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Short Communication

Partial purification of α-amylase expressed by a tropical mutant strain of *Aspergillus flavus* IFE 03

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A tropical mutant strain of *Aspergillus flavus* induced by ultraviolet radiation of a tropical wild type (*Aspergillus flavus* IFE 03) expressed α-amylase activity in a defined medium with starch as carbon source and peptone as nitrogen source of growth. The enzyme was subjected to partial purification by ammonium sulphate precipitation followed by dialysis. Specific activity of the enzyme increased 6.58 fold while recovery was 32.2% after dialysis.

Keywords: *Aspergillus flavus, partial purification, α-amylase, tropical, mutant strain*

Introduction

α-Amylases are hydrolytic enzymes which catalyze the degradation of starch into dextrins by cleaving α-1,4 glycosidic linkages randomly along the starch molecule (Aiyer, 2005). Vast numbers of fungi are capable of production of α-amylases when cultured on suitable substrates (Pandey, 2000). It was recently reported that *Aspergillus flavus* produced relatively highly active α-amylase in a defined medium with starch as carbon source and peptone as nitrogen source of growth (Adejuwon *et al*, 2015). In this present investigation, a tropical mutant strain of *Aspergillus flavus* IFE 03 was cultured in same defined medium with peptone as nitrogen source and starch as carbon source for fungal growth. Attempts were made to purify α-amylase expressed by the mutant.

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Materials and Methods

Sources and Identification of Isolate (Wild Type)

A tropical wild type strain of *Aspergillus flavus* (IFE 03) in this investigation was isolated from yam (*Dioscorea rotunda*) tuber at the Obafemi Awolowo University, Ile-Ife, Nigeria. It was identified at the Seed Health Unit of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Techniques contained in the illustrated Handbook of Fungi (Cannon and Kirk, 2007) were used in identification. Genetic sequencing (DNA) using Polymerase Chain Reaction was carried out to confirm identity.

Induction of Mutation

The isolate wild type strain *Aspergillus flavus* (IFE 03) was subjected to mutation by repeatedly culturing on potato dextrose agar at 25^{O}C and continual exposure to ultra violet radiation at 20^{O}C for a period of twelve months. Mutation was phenotypically discerned with a

permanent loss in greenish pigmentation after consecutive culturing on potato dextrose agar for a period of six months. nuDNA analysis of the two strains was determined to be different. Also the G + C ratio and DNA relatedness performed by Genomic DNA buffer Set and Genomic – tip 500/G (QIAGEN) were determined in the two strains to be different.

Culture Conditions and Inoculum

The mutant strain of isolate was sub-cultured and maintained on Potato Dextrose agar slants and plates. The fungus was further sub-cultured into test tubes of the same medium and incubated at 25^{o}C. Ninety-six hr-old cultures of the mutant *Aspergillus flavus* strain were used as inocula. Based on the modified method of Olutiola and Ayres (1973), culture was grown in a defined medium composed of: $MgSO_4.7H_20$ (0.1 g), K_2HPO_4 (2 g), KH_2PO_4 (0.5 g), L-cysteine (0.1 g), biotin (0.005 mg), thiamine (0.005 mg) and $FeSO_4.7H_20$ (1 mg) with starch (10 g) as carbon source and peptone (9.9 g) as nitrogen source (Sigma) in 1 litre of distilled water. Conical flasks (250 ml) containing 100 ml growth medium were inoculated with 1 ml of an aqueous spore suspension containing approximately $6x10^5$ spores per ml of isolate. Experimental flasks contained the inoculated sterilized medium while control flasks contained only the un-inoculated sterilized medium. Spores were counted using the Neubauer counting chamber (Olutiola *et al.*, 1991). Experimental and control flasks were incubated without shaking at 25^{0}C (Adejuwon and Ladokun, 2015).

Extraction of Enzyme

On the ninth day of optimum α-amylase activity (Pfueller and Elliott, 1969) expressed by the isolate, the contents of flask were carefully filtered through glass fibre filter paper (Whatman GF/A). The protein content of the filtrates was determined using the Lowry *et al.* (1951) method.

Enzyme Assay

α-amylase

α-Amylase activity was determined using a modified method of Pfueller and Elliott (1969). Reaction mixtures consisted 2 ml of 0.2% (w/v) starch in 0.02 M citrate phosphate buffer, pH 6.0 as substrate and 0.5 ml of enzyme. Controls consisted only 2 ml of the prepared substrate. The contents of both experimental and control tubes were incubated at 35^{o}C for 20 min. Reaction in each tube was terminated with 3 ml of 1 N HCl. 0.5 ml enzyme was then added to the control tubes. 2ml mixture from each set of experimentals and controls was transferred into new sets of test tubes. 3ml of 0.1 N HCl was added into the contents of each test tube after which 0.1 ml of iodine solution was added. Optical density readings were taken at 670nm. One unit of α-amylase activity was defined as the amount of α-amylase that produced 0.1 percent reduction in the intensity of the blue colour of starch-iodine complex under assay conditions. Specific activity was determined and calculated as enzyme units per milligram protein.

Ammonium Sulphate Precipitation

The crude enzyme, on day of optimum activity was treated with ammonium sulphate (analytical grade, BDH) at 40-90% saturation. Precipitation was allowed at 4^{o}C for 24 h. Thereafter, the mixture was centrifuged at 6,000g for 30 minutes at 4^{o}C using a high-speed cold centrifuge (Optima LE-80K Ultracentrifuge, Beckman, USA) at the Central Laboratory, Obafemi Awolowo University, Ile-Ife, Nigeria. The supernatant was discarded. The precipitate was reconstituted in 0.02 M citrate phosphate buffer, pH 6.0. The protein content of the precipitated enzyme was determined (Lowry *et al.*, 1951). α-Amylase activity was also thereafter determined (Pfueller and Elliott, 1969).

Dialysis

With the aid of a multiple dialyzer and using acetylated dialysis tubings (Visking dialysis tubings, Sigma) (Whitaker *et al.* 1963), the ammonium sulphate precipitated enzyme was dialysed under several changes of 0.02 M citrate phosphate buffer, pH 6.0 at 4^{o}C for 24 h. The protein content of the dialysed enzyme was determined using the Lowry *et al.* (1951) method while α-amylase activity was determined using the modified method of Pfueller and Elliott (1969).

Results

The mutant strain of isolate *Aspergillus flavus* (IFE 03) expressed α-amylase activity within a period of nine days in the defined medium at 25^{O}C. The purification steps are represented in Table 1.Optimum activity of the crude was recorded as 44.4 Units/mg of protein. Total activity of crude extract was recorded as 4,265 Units with total protein being 96.0mg. After dialysis subsequent to ammonium sulphate precipitation, total activity reduced to 1,374 Units with total protein also reducing to 4.7mg. However, specific activity had increased from 44.4 Units/mg of protein to 292.3 Units/mg of protein. Upon ammonium sulphate precipitation and dialysis of the crude enzyme, specific activity had increased 6.58 fold

Table 1: Partial purification of α-amylase obtained from a mutant strain of *Aspergillus flavus* IFE 03

Fraction	Total Activity (Units)	Total Protein(mg)	Specific Activity (Units/mg of protein)	Yield %	Purification fold
Crude extract	4265	96.0	44.4	100	1
90% $(NH_4)_2SO_4$ Precipitation	1374	4.7	292.3	32.2	6.58

while recovery was 32.2%.

Discussion

In this investigation, the mutant strain of *Aspergillus flavus* IFE 03 isolated from yam (*Dioscorea rotunda*) expressed α-amylase activity in our defined medium having starch as carbon source and peptone as nitrogen source of fungal growth. The enzyme was expressed at 25 ^{o}C. Rodziewcz and Rymowicz (1999a) reported the biosynthesis of amylase from *Bacillus polymyxa* B-20 cells immobilized in calcium alginate and chitosane. In their investigation, optimum production medium consisted of yeast extract (5-10g/L), maltose, peptone and mineral salt. In another earlier study, *Aspergillus oryzae* transformants produced *Bacillus licheniformis* thermophilic α-amylase (optimum temperature, 76 ^{o}C; optimum pH, 9) bred using a strain of *Aspergillus niger* (Ozeki *et al.*, 1998). A UV-inducedmutant strain of *Aspergillus niger* (CFTR1-1105-U9) over-produced a starch hydrolyzing enzyme with properties characteristically different from the known amylases of the non-mutant strain (Suresh *et al.*, 1999).

The amino acid L-cysteine supported growth of the mutant *Aspergillus flavus* in this study. In a much previous investigation, rice medium containing 0.05% L-cysteine, and 0.2% yeast extract supported amylolytic expression by strains of *Bifidobacterium* (Hwang and Geun, 1999). Activity of the crude α-amylase expressed by our mutant strain of *A. flavus* was recorded as approximately 45 Units/mg of protein. This was the optimum activity expressed by the isolate in the defined medium and was at day nine of inoculation of medium. Raj *et al.* (1999) reported that *Micrococcus halobius* OR-1 from tapioca cultivar soil produced amylase with activity in cell-free supernatant reaching a maximum of 8.6 U/ml after 48 h. According to Adams (1997), *Sporothichum thermophile* Apinis produced amylase with activity reaching approximately 45.2M-Units after 5 days of growth.

The activity of amylases from free *Bacillus polymyxa* obtained under chemostat conditions were observed to be between 91 – 118 U/ml but 70 – 78 U/g beads from bacteria cell immobilized in chitosane gel beads within 96 h incubation (Rodziewcz and Rymowicz, 1999b).

Conclusively, the mutant strain of *Aspergillus flavus* used in this study is capable of production of α-amylase at 25 ^{o}C. This is an avenue for the industrial production of the enzyme in the tropics.

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