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Isolation and Identification of Two New Fungal Strains for Xylanase Production

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Abstract Fungi are well known for their ability to excrete enzymes into the environment. The aim of this work was to evaluate xylanase production by fungi isolated from soil. One hundred and thirty-six fungal isolates were screened for xylanase production. Two xylanase producing isolates, FSS117 and FSS129, were identified on the basis of analyses of 5,8S gene sequencing. The closest phylogenetic neighbors according to 5,8S gene sequence data for the two isolates were *Aspergillus tubingensis* and *Aspergillus terreus*, respectively. When birchwood xylan or corn cob hulls was used as a substrate for 5 days under submerged culture cultivation, xylanase production from *A. terreus* FSS129 was 113 and 174 IU ml^{-1}, respectively. The pH and temperature for optimum xylanase activity were 8 and 65ºC.

Keywords *Aspergillus terreus* · *A. tubingensis* · Xylanase · Submerged culture

Introduction

Xylans, the major structural heteropolysaccharides in plants, are β-1,4-linked polymers of xylopyranosyl units with a degree of polymerization ranging from 70 to 200. Depending on their origin, xylans may also contain variable amounts of arabinosyl- and 4-*O*-methylglucuronic acid residues and acetyl groups [1, 2]. Xylanolytic enzymes are a group of enzymes that hydrolyze xylan and arabinoxylan polymers. This enzyme group includes endo-β-1,4-xylanase, β-xylosidase, arabinofuranosidase, and acetylxylan esterase [3]. Xylanolytic enzymes are produced by a wide variety of microorganisms, among which the filamentous fungi are especially interesting as they secrete these enzymes into the medium, and their xylanase activities are much higher than those found in yeast and

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bacteria [4–6]. Endo-β-1,4-xylanase plays important roles in the animal feed and increases the body weight gains of the animals [7]. In pulp and paper industry, the xylanases are employed in the prebleaching process to reduce the use of the toxic chlorine chemicals [8]. In bread and bakery industry, xylanases are used to increase the dough viscosity, bread volume, and shelf life [9]. Other potential applications include the conversion of xylan in wastes from agriculture and food industries into xylose and the production of fuel and chemical feed stocks [10].

Due to their diversity, fungi have been recognized as a target for screening and as a source of new enzymes with useful and/or novel characteristics [11].

PCR amplification using universal primers targeted to conserved regions within the rRNA complex, followed by DNA sequencing of the internal transcribed spacer (ITS) regions, shows promise to identify a broad range of fungi to the species level [12–16].

Considering the industrial importance of xylanase, in this present study, fungi were isolated from soil and screened for xylanase production. The investigation led to the identification of two high producing xylanase isolates, FSS117 and FSS129.

Materials and Methods

Fungus Isolation

Soil samples were collected from different areas of Syria, cereal fields, olive fields, forests, and gardens. One gram of soil sample was dissolved in 100 ml of sterilized distilled water and then diluted up to $1/10^4$ time. From which, 0.1-ml volumes were pipetted onto potato dextrose agar (PDA) and incubated at 30ºC for 3 days. Fungi were isolated from each plate and subcultured on PDA. Subculturing was continued until a pure isolate was obtained. Stock cultures were maintained on potato dextrose agar at 4ºC.

Fungus Screening for Xylanase Production

One hundred and thirty-six fungal isolates were screened for xylanase production in Erlenmeyer flasks (100 ml) containing 25 ml of basal culture medium (grams per liter); 10.0, wheat bran; 5.0, yeast extract; 10.0, $Na_2HPO_4{\cdot}2H_2O$; 0.5, KCl, 0.15; $MgSO_4{\cdot}7H_2O$. The pH was adjusted to 7 before sterilization. The flasks were sterilized at 120ºC for 20 min. Fresh fungal spores have been used as inocula, and the flasks were incubated at 30ºC for 5 days in a rotary shaker (150 rpm). The supernatant filtrate was used as enzymes source.

Biomass

Mycelial dry weight was determined by filtering the culture medium through preweighed Whatman filter paper no. 44, dried to a constant weigh at 80ºC, and reweighed. The difference in weight denoted the mycelial growth of fungus.

Soluble Proteins

Soluble proteins were analyzed according to the method of Lowry et al. [17] after preliminary precipitation with 50% trichloroacetic acid (TCA). Bovine serum albumin (BSA) was used as standard.

Enzymatic Assays

Xylanase activity was determined as described by Bailey et al. [18] using 1% birchwood xylan as substrate. The solution of xylan and the enzyme at an appropriate dilution were incubated at 55°C for 5 min, and the reducing sugars were determined by the dinitrosalicylic acid (DNS) procedure with xylose as the standard [19]. The released xylose was measured spectrophotometrically at 540 nm. One unit of enzyme activity is defined as the amount of enzyme releasing 1 μmol xylose per milliliter per minute under the described assay conditions. *Carboxymethylcellulase activity* was assayed similar to xylanase activity, using 1% solution of carboxymethylcellulose (sodium salt, ultra-low viscosity). Reducing sugars were assayed as glucose, using DNS method. Filter paper activity was assayed according to the method recommended by Ghose [20]. One international unit of filter paper activity is the amount of enzyme which forms 1 μmol glucose (reducing sugar as glucose) per minute during the hydrolysis reaction. Results given are the mean of triplicate experiments.

Identification of the Selected Isolates

Genomic DNA Isolation Total genomic DNA of selected isolates was extracted from 48 h—growing cultures in medium (2% glucose, 1% yeast extract, and 1% peptone). Cultured cells (1.5 ml) was collected by centrifugation at 17,950×*g* for 5 min. Cells were washed with distilled water and digested by 750 μl enzymatic lyses solution (10 μl of proteinase K 20 mg/ml, 2% SDS, 1% 2-mercaptoethanol, 1% CTAB, and 10 mM EDTA in 50 mM Tris pH8 buffer) and incubated for 30 min at 60°C. The lysate was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) three times. The extract was purified by adding 1/10 of the volume of sodium acetate 3 M and 1 ml ethanol. The mix was vortexed and placed for 15 min on ice. Sediment high molecular weight DNA was obtained after 5 min of centrifugation at 17,950×*g*, was washed with 70% ethanol, and air-dried. The final DNA pellet was dissolved in 50-μl hydration solution and stored at −20°C. DNA concentration was estimated by measuring the absorbance at 260 nm. The quality of the isolated genomic DNA was calculated by the ratio OD_{260nm}/OD_{280nm}.

PCR Amplification of the 5,8S rDNA PCR was used to amplify 5,8S rDNA gene of two xylanase-producing isolates. Primers used for PCR and DNA sequencing are ITS1 (5′-TCC GTA GGT GAA CCTGCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TATGC-3′). The standardized PCR conditions were as follows: 1 cycle of denaturation at 95ºC for 5 min followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 2 min, and 1 cycle of extension at 72°C for 10 min. PCR products were visualized by electrophoresis in 1% (*w*/*v*) agarose gel stained by ethidium bromide.

5,8S DNA Sequencing PCR amplicons were purified using Microcon Y-100 filters (Millipore) and sequenced using ABI Prism® Big Dye® terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, California) according to manufacturer's instructions. The sequencing products were purified by ethanol precipitation. Sequencing reactions were carried out on an ABI PRISMA™ DNA Sequencer (Perkin-Elmer, Gembloux, Belgium). The sequences obtained (length, approximately 500 bp) were then assembled in silico (Vector NTI) using overlapping zones to form contiguous sequence.

Phylogenetic Analysis Phylogenetic analysis was realized by an alignment of consensus sequences of 5,8S genes collected in an international database (Genebank). The resultants were then expressed in percentage of homology between the submitted sequence and the most relevant sequences from the database.

Results and Discussion

Screening of Xylanase Producing Fungus One hundred and thirty-six fungal isolates from soil samples collected from different areas of Syria, cereal fields, olive fields, forests, and gardens were evaluated for xylanase production. Of 136 isolates, nine isolates were able to produce >50 IU/ml in basal medium containing 1% wheat bran (Table 1). From this group, two isolates FSS117 and FSS129 exhibited >100 IU/ml xylanase production and were selected for further studies. These isolates did not show detectable levels of filter paper cellulose activity and also carboxymethyl cellulase activity.

PCR Amplification of the 5,8S rDNA Amplification of the 5,8S rDNA gene of two xylanase producing isolates by 5,8S rDNA gene primers (ITS1 and ITS4). Results of DNA electrophoresis in 1% agarose gel are demonstrated in Fig. 1a.

Identification of the Selected Isolates: A Phylogenetic Study Based on the Analysis 5,8S DNA Gene Sequence The phylogenetic analysis is based on the sequence gene 5,8S DNA and was carried out by alignment of sequences consensus of genes 5,8S DNA collected in the Gene Bank database. An algorithm making it possible to calculate the percentage of homology of sequence carried out this alignment per pairs of nucleotide. FSS129 were closely related to *Aspergillus terreus* with 100% of homology. For FSS117, the test sequence compared to the reference sequences in Genbank demonstrated that the majority was closely related to *Aspergillus tubingensis* with 100% of homology and some reference sequences indicated to relate to *Aspergillus niger*. To confirm FSS117 identification, DNA was extracted, and two primers pairs, one specific to *A. tubingensis* and the other to *A. niger*, were used in PCR reactions. The electrophoresis results in Fig. 1b demonstrated that FSS117 is related to *A. tubingensis* since amplicons were only seen with *A. tubingensis*-specific primers but not with *A. niger* primers.

Table 1 Extracellular enzymes production from the nine selected strains of filamentous fungus.

Isolate no.	Dry wt. (mg/ml)	Protein (μg/ml)	Xylanase (IU/ml)	Fpase (IU/ml)	CMCase (IU/ml)
FSS7	4.65 (±0.69)	393 (±25.38)	58 (±8.12)	0.21 (±0.01)	0.32 (±0.03)
FSS10	6.39 (±0.92)	455 (±39.24)	52 (±7.79)	0.19 (±0.01)	2.15 (±0.19)
FSS17	7.54 (±0.1.28)	438 (±36.16)	79 (±10.48)	0.23 (±0.01)	1.06 (±0.11)
FSS45	5.19 (±0.77)	1003 (±82.61)	71 (±10.23)	0.26 (±0.02)	2.58 (±0.21)
FSS79	17.18 (±2.83)	789 (±43.93)	66 (±9.24)	0.15 (±0.01)	0.34 (±0.03)
FSS114	4.84 (±0.73)	509 (±25.43)	52 (±7.28)	0.26 (±0.02)	3.04 (±0.25)
FSS117	5.78 (±0.87)	561 (±53.31)	104 (±13.22)	0.25 (±0.02)	1.25 (±0.15)
FSS129	9.06 (±1.08)	534 (±51.49)	119 (±16.29)	0.19 (±0.01)	1.08 (±0.12)
FSS133	6.71 (±0.94)	483 (±33.72)	88 (±12.51)	0.23 (±0.01)	1.52 (±0.17)

Values represent means and standard deviations (in parentheses) of three experiments

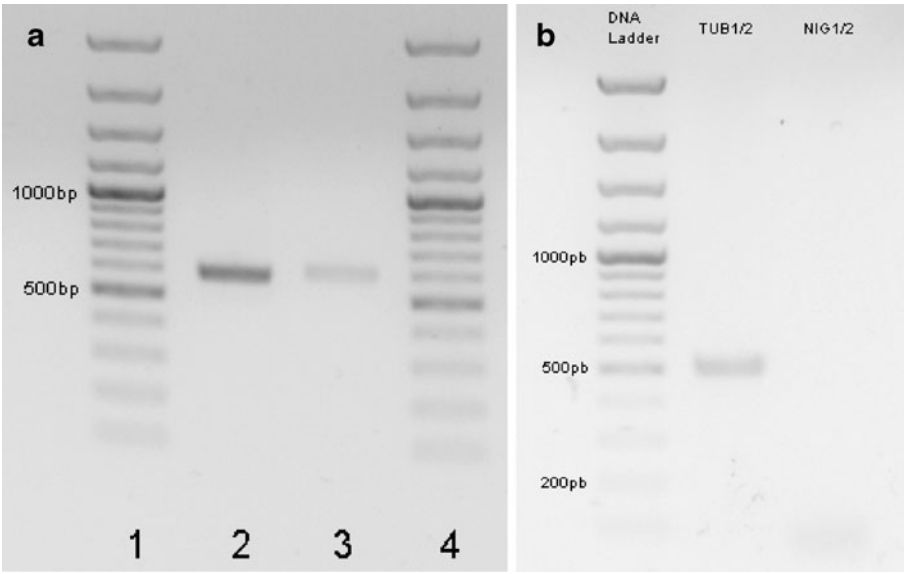

Fig. 1 **a** PCR of 5,8S rDNA fragments of two isolates; *lane 2* FSS129 and *lane 3* FSS117 using ITS1 and ITS4 primers. *Lanes 1* and *4* 100-bp DNA marker (O'GeneRuler 100pb plus DNA Ladder, Fermentas, Allemagne). **b** PCR of 5,8S rDNA fragments of FSS117 isolate using primers specific *for A. tubingensis* and *A. niger*

Influence of Initial pH on Xylanase Production from *A. terreus* FSS129

Xylanase production by this isolate was observed in the range 3.0–10.0 pH (Fig. 2). The organism showed a greater xylanase production at a neutral and slightly alkaline pH (pH8) than at an acidic pH and high alkaline pH. High xylanase production was (147.63 IU/ml) at pH8, and minimum was observed at pH3 (31.49). At pH7 (119 IU/ml), the production of xylanase was slightly decreased. In the case of fungi, the majority of researchers have reported an acidic pH to be the most appropriate for maximum enzyme production [21, 22]. However, Senthilkumar et al. [23] obtained maximum xylanase production by *Aspergillus fischeri* Fxn 1 in pH9 medium, while Sudan and Bajaj [24] found that *Aspergillus niveus* RS2 showed a better xylanase production at pH8.

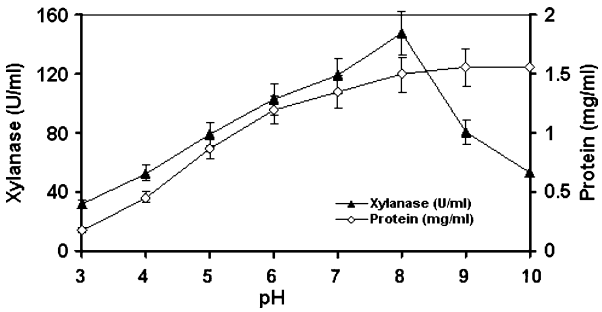

Fig. 2 Influence of pH of the medium on xylanase production by *A. terreus* FSS129 at 30ºC under submerged fermentation

Effect of Carbon Source on Xylanase Production from *A. terreus* FSS129

To select a suitable carbon source for xylanase production, *A. terreus* FSS129 was cultivated in a basal medium containing various carbon sources (1%). We observed that maximum enzyme activity (174 U/ml) was obtained by using corn cob hulls (Table 2). Table 2 shows a very low xylanase activity detected in the medium containing lactose, maltose, sucrose, glucose, starch, or xylose as carbon source. This indicated that the choice of an appropriate substrate is of great importance for the successful xylanase production. The substrate not only serves as a carbon and energy source, but also provides the necessary inducing compounds for the organism. Since the cost of the substrate plays a crucial role in the economics of xylanase production process, the expensive substrate (pure xylan) is not suited for larger-scale production processes due to its high cost. Insoluble lignocellulosic materials offer cost-effective substrate for xylanase production [16, 25]. Corn cob hulls proved to be the best carbon source followed by wheat straw. The volumetric productivity, which is an important parameter to assess the effectiveness of a process, was calculated from the xylanase activities reported for these processes in the literature (Table 3). Table 3 shows that this isolate have important level of xylanase production. The yields of xylanase productivity from FSS129 observed in this work were approximately twofold higher than optimum productivities reported in the literature for other fungi.

Effect of pH on Xylanase Activity

Enzyme activity is markedly affected by pH. This is because substrate binding and catalysis are often dependent on charge distribution on both, substrate and, in particular, enzyme molecules [22]. A pH range from 3 to 10 was used to study the effect of pH on xylanase activity, and the results are given in Fig. 3. The favorable pH range for xylanase activity of *A. terreus* FSS129 was 5.0–7.0, with optimum pH at 6.0. A significant drop in enzyme activity was observed below pH5.0 and above pH7.0. A sharp decrease of xylanase activity was observed between pH7.0 (93.28%) and pH8.0 (34.33%). At acidic pH, (23.13%) and (42.54%) activity was retained at pH3.0 and 4.0, respectively. The enzyme behavior clearly indicates that it is more suitable for any application in the pH range of 5.0–7.0. Similar optimum pH was reported for filamentous fungus [16].

Table 2 Effect of different carbon sources on xylanase activity by *Aspergillus terreus* FSS 129.

Carbon sources (1%)	Protein (mg/ml)	Xylanase (IU/ml)
Birch wood xylan	286 (±34.32)	113 (±13.43)
Oat xylan	181 (±21.12)	77 (±9.24)
Beech wood xylan	213 (±24.56)	73 (±8.03)
Wheat bran	480 (±52.8)	108 (±10.88)
Wheat straw	438 (±48.18)	111(±11.21)
Corn cob hulls	605 (±66.55)	174 (±21.88)
Lactose	28 (±3.61)	0.12 (±0.01)
Maltose	75 (±9.1)	0.19 (±0.02)
Sucrose	40 (±4.8)	1.38 (±0.134)
Glucose	97 (±11.64)	0.47 (±0.05)
Starch	17 (±2.21)	1.59 (±0.17)
Xylose	287 (±34.44)	1.82 (±0.2)

Values represent means and standard deviations (in parentheses) of three experiments

Table 3 Optimum xylanase production in submerged culture by filamentous fungus.

Microorganism	Substrate	Cultural condition	Enzyme activity (IU/ml)	Productivity (IU/l/h)	References
Aspergillus terreus FSS129	Wheat bran 10 g/l	Flasks 5 days	119	991.67	This work
	Corn cob hulls 10 g/l	–	174	1450	
Aspergillus nidulans	Xylan 10 g/l	Flasks 2 days	14.7	306.25	[21]
	Wheat bran 10 g/l	Flasks 5 days	73	608.33	[26]
Aspergillus fisheri FXn1	Wheat bran 5 g/l	Flasks 7 days	45	267.85	[27]
Aspergillus sp.	Xylan 10 g/l	Flasks 3 days	10.6	147	[28]
Aspergillus niger	Xylan 10 g/l	Flasks 4 days	37.7	392.7	[29]
Aspergillus awamori	Sugar can bagasse 20 g/l	Flasks 5 days	30	250	[30]
	Xylan 10 g/l	Flasks 4 days	49.5	515	
Gliocladium viride					[31]
	Wheat bran 30 g/l	Flasks 4 days	32	333	
Penicillium kloecheri	Xylan 10 g/l	Flasks 5 days	12.2	102	[32]
	Xylan 10 g/l	Flasks 3 days	13.3	184.7	
Penicillium oxalicum ZH-30	Wheat bran 30 g/l	Flasks 3 days	5.3	73.6	[16]
Trichoderma harzianum	Maltose 10 g/l	Flasks 10 days	50.6	210.83	[33]
Trichoderma harzianum	Xylan 10 g/l	Flasks 5 days	1.21	10.08	[34]

Effect of Temperature on Xylanase Activity and Stability

The effect of temperature on the xylanase activity from *A. terreus* FSS129 is shown in Fig. 4. The optimum temperature was 65ºC. When the temperature reached 80ºC, relative xylanase activity was only about 22%.

Thermal stability tests were carried out by preincubating xylanase up to 3 h in the range 30–65ºC (Fig. 5). There was no significant decrease in xylanase activity during 3-h incubation

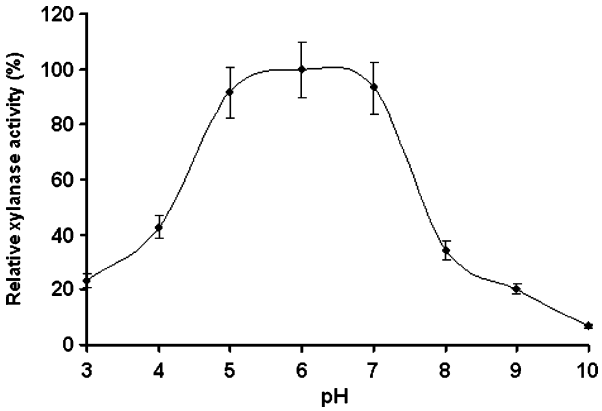

Fig. 3 Optimum pH activity of xylanase produced by *A. terreus* FSS129 grown on corn cob hulls under submerged culture. Relative activity was determined at 55ºC

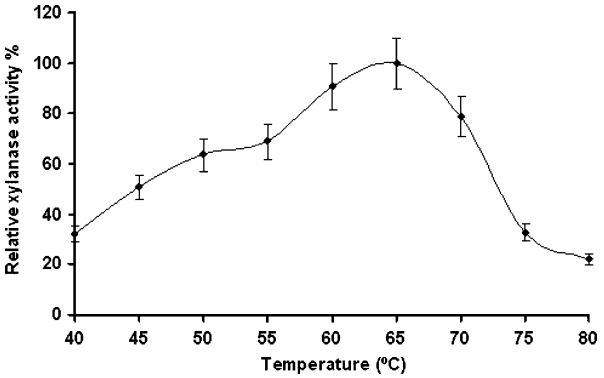

Fig. 4 Optimal temperature of xylanase produced by *A. terreus* FSS129 in submerged culture. Relative activity was determined at pH6

at 30–45ºC, while at 50ºC, the residual xylanase activity was 85.9%, after 1 h and 65.4% after 3 h. The enzyme was sensitive at 55ºC, retaining 25.4% activity after 1 h exposure and only 10.5% activity after 3 h. At 65ºC, the residual xylanase activity was only 6.8% after 1 h, and 4.2% activity was observed after 3 h. These results indicated that the suitable temperature range for industrial application for xylanase from *A. terreus* FSS129 was 30–50ºC.

Conclusion

In conclusion, homology analysis of the 5,8S gene provides suitable phonetic data that can be used to determine both close and very distant relationships. In the present study, this approach has allowed the identification of industrially important xylanase-producing organisms, FSS117 and FSS129. The closest phylogenetic neighbors according to the 5,8S gene sequence data for the two isolates were *A. tubingensis* and *A. terreus*, respectively. The results indicated that xylanase production from *A. terreus* FSS129 was highest (174 Uml^{-1}) when 1% corn cob hulls was added as carbon source. The yields of xylanase productivity from FSS129 observed in this work were approximately twofold higher than optimum productivities reported in the literature for some fungi.

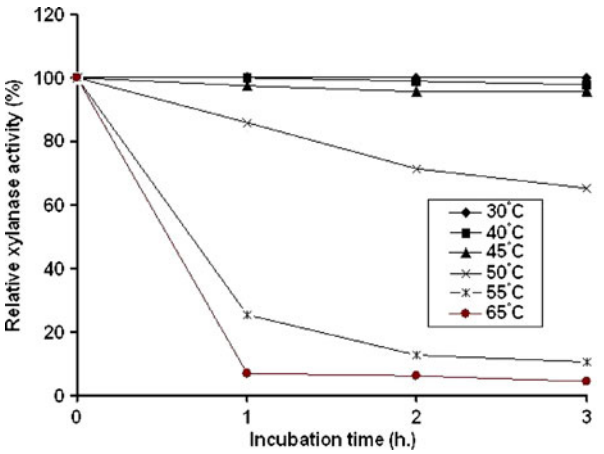

Fig. 5 Thermostability of xylanase from *A. terreus* FSS129

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