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Characterization and valuable applications of xylanase from endophytic fungus *Aspergillus terreus* KP900973 isolated from *Corchorus olitorius*

Samia A. Ahmed [a,*], Shireen A.A. Saleh [a], Faten A. Mostafa [a], Abeer A. Abd El Aty [a], Hala A.M. Ammar [b]

[a] Department of Chemistry of Natural and Microbial Products, National Research Centre, Dokki, Cairo, Egypt
[b] Department of Botany, faculty of Science, Zagazig University, Cairo, Egypt

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ABSTRACT

Isolated fungal strains from molokhia stalks (*Corchorus olitorius*) were screened for the production of xylanase enzyme. *Aspergillus terreus* KP900973 was selected as potential xylanase producer and was identified on the basis of 18S rDNA gene homology. Screening was done by Congo red test for xylanase, based on the light coloured zone around the sample in xylan agar plates. Optimization of xylanase production was performed using low cost substrates through submerged fermentation (SMF). Using a mixture of untreated molokhia stalks and pea peel with no nutrients added enhanced enzyme production by 100.73%. Optimal assay temperature and pH were 50 °C and 5.5, respectively. At pH 4.0 the activity decreased to 91.23%, moreover, at pH 9.0 the enzyme had 67.25% of its activity. The enzyme retained about 97% of its activity after being stored for 46 days at −18 °C. *A. terreus* is free from tested mycotoxins and considered as a non-toxigenic strain, so, some industrial applications of crude xylanase were tested. Wheat bran was found to be the most susceptible substrate for hydrolysis with the highest level of fermentable sugars and saccharification yield (16%) after 48 h. The greatest clarification degree of orange juice (64%) was observed after 72 h using crude enzyme preparation. Xylanase can even substitute the addition of emulsifiers and additives used in bread production enhancing dough-raising as compared to the control about 2-fold.

1. Introduction

Xylanses (EC. 3.2.1.8) are responsible for xylan hydrolysis, which is the main polysaccharide component of hemicelluloses in agro-residues (Farinas et al., 2011). Xylan's basic molecular structure is a linear backbone that is comprised of β-1, 4-D-xylopyranose residues (Sorgatto et al., 2012). There is a great interest in xylanase due to its applications in bioconversion of lignocellulosic materials to fermentable sugars, textile, animal feed, pulping and bleaching processes (Arabi et al., 2011; Ghoshal et al., 2015; Goluguri et al., 2016). They can be used for improvement of bread-quality, clarification of juices and wines, silage production, etc. (Bajaj and Manhas, 2012; Kaltsa et al., 2013). Xylanases may also be applicable to the production of cellophane, rayon and several chemicals such as cellulose ethers and esters (Subramaniyan and Prema, 2002). Researchers have focused on the isolation of new microbial strains that produce a large amount of xylanase enzymes (Herández- Domínguez et al., 2014). Xylanases have been isolated from a wide range of microorganisms including fungi, actinomycetes and bacteria (Patel and Prajapati, 2014). Fungi are important producers of xylanase and *Aspergillus* and *Trichoderma* species are the most commonly used ones (Herández- Domínguez et al., 2014). Mycotoxins are natural poisons produced as secondary metabolites by growing. For food applications, It is necessary to test for mycotoxin production in isolated strains. Most studies on the production of xylanases have been investigated in submerged fermentation (SMF) or on a solid state fermentation (SSF) using agricultural residues (Kaur et al., 2011). About 30–40% of the production cost of many industrial enzymes is associated with the cost of growth substrate (Khandeparkar and Bhosle, 2006). Therefore, it is necessary to explore cheap substrates for cost- effective enzyme production using solid agro-residues. Large quantities of agro-residues tend to dominate and pollute the environment (Ajani et al., 2011). Using agro-residues in the production of enzymes help to solve modern disposal problems and reduce

* Corresponding author.
E-mail address: dr_Sa_Ahmed@yahoo.com (S.A. Ahmed).

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pollution of the environment and providing a convenient and renewable source of energy in the form of bioethanol (Irfan et al., 2014). Generally, agro-residues have different physical properties and chemical composition that could hinder the accessibility of polysaccharides for hydrolysis (Ajani et al., 2011). Therefore, agro-residues may be pretreated to improve the enzymatic hydrolysis and help fungi have easier access to biomass (Kim et al., 2014). Molokhia plant (*Corchorus olitorius*) is an annual herb and their leaves have been a staple in Egyptian food since the time of the Pharaohs. Molokhia stalks (Ms) are one of the agro-residues rich in mineral salts, proteins, vitamins, and fiber. However, there is no literature available on the utilization of Ms for xylanase production by microorganisms. In the present investigation, non-toxic isolated fungi *Aspergillus terreus*, utilized Ms as a new substrate in the production of xylanase enzyme without rich media. The enzyme was extracted, characterized and examined for some industrial applications such as saccharification, juice clarification and bakery processes.

2. Materials and methods

2.1. Microorganisms

2.1.1. Isolation of endophytic fungi from molokhia stalks

By the method described by Selim et al. (2011), the plant material (Ms) was washed in running tap water, cut randomly into small pieces (5 mm long). The pieces were surface sterilized by sequentially dipping into 0.5% sodium hypochlorite (2 min) and 70% ethanol (2 min), then rinsed with sterile water and allowed to dry by blotting between two fold of sterilized filter paper under sterile conditions. The plant pieces were placed on potato-dextrose-agar (PDA) medium. Media was supplemented with chloramphenicol (50 mg/l) to suppress bacterial growth and incubated at 30 °C. They were checked every day for 2 weeks and individual fungal colonies were transferred to fresh PDA plates for purification and identification purposes. All fungal isolates were maintained at 4 °C on PDA slants and deposited at Chemistry of Natural and Microbial Product Department, National Research Center, Egypt.

2.1.2. Identification of endophytic fungi

Taxonomic identification of the most potent endophytic isolate for xylanase production was performed on the basis of morphological characteristics of fungal culture, colony and hyphae, comparing with fungal species described in identification keys (Botton et al., 1985; Pitt and Hocking, 1985). Genetic identification using molecular taxonomy was performed to validate and confirm the morphological identification and to study the phylogeny. Molecular identification of the selected fungal isolate was performed based on its internal transcribed spacer ribosomal DNA (ITS-rDNA) sequences. Mycelia were collected by centrifugation (at 4 °C and 5000 rpm for 20 min) and DNA was extracted by using protocol of Gene Jet Plant genomic DNA purification Kit (Thermo # K0791). PCR was performed by using Maxima Hot Start PCR Master Mix (Thermo# K0221), and PCR clean up to the PCR product made by using Gene JET™ PCR Purification Kit (Thermo# K0701) in Sigma Company of Scientific Services, Egypt (www.sigma-co-eg.com). Finally, sequencing of the PCR product was performed at GATC Company (German) by using ABI 3730xl DNA sequencer, using forward and reverse primers, and by combining the traditional Sanger technology with the new 454 technology. Purified DNA was subjected to PCR amplification using a pair of ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primers for ITS-rDNA amplification (White et al., 1990). Sequence data was analyzed in the Gene Bank database by using the BLAST program available on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The unknown sequence was compared to all of the sequences in the database to assess the DNA similarities (Altschul et al., 1997). The nucleotide sequence of the *endo*phytic fungus was submitted to the Gen Bank and assigned accession number KP900973. Multiple sequence alignment and molecular phylogeny were performed using Bio Edit software. The phylogenetic tree was displayed using the TREEVIEW program (Page, 1996).

2.2. Raw material

Agro-residues (molokhia stalks, corn cobs, wheat bran, saw dust, sugar cane, lemon skin and pea peel) were collected from local market in Egypt. They were washed with distilled water to remove unwanted dust particles and then dried in an oven (50 °C for 24 h). The dried materials were ground in an electric grinder, separated by 1 cm sieve and packed in air-tight containers for use as the substrate in SMF.

2.3. Pretreatment of molokhia stalks

2.3.1. Wet oxidation

Raw Ms were slurred in distilled water with solid-liquid ratio 1:20 (w/v) in Erlenmeyer flask (250 ml), and cooked for 1 h at 100 °C. After cooling, the residues were washed with distilled water, followed by centrifugation at 4 °C and 5000 rpm, for 20 min. The residues were dried in an oven over night at 50 °C.

2.3.2. Liquid hot water (LHW)

Molokhia stalks (5 g) were mixed with distilled water (100 ml), then autoclaved at 121 °C, 15 psi for 1 h (Arumugam and Manikandan, 2011). Samples were cooled to room temperature, centrifuged at 4 °C and 5000 rpm for 20 min, and then dried in an oven at 50 °C for 24 h.

2.3.3. Acid and alkali

Molokhia stalks were assessed using 1% (v/v) of H_2SO_4 or NaOH with a solid-liquid ratio1:20 (w/v) at 30 °C and shaking at 150 rpm for 1 h (Begum and Alimon, 2011). Samples were collected by filtration through a nylon cloth, followed by washing with running tap water until neutral pH. Then the residues were collected, placed in an oven at 50 °C for 24 h to be dried.

2.3.4. Acid and alkali with autoclaving

Five grams of MS in 100 ml of 1% H_2SO_4 or NaOH was autoclaved at 121 °C, 15 psi for 1 h. After cooling, treated materials were collected by filtration through a nylon cloth, followed by washing with running tap water to neutralize the pH. Then the residues were collected, and dried in an oven at 50 °C for 24 h.

2.4. Chemical analysis

The moisture content was determined by heating the sample to a constant weight at 105 °C for 3 h. Ash content was determined by burning the dried samples at 800 °C until constant weight and get fully white ash. Total carbohydrate was estimated by the phenol-sulfuric acid method with glucose as a standard (Dubois et al., 1956).

2.5. Selection of potent xylanase producing fungal strain

Fungal strains isolated from Ms were inoculated onto an agar plate for xylanase activity using birch wood xylan (0.2%) as the carbon source. The plates were incubated at 30 °C for 7 days. Congo red (0.2%) was added for 30 min at room temperature,