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Screening, isolation, and characterization of glycosyl-hydrolase-producing fungi from desert halophyte plants

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Summary. Fungal strains naturally occurring on the wood and leaves of the salt-excreting desert tree *Tamarix* were isolated and characterized for their ability to produce cellulose- and starch-degrading enzymes. Of the 100 isolates, six fungal species were identified by ITS1 sequence analysis. No significant differences were observed among taxa isolated from wood samples of different *Tamarix* species, while highly salt-tolerant forms related to the genus *Scopulariopsis* (an anamorphic ascomycete) occurred only on the phylloplane of *T. aphylla*. All strains had cellulase and amylase activities, but the production of these enzymes was highest in strain D, a *Schizophyllum-commune*-related form. This strain, when grown on pretreated *Tamarix* biomass, produced an enzymatic complex containing levels of filter paperase (414 ± 16 IU/ml) that were higher than those of other *S. commune* strains. The enzyme complex was used to hydrolyze different lignocellulosic substrates, resulting in a saccharification rate of pretreated milk thistle (73.5 ± 1.2 %) that was only 10 % lower than that obtained with commercial cellulases. Our results support the use of *Tamarix* biomass as a useful source of cellulolytic and amylolytic fungi and as a good feedstock for the economical production of commercially relevant cellulases and amylases. [**Int Microbiol** 2014; 17(1):41-48]

Keywords: *Schizophyllum commune* · *Tamarix* ssp. · cellulase activity · amylase activity

Introduction

Over the last few decades, shortages of fossil fuels and the increasing demand for renewable energy sources have led to a strong interest in biomass-based products such as biofuels, chemicals, and biomaterials [4]. For example, there is strong market demand for cellulases, given that the production costs for this class of enzymes have a major impact on ethanol production (40–49 % of net production costs) [Technical report NREL/TP-580-28893, National Renewable Energy Laboratory, Golden, CO, USA: http://www.nrel.gov/docs/fy01osti/28893.pdf] and pose a bottleneck for the commercialization of cellulosic ethanol [11,20]. One of the limiting factors in the development of an economical process for cellulase production is the paucity of inexpensive substrates [12,17]. At present, commercial cellulases are mainly produced, as a multi-enzyme complex, by both *Aspergillus niger* and mutant strains of *Trichoderma* including *T. reesei*, *T. viride*, and *T. longibrachium* [25]. The hydrolytic efficiency of these enzymes in the saccharification of lignocellulosic material is strongly dependent on the relative ratios of the enzymatic components, endo-β-1,4-glucanase (EG, EC 3.2.1.4), cellobiohydrolase

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(CBH, EC 3.2.1.91) and β-glucosidase (BG, EC 3.2.1.21), and the synergism among them [8,15].

Commercially available cellulase preparations from *T. reesei* (i.e., Celluclast 1.5L from Novozymes) are generally characterized by their very low β-glucosidase activity, such that their use results in incomplete saccharification of the substrate due to the accumulation of cellobiose, an inhibitor of both CBH and EG [18]. Hence, the use of commercial enzyme mixtures for specific substrate hydrolysis (e.g., different types of biomass) is conditioned and limited by the difficulty in varying the ratio of each specific hydrolase in order to obtain an optimized biomass-degrading enzyme system. An important advantage of optimizing the cellulase enzyme system for specific types of pretreated biomass is a significant reduction in enzyme loading without sacrificing hydrolysis yield [2].

Among feedstocks for biofuel production, higher plant species able to grow on marginal soils in arid or semiarid environments are of particular interest [5,30,35]. *Tamarix* (Tamaricaceae) species, which include small trees or shrubs, are desert halophytes adapted to grow on non-arable soils and highly tolerant of abiotic stresses, including salinity, drought, and defoliation [5,13]. These plants have several salinity tolerance mechanisms, one of which is their ability to secrete solutes onto the leaf surface through specialized salt glands [28].

Microorganisms living on *Tamarix* leaves are likewise exposed to multiple concurrent stresses, including high and fluctuating salinity, periodic desiccation, high temperatures, high levels of UV radiation, and high alkalinity [21,31]. However, the development of a diverse microbial community, even under extreme desert environments, is supported by the phylloplane of *Tamarix* spp., which provides a habitat that is rich in organic carbon (>3 g C/l) [21], phosphorus, and nitrogen (mostly available as phosphate and nitrate, respectively), and characterized by adequate moisture levels [7].

The aim of this study was to identify biomass-degrading fungi from leaves and wood of high-biomass-yielding genotypes of *Tamarix aphylla* "Erect" type and *Tamarix jordanis*, and to use these strains to produce a multi-enzyme system consisting of cellulases and amylases.

Materials and methods

Plant biomass. *Tamarix aphylla* "Erect" type and *T. jordanis* biomass (wood and leaves) were kindly provided by Prof. Amram Eshel, Dept. of Plant Sciences, Tel Aviv University, Israel, and were grown in the southern Aravah Valley in the Negev Desert in Israel. The material was sun dried and mechanically ground to yield particles within the 0.5- to 2.0-mm range and then stored in gunny bags. Poplar (*Populus nigra* L.) wood was kindly provided by Prof. Paolo De Angelis, DIBAF, University of Tuscia (Viterbo, Italy). Hazelnut (*Coriolus avellana*) shell waste and milk thistle (*Silybum marianum*) biomass were provided by Stelliferi SpA (Caprarola, Italy) and Novamont SpA (Novara, Italy), respectively.

Isolation of fungi. All chemicals were reagent grade and were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated. *Tamarix* wood and leaf pieces were incubated at 30 °C and 180 rpm in 200 ml of sterile saline solution containing 100 mg chloramphenicol/l. After 24 h, appropriate dilutions were inoculated onto potato dextrose agar (PDA, BD Difco Lab., Detroit, MI, USA) and incubated at 25 °C for 7–10 days. Each fungal strain was isolated to obtain a pure culture, as determined on the basis of the microscopic and macroscopic features of the colonies, which were subsequently maintained and transferred every month in malt extract agar. *Trichoderma viride* DIBAF-10, used as the reference strain in comparisons of the hydrolytic activities of cellulose, was maintained in the same way.

Fungal DNA extraction. Agar plugs (1 cm^2) from 15-day-old cultures were transferred into 500-ml Erlenmeyer flasks containing 100 ml of potato dextrose broth medium (PDB, Difco). After 72 h of growth at 30 °C, the mycelium was recovered by filtration and *ca.* 100 mg dry weight was used for DNA extraction as described by Cassago et al. [3].

PCR and sequencing. The internal transcribed spacer (ITS) sequence was PCR-amplified using the primers ITS1 (5′-TCCGTAGGTGAACCT CGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [1,35]. Amplification was carried out using the GeneAmp PCR system 9700 (Life Techn., Monza, Italy) with the following thermal conditions: 95 °C for 5 min, followed by 35 cycles of 30 s at 95 °C, 1 min at 55 °C, and 1.5 min at 72 °C, with a final extension for 10 min. The PCR product was cloned into the pGEM-T-easy vector (Promega, Madison, WI, USA) and the resultant recombinant plasmid DNA was sequenced.

Identification and phylogenetic analysis of fungal strains. The ITS sequences were compared with those of all known fungal species available in the GenBank database [http://www.ncbi.nlm.nih.gov] to identify potential phylogenetic relationships. All sequences were aligned using the multiple sequence alignment program ClustalW2 [14]. An unrooted phylogenetic tree was constructed using the neighbor-joining program contained in the PHYLIP phylogeny inference package, version 3.6, and the confidence values of the branches were determined by performing a bootstrap analysis based on 100 replicates [http://evolution.gs.washington.edu/phylip.html]. Molecular characterization of strain A and strain B indicated that these strains were virtually identical; thus only strain B was further characterized.

Substrate pretreatment. The *Tamarix jordanis* suspension (16 %) was pretreated under acidic conditions (H_2SO_4 0.5 %) at 120 °C for 30 min. Solid material was recovered by centrifugation (6000 rpm, 10 min), neutralized, and used for media preparation. Milk thistle dry biomass was pretreated using the lab-scale direct steam apparatus described by Santi et al [23]. Ground *Tamarix* wood was pretreated with sodium hydroxide (NaOH) before enzymatic hydrolysis. Briefly, a mixture of the *Tamarix* particles (0.5–2 mm) and NaOH (0.8 % w/v) was incubated at room temperature for 18 h under a solid loading condition of 5 % w/v. The pretreated solids were washed with 800 ml of hot deionized water and total solids were then determined. The solids were dried at 55 ºC and weighed.

Carbohydrates in biomass feedstocks and in pretreated biomass samples were analyzed by hydrolyzing 500 mg of dried material with 72 % sulfuric acid at 121 °C for 60 min, which allowed complete carbohydrate hydrolysis. The monomeric sugars (glucose and xylose) from completely acid-hydrolyzed biomass were analyzed quantitatively using the Enzytec D-Glucose (R-Biopharm AG, Darmstadt, Germany), and K-xylose (Megazyme International Ireland Ltd, Wicklow, Ireland) kits following the manufacturers' instructions.

Culture media, inoculum preparation, and culture conditions. Preliminary screening for the selection of cellulolytic fungal isolates was performed in the following medium (g/l): $NH_4H_2PO_4$, 2; KH_2PO_4, 0.6; K_2HPO_4, 0.4; $MgSO_4 \cdot 7H_2O$, 0.8; thiamine, 0.01; adenine, 0.004; yeast extract, 0.5; Avicel PH-101, 5. The pH of the medium was adjusted to 6.0 prior to sterilization (121 °C, 15 min).

The cellulase production medium had the following composition (g/l): $NaNO_3$, 3; KCl, 0.5; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2$, 0.1; yeast extract, 5; Avicel PH-101, 5. Before the medium was sterilized (121°C for 15 min), the pH was adjusted to 7.0. Pre-culture was carried out by inoculating 15-day-old mycelium fragments into 500 ml baffled flasks containing 100 ml of PDB. After 72 h of incubation at 30 °C under orbital shaking (180 rpm), 10 ml of pre-culture (ca. 0.13 g dry biomass) was transferred to 200 ml of production medium.

To evaluate the stimulatory effect of *Tamarix* biomass on the production of specific cellulases, Avicel was replaced with *T.-jordanis*-pretreated biomass at a concentration of 1 % (w/v). Parallel experiments, under the same culture conditions, were carried out using *T. viride* DIBAF-10 as the reference strain. All experiments were done in triplicate at 30 °C and 180 rpm. The results are reported as the mean values. Significant differences ($P < 0.05$) were analyzed using Tukey's honestly significant difference (HSD) test.

Enzymatic assay. Filter-paper-degrading activity (FPase), exocellulase (avicelase), endocellulase active against carboxymethylcellulose (CMC), and α-amylase activities were determined using Whatman filter paper no.1 (1 × 6 cm strip, 50 mg), Avicel PH-101, CMC, and starch as the respective substrates according to the methods described by Ghose [9]. Activities of α- and β-glucosidase were measured by monitoring the formation of glucose from maltose and cellobiose, respectively; glucose was quantified using an analysis kit (D-glucose) based on the enzymes glucose oxidase and peroxidase (R-Biopharm Roche, Darmstadt, Germany). Activities were expressed in international units (IU), defined as the amount of enzyme activity releasing 1 micromole of glucose reducing-sugar equivalents per ml of the sample per min under standard conditions.

Determination of temperature-dependent activity profile and storage stability. The effect of temperature on FPase activity was determined by incubating the crude extract in citrate buffer (0.05 M, pH 4.8) within a 30–70 °C range and the standard assay was performed as described above. The results were expressed as percent relative activity with respect to the optimum (50 °C), taken as 100. To determine storage stability, residual FPase, β-glucosidase, α-amylase, and α-glucosidase activities in the crude preparation were measured after incubation of the enzymes at 4 °C for 30 days. The amount of retained activity was expressed as a percentage of the zero-time control.

Enzymatic hydrolysis of cellulosic substrates. To evaluate the performance of the *S. commune* cellulolytic enzyme system in the hydrolysis of lignocellulosic substrates, saccharification experiments were conducted using *Tamarix* wood (untreated and alkaline-treated), poplar wood (untreated), hazelnut shell wastes (untreated), and milk thistle biomass (untreated and steam-exploded) as substrates. Substrate hydrolysis was catalyzed using the supernatant collected from *S. commune* strain D cultures grown on acid-pretreated *Tamarix* biomass (FPase activity, 96.1 IU/ml; CMCase activity, 180 IU/ml). An enzyme cocktail (Novozymes' cellulosic ethanol enzyme kit) containing cellulase complex (NS22086), xylanase (NS22083), β-glucosidase (NS22118), hemicellulase (NS22002), and glucoamylase (NS22035) was used for comparison (FPase activity, 1500 IU/ml; CMCase activity, 2900 IU/ml). Enzyme preparations were diluted in 50 mM sodium citrate buffer at pH 4.8 with 1 mM sodium azide to prevent microbial contamination and loaded at a rate of 5 IU/g of dry material. The hydrolysis experiments were performed using a substrate concentration of 3 % (w/v) in citrate buffer (0.05 M, pH 4.8), incubating the samples at 50 °C on an orbital shaker (180 rpm), for 24–48 h. Negative controls were produced by replacing enzyme preparations with the buffer. Samples were collected after defined time intervals, and the total reducing groups were quantified according to the DNS method [10]. Hydrolysis experiments with the addition of xylanases were conducted in the same way, except that a combination of two xylanase preparations (NS22083 and NS22002; Novozymes) was added to the reaction mix. Enzyme loading of the different xylanase preparations was the same used in control experiments carried out with Novozymes' cellulosic ethanol enzyme kit. The saccharification yield (%), i.e., the amount of substrate converted to reducing sugars, was calculated as follows: yield (%) = reducing sugar concentration (g/l) × working volume (l) × 0.9 × 100/dry substrate wt (g) × cellulose in biomass (%) [9].

Nucleotide sequence accession numbers. The ITS sequences obtained in this study were deposited in GenBank under the sequential accession numbers KF028368 through KF028373.

Results

Molecular and biochemical characterization. More than 100 isolates were obtained from wood and leaf samples from *T. aphylla* "Erect" type and *T. jordanis*. Based on colony morphology, hyphal morphology, and spore characteristics, these isolates were classified into six different morphological taxa, with one isolate from each group characterized at the molecular level using the ITS rRNA region as the DNA barcode (see Materials and methods). Sequence data of ITS fragments were used to generate a phylogenetic tree for comparison of the relatedness among these fungi and known species. The results of this analysis indicated that five of these isolates belonged to the Ascomycota phylum and one, closely-related to *Schizophyllum commune,* belonged to the Basidiomycota (Fig. 1). Major forms isolated from wood and leaves were assigned to different taxa, based on their high bootstrap values (Fig. 1). All forms from decaying wood occurred on both *T. aphylla* "Erect" type and *T. jordanis*. *Penicillium*-related form (strain F) occurred on leaves of both *Tamarix* species, while a *Scopuraliopsis*-related form (strain G) occurred only on *T. aphylla* "Erect" type leaves (Fig. 1).

The ability of single isolates to produce extracellular hydrolytic enzymes involved in cellulose and starch degradation was evaluated in cultures grown in liquid synthetic medium containing Avicel PH-101 as the sole carbon source. As shown in Table 1, all strains were able to produce cellulose-degrading enzymes in secreted form, but they differed greatly in the total amounts of β-glucosidase (which varied from 30 ± 1 to 2640 ± 40 IU/ml) and FPase (from 10 ± 2 to 400 ± 8 IU/ml) activity. Under the same culture conditions, all strains produced a basal level of α-amylase activity, ran-

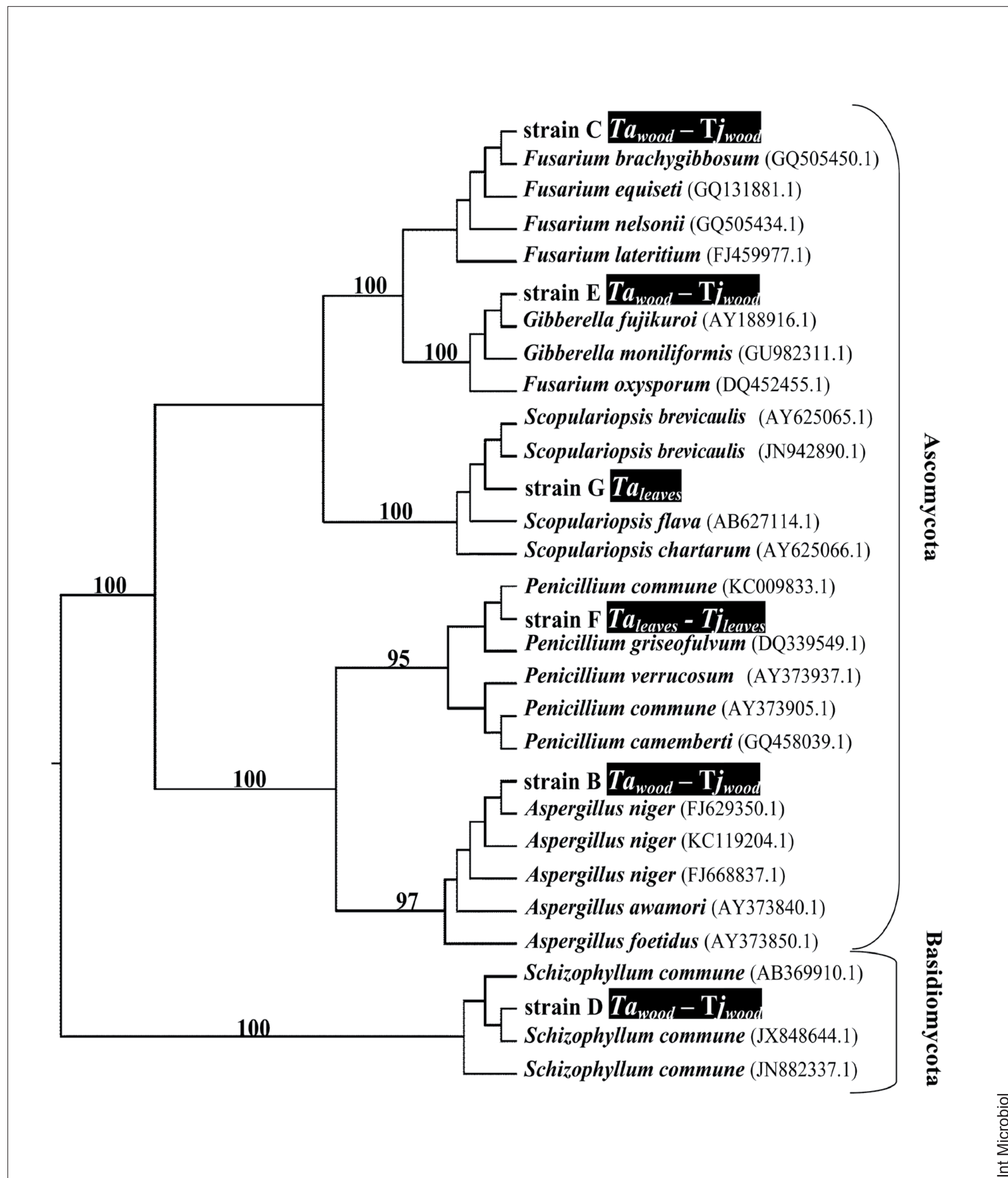

Fig. 1. Molecular identification of the six morphotypes isolated from leaves and wood of *Tamarix aphylla* "Erect" type (*Ta*) and *T. jordanis* (*Tj*) based on ITS rDNA sequence analysis. The source of each strain is indicated on the right. Phylogenetic analysis was performed using the neighbor-joining method, with 100 bootstrap replicates. Full-length ITS nucleotide sequences were retrieved from the NCBI database (GenBank Acc. Nos. in parentheses). Phyla are indicated by parentheses on the right. Numbers at nodes represent bootstrap values >70 %; values for some terminal nodes were omitted for clarity.

ging from 150 ± 11 to 410 ± 20 IU/ml (Table 1). Among the tested isolates, strain D was the best producer of FPase, β-glucosidase, and α-amylase activities (400, 2640, and 410 IU/ml, respectively).

Optimization of enzyme activity production on pretreated *Tamarix* biomass. To determine whether *Tamarix* biomass could be used as feedstock for the production of cellulose-degrading enzymes, further experiments were conducted with *S. commune* strain D on medium containing steam-acid pretreated *T. jordanis* biomass (1.6 % w/v) and glucose (0.5 % w/v) as carbon sources.

Under this condition, significant levels of cellulolytic and amylolytic enzymes were produced by strain D. The onset of all activities occurred 72 h after the inoculation, except for α-glucosidase activity, which was detected earlier (Fig. 2).

Table 1. Maximal avicelase, FPase, β-glucosidase, and α-amylase activities of fungal strains isolated from *Tamarix aphylla* "Erect" type and *T. jordanis* wood and leaves. Strains were grown on synthetic medium containing Avicel PH-101 microcrystalline cellulose. *Tamarix viride* DIBAF-10 was included as a reference strain

Strain	Enzymatic activity* (IU/l)			
	Avicelase	FPase	β-glucosidase	α-amylase
Aspergillum niger strain B	60 ± 22^{a}	70 ± 11^{b}	180 ± 8^{b}	250 ± 21^{ab}
Fusarium spp. strain C	60 ± 10^{a}	120 ± 9^{c}	160 ± 10^{b}	250 ± 12^{ab}
Schizophyllum commune strain D	60 ± 19^{a}	400 ± 8^{f}	2640 ± 40^{f}	410 ± 20^{c}
Gibberella spp. strain E	60 ± 12^{a}	100 ± 13^{bc}	250 ± 12^{c}	210 ± 20^{a}
Schizophyllum commune strain F	60 ± 8^{a}	250 ± 20^{e}	460 ± 20^{d}	150 ± 11^{a}
Scopulariopsis spp. strain G	60 ± 21^{a}	10 ± 2^{a}	30 ± 1^{a}	170 ± 9^{a}
T. viride DIBAF-10	80 ± 30^{a}	200 ± 10^{d}	1170 ± 7^{e}	430 ± 21^{c}

*Data are the mean ± standard deviation of three replicates.
[a–e]Column means followed by the same superscript letter are not significantly different. ($P < 0.05$) according to Tukey's HSD test.

Effect of temperature on activity and stability of cellulase complex. The extracellular cellulase complex from *S. commune* strain D grown on *Tamarix* biomass showed maximal FPase activity (100 %) at 50 °C. Enzyme activity decreased in samples incubated at higher or lower temperatures but 59 % (60°C) to 47 % (70 °C) of the maximal activity was retained at the higher temperature and 62 % (30 °C) to 90 % (40 °C) at the lower temperature (data not shown). To assess the storage stability of both the cellulase and the amylase complex from *S. commune* strain D, the crude preparation was kept at 4 °C and residual activity was determined after 1 month. Table 2 shows that the amylase complex retained al-

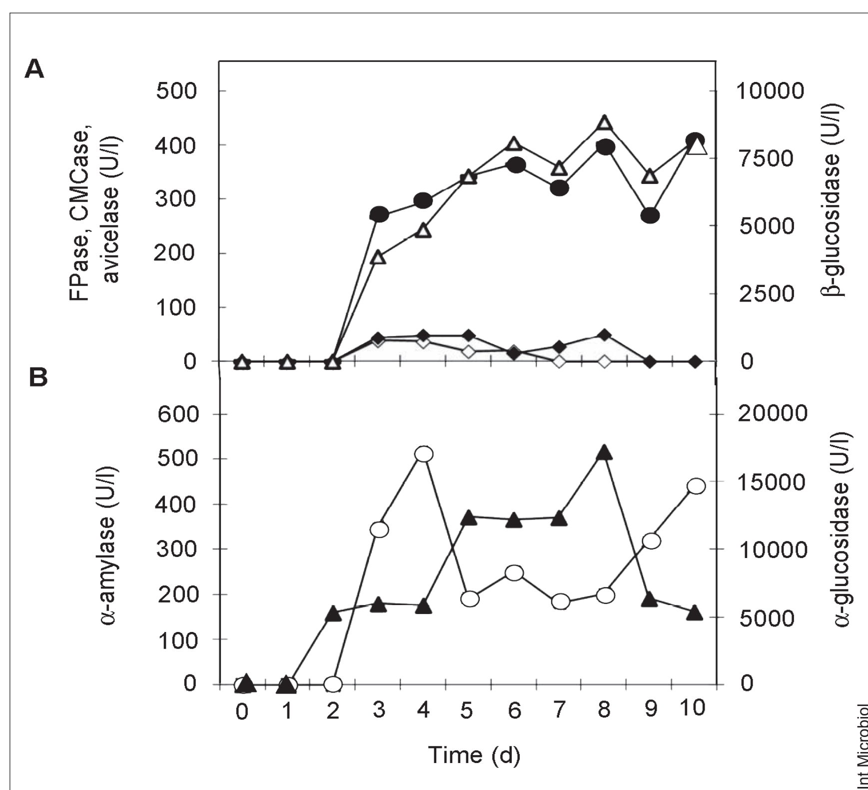

Fig. 2. Time courses of glycosyl hydrolases from *Schizophyllum commune* strain D grown on pretreated *Tamarix jordanis* biomass. (**A**) Endocellulase (CMCase, closed diamond); exocellulase (avicelase, open diamond); β-glucosidase (open triangle); FPase activity (closed circle). (**B**) α-glucosidase (closed triangle); α-amylase (open circle).

Table 2. Residual cellulolytic and amylolytic activities of the multi-enzyme complex system from *S. commune* strain D after storage at 4 °C for 30 days

Enzyme	Residual activity* (%)
α-amylase	100 ± 1[a]
α-glucosidase	96 ± 1[a]
FPase	81 ± 2[b]
β-glucosidase	81 ± 1[b]

*Data are the mean ± SD of three replicates.
[a,b]Values with the same superscript letter are not significantly different ($P < 0.05$) according to Tukey's HSD test.

most full activity (96–100 % residual activity) while the cellulase complex lost about 20 % of its activity under the same conditions.

Enzymatic hydrolysis of cellulosic biomass. *Schizophyllum commune*'s multienzyme complex produced on pretreated *Tamarix* biomass was then used to hydrolyze different untreated and pretreated lignocellulosic substrates. The amount of reducing sugars released during 24–48 h of saccharification, using an enzyme load of 5 IU/g of biomass, ranged from 3.2 ± 0.1 to 8.6 ± 0.3 mg/g of biomass (Table 3), corresponding to less than 2.0 % cellulose hydrolysis when untreated biomass was used as the substrate. As expected, biomass hydrolysis significantly increased upon pretreatment, reaching a saccharification yield of 48.7 ± 1.2 % when pretreated milk thistle was the substrate and 14.5 ± 1.0 % in the case of alkaline-pretreated *Tamarix* wood. Comparable values for the Novozymes multienzyme mixture were 81.9 ± 2.3 % and 47.8 ± 1.8 %, respectively. However, the differences in the performances of *S. commune* and commercial cellulase preparations on pretreated herbaceous biomass (milk thistle) were markedly lower when the multienzyme complex from *S. commune* strain D was supplemented with commercial xylanases commonly used to improve cellulase performance (Table 3).

Table 3. Reducing-sugar release and saccharification rate from different lignocellulosic substrates by the supernatant collected from *Schizophyllum commune* strain D cultures grown on acid-pretreated *Tamarix* biomass

Source	Enzyme mix (5 FPU/g biomass)	Pretreatment	Glucose equivalent* (mg/g biomass)	Saccharification rate* (% g glucose equivalent/g cellulose)
Milk thistle	*S. commune*	None	8.3 ± 0.4	1.9 ±0.1[a2]
Hazelnut	*S. commune*	None	3.2 ± 0.1	1.4 ± 0.1
Poplar	*S. commune*	None	8.6 ± 0.3	1.7 ± 0.1
Tamarix	*S. commune*	None	7.4 ± 0.1	1.9 ± 0.1[a1]
Milk thistle	*S. commune*	Steam-explosion	222.6 ± 2.4	48.7 ± 1.2[b2]
Tamarix	*S. commune*	Alkaline	63.5 ± 1.2	14.5 ± 1.0[b1]
Milk thistle	Commercial[1]	Steam-explosion	373.8 ± 4.2	81.9 ± 2.3[c2]
Tamarix	Commercial[1]	Alkaline	207.6 ± 5.5	47.8 ± 1.8[c1]
Milk thistle	*S. commune* + xylanases[2]	Steam-explosion	335.6 ± 2.2	73.5 ± 1.2[d2]

*Average ± SD from three replicates.
[a–d]Values with the same superscript letter are not significantly different ($P < 0.05$) according to Tukey's HSD test: [a1–c1]*Tamarix* biomass; [a2–d2]Milk thistle biomass.
Enzyme components in the commercial cocktail[1] and xylanases[2] were from Novozymes (see Materials and methods).

Discussion

In this study, fungi occurring on the wood and leaves of two *Tamarix* species cultivated in the Negev Desert (Israel), namely, *T. aphylla* "Erect" type and *T. jordanis*, were studied for their phylogenetic affinities and their ability to produce extracellular cellulolytic and amylolytic enzymes. Six species were identified by phylogenetic analysis of ITS sequences of nuclear ribosomal DNA: five ascomycetes and one basidiomycete (Fig. 1). The prevalence of Ascomycota in the phyllosphere microbial community of *T. aphylla* was also reported by Finkel et al. [7], who analyzed the effect of geographic location on the structure of the microbial community on leaf surfaces. Our data also showed that Ascomycota occurred on both the plant's woody material and its leaves, whereas the basidiomycete *S. commune* (strain D; Fig. 1) was found only on decaying wood.

In both *Tamarix* species, the wood and leaves were differentially colonized by fungal strains belonging to different taxa, which might explain the observed differences in fungal activity. There were no significant differences among taxa isolated from wood samples from *T. aphylla* "Erect" type and *T. jordanis*, in agreement with the observation that the microbial communities on different *Tamarix* species grown in the same location are highly similar [8]. Quite different results were obtained in our analysis of the leaves of the two *Tamarix* species, where moulds belonging to *Penicillium* genus (strain F) were found on the leaf surfaces of both species, whereas fungi belonging to *Scopulariopsis* genus (strain G) occurred only on *T. aphylla* "Erect" type leaves (Fig. 1). The latter data are in agreement with the observation that stems and leaves of mature *T. aphylla* "Erect" type plants secrete higher amounts of salt than those of *T. jordanis* [Abbruzzese and Kuzminsky, http://hdl.handle.net/2067/2366], which may generate a more favorable environment for slow-growing, highly salt-tolerant fungal genera such as *Scopulariopsis* [16,27].

Biochemical data indicated that all strains produced exoglucanase (avicelase), total cellulase (FPase), β-glucosidase, and α-amylase activities (Table 1). These enzyme activities were highest in *S. commune* strain D, as determined in its culture broth, in which enzyme activity was twice as high as in the cellulase-producing reference strain *T. viride* DIBAF-10 (Table 1).

Schizophyllum commune, a ubiquitous wood-degrading white-rot fungus with a worldwide distribution [24], is not known to be a pathogen for *Tamarix* species. This fungal species is considered to be both a genetically tractable model for studying mushroom development [34] and a likely source of enzymes capable of efficiently degrading lignocellulosic biomass [6,33]. Comparative analyses of the 38.5-Mb genome of *S. commune* have shown that, among basidiomycetes, this fungus has one of the most extensive enzymatic machineries for the degradation of cellulose and hemicellulose [19]. In our study, *S. commune* strain D produced significant amounts of cellulolytic and amylolytic activities on medium containing either Avicel or pretreated *Tamarix* biomass. Induction of cellulase production in fungi is usually mediated by low-molecular-weight soluble oligosaccharides that are released from complex substrates as a result of hydrolysis. These metabolites enter the cell, where they signal the presence of extracellular substrates and stimulate the accelerated synthesis of constituent enzymes of the cellulase complex [26]. Various mono-, oligo-, and polysaccharides have been shown to enhance the production of cellulase by *S. commune* [12,24].

The efficacy of cellulases from *S. commune* strain D in the hydrolysis of pretreated biomass from both woody and herbaceous crops and, when supplemented with extraneous commercial β-xylanase, the enhanced hydrolytic performance of these enzymes on pretreated biomass were shown in the enzymatic hydrolysis assay using untreated, alkaline (NaOH)-pretreated, and steam-explosion-pretreated lignocellulosic substrates (Table 3). When the hydrolytic capacities of the cellulase complex from *S. commune* strain D and a commercial cellulase mixture were investigated at the same enzyme loading on milk thistle, the glucose yield correlated with the β-xylanase level in the mixture (Table 3). These data are in agreement with the observation that cellulose digestion generally improves following xylan and lignin removal by chemical or enzymatic treatment. In conclusion, *Tamarix* biomass is a useful source to isolate novel fungal strains able to produce enzymes for biomass saccharification. Using *S. commune* strain D and pretreated *Tamarix* biomass, cellulases and amylases can be obtained with the potential to compete with commercial enzymes.

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Competing interests. None declared.

References

1. Begerow D, Nilsson H, Unterseher M, Maier W (2010) Current state and perspective of fungal DNA barcoding and rapid identification procedures. Appl Microbiol Biotechnol 87:99-108
2. Billard H, Faraj A, Ferreira NL, Menir S, Heiss-Blanquet S (2012) Optimization of a synthetic mixture composed of major *Trichoderma reesei* enzymes for the hydrolysis of steam-exploded wheat straw. Biotechnol Biofuels 5:9
3. Cassago A, Panepucci RA, Baião AMT, Silva FH (2002) Cellophane based mini-prep method for DNA extraction from the filamentous fungus *Trichoderma reesei*. BMC Microbiology 2:14
4. Clark JH, Luque R, Matharu AS (2012) Green chemistry, biofuels, and biorefinery. Ann Rev Chem Biomol Eng 3:183-207
5. Eshel A, Oren I, Alekperov C, Eilam T, Zilberstein A (2011) Biomass production by desert halophytes: alleviating the pressure on the scarce resources of arable soil and fresh water. Eur J Plant Sci Biotech 5:48-53
6. Fang J, Huang F, Gao P (1999) Optimization of cellobiose dehydrogenase production by *Schizophyllum commune* and effect of the enzyme on kraft pulp bleaching by ligninases. Proc Biochem 34:957-961
7. Finkel OM, Burch AY, Lindow SE, Post AF, Belkin S (2011) Geographical location determines the population structure in phyllosphere microbial communities of a salt excreting desert tree. Appl Environ Microbiol 77:7647-7655
8. Galbe M, Zacchi G (2002) A review of the production of ethanol from softwood. Appl Microbiol Biotechnol 59:618-628
9. Ghose TK (1987) Measurements of cellulose activity. Pure Appl Chem 59:257-268
10. Haltrich D, Sebesta B, Steiner W (1996) Induction of xylanase and cellulase in *Schizophyllum commune*. In: Saddler JN, Penner MH (eds) Enzymatic degradation of insoluble carbohydrates. ACS Symposium Series 618, American Chemical Society, Washington, pp 305-318
11. Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Lidén G, Zacchi G (2006) Bio-ethanol—the fuel of tomorrow from the residues of today. Trends Biotechnol 24:549-556
12. Hayes DJ (2009) An examination of biorefining processes, catalysts and challenges. Catal Today 145:138–151
13. Hudgeons JL, Knutson AE, Heinz KM, DeLoach CJ, Dudley TL, Pattison RR, Kiniry JR (2007) Defoliation by introduced *Diorhabda elongata* leaf beetles (Coleoptera: Chrysomelidae) reduces carbohydrate reserves and regrowth of *Tamarix* (Tamaricaceae). Biological Control 43:213-221
14. Larkin MA, Blackshields G, Brown NP, et al. (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947-2948
15. Lynd LR, Weimer PJ, Van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: Fundamentals and biotechnology. Microbiol Mol Biol Rev 66:506-577
16. Mudau MM, Setati ME (2006) Screening and identification of endomannanase-producing microfungi from hypersaline environments. Curr Microbiol 52:477-481
17. Naik SN, Goud VV, Rout PK, Dalai AK (2010) Production of first and second-generation biofuels: a comprehensive review. Renew Sust Energ Rev 14:578-597
18. Nidetzky B, Steiner W, Hayn M, Claeyssens M (1994) Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: a new model for synergistic interaction. Biochem J 298:705-710
19. Ohm RA, De Jong JF, Lugones LG, Aerts A, Kothe E, Stajich JE, De Vries RP (2010) Genome sequence of the model mushroom *Schizophyllum commune.* Nat Biotechnol 28:957-963
20. Percival Zhang Y-H, Himmel ME, Mielenz JR (2006) Outlook for cellulase improvement: screening and selection strategies*.* Biotechnol Adv 24:452-481
21. Qvit-Raz N, Jurkevitch E, Belkin S (2008) Drop-size soda lakes: transient microbial habitats on a salt-secreting desert tree. Genetics 178:1615-1622
22. Rho D, Desrochers M, Jurasek L, Driguez H, Defaye J (1992) Induction of cellulose in *Schizophyllum commune*: thiocellobiose as a new inducer. J Bacteriol 149:47-53
23. Santi G, D'Annibale A, Petruccioli M, Crognale S, Ruzzi M, Valentini R, Moresi M (2012) Development and testing of a novel lab-scale direct steam-injection apparatus to hydrolyse model and saline crop slurries. J Biotechnol 157:590-597
24. Schmidt O, Liese W (1980) Variability of wood degrading enzymes of *Schizophyllum commune.* Holzforschung 34:67-72
25. Singhania RR, Sukumaran RK, Patel AK, Larroche C, Pandey A (2010) Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. Enzyme Microb Tech 46:541-549
26. Singhania RR, Patel AK, Sukumaran RK, Larroche C, Pandey A (2013) Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Biores Technol 127:500-507
27. Steiman R, Guiraud P, Sage L, Seigle-Murandi F (1997) Soil mycoflora from the Dead Sea oases of Ein Gedi and Einot Zuqim (Israel). Anton Leeuw 72:261-270
28. Thomson WW, Berry WL, Liull L (1969) Localization and secretion of salt by the salt glands of *Tamarix aphylla*. Proc Natl Acad Sci USA 63:310-317
29. Tsujiyama S, Ueno H (2011) Production of cellulolytic enzymes containing cinnamic acid esterase from *Schizophyllum commune.* J Gen Appl Microbiol 57:309-317
30. Xiao LP, Shia ZJ, Xua F, Sun RC (2013) Hydrothermal treatment and enzymatic hydrolysis of *Tamarix ramosissima*: evaluation of the process as a conversion method in a biorefinery concept. Biores Technol 135:73-81
31. Waisel Y (1991) The glands of *Tamarix aphylla*: a system for salt secretion or for carbon concentration? Physiol Plant 83:506-510
32. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, New York, pp 315-322
33. Willick GE, Seligy VL (1985) Multiplicity in cellulases of *Schizophyllum commune*. Derivation partly from heterogeneity in transcription and glycosylation. Eur J Biochem 151:89-96
34. Wösten HAB, Wessels JGH (2006) The emergence of fruiting bodies in basidiomycetes. In: Kües U, Fisher R (eds) The Mycota. Part I: Growth, differentiation and sexuality. Springer, Berlin, pp 393-414
35. Zheng Y, Zhongli P, Zhang R, Labavitch JM, Wang D, Teter SA, Jenkins BM (2007) Evaluation of different biomass materials as feedstock for fermentable sugar production. J Appl Biochem Biotech 136–140:423-435