RESEARCH ARTICLE

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Characterization of α-amylase and antimicrobial activity of *Penicillium chrysogenum*

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ABSTRACT

Fungi have been used to produce a number of bioactive compounds that include enzymes and antibacterial agents with unique characteristics *in vitro*. *Penicillium chrysogenum* is a mould with great potential for producing important secondary metabolites. This study aimed to characterize α-amylase and antimicrobial activity of *P. chrysogenum*. The organism was grown in a solid-state fermentation (SSF) medium using cassava peels as the only carbon source. The molecular mass of the amylase was estimated to be 59 kDa by combined results of Amicon Ultra-15 filter unit (Sigma) 50 kDa MWCO and polyacrylamide gel electrophoresis (PAGE). The optimum activity of 93.1 U/mL was noticed at 30ºC and pH of 5.8. A stability assay revealed that the enzyme was stable at 40°C and 50°C by retaining 66% and 37% residual activities, respectively. Also, crude extract of *P. chrysogenum* was found to inhibit *Pseudomonas aeruginosa* and *Staphylococcus aureus* with minimum inhibitory concentrations (MIC) of 25 and 12.5 mg/mL respectively. Therefore, these properties make *P. chrysogenum* an interesting fungus that has the potential of being used to produce both antibacterial agents and α-amylase for the bio-economy.

Key words: hydrolytic enzymes; molecular mass; fungal species; bacterial culture; enzyme assay

Introduction

Increasing demand, the need for cost reduction, natural resources depletion, and safety are among the factors responsible for microbial enzymes production (Choi et al., 2015), as well as antimicrobial agents (Adeoyo et al., 2019). Microbial enzymes are receiving attention and production technology has developed greatly (Ferraz-Almedia et al., 2015). Microbial enzymes are most preferred due to their economic feasibility, improved yields, consistency, ease of product modification and optimization, regular supply due to lack of seasonal fluctuations, rapid growth of microbes on inexpensive media, stability, and greater catalytic activity. Microbial enzymes play an essential role in the diagnosis, treatment, biochemical investigation, and monitoring of various diseases. Similarly, the global market for industrial enzymes was estimated at $6.95 billion in 2022 and is expected to develop at a compound annual growth rate (CAGR) of approximately 6.4 % from 2023 to 2030 (GVR, 2020). Generally, enzymes are highly specific; and only accelerate the rate of particular reactions by lowering the activation energy without undergoing any permanent change in them. Thus, enzymes are vital biomolecules that support life (Aldridge, 2013; Piccolino, 2000).

There are some drawbacks to using enzymes in healthcare and other industries. For some enzymes, 37°C and 7.4 are the optimal temperature and pH, respectively, and their activity is highly sensitive to any change in these parameters. High temperature (usually above 40°C) and large deviation from the physiological pH (7.4) lead to denaturation of enzymes, which limits the use of these macromolecules in non-physiological conditions. Additionally, they are susceptible to substrate or product inhibition and their products may cause allergic reactions. The high cost of isolation and purification of enzymes, and their difficult recovery for subsequent reuse are among the drawbacks that discourage their use (Johannes et al., 2006).

α-amylases are enzymes that help in the hydrolysis of internal α-1,4-glycosidic linkages in starch in low molecular weight products, such as glucose, maltose, and maltotriose units. Amylases are found in seeds containing starch as a food reserve and are secreted by many bacteria and fungi. β-amylase (1, 4-α-D-glucan maltohydrolase; glycogenase; saccharogen amylase) is produced by bacteria, fungi, and plants. Working from the nonreducing end, β-amylase catalyzes the hydrolysis of the second α-1,4 glycosidic bond, cleaving off two glucose units (maltose) at a time (Keshwani et al., 2015). γ-Amylase (alternative names: Glucan 1,4-α-

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glucosidase; amyloglucosidase (glucoamylase); exo-1,4-α-glucosidase; lysosomal α-glucosidase; 1,4-α-D-glucan glucohydrolase) cleaves α (1-6) glycosidic linkages, as well as the last α(1-4) glycosidic linkages at the nonreducing end of amylose and amylopectin, yielding glucose (Sivaramakrishnan et al., 2006). The γ-amylase has the most acidic pH optimum because it is most active around pH 3 (Law, 2002).

Antimicrobial resistance is a major problem impeding the success of disease control. Efforts are ongoing to discover new active drugs from novel natural sources that will help to improve a healthy society. Antibiotic resistance has been noted with some strains of bacteria such as *Staphylococcus aureus* and *Escherichia coli* (Adeoyo et al., 2019). Bioactive compounds of fungal origin have been found to have both antioxidant and antibacterial effects on some bacterial species.

Moreover, microbial enzyme production when well harnessed will offer safe performance and speed up reaction rates in bio-processes in an economical and environmentally-friendly way (Nigam, 2013; Adeoyo et al., 2021). The majority of currently used industrial enzymes have hydrolytic actions, that is, they catalyze the breakdown of molecules (Gurung et al., 2013). Consequently, this study aimed at characterizing α-amylase and the antimicrobial activity of *P. chrysogenum.*

Materials and Methods

Sample collection

A 50 g soil sample was collected from the agricultural soil of Adekunle Ajasin University, Akungba-Akoko in Ondo state. The sample was transported to the Microbiology laboratory of Adekunle Ajasin University, Akungba-Akoko, and stored at 4ºC throughout the study period.

Culture

Potato dextrose agar was prepared according to the manufacturer's instruction whereby 7.8 g of PDA is to be suspended in 200 mL distilled. The medium was homogenized and then sterilized in an autoclave at 121°C for 15 min. A 20 mL medium was poured into 3 Petri plates using a serially diluted soil sample (10^{-5} dilution) after cooling to a temperature of about 42ºC. Chloramphenicol was added to inhibit bacteria growth and then allowed to set. After 5 days of incubation at 28ºC, isolates were subcultured to obtain pure cultures.

Identification of fungal isolates

The Identification of the isolates was based on their cultural features and microscopic characteristics. The cultural features (colony texture, shape, margin, and diffusible pigment with colour) were observed macroscopically. The microscopic characteristics (shape of hypha, presence of rhizoid, presence of sporangium, presence, and absence of cross-walls, and type of spores) were observed under a compound microscope after staining with lactophenol cotton-blue stain technique (Adeniran & Abiose, 2009; Ogbonna et al., 2014). The isolated organisms were identified using a compendium of soil fungi.

Fermentation and enzyme extraction

Fermentation was performed according to modify procedure of Kareem and Akpan (2003) using soluble starch 5 g/L; peptone 5 g/L; yeast extract 5 g/L; $MgSO_4.7H_2O$ 0.5 g/L; $FeSO_4.7H_2O$ 0.01 g/L; NaCl 0.01 g/L; and 2.5 g of cassava peels. Under aseptic conditions, each culture flask that contained 100 mL was inoculated with 2 disc mycelia mats (9 mm diameter), shaken continuously on a rotary incubator shaker (150 rpm), at room temperature for 14 days. Extraction of the enzyme was done by simple filtration of media using Whatman filter paper to obtain a mycelia-free enzyme extract. A 10 mL filtrate was withdrawn and centrifuged at 10,000 x *g* for 20 min at 4°C. The clear supernatant was used as a crude enzyme.

Enzyme purification

Crude enzyme filtrate was purified using a method described by Adeoyo et al. (2018). The cell-free crude filtrate was concentrated and optimised using the ammonium sulphate $[(NH_4)_2SO_4]$ and centrifuged at 6000 x *g* for 15 min at 4ºC. The precipitated enzyme was diluted with 10 mL acetate buffer (pH 5.0) and the enzyme (10 mL) was dialyzed against 0.1 M acetate buffer (pH 5.0) at 4ºC.

Enzyme assay

The dinitrosalicylic acid (DNSA) method described by Adeoyo et al. (2019) was used. A 1 g of soluble starch was diluted into 100 mL of distilled water. Using a test tube, a mixture of 1 mL of starch and 1 mL of the purified enzyme was incubated for 30 mins at 30°C. The reaction was terminated by the addition of 2 mL of DNSA reagent; the enzyme mixture was then boiled for 5 min and put into ice immediately for another 5 min. The absorbance was measured at 540 nm using a spectrophotometer. A unit of enzyme activity was expressed as 1 U/mL of glucose equivalent released per minute per milliliter of enzyme solution.

Determination of molecular weight of the enzyme

The molecular mass of the amylase was determined by Amicon Ultra-15 filter unit (Sigma) 50 kDa MWCO and polyacrylamide gel electrophoresis (PAGE). The molecular weight of the crude extract was determined in 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and the gel was stained with Coomassie brilliant blue to visualize the protein (Okoli, 2011, Martinez et al., 2000). The native gel was stained with Lugol solution (6.7 mg/ml KI and 3.3 ml/ml I_2), after which the photograph was taken.

Substrate specificity and temperature stability tests

A 10 mg/mL each of either soluble starch, carboxymethyl cellulose (CMC), Avicel (microcrystalline cellulose), BW xylan, CMC, cellulose, sucrose, chitin or blocked *p*-nitrophenyl-α-D-maltoheptaoside (B*p*NPG7) was used to determine substrate specific for enzyme action. Each substrate was incubated with the enzyme extract at 30ºC for 30 min (pH 5.8). Temperature stability was determined by subjecting the enzyme to pre-incubating heating at different temperatures (30, 40, 50, and 60°C) for 10, 20, 30, 40, 50, and 60 min prior to assaying for residual enzyme activity.

Extraction of crude antibacterial compound

The bioactive compound of the *P. chrysogenum* was extracted by solvent extraction method using ethyl acetate as solvent. An equal volume of ethyl acetate was added to the fungal filtrate at a ratio of 1:1, this was mixed thoroughly for 10 min and kept for 5 min to obtain two clear immiscible layers. The upper tier was separated using a separating funnel. The extracting solvent was then evaporated, and the resultant compound was dried in a rotary vacuum evaporator to produce the crude metabolite. The crude extract was then dissolved in distilled water and kept at 4°C. The crude extract was dissolved in distilled water to obtain a concentration of 50 mg/mL (Adeoyo et al., 2019).

Antibacterial susceptibility test

The crude extract of each fungal isolate was screened for antibacterial activity using some clinical bacterial isolates (*Bacillus* spp, *Staphylococcus aureus, Pseudomonas aeruginosa* and *Proteus vulgaris*) obtained from the Department of Microbiology Laboratory, Adekunle Ajasin University, Akungba-Akoko. All bacterial cultures were adjusted to 0.5 McFarland standards, which is visually comparable to a microbial suspension of approximately 1.5 × 10^8 CFU/mL. The screening for antibacterial activity was conducted using a well-dilution method. Mueller Hinton (MH) agar was prepared, poured into Petri plates, and inoculated with 50 µL of the bacterial suspension (1.5 × 10^8 CFU/mL, spread uniformly using a sterile glass spreader). Wells (9 mm) were made on the agar media with a sterile cork borer and 100 µL filtrate was placed into each separate well. Positive control (chloramphenicol) and negative control (sterile distilled water) were used. The plates were incubated at 37°C for 24 h. Petri plates showing inhibition zones were measured using a transparent ruler calibrated in millimeters (mm).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

The concentrated crude extract was adjusted to a final concentration of 50 mg/mL using sterile distilled water. The MIC was determined using the macrodilution method. The crude extract preparation was serially diluted in two-fold dilutions to form different concentrations ranging from 50, 25, 12.5, 6.25, 3.125 to 1.5625 mg/mL dilutions. A 100 µL bacterial liquid culture (10^8 CFU/mL, 0.5 McFarland's standard) was used to inoculate 10 mL broth (containing 1:100 mixture of extract and MH broth) and grown for 24 h at 37°C. After incubation, 500 µL (0.2 mg/mL) thiazolyl blue tetrazolium bromide (tetrazolium salt) was added to detect the MIC. After the MIC determination, 100 µL from each tube that showed no visible bacterial growth were seeded on a nutrient agar plate and incubated for 24 h at 37°C. MBC revealed the lowest concentration with no growth after culturing.

Statistical analysis

All experiments were conducted in triplicate and analysed using one-way ANOVA. Error bars were represented as the standard errors of the means (SEM).

Results

Identification

The study revealed the presence of three major fungal species that include *Penicillium chrysogenum Aspergillus flavus*, and *Byssochlamys nivea*. *Penicillium chrysogenum* was then selected for further studies based on its ability to produce metabolites with both enzymatic and antibacterial properties (Table 1).

Table 1. *Amylase activities of some fungi.*

S/N	Organism	Enzyme activity (U/mL)
1	*Penicillium chrysogenum*	93.10
2	*Aspergillus flavus*	12.24
3	*Byssochlamys nivea*	5.05

Enzyme activity

The enzyme activity of the tested organisms ranged from 93.10 to 5.05 U/mL. *P. chrysogenum* had the highest enzyme activity of 93.10 U/mL, followed by *Aspergillus flavus* at 12.24 U/mL, while *Byssochlamys nivea* had the least activity (5.05 U/mL) (Table 1).

Determination of MIC and MBC (cell viability assay using MTT)

Table 2 shows that the MIC of the extracts ranged between 3.125 and 12.5 mg/mL. *B. subtilis* had the lowest MIC (3.125 mg/mL), followed by *S. aureus* (12.5 mg/mL), and *P. aeruginosa* (25 mg/mL). The results of the MBC showed that the extract completely inhibited *B. subtilis, S. aureus,* and *P. aeruginosa* at the concentrations of 6.25, 25, and 50 mg/mL, respectively, while in the case of *P. vulgaris*, there was no activity. The MBC was confirmed by sampling from tubes

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showing no visible growth as indicated by colour change and when subcultured on LB agar medium.

Table 2. *MIC and MBC values of extract against four bacterial isolates.*

Test organism	MIC (mg/mL)	MBC (mg/mL)
Staphylococcus aureus	12.5	25
Bacillus spp.	3.125	6.25
Pseudomonas aeruginosa	25	50
Proteus vulgaris	0	0

Enzyme characterization and temperature stability of α-amylase

The enzyme utilizes each substrate (1%) of either soluble starch, CMC, Avicel, BW xylan, CMC, cellulose, sucrose, chitin, or BPNPG7 differently. Figure 1 shows that α-amylase was specific for two substrates; soluble starch and BPNPG7 with values of 93.1 and 100.6 U/mL, respectively. The enzyme could not degrade CMC, Avicel, BW xylan, CMC, cellulose, sucrose, and chitin. Also, a substrate specificity study showed that the enzyme utilizes BPNPG7 which is a substrate specific for α-amylase enzyme production.

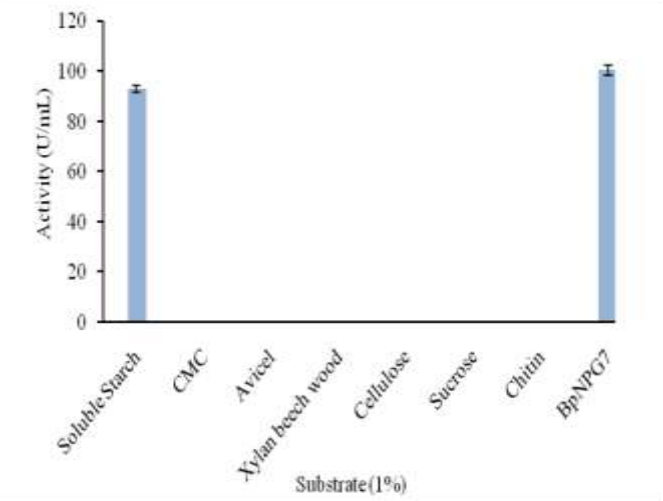

Figure 1. *Substrate specificity test for α-amylase. All error bars are represented as the standard errors of the means (SEM).*

The Zymogram and SDS-PAGE analysis of the α-amylase revealed its molecular weight (Figure 2). Temperature stability assay revealed that the enzyme was stable at 30°C, retaining about 75% activity after 30 min of pretreatment (Figure 3). Also, the enzyme was stable under 40°C and 50°C temperature pretreatment by retaining 66% and 37% residual activities 65% of its original activity for up to 30 min, while less than 37% activity was retained up to 30 min at 60°C (Figure 3).

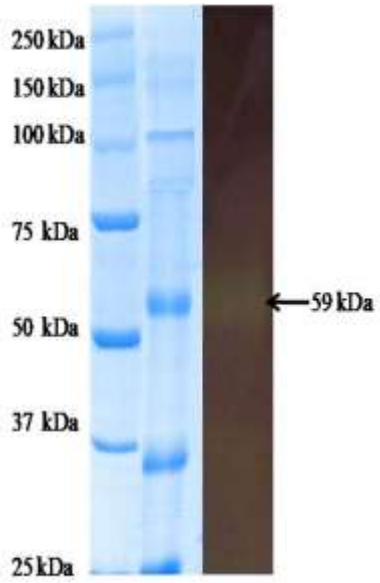

Figure 2. *A 12% zymogram and SDS-PAGE analysis. Lane 1 is precision plus unstained protein standard (BIO-RAD); Lane 2 is SDS-PAGE of crude AMG; Lane 3 is a crude*

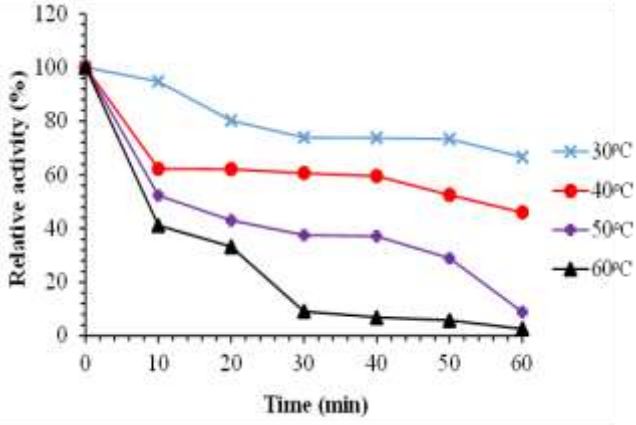

Figure 3. *Effect of temperature on the stability of the α-amylase activity. The enzyme was pre-incubated for 10, 20, 30, 40, 50 and 60 min before the assay was carried out at 30°C for 30 min (pH 5.8)*

Discussion

This study revealed that some fungal species in the soil are sources of enzymes and antimicrobial agents. In the present study, 3 fungal isolates (*Aspergillus flavus* and *Byssochlamys nivea* and *Penicillium chrysogenum*) were obtained from agricultural soil and tested for amylolytic and antibacterial properties. Ominyi et al. (2013) identified fungi on the basis of morphological and microscopic characteristics and revealed the presence of *Aspergillus* spp., *Mucor* spp. and *Rhizopus* spp while working on α-amylase and glucoamylase producing fungi for bioethanol production. In another report, Sohail et al. (2005) identified fungal isolates on the basis of morphological

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characters and reported *Aspergillus niger, Aspergillus flavus, Fusarium* spp., *Alternaria* spp., *Trichoderma* spp., *Penicillium* spp., *Curvularia* spp. and *Cladosporium* spp.

Similarly, some reports showed isolation of fungi from soil samples of different regions for the study of amylase (Lawal et al., 2014). In another study, Singh et al. (2009) reported isolation of fungal strains from soil samples collected from different sites in Jalandhar region of Punjab for the study of amylase activity and the production of fungal amylase was studied using cheap readily available agricultural residues. The variation in diversity index of collection sites indicates that soil harbors plethora of fungal population which can be used for industrial applications.

Amylase-producing fungal genera (*Aspergillus* and *Byssochlamys* and *Penicillium*) were encountered in the fungal genera isolated from soil. This agrees with earlier studies carried out by Ogbonna et al. (2014) and Mishra et al. (2010) that isolated some filamentous fungi from soil. In a similar observation, Ominyi *et al.* (2013) screened the isolates from the soil of rice mill industrial areas, corn processing industries, rice milling, and dumping ground soil and reported 26 isolates to show maximum amylase production in starch hydrolysis test of potassium iodide method. Sohail et al. (2005) studied the screening of microbial populations for their ability to possess amylolytic potential and reported that a total of 130 fungal species were screened and revealed that 21 fungal species were amylase producers.

After submerged fermentation (SmF), the enzyme assay result showed that *P. chrysogenum* had 93.1 U/mL of amylase activity and demonstrated greater potential in the production of amylase than the other two fungal species tested in this study. The result is similar to what was observed by Balkan and Ertan (2005) who reported high enzymatic activity of 155 U/mL for the same organism. Selim et al. (2017) also worked on eight local fungal species using some agricultural by-products and reported maximum production of amylase by *Penicillium* spp. after 7 days of incubation. Some researchers have expressed contrary opinions by reporting that *A. flavus* is the best amylase producer with an enzyme activity of 11.13 U/mL (Bakri et al., 2009). Geetha et al. (2011) observed that 13.03 U/mL was the highest quantity of amylase produced by *A. flavus* at 96 h of incubation. Fadahunsi and Garuba (2012) also reported that *A. flavus* was the best producer of amylase among the fungal isolates screened. Overall, fungal species have demonstrated unique enzyme productivity over other organisms.

For the antibacterial activity study, the results obtained are promising. It was observed that the extracts did show antibacterial activity against *S. aureus, Bacillus* spp, and *P. aeruginosa* but no effect on *P. vulgaris*. This corroborates the results reported on *S. aureus, Bacillus* spp, *E. coli, K. pneumonia, P. aeruginosa* and *P. vulgaris* (Alves et al., 2012; Adeoyo et al., 2018) who reported similar activities against some of these organisms. The MIC and MBC results showed that the fungus contains antibacterial compounds which should be explored to complement the already available drugs for active disease treatment.

Conclusion

Bioactive compounds produced by *P. chrysogenm* are highly rated because they possess antimicrobial and enzymatic capabilities. The continuous exploration of new and novel microbial compounds is a primary target for overcoming the difficulties associated with a resistant pathogen and the production of industrial useful enzymes. Thus, the fungus should be extensively studied and considered as additional sources of new bioactive metabolites for industrial use.

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