Effect of some nitrogen sources of growth medium on α-amylase production by *Penicillium solitum* and *Aspergillus rubrum* isolated from yam (*Dioscorea alata*)

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Abstract: *Penicillium solitum* and *Aspergillus rubrum* isolated from deteriorated yam (*Dioscorea alata*) using potato dextrose agar grew and sporulated at 25°C. They expressed Ŭ-amylase activity in a growth medium with starch as carbon source and certain nitrogen sources. The nitrogen sources were varied and were ammonium sulphate, glycine, potassium nitrate, ammonium chloride, peptone, sodium nitrate, tryptone and urea. *Aspergillus ribrum* seemed a better inducer of Ŭ-amylase as expressed by the tenth day of inoculation of medium.
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1. Introduction

Aspergilli and Penicilli are both of the taxanomic Family Trichomaceae (Alexopoulos, 1962). They infect crops and crop products invariably causing infection in humans and animals after consumption (Brock and Madigan, 1991). Food infected by certain species of Aspergilli may be carcinogenic to humans and animals (Willey *et al*., 2008). However, species of Penicilli produce antibiotics during crop infection, inhibiting bacteria growth (Tortora *et al.*, 2004).

Amylases are hydrolytic enzymes that speed up the degradation of starch molecules (Adejuwon, 2010). Ŭ-amylases cleave Ŭ-1,4-glycosidic bonds yielding dextrins. They have been implicated in yam deterioration (Adejuwon, 2011).

Ŭ-Amylases are applicable, industrially. The present study reveals the induction of this enzyme in yam (*Dioscorea alata*) phytopathogens, *Penicillium solitum* and *Aspergillus rubrum* by certain nitrogenous compounds of a growth medium, expressed within a time frame, and a possible industrial innovation.

2. Materials and Methods

2.1 Materials

Starch (Sigma), potassium nitrate, glycine, tryptone, peptone, sodium nitrate, ammonium sulphate, urea, ammonium chloride, 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). 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 were bought from British Drug Houses (BDH) Chemical Limited, Poole, England. Glass fibre filter papers were from Whatman.

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), water bath, Neubauer counting chamber (Gallenhamp), multiple dialyser (Pope Scientific Inc. Model 220, USA), portable autoclave (Express Equipment, Dixonôs Surgical Instruments Ltd, USA), heater (Mettler), inoculating chamber (Beckman, USA).

2.2 Methods

2.2.1 Sources and Identification of Isolates

The isolates, *Penicillium solitum* and *Aspergillus rubrum* for this research were from deteriorated yam (*Dioscorea alata*) 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 samples were smeared on glass slides and stained with lactophenol cotton blue.

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 culture of *Aspergillus rubrum* and one hundred and twenty-hr-old culture of *Penicillium solitum* 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 nitrogen sources used were varied and were ammonium sulphate, ammonium chloride, glycine, urea, potassium nitrate, tryptone, peptone and sodium nitrate. The carbon source was starch. Conical flasks (250 ml) containing 50 ml growth medium were inoculated with 1 ml of an aqueous spore suspension containing approximately $5x10^6$ 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 25^0C (Olutiola and Nwaogwugwu, 1982).

2.2.3 Enzyme and Protein Assays

2.2.3.1 α-Amylase

Ŭ-Amylase activity was determined using the method of Pfueller and Elliott (1969) . The reaction mixtures consisted of 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 of only 2 ml of the prepared substrate. The contents of both experimental and control tubes were incubated at 35°C for 20 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 670 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. Specific activity was expressed as enzyme unit per mg protein.

2.2.4 Ammonium Sulphate Fractionation

The crude enzymes, on the tenth day of inoculation of medium, were treated with ammonium sulphate (analytical grade) at 90% saturation. Precipitation was allowed to continue at 4°C for 24 h. The mixtures were centrifuged at 6,000 g 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 0.02 M citrate phosphate buffer, pH 6.0. Ŭ-Amylase activity was determined using the method of Pfueller and Elliott (1969). Protein content was determined using the Lowry *et al.* (1951) method.

2.2.5 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 0.02 M citrate phosphate buffer, pH 6.0 at 4°C for 24 h. Ŭ-Amylase activity was determined using the method of Pfueller and Elliott (1969). Protein content was determined using the Lowry *et al.* (1951) method.

3. Results

3.1 Amylase activities of isolates on growth media

Penicillium solitum and *Aspergillus rubrum* grew and expressed amylase activity in a modified growth medium used in this investigation. Some nitrogen sources (ammonium sulphate, glycine, potassium nitrate, sodium nitrate, ammonium chloride, urea, tryptone and peptone) of growth medium, with starch as carbon source induced activity, varyingly, as expressed by the tenth day of incubation (Table 1).

Table 1: Effect of nitrogen sources on activity of Ŭ-amylase produced by isolates

Nitrogen source	Isolate	Amylase activity (Specific Activity) (Units/mg Protein)
Ammonium sulphate	*Penicillium solitum*	0.38 ± 0.13
	Aspergillus rubrum	0.13 ± 0.03
Glycine	*Penicillium solitum*	0.00 ± 0.00
	Aspergillus rubrum	1.53 ± 0.48
Potassium nitrate	*Penicillium solitum*	0.25 ± 0.05
	Aspergillus rubrum	0.68 ± 0.03
Ammonium chloride	*Penicillium solitum*	0.13 ± 0.06
	Aspergillus rubrum	0.13 ± 0.03
Peptone	*Penicillium solitum*	0.13 ± 0.04
	Aspergillus rubrum	2.48 ± 0.03
Sodium nitrate	*Penicillium solitum*	0.25 ± 0.01
	Aspergillus rubrum	0.93 ± 0.07
Tryptone	*Penicillium solitum*	0.38 ± 0.03
	Aspergillus rubrum	0.24 ± 0.01
Urea	*Penicillium solitum*	0.00 ± 0.00
	Aspergillus rubrum	2.33 ± 0.08

Each value represents the mean of three replicates with standard error

4. Discussion

The results of this investigation show that Ŭ-amylase was not detected in the growth medium on the tenth day of inoculation, when glycine and urea were nitrogen sources of growth, for *Penicillium solitum*. Starch is expected to induce Ŭ-amylase production by these isolates. Earlier investigation, Adejuwon (2011), showed that starch is able to induce Ŭ-amylase in *Lasiodiplodia theobromae*, with potassium nitrate as source of nitrogen in medium of growth of similar composition. We are therefore not ruling out the possibility of repression of Ŭ-amylase in both situations or the degradation of Ŭ-amylase by a protease, induced by a specific defined nitrogen source. These postulations are therefore inferences of further investigations.

Also, amylases are industrially viable enzymes and the possibility of using these isolates as bio-markers of industrial production can be explored.

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