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REGULAR ARTICLE

ANTIMICROBIAL EFFECTS OF SOME DICOTYLEDONOUS PLANTS ON FUNGAL ISOLATES OF *CANDIDA albicans* AND *TRICHOPHYTON mentagrophyte*

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ABSTRACT

The preliminary phytochemical screening revealed that, antimicrobial properties of the leaf extracts were due to secondary metabolites such as amino acids, essential oils, flavonoids and saponins contained. The antimicrobial activities of alcoholic extracts were tested against pathogenic fungal isolates of *Candida albicans* and *Trichophyton mentagrophyte*. This was performed by inoculating the isolates into the pure extract, spread onto petri plates containing Sabouraud dextrose agar (SDA) media, observed for growth at stipulated standards. The sensitivity test was done by the disk diffusion method to test the effectiveness of an antimycotic (Griseofulvin) applied on the specific isolates. The minimum inhibitory concentration (MIC) was determined to ascertain the lowest drug concentrations that inhibited the fungal growths. The antimicrobial test revealed that, the leaf extracts of *Eupatorium odoratum* and *Canjanus cajan* inhibited the growths of the organisms while extracts of *Citrus aurantifolia* and *Eucalyptus citriodora* only prevented the growth of *Candida albicans*. The sensitivity test recorded the inhibition zone to range from 11 mm to 32 mm, with the lowest cleared area reported in the extract of *E. citriodora* and the highest in *E, odoratum*. Consequently, the MIC values of extracts at dilution levels were; *E. odoratum*: 1: 10000; 1: 1000, *C. cajan*: 1: 1000; 1: 10000, E. *citriodora*: 1:1000; 1:100 and *C. aurantifolia*: 1: 100000; 1: 100 respectively. This work has confirmed the progressive utilization of plants as antimicrobials for the benefit of mankind, to have originated from microbial sources.

Keywords: Antimicrobial, fungal isolates, leaf extracts Lam, minimum inhibitory concentration and sensitivity test

INTRODUCTION

Antimicrobial plants are described as those with therapeutic active principles or inhibitory chemical substances known to cure ailments or stop the growth and proliferation of microorganisms, by the administration of extracts from whole, parts, juices and exudes of plants. This act of medicinal plants as nature's remedies dates back to creation and first awakening of man when he sought to fight, control and treat infectious diseases and pains. In this concept, herbs form interface between two realms of nature; when humanity and plants meet, a synergistic energy can be created and exchanged (**Lam, 2007**).

The antimicrobial properties are conferred in plants in part by the compounds synthesized by secondary metabolism of plants that might act individually, additively, or in synergy (**Akpoka *et al*., 2019**). **Malhadas *et al*. (2017)** highlighted some compounds with antimicrobial effects to include: protocathechol, a phenolic compound present in onions, avenacin in oat plant, hondatin in barley (*Hordium vulgare)*) linamarin in cassava (*Manihot esculenta*) and dihydroxymethoxybenzonone (DIMBZO) in wheat (*Triticum aestivum)*. Other compounds that have established therapeutic actions are alkaloids, glycosides, tannin, essential oils, saponins, anthraquinones and polyphenols.

Globally, important drugs elaborated from crude plant's extracts are being applied to treat diseases through various mechanisms such as osmotic pressure, disruptions of cell membrane and cell wall by hydrolysis of glycosidic bond, inhibition of protein synthesis by non-transcription of the mRNA. The other modes are, inhibition of nucleic acid synthesis and formation of curling effect of terminal hyphae of fungi, leading to growth retardation and death (**Lam, 2007; Jia *et al*., 2016**).

Consequently, it has been reported that, aqueous alcoholic extracts of *Diospyros bateri* and *Ziziphus abyssinica* showed strong antimicrobial prowess against *Aspergillus niger* and *Candida albicans*. Additionally, aqueous petroleum ether and dichloromethane extracts of bark and leaves of *Citrus aurantifolia*, *Canjanus cajan* and *Vernonia amygdalina* had great antimicrobial actions against *Pseudomonas aeruginosa, Staphylococcus aureus, Aspergillus niger, Trichophyton mentagrophytes* and *Candida albicans* (**Doddmna, *et al*., 2013**).

The value for medicinal plants is gaining more attention today due to huge plant's based active principles, increasing cost of orthodox medicines, ability to respond to specific organ or system of the body (**Mishra, *et al*., 2016**). More so, other needs that promote herbal use include; resistance of certain organisms to drugs, herbal preparations retaining life-giving nutrients such as vitamins and minerals contained in the original plant's composition. These valuable substances cannot be supplied by a single drug, rather one must resort to combined drug intake with its toxicological potential hazards.

In determining the high potency of extracts, sterile water was chosen instead of (or used along) the antimycotic as a negative control. However, such inclusion showed that, any reported antimicrobial activities are a direct product of the extract and not a constituent within the plate and disc. This indicated that, if the test plant used in this study were unavailable, other plants known to possess high antimicrobial active principles could conceivably be considered. This study was aimed to validate the claims by herbal medicine practitioners in their folklores, as to the efficacy of plants' materials in the treatment of various infectious diseases. To formally bring to public domain, the need for people around the globe without access or opportunity to orthodox or foreign drugs that, they could reliably apply herbal extracts as an option. The outcome of this study was to compare the growth inhibitory effects and the lowest concentration of extract dilutions and known antimycotic, on test organisms.

MATERIALS METHODS

Sample Collections

The Test Plants

The test plants screened were: *Vernonia amygdalina, Azadirachta indica, Persia americana, Citrus aurantifolia, Magnifera indica, Eucalyptus citriodora, Eupatorium odoratum,* and *Canjanus cajan*. The leaves used in this investigation were collected from the University of Benin botanical garden and identified in the Department of Pharmacognosy, University of Benin, Nigeria

The Test Cultures

Pure cultures of the test organisms; *Candida albicans* and *Trichophyton mentagrophyte* were obtained from Medical Laboratory Science Department, University of Benin Teaching Hospital (UBTH) Nigeria. The sub-culturing of pure culture was carried out aseptically by transferring a loopful of each of the test organisms into sterile test tubes containing Sabouraud dextrose agar (SDA) and stored at 4 ^{o}C.

Extract Preparation

Fifty grams (50 g) of fresh leaves of each collected plants were washed in distilled water to remove dust particles and insect larvae, dried in an oven at temperature of 40 ^{o}C for 8 hrs. The dried leaves were made to powdered form by using mortar and pestle. Ten Milliliter (10 ml) of 75 % ethanol was added to each of the test powdered dried leaves in each test tube and were placed in hot

water bath to evaporate the ethanol content at temperature of 60 ºC. This process eventually left paste-like substance at the bottom of the test tube. This substance was then diluted with moderate amount of distilled water, shaken thoroughly and finally filtered with Whatman No. 1 filter paper to obtain the complete pure extract into sterile McCarthy bottles and stored at 4 ºC for subsequent use (**Nayan & Shukla, 2011**).

Phytochemical Testing

The extracts were subjected to preliminary testing to detect for the presence of different chemical groups of compounds. Air dried and powdered plant materials were screened for the presence of amino acids, phenols, saponins, glycosides, anthraquinone, tannins and isoflavones as described by (**Jia, *et al.*, 2016**) and modified according to (**AOAC, 2019**).

Antimicrobial Test Properties

The antimicrobial tests were conducted using the test tube technique: Five milliliter of each leaf extract was measured into 2 test tubes, 1 ml each of the 2 test organisms was used to inoculate each of the test tubes and left for 24 hr. Sterile SDA was poured onto pre -sterilized petri plates and allowed to set. These agar plates were then seeded with 0.2 ml of the test organism which was inoculated into the pure extract in the test tubes and spread evenly with a flamed, but cool glass spreader to derive effective growth of a smooth fungal lawn. Finally, the plates were then incubated at 37 ºC for 24 to 48 hr. However, control plates were prepared using distilled water and 2.5 % phenol as negative and positive controls respectively (**Malhades, *et al.*, 2007**).

Sensitivity Test

The extracts with high spectra of activities against the test organisms obtained from the test tube approach of plants' screening were further confirmed for their extent of inhibiting the growth of prevailing organisms by employing the Disc diffusion method. This was conducted by sterilizing the filter paper soaked in an appropriate extract before each was inserted at middle of the plates previously flooded with test organisms. The plates were incubated at 37 ºC for 24 to 48 hrs and the cleared zones of inhibition produced around the epicenter of the plates were observed and measured in diameter (**Cassini *et al.*, 2016; Hans, *et al.*, 2017**).

Minimum Inhibitory Concentration (Mic)

Five milliliters (5 ml) of varying dilutions of the extract was prepared using peptone water as diluents; the serial dilution made ranged from 10^{-1} to 10^{-5} (1: 10 to 1: 100000). In preparing the dilution, 8 test tubes containing antibiotic concentration was prepared and inoculated with standard quantity of each extract. These dilutions were aseptically inoculated with the test organisms and incubated at 37 ºC for 24 to 48 hr. Thereafter, 1 ml of each dilution was pipetted into the 8 SDA media plates, incubated at 37 ºC for 24 to 48 hrs (**Barrow & Felham, 2003**).

RESULTS

The results of the phytochemical screening showed that, the alcoholic extracts of the test plants contained saponins, tannins, glycosides and isoflavones. The antimicrobial activities of the extracts of the test plants were studied against pathogenic fungal strains of *Candida. albicans* and *Trichophyton mentagrophyte.* The leaf extracts of *Eupatorium odoratum* and *Canjanus cajan* inhibited fungal growth (-) or no colonies were observed, while the extracts of *Citrus aurantifolia and Eucalyptus citriodora* inhibited the growth of *Candida albicans only,* with mild growth recorded for *Trichophyton mentagrophyte.* High fungal growth was reported in the extract of *Magnifera indica* against the test isolates (++) (Table 1).

Table 1 Effects of plant leaf extracts on growth rate of fungal isolates

Extracts	*C. albicans*	*T. mentagrophyte*
Vernonia amygdalina	+	+
Azadaricta indica	+	+
Persea americana	++	–s
Citrus aurantifolia	–	+
Magnifera indica	++	++
Eucalyptus citriodora	–	+
Eupatorium odoratum	–	–
Canjanus cajan	–	–
Sterile water	++	++
2.5 % Phenol	–	–

Key: No fungal growth (-) Slight growth (+) High growth (++)

The plant's leaf extracts that indicated high potential of antimicrobial properties; *E. odoratum, C. cajan, E. citriodera,* and *C. aurantifolia* were further screened to determine the level of clearing zone of test fungal isolates using antimycotic drug (griseofulvin) and measured in millimeters (mm).

Table 2 Inhibition zones (mm) of the potential leaf extracts on test fungal isolates

Extracts	*C. albicans*	*T. mentagrophyte*
C. aurantifolia	22	16
E. citriodora	16	11
E. odoratum	32	28
C. cajan	26	28
Distilled water	2.0	1.5
2.5 % Phenol	40	43

The minimum inhibitory concentration (MIC) of a leaf extract is the lowest concentration of the therapeutic agent or chemical substance present in plant, that prevents visible growth of an organism. In the determination of MIC for leaf extracts on the test fungal isolates; *Candida albicans* and *Trichophyton metagrophyte* at lowest extract dilution factor, the MIC was reported as followed: *E. odoratum*: 1:10000; 1:1000, *Cajanus cajan*: 1:10000; 1:10000, *E. citriodora*: 1: 1000; 1: 100 and *C aurantifolia*: 1:100000; 1:100 respectively (Tables 3 - 6).

Table 3 Minimum inhibitory concentration (MIC) of leaf extract (*E. odoratum*) for test fungal isolates

Extracts dilutions	*C. albicans*	*T. mentagrophyte*
1: 10	–	–
1: 100	–	–
1: 1000	–	–
1: 10000	++	–
1: 100000	++	+
MIC	1: 0000	1: 000

Key: No fungal growth (–) Slight growth (+) High growth (++)

Table 4 Minimum inhibitory concentration (MIC) of leaf extract (*C. cajan*) for test fungal isolates

Extract dilutions	*C. albicans*	*T. mentagrophyte*
1: 10	–	–
1: 100	–	–
1: 000	–	–
1: 10000	–	–
1: 100000	+	++
MIC	1: 10000	1: 10000

Key: No fungal growth (–) Slight growth (+) High growth (++)

Table 5 Minimum inhibitory concentration (MIC) of leaf extract (*E. citriodora*) for test fungal isolates

Extract dilutions	*C. albicans*	*T. mentagrophyte*
1: 10	–	–
1: 00	–	–
1: 1000	–	+
1:10000	++	+
1: 00000	++	++
MIC	1: 1000	1: 100

Key: No fungal growth (–) Slight growth (+) High growth (++)

Table 6 Minimum inhibitory concentration (MIC) of leaf extract (*C. aurantifolia*) for test fungal isolates

Extract dilutions	*C. albicans*	*T. mentagrophyte*
1: 10	–	–
1; 100	–	–
1:1000	–	+
1: 10000	–	++
1: 100000	–	++
MIC	1: 100000	1: 100

Key: No fungal growth (–) Slight growth (+) High growth (++)

DISCUSSION

The plants around man's surrounding attracted its attention with the various parts; bark, flowers, fruits, leaves and roots in the long history of human civilization and became known for their nutritional and therapeutic properties, hence formed the basis of medicine. The present study justified the claimed uses of leaves in the traditional approach to curb various ailments (**Akpoka, 2019**).

The results obtained from the phytochemical screening showed that, alcoholic extracts of leaves contained amino acids, essential oils, flavonoids, glycosides and saponins. This confirmed the previous report that, presence of various phytochemicals with active biological principles can be of imperative therapeutic index (**AOAC, 2019**).

In this study, eight dicotyledonous plants were screened against fungal isolates of *Candida albicans* and *Trichophyton mentagrophyte*. The results (Table 1) showed that, the leaf extracts of *Eupatorium odoratum* and *Canjanus cajan* completely inhibited the growths of tested organisms; *C. albicans* and *Trichophyton mentagrophyte*, indicating their effectiveness in preventing the test organisms related infections.

However, the leaf extracts of *Citrus aurantifolia* and *Eucalyptus citriodora* prevented their growths, while slight or moderate growths were noticed for tested organisms. Meanwhile, high growth rate recorded in the extract of *Magnifera indica*, demonstrated the resistance of the fungal isolates to the tested extract. This indicated that, the extract cannot be applied as preventive or treatment measure against pathogenic effects of the test organisms.

The high antimycotic activities of *E. odoratum* (bitter bush) could be due to the endowed Beta cuberine and cadinene and useful in the treatment of wounds, burns, skin diseases and applied to decrease cholesterol and blood pressure levels (**Hamzah, *et al*., 2018**). The *Cajanus cajan* has also been reported to contain dry matter, crude protein and minerals and used in the cure of cough, bronchitis, sore throat infections and diabetics.

Similarly, the *E. citriodora* extract contained 80 % citriodellal, an aldehyde, responsible for its antimicrobial properties, essential oil, a valuable constituent in the treatment of athletic foot disease. The *C. aurantifolia* (Key lime) serves as anticancer, antifungal and antioxidant, protects the liver and heart, prevents urinary tract infections (UTIs) and neutralizes odours (**Nayan & Shukla, 2011; Jia, *et al*., 2016**). Consequently, the *C. albicans* is responsible for vulvovaginal infections, invasive and oropharyngeal candidiasis, while the T. mentagrophyte causes dermatophytosis, impetigo and athletic foot disease (*Tinea pedis*) (**Doddmna, *et al*., 2013**).

Avelar-Pires, *et al*. (2004) had previously indicated the ability of hydroalcoholic extract of the whole plant of *Xanthoriza simplissima* to exhibit good activity against the acquired immune deficiency disease syndrome (AIDS) related opportunistic pathogens; *Candida albicans, Mycobacterium intracellularae* and *Crystococcus neoformatis*. However, the bioassay fractionation of the extract led to the isolation of the known alkaloid as the major active component.

The growth inhibition zone measured ranged from 11 to 32 mm for the test organisms. However, the lowest cleared area on the plate was recorded in the extract of *E. Citriodora* (11 mm) and the highest approximated killed zones were in the extracts of *E. odoratum* and *C. cajan* (32 mm and 28 mm respectively (Table 2). This illustrated the rich potent chemical principles contained in extracts of these leaves, as the most active agents in curbing the spread and proliferation of the tested isolates amongst the surveyed extracts. Nevertheless, the low and high inhibition levels obtained from the controls: sterile water (negative control) and 2.5 % Phenol (positive control) relatively confirmed the presence of compounds to be responsible for antimicrobial activities.

The minimum inhibitory concentration (MIC) described as the lowest concentration of chemical (drug) that prevents visible growth of an organism is considered to indicate that; lower MIC values meant that, less drug is required to inhibit microbial growth. Therefore, drugs with lower MIC scores are more effective antimicrobial agents (**Lam, 2007; Mishra., 2016).**

The findings revealed that, *E. odoratum* and *Canjanus cajan* were considered extracts, with high potential antimicrobial properties against *C. albicans* and *T. mentagrophyte* (1:1000; 1:10000) extract dilutions respectively) (Tables 3 and 4). Consequently, the MIC for *E. citriodora* against test organisms were 1:1000; 1:00 (Table 5). This showed that, this extract concentration is more potent on *C. albicans* (lower value) than the *T. mentagrophyte*. Succinctly put, the extract of *C. aurantifolia* exhibited MIC at the lowest dilution level (1: 100000) and completely cleared the *C. albicans* isolate (Table6). This showed that, low concentration of this extract is very effective and therefore can be considered for the treatment of infectious diseases caused by this organism.

CONCLUSION

The alcoholic extracts of *E. odoratum* and *C. cajan* could be administered to cure the clinically pathogenic effects of the isolates, while the extract of *C. aurantifolia* is recommended for the treatment of candidiasis associated diseases. Hence, this study has validated the claimed importance of plants in the herbal system of health care services to humanity. It will also form the primary criterium for selection of plant species in further analysis, for the potential need of recent natural bioactive components. It is recommended that the use of plant extracts from *Eupatorium odoratum, Cajanus cajan* and *Citrus aurantifolia* should be considered as therapeutics in the cure for candidiasis and dermatophytosis diseases caused by the test organisms.

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REGULAR ARTICLE

ISOLATION OF AMYLASE PRODUCING FUNGI FROM CASSAVA FLOUR

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ABSTRACT

Amylases are among the most important enzymes with potential applications in the present-day industry. Thus, isolating pure culture from cassava as the cheap source has manifold importance for food industries. In the present study, eleven amylase producing fungal strains were isolated from cassava flour and growth pattern, as well as optimum growth condition, was determined. All isolates showed amylases activity but isolate BR005, BR001 and GR003 recorded maximum clear zone diameters of 54.75 ± 0.957 mm, 53.25 ±0.645 mm 51.5 ± 1.414 mm, respectively. The submerged fermentation method was employed for crude amylase and biomass production. There were significant differences ($p<0.05$) in starch concentrations and growth rates between the three isolates. GR003 and BR005 attained their optimal amylase activities of 4.23±0.25 U/mL and 3.75±3.16 U/mL at 50 °C, respectively, whiles BR001 attained its optimum amylase activity of 3.43±0.77 at 60 °C. Whereas, BR005, BR001 and GR003 attained their optimal amylase activity of 5.14±1.99 U/mL, 4.53±0.01 U/mL and 1.25±1.11 U/mL at a pH range of 6 to 7, making them neutrophilic fungi. Moreover, isolates BR005, BR001 and GR003 recorded the highest amylase activities of 4.31±0.14U/mL, 4.67±1.16±U/mL and 3.16±0.12U/mL at the starch concentrations of 3%, 2%, and 2.5% and fermentation period of 48 h, 66 h and 42 h, respectively. Lastly, BR005, BR001 and GR003 achieved their optimal amylase activities of 5.41±0.11U/mL, 6.24±0.14 U/mL and 6.22±0.12 U/mL at 48 h, 66 h and 42 h of incubation, accordingly. Indicating that cassava flour is a good source of amylolytic fungi with a potential application under wide conditions.

Keywords: Amylase; fungi species; cassava flour; optimum activity

INTRODUCTION

Amylases are a family of enzymes that degrade starch into simple sugars and lower molecular weight polysaccharides. This enzyme has diverse applications in a wide variety of industries such as food fermentation, textile, paper, detergent, pharmaceutical, and sugar industries. As starch degrading enzymes, amylase have received a great deal of attention because of their perceived technological significance and economic benefits (**Gupta et al., 2008; De Carvalho et al., 2018**).

Amylases are classified into three types namely α-amylase, ß-amylase, and γ.-amylase based on their catalytic mechanisms **(Akpan et al., 1999)**. α -Amylase (EC 3.2.1.1), catalysis the hydrolysis of internal α -1,4-glycosidic linkages in starch into low molecular weight products, such as glucose, maltose units. ß-amylase (EC 3.2.1.2,) catalysis the hydrolysis of α -1,4-glycosidic linkage of starch and ß-anomeric maltose from the non-reducing ends **(David et al., 2000)**. γ -Amylase (EC 3.2.1.3), hydrolysis α -1,6 glycosidic linkages, in addition to the last α -1,4 glycosidic linkages at the non-reducing end of amylose and amylopectin, yielding glucose. The major importance of using fungi for amylase production is the economical bulk production capacity. Amylase constitutes a class of industrial enzymes accounting for approximately 25% of the enzyme market **(Khoo et al., 1994)**. Amylases are widely produced by microbes, plant and animals. The application of an amylase depends on its unique characteristics, such as its action pattern, substrate specificity, optimal temperature, and optimal pH. Thus, amylases from microbes are much preferred because microbes are much easier to manipulate to facilitate enzyme production with desire characteristics **(Souza 2010; Sundarram and Murphy, 2014)**.

Cassava (*Manihot esculenta*) is an important starchy root crop that contains starch content of about 60-70 %. Fermentation techniques to produce microbial protein using either cassava flour or cassava wastes or enriching cassava flour or cassava wastes have widely been used **(Sundarram and Murphy, 2014)**. Cassava is a low-cost starch material for microbial enzyme production. A dried fermented cassava product, gari, has been reported to contain *Aspergillus niger*, *Aspergillus flavus* and *Penicillium* species **(Thoha et al., 2012)**. Amylase producing fungi from cassava flour has been reported. Therefore, this study reports the isolation and optimization of novel amylase producing fungi from cassava flour. Production conditions were optimized (temperature, pH, substrate concentration) to achieve high enzyme production and better enzyme activity.

MATERIALS AND METHODS

Sample Collection

Forty (40) grams of ten samples of cassava flour were purchased from Aboabo and Nyankpala markets Tamale, the Northern part of Ghana. Samples were kept in sterile resealable bags at 4 °C before the experiments.

Isolation of Amylolytic Fungi Isolates

A starch-peptone agar containing the following chemical composition (starch, 20; peptone, 10; streptomycin 0.05 and agar, 20) in g/L was used to isolate the amylolytic fungi. Composite samples were made by mixing 20 g of each cassava flour, then 10 g of the bulked samples were weighed into a 250 mL beaker containing 90 mL of sterilized distilled water. The suspension was gently stirred to obtain homogenous mixture. The homogenized suspension was serially diluted and inoculated onto starch-peptone agar by a spread plating method, and incubated at 30 °C for 4 days. Colonies were randomly sampled from the mixed cultures and sub-cultured to obtain pure cultures. Pure isolates obtained were kept on starch peptone agar and stored at 4 °C.

Screening for Amylase Activity

A cock borer, 4 mm in diameter was used to transfer 7 days-old pure culture of each of the eleven isolates and placed on the middle of replica solidified starch peptone agar plates and incubated at 30 °C. After 4 days of incubation, the isolates were flooded with Lugol's iodine solution. The clearing zone diameter formed around the fungal colonies were measured and taken to represent the amylolytic activity of the fungal isolates as illustrated in Fig. 1.

Fermentation and assay of crude amylase

Ten milliliters of sterile distilled water were poured onto a 7 days old sporulating slant cultures of selected fungi strains; BR005, BR001, and GR003 and agitated to obtain homogenized spore suspensions. Haemocytometer (Marienfeld, Neubauer) was used for spores counting and to determine spore concentration of the suspension. An average inoculum of $1.47x10^7$ spores was used to count the inoculate 100 mL of fermentation broth and incubated at 30 °C for 96 hours in a water bath shaker (Unitronic Orbital Selecta- J.P) at 100 rpm. Broth of 5 mL was sampled at regular intervals of 6 hours and centrifuged at 10, 000 rpm for 10 minutes at 4 °C in centrifuge (Centrolit-II, Selecta-J. P). Supernatants were stored at -20 °C for amylase test.

The enzyme assay was determined as described by **(Yoo et al., 1987**), with some modifications. To 0.1 mL of starch solution in an assay test tube, 100 µL of

crude enzyme extract was added and incubated for 5 minutes. Approximately 2 ml of acidified iodine solution (0.28 g KI + 0.03 g I_2 in 500ml of water + 6 mL of conc. HCl) was added to terminate the reaction. To the control, 0.1 ml of the 1 % starch solution was mixed with 100 µL of 0.02M sodium phosphate buffer of pH 7. The control was also incubated for 5 minutes together with the assay tube after which 2 mL of acidified iodine solution was added. The tubes were kept on the ice cool for 5 minutes before measuring the absorbance at A590 after the Spectrophotometer (Jenway, 6405 UV/ Vis) was calibrated with distilled water. The amount of starch degraded was estimated from the standard curve and corresponding amylase activity was determined. One amylase unit (U) was defined as the amount that degraded 1 mg of starch in one minute under assay condition.

Optimization Test for Amylase Activity

The optimal temperature for amylase activity was determined by incubating the enzyme-substrate reaction mixture of 0.1 mL of 1% starch and control containing 0.1 mL of starch and phosphate buffer solution at different temperature 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C and 80 °C for 5 minutes. A volume of 2mL of iodine solution was used to stop the reaction. The absorbance was measured at 590 nm after it was cooled to room temperature. The optimum pH for the enzyme activity was determined by preparing 1 % starch solutions of pH 3, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10. Citrate phosphate buffer was used for pH 3 to 8 and glycine-NaOH buffer for pH 9 and 10. A volume of 0.1 mL of crude enzyme solution and 0.1 mL Phosphate buffer of pH 7 was added to replicate and control tubes respectively containing 0.1 mL of starch solution each. The reaction was incubated for 5 minutes. A volume of 2 mL of acidifying iodine reagent was added to stop the reaction and the absorbance at A590nm was measured and recorded using a spectrophotometer.

Different concentrations of (0.5 -5%) starch solutions were prepared. To 0.1 mL of starch in a test tube, 0.1 m/L of phosphate buffer was added, and 0.1 m/L of volume crude enzyme was added and 2 m/L of acidified iodine the reaction but not the control. 2 mL of acidified iodine solution was added and kept on ice for 5 minutes. The absorbance values at $A_{540\ nm}$ were measured and recorded.

Estimation of Growth Rate

Aliquots of 6m/L were a sample from triplicate fermentation flask of isolates at regular intervals of 6 hours and centrifuged at 10,000 rpm for 10 minutes at 4 °C in a centrifuge (centrolit-II selecta-J. P. The supernatants were decanted, and filtrates transferred onto Whiteman No.1 filter paper. The filter papers were kept in hot air oven at 80 °C for 2 hours after which their dry biomass weights were determined with electronic balance

Experimental Design

Genstat discovery (edition 7) was used to determine the significant difference between isolates at a significance level of 5 % and Microsoft Excel was also used to compute the various graphs.

RESULTS AND DISCUSSION

Fungi Isolation and Screening

Starch degrading microorganisms were isolated from ten samples of cassava flour. Eleven fungi isolates were randomly picked from starch-peptone agar plates prepared based on their distinct colony morphologies. There was a positive correlation between the radial growth of the fungal colony on media containing starch as sole carbon and energy source and amylase activity. The screening of eleven fungi isolates on starch-peptone agar, selective media was done to obtain efficient amylolytic isolates. The clear zone diameter of the eleven isolates was significantly different (P<0.01). All isolates showed amylases activity, but BR005, BR001 and GR003 exhibited the highest amylase activities with maximum diameters of 54.75 ± 0.957 mm, 53.25 ±0.645 mm and 51.5 ± 1.414 mm, respectively (Fig. 2). The maximum clear zone formation on the starch peptone agar media by isolates evidence their potential as amylase producers.

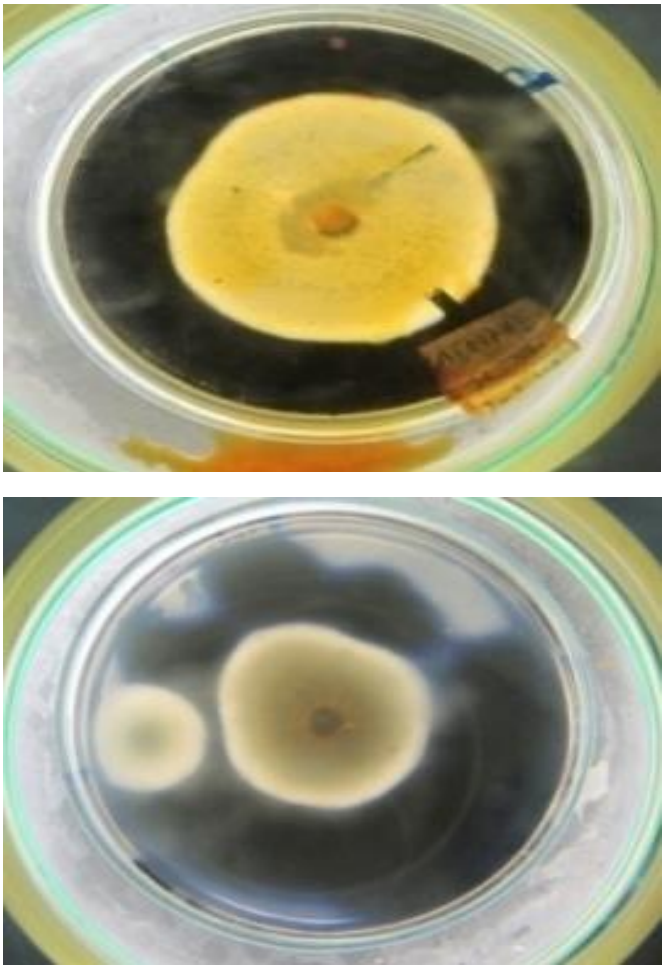

Figure 1 Pictures of clearing zone diameter of isolates

Effect of Incubation Period on Fungal Growth and Amylase Activity

As shown in Fig. 4, the growth rate of isolates BR005, BR001, and GR003 varied significantly (P<0.05). BR005 achieved the highest growth rate of 1.406±0.19mg/h followed by BR001, which recorded 1.095±0.12mg/h and lastly, GR003 obtaining 1.032±0.10mg/h on dry biomass basis during the 96 hours of incubation. In a submerged fermentation, if amylase composition and activity are kept constant, the physiological adaptation to fermentation factors such as bulk oxygen transfer, uniformity of nutrient distribution by agitation, altering pH and excreted metabolites significantly affect microbial growth.

Optimization of the incubation period is an important parameter for the maximum growth of the fungal isolates and enzyme production. Isolates BR001, GR003, and BR005 obtained their highest amylase activity of 6.26±0.14 U/ml, 6.23±0.12 U/mL and 5.41±0.11 U/mL at 66 hours, 42 hours and 48 hours of incubation, respectively. Generally, there was an increased in amylolytic activity with increasing incubation time, up to the optimal yield point where enzyme activity decreased with an increased incubation period. Activation of genes which codes for amylases might account for the increased, while glucose repression and instability of the enzyme might cause the decreased with the incubation period **(Souza, 2010)**. As shown in Fig. 3, the optimal activities of the isolates achieved at different incubation periods might be attributed to their intrinsic isolates.

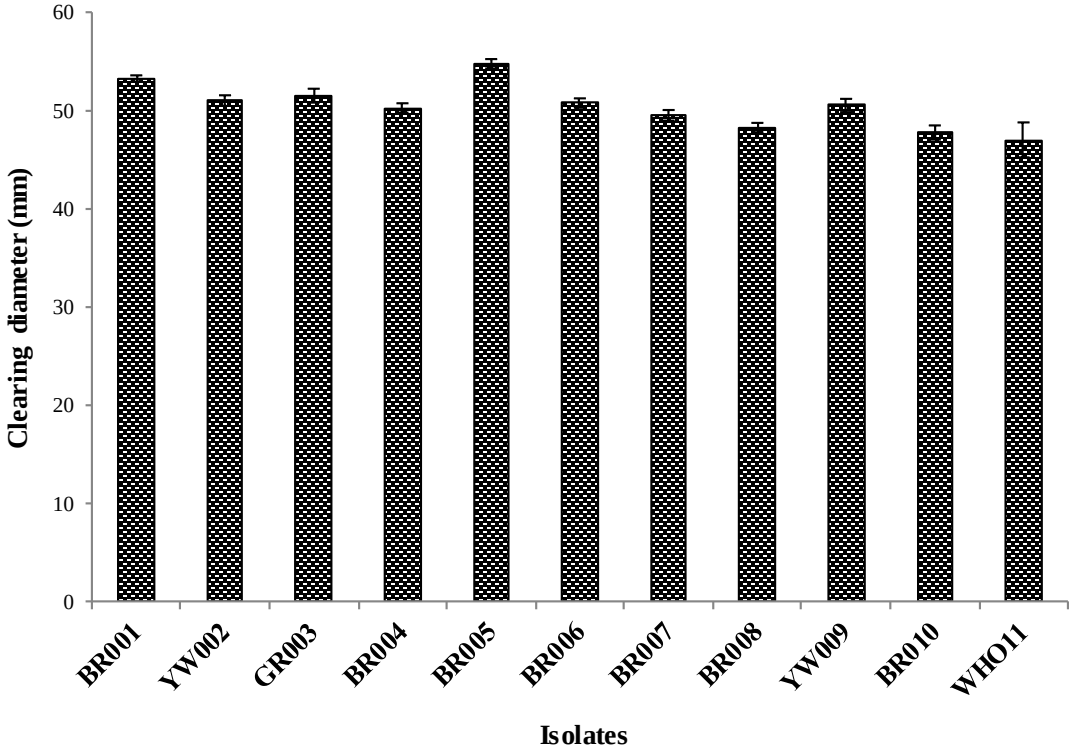

Figure 2 Clearing zone diameter of the fungal isolates after 96 hours incubation period.

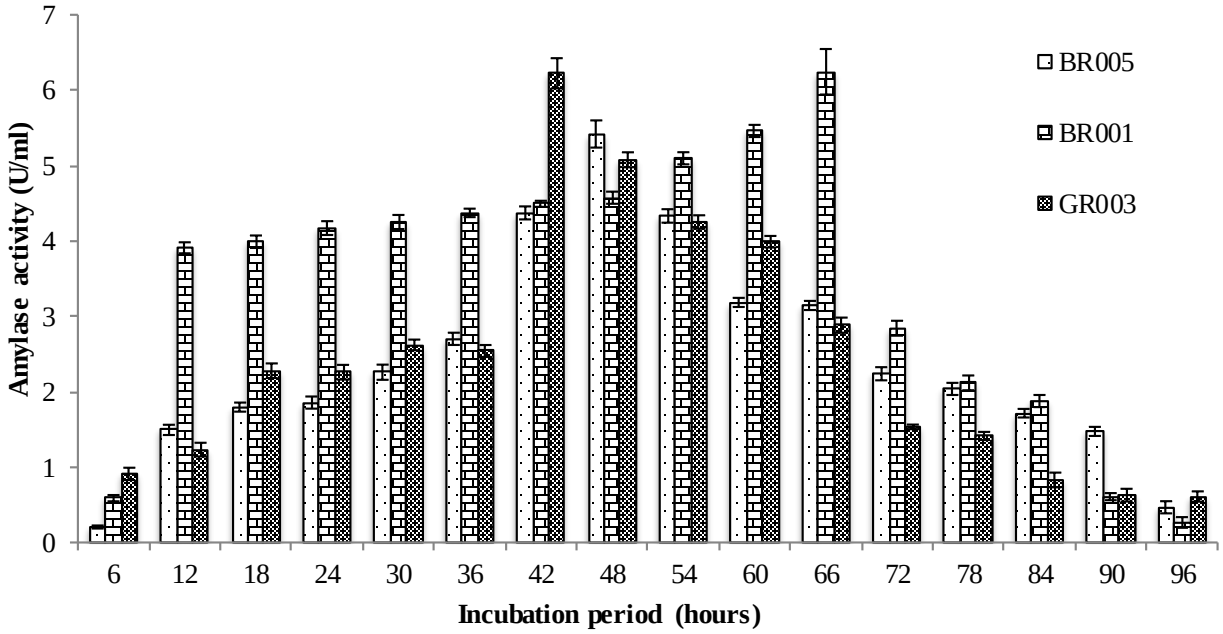

Figure 3 Time course of amylase activity (U/ml) during 96 hours incubation at 30 °C, pH 7.

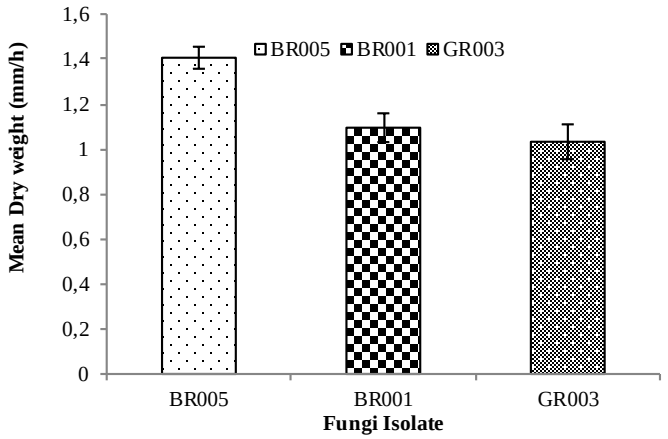

Figure 4 The growth rate of isolates over 96 hours of submerged fermentation at 30 ºC, pH 7

Effect of Temperature, pH and Substrate Concentration on Amylase Activity

In this work temperature affected the growth/yield and metabolic activities of the isolates. BR005 and GR003 attained their optima amylase activities of 3.75±3.16 U/mL and 4.23±0.25 U/mL at 50 ºC respectively. Whereas, BR001 reached its optimum amylase activity of 3.43±0.26 at 60 ºC, showing that their amylases are thermostable as shown in Fig. 5. It was found that amylase obtained from *Aspergillus tamarii* was stable for several hours at temperature up to 65 °C (**Moreira et al., 2004**).

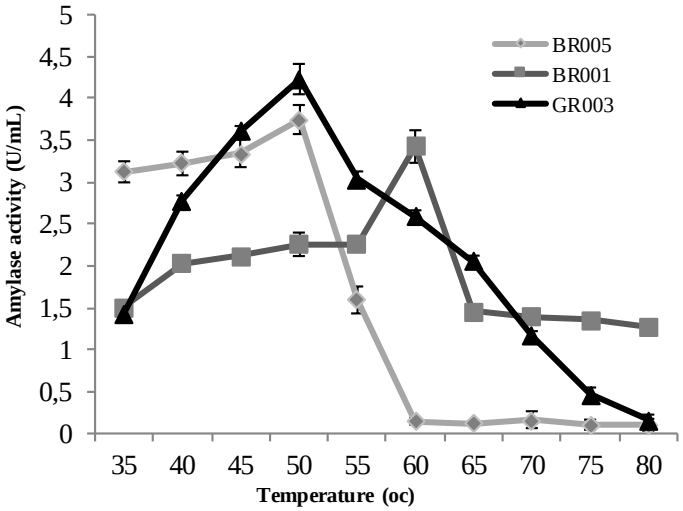

Figure 5 Effect of temperature on amylase activity

The effect of pH on amylase activity was investigated at varying of pH 3 to 10. Isolates BR005 achieved its optimum amylase activity of 5.14±1.20 U/ml at pH 7, which was followed by BR001 and then, GR003 with their amylase activities of 4.53±0.01 U/ml and 1.25±1.11 U/mL at pH 4, respectfully, thus behaving as neutrophilic. As shown in Fig. 6, their amylase activities decrease with an increased in pH after a pH 6 and pH 7. Shafique and others observed a maximum fungal amylase production at pH 7 from bagasse (**Shafique et al., 2009**). It was also found that the optimum conditions of pH for amylase activity ranging from pH 6 to pH 8 (**Keharom at al., 2016; Silverman 2002**). This means that BR005 could provide an easy condition to produce amylase.

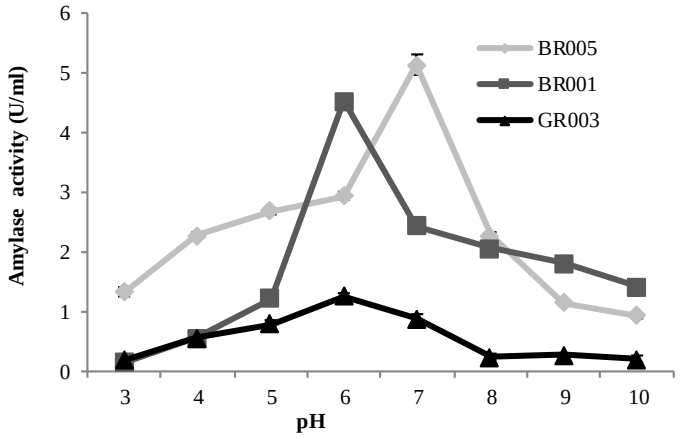

Figure 6 Effect of pH on amylase activity (U/mL) of isolates

The results in Fig. 7 showed that amylase activity increased with respect to increasing starch concentration. Thereto, BR001 recorded the highest amylase activity of 4.67±1.16 U/mL at 2% of starch, while BR005 attained its optimum substrate concentration at 3% with amylase activity of 4.31±0.14 U/mL, and also GR003 achieved its optimum amylase activity of 3.16±0.12 U/mL with 2.5% of starch. **Adekoya *et al.* (2018)** reported fungal amylase with optimal starch concentration of 2%. Hence, BR001 could offer potential means of producing high amylase at a low cost-based substrate the concentration.

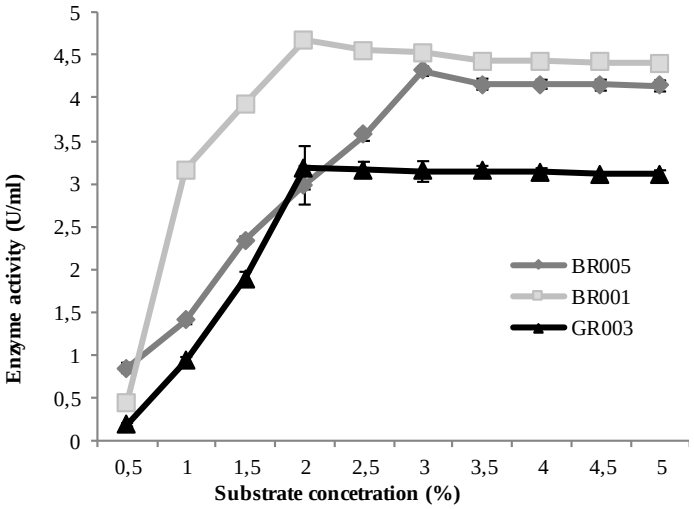

Figure 7 Effect of substrate concentration on amylase activity of isolates

CONCLUSION

In conclusion, all the isolates showed amylase activity, but BR005, BR001 and GR003 exhibited substantial amylase activities with maximum clear zone diameters of 54.75 ± 0.957 mm, 53.25 ±0.645 mm and 51.5 ± 1.414 mm, respectively. The effect of temperature and pH indicated that isolates BR005, BR001 and GR003 had their optimal temperatures at 55 °C, 60 °C and 65 °C, respectively, and optimal performances from pH 6 to pH 7. As a result, they are thermophilic and neutrophilic fungi. This study revealed that cassava flour is a good source of amylolytic fungi.

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Conflict of Interest Statement: The authors declare that there is no conflict of interest

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