Isolation and Identification Of Ascomycetous Yeast Producing Phytase

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Abstract. The objective of the study was to isolate, identify and characterize phytase producing yeast. The yeast was isolated from various resources, and the identification of yeast was conducted by the sequencing of the ITS region. Of 41 isolates, 24 isolated produced phytase. The highest phytase producing yeast was identified as Candida natalensis YEG 132, Saturnispora quitensis YEG 135, and Candida albicans YEG 226. These isolates were able to use various carbon and nitrogen sources. The maximum phytase production depends on isolates, media composition, pH and incubation temperature. Temperature affected phytase production. All isolates produce the highest phytase was at 30°C. Increased temperature to 35°C and 40°C inhibits phytase production by all strains. S. quitensis was the highest isolate inhibited by an increase of incubation temperature. The pH of cultures affects phytase production and varies depending on isolate. S. quitensis and Candida natalensis produced the highest phytase activities at pH 5.0, while C. albicans was at pH 6.0. All isolates were able to assimilate glucose, dextrose, sucrose, xylose and lactose. In general, glucose was the best carbon source for phytase activity. But each isolate has its preferential on carbon sources. S. quitensis produced the highest phytase on glucose, but not on dextrose. Each isolate has preferential on nitrogen sources. Yeast extract was the best N-sources for C. albicans, while ammonium sulfate was best for C. natalensis, S. quitensis. Ammonium nitrate was not favorable for phytase production by C. natalensis, Candida natalensis YEG 132, Saturnispora quitensis YEG 135, and Candida albicans YEG 226 was a good candidate for phytase production.

1. Introduction

Yeast has been exploited for many industrial purposes. The exploration of yeast in the tropical region especially Indonesia have been started in the 1960s [1], but few studies on phytase producing yeast. Yeast is a unicellular fungus not only important keeping and accelerating nutrient cycle, producing plant growth, an important diet for certain insects, but also has tremendous economic potential for enzyme production. Phytase is economically potential and has a very good market on food, feed and pharmaceutical industry. In the industrial scale, phytase is mostly produced by the microorganism. Recently there is a tremendous interest in developing phytase for the feed industry.

Most of cattle feed is a cereal grain. It is estimated about 60–90% of the phosphorus in cereal grains, a major constituent of animal feeds worldwide, as well as in oilseeds and legumes, is present in the form of phytic acid or phytate [2]. Degradation of phytate in the gastrointestinal tract of monogastric animals such as poultry, pigs and fish, as these animals are very poor since these animals...
are having a lack of phytase [3]. This then results in phytate waste excretion into the environment, and leading to eutrophication of receiving water bodies [4]. To ensure that the phosphorus needs of animals are met, diets are commonly fortified with additional inorganic phosphates, which may further increase phosphorus excretion. Complete dephosphorylating of the phytate complex in poultry and animal has double benefits i.e. increase feed nutrition and protecting water bodies from eutrophication [5].

The bioavailability of other minerals is also considerably reduced by the presence of phytate, which forms insoluble complexes with numerous cations, such as copper, zinc, calcium, magnesium, iron, and potassium. Phytate may also bond on protein, and thus reduce protein availability for animal growth [6]. Phytase (myoinositol-hexakisphosphate 6-phosphohydrolases), are nonspecific phosphatase enzymes, which release free inorganic phosphate from myoinositol hexakisphosphate (IP6), as well as from a variety of other phosphorylated substrates [7]. The addition of phytase to animal feed increases the availability of phosphorus for animal digestion by the degradation of the phytate and decreases the phosphorus load in animal manure by up to 50% [3]. The high price of commercial phytase currently restricts the widespread application of phytase as a feed supplement. The enzyme is produced by conventional submerged fermentation, an expensive high-technology process. A more economical alternative for phytase addition would be solid substrate fermentation (SSF) of the feed, whereby phytase is produced in situ during SSF by the growth of yeasts on selected feed components [8]. *Arxula adeninivorans* and *Pichia anomala* were reported as phytase producing yeast [9]. *Kodamaea ohmeri* BG3 produce maximum enzyme phytase activity in media contained oats 1.0%, ammonium sulfate 2.3%, glucose 2.0%, NaCl 2.0% and initial pH 6.3 [8].

Classical screening of phytase producing microbes are normally conducted on solid media which contained phytate [10], but this method is laborious, and therefore we follow recent methods proposed by Department of Microbiology, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden [9], to search for new resources of phytase producing microorganism. We conducted a microbial survey in South East Sulawesi as one hot spot biodiversity area in Indonesia. The forest vegetation was dominated by *Palaquium obwatum*, *Santria laevigata*, *Kjellbergiodendron celebicum*, *Baccaurea javanica*, *Santria laevigata*, *Calophyllum soulatri*, *Xanthostemon confertiflorum*, *Podocarpus neriifolius*, and *Garcinia celebica* [11]. Previous microbial survey in this area is very limited, yet many endemic plant and animal are found in this area. The objective of study was to isolate, and identify phytase producing yeast isolated from varying Indonesian resources. The objective of study was to isolate and identify Ascomycetous yeast that produce phytase.

2. Materials and Methods
2.1. Isolation and molecular identification of yeasts
Yeasts were isolated from samples using published methods [12]. One g of soil or leaf litter was added to 25 mL of saline/Tween (0.85% NaCl, 0.01% Tween 80) in a 7 oz. Whirl-Pak filter bag (Nasco) and shaken to suspend the microbes. Two hundred μL of liquid from the other side of the filter were plated on agar plates.

2.2. Culture maintenance
The yeast strains used in this study (Table 1) were accessioned into the culture collection of the InaCC (Indonesian Culture Collection) of the Research Center for Biology-Indonesian Institute of Science (LIPI), held as 50% glycerol stocks at -80 °C.

2.3. rDNA sequence determination
Yeast DNA template was prepared from freshly-grown cells on the Potato Dextrose Broth (PDB) and used for extracted the DNA [13]. PCR amplification of the partial Internal Transcribed Spacer (ITS) ribosomal subunit with primers ITS 4: 5'– TCC TCG GCT TAT TGA TAT GC - 3' and Primer ITS 5: 5'– GGA AGT AAA AGT CGT AAC GAG G –3' (White et al., 1990) using GoTaq master mix (Promega, M7122). PCR products were visualized on 2% agarose and sequenced with both primers
using Big Dye terminator v3.1. Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer’s instructions. The partial 26S sequences determined in this study were compared to those in the EMBL/GenBank/DDBJ databases using the Nucleotide Basic Local Alignment Search Tool (BLASTn).

2.4. Growth media
Phosphate containing minimal medium (MMP) contained 6.7 g L\(^{-1}\) yeast nitrogen base (YNB) without amino acids (Difco Laboratories, Detroit) and 20 g L\(^{-1}\) D-(1)-glucose (BDH Chemicals Ltd, Poole, UK). To prepare phosphate-free minimal medium (MM\(-\)), the YNB was replaced by YNB without amino acids and without KH\(_2\)PO\(_4\) (Formedium Ltd, Norwich, UK). The phytic acid (MMPa) containing medium was prepared from MM- supplemented with 2 g L\(^{-1}\) phytic acid dipotassium salt of the highest available purity (Sigma-Aldrich Inc., St. Louis). The amount of free phosphate in the phytic acid was determined to be 0.5% (w/w) in all preparations used, in accordance with the provider’s statement. All media were prepared as 10-fold concentrated stock solutions and filter sterilized (Filtropur S, 0.45 mm, Sarstedt, Numbrecht, Germany) because autoclaving released orthophosphate from phytate (Bae et al., 1999; Fredriksson et al., 2002). To obtain the corresponding solid medium, Bacteriological Agar without phosphorus (15 g L\(^{-1}\)) (Oxoid Ltd, Basingstoke, Hampshire, UK) was suspended in water (90% of the final volume) and autoclaved at 121 °C for 15 min. After cooling to 45 °C, 10-fold concentrated MMP, MM- or MMPa were added. All media were formulated with ultrapure water.

2.5. Preparation of yeast cells for growth experiments
Yeast inoculant was prepared from cultures grown in 10mL MMP on a rotary shaker at 30°C overnight. Cells were harvested by centrifugation (H-15FR Kokusan) at 4000 g for 5 min and washed three times with 10 mL sterile NaCl solution (9 g L\(^{-1}\)). The final suspension was accordingly diluted to an OD\(_{540}\) nm of c. 2.0.

2.6. Growth test on solid media
The washed yeast pellet from the prepared inoculum was resuspended in NaCl solution. The cells were counted in a Haemacytometer, depth 0.100 mm, 0.0025 mm\(^2\) (Marienfeld, Germany), and diluted to obtain 25, 125 and 250 cells mL\(^{-1}\). From each cell suspension, 2 mL was inoculated on MM-, MMP and MMPa agar plates. Triplicate plates were incubated at 25 °C for 5 days. Thereafter, the plates were photographed, and the ability of the strains to grow with phytic acid as sole phosphorus source was determined by comparing the growth on the MMPa plate with that on MMP (positive control) and MM- (negative control).

2.6.1. Micro titer plate growth test in liquid cultures. For each yeast strain, cells were inoculated in triplicate into 200 µL of MMP, MM- or MMPa in the wells of micro titer plates (Thermo Scientific Cat No. 174925). The wells were inoculated with 10 µL of the washed and diluted cell suspension to an initial OD at 540 nm of c. 0.01. Growth at 30°C was measured every 15 min in Varioskan Flash Spectral Scanning Multimode Reader (Varioskan flash Thermo scientific) for 48 h. Before the OD measurements, the plate was agitated for 3s with an amplitude of 3 mm. The mean values of the triplicates were calculated and growth curves were drawn for yeasts growing on MMP, MM- and MMPa.

2.6.2. The effect of incubation time on phytase activities. To evaluate the optimum incubation for phytase production, yeasts were pregrown in Erlenmeyer flasks containing 100 mL MMP on a rotary shaker at 25 °C for 24 h. Cells were harvested by centrifugation at 4000 g for 5 min and washed three times with 50 mL sterile NaCl solution. The pellet was resuspended in an Erlenmeyer flask containing 100mL MPPor 100mL MMPa, with additional incubation on a rotary shaker at 30 °C for 0-5 days. OD\(_{600}\) was measured with a spectrophotometer (UV Mini 1240 UV-Vis Spectrophotometer Shimadzu, Japan), before and after incubation. To measure extracellular phytase activities (i.e. the activity of all
enzymes that are dissolved in the medium and not bound to cells), the cultures were centrifuged at 8000 rpm at 4°C for 10 minutes. The extracellular enzyme was kept on ice until activity measurements were performed.

2.6.3. The effect of carbon sources on phytase activity. To evaluate the effect of carbon sources, 1.0 % (w/v) of either glucose, xylose, lactose, dextrose, or sucrose was added to MMPa. The pH of cultures and time when phytase was measured depending on the result of optimum pH at which the highest phytase activities achieved by each isolate.

2.6.4. The effect of pH cultures on phytase activities. To evaluate the optimum pH of culture on phytase production, yeasts were pregrown in Erlenmeyer flasks containing 100 mL MMP on a rotary shaker at 25 °C for 24 h. Cells were harvested by centrifugation at 4000 g for 5 min and washed three times with 50 mL sterile NaCl solution. The pellet was resuspended in an Erlenmeyer flask containing 100mL MMPa with the pH adjusted to 4, 5, 6 and 7. The cultures were incubated on a rotary shaker at 30°C. OD<sub>600</sub> was measured with a spectrophotometer (UV Mini 1240 UV-Vis Spectrophotometer Shimadzu, Japan), before and after incubation. The cultures were centrifuged at 8000 rpm at 4°C for 10 minutes. The extracellular enzyme was kept on ice until activity measurements were performed.

2.6.5. The effect of N-sources on phytase activity. To evaluate the nitrogen sources 0.5 % of either yeast extract, peptone, ammonium sulfate, ammonium nitrate or urea were supplemented to MMPa. The pH of cultures, incubation time at which phytase was measured depending on the result of optimum pH, and incubation temperature and incubation time at which the highest phytase activities achieved by each isolate.

2.7. Enzyme assay
Sample (10 mL) was mixed in an Eppendorf tube with 40 mL 0.1M sodium acetate buffer (0.2 M acetic acid and 0.2 M sodium acetate, pH 5.0), supplemented with phytic acid dipotassium salt (final phytic acid concentration 2 g L<sup>-1</sup>). Negative controls were prepared from samples mixed with sodium acetate buffer without phytic acid. Samples were incubated at 30°C and the reactions were stopped immediately (t0), and after 5, 10, 15, 30 and 60 min by adding 50 mL 10% trichloroacetic acid. Determination of liberated phosphate was performed following methods described previously [14] by adding 800 mL acid molybdate reagent (1 volume of 10 mM ammonium heptamolybdate-tetrahydrate, 1 volume of 2.5M sulphuric acid and 2 volumes of acetone). Thereafter OD at 355 nm was measured, using sodium acetate buffer (without phytic acid) and trichloroacetic acid as blank. A standard curve of phosphate (0–3 mmol mL<sup>-1</sup>) was prepared with sodium acetate buffer and measured in the same conditions as the enzyme samples.

2.7.1. Effect of temperature on phytase Activity. Yeasts were pregrown in Erlenmeyer flasks containing 100mL MMP on a rotary shaker at 25-40 °C for 24 h. Cells were harvested by centrifugation at 4000 g for 5 min and washed three times with 50 mL sterile NaCl solution. The pellet was resuspended in an Erlenmeyer flask containing 100mL MMP or 100 mL MMPa, with additional incubation on a rotary shaker at 30 °C for 24h. OD<sub>600</sub> was measured with a spectrophotometer (UV Mini 1240 UV-Vis Spectrophotometer Shimadzu, Japan), before and after incubation. The cultures were centrifuged at 8000 rpm at 4°C for 10 minutes. The extracellular enzyme was kept on ice until activity measurements were performed.

2.7.2. Effect of pH on phytase activity. Yeasts were pregrown in Erlenmeyer flasks containing 100 mL MMP on a rotary shaker at 30 °C for 24 h. Cells were harvested by centrifugation at 4000 g for 5 min and washed three times with 50 mL sterile NaCl solution. The pellet was resuspended in an Erlenmeyer flask containing 100 mL MMP or 100 mL MMPa, with additional incubation on a rotary shaker at 30 °C for c. 24h. OD<sub>600</sub> was measured with a spectrophotometer (UV Mini 1240 UV-Vis Spectrophotometer Shimadzu, Japan), before and after incubation. The cultures were centrifuged at
8000 rpm at 4°C for 10 minutes. The extracellular enzyme was kept on ice until activity measurements were performed.

2.7.3. Activity calculation
One unit of phytase activity is defined as the amount of protein that releases 1 mmol inorganic phosphate within 1 min. Activities were calculated as both specific activities (Umg⁻¹ protein) and as volumetric activity (UmL⁻¹ of yeast culture). All assays were performed in triplicates and the mean values presented. The activities were calculated according to Bergmeyer [15]. Specific activity (Umg⁻¹ protein).

3. Result and Discussion

3.1. Isolated Ascomyceteous yeast
Isolated Ascomyceteous yeast mostly belongs to Candida, Debaryomyces, Pichia, Yamadazyma (table 1). Ascomyceteous yeasts could be isolated from wide sources include soil, leaf litter, insect, insect larvae (Table 1). Out of 41 isolates, 24 isolates produced phytase (table 1). They occupy wide range of habitat from the Arctic [16], deep sea, forest, marine invertebrate, mountain, even extreme environment [13], [17]. Among the isolates obtained from hypersaline waters, *Pichia guilliermondii*, *Debaryomyces hansenii*, *Yarrowia lipolytica* and *Candida parapsilosis*, *Rhodosporidium sphaeroarum*, *R. babjevae*, *Rhodotorula laryngis*, *Trichosporon mucoides*, *Metschnikowia bicuspidata*. They are able to use a wide variety of carbon sources include alkanes, amines and benzene compounds. *Trichosporon* sp. is able to catabolize 4-hydroxybenzoic acid via protocatechuate and hydroxyhydroquinone [18]. The ability of Ascomyceteous to use various C-sources which include persistent organic substances are greatly important for the bioremediation process. Production of extracellular phytase is important for yeast to survive in the environment with low P.

| No | Isolate Code | Name of Isolates   | Sample sources | Phytolitic Index |
|----|--------------|--------------------|----------------|-----------------|
| 1  | YEg266       | *Candida albicans* | soil           | 4.4             |
| 2  | YEg035       | *C. dosseyi*       | soil           | 2.9             |
| 3  | YEg033       | *C. insectorum*    | soil           | 3.4             |
| 4  | YEg173       | *C. intermedia*    | Leaf litter    | 0               |
| 5  | YEg264       | *C. intermedia*    | Leaf litter    | 0               |
| 6  | YEg303       | *C. intermedia*    | Leaf litter    | 0               |
| 7  | YEg262       | *C. mesorugosa*    | Leaf litter    | 2.2             |
| 8  | YEg225       | *C. metapsilosis*  | Leaf litter    | 0               |
| 9  | YEg036       | *C. natalensis*    | soil           | 1.7             |
| 10 | YEg132       | *C. natalensis*    | soil           | 4.3             |
| 11 | YEg301       | *C. phangngensis*  | soil           | 0               |
| 12 | YEg165       | *C. pseudolambica* | soil           | 1.6             |
| 13 | YEg256       | *C. pseudolambica* | Insect frass   | 1.7             |
| 14 | YEg032       | *C. quercitrusa*   |                | 2.3             |
| No | Isolate Code | Name of Isolates       | Sample sources | Phytolitic Index |
|----|--------------|------------------------|----------------|-----------------|
| 15 | YEg254       | *C. saturnus*          | Insect frass   | 1.4             |
| 16 | YEg029       | *Candida sp.*          | Insect frass   | 0               |
| 17 | YEg059       | *C. tolerans*          | Insect frass   | 1.6             |
| 18 | YEg148       | *C. tropicalis*        | Insect frass   | 4.0             |
| 19 | YEg217       | *C. tropicalis*        | Leaf litter    | 1.6             |
| 20 | YEg199       | *C. yuanshanica*       | Leaf litter    | 0               |
| 21 | YEg252       | *C. yuanshanica*       | Leaf litter    | 4.4             |
| 22 | YEg153       | *Cyberlindnera fabianii* | Soil         | 2.9             |
| 23 | YEg141       | *C. saturnus*          | Leaf litter    | 3.4             |
| 24 | YEg123       | *Debaryomyces hansenii* | Soil         | 0               |
| 25 | YEg157       | *Galactomyces pseudocandidum* | Soil         | 0               |
| 26 | YEg086       | *Kodamaea ohmeri*      | Soil           | 0               |
| 27 | YEg259       | *Kluveromyces hubeiensis* | Soil         | 2.2             |
| 28 | YEg269       | *K. hubeiensis*        | Leaf litter    | 0               |
| 29 | YEg297       | *Meyerozyma carribica* | Leaf litter    | 1.7             |
| 30 | YEg122       | *Pichia burtonii*      | Leaf litter    | 4.3             |
| 31 | YEg170       | *P. manshurica*        | Leaf litter    | 0               |
| 32 | YEg226       | *Sarcocladium bactrocephalum* | Leaf litter   | 2.0             |
| 33 | YEg227       | *S. bactrocephalum*    | Insect tunnel  | 1.4             |
| 34 | YEg135       | *Saturnispora quitensis* | Insect tunnel | 1.7             |
| 35 | YEg094       | *S. silvae*            | Insect tunnel  | 0               |
| 36 | YEg126       | *S. silvae*            | Insect tunnel  | 1.1             |

Table 1. Ascomyceteous yeast isolated from various resources (Continued)

3.2. Growth on solid medium with phytate as the sole phosphorus source
Most of the isolates can grow when they are cultured on MMP, MM- and MMPa agar plates to which implies that they can use phytic acid as the sole phosphorus source. However, in every case, growth was also observed on the MM- plates (negative control), although the colonies were slightly smaller than those on MMP and MMPa agar plates (figure 1). The highest clear zone formation was by *Candida natalensis* YEG 132, *Saturnispora quitensis* YEG 135, and *Candida albicans* YEG 226 (figure 1).
The growth of pytase producing yeast on MMPa YEg132 (C. natalensis), YEg135 (S. quitensis) and YEg266 (C. albicans) after 3 days incubation at 30°C.

Phylogenetic tree analysis shows the position of Candida natalensis YEG 132, Saturnispora quitensis YEg 135, and Candida albicans YEg 226 (figure 2). The numbers after the species name represent the accession numbers of isolates in GenBank. The numbers in each branch points denote the percentages supported by bootstrap.

Figure 1. The growth of pytase producing yeast on MMPa YEg132 (C. natalensis), YEg135 (S. quitensis) and YEg266 (C. albicans) after 3 days incubation at 30°C.

Figure 2. An rDNA-ITS-based phylogenetic tree of Candida natalensis YEG 132, Saturnispora quitensis YEg 135, and Candida albicans YEg 226 showing the position of the isolate.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates [19] is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The
analysis involved 15 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 [19].

![Figure 3](image)

**Figure 3.** Profile of phytase activity of *C. natalensis*, *S. quitensis* and *C. albicans* as affected by incubation time

3.3. *Growth in phytic acid liquid culture*

Growth tests on solid medium a clear phytase activity was observed (figure 1). To study the effect incubation time (figure 3), and abiotic factor then the cultures were grown at varying temperatures (figure 4), pH (figure 5), carbon sources (figure 6) and nitrogen sources (figure 7).

3.3.1. *Phytase activity measurements.* Incubation time affects phytase activities. Optimum incubation time for all isolates was 4 days (figure 3). *S. quitensis* showed good performance on phytase production. This isolates rapidly start phytase production after 2 days of incubation. While *C. natalensis* start producing phytase after 3 days of incubation (figure 3), but these isolates also reach maximum phytase production after 4 days of incubation. Therefore, we select 4 days as optimum production of phytase for all isolates.

3.3.2. *Effect of incubation temperature.* Temperature affected phytase production. All isolates produce the highest phytase was at 30 °C. Increased temperature to 35 °C and 40 °C inhibits phytase production by all strains (figure 4). *S. quitensis* was the highest isolate inhibited by an increase of incubation temperature.
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Figure 4. The Effect of incubation temperature on phytase activity of C. natalensis, S. quitensis, and C. albicans, under shaking condition at 125 rpm

Temperature affecting the phytase production profile was reported in earlier studies [20]. Aspergillus sp. 5990 produces a 5-fold higher activity in liquid culture when compared with cultures of Aspergillus ficuum NRRL 3135. The optimum fermentation conditions were determined to be 35°C, neutral pH, and 4 days incubation. The phytase had a higher optimum temperature for its activity than the commercial enzyme, Natuphos, from Aspergillus ficuum NRRL 3135 [20]. Our isolates C. natalensis, S. quitensis and C. albicans, under shaking condition at 125 rpm produce higher phytase than that of Aspergillus ficuum (NRRL 3135). This strain, when grown at 33°C, pH 4.5, with aeration of 0.9 vvm, in 2-l batch submerged phytase productions, it produced about 2.27 U/ml [21].

3.3.3. The effect of pH on phytase production. The pH of cultures affects phytase production and varies depending on the isolate (figure 5). S. quitensis and Candida natalesis produced the highest phytase activities at pH 5.0, while C. albicans was at pH 6.0. Earlier studies on Aspergillus ficuum (NRRL 3135) showed that the highest activities of phytase activities were obtained at pH 4.5 [21]. But Bacillus subtilis produces the highest phytase at pH 5.5 [22]. Kodamaea ohmeri BG3 produces maximum enzyme phytase activity at pH 6.3 [8].
3.3.4. Effect of carbon sources on phytase activity. All isolates were able to assimilate glucose, dextrose, sucrose, xylose, and lactose (figure 6). In general, glucose was the best carbon source for phytase activity. But each isolate has its preferential on carbon sources. *S. quitensis* produced the highest phytase on glucose, but not on dextrose. These isolates produce almost the same amount of phytase when grown on sucrose, xylose and lactose. *C. natalensis* also produces the highest phytase on glucose, but they produce almost the same amount of phytase when grown on dextrose, xylose, lactose and sucrose. *C. albicans* produced the highest phytase when they were grown on glucose, dextrose and sucrose. This implies phytase production profile was dependent on the species and type of carbon sources.

**Figure 5.** The effect of pH on phytase activity of *C. natalensis, S. quitensis, and C. albicans*
Figure 6. Effect of Carbon sources on the phytase activity of *C. natalensis*, *S. quitensis*, and *C. albicans*

Figure 7. The effect Nitrogen sources on phytase activity of *C. natalensis*, *S. quitensis*, and *C. albicans*
3.3.5. Effect of N-sources
Each isolate has preferential on nitrogen sources. Yeast extract was the best N-sources for *C. albicans*, while ammonium sulfate was best for *C. natalensis*, *S. quitensis*. Ammonium nitrate was not favorable for phytase production by *C. natalensis* (figure 7). Earlier bacterial phytase produced by *Bacillus subtilis* was optimal when yeast extract was used as nitrogen sources [22].

3.4. Biomass growth and phytase production profile
There was a correlation between biomass growth and phytase production in *C. natalensis*, *S. quitensis* and *C. albicans*, but each isolate has its own profile (Figures 8, 9, and 10). For all isolates, maximum biomass growth was observed at 4 days, and the enzyme activities were also optimum on the same days. Earlier studies on *Aspergillus niger* also found that 4 days incubation was maximum for phytase production [23]. When grown under solid-state fermentation *Aspergillus niger* NCIM 563, obtained maximum enzyme activity (108 U g(-1) dry moldy bran, on cowpea meal, which was achieved after 7 days incubation [24]. Our strain produce phytase much faster than *Aspergillus niger* NCIM 563.

![Figure 8](image_url)

*Figure 8*. Biomass growth and phytase activity of *C. natalensis* (at their optimum when glucose as carbon sources at their optimum N-sources)
Figure 9. Biomass growth and phytase activity of *S. quitensis* at their optimum when glucose as carbon sources at their optimum N-sources.

Figure 10. Biomass growth and phytase activity of *C. albicans* (C) at their optimum when glucose as carbon sources at their optimum N-sources.

Phytase producing yeast offer several advantages than other organisms which include higher phytase production. It can be produced by submerge fermentation as well as solid-state fermentation. We found *C. natalensis, S. quitensis,* and *C. albicans* produced high phytase when grown on glucose.
All strains also able to use varying carbon sources include xylose, dextrose, sucrose and lactose. Yeast extract was good nitrogen sources, but all isolates also able to use varying nitrogen sources which include peptone, ammonium sulfate, and ammonium nitrate. All isolates also able to produce phytase at pH 4-7, which implies these isolates are versatile microbes for phytase production. As a concern of many scientists, fed sources are a very important factor in reducing production cost [25], then our strain could be a candidate for phytase production using various substrate composition.

4. Conclusion
We isolated pytase producing yeast which was identified Candida natalensis YEG 132, Saturnispora quitensis YEg 135, and Candida albicans YEg 226. These isolates were able to use various carbon and nitrogen sources. The maximum phytase production depends on isolates, media composition, pH and incubation temperature. These isolates are potential for phytase production on various carbon sources. Isolates selection before phytase production is always advisable.

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