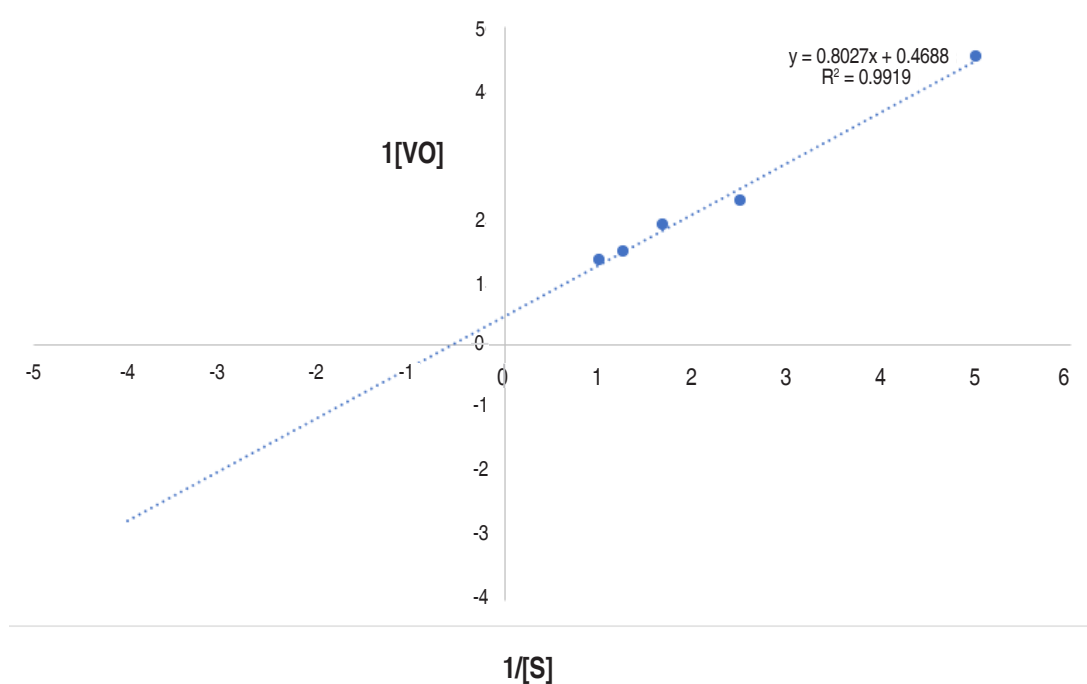

Figure 9. Line weaver-Burk plot of reaction velocity against substrate concentration for purified α-amylase using starch as substrate.

This research documented the apparent K_m and V_{max} of the purified α-amylase to be 1.71 mg mL^{-1} and 2,133 µmol min mL^{-1} respectively. Higher K_m and V_{max} of α-amylase from *Bacillus* sp. MB6 were documented as 5.45 mg mL^{-1} and 24.15 µmol min mL^{-1} (Paul *et al.*, 2017). Abdulaal (2018) also reported the K_m and V_{max} values for purified α-amylase from *Trichoderma pseudokoningii* to be 4.0 mg mL^{-1} and 0.74 µmol min mL^{-1}. K_m measures the affinity of the enzyme for substrates i.e., the enzyme substrates complex, while the V_{max} of an enzyme is the measure of the maximum rate of reaction. The K_m of an enzyme, relative to the concentration of its substrate under normal assay conditions allows prediction of whether or not the rate of product formation, will be affected by substrate concentration (Badejo *et al.*, 2021). From this study, the higher V_{max} and lower K_m values suggest a reasonable bonding between the substrate and enzyme. It also confirms the efficiency of the purified α-amylase for diverse industrial applications (Badejo *et al.*, 2021) such as in food industries.

Molecular weight estimation

The most active fraction from the purified amylase subjected to SDS-PAGE for molecular weight determination was estimated to be 45 kDa. SDS-PAGE is considered to determine the molecular mass of the purified enzyme as well as a method to indicate the purity of an enzyme.

Maalej *et al.* (2021) similarly documented 45 kDa in the study of a novel digestive α-amylase from Blue Crab. Xian *et al.* (2015) reported higher molecular weight of 70 kDa from purified α-amylase obtained from *Talaromyces pinophilus*. The variations in molecular weights obtained from different organisms might be related to isozymic properties as well as types of amino acid residues of the enzyme (Maalej *et al.*, 2021).

CONCLUSION

The fungus *A. flavus* isolated from fermented cereals cultivated on low-cost feedstock via SSF demonstrated α-amylase activity with excellent biochemical properties. The biochemical properties of the purified α-amylase showed that it is acidophilic and thermotolerant with a high affinity for the substrate. These are the properties required in a wide range of industries. Therefore, the combination of rice and millet as substrate serves as a good medium for higher amylase production for various industrial applications.

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Rev. Fac. Nac. Agron. Medellín 76(1): 10213-10225. 2023

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