Effect of certain carbon sources of growth medium on α-amylase production by *Aspergillus versicolor* and *Aspergillus terreus*

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Abstract: *Aspergillus versicolor and Aspergillus terreus* isolated from deteriorated rice using potato dextrose agar grew at 25°C and expressed -amylase activity in a growth medium with potassium nitrate as nitrogen source and various carbon sources. The carbon sources which were varied were lactose, maltose, starch, sucrose, galactose and glucose. Rice also supported growth and induced -amylase production by these isolates. *Aspergillus terreus* seemed a better inducer.
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1. Introduction

The genus Aspergillus includes over 185 species and about 20 species have so far been reported as causing opportunistic infections in man (Tortora *et al*., 2004). Among these species, *Aspergillus fumigatus* is the most commonly isolated species followed by *Aspergillus flavus* and *Aspergillus niger. Aspergillus clavatus, Aspergillus glancus, Aspergillus oryzae, Aspergillus terreus, Aspergillus ustus* and *Aspergillus versicolor* are among the other species less commonly isolated as opportunistic pathogens (Brock and Madigan, 1991). Food infected by certain species of Aspergilli may be carcinogenic to humans and animals (Willey *et al*., 2008).

Amylases are hydrolytic enzymes that catalyse the degradation of starch molecules and other carbohydrates to yield dextrins and progressively smaller polymers composed of glucose units (Bohinski, 1983). The two types of amylases commonly encountered in microbial degradation of starch are and amylases.

The present investigation relates the inductive production of -amylases by the phytopathogens *Aspergillus versicolor* and *Aspergillus terreus* isolated from deteriorated rice with nature of supporting medium of growth and the possible exploration of this innovation in industrial application.

2. Materials and Methods

2.1 Materials

Starch (Sigma), maltose, sucrose, glucose, galactose, lactose, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, hydrated magnesium sulphate, hydrated iron sulphate, thiamine, biotin, L-cysteine, lactophenol cotton blue and potato dextrose agar (PDA) were products of Sigma and British Drug Houses (BDH). Sephadex G-100 (particle size 40-120µ), CM-Sephadex C-25 and CM-Sephadex C-50 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Ethanol, sodium azide, sodium carbonate, sodium hydroxide, hydrated copper sulphate, potassium sodium tartrate, iodine, potassium iodide, absolute ethanol , diethyl ether, pyridine, benzene, acetic anhydride, hydrochloric acid, citric acid, disodium hydrogen orthophosphate, catalase, glucose oxidase, human haemoglobin, egg albumin, horse myoglobin and cytochrome C were bought from British Drug Houses (BDH) Chemical Limited, Poole, England. Glass fibre filter papers were from Whatman. Rice (Caprice) was purchased from the Main Market, Ile-Ife, Nigeria.

Apparatus used included, top load weighing balance (Mettler PB 153), pH meter (Jenway 3015), electric stirrer, cold centrifuge (Optima LE-80K ultracentrifuge, Beckman, USA), UV/VIS spectrophotometer (Cecil 2041), automated LKB fraction collector (700A Ultro Rac), glass tube chromatographic columns (internal dimensions of 2.5 cm x 70 cm and 2.5 cm x 40 cm, Pharmacia, Sweden), water bath, Neubauer counting chamber (Gallenhamp), multiple dialyser (Pope Scientific Inc. Model 220, USA), portable autoclave (Express Equipment, Dixonøs Surgical Instruments Ltd, USA), heater (Mettler), inoculating chamber (Beckman, USA).

2.2 Methods

2.2.1 Sources and Identification of Isolates

The isolates of *Aspergillus versicolor* and *Aspergillus terreus* for this research were from

deteriorated rice and identified at the Seed Health Unit of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, using techniques contained in the illustrated Handbook of Fungi (Hanlin, 1990). The identification was done by observing cultural and morphological characteristics. Each isolate was cultured on Potato Dextrose agar. The nature of growth, rate of growth, colony colour and sporulation patterns were carefully observed. Sporulating mature cultures were used in microscopic examination. Fungal samples were taken from advancing margins and centres of the growth regions with the aid of sterile inoculating needle. The samples were smeared on glass slides and stained with lactophenol cotton blue. Macroscopic and microscopic morphological characteristics like arrangement and shape of spores, type of sporangia, type of hyphae, presence or absence of septa on hyphae were examined under the high power objective of a compound binocular microscope.

2.2.2 Culture Conditions and Preparation of Inocula

The isolates were subcultured and maintained on Potato Dextrose agar plates and slants. The fungi were further subcultured into test tubes of the same medium and incubated at 25°C. Ninety-six hr-old cultures were used in this investigation. According to the modified method of Olutiola and Ayres (1973), cultures were grown in a defined medium of the following composition: $MgSO_4.7H_20$ (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 added carbon (10 g) and nitrogen (9.9 g) sources (Sigma) in 1 litre of distilled water. The carbon sources used were varied and were starch (soluble), maltose, sucrose, glucose, galactose, lactose. The nitrogen source was potassium nitrate. Conical flasks (250 ml) containing 100 ml growth medium were inoculated with 1 ml of an aqueous spore suspension containing approximately $6x10^4$ spores per ml of isolate. Spores were counted using the Neubauer counting chamber (Olutiola *et al.*, 1991). Experimental and control flasks were incubated without shaking at 25^{0}C (Olutiola and Nwaogwugwu, 1982).

2.2.3 Rice as a Source of Carbon

Rice (Caprice) was bought at the Main Market, Ile-Ife, Nigeria. The rice was added to distilled water (1% w/v) and autoclaved at 15Ib/in^2 (121°C) for 15 minutes. 100 ml of the rice medium in conical flasks (250 ml) was inoculated with 1 ml of aqueous spore suspension containing approximately $6x10^4$ spores per ml of each isolate.

On a daily basis, the contents of each flask were filtered through glass fibre filter paper (Whatman GF/A). The protein content of the filtrates was determined as earlier described. The filtrates were assayed for -amylase activity using the method of Pfueller and Elliott (1969).

2.2.4 Enzyme Assay

2,2,4.1 α-Amylase

-Amylase activity was determined using the method of Pfueller and Elliott (1969) . The reaction mixtures consisted of 2 ml of 0.1% (w/v) starch in 0.2 M citrate phosphate buffer, pH 6.0 as substrate and 0.5 ml of enzyme. Controls consisted of only 2 ml of the prepared substrate. The contents of both experimental and control tubes were incubated at 35°C for 30 min. The reaction in each tube was terminated with 3 ml of 1 N HCl. Enzyme (0.5 ml) was then added to the control tube. Two millilitre of the mixture from each of the sets of experimentals and controls was transferred into new sets of clean test tubes. Three millilitre 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 620 nm. One unit of enzyme activity was defined as the amount of enzyme which produced 0.1 percent reduction in the intensity of the blue colour of starch-iodine complex under conditions of the assay.

2.2.5 Ammonium Sulphate Fractionation

The crude enzymes, on the days of optimum activity during daily basis samplings, were treated with ammonium sulphate (analytical grade) at 90% saturation (662 g/L). Precipitation was allowed to continue at 4°C for 24 h. The mixtures were centrifuged at 4,000 rpm for 30 minutes at 4°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 were discarded. The precipitate was redissolved in 0.2 M citrate phosphate buffer, pH 6.0. -Amylase activity was determined using the method of Pfueller and Elliott (1969).

2.2.6 Dialysis

Using acetylated dialysis tubings (Visking dialysis tubings, Sigma) (Whitaker *et al.* 1963) and a multiple dialyser, the enzyme preparations were dialysed under several changes of 0.2 M citrate phosphate buffer, pH 6.0 at 4°C for 24 h. -Amylase activity was determined using the method of Pfueller and Elliott (1969).

2.2.7 Fractionation of Enzyme using Sephadex G-100

Amylase from the isolate was subjected to further purification using gel filtration.

(a) Preparation and Packing of Sephadex G-100 Column

The Sephadex G-100 resin was suspended in buffer (0.2 M citrate phosphate, pH 6.0 containing 5 mM sodium azide) and allowed to swell for seventy two hours. Fine particles on the suspension were decanted. The suspension was deaerated under

vacuum pressure until a slurry of air-free gel particles was obtained. The buffer was also deaerated. The column was half filled with the buffer and the gel slurry added until the column was almost filled. The gel was left to settle in the column. The top of the column was connected to a reservoir and more gel added until the column was filled. The buffer was allowed to flow continuously through the gel throughout the process. When the column had been well equilibrated with the same buffer and resin and a constant bed height obtained, the enzymes were applied. A sample applicator (2.3 cm x 5.0 cm) was placed on top of the gel to prevent distortion when applying sample. Fractions of 5 ml were collected at a flow rate of 10 ml per hour.

(b) Calibration of Sephadex G-100 Column

The column was calibrated with proteins of known molecular weight (Andrews, 1964; Olutiola and Cole, 1976). Five milligram per ml each of the standard proteins was dissolved in 0.2 M citrate phosphate buffer, pH 6.0 containing 5mM sodium azide. The total sample volume of each standard protein was 5ml. The solution was applied to the column. Fractions (5 ml/tube) were collected in tubes using an automated LKB fraction collector (700A Ultro Rac). Protein of eluted fractions was monitored at 280 nm. The elution volume of each of the standard protein was plotted against the logarithm of its molecular weight to obtain a standard calibration curve (Andrews, 1964).

(c) Application of Enzyme to Sephadex G-100 Column

Ten milliliter of the dialysed enzyme was applied to the column and eluted with 0.2 M citrate phosphate buffer, pH 6.0 containing 5 mM of sodium azide. Fractions (5 ml/tube) were collected. Protein was monitored at 280nm. -Amylase activity was determined. The molecular weights of the unknown enzymes were extrapolated from the standard curve.

2.2.8 Further Fractionation using Ion-Exchange Chromatography

CM-Sephadex C-25 and CM-Sephadex C-50 columns were prepared as described in the Pharmacia manual. The resin were swollen in distilled water and equilibrated in the elution buffer. A column (2.5 x 40 cm) was used. Ten milliliter of pooled fractions from the Sephadex G-100 column which exhibited activity were applied to the prepared columns of CM-Sephadex C-50 and CM-Sephadex C-25. The columns were first washed with 0.2 M citrate phosphate buffer pH 6.0 containing 5 mM sodium azide to remove unbound proteins followed by elution with 0.2 M citrate phosphate buffer, pH 6.0, with linear gradients of 0.1 ó 0.5 M NaCl. Fractions (5 ml/tube) collected were monitored spectrophotometrically at 280nm. Amylase activity was determined using the method of Pfueller and Elliott (1969).

3. Results

3.1 Amylase activities of isolates on growth media

Aspergillus versicolor and *Aspergillus terreus* grew and exhibited amylase activities, varyingly, in modified growth medium used for this research. Using different carbon sources (rice, starch, maltose, sucrose, lactose, glucose and galactose) in the growth medium, with potassium nitrate as nitrogen source, amylase activity expressed by the isolates on the tenth day of incubation are shown in Table 1.

Table 1: Effect of carbon source on activity of amylase produced by isolates

Carbon source	Isolate	Amylase activity (Units) x 10^2
Rice	*Aspergillus versicolor* *Aspergillus terreus*	0.54 $\pm$ 0.01 0.73 $\pm$ 0.02
Galactose	*Aspergillus versicolor* *Aspergillus terreus*	0.06 $\pm$ 0.01 0.53 $\pm$ 0.13
Glucose	*Aspergillus versicolor* *Aspergillus terreus*	0.50 $\pm$ 0.04 0.63 $\pm$ 0.08
Lactose	*Aspergillus versicolor* *Aspergillus terreus*	0.10 $\pm$ 0.00 0.66 $\pm$ 0.10
Maltose	*Aspergillus versicolor* *Aspergillus terreus*	0.52 $\pm$ 0.03 0.68 $\pm$ 0.04
Starch	*Aspergillus versicolor* *Aspergillus terreus*	0.45 $\pm$ 0.04 0.57 $\pm$ 0.12
Sucrose	*Aspergillus versicolor* *Aspergillus terreus*	0.46 $\pm$ 0.05 0.69 $\pm$ 0.03

Each value represents the mean of three replicates with standard error

4. Discussion

With potassium nitrate as nitrogen source, the results of this investigation show that rice, glucose and maltose were best carbon sources for -amylase production by *Aspergillus versicolor.* Amylase production by *Aspergillus versicolor* was poor with galactose. Rice and sucrose were best inducers of -amylase by *Aspergillus terreus* with galactose being least inductive. All the carbon sources were relatively better inducers of -amylase with *Aspergillus terreus* than with *Aspergillus versicolor*. According to Coleman (1967), extracellular -amylase was secreted by *Bacillus subtilis* in a complex medium

containing maltose, starch, glycerol or glucose as carbon source. However, glucose best supported growth of *Penicillium steckii* from a variety of sources of carbon (Famurewa and Olutiola, 1991).

Amylases are industrially produced and are useful in detergents and as anti-staling agents. From the data generated from this investigation, *A. versicolor* and *A. terreus* are isolates that can be explored for the industrial production of -amylases. These enzymes can be conveniently purified, engaging the best procedures and marketed.

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