RESEARCH ARTICLE

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Isolation and screening of phytase-producing fungi for phytase production by solid state fermentation using agro wastes

ABSTRACT

Phytases are phosphatase enzymes that catalyze the hydrolysis of phytic acid and its salts. This study aimed to isolate and screen for phytase-producing fungi from cereals, fruits, palm kernel cake and soil samples by solid state fermentation. Isolation and identification was done using standard methods. The fungal isolates were screened for phytase production using phytase screening medium (PSM) agar. The isolates with the highest and consistent zone of hydrolysis were used. Eighty-seven (87) fungal isolates were obtained while eighteen showed consistent zone of hydrolysis. These were screened to five (5) isolates: *Aspergillus niger* PKruw7, *Aspergillus awamori* Pkruw5, *Aspergillus flavus* PBDJ7, *Aspergillus niger* MOJ5b and *Penicillium chrysogenum* OBDJ1.They were used for solid state fermentation using rice bran, soy bean and wheat bran for phytase production. The optimized conditions for phytase production were: 40ºC temperature, 5.5 pH, 1% w/w fructose and 0.5% w/w yeast extract by both *Aspergillus niger* PKruw7 and *Aspergillus flavus* PBDJ7, 40ºC, 4.5 pH, 1% w/w fructose and 0.5% w/w NH_4NO_3: *Aspergillus awamori* Pkruw5, 25ºC, 6.5 pH, 1% w/w fructose and 0.5% w/w NH_4NO_3: *Aspergillus niger* MOJ5b and 40ºC, 4.5 pH, 1% w/w sucrose and 0.5% w/w $(NH_4)_2SO_4$: *Penicillium chrysogenum* OBDJ1with incubation period of 120 hours optimal for all the isolates. Maximum phytase production from optimized culture conditions include; incubation period of 5 days, temperature of 40°C, pH of 4.5 to 6.5, fructose (1% w/w), yeast extract and ammonium nitrate (0.5% w/w). Phytase can be applied in animal feed to enhance digestibility and nutrient availability.

Key words: Phytate, phytase, hydrolysis, fermentation, optimization

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Introduction

Phytases are phosphatase enzymes that catalyze the hydrolysis of phytic acid and its salts (phytate) to yield inositol monophosphate, inositol and inorganic phosphate (Bohn et al., 2008; Azeke et al., 2011; Badamchi et al., 2013). Phytases also known as myo-inositol hexakisphosphate phosphohydrolase (EC 3.1.3.8), belong to the histidine acid phosphatase (HAP) family. They catalyze the hydrolysis of phytic acid into myo-inositol and phosphoric acid, converting penta- to mono-phosphates. This process enhances the bioavailability of essential minerals and reduces environmental pollution from excessive use of inorganic phosphorus supplements (Scott et al., 2024). Phytase enzymes are found in plants, animals, bacteria, yeast and filamentous fungi. Phytase activity is abundant in filamentous fungi, mostly in *Aspergillus* species (Jafari-Tapeh et al., 2012; Akturk et al., 2013; Bhavsar et al., 2013; Bakri et al., 2018). The use of filamentous fungi, *Aspergillus niger* for example that is used for the production of phytase through solid state fermentation (SSF) by agro-industrial wastes has been useful in research for some years now (Pandey et al., 2001) because SSF system offers several advantages including: high products concentration, improved products recovery, simple cultivation equipment and lower plant operational costs (Bakri et al., 2018). In the production of phytase, the substrate diversity comprises of agricultural residues like; citrus peels, rice bran, corn cobs, soybean meal, wheat bran, oil cakes, corn bran, and sugarcane bagasse, which can be utilized by different microorganisms, especially fungi, to produce the phytase enzyme; with sodium phytate being the optimal substrate for maximizing phytase activity

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(Sadh et al.,2018). The major components in animal feed are cereal grains and oilseed meals. They are known sources of phosphorus, which is an essential macro-element required for the growth of animal (Selle and Ravindran, 2008). The physico-chemical characteristics and catalytic properties of phytase depend upon the different fungal strains that serve as their source. Therefore, phytase production depends on different optimum temperatures and pH values that ranging from neutral to acidic (Yao et al., 2011; Singh and Satyanarayana, 2015). This research work was therefore aimed to isolate and screen for phytase- producing fungi for phytate production by solid state fermentation and optimal condition to produce phytase enzyme by selected isolates

Materials and Methods

Sample collection

Maize, sorghum, orange, water melon, pineapple samples were purchased from Bodija market and Ojoo, in Ibadan. Palm kernel cake was purchased from Eruwa (Ibarapa East area of Oyo State) while soil samples were taken from Ikire (Osun State).

Agro wastes which includes; rice bran, wheat bran and soy bean were also purchased from Bodija market in Ibadan, Oyo State. All the samples were brought to the Laboratory in sterile Ziploc bags.

Processing of samples

Maize and sorghum were wetted with sterile distilled water and kept in aluminium foil for 24 hours and then blended with mortar and pestle. Spoilt orange, pineapple and water melon were cut with sterile knife and individual representative sample was blended using sterile mortar and pestle. A small fraction of the palm kernel cake was also blended to fine particle size.

Isolation of fungal isolates

Potato dextrose agar (PDA, LAB M) was prepared according to manufacturer's instruction and used for the isolation and enumeration of fungi according to the method of Aneja (2005), using serial dilution and pour plate methods. One gram and 1mL of each of the processed solid samples; (maize, sorghum, palm kernel cake and soil) and liquid samples (orange, pineapple and water melon) were added into sterile distilled water to obtain 10-1 dilution. One mL of dilution levels 10^5 and 10^{-7} were dispensed into petri-dishes after which about 10 mls of sterilized molten PDA supplemented with 0.05mg/ml streptomycin was added to each plate to avoid bacterial contamination. Plates were then incubated at 25°C for 7 days. At the end of incubation, the plates were observed for colonial development. Pure cultures of the fungi were stored on PDA slants in the refrigerator at 4^0C for further studies.

Morphological and microscopic characterization of isolates

The colonies that developed on the different plates were grouped on the basis of their morphology. The isolates were observed and recorded with reference to Mycology Atlas. Leslie and Summerell (2006); Sharma and Pandey (2010).

Screening for phytase producing fungi

This was done by using phytase screening medium (PSM) by modified method of Howson and Davis, (1983). Screening was carried out on agar plates containing sodium phytate (4 g L^{-1}) as sole phosphorus source. The fungi were selected based on their ability to form halos. Positive results from the screening were confirmed by growing the fungi in PSM medium using submerged fermentation. The composition of PSM in Litres was: 20 g glucose, 4 g sodium phytate, 2 g $CaCl_2$, 5 g NH_4NO_3, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, 0.01 g $MnSO_4.H_2O$ and 0.01 g $FeSO_4.7H_2O$.The pH was adjusted to 5.5 by drop wise addition of 0.1M HCl. Thirty (30) mL aliquots was sterilized at 121ºC, at 15 psi for 15 minutes and allowed to cool before being poured into the petri-dishes and allowed to solidify. It was then incubated at 30°C for 7 days using orbital shaker at 150 rpm. Secondary screening was also done using the same PSM medium to ascertain the possibly probable best phytase producing fungal isolates. The isolates with higher clear zone diameter were selected for further work.

Inoculum preparation

Stock cultures were maintained on Potato Dextrose Agar (PDA) slants and stored at 4 °C. Spore suspension was prepared using well sporulated cultures. The concentration of spore suspension was measured using a Haemocytometer (Neubauer, Germany).

Production of phytase using solid state fermentation

Solid state fermentation was carried out using three different substrates which include; wheat bran, rice bran and soy bean. The substrates were grounded to particle sizes and then moistened with suitable amount of diluents (distilled water), having a pH adjusted to 5.5 using concentrated hydrochloric acid. The flasks were then sterilized at 121°C for 15 minutes and then cooled at room temperature. One millilitre of fungal spore suspension containing $1x10^7$ spores of each of the five phytase-producing fungal isolates were used to inoculate the fermentation media and incubated at room temperature for 7 days with samples being taken at every 24 hours.

Extraction of phytase enzyme

For crude phytase extraction, 10mL of citrate buffer (0.2M, pH 5.5) was added to each flask containing 2 g of the fermented medium. The flask containing the fermented media was then agitated in a water bath shaker at 200 rpm for 90 minutes. The suspension was filtered and centrifuged at 4,000

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rpm for 15 minutes at 4 °C. The clear supernatant was then used as crude enzyme extract for the estimation of phytase.

Assay for phytase enzyme

Enzyme activity was determined by using the modified procedure of McKie and McCleary (2016). One percent (w/v) phytic acid solution and enzyme extract 0.2 mL each was taken in a test tube and incubated at 37 °C for 15 minutes. Then, 0.4 mL of 15 % trichloroacetic acid (TCA) was added to stop the reaction. The mixture was then incubated at 50 °C for 15 minutes after adding colour reagent. A spectrophotometer (T70UV/VIS Spectrometer, PG Instrument Ltd) was used to determine the absorbance of reaction mixture at 655 nm against blank. One unit (U) of phytase is defined as the amount of enzyme releasing 1 µmol of inorganic phosphorus per ml per minute under the described assay conditions.

Statistical analysis

The data were analysed using Analysis of Variance (ANOVA) to determine the means with SPSS version 23 and the level of significance was set at $\alpha_{0.05}$. Bar chart was also used to compare the effects of different parameters on phytase production by the fungal isolates

Results

Counts and characterization of Fungi

A total of eighty-seven (87) fungal isolates were obtained from all the samples including maize, sorghum, palm kernel cake and soil. These isolates included species of: *Aspergillus* as the predominant genera, *Rhizopus, Fusarium, Trichoderma, Penicillium* and *Rhizoctonia.* Eighteen (18) of the fungal isolates including the species of *Aspergillus, Penicillium* and *Rhizoctonia* showed zone of hydrolysis on phytase screening medium (PSM). Fourteen (14) phytase positive fungal isolates were identified to be of the genera *Aspergillus*, one *Penicillium chrysogenum* and one *Rhizoctonia species* as shown in Table 1. These eighteen (18) isolates were further screened to five (5) using phytase screening medium (PSM). Four (4) of these fungal isolates were species of *Aspergillus* (including *Aspergillus niger* PKruw7, *Aspergillus awamori* Pkruw5, *Aspergillus flavus* PBDJ7 and *Aspergillus niger* MOJ5b) and one being *Penicillium chrysogenum* OBDJ1.

Production of phytase using solid state fermentation

Table 2 shows that soy bean had highest phytase production of 4.29 IU/mL, 4.08 IU/mL and 4.08 IU/mL for isolates; *Aspergillus niger* PKruw7, *Aspergillus awamori* PKruw5 and *Aspergillus niger* MOJ5b respectively while highest phytase production of 4.30 IU/mL and 4.21 IU/mL was obtained for isolates *Aspergillus flavus* PBDJ7 and *Penicillium chrysogenum* OBDJ1 respectively.

Maximum phytase production was observed at 120 hours of incubation for all the isolates as shown in table 4. Table 5 shows that incubation temperature of 40 °C is optimal for phytase production for all the isolates except *Aspergillus niger* MOJ5b which had optimal temperature of 25 °C.

The effect of pH on phytase production by the five (5) isolates is shown in Table 6. Here, *Aspergillus niger* PKruw7 and *Aspergillus flavus* PBDJ7 had higher phytase production of 2.97 IU/mL and 3.37 IU/mL respectively at optimal pH of 5.5. Isolates; *Aspergillus awamori* PKruw5 and *Penicillium chrysogenum* had highest phytase production of 2.65 IU/mL and 2.86 IU/mL respectively at optimal pH of 4.5. However, *Aspergillus niger* MOJ5b had its highest phytase production of 2.67 IU/mL at optimal pH of 6.5. Table 7 shows that fructose had highest phytase production for all the isolates except *Penicillium chrysogenum* OBDJ1 which had highest phytase production with sucrose. Yeast extract was found to produce highest phytase with isolates; *Aspergillus niger* PKruw7 and *Aspergillus flavus* PBDJ7 with enzyme activity 3.63 IU/mL and 3.53 IU/mL respectively, as shown in Table 8. However, with ammonium nitrate, highest phytase production of 3.62 IU/mL and 3.81 IU/mL was obtained for isolates; *Aspergillus awamori* PKruw5 and *Aspergillus niger* MOJ5b respectively while highest phytase production of 3.95 IU/mL was obtained for *Penicillium chrysogenum* OBDJ1.

In the determination of the best of the three (3) substrates which include; rice bran, soy bean and wheat bran for phytase production with characteristic industrial and economic value as shown in Figure 1. Rice bran and soy bean had higher phytase activity than wheat bran with respect to each of the isolates (*Aspergillus niger* PKruw7, *Aspergillus awamori* PKruw5, *Aspergillus flavus* PBDJ7, *Aspergillus niger* MOJ5b and *Penicillium chrysogenum* OBDJ1) used. After 5 days of incubation, for isolate *Aspergillus niger* PKruw7, the enzyme activity of the phytase produced from rice bran and soy bean is 4.04 IU/mL and 4.29 IU/mL respectively. For isolate *Aspergillus awamori* PKruw5, the enzyme activity of the phytase produced from rice bran and soy bean is 4.04 IU/mL and 4.08 IU/mL respectively. For isolate *Aspergillus flavus* PBDJ7, the enzyme activity of the phytase produced from rice bran and soy bean is 4.30 IU/mL and 4.06 IU/mL respectively. For isolate *Aspergillus niger* MOJ5b, the enzyme activity of the phytase produced from rice bran and soy bean is 3.93 IU/mL and 4.08 IU/mL respectively. For isolate *Penicillium chrysogenum* OBDJ1, the enzyme activity of the phytase produced from rice bran and soy bean is 4.21 IU/mL and 4.08 IU/mL respectively.

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Table 1. *Zone of hydrolysis of the fungal isolates on phytase screening medium (PSM)*

Isolate code	Source	Isolate name	Halo diameter (mm) mean ± SD	Colony diameter (mm) mean ± SD	Hydrolysis index mean ± SD
MOJ1	Maize	*A. tamari*	60±0.5	36±0.4	1.7±0.2
MOJ5b	**Maize**	***A. niger***	**75±0.4**	**43±0.4**	**1.7±0.2**
SOJ4	Sorghum	*Aspergillus* sp	72±0.4	42±0.4	1.7±0.1
MBDJ9	Maize	ND	46±0.3	30±0.5	1.5±0.2
SBDJ1	Sorghum	*Aspergillus* sp	51±0.5	31±0.3	1.6±0.2
OOJ4	Orange	*Aspergillus* sp	65±0.4	38±0.2	1.7±0.3
PBDJ7	**Pineapple**	***A. flavus***	**86±0.6**	**40±0.4**	**2.2±0.2**
WmBDJ1	Water melon	*Aspergillus* sp	74±0.5	43±0.4	1.7±0.2
WmBDJ2	Water melon	*Aspergillus* sp	67±0.5	40±0.3	1.7±0.2
PKruw5	**Palm kernel cake**	***A. awamori***	**70±0.5**	**34±0.5**	**2.1±0.2**
PKruw7	**Palm kernel cake**	***A. niger***	**76±0.4**	**30±0.3**	**2.5±0.1**
PKruw10	Palm kernel cake	*Aspergillus* sp	69±0.6	42±0.3	1.6±0.1
SlIK6	Soil	*Rhizoctonia* sp	42±0.6	26±0.3	1.6±0.2
SlIK10	Soil	*A. flavus*	70±0.4	40±0.4	1.7±0.3
SlUI10	Soil	*Aspergillus* sp	53±0.4	33±0.4	1.6±0.2
OBDJ1	**Orange**	***P. chrysogenum***	**40±0.3**	**10±0.5**	**4.0±0.2**
WmBDJ3	Water melon	ND	25±0.5	16±0.3	1.6±0.2
SlUI13	Soil	*Aspergillus* sp	44±0.4	26±0.3	1.7±0.2

Legend: MOJ: Maize from Ojoo, SOJ: Sorghum from Ojoo, MBDJ: Maize from Bodija, SBDJ: Sorghum from Bodija, OOJ: Orange from Ojoo, PBDJ: Pineapple from Bodija, WmBDJ: Water melon from Bodija, PKruw: Palm kernel cake from Eruwa, SlIK: Soil from Ikire, SlUI: Soil from University of Ibadan, OBDJ: Orange from Bodija**.**

Figure 2 shows the effect of incubation period on the phytase production using rice bran as fermentation medium with respect to each of the five (5) isolates. All the isolates reached the peak of their phytase production at day 5 (120 hours) then there was a decrease in production. All the five (5) fungal isolates; *Aspergillus niger* PKruw7, *Aspergillus awamori* PKruw5, *Aspergillus flavus* PBDJ7, *Aspergillus niger* MOJ5b and *Penicillium chrysogenum* OBDJ1 had enzyme activity of 4.04 IU/mL, 4.04 IU/mL, 4.30 IU/mL, 3.93 IU/mL and 4.21 IU/mL respectively which are higher than the phytase activity for other periods of incubation which had average of 2.0 IU/mL.

Figure 3 shows the effect of temperature on phytase production by the five (5) fungal isolates using rice bran as fermentation medium. The fermentation medium was subjected to varying temperatures ranging from 25, 30, 35, 40, 45 and 50ºC. It was observed that all the five (5) isolates except *Aspergillus niger* MOJ5b had their optimal temperature for phytase production to be 40ºC. However, *Aspergillus niger* MOJ5b had optimal production at 25ºC.

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Table 2. *Effect of different substrates on phytase production by the fungal isolates*

	Phytase production (IU/mL)		
Isolates	**Rice bran**	**Soy bean**	**Wheat**
***Aspergillus niger* PKruw7**	4.04±0.02	**4.29**±0.02	2.23±0.05
***Aspergillus awamori* PKruw5**	4.04±0.03	**4.08**±0.05	2.64±0.03
***Aspergillus flavus* PBDJ7**	**4.30**±0.04	4.06±0.05	3.78±0.02
***Aspergillus niger* MOJ5b**	3.93±0.05	**4.08**±0.05	2.70±0.05
***Penicillium chrysogenum* OBDJ1**	**4.21**±0.04	4.08±0.04	3.43±0.05
Control	**3.18**±0.02	3.15±0.02	0.51±0.04

*Values are means of duplicates; (±): represent the standard deviation, $p \leq 0.05$

Table 3. *Effect of incubation period on phytase production by the fungal isolates using rice bran*

	Phytase production (IU/mL)						
Isolates	**24hr**	**48hr**	**72hr**	**96hr**	**120hr**	**144hr**	**168hr**
***Aspergillus niger* PKruw7**	1.97±0.04	2.16±0.04	1.96±0.02	3.71±0.05	**4.04**±0.04	2.36±0.05	1.78±0.04
***Aspergillus awamori* PKruw5**	2.00±0.06	3.13±0.03	2.52±0.05	2.30±0.02	**4.04**±0.02	1.26±0.04	1.64±0.05
***Aspergillus flavus* PBDJ7**	2.75±0.03	2.06±0.04	2.50±0.04	3.11±0.04	**4.30**±0.04	2.33±0.04	2.32±0.06
***Aspergillus niger* MOJ5b**	3.33±0.02	2.30±0.04	2.35±0.02	2.99±0.04	**3.93**±0.05	2.81±0.05	2.95±0.02
***Penicillium chrysogenum* OBDJ1**	2.00±0.02	2.58±0.05	2.18±0.05	2.50±0.06	**4.21**±0.02	2.0±0.02	1.66±0.02

*Values are means of duplicates; (±): represent the standard deviation, $p \leq 0.05$

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Table 4. *Effect of incubation temperature on phytase production by the fungal isolates using rice bran*

Isolates	Phytase production (IU/mL)					
	25 ºC	**30 ºC**	**35 ºC**	**40 ºC**	**45 ºC**	**50 ºC**
***Aspergillus niger* PKruw7**	2.95±0.04	3.36±0.04	3.31±0.02	**3.94**±0.04	3.19±0.05	3.72±0.05
***Aspergillus awamori* PKruw5**	3.19±0.06	2.82±0.02	3.17±0.04	**3.25**±0.02	3.17±0.02	3.22±0.04
***Aspergillus flavus* PBDJ7**	3.11±0.04	2.74±0.02	3.10±0.04	**3.18**±0.02	2.84±0.04	2.93±0.02
***Aspergillus niger* MOJ5b**	**3.42**±0.04	2.79±0.04	2.96±0.04	3.35±0.04	2.89±0.03	2.72±0.02
***Penicillium chrysogenum* OBDJ1**	3.67±0.02	2.16±0.05	2.96±0.05	**3.82**±0.02	2.71±0.02	3.43±0.04

*Values are means of duplicate; (±): represent the standard deviation, $p \leq 0.05$

Table 5. *Effect of pH on phytase production by the fungal isolates using rice bran*

Isolates	Phytase production (IU/mL)						
	2.5	**3.5**	**4.5**	**5.5**	**6.5**	**7.5**	**8.5**
***Aspergillus niger* PKruw7**	1.89±0.04	2.40±0.04	2.00±0.04	**2.97**±0.04	2.08±0.04	1.88±0.04	2.29±0.02
***Aspergillus awamori* PKruw5**	2.15±0.02	2.42±0.04	**2.65**±0.04	2.32±0.02	2.23±0.02	2.22±0.04	2.14±0.04
***Aspergillus flavus* PBDJ7**	2.10±0.06	2.27±0.03	2.51±0.05	**3.37**±0.02	2.40±0.05	1.49±0.02	2.31±0.02
***Aspergillus niger* MOJ5b**	2.00±0.04	2.43±0.02	1.76±0.03	2.32±0.06	**2.67**±0.04	1.48±0.02	2.59±0.04
***Penicillium chrysogenum* OBDJ1**	1.57±0.02	2.01±0.04	**2.86**±0.04	2.58±0.02	2.64±0.02	2.41±0.06	2.35±0.04

*Values are means of duplicate; (±): represent the standard deviation, $p \leq 0.05$

Figure 4 shows the effect of pH as determined by adjusting the pH of the fermentation medium in the range of 2.5, 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5. Here, it was observed that *Aspergillus niger* PKruw7 and *Aspergillus flavus* PBDJ7 had optimal pH at 5.5 for phytase production with enzyme activity of 2.97 IU/mL and 3.37 IU/mL respectively while *Aspergillus awamori* Pkruw5 and *Penicillium chrysogenum* OBDJ1 had optimal pH of 4.5 with enzyme activity of 2.65 IU/mL and 2.86 IU/mL respectively and *Aspergillus niger* MOJ5b showing optimal pH of 6.5 with enzyme activity of 2.67 IU/mL.

The effect of carbon sources on phytase production by the five (5) fungal isolates using rice bran as fermentation substrate is shown in Figure 5. The different carbon sources include; 1%w/w each of glucose, sucrose, fructose, lactose and maltose. It was observed that fructose yielded highest phytase production in four (4); (*Aspergillus niger* PKruw7, *Aspergillus awamori* Pkruw5, *Aspergillus flavus* PBDJ7 and *Aspergillus niger* MOJ5b) out of the five (5) fungal isolates with enzyme activity of 3.95 IU/mL, 4.15 IU/mL, 3.5 IU/mL and 3.8 IU/mL respectively while *Penicillium chrysogenum*

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OBDJ1 showed highest phytase production with enzyme activity of 3.57 IU/mL when supplemented with sucrose.

The effect of nitrogen sources on phytase production by the five (5) fungal isolates using rice bran as fermentation medium is shown in Figure 6. The different nitrogen sources used were 0.5% w/w each of ammonium sulphate, ammonium nitrate, potassium nitrate, peptone and yeast extract. Yeast extract was found to be the optimal nitrogen source for phytase production by *Aspergillus niger* PKruw7 and *Aspergillus flavus* PBDJ7 with enzyme activity of 3.63 IU/mL and 3.53 IU/mL respectively while ammonium nitrate was found to be the optimal nitrogen source for *Aspergillus awamori* Pkruw5 and *Aspergillus niger* MOJ5b with enzyme activity of 3.62 IU/mL and 3.81 IU/mL respectively, however, ammonium sulphate was found to be the optimal nitrogen source for *Penicillium chrysogenum* OBDJ1 with enzyme activity of 3.84 IU/mL.

Table 6. *Effect of different carbon sources on phytase production by the fungal isolates using rice bran*

	Phytase production (IU/mL)					
Isolates	**Glucose**	**Sucrose**	**Fructose**	**Lactose**	**Maltose**	**Control**
***Aspergillus niger* PKruw7**	3.56±0.02	2.94±0.02	**3.95**±0.02	3.15±0.04	3.65±0.05	3.35±0.02
***Aspergillus awamori* PKruw5**	3.60±0.04	3.45±0.04	**4.15**±0.04	3.22±0.05	3.43±0.05	2.93±0.04
***Aspergillus flavus* PBDJ7**	3.04±0.02	3.09±0.04	**3.50**±0.06	3.39±0.02	3.45±0.06	2.81±0.04
***Aspergillus niger* MOJ5b**	3.27±0.06	3.74±0.05	**3.84**±0.05	3.08±0.02	3.04±0.04	3.46±0.04
***Penicillium chrysogenum* OBDJ1**	3.08±0.05	**3.57**±0.05	3.19±0.02	3.05±0.05	3.55±0.02	2.32±0.02

*Values are means of duplicates; (±): represent the standard deviation, $p \leq 0.05$

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Table 7. *Effect of different nitrogen sources on phytase production by the fungal isolates using rice bran*

	Phytase production (IU/mL)					
Isolates	**$(NH_4)_2SO_4$**	**$NH_4(NO_3)$**	**KNO_3**	**Peptone**	**Yeast Extract**	**Control**
***Aspergillus niger* PKruw7**	3.15±0.04	3.20±0.06	2.96±0.02	3.19±0.05	**3.63**±0.05	2.98±0.04
***Aspergillus awamori* PKruw5**	3.51±0.02	**3.62**±0.04	3.24±0.05	3.54±0.02	3.35±0.03	2.93±0.04
***Aspergillus flavus* PBDJ7**	3.30±0.05	3.27±0.04	3.13±0.04	3.45±0.04	**3.53**±0.02	2.81±0.02
***Aspergillus niger* MOJ5b**	3.19±0.02	**3.81**±0.04	3.59±0.02	2.70±0.02	3.70±0.04	3.06±0.05
***Penicillium chrysogenum* OBDJ1**	**3.95**±0.04	3.79±0.04	3.76±0.06	3.84±0.06	2.84±0.02	2.32±0.04

***Values are means of duplicates; (±): represent the standard deviation, $p \leq 0.05$**

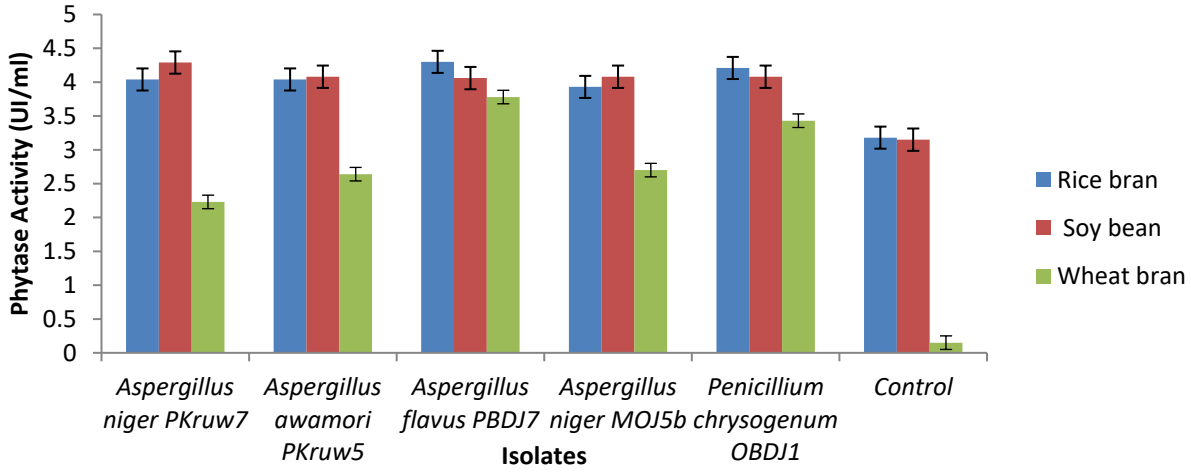

Figure 1. *Effect of different substrates on phytase production by fungal isolates.*

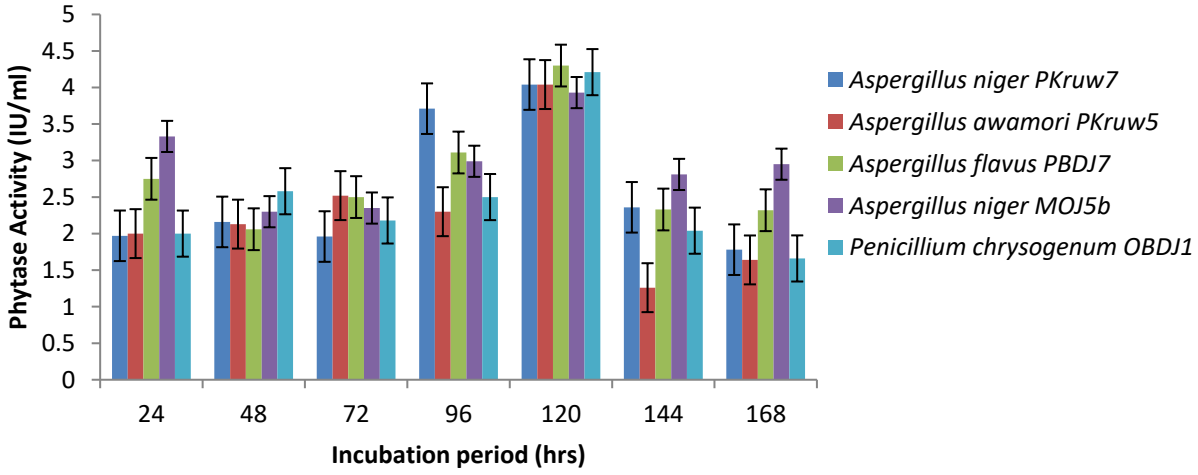

Figure 2. *Effect of incubation period on phytase production by the fungal isolates*

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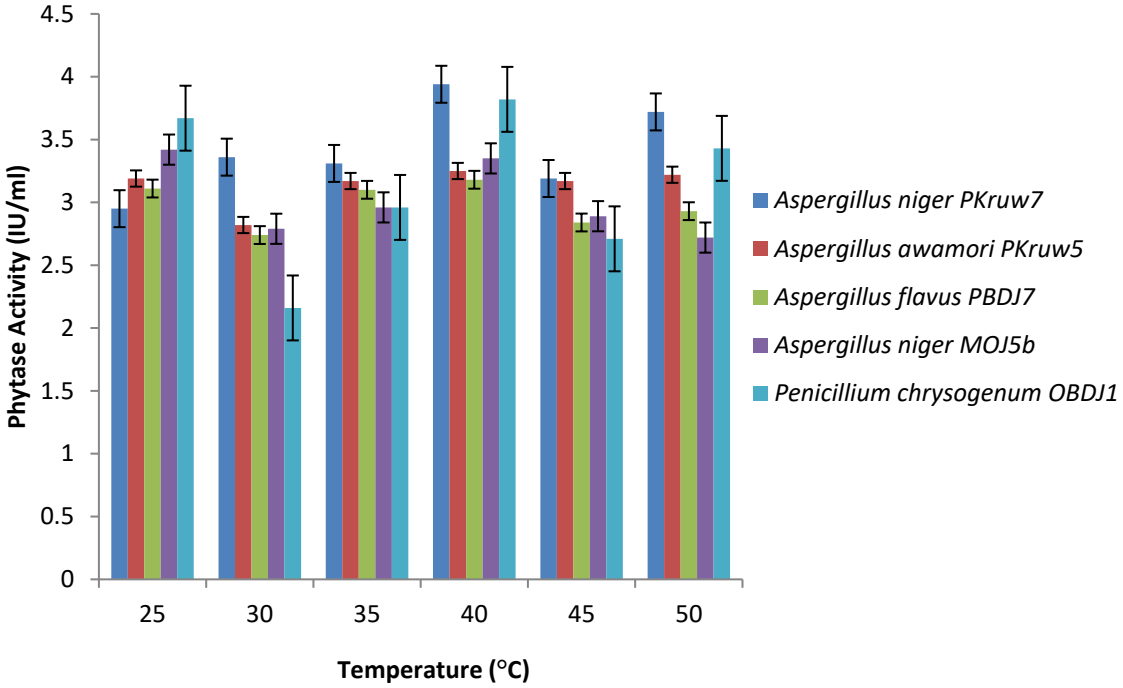

Figure 3. *Effect of varying temperature on phytase production by the fungal isolates.*

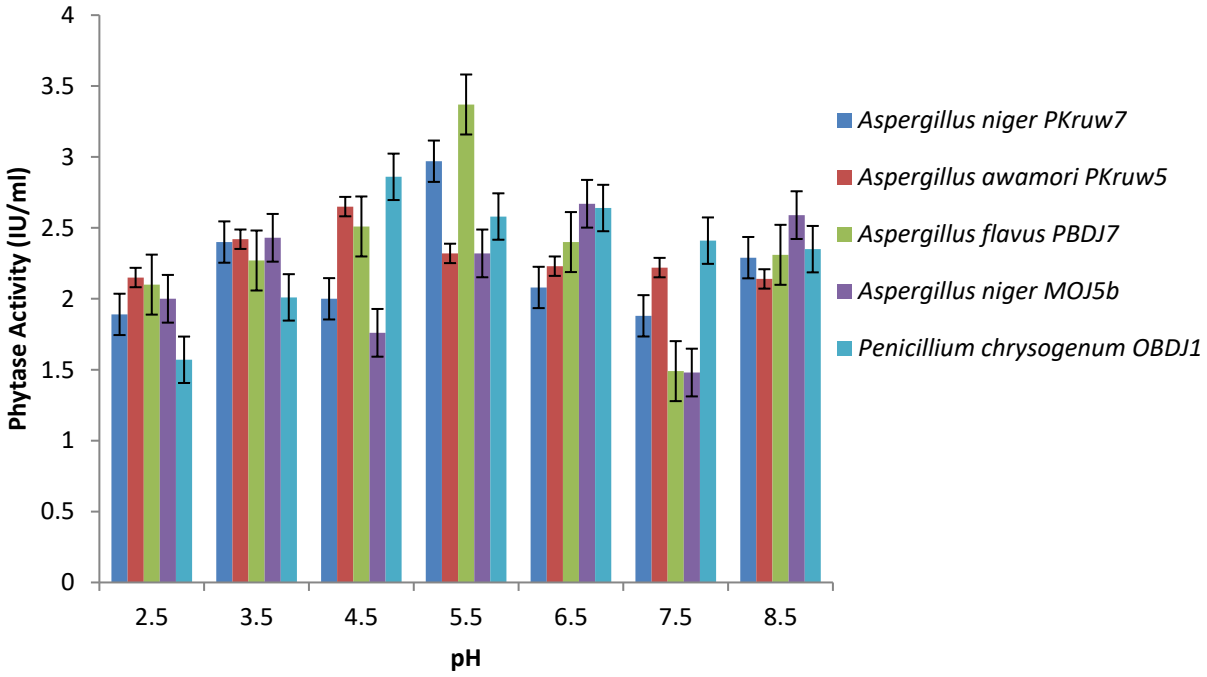

Figure 4. *Effect of varying pH on phytase production by the fungal isolates*

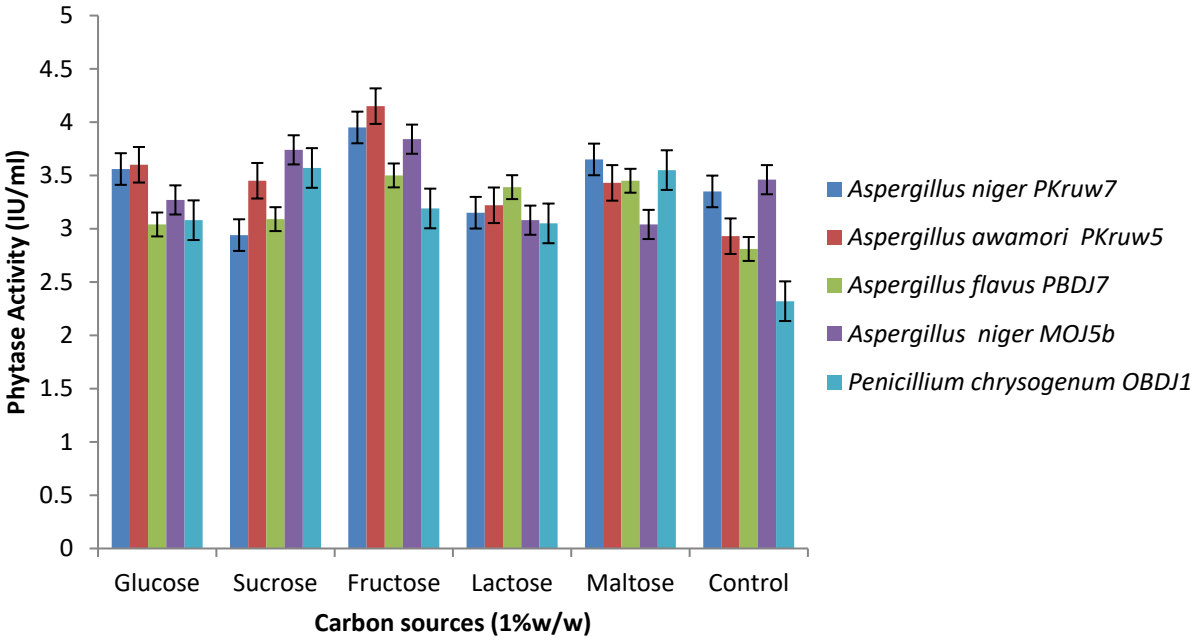

Figure 5. *Effect of different carbon sources on phytase production by the fungal isolates.*

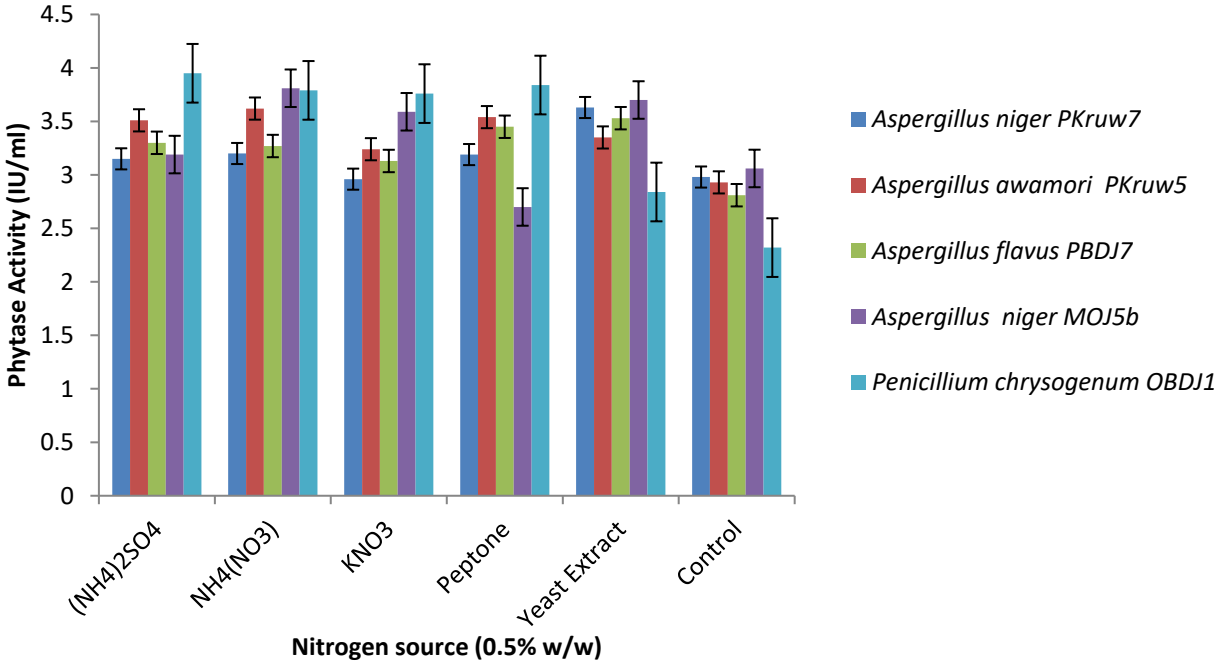

Figure 6. *Effect of different nitrogen sources on phytase production by the fungal isolates.*

http://www.jbb.uni-plovdiv.bg DOI: 10.69085/jbb20251037

Discussion

Eighty-seven (87) fungal isolates were obtained from different samples including: maize, sorghum, orange, pineapple, water melon, palm kernel cake and soil. This is in agreement with the findings of Arnold, (2007) and Arnold and Lutzoni, (2007) who also isolated similar fungi from the same sources. The fungal isolates were of the genera: *Aspergillus*, *Rhizopus, Fusarium, Trichoderma, Penicillium* and *Rhizoctonia.* None of the species of *Rhizopus, Fusarium* and *Trichoderma* showed zone of hydrolysis when screened for phytase production using phytase screening medium (PSM). According to Maciel et al., (2013), screening is the first step for selecting the microorganisms that has the essential features for industrial applications. However, eighteen (18) of the fungal isolates including the species of *Aspergillus, Penicillium* and *Rhizoctonia* showed zone of hydrolysis on phytase screening medium (PSM). This is in line with the report of Singh and Satyanarayana, (2015) who reported that phytases have been commonly detected in many fungal species and are most often characterized by their presence in those fungal species.

These eighteen (18) fungal isolates were further screened to five (5) using phytase screening medium (PSM. Four (4) out of these fungal isolates were species of *Aspergillus* (including *Aspergillus niger* PKruw7, *Aspergillus awamori* Pkruw5, *Aspergillus flavus* PBDJ7 and *Aspergillus niger* MOJ5b) and one being *Penicillium chrysogenum* OBDJ1 based on their consistency coupled with the wideness of their zone of clearance. *Aspergillus* spp has the highest occurrence (77.78%) of the eighteen (18) fungal isolates. This is in agreement with the work of Gunashree and Venkateswaran, (2014) who reported *Aspergillus niger* as the best fungi when compared with others that is: *Penicillium commune, Rhizopus oligosporus, Trichoderma viride and Saccharomyces cerevisiae* for phytase production by solid state fermentation process. Buddhiwant et al., (2016); Thakur et al., (2017); Neira-Vielma et al., (2018); Sarita et al., (2018) reported many other species of genus *Aspergillus* for the production of phytase.

These substrates i.e., rice bran, soy bean and wheat bran has been reported by Das and Ghosh (2014) to be suitable for phytase production. The five (5) isolates (*Aspergillus niger* PKruw7, *Aspergillus awamori* Pkruw5, *Aspergillus flavus* PBDJ7 and *Aspergillus niger* MOJ5b) and one being *Penicillium chrysogenum* OBDJ1) were selected and used for solid state fermentation. The highest yield of phytase production by *Aspergillus flavus* PBDJ7 (4.30 IU/mL) and *Penicillium chrysogenum* OBDJ1 (4.21 IU/mL) with rice bran over that with soy bean and wheat bran agreed with the work of Gunashree and Venkateswaran (2014) who reported rice bran as good substrate for phytase production by *Aspergillus niger, Rhizopus oligosporus* and *Aspergillus ficcum*. This also was based on the consideration by (Kumar and Kanwar, 2012) who noted the cost problem as one of the major hinderances in phytase production.

The best incubation period for phytase production using rice bran as fermentation medium with respect to each of the five (5) fungal isolates showed that all the isolates reached the peak of their phytase production at day 5 (120 hours) after when they began to have decrease in production. This corroborates the work of Mahmood et al., (2021); Gupta et al., (2014); Gunashree and Venkateswaran (2014) and Bakri et al., (2018) who reported that phytase production started after 48 hours of inoculation and increased with the increase in incubation time up to 120 hours, reaching its maximum and afterward, a decrease in phytase production. However, this is in disagreement with the report by Sandhya et al., (2015), Tian and Yuan (2016) who reported 4 days and 6 days as optimum incubation time for phytase production by *Aspergillus niger* and *Aspergillus ficuum*, respectively and also Gaind and Singh (2015) who reported maximum phytase activity in fermented substrate by *A. flavus* at 96 hours.

All the five (5) fungal isolates: *Aspergillus niger* PKruw7, *Aspergillus awamori* PKruw5, *Aspergillus flavus* PBDJ7, *Aspergillus niger* MOJ5b and *Penicillium chrysogenum* OBDJ1 had high enzyme activity of 4.04 IU/mL, 4.04 IU/mL, 4.30 IU/mL, 3.93 IU/mL and 4.21 IU/mL respectively as against the values for other periods of incubation with average of 2.0 IU/mL. The lower phytase activity before day 5 may be due to the fungal isolates inactivation on the fermentation medium while that after day 5 may be due to the inhibition of the fermentation process due to the exhaustion of nutrients. This agrees with the report by Ramachandran et al., (2005); Singh and Satyanarayana, (2006) who reported that longer incubation period did not result into increase of enzyme production due to reduction in nutrients in the substrate, denaturation of the enzyme, catabolic repression or due to production of other by-products.

The range of temperature from 25 °C to 50 °C used in this study showed activity for phytase production which invariably showed wider temperature adaptability by fungi. The most suitable temperature for phytase production as shown by four (4) of the isolates; *Aspergillus niger* PKruw7, *Aspergillus awamori* Pkruw5, *Aspergillus flavus* PBDJ7 and *Penicillium chrysogenum* OBDJ1 is 40 °C while that of *Aspergillus niger* MOJ5b was observed to be 25 °C. This is in agreement with the findings by Hillebrand, (2004) and Arnold and Lutzoni, (2007) among other researchers that tropical regions of the world are considered to have the highest diversity for most groups of organisms and specifically fungi.

In this research, the pH for maximum phytase production falls between acidic range and near neutral (4.5 and 6.5) across the 5 isolates. It was observed that *Aspergillus niger* PKruw7 and *Aspergillus flavus* PBDJ7 showed optimal pH of 5.5 for phytase production with enzyme activity of 2.97 IU/mL and 3.37 IU/mL respectively.

This is in line with the report of the research findings by Vohra and Satyanarayana, (2003); Pardo et al., (2006); Yao et al., (2011); Zhang et al., (2013b) and Qasim et al., (2017) who reported that most fungal phytases are active under acidic pH conditions in the optimum pH range of 4.5–6.0.

The effect of carbon sources on phytase production by the fungal isolates showed that *Penicillium chrysogenum* OBDJ1 showed highest phytase production with enzyme activity of 3.57 IU/mL when supplemented with sucrose. This is in agreement with the work of Mahmood et al., (2021) whose results showed sucrose (200 IU/g) as the next best carbon source for phytase production after glucose, though reported for *Aspergillus niger*.

The best nitrogen sources for phytase production by the five (5) fungal isolates using five (5) different nitrogen sources including; ammonium sulphate, ammonium nitrate, potassium nitrate, peptone and yeast extract showed that Ammonium nitrate was found to be the best nitrogen source for *Aspergillus awamori* Pkruw5 and *Aspergillus niger* MOJ5b with enzyme activity of 3.62 IU/mL and 3.81 IU/mL respectively. This is in agreement with Ramachandran et al., (2005); Sandhya et al., (2015); Suresh and Radha, (2016) who reported that NH_4NO_3 at a concentration level of 0.5% (w/w) gave a maximum phytase activity (243.10±5.06 IU/g) by *Aspergillus niger*.

Conclusion

In this study, it was observed that there was high phytase production by rice bran compared with soy bean and wheat bran for optimal phytase production using each of the fungal isolates; *Aspergillus niger* PKruw7, *Aspergillus awamori* PKruw5, *Aspergillus flavus* PBDJ7, *Aspergillus niger* MOJ5b and *Penicillium chrysogenum* OBDJ1. This suggested a highly cost effective source of phytase with characteristic benefit to animals as it aids their feed digestibility. This research also suggests that maximum phytase production can be enhanced by optimizing the culture conditions which include; culture incubation period of five (5) days, incubation temperature of 40°C, pH of 4.5 to 6.5, fructose (1% w/w) and yeast extract or ammonium nitrate (0.5% w/w). Phytase can be applied in animal feed to enhance digestibility and nutrient availability.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request

Competing interests

The authors declare that they have no competing interests

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No external source of funding was received for this work.

Authors' contributions

FTA designed the work. SOA collected the data and also prepared the manuscript together with FTA. FTA analyzed some of the data. All authors read and approved the final manuscript.

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