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Optimizing parameters for production of cell wall hydrolyzing enzymes by *Aspergillus eucalypticola* AUMC 15402 under submerged and solid-state conditions

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ABSTRACT

In this study, a promising fungal isolate has been isolated from sugarcane bagasse, and it has demonstrated a high potential for producing endoglucanase, exoglucanase, and xylanase. The fungus was identified as *Aspergillus eucalypticola* by using sequencing of the internal transcribed spacer region. At 30 C, sodium nitrate was the best nitrogen source for the maximum activity of endoglucanase (21.0±2.2 U/mg at pH 8.0 after 8 days), exoglucanase (37.8±4.0 U/mg at pH 5.0 after 9 days), and xylanase (42.0±4.0 U/mg at pH 8.0 after 6 days). Endoglucanase and exoglucanase presented their highest activity of 0.362 and 4.25 U/mg, respectively, at pH 8.0 and 45 ºC, while pectinase and xylanase showed their activity maxima of 4.0 and 2.94 U/mg, respectively, at pH 8.0 and 50 ºC. Co^{2+}, Mn^{2+}, SDS, Ca^{2+}, and Ni^{2+} boosted endoglucanase activity by 138.72, 126.65, 125.24, 110.1, and 104.85 %, respectively. Every material under investigation boosted the activity of exoglucanase, except for Ni^{2+} and SDS. The increased impact ranged from 101.43% for EDTA to 146.16% for Mn^{2+}. Co^{2+}, Mn^{2+}, SDS, and Fe^{2+} increased pectinase activity by 123, 118.34, 107.4, and 101.9 %. Mn^{2+} followed by Co^{2+}, SDS, Fe^{2+}, and Ca^{2+} improved xylanase activity by 179.4, 167.75, 152.0, 123.35, and 102.0 %, respectively. The strain fermented date palm leaves, rice straw, and sugarcane bagasse under solid state fermentation into endoglucanase (18.0, 16.0, and 9.0 U/g), exoglucanase (20.0±1.64, 25.0±2.0, and 15.0±1.0), pectinase (34.0±2.84, 38.0±2.85, and 38.0±3.0 U/g), and xylanase (25.0±1.8, 28.0±2.0, and 17.0±1.1 U/g), respectively.

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Introduction

Presently, lignocellulosic biomass is the most prevalent type of renewable biomass on Earth, serving as an inexpensive and conveniently obtainable raw material for the manufacturing of several biotechnologically significant products (Chandra and Madakka 2019). Every year, agriculture produces about 200 billion metric tons of lignocellulosic biomass worldwide which represents about 90 % of plant material global production (Singh et al. 2012, Ilić et al. 2023). These biomass leftover are

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made up of bran and straw of rice, wheat, maize, corn, rice straw, sugarcane bagasse, date palm leaves, fruit and vegetable scraps, cotton leaf scraps, etc. Therefore, it is crucial to decrease these wastes' detrimental impacts on the environment and to efficiently and effectively transform them into valuable products with industrial and commercial value (Wang et al. 2016, Al-Kolaibe et al. 2021). Lignocellulose biomass is composed of 35–50 % cellulose, 20–35 % hemicellulose, and 15–25 % lignin, all of which are tightly bonded together by a combination of non-covalent and covalent connections (Limayem and Ricke 2012, Ismail et al. 2018). They are still mostly undiscovered in Egypt, where burning them in the fields poses serious health concerns and pollution problems. Fortunately, these residues shouldn't be considered "waste" but rather "natural resources" that can be utilized in other industrial processes because they are frequently rich in proteins, carbs, and minerals (Mussatto *et al.* 2012, Ismail *et al.* 2018). Due to their complexity, microorganisms require a variety of enzymes to biodegrade these biomass residues.

The cellulases enzyme system is responsible for bioconverting cellulose to glucose. For effective cellulose hydrolysis, the three enzymes that make up the cellulase enzyme complex—endoglucanase (endo β-1,4-D glucan glucanohydrolase, CMCase, EC 3.2.1.4); exoglucanase, cellobiohydrolase, Avicellase (β-1,4-D glucan cellobiohydrolase, EC 3.2.1.91); and β-glucosidase or cellobiase (β-D-glucoside glucohydrolase, EC3.2.1.21)—must work together synergistically (Gupta and Bisaria 2018, Srivastava et al. 2020, Mihajlovski and Milić 2022). The global cellulase market is projected to have increased at a compound annual growth rate (CAGR) of 6.9 % by 2032, or about USD 3.1531 billion. Numerous industries, including the textile, bioethanol, pharmaceutical, cosmetic, pulp and paper, and agriculture sectors, are using cellulase more frequently (Ilić et al. 2023).

Hemicellulases (ex: xylanases) and ligninases (ex: laccases) are also necessary enzymes for efficient lignocellulose biomass breakdown because cellulose is encircled by a network of hemicellulose and lignin. Xylanases are a class of enzymes that catalyze the degradation of the linear polysaccharide β-1,4-xylan, which is a crucial part of the plant cell wall, into xylose (Moubasher et al. 2019, Sarangi and Thatoi 2024). Xylanase exhibits significant promise in numerous industrial processes, specifically in the areas of textiles, leather, detergents, and baking. Further biotechnological uses for xylanase include the biopulping of wood, pulp bleaching, providing animal feed to improve digestibility, and processing food to promote clarity (Al-Kolaibe et al. 2021, Sarangi and Thatoi 2024).

Many enzymes can be produced by either solid-state fermentation (SSF) or submerged fermentation (SmF). SSF is a widely used technique for producing enzymes because of its inexpensive cost and lack of complicated technology. Date palm leaves, rice straw, and sugarcane bagasse are attractive sources for the profitable synthesis of important enzymes in Egypt. The current study therefore focused on optimizing the fermentation conditions for a wild strain of *Aspergillus eucalypticola* AUMC 15402 to produce endoglucanase, exoglucanase, and xylanase in submerged fermentation. Second: exploitation of the date palm leaves (DPL), rice straw (RS), and sugarcane bagasse (SB) as substrates to produce high-activity endoglucanase, exoglucanase, pectinase, and xylanase in SSF by *A. eucalypticola* AUMC 15402.

Materials and Methods

Isolation of fungal strain

This study's fungal strain was isolated from sugarcane bagasse that was gathered from local markets in the Assiut Governorate, Egypt. The direct plate technique (Al-Bedak *et al.* 2021) was used, wherein five segments of the sugarcane bagasse sample were placed on the surface of Petri dishes containing Cz agar supplemented with 50 mg/L Rose Bengal. The plates were then incubated at 25 °C for seven days. The developed fungi were then isolated, purified, and kept as pure cultures at -86°C in 20% glycerol/water and on cotton balls (Al-Bedak *et al.* 2019).

Fermentation medium

Sucrose-free Czapek's mineral medium was used as fermentation medium. The medium has the following composition (g/L): Na_2NO_3, 2.0; K_2HPO_4, 1.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4$, 0.01; $ZnSO_4$, 0.01; $CuSO_4$, 0.005.

Extraction of xylan from oat spelts

With few modifications, the alkaline extraction technique recommended by (Puls *et al.* 2005, Al-Kolaibe *et al.* 2021) was used to extract xylan from oat spelts. An oat spelt weighing one hundred grams was soaked in one liter of 5.0 % NaOH. The mixture was brought to 90 °C by steam heating for 120 minutes. The supernatant was produced by centrifuging the mixture at 5,000 rpm for 30 minutes. When double the volume of isopropanol was added, xylan precipitated. Following a bleaching process using 0.4% hydrogen peroxide and an isopropanol wash, the residual lignin was broken down. After centrifugation, the xylan was dried in a hot air oven at 55°C, and used in the assay test.

Quantitative assessment of enzymatic capacity in SmF

Fungal strain was grown in 250 mL Erlenmeyer conical flasks each containing 50 ml of sucrose free-Czapek's broth medium, complemented by 1% oat spelt xylan (for endoglucanase, exoglucanase and xylanase production). Every flask was inoculated with 2.0 mL of spore suspension (1.5×10^8 spores/mL) from 7-day-old cultures.

Extraction of cocktail enzymes

After incubation, the cell-free supernatant was collected through centrifugation (10,000 rpm at 4 °C for 10 min) and used as a source of the cocktail enzyme.

Enzymes assay and protein determination

Endoglucanase, exoglucanase, and xylanase activity were determined by mixing 0.5 ml filtered crude enzyme with 0.5 ml of 1% of each carboxymethyl cellulose (CMC), microcrystalline cellulose (MCC), or oat spelts xylan, respectively (each prepared in 50 mM Na-citrate buffer, pH 5.0). The reaction mixture was incubated at 50°C for 15 min and the process was stopped by applying 2 mL of 3, 5-dinitrosalicylic acid (DNS) and boiling in a water bath for 10 min (Miller 1959). After cooling, the color absorbance was measured at 540 nm using UV-Visible spectrophotometer (T80+, UK). The amount of reducing sugar liberated was quantified using standard curves of glucose (for endoglucanase and exoglucanase), or xylose (for xylanase). One unit of the enzyme is defined as the amount of enzyme that liberates 1 µmol of the reducing sugar (glucose or xylose) equivalent per minute under the standard assay conditions (Ghose 1987, Ghose and Bisaria 1987). Total protein content was measured by the method suggested by Lowry *et al.* (1951) using bovine serum albumin (BSA) as the standard. Enzyme activity of endoglucanase, exoglucanase or xylanase can be calculated according to the following Equation:

$$\textbf{Enzyme activity} = \left(\frac{\text{Absorbance x DF}}{(X)(Y)(t)(\text{slope})}\right) \quad \text{U/mL}$$

Where: DF = the dilution factor for enzyme; x = the volume of enzyme used; y = the volume of hydrolysate used for assay of reducing sugars; t = the time of hydrolysis; slope was determined by the standard curves of glucose or xylose.

Morphological and molecular identification of the fungal strain

Using an inoculum size of 1.0 µL/spot, fungal spore suspension (prepared in a 30% glycerol, 0.2% agar, and 0.05 % Tween 80 solution) were used to inoculate Petri plates containing Cz, MEA, and CYA (Samson *et al.* 2014). Microscopic features were examined using a Zeiss microscope (Axio Star, Germany) and lacto-phenol cotton blue after seven days of incubation at 25 °C.

Optimization of fermentation parameters

In order to maximize the output of endoglucanase, exoglucanase, and xylanases, the fermentation medium' respective pH, nitrogen supply, temperature, and fermentation duration were varied under one factor at a time (OFAT) conditions. The experiments were carried out in 250 mL Erlenmeyer flasks with 50 mL of the fermentation medium supplemented with 1.0 % oat spelt xylan as an only carbon source. Spore suspension containing 1.5×10^8 spore/mL of Aspergillus eucalypticola AUMC 15402 strain that was 7-day-old was used to individually inoculate the flasks. The flasks were then incubated for 1 to 10 days under various operating conditions, including pH (3-10), nitrogen source (peptone, yeast extract, sodium nitrate, sodium nitrite, ammonium sulphate, ammonium chloride, and urea; each at 0.2 %), temperature (25, 30, 35, 40, 45, and 50 °C), and incubation duration (1–10) days. Three different experiments were conducted**.**

Production of cell wall hydrolyzing enzymes in SmF

Using the optimum fermentation conditions for each enzyme, the experiment was conducted in 500 mL Erlenmeyer flasks each containing 100 mL of the fermentation medium that was supplemented separately with 1.0 % of CMC, MCC, pectin, or oat spelt xylan as the only carbon source for the production of endoglucanase, exoglucanase, pectinase, and xylanase, respectively. Each flask was inoculated individually with 5.0 mL of the A. eucalypticola AUMC 15402' spore suspension that containing 1.5×10^8 spores/mL of 7-day-old culture. Three distinct experiments were carried out.

Impact of pH, temperature, ions, and inhibitors on the pure xylanase activity

The impact of pH (3.0–11.0) at 25-60 ºC on the activity of endoglucanase, exoglucanase, pectinase, and xylanase produced by *A. eucalypticola* AUMC 15402 was investigated. Citrate buffer (pH 3.0-6.0), phosphate buffer (pH 7.0-8.0), and glycine/NaOH buffer (pH 9.0–11.0) were the buffers employed. The reaction mixture contained 0.01 g enzyme powder and 0.01 g CMC, MCC, pectin, or oat spelt xylan, for testing the enzymes, respectively (each dissolved in 1.0 mL of 50 mM buffer solution). After the reaction time (20 min), the reaction was terminated by introducing 2.0 mL of 3,5-dinitrosalicylic acid (DNS) (Miller 1959), and the enzyme activity was determined as previously

mentioned. Additionally, ions such as Na^{+}, K^{+}, Ca^{+2}, Mg^{+2}, Fe^{+2}, Cu^{+2}, Mn^{+2}, Zn^{+2}, Co^{+2}, and Ni^{+2} were tested by adding them to the reaction mixture at 5 mM/mL concentration as NaCl, KCl, $CaCl_2$, $MgSO_4$, $FeSO_4$, $CuSO_4$, $MnSO_4$, $ZnSO_4$, $CoCl_2$, and $NiSO_4$. In order to test an enzyme inhibitor, 5 mM/mL ethylenediaminetetraacetic acid (EDTA) was also utilized. Under the optimum conditions of each enzyme, the enzyme's activity without the presence of metal ions or EDTA, was assessed to determine the residual activity. The experiment was carried out three times.

Production of cocktail enzymes in solid-state fermentation (SSF)

Substrate pretreatment

To produce cocktail enzymes under the solid state fermentation (SSF), three distinct agricultural residues were selected: date palm leaves (DPL), rice straw (RS), and sugarcane bagasse (SB). Every substrate was acquired from public markets in Egypt's Governorates of Assiut and New Valley. After being cleaned with distilled water, they were ground into tiny particles that could fit through a 2.0 mm filter and oven dried at 50 ºC to maintain a constant weight.

Set Up the Fermentation Conditions

Erlenmeyer flasks (250-mL) each holding 10 g of agricultural residues, were prepared in triplicate. Ten mL of the fermentation medium supplemented with 0.1% oat spelt xylan were used to moisten each agricultural residues. The flasks were subsequently autoclaved at 121 ºC for 20 minutes. Following cooling, 5.0 mL of spore suspension containing 1.5×10^8 (spore/mL) from a 7-day-old culture of *A. eucalypticola* AUMC 15402 was added to each flask. Sodium nitrate was used as a source of nitrogen and the fermentation conditions were adjusted to pH 8.0 and the inoculated flasks were kept in a static environment at 30 ºC for ten days. After the fermentation period, a 100 mL of 50 mmol sodium citrate buffer (pH 5.0) was added to each flask to harvest the fermented slurry. The cell-free supernatant was then obtained by centrifugation at 10,000 rpm for 20 min at 4 ºC. The cell-free supernatants were used for cocktail enzymes assay.

Statistical analysis

The mean and standard deviation (SD) of the tentative study performed in triplicate were used to express all data. Analysis of the statistical significance was conducted according to (Gomez 1984, Stahle and Wold 1989). It was deemed significant at $p \leq 0.05$.

Results

Morphlogical and molecular identification of the Aspergillus isolate

The *Aspergillus* isolate in this study showed the identical morphological features of *Aspergillus eucalypticola*. Conidiophores biseriate with globose vesicles 30–55 µm, stipe smooth-walled to finely roughened, hyaline, 8–14 µm width. Conidia globose, 2.5–3.5 µm, brown, smooth-walled to coarsely roughened. Sclerotia not observed (Fig. 1).

After conducting a megablast search in the NCBI database and comparing it to the type materials, it had been found that the Aspergillus isolate AUMC 15402's ITS sequence was most similar to those of *Aspergillus costaricensis* CBS 115574 (holotype) and *Aspergillus eucalypticola* CBS 122712 (holotype) [(GenBank accession number NR_103604 and OQ135173, respectively; identities = 567/567 (100%); gaps = 0/567 (0%)]. The Aspergillus strain utilized in this investigation was molecularly identified using ITS sequencing-based phylogenetic analysis. In the final analysis, the ITS data set had 596 characters overall from 25 sequences, 459 of which could be accurately aligned, 51 (11.1%) of which were categorized as variable, and 26 (5.7%) of which were rated as informative. Tamura's 3-parameter model, which used a discontinuous Gamma distribution (T92+G), functioned adequately for representing the relationship between taxa. The Maximum Parsimony method produced ten trees. Tree length (104 steps), greatest log likelihood (-1411.41), consistency index (0.796296), retention index (0.902655), and composite index (0.718781) are the attributes of the most parsimonious tree. The strain used in this investigation was found at the same branch as *A. eucalypticola* CBS 122712 (holotype). As a result, it is designated as *A. eucalypticola* here, and PQ222663, the ITS sequence for it, was uploaded to GenBank (Fig. 2).

Optimization of cocktail enzymes production

Effect of medium's pH and nitrogen source

After adjusting the pH of the fermentation medium, it was found that endoglucanase activity was best at pH 8.0, producing a significant ($p < 0.05$) high specific activity of 5.1±0.6 U/mg (Fig. 3A). It became apparent that exoglucanase was most active at pH values between 4.0 and 5.0, with pH 5.0 being the most significant ideal ($p < 0.05$), showing 6.98±0.88 U/mg of specific activity (Fig. 3B).

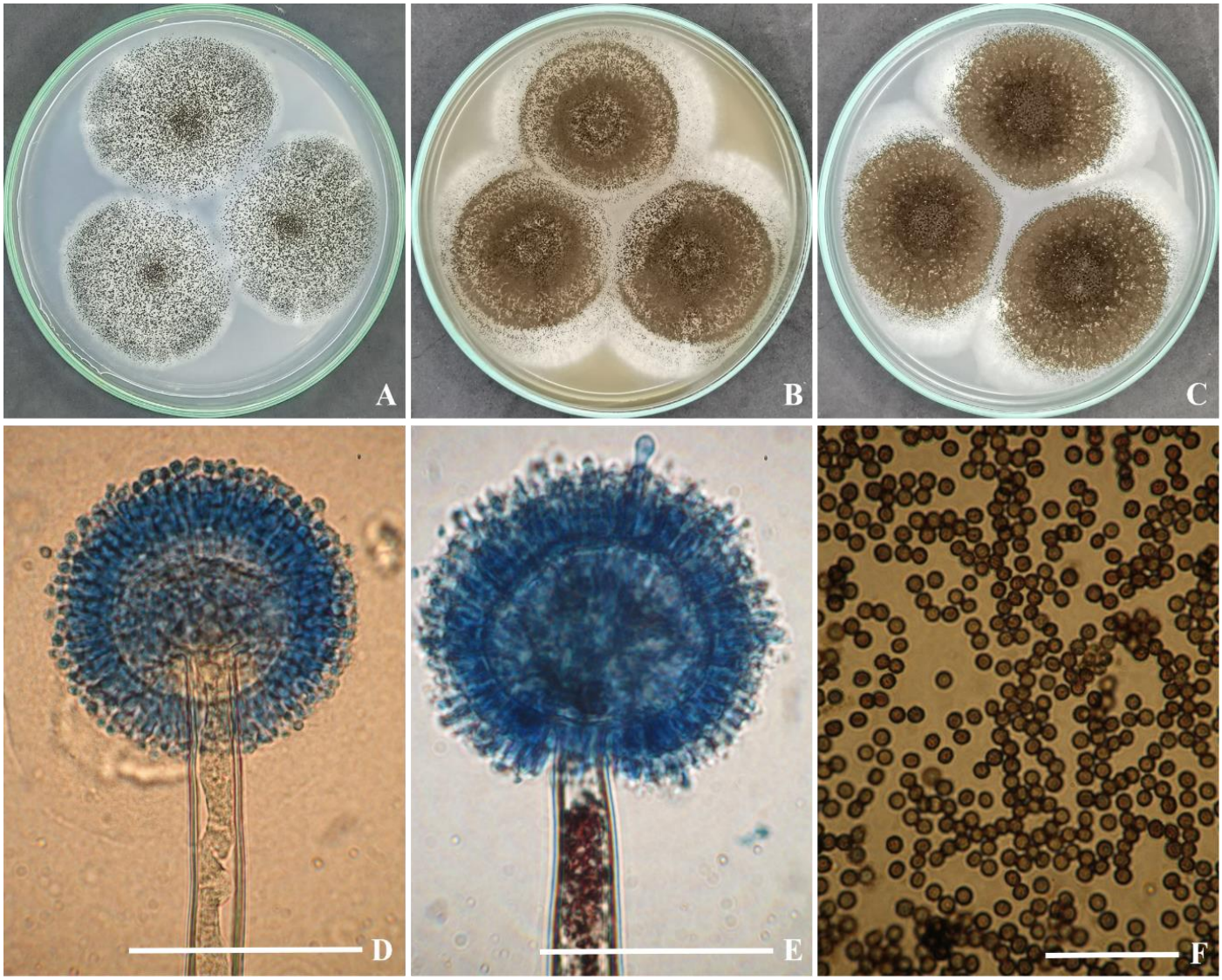

Fig 1. (A–C) Seven-day-old colonies of *Aspergillus eucalypticola* AUMC 15402 on Cz, MEA, and CYA at 25 C. (D–E) Smooth-walled to finely roughened, hyaline, biseriate conidiophores with globose vesicles. (F) Globose, brown, smooth-walled to coarsely roughened conidia (Scale bar: D–E = 50 µm; F = 20 µm).

At pH 8.0, xylanase significantly ($p < 0.05$) produced its highest specific activity of 41.1±4.2 U/mg (Fig. 3C). Following medium nitrogen supply optimization, endoglucanase, exoglucanase, and xylanase activity peaked with using sodium nitrate, yielding the significant ($p < 0.05$) highest specific activity of 21.0±2.2, 37.8±4.0, and 42.0±4.0 U/mg, respectively (Fig. 3 D–F).

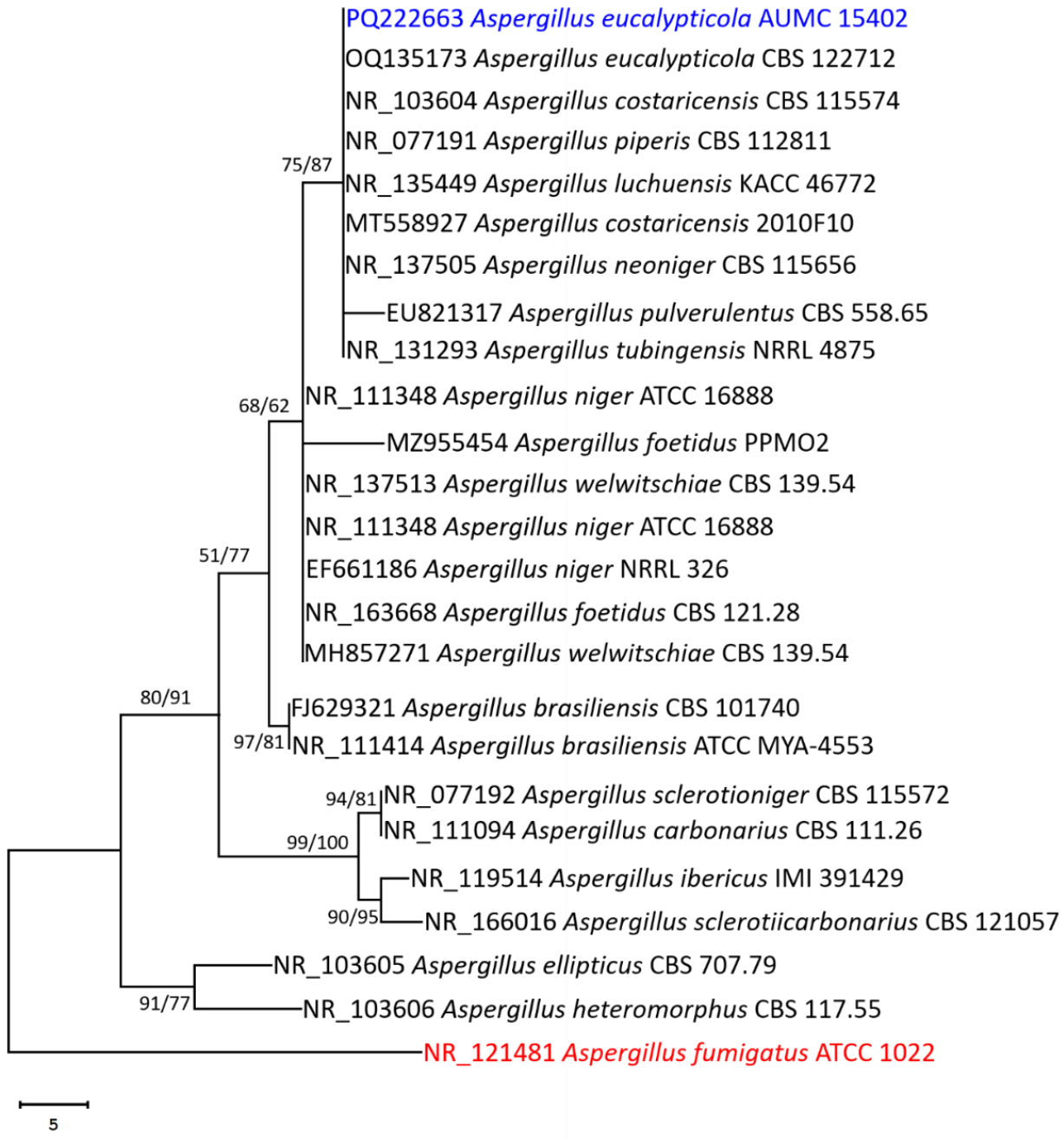

Fig 2. The most parsimonious evolutionary tree obtained from ML/MP analysis of ITS sequences of *A. eucalypticola* AUMC 15402 in this study (in blue) compared to the most similar species of *Aspergillus niger* group in GenBank. Bootstraps (1000 replications) for ML/MP ≥ 50% are indicated near the respective nodes. The tree is rooted to *Aspergillus fumigatus* ATCC 1022 (in red).

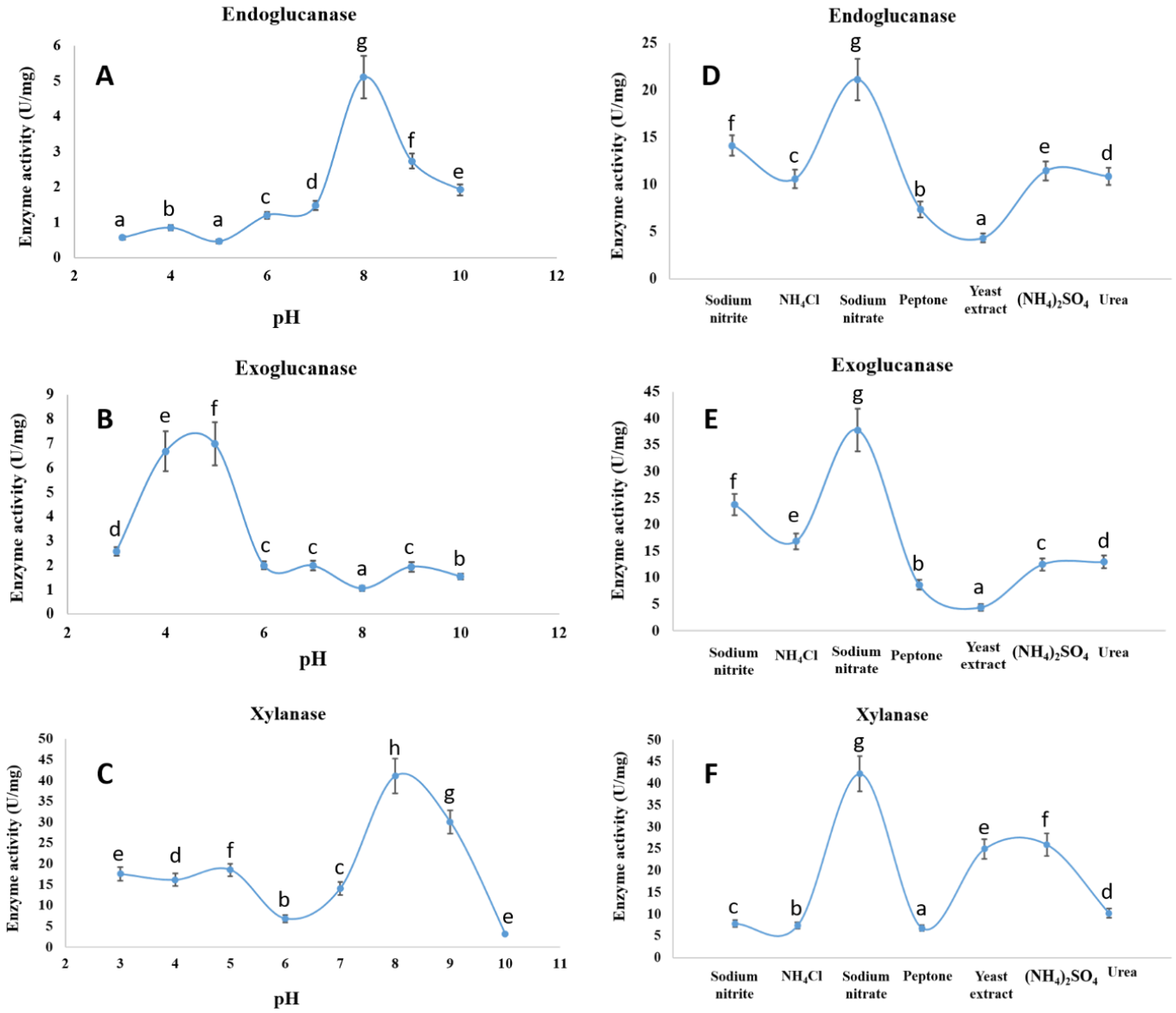

Fig 3. (A–C) Effect of medium's pH and **(D–F)** nitrogen source on the activity of endoglucanase, exoglucanase, and xylanase, respectively produced by *A. eucalypticola* AUMC 15402 in SmF (Mean values±SD with different letters are significantly different; $p < 0.05$; $n = 3$).

Effect of incubation temperature and fermentation period

A. eucalypticola AUMC 15402 produced the highest activity of endoglucanase (21.5±2.0 U/mg), exoglucanase (38.2±4.0 U/mg), and xylanase (42.6±4.0 U/mg), at 30 ºC ($p < 0.05$), when incubated at different temperatures (Fig. 4 A–C). On the eighth, ninth, and sixth day of incubation, the maximal values ($p < 0.05$) of endoglucanase, exoglucanase, and xylanase activities were 22.5±2.1, 38.6±4.0, and 44.2±3.8 U/mg, respectively (Fig. 4 D–F).

Production of cocktail enzymes in SmF

A. eucalypticola AUMC 15402 yielded a comparatively high amount of endoglucanase (1.2 g), exoglucanase (2.3 g), pectinase (4.0 g), and xylanase (2.9 g) per liter of fermentation media in submerged fermentation.

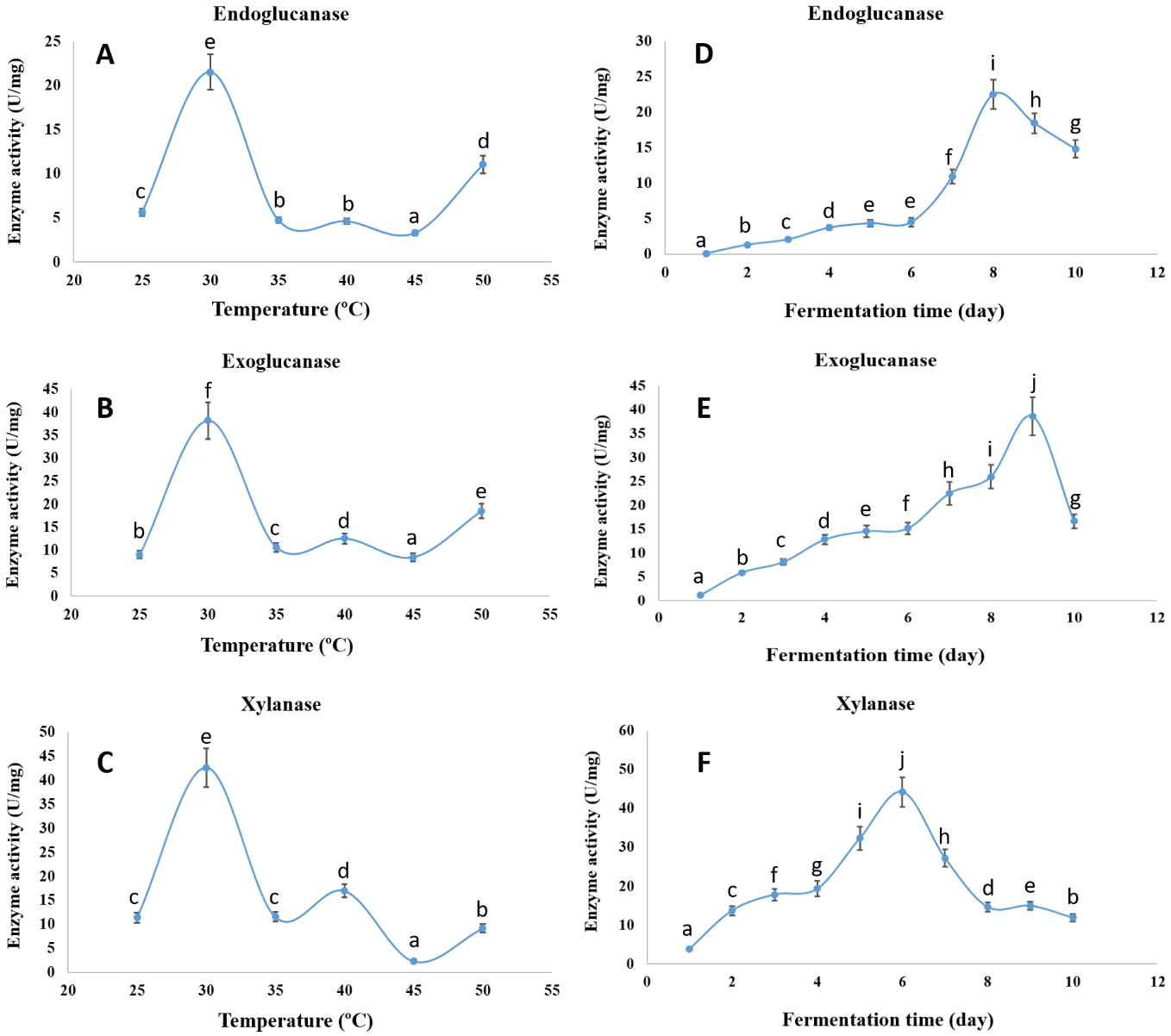

Fig 4. (A–C) Effect of medium's temperature and **(D–F)** fermentation time on the activity of endoglucanase, exoglucanase, and xylanase, respectively produced by *A. eucalypticola* AUMC 15402 in SmF (Mean values±SD with different letters are significantly different; $p < 0.05$; $n = 3$).

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Effect of pH and temperature on the enzymes activities

The activities of endoglucanase, exoglucanase, pectinase, and xylanase were assessed at different pH values ranged from 3 to 11. The four enzymes showed their maximum specific activities of 0.27, 3.775, 4.0, and 2.9 U/mg, respectively at pH 8.0 which was determined to be the optimal value (Fig. 5). When temperature was changed from 25 to 60 ºC, endoglucanase and exoglucanase presented their highest activity of 0.362 and 4.25 U/mg, respectively, at 45 ºC, while pectinase and xylanase showed their activity maxima of 4.0 and 2.94 U/mg, respectively, at 50 ºC (Fig. 6).

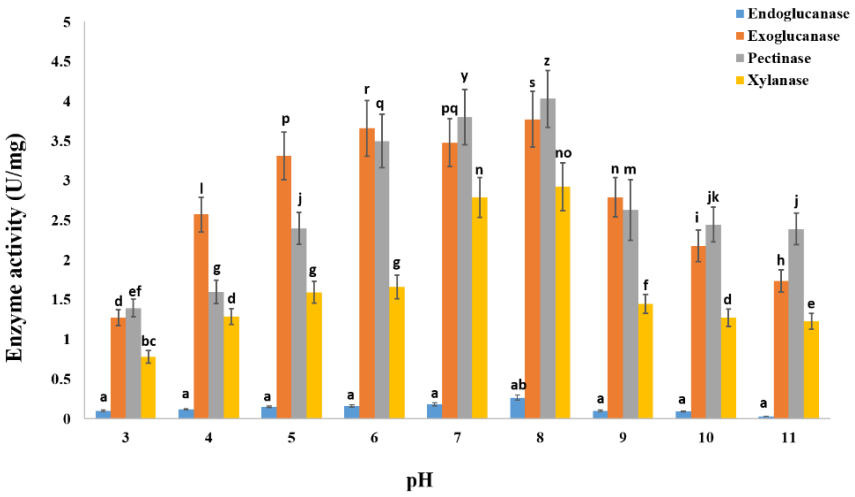

Fig 5. Effect of pH at 50 ºC on the activity of endoglucanase, exoglucanase, pectinase, and xylanase produced by *A. eucalypticola* AUMC 15402 in SmF (Mean values±SD with different letters are significantly different; $p < 0.05$; $n = 3$).

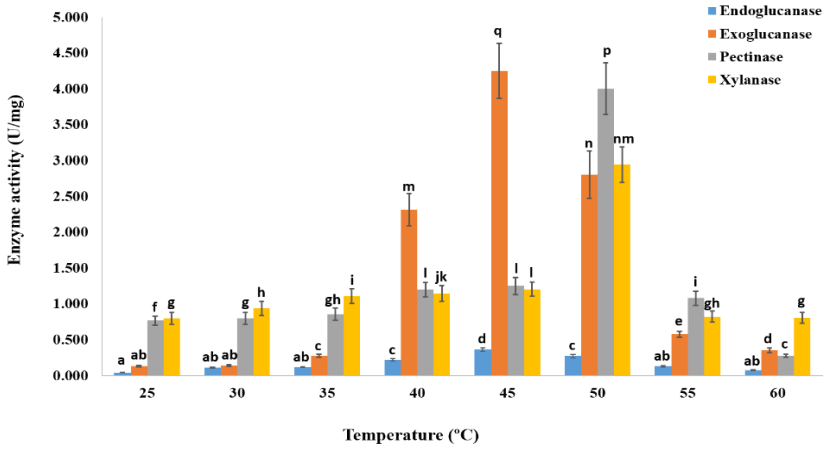

Fig 6. Effect of temperature on the activity of endoglucanase, exoglucanase, pectinase, and xylanase produced by *A. eucalypticola* AUMC 15402 in SmF (Mean values±SD with different letters are significantly different; $p < 0.05$; $n = 3$).

Effect of metal ions and inhibitors on the enzymes activities

The investigated compounds had a spectrum of effects, from enhancement to inhibition. Co^{2+}, Mn^{2+}, SDS, Ca^{2+}, and Ni^{2+} boosted the endoglucanase activity by 138.72, 126.65, 125.24, 110.1, and 104.85%, respectively. In contrast, Na^{+} had the most inhibitory effect resulted in 21% of the endoglucanase residual activity. Na^{+} was followed by Zn^{2+}, Fe^{2+}, EDTA, Mg^{2+}, and K^{+} in decreasing order of effectiveness (81.27, 89.5, 92.93, 96.77, and 97.0 % residual activity, respectively). Exoglucanase activity was increased by all substances examined, except for Ni and SDS, which reduced it to 96.4 and 95.67% of its activity, respectively. The range of the enhanced impact was 101.43% for EDTA to 146.16% for Mn^{2+}. The addition of Co^{2+}, Mn^{2+}, SDS, and Fe^{2+} to the reaction increased pectinase activity by 123, 118.34, 107.4, and 101.9 %, while the remaining compounds inhibited the pectinase activity at varying levels yielded residual activities ranging from 5.56 % for Na^{+} to 96.9 % for K^{+}. Mn^{2+} followed by Co^{2+}, SDS, Fe^{2+}, and Ca^{2+} improved the xylanase activity by 179.4, 167.75, 152.0, 123.35, and 102.0 %, respectively. The remaining compounds decreased the xylanase activity with the highest inhibition being caused by EDTA and the lowest by Ni^{2+} which resulted in residual activities of 64.7 % and 93.0 %, respectively (Table 1).

Table 1. Effect of metal ions and inhibitors (5 mM) on the activity of endoglucanase and exoglucanase (at pH 8.0 and 45 ºC), pectinase and xylanase (at pH 8.0 and 50 ºC) produced by *A. eucalypticola* AUMC 15402. The results are expressed as the proportion of the enzyme activity in the tested inhibitory conditions from the enzyme activity in the control without inhibitors (Mean values ± SD with different letters are significantly different; $p < 0.05$; $n = 3$).

Metal ions	Endoglucanase		Exoglucanase		Pectinase		Xylanase	
	Activity (U/mg)	Residual activity (%)	Activity (U/mg)	Residual activity (%)	Activity (U/mg)	Residual activity (%)	Activity (U/mg)	Residual activity (%)
Control	**0.362±0.3 ^{g}**	**100.00**	**4.25±0.35 ^{c}**	**100.00**	**4.0±0.34 ^{g}**	**100.00**	**2.94±0.25^{h}**	**100.00**
Na	0.076±0.01 ^{a}	21.0	4.55±0.38 ^{e}	107.18	0.22±0.02 ^{a}	5.56	2.69±0.22 ^{e}	91.4
K	0.35±0.03 ^{f}	97.0	5.2±0.44 ^{i}	122	3.87±0.35 ^{f}	96.9	2.5±0.2 ^{c}	86
Ca	0.4±0.03 ^{j}	110.1	5.0±0.4 ^{g}	118.33	2.72±0.22 ^{b}	68.0	3.0±0.28 ^{i}	102
Mg	0.35±0.03 ^{e}	96.77	5.2±0.5 ^{j}	122.26	3.0±0.24 ^{c}	75.5	2.4±0.2 ^{b}	82.5
Fe	0.32±0.022 ^{c}	89.5	5.0±0.46 ^{h}	118.51	4.1±0.36 ^{j}	101.9	3.6±0.32 ^{j}	123.35
Cu	0.36±0.025 ^{h}	100.3	4.77±0.38 ^{f}	112.18	4.0±0.32 ^{i}	100	2.6±0.2 ^{d}	87.54
Mn	0.46±0.036 ^{l}	126.65	6.2±0.52 ^{m}	146.16	4.7±0.4 ^{l}	118.34	5.3±0.4 ^{m}	179.4
Zn	0.294±0.02 ^{b}	81.27	5.4±0.5 ^{k}	127.16	4.0±0.34 ^{h}	100	2.7±0.18 ^{f}	92.25
Co	0.5±0.04 ^{m}	138.72	6.0±0.55 ^{l}	143.0	4.92±0.5 ^{m}	123	4.93±0.32 ^{l}	167.75
Ni	0.38±0.033 ^{i}	104.85	4.14±0.36 ^{b}	97.4	3.8±0.3 ^{e}	94.7	2.73±0.16 ^{g}	93
EDTA	0.336±0.025 ^{d}	92.93	4.3±0.35 ^{d}	101.43	3.63±0.26 ^{d}	90.9	1.9±0.12 ^{a}	64.7
SDS	0.45±0.035 ^{k}	125.24	4.0±0.27 ^{a}	95.67	4.3±0.35 ^{k}	107.4	4.47±0.5 ^{k}	152

Production of cocktail enzyme under solid-state fermentation (SSF)

A. eucalypticola AUMC 15402 was cultivated on date palm leaves (DPL), rice straw (RS), and sugarcane bagasse (SB) under SSF. The strain demonstrated an aptitude to convert date palm leaves (DPL), rice straw (RS), and sugarcane bagasse (SB) under SSF into endoglucanase (18.0, 16.0, and 9.0 U/g), exoglucanase (20.0±1.64, 25.0±2.0, and 15.0±1.0), pectinase (34.0±2.84, 38.0±2.85, and 38.0±3.0 U/g), and xylanase (25.0±1.8, 28.0±2.0, and 17.0±1.1 U/g), respectively (Fig. 7).

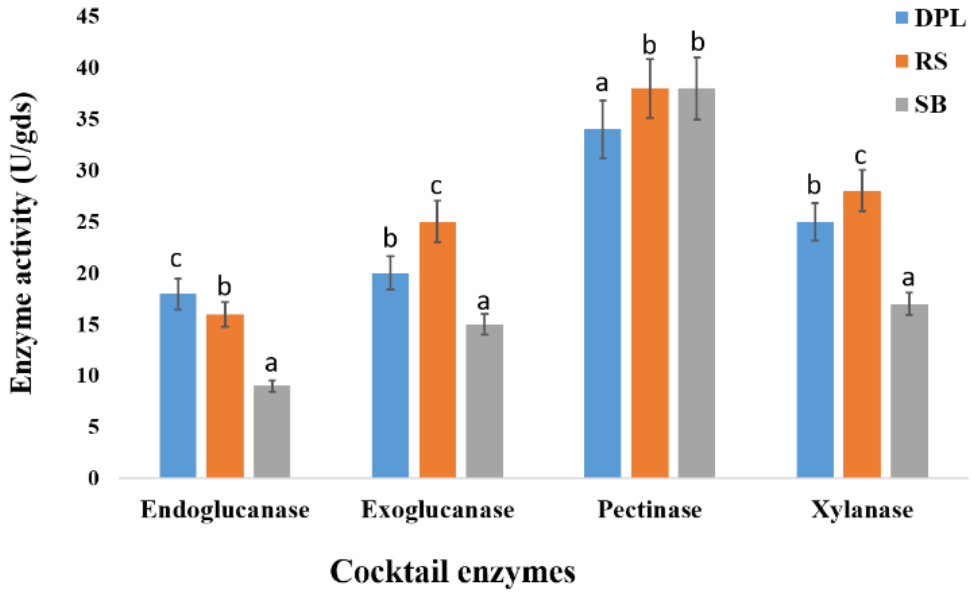

Fig 7. Endoglucanase, exoglucanase, pectinase, and xylanase production by *Aspergillus eucalypticola* AUMC 15402 utilizing DPL, RS, and SB under SSF (Mean values±SD on bar graphs with different letters are significantly different; $p < 0.05$; $n = 3$).

Discussion

Due to the enormous amounts of agricultural residues produced annually worldwide, environmentally appropriate biomass disposal methods must now be utilized. Using agricultural wastes as a substrate to produce industrially needed products including enzymes, polysaccharides, organic acids, fragrances, and taste compounds, is a profitable approach with significant economic benefits. Researching efficient strains of microbes that capable of breaking down cellulose directly, without the requirement for enzymatic or acidic breakdown, is crucial. These powerful strains make the process affordable and environmentally friendly. Improvements to the strain would boost its production of sugars and enzymes, increasing the viability and competitiveness of large-scale manufacturing.

This study used *A. eucalypticola* AUMC 15402 strain that was isolated from sugarcane bagasse to produce cocktail enzymes in SmF. *A. eucalypticola* AUMC 15402 could generate endoglucanase, exoglucanase, and xylanase. The optimum fermentation parameters for each enzyme was estimated. *A. eucalypticola* AUMC 15402 produced the highest specific activity of endoglucanase (22.5±2.1 U/mg), exoglucanase (38.6±4.0 U/mg), and xylanase (44.2±3.8 U/mg), at 30 ºC after 8, 9, and 6 days of incubation using sodium nitrate as nitrogen supply, respectively. Numerous investigations have been carried out to generate cocktail enzymes from different species of fungi. With respect to this be concerned, *A. nidulans* demonstrated the highest specific activity of endoglucanase (68.58 U/mg), FPase (12.0 U/mg), xylanase (27.17 U/mg), and β-glucosidase (1.89 U/mg) using solid-state fermentation (SSF) at 30 °C and pH 6.0 after 216 hours (Naitam et al. 2022). In a 3-day culture of *Penicillium chrysogenum* strain PCL 501, crude extracellular enzyme produced 0.67±0.03, 19.94±1.30, and 8.50±0.50 U/mg of endoglucanase, β-glucosidase, and xylanase activity, respectively (Chinedu et al. 2008).

Aspergillus niger ANL 301 in basal medium containing cellulose as sole carbon source, yielded crude extracellular endoglucanase activity of 0.54 ± 0.02 U/mg (Chinedu et al. 2011). *Aspergillus niger* and *Bacillus sp*. generated xylanase, which reached its maximum activity (3.67 U/mL and 3.36 U/mL) at 40 °C, respectively. *A. niger* and *Bacillus sp*. showed optimal xylanase activity (4.58 and 3.58 U/mL) at pH 5.0 and 6.2, respectively (Fasiku et al. 2022). *Fusarium oxysporum* showed endoglucanase and exoglucanase activity of 0.33 and 3.33 U/mg after 5 days of cultivation while *F. verticillioides* displayed 0.55 U/mg of exoglucanase activity. Maximum xylanase activity was achieved after 7 days of cultivation of *F. verticilliodes* (16 U/mg), while *F. oxysporum* showed maximum activity after 9 days that was 8.0 U/mg (Marđetko et al. 2021).

In the present study, activities of endoglucanase, exoglucanase, pectinase, and xylanase produced by *A. eucalypticola* AUMC 15402 in SmF were assessed at different pH values ranged from 3.0 to 11. The four enzymes showed their maximum specific activities of 0.27, 3.775, 4.0, and 2.9 U/mg, respectively at pH 8.0. Endoglucanase and exoglucanase displayed their highest activity of 0.362 and 4.25 U/mg, respectively, at 45 ºC, while pectinase and xylanase showed their activity maxima of 4.0 and 2.94 U/mg, respectively, at 50 ºC. The activity of xylanase produced by *A. fumigatus* KSA-2 activity was at its maximum at pH 6.0 and 45 ºC (Ameen 2023). For xylanases of the Aspergillus species, the pH range of optimum action is typically between pH 3.0 and 6.0 (Gupta et al. 2019). The optimal pH for xylanase generated by *A. oryzae* LC1 was found to be 5.0 (Bhardwaj et al. 2019), but *A. fumigatus* SK1 gave superior results at a pH of 4.0 (Ang et al. 2013).

Under SSF, *A. eucalypticola* AUMC 15402 was grown on date palm leaves (DPL), rice straw (RS), and sugarcane bagasse (SB). The strain showed the ability to convert agricultural biomass into pectinase, xylanase, endoglucanase, and exoglucanase at varying concentrations. Pectinase was the most productive enzyme, producing significant activity levels for DPL, RS, and SB of 34.0±2.84, 38.0±2.85, and 38.0±3.0 U/g, respectively. Xylanase demonstrated a maximal activity of 28.0±2.0 U/g from RS, outperforming pectinase. The activity peaks for DPL and SB were 25.0±1.8 and 17.0±1.1 U/g, respectively, following RS. Endoglucanase came in fourth place, whereas exoglucanase came in third. With 25.0±2.0 U/g, RS produced the most exoglucanase, followed by DPL and SB with 20.0±1.64 and 15.0±1.0 U/g, respectively. Endoglucanase, which had the highest amount (18.0 U/g), was the least amount of enzyme produced from DPL. It was followed by RS (16.0 U/g) and SB (9.0 U/g).

Because of small variations in methodology, it is generally challenging to compare the values of enzyme activity between different research. As a result, care should be taken while making comparisons. Regarding this concern, lignocellulolytic enzymes were produced from palm empty fruit bunches by *Aspergillus tubingensis* TSIP9 and *Trichoderma reesei* QM 9414. *A. tubingensis* TSIP9 generated enzymatic cocktails with the highest cellulase (89.6 ± 5.7 U/g) and xylanase (196.8 ± 3.6 U/g) activities, whereas *T. reesei* QM 9414 showed the highest β-glucosidase activity of 47.9 ± 0.9 U/g (Intasit et al. 2021). Activities of CMCase (126.87 U/g), FPase (85.53 U/g) and xylanase (215.42 U/g) achieved the maximum under optimized SSF conditions (pH 6.0 and 30 °C) by *Trichoderma asperellum* UC1, whereas The best β-glucosidase activity (131.76 U/g) produced by *Rhizopus oryzae* UC2 was obtained at pH 12 and 32 ºC (Ezeilo et al. 2022). The highest concentrations of amylase (30 U/g), cellulase (27 U/g), pectinase (21 U/g), xylanase (98 U/g), and protease (108,000 U/g) were detected at 30 °C after 96 hours in the mixed culture of A. niger and *R. oryzae* (Morilla et al. 2023). Significantly, at pH 6.4, *Neurospora sitophila* strain BDJ-1I converted agave bagasse to produce high levels of cellulase (39.3 U/g), pectinase (96.8 U/g), and xylanase (26.6 U/g) (Valle-Pérez et al. 2024). Fermentation was carried out with *Trichoderma koningii* using untreated and pretreated corn cob supplemented with pineapple peel powder showed higher production of xylanase (2,869.8 ± 0.4 U/g) at pH 6.5 and incubation period for 96 h (Bandikari et al. 2014). *Aspergillus fumigatus* produced exoglucanase having high enzymatic activity (83.0 U/g) during the solid-state fermentation of wheat straw under optimum conditions. Maximum production was obtained after 72 h of fermentation, at 55 °C temperature, pH 5.5 (Mahmood et al. 2013).

Date palm leaves, rice straw, and sugarcane bagasse are attractive sources for the profitable synthesis of important enzymes in Egypt. *Aspergillus eucalypticola* AUMC 15402, which was isolated from sugarcane bagasse, has shown outstanding ability to break down all residues used and produce high yields of valuable enzymes namely endoglucanase, exoglucanase, pectinase, and xylanase under SSF. It is intended to create industrial bioprocesses in the near future for the production of industrial products (Bhatia and Paliwal 2011). Despite the significant lignocellulosic biomass, to the best of our knowledge, only Al-Kolaibe et al. (2021) published an article on the synthesis of enzyme from lignocellulosic date palm leaves in Egypt. One of the most important fruit crops in the Egyptian Governorate of the New Valley is the date palm (Phoenix dactylifera L.). As a significant crop in agriculture, it has historically been associated with the preservation of human life and the ancestry of the

people who live in the New Valley. The primary constituents of date palm leaf (DPL) residues are cellulose (27–41%), hemicellulose (16–18%), and lignin (10–19%) (Bahman et al. 1997, Pascual et al. 2000, Arhab et al. 2009, Rad et al. 2015). DPL residues are a pruning waste. A naturally dried leaf, with leaflets and rachis, typically weighs between two and three kilograms (Rezende et al. 2011). Egypt produces over 18.0% of the dates produced worldwide and 23.0% of the dates produced in the Arab world (Rageh et al. 2020). The New Valley Government is home to around 2.5 million date palm trees. As a result, it is estimated that every date palm produces about 50 kg of leaf residue year (Rad et al. 2015). This indicates that date palm leaf wastes yield more than 125,000 tons annually, but regrettably, much of this waste is still completely unused, which could lead to disposal issues.

According to the present research, rice straw was an ideal substrate for *A. eucalypticola* AUMC 15402 to use in SSF, where it was able to create large amounts of enzymes. One of the most common lignocellulosic waste products in the world is rice straw. An estimated 731 million tons are generated each year, with 20 million tons produced in Africa, 667 million tons in Asia, 3,9 million tons in Europe, and 37.2 million tons in America (Karimi et al. 2006). With about 1,428,600 feddan rice cultivated, Egypt is the leading producer of rice in the Near East (Hammoud et al. 2020). Straw production is expected to be 2.4 tons/feddan (Sabaa and Sharaf 2000). The country's farm yield is estimated to be 6.0 tons total. Thus, rice straw residues are employed to produce 3,428,640 million tons of wasted lignocellulosic biomass annually. Large volumes of agricultural waste rich in cellulose, hemicellulose, and lignin are produced in Egypt as a result of the construction and rice farming industries. These materials are made available year-round for free. To get rid of so much post-harvest rice residue, the main method is to burn it outdoors in public on farms. Although field burning effectively eliminates pathogenic microbe spores and weed seeds, the black smoke it emanates poses a health risk to the public at large (Sherief et al. 2010, Ragab et al. 2014).

In the current investigation, *A. eucalypticola* AUMC 15402 fermented sugarcane bagasse and this organism was capable to producing substantial amounts of the enzymes endoglucanase, exoglucanase, pectinase, and xylanase in SSF. More than fifty million tons of dry bagasse are generated worldwide each year (Moubarik and Grimi 2015), with around 4.7 million tons being produced in Egypt (Mohamed et al. 2015). Therefore, the search for a suitable use for this waste is a field of study that contributes to ecosystem protection. As one of the lignocellulosic waste materials, sugarcane bagasse has drawn a lot of attention due to its potential applications as a bioadsorbent for wastewater treatment (Peñafiel et al. 2021, Tony 2021), a promising substrate for ethanol production, and a secure source for the production of enzymes using microorganisms (Cardona et al. 2010, Bhatia and Paliwal 2011, Faisal and Saeed 2021, Ntimbani et al. 2021).

The present study evaluated the effects of certain metal ions and some inhibitors on the activity of endoglucanase, exoglucanase, pectinase, and xylanase. The chemicals under investigation demonstrated a range of effects, spanning from inhibition to augmentation. The endoglucanase activity was boosted by Co^{2+}, Mn^{2+}, SDS, Ca^{2+}, and Ni^{2+}, but Na^{+} had the greatest inhibitory effect. All chemicals investigated showed an increase of exoglucanase activity, with the exception of Ni^{2+} and SDS. Pectinase activity had been improved by the addition of Co^{2+}, Mn^{2+}, SDS, and Fe^{2+} to the reaction. The activity of xylanase had been improved by Mn^{2+}, Co^{2+}, SDS, Fe^{2+}, and Ca^{2+} in that order. Regarding the ion effect on an enzyme activity, ions may interact (or chelate) with proteins to form complexes that may influence protein stability (Bauduin et al. 2004). It had previously been thought that an ion's ion selectivity was mostly caused by the ability it has to modify the structure of water, also known as a "physical" effect (Bauduin et al. 2004, Boström et al. 2004). Water ions with strong hydration that reinforce its structure are called kosmotropes, while water ions with weak hydration that break down its structure are called chaotropes, or "structure-breakers" (Krestov 1991, Zhao 2005). Divalent metal ions and EDTA at 2.0 mM concentrations affected the enzyme activity produced by P. chrysogemum PCL501. Mn^{2+} and Fe^{2+} had stimulatory effects on the enzyme whereas Mg^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+} and EDTA inhibited the enzyme activity. The effect of Ca^{2+} was not significant. Over 3-fold increase in the enzyme activity was recorded with Mn^{2+}. Inhibition of 65.9 and 79.7 % respectively was obtained with Hg^{2+} and EDTA (Chinedu et al. 2008). Similar results have been reported for xylanase produced by *Penicillium roquefortii* compared to a commercial xylanase. The presence of ionic species in the reaction medium was reported to inhibit enzymatic activity (Souza et al. 2018). On the other hand, xylanase produced by SSF on yellow mombin residue (*Spondias mombin* L.) showed 40 % higher enzymatic activity in the presence of Mn^{2+}, while the addition of Cu^{2+} led to 50 % reduction (de Almeida Antunes Ferraz et al. 2020). Enzymes can interact with ionic species present added to the reaction medium. Ions can act as co factors, either enhancing or inhibiting enzymatic activity as a result of the formation of non-active complexes with the enzyme (Ferraz et al. 2018).

Conclusions

Products with additional value that are produced from biomass sources which satisfy both increasing energy requirements and decreased energy supply include enzymes, fermentable sugars, and organic acids. The morphological diversity and crystallinity of lignocellulosic biomass are the two primary obstacles to bioconversion processes. Because agricultural resources are processed, the cost of manufacturing enzymes has decreased. With thousands of new employment expected to be created as a result, the 2030 vision gives the possibility to begin recycling such waste in the creation of industrial products with major financial benefits for the national economy. A promising fungal strain was isolated in this study from sugarcane bagasse, and it has been shown to have a high potential for producing endoglucanase, exoglucanase, and xylanase, during submerged fermentation (SmF). By employing the internal transcribed spacer region (ITS) sequencing method, the fungal strain was identified as *A. eucalypticola*. One factor at a time (OFAT) approach was used to optimize the pH, nitrogen supply, incubation temperature, and the fermentation time, for the three enzymes. Under solid-state conditions (SSF), the strain produced valuable endoglucanase, exoglucanase, pectinase, and xylanase with high enzyme activity from date palm leaves, rice straw, and sugarcane bagasse.

Competing interests

The authors declare no competing interests.

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