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ISOLATION AND SCREENING OF PHYTATE-DEGRADING YEASTS FROM CEREALS

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ABSTRACT

Application of phytase (myo-inositol hexakisphosphate phosphohydrolase) to catalyze the release of phosphate from phytates contained on grain-based feed has been used widely. This study was carried out to isolate, identify, screen and produce yeast phytase from cereals using submerged fermentation. Two hundred and twenty seven (227) yeast isolates were obtained from maize, sorghum and millet and identified based on various characteristics such as colony morphology, microscopy, biochemical tests, sugar fermentation tests and molecular analysis. The isolates were then screened for phytase production by growing them on Phytase Screening Medium (PSM) and observing the formation of a clear zone around their colonies, indicating their ability to degrade phytate. It was found that yeast species such as *Pichia membranefaciens, Meyerozyma guilliermondii* SWS81*, Candida krusei, M. guilliermondii* M122*, Pichia fermentans, M. guilliermondii* WM226 and *Schwanniomyces occidentalis*, were capable of phytate degradation. *M. guilliermondii* M122*, M. guilliermondii* SWS81, and *M. guilliermondii* WM226 with higher solubilizing indices (4.52 mm, 3.64 mm and 5.14 mm respectively) were selected for production and assay. The results showed that crude enzymes from these yeast strains had phytase activity ranging from 44.70 U/mL to 97.70 U/mL, making them potential supplements for animal feeds to improve nutritional status and combat environmental phosphorus pollution.

Keywords: Phytase, Phytate, Yeast, Submerged, Fermentation, Optimization

INTRODUCTION

Micronutrients play important role in the growth and development of plants, and it also promotes good health in humans and animals (Bashir et al. 2013). Nitrogen and phosphorus (P) are also vital components necessary for microorganisms' growth and development. It serves multiple functions, which includes energy generation, metabolic control, and forms a portion of the macromolecular structure. However, due to its low concentration and poor mobility in soil, the availability of phosphorus to plants is limited by strong interactions with soil constituents (Shen et al. 2011). The supplementation of inorganic phosphorus in non-ruminant animal feed and the excretion of phosphorus from plant phytate into effluents are contributing to a global ecological issue by causing eutrophication in water bodies (Azeem et al. 2015).

Sustainability is essential when it comes to safeguarding the environment and enhancing the quality of phosphorus in livestock (Prasad et al. 2015). Phytic acid, in grain-based feed, and contains 60-90% of the total phosphorus in various cereals (Gao et al. 2021). Phytate, a polyanionic molecule, binds with positively charged nutrients like Ca_2C, Mg_2C, Zn_2C, and Fe_2C, and acts as an anti-nutritional factor by forming protein complexes that interfere with digestion and block specific digestive enzymes, such as a-amylase, trypsin, phosphatase acid, and tyrosinase (Song et al. 2021). Some animals, such as swine, poultry, and pre-ruminant calves, do not have phytate-phosphorus hydrolyzing enzymes in their digestive systems, which can lead to reduced phosphorus bioavailability (Wu et al. 2022). Thus, a sustainable strategy is essential to address these challenges and safeguard the environment while improving phosphorus quality in livestock.

Phytate-degrading activity has been observed in different microorganisms, plants and animal tissues, resulting in the isolation, purification, and characterization of the enzymes, which have subsequently been produced on a large scale (Brautaset et al. 2010; Guo et al. 2016). The filamentous fungi phytases, particularly those from *Aspergillus niger*, have been extensively studied and are commonly used as a microbial source of phytase in feed applications (Lei et al. 2018). Information on various aspects of phytases (such as the characteristics of phytate-degrading enzymes or phytases, their identification, and production), as well as their applications in the food and feed industries, have been published in the past (Selle and Ravindran, 2007; Lei et al. 2013; Ullah et al. 2015; Wu et al. 2021). Phytate hydrolysis using *Saccharomyces cerevisiae* var. *boulardii*, also increased the functional value of plant-based food products (Menezes et al., 2020). Some research revealed that yeast group such as S. cerevisiae yeast produced phytase of high specific activity. The use of phytase produced by yeast as feed additive becomes a solution for nutritional efficiency in poultry because of the high content of phytic acid as anti-nutrient in poultry feed. However, there is a dearth of information on the production of phytase from yeast. This research therefore aimed at the isolation, identification and screening of phytase-producing yeast from cereals

MATERIALS AND METHODS

Sample Collection

Two (2) kg samples of various cereals such as millet (brown variety), sorghum (white variety and maize (white variety) were bought from Bodija Market in Ibadan and transported in sterile bags to the Department of Microbiology, University of Ibadan, for further investigation. Ground samples were fermented for five days in 500 ml Erlenmeyer flasks and then incubated at 30°C at static condition, after which the aforementioned methods were employed (Kurtzman et al. 2011).

Isolation of Yeast

Serial dilutions of the stock samples of the milled samples were conducted. To achieve this, 1.0 ml of the milled sample was placed in a test tube containing 9 ml of sterile distilled water, and mixed thoroughly. This dilution procedure was repeated until a 10^{-9} factor was achieved. This was used for the isolation of yeast from the milled samples after each day

of fermentation for five consecutive days. One milliliter of the serial dilutions (10^{-3}, 10^{-5} and 10^{-7}) was transferred aseptically into dishes in 3 replicates. About ten mls of Yeast Extract Agar (YEA) ((LAB M) was then poured into the plates at about 45ºC and then allowed to solidify after which the plates were also incubated at room temperature in the laboratory (Kurtzman et al. 2011).

Identification of Isolates

The yeast isolates were identified based on their colonial morphology, microscopy, biochemical tests and sugar fermentation and with the aid of photomicrographs (Kurtzman et al. 2011). Isolates were also identified using molecular analysis. The molecular identification of the yeast isolates was done using 18SrRNA analysis and Polymerase chain reaction (PCR) amplification of internal transcribed spacer (ITS) region.

Screening of Phytate-Degrading Yeast

The isolated pure strains were screened for the production of extra cellular phytase using Phytase Screening Medium (Howson & Davis, 1983) as a screening medium (Glucose - 20.0 g, Ammonium nitrate - 5.0 g, Magnesium sulphate - 0.5 g, Potassium chloride - 0.5 g, Calcium chloride – 0.5g, Ferrous sulphate - 0.01 g, Manganese sulphate - 0.01 g, Sodium phytate - 4.0 g, Agar - 15.0 g, pH - 5.0. The pure cultures were streaked at the centre of the plate and the plates were incubated at 37° C for 62 h. The observation was made to see the phytase solubilisation zone around the colony. Only positive and better zone formed strain was taken for further study.

Phytase Production

The inoculum for further production of enzymes and other studies was prepared. Isolates was cultivated in 250 mL Erlenmeyer flask containing 100 mL Phytase Screening Broth (PSB). PSB was made up of Glucose-5 g, NH_4NO_3- 1.25 g, KCl- 0.125 g, $CaCl_2$- 0.5 g, $FeSO_4.7H_2O$- 0.0005 g, $MgSO_4.H_2O$- 0.0005 g, $MgSO_4.7H_2O$-0.125 g and Sodium Phytate- 1.0 g. This was autoclaved at 121ºC for 20 minutes and after cooling, the various components were added separately. Then an inoculum density of 1 x 10^6 cells/mL was used for each isolate. This was then be incubated at 30º C for 5 days in an orbital shaker. The yeast biomass was separated from the medium through filtration and the cell-free filtrate was used for phytase assay.

Phytase Enzyme Assay

At the end of the incubation period, crude enzyme (phytase) was obtained by adding 50ml of acetate buffer (0.2 M, pH 5.5) to the samples in 250 mL Erlenmeyer flasks. The flasks were then placed in a rotary shaker at 200 rpm and kept at room temperature for one hour. The solid biomass was separated from the mixture through filtration using filter paper (Whatman No.4). The resulting filtrate was then centrifuged at 10,000 rpm for 10 minutes at 4º C. The supernatant was used as a source of crude enzyme for the phytase assay. To determine the enzyme activity, the liberated inorganic phosphate was measured according to Award et al. (2014). The enzyme assay mixture contained 0.9 mL of acetate buffer, 1mM phytate, and 0.1ml of the crushed enzyme supernatant. The mixture was then incubated for 30 minutes at 37º C, after which 1 mL of 10% trichloroacetic acid was added to stop the reaction. One unit of phytase was defined as the amount of enzyme that releases one µg inorganic phosphate per mL per minute under the assay conditions.

Statistical Analysis

The effects of physicochemical parameters were compared using Statistical Package for Social Sciences (SPSS) and Duncan Multiple Range New Test. Values were considered significant at $P< 0.05$.

RESULTS AND DISCUSSION

Isolation and Identification

Two hundred and twenty seven (227) isolates were obtained from different cereal samples (millet, sorghum and maize). The yeast cultures were maintained on slants and stored at -20ºC until phytase production and assay. The selected yeast isolates were identified up to the genus level based on the colonial morphology, microscopic observation, carbohydrate fermentation biochemical test and molecularly (Tables 1- 4).

Colonial Morphology

The morphological characteristics of the selected yeast isolates such as margin, surface, colour, shape and elevation from each sample were observed as shown in Table 1.

Microscopic Observation

Cell morphology was observed under a Microscope using X40 objective. The microscopic characteristics included ovoid/elongated cells with budding or clustered arrangement (Table 1).

Carbohydrate Fermentation

The ability of the selected yeast isolates to utilize different sugars was observed. Thirteen (13) sugars were used in the study (glucose, dextrose, trehalose, fructose, raffinose, lactose, sucrose, maltose, galactose, mannitol, mellibiose, sorbose and sorbitol). Most of the seven (7) positively screened isolates were able utilise glucose, dextrose, trehalose, fructose and raffinose. While none was able to utilise lactose, sucrose, maltose, galactose, mannitol and sorbitol (Table 2).

Biochemical Test

In Table 3, the biochemical tests of the yeast isolates was described. These included urease hydrolysis, tolerance to 1% acetic acid, gelatin hydrolysis and acid production from glucose. None of the isolates could tolerate 1% acetic acid. Acid production from glucose was also not observed.

Molecular Analysis

The phylogenetic tree shows that both organisms are from the same ancestor, although there is a little relationship between them as shown in figure 1. Table 4 shows the molecular identity of the yeast isolates and the accession numbers for *M. guilliermondii* M122, *M. guilliermondii* SWS81and *M. guilliermondii* WM226 which are JX993812, KF293846 and MT534187 respectively.

Screening of Phytate Degrading Yeast

Out of the two hundred and twenty seven (227) isolates screened on PSM, seven (7) showed positive result with maximum clear halo zone around the colonies after growing them for 5 days (Plate 2). *M. guilliermondii* WM226, *M. guilliermondii* M122 and *M. guilliermondii* SWS81 with higher solubilisation indexes of 5.14 mm, 4.52 mm and 3.64 mm respectively, were used for production, assay and optimisation (Plate 1, Table 5).

Phytase Production and Assay

Phytase extracted from the culture of the selected isolates (*M. guilliermondii* M122, *M. guilliermondii* WM226 and *M.*

guilliermondii SWS81) showed differences in yield. Higher enzyme activity was observed across the three (3) isolates after an incubation period of 1 day (79.90U/mL, 97.70U/mL and 75.90U/mL respectively). A decrease in enzyme activity was however observed after day 2 and 3 across all three isolates (Table 6).

Table 1: Macroscopic and microscopic characteristics of the yeast isolates obtained from different fermented cereals

Organism	Source	Colonial Morphology					Microscopy
		Shape	Colour	Surface	Elevation	Margin	
M. guilliermondii M122	Millet	Circular	Shiny	Smooth	Raised	Entire	Ovoid
Pichia fermentans	Sorghum	Circular	Shiny	Smooth	Flat	Entire	Elongated with branching filaments
M. guilliermondii SWS81	Sorghum	Circular	Dull	Dry	Flat	Entire	Elongated and Clustered
Candida krusei	Sorghum	Circular	Dull	Dry	Flat	Entire	Ovoid and budded.
Pichia membranefaciens	Maize	Circular	Shiny	Smooth	Flat	Entire	Elongated
M. guilliermondii WM226	Maize	Circular	Dull	Dry	Raised	Entire	Ovoid and budded
Schwanniomyces occidentalis	Sorghum	Circular	Dull	Dry	Raised	Entire	Elongated and budded

Table 2: Carbohydrate fermentative pattern of the yeast isolates obtained from different fermented cereals

Isolate	Sugar												
	Glu	Gal	Sorb	Suc	Dex	Lac	Mel	Mal	Tre	Fru	Sor	Man	Raff
M. guilliermondii M122	+/A	-	-	-	+/A	-	-	-	+/AG	+/A	-	-	+/AG
Pichia fermentans	-	-	+/AG	-	-	-	-	-	-	-	-	-	-
M. guilliermondii SWS81	+/A	-	-	-	+/A	-	-	-	+/AG	+/A	-	-	+/AG
Candida krusei	+/A	-	-	-	+/A	-	-	-	-	+/A	-	-	+/AG
Pichia membranefaciens	-	-	-	-	-	-	+/AG	-	+/AG	-	-	-	-
M. guilliermondii WM226	+/A	-	-	-	+/A	-	+/AG	-	-	+/A	-	-	+/AG
Schwanniomyces occidentalis	-	-	-	-	-	-	-	-	-	-	-	-	+/AG

Key: GLU: Glucose, FRU: Fructose, GAL: Galactose, MAL: Maltose, MAN: Mannitol, LAC: Lactose, SUC: Sucrose, SOR: Sorbitol, DEX: Dextrose, SORB: Sorbose, MEL: Mellibose, RAFF: Raffinose, TRE: Trehalose, +AG: Positive with acid and gas, +A: Positive, -: Negative.

Table 3: Biochemical reactions of the yeast isolates obtained from different fermented cereals

Organism	Urease	Tolerance to 1% Acetic Acid	Gelatin Hydrolysis	Acid Production from Glucose
***M. guilliermondii* M122**	+	-	-	-
Pichia fermentans	+	-	+	-
***M. guilliermondii* SWS81**	-	-	-	-
Candida krusei	-	-	-	-
Pichia membranefaciens	+	-	-	-
***M. guilliermondii* WM226**	-	-	-	-
Schwanniomyces occidentalis	-	-	-	-

Key: + = Growth. - = No Growth

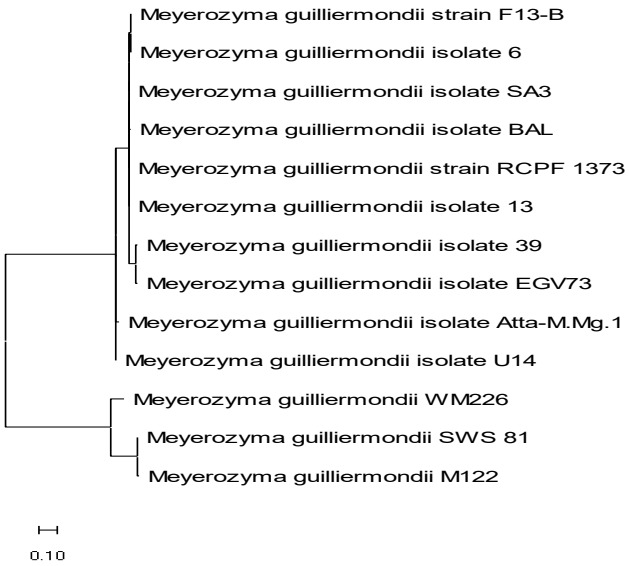

Figure 1: Phylogenetic Tree of yeast isolates obtained from different fermented cereals

Table 4: Molecular identity of the yeast isolates obtained from different fermented cereals

Isolate code	Identity	Accession number
M122	*M. guilliermondii*	JX993812
SWS81	*M. guilliermondii*	KF293846
WM226	*M. guilliermondii*	MT534187

Table 5: Screening of the yeast isolates obtained from different fermented cereals on Phytase Screening Medium (PSM)

Organism	Colony Diameter (mm)	Halo Zone Diameter (mm)	Solubilisation Index (mm) (colony index + halo zone diameter/ colony diameter)
***M. guilliermondii* M122**	27	95	4.52
P. fermentans	31	70	3.25
***M. guilliermondii* SWS81**	34	90	3.64
Candida krusei	38	85	3.23
P. membranefaciens	36	75	2.80
***M. guilliermondii* WM226**	28	120	5.14
Schwanniomyces occidentalis	30	65	2.87

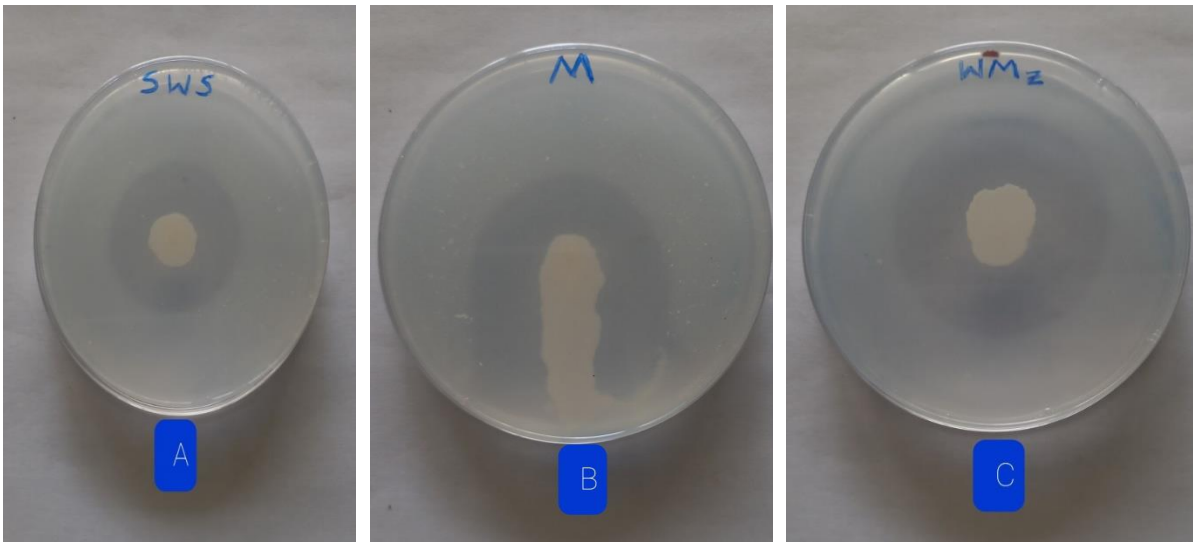

Plate 1: Screening showing positive isolates (A:*M. guilliermondii*SWS81, B: *M. guilliermondii M*122 and C: *M. guilliermondii* WM226)

Table 6: Phytase production and assay by the yeast isolates obtained from different fermented cereals

Incubation Period (Days)	Isolate/Enzyme Activity (U/mL)		
	***M. guilliermondii* M122**	***M. guilliermondii* WM226**	***M. guilliermondii* SWS81**
1	79.90±0.85[a]	97.70±0.71[a]	75.90±0.71[a]
2	63.65±0.78[b]	84.45±0.92[b]	62.65±0.78[b]
3	59.55±0.64[c]	75.65±0.64[c]	44.70±0.85[c]

Note: Values are Means ± Standard Deviations of duplicate observations. Means with different superscript letter down each column are significantly different from each other at $P \leq 0.05$ using the Independent Samples *t*-test

Discussion

In this study, two hundred and twenty seven (227) yeast species were isolated from different cereal sources (maize, sorghum and millet) and were identified. These species were then screened to determine their potential for degrading phytate. Among the seven isolates that displayed positive results (*M. guilliermondii* M122*, M. guilliermondii* SWS81*, Candida krusei, Pichia fermentans, M. guilliermondii* WM226*, Pichia membranefaciens, and Schwanniomyces occidentalis),* three *(M. guilliermondii* M122*, M. guilliermondii* SWS81*,* and *M. guilliermondii* WM226) had the highest solubilisation indices (3.64mm, 5.14mm, and 4.52mm respectively). These three isolates were subsequently used for further assay and production of the enzyme phytase. The solubilisation indices shown by *M. guilliermondi* showed that were the best strains that had good zone of solubilisation. The screening of yeast phytase from cereals has been investigated in several studies. Several authors have reported the successful screening of yeast phytase from different cereal sources, including wheat, barley, and maize (Zhou et al. 2019; Juturu & Wu, 2020; Guo et al. 2021; Joshi, 2021). These studies have highlighted the potential of yeast phytase as a cost-effective and sustainable alternative to traditional sources of phytase. In a previous study, Razan and Sahay (2013) isolated 61 yeast isolates from various samples, with 12 demonstrating the ability to produce phytase. Out of those 12, five exhibited maximum phytase activity and were utilized for further production and assay.

Similarly, Wang et al. (2021) screened for phytase-producing yeasts from various cereals, including rice, corn, wheat, and barley. They found that 13 out of 54 yeast strains isolated from these cereals were capable of producing phytase. Among these strains, one yeast strain isolated from barley, identified as *Saccharomyces cerevisiae*, showed the highest phytase activity. Another study by Li et al. (2021) aimed to screen for phytase-producing yeasts specifically from wheat bran. The researchers isolated 12 yeast strains from wheat bran and screened them for phytase activity. They found that one strain, identified as *Candida utilis*, showed the highest phytase activity. Phytase Screening Medium (PSM) that was used is a chemically defined minimal nutrient medium with phytate as the main organic compound. The PSM induced phytase activity had increased the phytase enzyme. This may be due to availability of nutrients as stated by Kim et al., 1999. Yeasts are associated with cereals and are predominantly involved in the fermentation of sprouted-grains and grain-flours fermentation. Phytate solubilizing yeasts have been isolated from different cereals using medium enriched with phytate as it is known to induce phytase activity in the growth medium (Raghavendra & Halami, 2009).

The high growth rate of microbial biomass during culture and the production of enzyme proteins depend primarily on the availability of optimum physical and nutrient conditions. The proper selection of culture conditions guarantees a highly efficient production of biocatalysts and a high degree of bioconversion of the substrate using enzymatic proteins. In this study, *M. guilliermondii* SWS81*, M. guilliermondii* WM226 and *M. guilliermondii* M122 had phytase activity of 75.90 U/mL, 97.70 U/mL and 79.90 U/mL respectively. One study by Kumar and Singh (2022) investigated the use of a yeast strain, *Saccharomyces cerevisiae*, for the production of phytase from different cereals, including maize, wheat, and rice. The researchers found that the production of phytase was highest in maize-based medium, with a yield of 65 U/mL after 96 hours of fermentation. The wheat-based medium produced a yield of 55 U/mL, while the rice-based medium produced a yield of 40 U/mL. Another study by Rajagopal et al. (2020) investigated the production of phytase from a novel yeast strain, *Debaryomyces hansenii*, using rice bran as the substrate. The researchers found that the phytase yield was highest at pH 5.5 and a temperature of 30°C, with a yield of 9.43 U/mL after 72 hours of fermentation.

CONCLUSION

M. guilliermondii M122*, M. guilliermondii* SWS81*,* and *M. guilliermondii* WM226 were able to form clear zone indicating their abilities to degrade phytate. Findings revealed that phytase activity of the crude enzyme from *M. guilliermondii* SWS81*, M. guilliermondii* WM226 and *M. guilliermondii* M122 were 75.90 U/mL, 97.70 U/mL and 79.90 U/mL respectively. *M. guilliermondii* was the best yeast with high phytase producing ability making them potential supplements for animal feeds to improve nutritional status and combat environmental phosphorus pollution.

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