Biochemical and Molecular Characterization of Phytase Producing Yeast Isolated From Wastewater

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Abstracts

Background: Most animal feeds are cereal or legume-based, and they have phytate as their major component. Phytate chelates and precipitates minerals such as zinc, iron, calcium, and magnesium in such feeds, reducing the bioavailability of these micronutrients. Phytase breaks down phytate by hydrolyzing its phosphate groups and releasing the chelated minerals and proteins in them. The degradation of phytate is, therefore, necessary for the animals to assimilate the nutrient bound in their feed. **Materials and Methods**: This study evaluated the probiotic potentials (phytase production) from isolated yeasts species, the biochemical and molecular characterization of the phytase. Biochemical tests such as urea hydrolysis, catalase and oxidase were done following standard procedures and molecular characterization was done by amplifying the ITS gene of the isolates. **Results:** The optimum temperature and pH for maximal enzyme production was 30°°C and 6.5 respectively. Best carbon source for the enzyme production was the simple sugars; the best nitrogen source was casein. Highest enzyme production was achieved after 72 hours of incubation; Molecular characterization of the isolates revealed their identity as *Candida albicans, Geotrichum candidum, Candida tropicalis,* and *Saccharomyces cerevisiae.* Electrophoresis gel of the amplified ITS gene from these isolates showed that *Candida albicans* bands at 600bp, *Geotrichum candidum* bands at 650bp, *Candida tropicalis* bands at 540bp, and *Saccharomyces cerevisiae* bands at 630bp. **Conclusion:** The isolated yeasts has the potential to produce extracellular phytase, an enzyme that has ability to hydrolyze phytate

Keywords: Probiotic potential, phytate, phytase, yeast Species, waste and chelators

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1.0 Introduction

Yeasts have a lot of potentials among which are phytase production; phytase hydrolyze phytate to liberate soluble and utilizable inorganic phosphate. Phytase are produced by various groups of microbes, yeasts being simple eukaryotes and mostly non-pathogenic with proven probiotic benefits can serve as ideal candidates for phytase research. With awareness that the full potential of yeast phytase has not, been exploited. Phytic acid is a major component of all plant seeds, constituting 1-3% by weight of many cereals and oilseeds and typically accounting for 60-90% of the total phosphorus (1). Phytate serve several physiological functions, especially in seed germination. Historically, phytate have been considered solely as anti nutrients because they are known as strong chelators of divalent minerals such as Ca^{2+}, Mg^{2+} , Zn^{2+}, and Fe^{2+}. Moreover, phytate are also capable of binding with starch and proteins while preventing their assimilation through the digestive system (2). Conversely, as a strong chelator of iron and zinc, phytate in plant foods actually can serve as an antioxidant to reduce free radical formation mediated by these metals (3). Most animal feeds are cereal/legume based; having a major limitation of containing phytate that has been shown to chelates micronutrients thereby reducing the bioavailability of those nutrients to the animals.

This study aimed at screening and characterizing phytase producing yeast using biochemical and molecular methods.

2.0 Materials and Methods

2.1 Collection of samples

Four different samples (whey wastewater, cassava wastewater, human urine, and rabbit dung) were used for this study. Whey wastewater was collected from a Fulani settlement at Aduramigba in Osogbo, cassava processing wastewater was collected from a local fufu producer at Ofatedo in Osogbo, human urine was from a student donor and rabbit dung was collected from an animal house at Mercy-land unit of LAUTECH campus, Osogbo.

2.2 Reagent Preparation

All the reagents and chemicals used for this study were of analytical grade and were procured from accredited agents of the manufacturers. All the media and reagents were prepared according to the manufacturers' instructions.

2.3 Isolation of organisms (yeasts) from the various wastes

Isolation of organisms was done according to standard procedure as described by (4). The animal dung was treated before use for this study. They were dried in an oven at 50 ºC, pulverized in a mortar, and kept in an air-tight container.

One (1) gram of the rabbit dung was weighed into a 10-ml universal bottle, emulsified with 5-ml physiological saline, and made up to 10 ml with physiological saline to form the stock solution. Ten milliliters (10 ml) each of human urine, cassava wastewater, and whey wastewater were put into different universal bottles and labeled appropriately to serve as stock solutions for each of these samples. The stock sample solutions were serially diluted to the power of 10^{-7} dilution and inoculated on potato dextrose agar using the spread plate method. Chloramphenicol was added to the medium to inhibit bacterial growth. The plates were incubated at 37 ºC for 24 hours. Organisms with different cultural morphological features were selected and sub-cultured on separate plates to obtain pure cultures. Isolated organisms were maintained on a PDA slant at 4 ºC until further use.

2.4 Characterization and identification of isolated organisms

Characterization and identification of isolated organisms were done using phenotypic and molecular protocols.

2.5 Phenotypic identification scheme

Phenotypic characterization and identification of the isolated organisms were done based on their cultural, morphological, microscopic features, and various biochemical and sugar fermentation tests. The further identification process was done using API 1D 32 C kit. Colony morphological features (form, size, elevation, margin/edge, colony color) were observed and recorded. Cell morphology was observed microscopically on the wet mount and direct mounts preparations stained with lacto phenol blue and viewed under the microscope. A germ tube test was also done. Small portion of fresh culture (12-24 hours) of the various isolates were emulsified in 0.5 ml of human serum in a test tube. The tubes were then incubated at 37 ˚C for three hours, after which a drop of the isolate suspension was put on a slide, covered with a coverslip, and viewed under the microscope.

The following biochemical tests; urea hydrolysis, catalase, urease, and oxidase were done following standard microbiological protocol as described [3] For the sugar fermentation tests, 10 sugars (lactose, fructose, maltose, galactose, xylose, raffinose, mannitol, sorbitol, sucrose, and arabinose) were tested at 1 % concentration. The tests were done following standard microbiological protocol as described [3]. Further characterization of the isolates was done using the analytical profile index kit (API 1D 32 C kit), according to the manufacturer's protocol.

2.6 Molecular Characterization of isolates

2.6.1 DNA Extraction

Genomic DNA was extracted from each isolate using the Zymo Fungal/Bacteria DNA extraction kit. The extraction procedure was according to the manufacturer's protocol.

PCR Amplification of extracted DNA fragments

The PCR amplification reaction was carried on the isolated genomic DNAs to amplify the ITS gene of the isolates using the Primer sequence pair; Forward: ITS 5'TCCGTAGGTGAACCTGCGG3 and Reverse: ITS 5'TCCTCCGCTTATTGATATGC. Amplification was performed in a thermal cycler (Eppendorf Vapo protect) (Nexus Series) in a 25 µl reaction volume consisting of 12.5 µl of 5X HOT FIREPol Blend 1XBlend Master mix buffer (Solis Biodyne), 2.5 µl pMol each of forward and reverse primers (BIOMERS, Germany),1.5 mM $MgCl_2$, 2.0 µl of each deoxynucleoside triphosphates (dNTP) (Solis Biodyne), 2 unit of HotFIREPol DNA polymerase (Solis Biodyne), 2.5 units of Taq DNA polymerase, Proofreading Enzyme, 2 µl of the extracted DNA, and sterile distilled water was used to make up the reaction volume. Thermal cycling conditions were; an initial denature at 95 °C for 15 minutes, followed by annealing at 58 °C for 1 minute 30 Seconds and extension at 72 °C for 10 minutes. Amplification cycles were 35.

2.6.2 Gel electrophoresis

The amplified products were stained with ethidium bromide and loaded into a 1.5 % agarose gel and electrophoresis was carried out at 80V for 1 hour 30 minutes to check the success of the PCR protocol and to separate the amplicons by size. One hundred (100) base pair (bp) DNA ladder was loaded as a size marker. Amplified DNA fragments were viewed with UVP Gel Doc-It imaging system.

2.6.3 Induction of phytase producing properties in isolated yeasts

Before the screening of the isolates for phytase production, they were induced for enzyme production. The yeast isolates were inoculated into a broth medium composed as follows; 2 g of yeast extract, 20 g of peptone, 10 g of glucose, 0.65 g sodium phytate in a litre of water. They were incubated at 37°C for 24 hours and centrifuged at 12,000 rpm for 15 minutes. Cell pellets were harvested and inoculated into 20 ml of sterile normal saline and used as stock for further work.

2.6.4 Qualitative screening of yeasts isolates for phytase production

The induced isolates were screened for phytase production using the agar well diffusion method. The agar medium for the experiment was composed as follows: 15 g of glucose, 5 g of ammonium nitrate, 2 g of calcium chloride, 0.5 g of magnesium sulphate, 0.5 g of potassium chloride, 0.01 g iron sulphate, 0.01 g manganese sulphate, 2 g of sodium phytate, and 15 g of agar in 1 litre of water. The negative control medium had no sodium phytate in it while the positive control contained KH_2PO_4 in the place of sodium phytate. Media preparation and procedures for the experiment were according to standard microbiological protocols as described by (4). Inoculated plates were incubated at 37^{o}C for 48 hours. A clear zone around each isolate indicated positive phytase production.

2.6.5 Quantitative assay for phytase production

Isolates that tested positive for phytase production were selected and used for further experiments. They were assayed for phytase production using the colorimetric method. Selected isolates were cultured in a broth medium between 18-24 hrs**, (**2 g of yeast extract, 20 g of peptone, 10 g of glucose, 0.65 g sodium phytate in a litre of water) without the agar component. The cultured isolates were centrifuged and the supernatant assayed for phytase. 150 μL of enzyme solution was mixed with 600 μL of 0.1 M Tris-HCl (pH 7.0) supplemented with 2 mM sodium phytate and 2 mM of $CaCl_2$, and incubated at 37 °C for 30 min. The reaction was then stopped by the addition of 750 μL of 5 % trichloroacetic acid, after which 1.5 μL of the color reagent was added to generate phosphomolybdate.

The concentration of inorganic orthophosphate (Pi) in the mixture was determined colorimetrically by measuring the absorbance of the solution at 700 nm using a Beckman Coulter DU640 Spectrophotometer (Fullerton, CA, USA). The color reagent was prepared fresh by mixing 4 volumes of 1.5 % (w/v) ammonium molybdate solution supplemented with 5.5 % (v/v) sulphuric acid and 1 volume of 2.7 % (w/v) ferrous sulphate solution. The amount of phytase produced was extrapolated from a standard curve prepared using K_2HPO_4 as a source of inorganic phosphate at concentrations ranging from 0.0448 to 2.8706 μM. One unit (U) of phytase activity was defined as the concentration of inorganic phosphate in μ mol, released per min per ml of enzyme preparation (U/ml) under defined reaction conditions.

3.0 Results

3.1 Isolation of yeasts from various wastes

Phenotypic characterization of isolated organisms revealed their identity as *Candida albicans, Geotrichum candidum, Candida tropicalis,* and *Saccharomyces cerevisiae* (Table1).*Candida albicans* was isolated from human urine, *Geotrichum candidum* was isolated from rabbit dung, *Candida tropicalis* was isolated from whey wastewater while *Saccharomyces cerevisiae* was isolated from cassava wastewater.

3.2 Molecular Characterization of isolates

Molecular characterization of the same isolates as mentioned in section revealed their identity as *Candida albicans, Geotrichum candidum, Candida tropicalis,* and *Saccharomyces cerevisiae.* Electrophoresis gel of the amplified ITS gene from these isolates revealed that *Candida albicans* bands 600bp, *Geotrichum candidum* bands at 650bp, *Candida tropicalis* at 540bp; and *Saccharomyces cerevisiae* at 630bp (Figure1). The phylogenetic relationship of the organisms is as shown in Figure 1. There are four main clusters, (A, B, C & D). Organisms in the same clade in the phylogenetic tree are assumed to be closely related. They are believed to have evolved from the same parent and could have even been the same organism or different strains of the same organism**.**

3.3 Characterization of Phytase activity

The kinetics of the enzyme was determined (Figure 2). The Line weaver-Burk plot showed the effect of varying concentrations of phytate on the initial reaction velocity of the enzyme. Enzyme activity is expressed in micromole per min. The *Km* and *Vmax* values were 4.03 mM and 16.06 µmoles /ml respectively.

The optimum pH and temperature for maximal activity of the enzyme were at 5.5 (Figure 3) and 50 – 60^{o} C (Figure 4) respectively. The enzyme loses its activity/stability as the temperature rises. The presence of urea, EDTA, and mercaptor ethanol negatively affected the activity of the enzyme. EDTA hurt the enzyme activity with the enzyme completely losing the activity in its presence, while the least effect was from urea (Figure: 5)

Table 1: Sugar fermentation reaction of isolates to different sugars using analytical Profile Index (API)

S/NO	SUGAR	*Yeast 1*	*Yeast 2*	*Yeast 3*	*Yeast 4*
S	**Code**	**5265040001**	**5777375337**	**7771774257**	**7367340315**
1	D-Galactose	+	+	+	+
2	Cycloheximide	_	_	+	+
3	D-Saccharose	+	+	+	+
4	N-AcetylGlucossamine	_	+	+	+
5	Lactic acid	+	+	+	+
6	L –arabinose	_	+	+	_
7	D-Celiobiose	_	+	+	+
8	D-Raffinose	+	+	+	_
9	D-Maltose	+	+	+	+
10	D-Trehalose	+	+	+	+
11	Potassium 2-Keto Gluconate	_	+	_	+
12	Methyl α D-Gluconate	+	+	_	+
13	D Sorbitol	_	+	+	+
14	XYL	_	+	+	+
15	D- Ribose	_	_	+	_
16	Glycerol	_	+	+	_
17	L-Rhamnose	_	+	+	_
18	Palatinose	+	+	+	+
19	Erythritol	_	+	_	_
20	D-Melibiose	_	_	_	_
21	Sodium Glucuronate	_	+	+	_
22	D-Melezitose	_	+	_	+
23	Potassium Gluconate	_	+	+	+
24	Levulinic acid	_	_	_	_
25	D- Mannose	_	+	+	+
26	D- lactose	_	+	_	_
27	D-innositol	_	_	+	_
28	Glucosamine	+	+	+	+
29	Escolin ferric citrate	_	+	+	_
30	GLN	_	+	+	+
	Total. Positive	9 (12%) *Saccharomyces Cerevisiae*	25 (33.3%) *Candida albicans*	23 (30.7%) *Geotrichum candidum*	18 (24%) *Candida Tropicalis*

Key: - = Negative, + = Positive

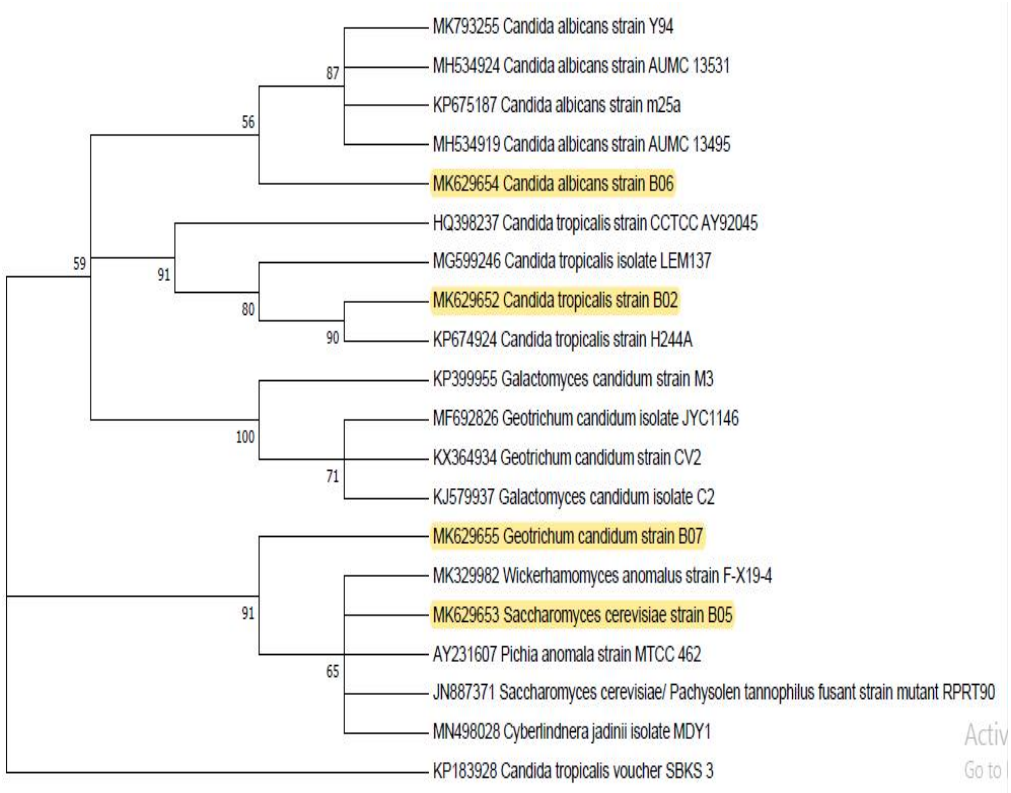


Figure 1: Evolutionary relationship of Yeast isolates sample
(1) *Candida tropicalis* (2) *Candida albicans* (3) *Geotrichum candidum and* (4) *Saccharomyces cerevisiae with* their GenBank Accession numbers. With other yeasts taxa from Gen Database

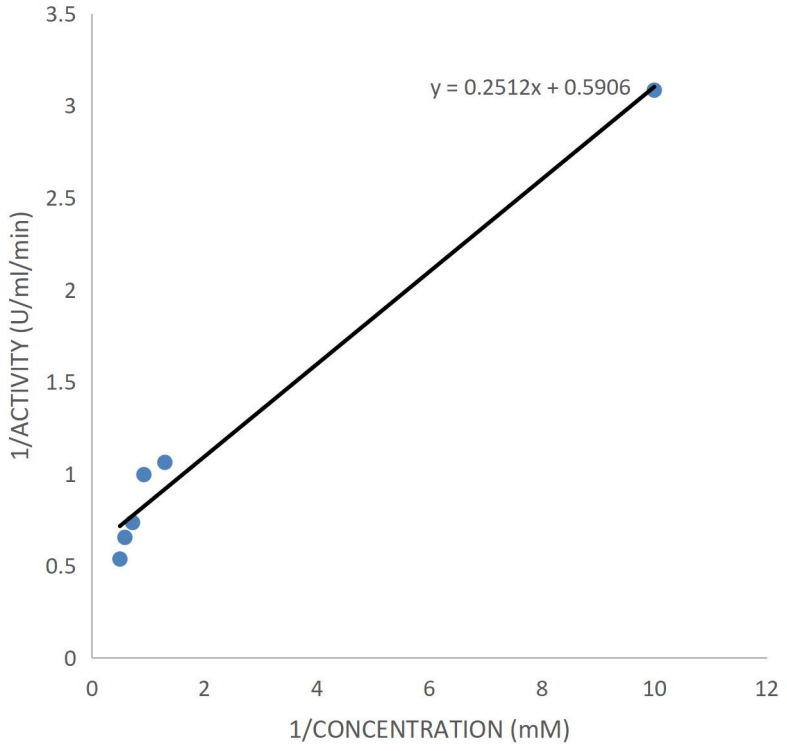


Figure 2: The kinetics of the Phytase activity

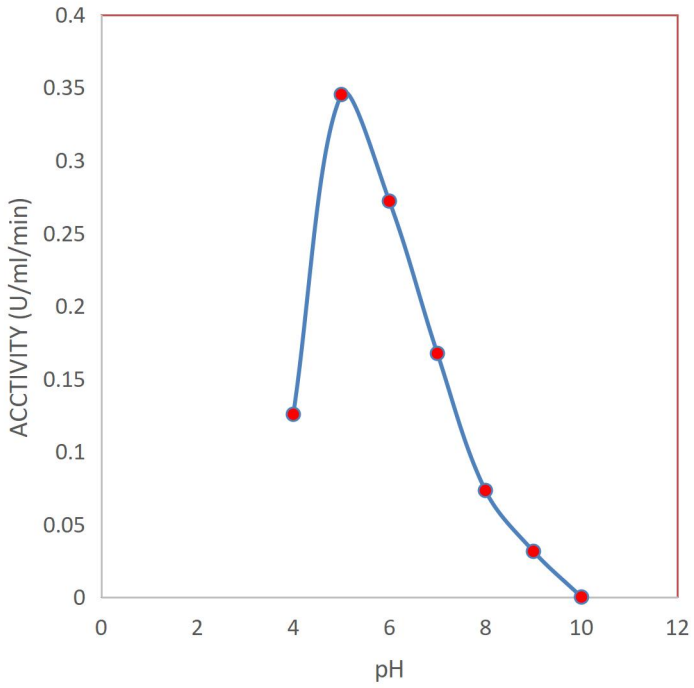
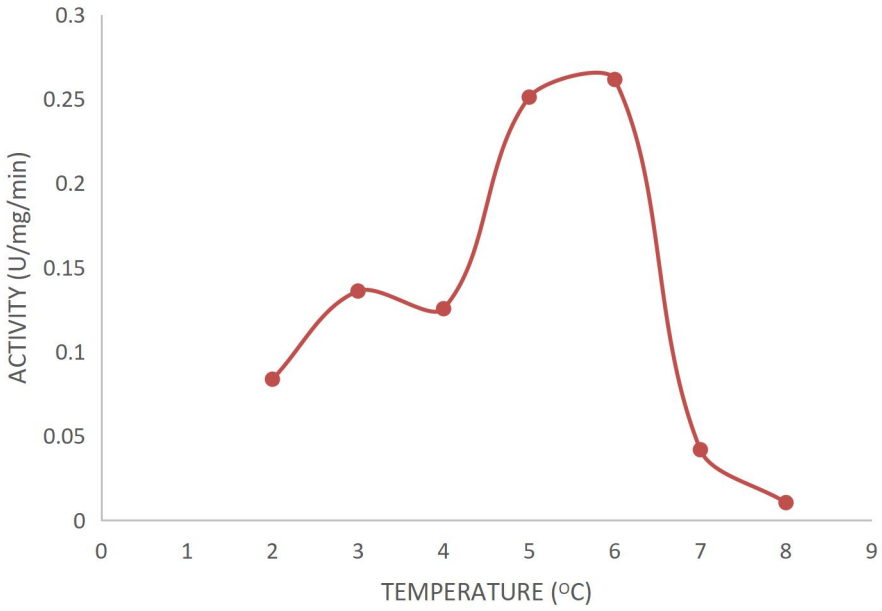


Figure 3: Effect of pH on phytase activity



Key: 1 rep 10◦C

Figure 4: Effect of Temperature on phytase activity

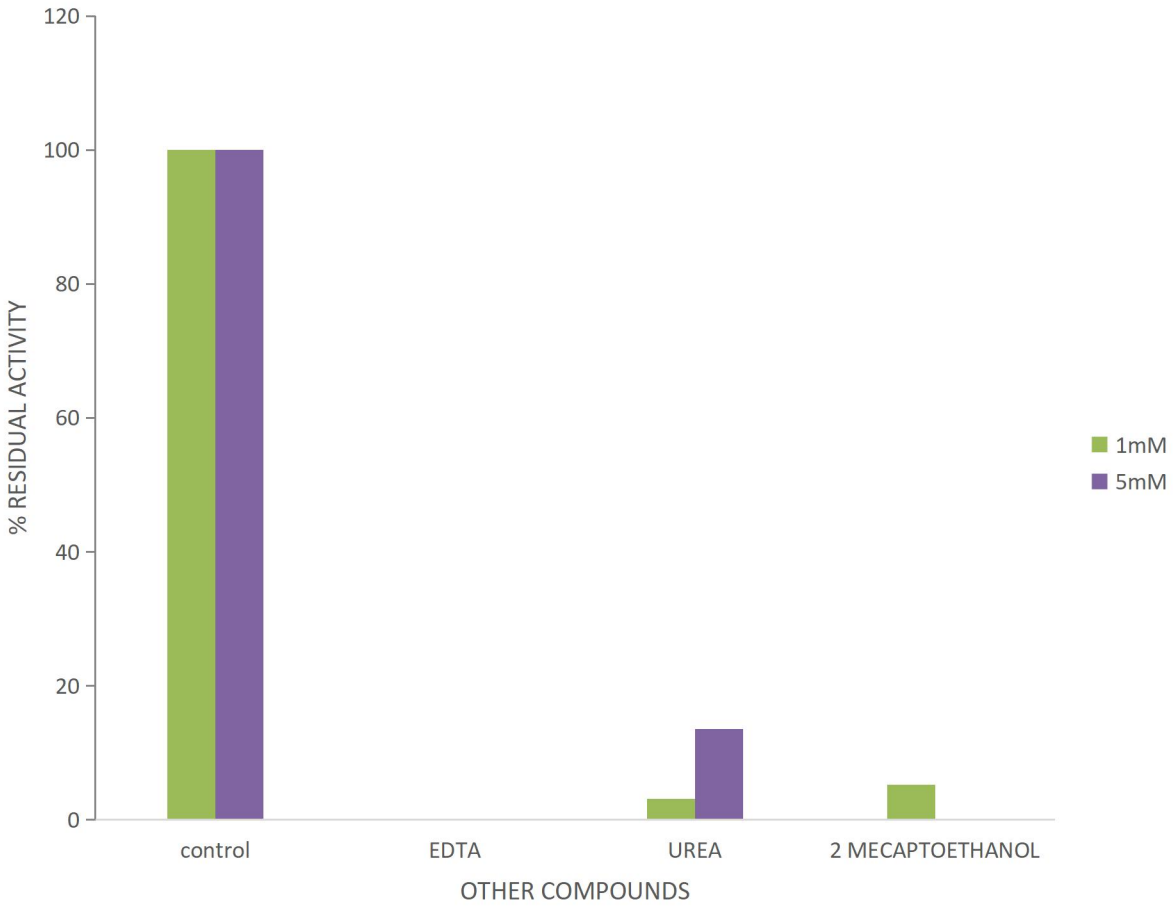


Figure 5: Effect of other compounds on phytase activity

4.0 Discussion

The four isolated yeast species were evaluated for their ability to produce phytase. Result obtained reveals that *Candida tropicalis* exhibited the highest potential for the enzyme (5) asserted that phytase is produced by various groups of microorganism including yeasts. In this study, *Candida tropicalis* exhibited the highest potential for enzyme production; this is not surprising as it may have been induced for the enzyme production, having been isolated from a protein/phytase rich source (whey).

Phytate (phytic acid) is the storage form of phosphorus (a mineral) bound to inositol (B vitamin) in foods high in fiber (all plant foods), and particularly the fiber of raw whole grains, legumes, seeds, and nuts. Although these foods have high phosphorus content, the phosphates in phytate are not released by human and animal digestion (6). The phytate, particularly in such raw foods as bran, is a course for concern because it can bind a portion of the iron, zinc, and calcium in foods, making the minerals unavailable for absorption. Enzyme called phytase which hydrolyzes phytate during fermentation processes thereby increasing the bio-availability of these nutrients to the body (7). The degradation of phytate is, therefore, necessary for monogastric animals to assimilate the nutrients bound to it in their feed.

The conditions that could affect the production of phytase were optimized in this study. Results obtained revealed that the optimum temperature and pH for the enzyme production were 30^{o} C and pH 6.9 respectively. These results are in agreement with what has been reported in the literature (8, 9).

The characterization of phytase was equally done, the optimum pH and temperature for

maximum activity of the enzyme were pH 5 which is slightly acidic and temperature of 58^{o} C. Thermal stability of the enzyme was achieved at 30^{o} C and similar results have been reported in the literature by (9).

Conclusion

This study has demonstrated that the isolated yeast species; particularly *Candida tropicalis* has the potential to produce extracellular phytase, this enzyme has been established to be able to hydrolyze phytate.

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Declaration of Conflict Interest

'The authors declare that there is no conflict of interest.

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