Chapter

α-Amylase Production by Toxigenic Strains of *Aspergillus* and *Penicillium*

Adekunle Odunayo Adejuwon
and Victoria Anatolyivna Tsygankova

Abstract

Aflatoxins are produced by a variety of fungal species and these have contributed to devastating health problems globally. However, apart from the capability of the production of aflatoxins, the productions of enzymes by like fungi have been explored. Aflatoxin B1-producing-toxigenic strains of *Aspergillus flavus* (A_1), *Aspergillus parasiticus* (A_2), *Penicillium citrinum* (P_1) and *Penicillium rubrum* (P_2) isolated from rice were grown on a defined medium with varying carbon and nitrogen sources. They were also grown on rice as sole carbon and nitrogen source for fungal growth. In an attempt to purify, the extracellular α-amylases produced were subjected to ammonium sulfate precipitation (40–90% saturation) followed by dialysis. The aflatoxin B1-producing toxigenic strains of *Aspergillus flavus* (A_1), *Aspergillus parasiticus* (A_2), *Penicillium citrinum* (P_1) and *Penicillium rubrum* (P_2) were able to produce α-amylases in both the growth medium with varying C and N sources of fungal and also in the rice medium. The most active α-amylase activity was produced by toxigenic *A. flavus* (A_1) with a value of 3.25 ± 0.15 Units and this was when ammonium sulfate was nitrogen source with starch as carbon source of fungal growth in the defined growth medium. These toxigenic fungal strains can be explored for the industrial production of α-amylases.

Keywords: α-amylase, toxigenic, fungi, aflatoxin B1

1. Introduction

1.1 *Aspergilli* and pathogenicity

Aspergilli are of the taxonomic Division Eumycota, Subdivision Eumycotina, Class Ascomycetes, Order Eurotiales, Family Trichocomaceae [1]. *Aspergillus* is a filamentous cosmopolitan and ubiquitous fungus commonly isolated from soil, plant debris and indoor air environment [2]. While the teleomorphic state exists for *Aspergillus species*, some are accepted to be mitosporic without any known sexual spore [3]. The genus *Aspergillus* includes over 185 species and about 20 species have so far been reported as causing opportunistic infections in man [4, 5]. Among these species, *Aspergillus fumigatus* is the most commonly isolated species followed by *Aspergillus flavus* and *Aspergillus niger. Aspergillus clavatus*, *Aspergillus glaucus*, *Aspergillus oryzae*, *Aspergillus terreus*, *Aspergillus ustus* and *Aspergillus versicolor* are

among the other species less commonly isolated as opportunistic pathogens [6, 7]. Food infected by *Aspergillus flavus* may be carcinogenic to humans and animals [8]. *Aspergillus flavus* is a saprophyte of grains. It produces mycotoxins in infected food [2]. Infection of peanuts (*Arachis hypogaea*) seeds by *Aspergillus flavus* and *Aspergillus parasiticus* is a serious problem that can result in aflatoxin contamination in the seed [9]. *Aspergillus flavus* produces aflatoxins B, G and cyclopiazonic acid CPA [10].

Beta-1,3-Glucanase activity in peanut seed is induced by infection with *Aspergillus flavus* [9]. Maize seeds are susceptible to *Aspergillus flavus* infection [11]. *Aspergillus flavus* causes Ear rot in corn with aflatoxin production. Resistance to aflatoxin production can be controlled by epistasis [12]. *Aspergillus flavus* causes kernel infection in maize, the Southwestern corn borer (SWCB) has been reported to substantially increase aflatoxin levels in such infection [13]. Kernels of corn genotype GT-MAS:gk are resistant to *Aspergillus flavus* [14]. A 14-KDa protein in corn kernel makes it resistant to *Aspergillus flavus* infection [15]. *Aspergillus flavus* found around corn storage cribs and bins are point sources of inoculum/infection with *Aspergillus flavus* in the corn agroecosystem [16]. Ear corn rot caused by *Aspergillus flavus* and *Aspergillus parasiticus* is severe in areas with high temperatures and drought [2]. *Aspergillus flavus* causes the post-harvest disease of *Arachis hypogaea* [3].

According to Norton [17], carotenoids in endosperm may decrease the amount of aflatoxin produced by *Aspergillus flavus. Aspergillus flavus* can be divided into S and L strains on the basis of sclerotial morphology [18]. Atoxigenic *Aspergillus flavus* L strain reduce formation of both sclerotia and aflatoxin when coinoculated with S strain isolate [18]. *Aspergillus flavus* L strain reduce formation of both sclerotia and aflatoxin when coinoculated with S strain isolate [18]. *Aspergillus flavus* produces aflatoxin in cotton seed with the S strain being highly toxigenic [19]. *Aspergillus parasiticus* isolated from soil from a corn field produced aflatoxin B(1) B(2) and G(1) G(2) [20]. *Aspergillus flavus* produces beta-glucuronidase [21]. *Aspergillus flavus* and *Aspergillus parasiticus* can contaminate agricultural crops with the production of toxic fungal metabolite aflatoxins. An endochitinase which is an inhibitory protein with M(r) of 29,000 is capable of inhibiting growth of *Aspergillus flavus* on maize [22]. Onion seeds stalk and flowers are susceptible to infection by *Aspergillus niger* Tiegh [23, 24]. The black-spored *Aspergillus* isolates that have been found to cause the disease fig smut are *Aspergillus niger* var. niger, *Aspergillus niger* var. awamori, *Aspergillus japonicus* and *Aspergillus carbonarius* [25]. Epiphytic fungi found on table grapes include *Epiccocum nigrum*, *Cladosporium herbarum*, *Alternaria alternata*, *Aspergillus niger* [26].

1.2 Penicilli and pathogenicity

Penicilli belongs to the taxonomic Division Eumycota, Subdivision Eumycotina, Class Ascomycetes, Order Eurotiales, Family Trichocomaceae [1]. Grape fruit green mold is caused by *Penicillium digitatum* [27]. *Penicillium* is common on citrus, gelly and preservatives. It is abundant in the soil and on decaying materials [3]. *Penicillium* spores are present in the air [3]. Studies have shown that *Penicillium* is important in the production of antibiotics such as Penicillin and Griseofluvin [5]. *Penicillium digitatum* causes the green mold of citrus fruits. Optimum temperature of their mycelia on such fruits is about 25°C [28]. The food borne pathogen *Listeria monocytogenes* has been observed to grow on apple infected with *Penicillium expansum* but not after 5 days [29]. The blue mold of decayed pear fruit is caused by *Penicillium expansum* [30]. *Penicillium expansum* has been observed to cause gray mold disease in apple and blue mold in pear [31]. *Penicillium* spp. have been isolated from pear stem [32] while *Penicillium italicum* cause

citrus blue mold and green mold [33, 34]. Postharvest green mold of oranges is caused by *Penicillium digitatum* [35, 36]. *Penicillium* sp. has been isolated from grapes [26]. *Penicillium expansum* causes the blue mold decay of pear [37]. *Penicillium digitatum* and *Penicillium italicum* cause postharvest green and blue molds of citrus fruits. Sporulation of both molds can be prevented or reduced by gaseous ozone without noticeable ozone phytotoxicity to the fruits [33, 34]. Apple fruits with stem pulls have been reported to be more susceptible to blue mold decay caused by *Penicillium expansum* than fruits with stems [38]. According to Spotts and Holz [39], *Penicillium expansum* can infect and cause disease in grape and plum fruits. Aqueous chlorine has been reported to reduce the viable spores of *Penicillium digitatum*, the causative fungi of the green mold and sour rot of citrus [40]. Sodium bicarbonate has been found to reduce postharvest decay of apple [41]. The level of resistance to decay of apple cultivars, caused by *Penicillium expansum*, varies from cultivar to cultivar [42]. Ziram but not calcium chloride control gray mold and bull's-eye rot, the postharvest decay of pear associated with the pathogen *Penicillium expansum* [43]. *Penicillium digitatum* has been associated with the postharvest green mold of oranges. Soda ash was observed to control this post-harvest disease [35, 36]. According to Smilanick et al. [35, 36], the effectiveness of imazalil for the control of citrus green mold caused by *Penicillium digitatum* improved significantly when the citrus fruits were treated with heated aqueous solutions of the fungicide as compared with the current commercial practice of spraying wax containing imazalil on the fruits. According to Smilanick et al. [44], fungicide applications with thiabendazole (TBZ) and sodium bicarbonate reduce green mold caused by *Penicillium digitatum* of citrus fruits and lemon fruits. It was also observed that pre harvest applications of thiophanate methyl to the fruits controlled postharvest green mold. Blue mold caused by *Penicillium* is an important postharvest disease of apple. *Penicillium expansum* and *Penicillium solitum* have been identified and isolated from rotten apple and pear fruits [45]. According to Sirois et al. [23, 24], onion seeds are affected by species of *Penicillium*.

1.2.1 α-Amylases

Amylases are hydrolytic enzymes that catalyze the degradation of starch molecules and other carbohydrates to yield dextrins and progressively smaller polymers composed of glucose units [46, 47]. Based on their pattern of catalysis and yield of products, amylases can be categorized as: alpha (α) amylase (endoamylase) (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1); beta (β) amylase (exoamylase) (1,4-α-D-glucan maltohydrolase, EC 3.2.1.2); glucoamylase (exohydrolase) (Glucan-1,4-α-glucosidase, EC 3.2.1.3); pullulanase (α-dextrin endo-1,6-α-glucosidase, EC 3.2.1.41); isoamylase (EC 3.2.1.68). Pullulanases and isoamylases are termed debranching enzymes [46, 48, 49]. They can be of plant and microbial sources [50–52].

1.2.2 Microbial α-amylases

α-Amylases are produced by bacteria and fungi [53–58]. The two types of amylases commonly encountered in microbial degradation of starch are α and β amylases.

Degradation of substrate is important in enzymatic hydrolysis [59]. Starch is the substrate used in microbial amylase assay [60]. The starch molecules are hydrolyzed into polymers of glucose units [47]. According to Vihinen and Mantsala [61], starch-degrading enzymes are widely distributed among microbes and several activities are required to hydrolyze the starch into glucose units. *Bacillus subtilis* isolated from flour mill wastes produced a thermostable α-amylase in a complex medium containing starch [62]. According to Ajayi and Fagade [63], corn starch

can be used as substrate for β-amylase production by *Bacillus macerans*, *Bacillus licheniformis*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus megaterium*, *Bacillus polymyxa*, *Bacillus cereus* and *Bacillus subtilis*. According to Reiss et al. [64], approximately 80% of potato starch and 40–50% of grain starch were hydrolyzed by alpha amylase of certain microbes. Lactic acid bacteria have been found to ferment starchy foods to recover RNA though digestion with alpha amylase did not improve extraction [65]. *Rhizopus oligosporus*, a prolific amylase producer can degrade cassava tuber containing 65% starch into glucose [66]. *Thermoactinomyces thalpophilus* isolated from flour mill waste has been found to be capable of hydrolyzing 2% soluble starch [62]. A thermostable α-amylase activity from *Bacillus subtilis* isolated from flour mill waste was found to be more strongly expressed with corn starch than soluble starch [62]. Alpha amylase from *Bacillus licheniformis*, an hyperthermostable enzyme, is able to hydrolyze starch to medium-size oligosaccharides [67]. *Fusarium moniliforme* was found to produce alpha amylase in a culture medium containing starch [68].

Certain environmental (physical) factors affect amylase activity [48]. Lactic acid was found to be produced from *Lactobacillus delbrueckii* subsp.delbrueckii and defatted rice bran powder containing starch with coupled saccharification with amylase at 37°C and pH 5.0 [69]. An α-amylase produced by *Bacillus* sp. isolated from soil sample was optimally active at 75–80°C [70]. Alpha-amylase from *Bacillus licheniformis* is able to hydrolyze soluble starch within a temperature range of 60–75°C [71]. A thermophilic, moderately halophilic anaerobic *Halothermothrix orenii* synthesized an amylase similar to *Bacillus megaterium* amylase with optimal activity at 65°C [72]. Thermophilic *Thermus* sp. was reported to produce an extracellular α-amylase able to degrade starch at 70°C [73]. *Bacillus stearothermophilus* was found to produce a thermostable α-amylase active at 43°C [74]. According to Saito [75], *Bacillus licheniformis* produced a thermophilic extracellular α-amylase stable at 25°C but more active at an optimum temperature of 76°C. Manning and Campbell [76] reported that *Bacillus stearothermophilus* synthesized a thermostable α-amylase. *Rhizopus arrhizus* and *Rhizopus oryzae* were found to be capable of hydrolyzing starch at 30°C [77]. *Bacillus halodurans* produced an alkaline active maltohexaose-forming α-amylase active at 60°C. According to Oh et al. [57], *Lactobacillus gasseri* is able to synthesize a maltogenic amylase exhibiting optimum activity for β-CD hydrolysis at 55°C. Based on studies carried out by Najafi and Kembhavi [50], a marine *Vibrio* sp. produced an extracellular α-amylase with maximum activity at 55–60°C. According to Ogasahara et al. [78], *Bacillus stearothermophilus* was able to produce a thermophilic α-amylase with optimum temperature range of 65–73°C.

α-Amylase from *Pyrococcus woesei* has maximal activity at pH 5.6 [79]. A *Bacillus* sp. isolated from piglet cacum produced an extracellular alpha amylase optimally active at pH 7.0 [80]. A microorganism from uncultured soil was observed to produce amylolytic enzyme with optimal pH of 9.0 [81]. A mutant of *Bacillus amyloliquefaciens* has been reported to synthesize alpha amylase with optimal activity at pH 7.0 [82]. An extracellular alpha amylase isolated from cell free broth of *Streptomyces megasporus* grown in glucose, soluble starch and raw starch was stable at a pH range of 5.5–8.5 but with optimum activity at pH 6.0 [83]. Amylases in culture supernatants of an environmentally derived microbial mixed culture selected for its ability to utilize starch-containing plastic films as sole carbon sources produced amylases active at pH 5.5 and 8.0 [84]. Alpha amylase from *Thermoactinomyces vulgaris* had optimum activity at pH 4.8–6.0 [85]. Starch degradation by *Rhizopus oryzae* was favorable at pH 6.0 [77]. Akindahunsi [86] reported that waste water from cassava mash fermented by pure strains of *Saccharomyces cerevisiae*, *Lactobacillus delbrueckii* and *Lactobacillus coryniformis* produced amylase after 3 days with maximal activity at pH 6.0.

According to Mijts and Patel [72], the thermophilic, moderately halophilic anaerobic *Halothermothrix orenii* is able to synthesize alpha amylase active with specific activity of 2232 U mg^{-1} requiring $CaCl_2$ for optimum activity and thermostability. The maltooligosaccharide-forming amylase from *Bacillus circulans* is enhanced by $C0^{2+}$ and Mg^{2+} [87]. Amylase synthesized by *Lipomyces starkeyi* was found to be actively stable in a commercial mouthwash [88]. A salt-tolerant thermostable amylase produced by *Bacillus megaterium* was reported to be stable at 5 M NaCl [89]. A thermophilic *Thermoascus aurantiacus* has been observed to produce amylase with thermostability enhanced by calcium chloride [90]. Amylase production from *Bacillus sphaericus* was reported to be maximum with 3 mM divalent cations Mg^{++} and Ca^{++} incorporated in a growth medium [91]. Cadmium, Cobalt, Copper, Manganese, Nickel and Lead incorporated into Czapek-Dox liquid medium supported growth and production of amylase by soil yeasts *Geotrichum capitatum* and *Geotrichum candidum* [92]. Activity of α-amylase from a marine *Vibrio* sp. was found to be restored by Fe^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+} and Cu^{2+} to nearly 25–55% [50]. A *Bacillus* sp. produced an alkaliphilic amylase which was enhanced by Na^{+} and Co^{2+} [93]. According to Mishra et al. [94], *Bacillus subtilis* produced an α-amylase. Herbizid has been reported to activate amylase production in culture of *Fusarium oxysporum*, *Mucor niemalis* and *Penicillium chrysogenum* [95].

Amylase from *Fusarium verticillioides* has been found to be inhibited by a hydrophobic 19.7-KDa inhibitor from corn kernel [96, 97]. *Fusarium moniliforme*, a mycotoxigenic fungus has been reported to produce an amylase inhibited by a specific amylase inhibitor found in corn [96, 97]. Alpha-amylase inhibitor has been isolated from culture medium of *Streptomyces parvullus* [98]. *Streptomyces aureofaciens* produces a novel polypeptide inhibitor [99]. A strain of *Streptomyces nigrifaciens* has been reported to produce an amylase inhibitor having inhibitory effects on alpha amylase and glucoamylase [100].

Bacillus subtilis isolated from soil produced a starch degrading amylase with molecular weight of 50 KDa and an isoelectric point of 4.9 [101]. *Streptomyces lividans* has been reported to have a molecular weight of 107,054 KDa [102]. Alpha amylases from some *Bacillus* spp. were detected to possess molecular weight of approximately 65,5854 and 49 KDa [103]. Alpha-amylase of *Clostridium thermosulfurogenes* has been reported to have a molecular mass of 75,112 Da [104]. According to Kang et al. [105], *Bacillus stearothermophilus* produces an alpha-amylase which was glycosylated and with molecular weights of approximately 61–75 KDa.

According to Lorentz [106], Protected 4-Nitrophenyl-1,4–1-D-maltoheptaoside can be used in routine amylase assay. A simple and rapid method using Remazol Brilliant Blue-starch as substrate which is non-destructive allows direct visualization and isolation of amylolytic microorganisms from the environment [107].

Alpha amylase can be used in improving anaerobic solid waste treatment [108]. Carbohydrate-hydrolyzing enzymes have long been used by industrial product markers as major catalysts to transform raw materials into end products in such areas as food processing, beverage production, animal nutrition, leather and textiles [109].

With the advent of new frontiers in biotechnology, the spectrum of amylase application has widened in many fields such as clinical, medicinal and fine-chemical industries, as well as a widespread application of starch saccharification in the textile, food, brewing and distilling industries [110].

1.2.3 Aflatoxin B_1 and α-amylase production

According to Mellon et al. [111], an aflatoxin B1 producer strain of *Aspergillus flavus* seem to possess the ability to produce numerous extracellular hydrolases

(α-amylase inclusive). Aflatoxin B1 have been detected in groundnut and maize contaminated with *Aspergillus flavus* [112]. The removal of lipids from ground substrates significantly reduced the substrate's potential for aflatoxin B1 (AFB1) production by *Aspergillus flavus*. However, maltose, glucose, arginine, glutamic acid, aspartic acid and zinc significantly induced the AFB1 production up to 1.7–26.6 fold [113]. Aflatoxins B_1, B_2, G_1 and G_2 and α-amylase were detected in *Aspergillus oryzae*, *Aspergillus flavus*, *Talaromyces spectabilis*, *Pacilomyces variotii* and *Lichtheimia* sp. isolated from nuruks in several regions of Korea [114]. According to Fakhoury and Woloshuk [115], a mutant strain of *Aspergillus flavus* failed to produce extracellular α-amylase when the Amy1 gene necessary for the production of α-amylase was disrupted in an aflatoxigenic strain (an aflatoxin B1 producing strain) of the fungus. Mycotoxigenic strains (aflatoxin B1 producing strains) of *Fusarium moniliforme* and *Aspergillus flavus* were capable of α-amylase production in a medium composed of 2% ground corn in milky stage corn [116]. In their attempt to increase aflatoxin B1 resistance in maize, Rajasekaran et al. [117] discovered that the α-amylase inhibitor-like protein (AILP) seem to play a role in the inhibition of *Aspergillus flavus* α-amylase and fungal growth. Fountain et al. [118] reviewed the nature of the interaction occurring between aflatoxin production by *Aspergillus flavus*, the environment in which the fungus thrives and its susceptibility to crop host before harvest. They proposed future directions for elucidating future relationship between resistance and susceptibility to the fungus' colonization, abiotic stress and its relationship to oxidative stress in which its aflatoxin B1 production may function as a form of antioxidant protection to the producing fungus. In a known positive transcriptomic database, E-probe Diagnostic for Nucleic acid Analysis (EDNA), a bioinformatic tool, originally developed to detect plant pathogens in mutagenomic databases, is capable of discriminating between production and non-production of aflatoxin B1 by *Aspergillus flavus* [119]. Substrate-induced lipase gene expression might be indirectly related to aflatoxin formation by providing the basic building block "acetate" for aflatoxin B1 synthesis in aflatoxin-producing *Aspergillus flavus* and *Aspergillus parasiticus* [120]. According to Smith et al. [121], silencing of the aflatoxin gene cluster in a certain strain of aflatoxin B1 producing *Aspergillus flavus* is suppressed by ectopic aflR gene (the transcriptional regulator of the aflatoxin biosynthetic gene cluster) expression.

1.2.4 Rice (Oryza sativa)

Rice (*Oryza sativa*) is a monocotyledonous cereal which belongs to the Grass family Gramineae or Poaceae [122]. With over 7000 varieties of rice, its pericarp and embryo contain 70–80% starch, 7% proteins, 1.5% oils, some vitamins (mostly A, B and C) and some essential minerals [3]. According to Sizer and Whitney [123], rice contains fiber and the vitamin folate and provides 80% of the calories consumed by humans worldwide [122]. It contains 12 chromosomes in a haploid set [124]. The domestication of rice formed part of the basis for civilization in the near East, far East and the New World [125]. Feeding more people worldwide than any other crop, rice is the only crop grown exclusively for human consumption [125]. Sedentary irrigated rice production in tropical lowlands can support hundreds of people per square kilometer, explaining the wide spread importance of rice crops in the tropics [126]. The discovery of Gibberellins arose from infected rice [122]. *Oryza sativa* is the main cultivated rice species but over 20 species in the genus are known [125].

This research was designed to examine the production and activity of α-amylases by some toxigenic aflatoxin B1-producing strains of *Aspergillus* and *Penicillium* isolated from deterioration rice. Attempts were made to purify the α-amylases.

1.2.5 Contribution to knowledge

The present research will establish the presence of α-amylases in rice during mycotic spoilage by toxigenic strains of *Aspergillus flavus* (A_1), *Aspergillus parasiticus* (A_2), *Penicillium citrinum* (P_1) and *Penicillium rubrum* (P_2). These fungi, being capable of producing these enzymes can be used in the production of amylases. Rice as substrate can be explored in such production.

Amylases are used in clinical chemistry most especially in diagnosis. Their combination with proteases and lipases are also employed industrially in the bioremediation of recalcitrants/organic pollutants and the hydrolytic digestion of the peptidoglycan layers of both gram positive and gram negative bacteria in wastewaters before chlorination [109].

2. Materials and methods

2.1 Sources and identification of isolates

The isolates of aflatoxin B1-producing-toxigenic strains of *Aspergillus flavus* (A_1), *Aspergillus parasiticus* (A_2), *Penicillium citrinum* (P_1) and *Penicillium rubrum* (P_2) for this research were from deteriorated rice and identified at the Seed Health Unit of the International Institute for Tropical Agriculture, Ibadan, Nigeria using techniques contained in the illustrated Handbook of fungi [127, 128].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 color and sporulation patterns were carefully observed. Sporulating mature cultures was used in microscopic examination. Fungal samples were taken from advancing margins and centers of the growth regions with the aid of sterile inoculating needle. The samples were smeared on glass slides and stained with lactophenol cotton blue. After placing the cover slips, macroscopic and microscopic morphological characteristics like arrangement and shape of spores, type of sporangia, type of hyphae, presence or absence of septa on hyphae was examined under the high power objective of a compound binocular microscope.

2.2 Culture conditions and preparation of inocula

The isolates were subcultured and maintained on Potato Dextrose agar plates and slants. Each fungus was further subcultured into test tubes of the same medium and incubated at 25°C. A 96-hr-old culture of toxigenic strains of *Aspergillus flavus* (A_1), *Aspergillus parasiticus* (A_2) and *Penicillium rubrum* (P_2) and 120-h-old culture of *Penicillium citrinum* (P_1) was used as inocula. According to the modified method of Olutiola and Ayres [129], cultures was grown in a defined medium of the underlisted composition: $MgSO_4.7H_20$, K_2HPO_4, KH_2PO_4, L-cysteine, biotin, thiamine and $FeSO_4.7H_20$ with added carbon and nitrogen sources (Sigma). Conical flasks (250 ml) containing 100 ml growth medium will be inoculated with 1 ml of an aqueous spore suspension containing approximately 6×10^4 spores per ml of each isolate. Experimental and control flasks was incubated without shaking at 25°C [130].

2.3 Rice as a source of carbon

Rice (Caprice) from Spain was bought at the main market, Bodija, Ibadan, Nigeria. The rice was added to distilled water (1% w/v) and autoclaved at 15Ib/in^2 at

121°C. Experimental Conical flasks (250 ml) containing 100 ml of the rice medium was inoculated with 1 ml of an aqueous spore suspension containing approximately 6×10^4 spores per ml of each isolate. Control flasks contained sterilized rice medium not inoculated with aqueous spore suspension of the isolate. Experimental and control flasks was incubated without shaking at 25°C.

On a daily basis, the contents of each flask was filtered through glass fiber filter paper (Whatman GF/A). The protein content of the filtrates was determined using the method of Lowry et al. [131]. The filtrates were analyzed for amylase activity using the modified methods of Pfueller and Elliott [132] and Xiao et al. [133]. The filtrates were used as crude preparation.

2.4 Ammonium sulfate fractionation

The crude enzymes were treated with ammonium sulfate (analytical grade) within the limits of 40–90% saturation. Precipitation was allowed to continue at 4°C for 24 h. The mixtures were then centrifuged 10,000 g for 30 min at 4°C using a high speed cold centrifuge (Optima LE-80 K Ultracentrifuge, Beckman, USA). The supernatant was discarded. The precipitate was re-dissolved in 0.2 M citrate phosphate buffer, pH 6.0. The protein contents were determined using the Lowry et al. [131] method while amylase activity was determined using the modified methods of Pfueller and Elliott [132] and Xiao et al. [133].

2.5 Dialysis

Using acetylated dialysis tubings (Visking dialysis tubings, Sigma) [134] and a multiple dialyser (Pope Scientific Inc. Model 220, USA), the enzyme preparations were dialysed under several changes of 0.2 M citrate phosphate buffer pH 6.0 at 4°C for 24 h. The protein contents of the dialysed enzymes were determined using the Lowry et al. [131] method while amylase activity was determined using the modified methods of Pfueller and Elliott [132] and Xiao et al. [133].

2.6 Enzyme assay

Both experimental (fungal isolate inoculated) and control (un-inoculated) flasks were assayed for amylase activity.

2.6.1 α-Amylase

α-Amylase activity was determined using the modified methods of Pfueller and Elliott [132] and Xiao et al. [133]. The reaction mixtures consisted 2 ml of 0.1% (w/v) starch (Sigma) in 0.2 M citrate phosphate buffer, pH 6.0 as substrate and 0.5 ml of enzyme. These were the experimentals in the assay procedure. The controls in the assay procedure consisted only 2 ml of the prepared substrate. The contents of both experimental and control tubes were incubated at 35°C for 30 min. The reactions were terminated with 3 ml of 1 N HCl. Enzyme (0.5 ml) was added to the contents of each control. About 2 ml of the mixture from each of the sets of experimentals and controls was transferred into new sets of clean test tubes. About 3 ml 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 spectrophotometrically at 620 nm. Enzyme activity was defined in units and specific activity as enzyme units per mg protein.

One unit of α-amylase activity was defined as the amount of enzyme which produced 0.1% reduction in the intensity of the blue color of starch-iodine complex under conditions of the assay.

3. Results

3.1 Amylase activities of isolates on growth media

Toxigenic strains of *Aspergillus flavus* (A_1), *Aspergillus parasiticus* (A_2), *Penicillium citrinum* (P_1) and *Penicillium rubrum* (P_2) 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, amylase activity expressed by each isolate on the tenth day of incubation is shown in **Table 1**.

With different sources of nitrogen (NH_4Cl, urea, KNO_3, ammonium sulfate, glycine, sodium nitrate, tryptone and peptone) in the growth medium, amylase activity expressed varyingly by each isolate on the tenth day of incubation is shown in **Table 2**.

Toxigenic *P. citrinum* (P_1) produced active α-amylase (0.75 ± 0.01 Units) and this was when potassium nitrate was nitrogen source with maltose as carbon source of the defined growth medium. Toxigenic *A. parasiticus* (A_2) also expressed an α-amylase activity value of 0.72 ± 0.04 Units when rice was both carbon and nitrogen source of medium for fungal growth (**Table 1**).

Carbon source	Isolate	Amylase activity (Units)
Rice	*Aspergillus flavus* (A_1)	0.54 ± 0.01
	Aspergillus parasiticus (A_2)	0.72 ± 0.04
	Penicillium citrinum (P_1)	0.43 ± 0.23
	Penicillium rubrum (P_2)	0.62 ± 0.06
Galactose	*Aspergillus flavus* (A_1)	0.06 ± 0.01
	Aspergillus parasiticus (A_2)	0.53 ± 0.13
	Penicillium citrinum (P_1)	0.36 ± 0.05
	Penicillium rubrum (P_2)	0.09 ± 0.04
Glucose	*Aspergillus flavus* (A_1)	0.50 ± 0.04
	Aspergillus parasiticus (A_2)	0.63 ± 0.08
	Penicillium citrinum (P_1)	0.44 ± 0.08
	Penicillium rubrum (P_2)	0.32 ± 0.11
Lactose	*Aspergillus flavus* (A_1)	0.10 ± 0.00
	Aspergillus parasiticus (A_2)	0.66 ± 0.10
	Penicillium citrinum (P_1)	0.40 ± 0.17
	Penicillium rubrum (P_2)	0.37 ± 0.08
Maltose	*Aspergillus flavus* (A_1)	0.52 ± 0.03
	Aspergillus parasiticus (A_2)	0.68 ± 0.04
	Penicillium citrinum (P_1)	0.75 ± 0.01
	Penicillium rubrum (P_2)	0.58 ± 0.12
Starch	*Aspergillus flavus* (A_1)	0.45 ± 0.04
	Aspergillus parasiticus (A_2)	0.57 ± 0.12
	Penicillium citrinum (P_1)	0.68 ± 0.03
	Penicillium rubrum (P_2)	0.60 ± 0.14
Sucrose	*Aspergillus flavus* (A_1)	0.46 ± 0.05
	Aspergillus parasiticus (A_2)	0.69 ± 0.03
	Penicillium citrinum (P_1)	0.59 ± 0.13
	Penicillium rubrum (P_2)	0.39 ± 0.06

Each value represents the mean of three replicates with standard error.

Table 1.
Effect of carbon sources on activity of amylase produced by isolates.

Nitrogen source	Isolate	Amylase activity (Units)
Ammonium sulfate	*Aspergillus flavus* (A_1)	3.25 ± 0.15
	Aspergillus parasiticus (A_2)	0.05 ± 0.00
	Penicillium citrinum (P_1)	0.38 ± 0.13
	Penicillium rubrum (P_2)	0.13 ± 0.03
Glycine	*Aspergillus flavus* (A_1)	0.38 ± 0.13
	Aspergillus parasiticus (A_2)	2.48 ± 0.03
	Penicillium citrinum (P_1)	0.00 ± 0.00
	Penicillium rubrum (P_2)	1.53 ± 0.48
Potassium nitrate	*Aspergillus flavus* (A_1)	0.50 ± 0.00
	Aspergillus parasiticus (A_2)	1.30 ± 0.10
	Penicillium citrinum (P_1)	0.25 ± 0.25
	Penicillium rubrum (P_2)	0.68 ± 0.03
Ammonium chloride	*Aspergillus flavus* (A_1)	3.02 ± 0.18
	Aspergillus parasiticus (A_2)	0.05 ± 0.05
	Penicillium citrinum (P_1)	0.13 ± 0.13
	Penicillium rubrum (P_2)	0.13 ± 0.03
Peptone	*Aspergillus flavus* (A_1)	0.25 ± 0.00
	Aspergillus parasiticus (A_2)	1.50 ± 0.15
	Penicillium citrinum (P_1)	0.13 ± 0.13
	Penicillium rubrum (P_2)	2.48 ± 0.03
Sodium nitrate	*Aspergillus flavus* (A_1)	0.38 ± 0.13
	Aspergillus parasiticus (A_2)	1.48 ± 0.13
	Penicillium citrinum (P_1)	0.25 ± 0.00
	Penicillium rubrum (P_2)	0.93 ± 0.73
Tryptone	*Aspergillus flavus* (A_1)	0.25 ± 0.00
	Aspergillus parasiticus (A_2)	0.20 ± 0.05
	Penicillium citrinum (P_1)	0.38 ± 0.13
	Penicillium rubrum (P_2)	2.40 ± 0.10
Urea	*Aspergillus flavus* (A_1)	0.15 ± 0.00
	Aspergillus parasiticus (A_2)	2.32 ± 0.03
	Penicillium citrinum (P_1)	0.00 ± 0.00
	Penicillium rubrum (P_2)	2.33 ± 0.08

Each value represents the mean of three replicates with standard error.

Table 2.
Effect of nitrogen sources on activity of amylase produced by isolates.

Toxigenic *A. flavus* (A_1) produced the most active α-amylase (3.25 ± 0.15 Units) and this was when ammonium sulfate was nitrogen source with starch as carbon source of the defined growth medium. Toxigenic *A. flavus* (A_1) also expressed an α-amylase activity value of 3.02 ± 0.18 Units when starch was carbon source and ammonium chloride was nitrogen source of the defined fungal growth medium (**Table 2**).

4. Discussion

The results of this investigation show that the toxigenic strains of *A. flavus* (A_1), *A. parasiticus* (A_2), *P. citrinum* (P_1) and *P. rubrum* (P_2) grew in a synthetic medium with varying carbon and nitrogen sources exhibiting α-amylase activities. α-Amylase activities were detected in the extracts of growth medium with rice as carbon source, infected with the toxigenic strains of *A. flavus* (A_1), *A.*

parasiticus (A_2), *P. citrinum* (P_1) and *P. rubrum* (P_2). When the carbon source was varied, potassium nitrate was the nitrogen source. When the nitrogen source was varied, starch was the carbon source for fungal growth. According to Olutiola [135], *Aspergillus chevalieri* from moldy maize produced extracellular amylase when grown in a liquid medium containing starch as carbon source. According to Barnett and Fergus [136], increasing the amount of starch-yeast extract medium increased the extracellular amylase produced by *Humicola lanuginosa*. Studies carried out by Okafor et al. [137] revealed that *Lactobacillus delbrueckii*, *Lactobacillus coryniformis* and *Saccharomyces* sp., isolated from cassava processing environments were high amylase producers. Among a series of starch sources of carbon, wheat and soluble starch were inducers of a thermostable amylase by a yeast strain isolated from starchy soil [138]. According to Bluhm and Woloshuk [139], amylopectin, an important constituent of starch, induces fumonisin B(1) production in *Fusarium verticillioides* during colonization of maize. According to Coleman [140], extracellular α-amylase was secreted by *Bacillus subtilis* in a complex medium containing maltose, starch, glycerol or glucose as carbon source; the general characteristics of secretion indicated a low but definite production of exoenzyme from the moment the cells of the organism started to grow until the end of the logarithmic phase after which, the rate of increase in cell mass decreased, the rate of enzyme secretion increased to a high linear value which was maintained even in the stationary phase.

4.1 Significance of study

Aflatoxin B1-producing-toxigenic strains of *Aspergillus flavus*, *Aspergillus parasiticus*, *Penicillium citrinum* and *Penicillium rubrum* can be explored industrially for α-amylase production using the specific growth medium and the rice medium used in this investigation. Varying the specific C and N source of this growth medium is of upmost significance in such an exploration.

4.2 Limitations

The genetic make-ups of these aflatoxin B1-producing-α-amylase-producing fungal strains are important in their ability to produce the enzyme α-amylase. Specific genes are necessary and important in the production of this enzyme. Mutant strains lacking the specific genes for α-amylase production will not be ideal in the exploration for production of the enzyme. More so, there seems to be a significant relationship between the ability to produce α-amylase and aflatoxin B1 production in mycotoxigenic fungi from literature.

5. Conclusion

The toxigenic strains of *A. flavus* (A_1), *A. parasiticus* (A_2), *P. citrinum* (P_1) and *P. rubrum* (P_2) can be explored in the industrial production of α-amylases.

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A. Appendix

A.1 Acetylation of cellophane tubings [134]

Material
Visking dialysis tubings (Sigma- Aldrich).
Reagents

(i) Aqueous ethanol (50% V/V)

(ii) Absolute ethanol

(iii) Diethyl ether

(iv) A mixture of benzene, acetic anhydride and pyridine in the ratio 5:4:2 (V/V)

(v) 10% KCl (10 g of KCl in 100 ml distilled water)

Procedure
The cellophane tubings were filled with distilled water and soaked in distilled water for 24 hours. The tubings were then soaked in turn, for 30 min each time in 50% ethanol, absolute ethanol and diethyl ether successively. The tubings were thereafter soaked in the mixture of benzene, acetic anhydride, and pyridine, prepared as described above, for 18 hours. Each tubing was then properly rinsed in distilled water and stored in 10% KCl solution at 4°C until required.

A.2 Protein content determination [131]

Reagents

(i) Reagent A—2% Na_2CO_3 in 0.1 N NaOH

(ii) Reagent B—0.5% $CuSO_4.5H_2O$ in 1% Sodium Potassium tartrate

(iii) Reagent C—50 ml of reagent A mixed with 1 ml of reagent B

(iv) Folin-Ciocalteu's phenol reagent (Sigma-Aldrich Chemie GmbH, Fluka Biochemika) diluted with distilled water in the ratio 1:1(V/V). This is labeled reagent D.

Procedure
5 ml of reagent C was added to 1 ml of the test sample. This was thoroughly mixed and left at room temperature for 10 min. Thereafter, 0.5 ml of reagent D was added and allowed to remain at room temperature for 30 min. Absorbance was determined at 620 nm.

Serial dilutions of Bovine serum albumin (Sigma) were treated likewise and used to plot standard graph. The unknown protein value in each test sample is meant to be extrapolated from the standard graph.

A.3 Iodine solution

(0.3% Iodine in 3% KI)

Reagents

(i) Iodine

(ii) Potassium iodide (KI)

Procedure

3 g of KI was dissolved in 100 ml of warm distilled water. 0.3 g of Iodine was thereafter added and allowed to dissolve in the solution by mixing and warming.

Author details

Adekunle Odunayo Adejuwon[1*] and Victoria Anatolyivna Tsygankova[2]

1 School of Health Information Management, University College Hospital, Ibadan, Nigeria

2 Department for Chemistry of Bioactive Nitrogen-Containing Heterocyclic Compounds, Institute of Bioorganic Chemistry and Petrochemistry of the National Academy of Sciences of Ukraine (NAS), Kyiv, Ukraine

*Address all correspondence to: ao_adejuwon@yahoo.ca

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