Production and Optimization of Xylanase and α-Amylase from Non-Saccharomyces Yeasts (*Pichia membranifaciens*)

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INTRODUCTION

Biotechnology became an alternative and conventional trend in the fields of industry and analysis. The incorporation of biotechnology in these fields has many advantages includes production of desirable products which could be used in ingredient substitutions, alleviates the product yields, less energy consumption and ecofriendly1. The microbial enzymes meet the industrial demand and now a large number of them are commercially available2.

Following cellulose, xylan is the second most available renewable polysaccharide. Xylanases are hydrolytic enzymes that cleave the β-1,4 glycosidic bonds of xylan. They are applied for the production of hydrolysates in food and beverage industries, bioethanol and azodye removal5. These are used to enhance the nutrients digestibility in animal feed4 as well as used for biobleaching of kraft pulp in pulp and paper industry7. It has also been reported that xylanases play a role in a production of natural sweetener6. The bioproduction of xylanases was performed by bacteria, actinomycetes, yeast and filamentous fungi7. However, xylanolytic yeasts have not been cited frequently8,9, such as Pichia stiptis10, Cryptococcus, Fellomyces 11 and Candida3. The optimum activity of fungal xylanases is neutral or slightly acidic pH's12.

α-Amylases (E.C.3.2.1.1) are enzymes that catalyze the hydrolysis of internal α-1,4-glycosidic linkages in starch13 to produce dextrin, maltose and glucose units. The enzymes are applied in starch liquefaction, paper, improving textile fabrics and preparing starch coatings of paints industries. In addition, α-amylases are applied in the detergent industry, breweries and in the production of syrups. The use of amylase has grown in many ways, including scientific, pharmaceutical and analytical chemistry2,14,15. Different types of amylases can be produced by various organisms16. However, microbial amylase is preferred due to its biochemical versatility, higher production rate, stability, and availability by large number of microbial species. α-Amylases is the major type of microbial amylases that produced by fungi, yeasts and bacteria1. The production of α-amylase by submerged fermentation (SmF) and solid-state fermentation (SSF) has been depend on some of physicochemical factors such as pH, temperature
and moisture\textsuperscript{17,18}. The pH is very effective in the industry, where α-amylases used in starch industry are stable at low pH and in the detergent industry the high pH is required\textsuperscript{19}.

Therefore, this study aimed to isolate xylanolytic and amylolytic yeast strain and detect the optimum parameter for xylanase any amylase production by isolated yeast.

MATERIALS AND METHODS

Yeast isolation, and purification

A weigh of 5 g of soil samples that were collected from various farmlands in Assiut region, Egypt were suspended on 45 mL of sterilized distilled water to form soil spore suspension. Ten-fold serial dilutions of spore suspension were prepared. An aliquot of 100µL was withdrawn and plated on sterilized yeast extract peptone dextrose (YEPD) agar plates as the method described by\textsuperscript{20}. The plates were incubated at 28°C for 72 h. The grown colonies were picked up, purified and preserved in YEPD slant agar tubes at 4°C.

Qualitative screening of xylanase and amylase production

For propagation of inoculum, the pure yeast culture was inoculated in liquid medium containing per liter glucose; 10 g, NaNO\textsubscript{3}; 0.3 g, KH\textsubscript{2}PO\textsubscript{4}; 3g, MgSO\textsubscript{4}; 0.3 g and yeast extract; g at 28°C, 140 rpm, for 48 h. Xylanase production was qualitatively confirmed by formation of clear halo zones around growing colonies that inoculated on Congo red-beechwood xylan (1%) agar plates after washing with 1M NaCl\textsuperscript{3}. While the screening of amylase production was qualitatively confirmed by formation of clear halo zones around growing colonies that inoculated on soluble starch (1%) agar plates after washing with iodine solution (10%)\textsuperscript{21}.

Molecular identification of Xylanase and α-amylase producing yeast

Total genomic DNA from xylanase and α-amylase producing yeast was isolated, according to 20. primers NL1 (5’-GCATATCAATAAGCGGAGGAAAAG-3’) and NL4 (5’-GGTCCGTGTTTCAAGACGG-3’)\textsuperscript{22} were used to amplify 26S rDNA D1/D2 domain region. PCR was carried out in a total volume of 50 µl consisting GoTaq green master mix (Promega, Madison, WI, USA), 1 µL DNA sample, and 1 µL of every primer (0.5 mM)\textsuperscript{23}. The amplification was performed under the following conditions: the denaturation at 95°C for 5 min, accompanied by 36 cycles at 94°C for 2 min, 52°C for 1 min, 72°C for 2 min; the extension at 72°C for 7 min; and then sustained at 4°C. A volume of 5 µl of PCR products was then analyzed using 1.5% 0.5× TBE agarose gel electrophoresis. A 100-bp DNA ladder has been used as a marker. The gel was stained with Ethidium bromide and images were captured under ultraviolet light.

Fig. 1. Phylogenetic relationships between strain AUN-02 and 26S rRNA gene sequences from other published Pichia spp. GenBank accession numbers are given in parentheses.
Purification of PCR products and determination of 26S rRNA gene D1/D2 domain sequences

With a Takara agarose gel DNA purification package, the PCR products (~600 bp) were purified, and then sequenced using ABI 3730 automated sequencer by Macrogen in both directions (Seoul, Korea).

Phylogenetic analysis and comparisons of 26S rRNA gene D1/D2 domain sequences

The 26S rRNA gene D1/D2 domain sequences yeast generating xylanase and α-amylase were searched in the GenBank and matched with recognized 26S rRNA gene sequences at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) using the basic local alignment search tool (BLAST). To identify the isolated yeast, percent identity scores were generated. The phylogenetic tree was also established using MEGA version 4.0 with the neighbor-joining algorithm and Jukes-Cantor distance estimation, with 1,000 bootstrap replicates, to validate the taxonomic classification of the xylanase and α-amylase producing yeast.

Quantitative Determination of enzymes activities

A percentage of 10% V/V of propagated culture were inoculated on new sterilized previously mentioned agar media supplemented with birchwood xylan or soluble starch instead of glucose and incubated at 150 rpm and 30°C for 7 days. Substrates used were beechwood xylan and starch for xylanase and α-amylase, respectively. One mL of each culture sample was centrifuged at 13000 rpm, 4°C, and the supernatants (enzyme crude extract) were stored at -20°C for analysis. Xylanase and α-amylase activities were assayed by determining the liberated reducing end products using xylose and maltose as standards, respectively. one percent of substrate, 0.05 M sodium acetate buffer pH 5.5 and 0.1mL enzyme crude extract were used in the reaction mixture (0.5 mL) contained. Assays were performed for an hour at 37°C. A volume of 0.5 mL 3,5-dinitrosalicylic acid (DNS) reagent was applied to each tube. Then the reaction mixture was vortexed and boiled in a water bath for 10 min. The absorbance was determined at 560 nm after cooling to room temperature. The reducing sugar was estimated from the standard calibration curve equation using xylose and D-glucose for xylanase and amylase, respectively. The quantity of enzyme that released one μmol of reduced sugar per min under optimum assay condition is defined as one-unit enzyme activity.

Optimization factors for xylanase and α-amylase production

The yeast isolate was cultivated in the minimal basal slat medium (MBS-g/L) NaNO₃; 3g, MgSO₄; 0.3 g, KH₂PO₄; 0.3 g supplemented with birchwood (1%) or soluble starch (1%) for 7 days at 150 rpm and 30°C. An aliquot of 1 mL of the culture was taken at regular intervals and centrifuged at 13000 rpm, 4°C for 10 min. The supernatant was used for the enzyme assays.

Fig. 2. Production of xylanase and α-amylase by fermentation of Pichia membranifaciens AUN-02 in Erlenmeyer flasks at 30°C and 150 rpm. The data were considered as means ± S.E. (n=3).
was withdrawn each 24 h interval to perform the enzymes assay in order to determine the optimum incubation time for maximum production of xylanase and α-amylase.

The effect of temperature on the studied enzymes production was determined by cultivating the isolate in (MBS) medium at varying temperatures (25, 30, 35, 40 and 45°C) accompanied by xylanase and α-amylase assays at optimum incubation period. Similarly, optimal pH for the xylanase isolate in the medium with pH changed to 4.0, 5.0, 6.0, 7.0 or 8.0 at the optimum temperature.

Similarly, the effect of the substrate source 1% (birchwood, oat split and beechwood xylan) and (potato starch, glycogen, amylose, α-Cyclodextrin and β-Cyclodextrin) on the activity of xylanase and α-amylase, respectively were investigated at optimum time, temperature and pH. All determinations were performed in triplicate.

### Statistical analysis
The data were statistically analyzed by a one-way ANOVA. The data were considered as means ± S.E. (n=3).

### RESULTS

**Isolation and selection of yeasts producing xylanase and α-amylases**

Twenty yeast isolates were collected from various soil samples in Assiut region, Egypt. All isolated yeasts were screened for xylanase and α-amylase production and one promising isolate designated as AUN-02 was selected. The AUN-02 isolate exhibited large halo zone around its growing colony on xylan supplemented medium colored with Congo red and soluble starch stained with iodine solution.

**Yeast Identification using 26S rRNA gene D1/D2 Region Sequencing and Phylogenetic Analyses**

The phylogenetic location of yeast AUN-02 isolate was established and determined through molecular techniques. The alignment of 26S rRNA gene sequences of the yeast AUN-02 with the reported 26S rRNA sequences from GenBank using BLAST indicates 100% with *Pichia membranifaciens*. Phylogenetic tree was developed for AUN-02 isolate along with other GenBank sequences of the same genus. As shown in (Fig. 1), strain AUN-02 and *Pichia membranifaciens* are a node with zero, or near zero, evolutionary distance of separation. Therefore, strain AUN-02 was identified as *Pichia membranifaciens*.

### Table 1.
Km values (mg/mL) of xylanase and α-amylase enzymes produced from *Pichia membranifaciens* at different pH values. The data were considered as means ± S.E. (n=3)

<table>
<thead>
<tr>
<th>pH</th>
<th>Xylanase (mg/mL)</th>
<th>α-amylase (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>0.85±0.05</td>
<td>0.22±0.01</td>
</tr>
<tr>
<td>6.0</td>
<td>1.6±0.1</td>
<td>0.43±0.02</td>
</tr>
<tr>
<td>4.0</td>
<td>3.4±0.18</td>
<td>2.8±0.13</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of the pH of the culture medium and the temperature of fermentation of *Pichia membranifaciens* AUN-02 on the production of xylanase and α-amylase. The data were considered as means ± S.E. (n=3).
Accession number of the nucleotide sequences

In this analysis, nucleotide sequences of the xylanase and α-amylase producing AUN-02 yeast were recorded in the DDBJ (www.ddbj.nig.ac.jp/), EMBL (www.embl.de/), and GenBank (http://www.ncbi.nlm.nih.gov/genbank/) databases of nucleotide sequences under accession number: MH560348.

Kinetics of xylanase and α-amylase production by the yeast Pichia membranifaciens AUN-02

The time of incubation has a significant impact on the stability of enzymes. A higher production of xylanase (38.8 U/mL) and α-amylase (28.7 U/mL) was obtained after 4 days of fermentation of the yeast Pichia membranifaciens in flasks stirred at 30°C and 150 rpm. After 5 days of fermentation, the activity of xylanase and α-amylase decreased to 31.2 and 26%, respectively (Fig. 2). Moreover, the significant decrease of xylanase and α-amylase to 44.5 and 40.6% after 7 days compared to the stability at 4 days of incubation (Fig. 2).

Characterization of pH and temperature profile of xylanase and α-amylase activity in crude enzyme extract

The effect of pH on the productivity of xylanase and α-amylase from Pichia membranifaciens strain AUN-02 was illustrated in (Fig. 3a). The activities of enzymes were dramatically increased from acidic pH 4.0 to neutral pH 7.0. Higher activity of xylanase (36.83 U/mL) and α-amylase (27.7 U/mL) was obtained in the fermentation of Pichia membranifaciens AUN-02 in a culture medium adjusted to pH 7.0. The activity was then significantly reduced to 21.2 and 16.3 U/mL for xylanase and α-amylase at alkaline pH 8.0, respectively. The results demonstrated that the yeast P. membranifaciensis suitable for production of acidic and neutral xylanase and α-amylase enzymes although the latter being better.

The temperature range between 25 and 45°C was used to study the influence of temperature on the studied enzymes (Fig. 3b). The temperature profile revealed that, the optimum temperature that showed maximum xylanase and α-amylase activity (42.6 and 32.5 units/mL/min, respectively) was estimated at 35°C. However, the xylanase and α-amylase activities showed significant 50% decrease at 25 and 45°C, compared to the values at 35°C.

Fig. 4. Substrate specificity of xylanase and α-amylase that produced from Pichia membranifaciens.
Assessment of xylanase and α-amylase activity on different substrates

In order to determine the extracellular xylanolytic and amyloytic potential of *P. membranifaciens* AUN-02, were grown in media containing (birchwood, oat split or beechwood xylan) and (potato starch, glycogen, amylose, α-Cyclodextrin and β-Cyclodextrin) (1% w/v carbohydrate content) for 7 days at 28°C. The xylanase and amylase activities of crude enzyme extracts from *P. membranifaciens* AUN-02 were estimated and compared for the different substrates tested (Fig. 4). The strain revealed 100% relative activity of xylanase and α-amylase on beechwood and potato starch, respectively. It also exhibited 75 and 65% of relative xylanase activity on oat spelt and birchwood xylan, respectively. On the other hand, it exhibited no significant difference in the relative α-amylase activity on glycogen and amylose (80 and 70%, respectively). Comparatively, the significant decrease in α-amylase activity was detected on α-Cyclodextrin and β-Cyclodextrin (22 and 18%), respectively.

Enzymes kinetics at different pH values

The Km values of xylanase and α-amylase from *Pichia mebranifaciens* strain AUN-02 at different pH values are shown in Table 1. The Km values of xylanase and α-amylase increased in the order of pH’s 7.0, 6.0 and 4.5 (0.85, 1.6 and 3.4 mg xylan/mL and 0.22, 0.43 and 2.8 mg starch/mL, respectively). The low Km indicated the high affinity toward xylan and starch. Therefore, the xylanase and α-amylase produced at pH 7.0 had high affinity toward xylan and starch.

**DISCUSSION**

Xylanase along with different enzymes are important for the lignocellulosic based biorefineries to convert complex substrates. Amylases and xylanases are amongst the most studied enzymes that attracted worldwide attention due to their physiological and biotechnological applications.

Screening xylanase and amylase with Congo red and Iodine solution, respectively are common methods in which the Congo red dye remains attached to xylan polymer while the iodine solution forms a blue complex with starch polymer. So, the formation of the halo zone around the growing colonies is indicating the hydrolysis of xylan or starch which are directly related to the region of action of the corresponding enzymes. Relatively less abundance 23 strains of yeasts out of 119 of yeasts strains has been reordered positive xylanolytic activity as described by. Xylanase producing yeasts can be used in biorefineries for subsequent steps. They are however, still the focus of intensive research. The xylanolytic and amyloytic yeast strain AUN-02 was isolated from farmland in this sense because it consists of various types of cellulosic, hemicellulosic, and lignocellulosic matters.

As a valuable identification tool, The 26S rRNA gene D1/D2 domain has acquired recognition in yeast taxonomy. D1/D2 domain sequence databases are available for all currently recognized ascomycetous and basidiomycetous yeasts. This makes it much easier to classify species and serves as a valid and functional criterion for identification of most recognized yeast. Yeast strain *Pichia membranifaciens* was isolated from different sources and it has many biotechnological applications. 1,3-dihydroxyacetone producing *P. membranifaciens* was isolated from soil sample and identifies based on ITS rDNA gene sequence analysis. *Pichia membranifaciens* was among the abundant yeast species that isolated from directly brined olives of Alorena reported the purified 1,4-β-xylosidase from *P. membranifaciens* that has been cultivated on xylan as a substrate and this enzyme is used in the bioethanol production process.

xylanase showed optimum at or near mesophilic temperatures between 30-60°C and slightly acidic pH. Similarly, the pH value for maximum production of α-amylase by microorganisms ranged from 6.0 to 7.0. Optimum neural pH was recorded for xylanase from *Aspergillus caespitosus* (pH 6.5–7.0). At pH 4.5, the maximum production of α-amylase was detected for *Trichoderma harzianum*. α-amylase producing yeast strains such as *Saccharomyces cerevisiae* and *S. kluyveri* exhibited maximum enzyme production at pH 5.0. The maximum amylase production from *Bacillus cereus* IND4 at 45°C on starch agar medium, whereas maximum amylase production at moderate temperature (30°C) from the fungal strain.

Depending upon the substrates, xylanolytic enzymes are normally inducible
under normal conditions. However, a few species demonstrate constitutive production of the enzyme and are consumed as carbon sources\textsuperscript{46}. In certain cases, xylan has been found to be the best inducer of xylanase production\textsuperscript{12}. Xylan, is a high molecular weight polymer, that is unable to enter the microbial cell directly. Low molecular weight of xylan fragments induce the production of enzymes\textsuperscript{1}. Xylanase induction can be performed on a number of low cost lignocellulosic ingredients, such as wheat bran, wheat straw, rice husk, rice bran, rice straw and corncob have been shown to be most appropriate substrates for fermentation in some microbes\textsuperscript{47}.

The induction of \(\alpha\)-amylase production by microorganisms is stimulated by potato and corn starch, and soluble starch. The rate of hydrolysis by \textit{Bacillus} sp. BCC 01-50 of soluble starch, wheat, potato, and corn starches at 1\% concentration was 73.43, 60.81, 55.26, and 67.81\%\textsuperscript{16}.

The \(K_m\) values of xylanase from \textit{P. membranifaciens} were similar to \(K_m\)’s of xylanases from \textit{Anoxybacillus kamchatkensis} (0.7 mg xylan/mL\textsuperscript{48}) and \textit{Aspergillus ficium} AF-98 (Km 3.267 mg xylan/mL\textsuperscript{49}). For the \(K_m\) values of \(\alpha\)-amylase from \textit{P. membranifaciens}, they similar to the \(K_m\) values of \(\alpha\)-amylase from \textit{Cryptococcus flavus} (0.056 mg starch/mL\textsuperscript{50}) and \textit{Lipomyces kononenkoe} CBS 5608 (Km 0.8 g starch/L\textsuperscript{51}).

It could be concluded that, the yeast isolate AUN-02 is a promising strain for production of xylanases and \(\alpha\)-amylase. The optimum production conditions were neutral \(pH\) and 35\(^\circ\)C. The induction of xylanases and \(\alpha\)-amylase are substrate dependent.

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None.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**AUTHORS’ CONTRIBUTION**

AH, AMM and HAS designed the experiments. HAT, HAS and NAS, KAM, KAA performed the experiments. SRZA, and JAA-H analyzed the data. AH, AMM wrote the manuscript. All authors read and approved the manuscript.

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None.

**DATA AVAILABILITY**

All datasets generated or analyzed during this study are included in the manuscript.

**ETHICS STATEMENT**

Not applicable.

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