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Isolation and Screening for Phytase-Producing Fungi for Phytase Production by Solid State Fermentation of Agro Wastes.

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ABSTRACT

Phytases are enzymes found in animals, plants, bacteria, yeast and filamentous fungi. This study aimed to isolate and screen for phytase-producing fungi from cereals, fruits, palm kernel cake and soil by solid state fermentation. The samples were purchased from Ojoo and Bodija in Ibadan, Nigeria. Isolation was done using Potato Dextrose Agar. The isolates were screened for phytase production using phytase screening medium agar and phytase activity was determined. Eighty-seven fungal isolates were obtained from the samples, out of which eighteen showed consistent zone of hydrolysis and were: *Aspergillus*, *Penicillium* and *Rhizoctonia*. Five fungal isolates; *Aspergillus niger* PKruw7, *Aspergillus awamori* Pkruw5, *Aspergillus flavus* PBDJ7, *Aspergillus niger* MOJ5b and *Penicillium chrysogenum* OBDJ1 were used for fermentation and phytase production. The optimized condition for phytase production were: 40ºC, 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 by *Aspergillus awamori* Pkruw5, 25ºC, 6.5 pH, 1% w/w fructose and 0.5% w/w NH_4NO_3 by *Aspergillus niger* MOJ5b and 40ºC, 4.5 pH, 1% w/w sucrose and 0.5% w/w $(NH_4)_2SO_4$ by *Penicillium chrysogenum* OBDJ1. Incubation period of 120 hours was optimal for all the isolates. In conclusion, optimized culture conditions for phytase production was; incubation period: 5 days, 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). This optimized phytase can thereby be applied in animal feed to enhance its digestibility and nutrient availability.

Keywords: Phytates, phytase, hydrolysis, fermentation, optimization.

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1. BACKGROUND OF STUDY

Phytases (myo-inositol hexakis phosphate phosphohydrolase) (EC 3.1.3.8) are phosphatase enzymes that catalyze the hydrolysis of phytic acid and its salts (phytate) and yield inositol monophosphate, inositol and inorganic phosphate (Azeke *et al*., 2011). Phytase enzyme can be found in animals, plants, bacteria, yeast and filamentous fungi. However, among microorganisms, phytase activity has been abundantly found in filamentous fungi, particularly in *Aspergillus* species (Bakri *et al*., 2018).
The use of filamentous fungi such as *Aspergillus niger* for the production of phytase through solid state fermentation (SSF) using agro-industrial wastes has gained many interests for research in the last years (Pandey *et al*., 2001). This is because SSF system offers several economical and practical advantages including high products concentration, improved products recovery, simple cultivation equipment and lower plant operational costs (Bakri *et al*., 2018). Cereal grains and oilseed meals are major ingredients in animal feed as they are known sources of phosphorus, an essential macro-element required for animal growth (Singh and Satyanarayana, 2014). However, the physico-chemical characteristics and catalytic properties of phytase depend upon the different fungal strains that serve as their source. Thus, the phytase production of fungi is dependent upon differing optimum temperatures and pH values that range from neutral to acidic (pH 1–6) or alkaline (pH 8–14) (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

2. STATEMENT OF STUDY

The use of chemical method in the production of phytase is expensive. Therefore, the use of biological methods using filamentous fungi such as *Aspergillus niger* for the production of phytase through solid state fermentation (SSF) using agro-industrial wastes has is being used in this research work because solid state fermentation (SSF) system offers several economical and practical advantages including high products concentration, improved products recovery, simple cultivation equipment and lower plant operational costs

3. OBJECTIVE

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

4. METHODOLOGY

4.1 Sample collection

Samples of maize, sorghum, orange, water melon, pineapple were purchased from Bodija market and Ojoo, Ibadan. Palm kernel cake was purchased from Eruwa (Ibarapa East area of Oyo State) while soil samples were taken from Ikire (Osun State) and University of Ibadan separately. Samples of agro wastes which includes; rice bran, wheat bran and soy bean were purchased from Bodija market in Ibadan, Oyo State. All the samples were brought to the Laboratory in sterile polythene bags.

4.2 Screening for phytase producing fungi

This was done by using phytase screening medium (PSM) by modified method of Howson and Davis, (1983). The medium was sterilized at 121°C, 15 psi for 15 minutes and allowed to cool before being poured into the petri-dishes and allowed to solidify. Point inoculation was done using flamed inoculating needle to transfer inoculums from pure plates of 3 days old fungal isolates unto the centre of the plates containing already set PSM agar medium. The plates were then incubated at room temperature for 3 days. After three days, the plates were observed for zones of clearance and individual diameter was measured and recorded.
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 next experiments.

4.3 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 substrates which were also supplemented with trace amount of growth factors were then taken separately into different Erlenmeyer flasks and mixed thoroughly.

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 (*Aspergillus niger* Pkruw7, *Aspergillus awamori* Pkruw5, *Aspergillus flavus* PBDJ7, *Aspergillus niger* MOJ5b and *Penicillium chrysogenum* OBDJ1) were used to inoculate the fermentation media (to make up for 80% moisture content of the media) by aseptically transferring each of the organisms into the respective flask containing the medium and then incubated at room temperature for 7 days with samples being taken at every 24 hours.

5. RESULTS

A total of eighty-seven (87) 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 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 based on their consistency coupled with the wideness of their zone of clearance.

The characteristic features of the five (5) fungal isolates showing consistent zone of hydrolysis on phytase screening medium (PSM) was shown in Table 2.

In the determination of the best of the three (3) substrates which include; rice bran, soy bean and wheat bran for phytase production as shown in Figure 1 with characteristic industrial and economic value. 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.

Table 1: Fungal isolates showing zone of hydrolysis 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
SIIK6	Soil	*Rhizoctonia* sp	42±0.6	26±0.3	1.6±0.2
SIIK10	Soil	*A. flavus*	70±0.4	40±0.4	1.7±0.3
SIUI10	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
SIUI13	Soil	*Aspergillus* sp	44±0.4	26±0.3	1.7±0.2

Key: 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, SIIK: Soil from Ikire, SIUI: Soil from University of Ibadan, OBDJ: Orange from Bodija.

Table 2: Characteristic features of fungal isolates showing consistent zone of hydrolysis on phytase screening medium (PSM)

ISOLATES	CHARACTERISTIC FEATURES
Aspergillus niger	Initially grows as white velvety mycelia forming ringed circular end which later produced heavy brownish black spore heads. Yellow reverse colour which appears wrinkled or heavily furrowed. It has hyaline, septate hyphae with conidial head with characteristic biseriate, large, globose, dark brown becoming radiate with phialides borne on metulae.
Aspergillus awamori	Conidial heads were white to brown, loosely globose, biseriate with phialides borne on metulae. Conidiophores were hyaline to pale brown and non-constricted below vesicles.
Aspergillus flavus	Initially grows as yellow mycelia but quickly become bright to darken yellow with age. It is a spreading yellow-green, granular, flat colony with

	radial grooves. It has characteristic rough walled stipes with mature vesicles bearing phialides over their entire surface and conspicuously echinulate conidia. Conidial heads were radiate and biseriate but with some phialides borne directly on the vesicle (becoming uniseriate). Conidiophore stipes were hyaline and coarsely rough with globose to subglobose, pale green and conspicuously echinulate conidia.
Penicillium chrysogenum	Slow growing green compacted dried surface which is raised with vertically wavy (trough and crest) margin. It has a golden yellow reverse colour.

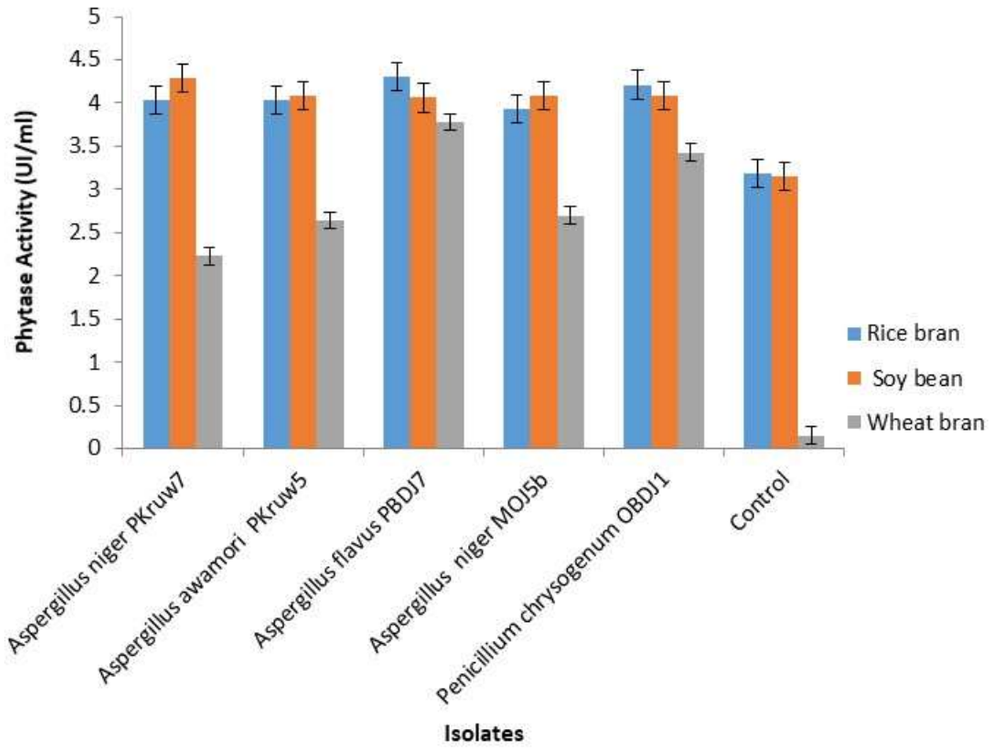

Figure 1: Effect of different substrates on phytase production by fungal isolates

6. DISCUSSION OF FINDINGS

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 and Lutzoni, (2007) that fungi appear to be common place and that every plant species examined till date harbours one or more endophytic fungi particularly in tropical regions of the world which are considered to have them in highest diversity. The fungal isolates included species of *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 by Singh and Satyanarayana, (2014) 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 amongst all the others which include: *Penicillium commune, Rhizopus oligosporus, Trichoderma viride and Saccharomyces cerevisiae* for phytase production by solid state fermentation process (Neira-Vielma *et al*., (2018).

As fungal phytases are commonly produced using solid-state fermentation (SSF) methods, in which agricultural waste and other cheap natural substrates are used as substrates (Huang *et al*., 2018). This study considered the use of three substrates which include; rice bran, soy bean and wheat bran for solid state fermentation. This is in agreement with Shahid and Nadeem, (2015) who noted that microorganism must be provided with a suitable growth medium in which it can grow and produce maximum amount of enzyme. This substrate 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 using the three different substrates which include; rice bran, soy bean and wheat bran to determine the best phytase-producing substrate with industrial and economic value. 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 agrees with the work of Sandhya *et al*., (2015) who reported rice bran as good substrate for phytase production by *Aspergillus niger, Rhizopus oligosporus* and *Aspergillus ficcum*.

7. CONCLUDING REMARKS

In this research, it was observed that there was high phytase production by rice bran over that of 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 suggests a highly cost effective source of phytase with characteristic benefit to animals as it aids their feed digestibility.

8. CONTRIBUTION TO KNOWLEDGE

Phytase can be applied in animal feed to enhance digestibility and nutrient availability.

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