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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.

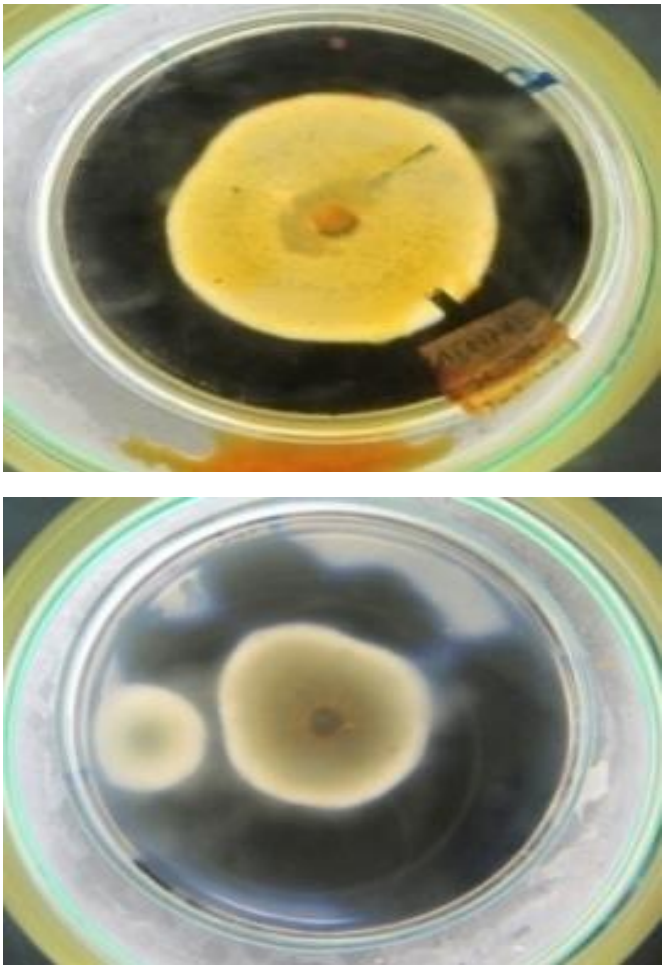

Figure 1 Pictures of clearing zone diameter of isolates

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.

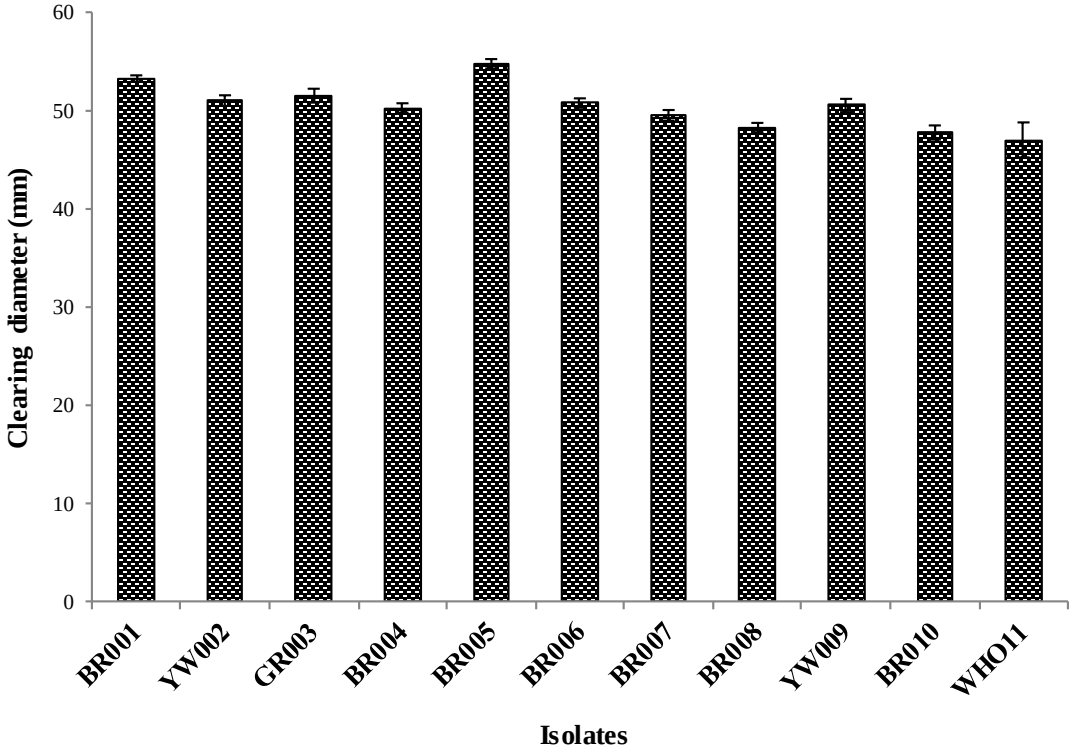

Figure 2 Clearing zone diameter of the fungal isolates after 96 hours incubation period.

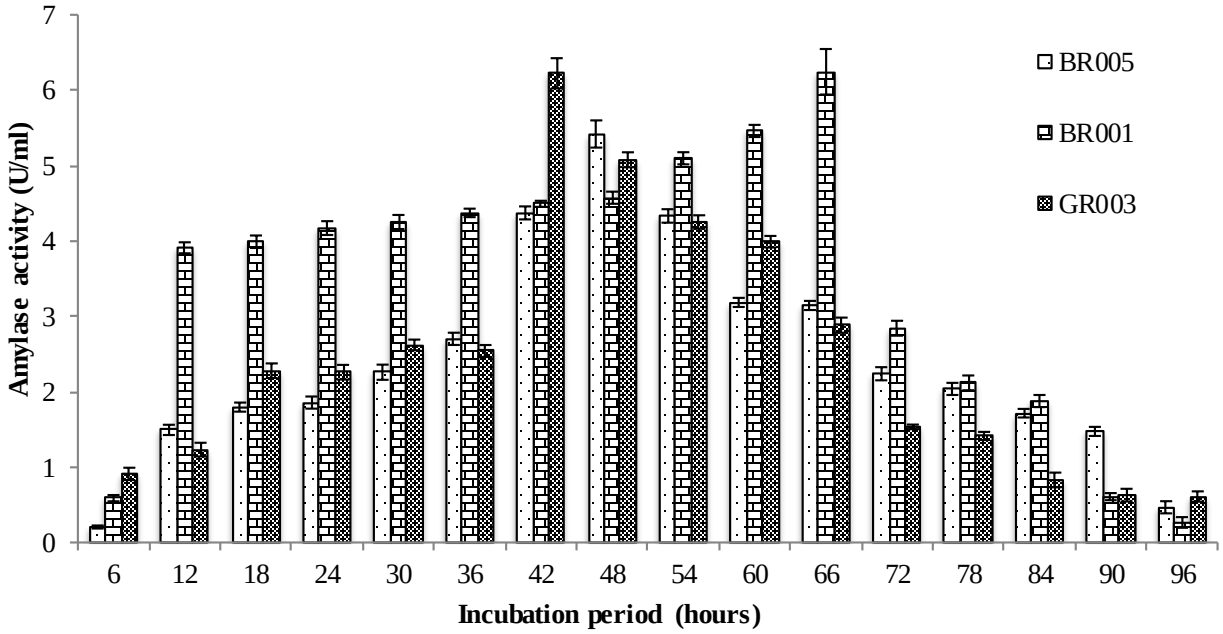

Figure 3 Time course of amylase activity (U/ml) during 96 hours incubation at 30 °C, pH 7.

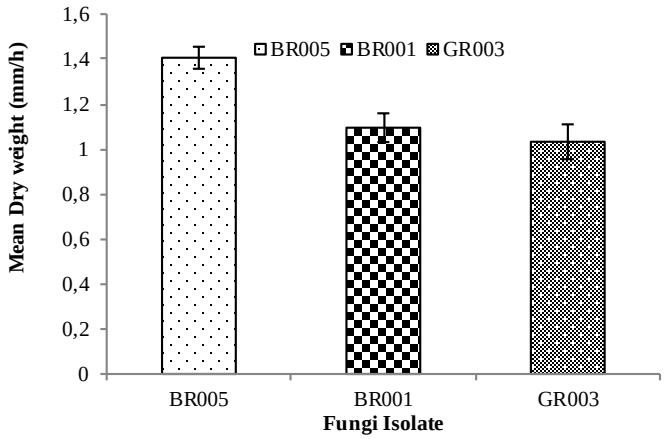

Figure 4 The growth rate of isolates over 96 hours of submerged fermentation at 30 ºC, pH 7

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**).

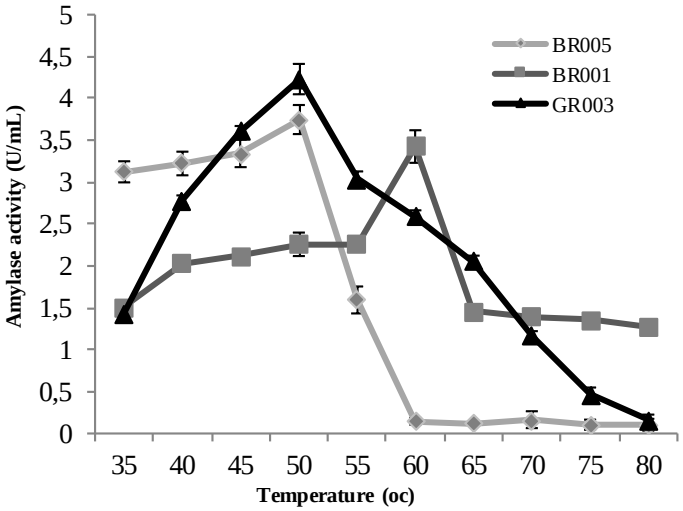

Figure 5 Effect of temperature on amylase activity

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.

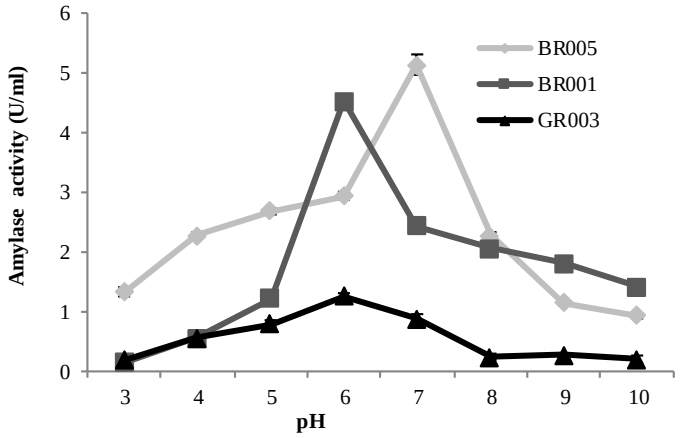

Figure 6 Effect of pH on amylase activity (U/mL) of isolates

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.

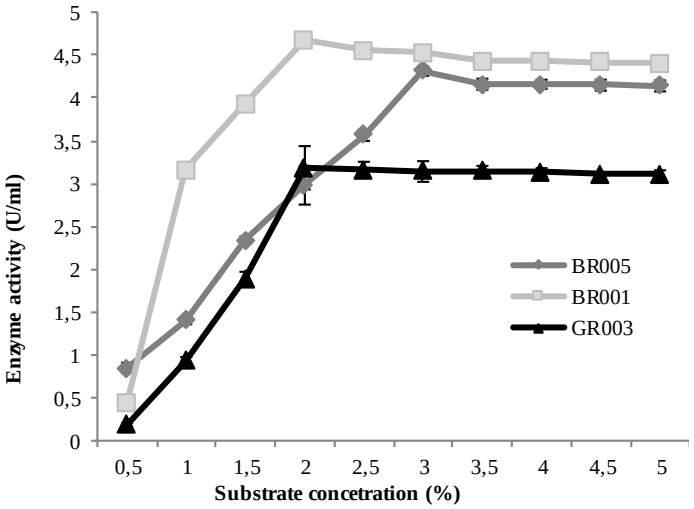

Figure 7 Effect of substrate concentration on amylase activity of isolates

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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