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Morphological and enzymatic response of the thermotolerant fungus Fomes sp. EUM1 in solid state fermentation under thermal stress

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ABSTRACT

Thermotolerance of the fungus Fomes sp. EUM1 was evaluated in solid state fermentation (SSF). This thermotolerant strain improved both hyphal invasiveness (38%) and length (17%) in adverse thermal conditions exceeding 30 ºC and to a maximum of 40 ºC. In contrast, hyphal branching decreased by 46% at 45 ºC. The production of cellulasres over corn stover increased 1.6-fold in 30 ºC culture conditions, xylanases increased 2.8-fold at 40 ºC, while laccase production improved 2.7-fold at 35 ºC. Maximum production of lignocellulolytic enzymes was obtained at elevated temperatures in shorter fermentation times (8–6 days), although the proteases appeared as a thermal stress response associated with a drop in lignocellulolitic activities. Novel and multiple isoenzymes of xylanase (four bands) and cellulase (six bands) were secreted in the range of 20–150 kDa during growth in adverse temperature conditions. However, only a single laccase isoenzyme (46 kDa) was detected. This is the first report describing the advantages of a thermotolerant white-rot fungus in SSF. These results have important implications for large-scale SSF, where effects of metabolic heat are detrimental to growth and enzyme production, which are severely affected by the formation of high temperature gradients.

Keywords: thermotolerance; white-rot fungi; solid state fermentation; lignocellulolytic enzymes

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INTRODUCTION

The majority of white-rot fungi are mesophiles that are able to produce lignocellulolytic enzymes either in solid state fermentation (SSF) or submerged fermentation (SF). *Fleuroutus, Lentinus, Trametes* and *Phanaerchaete* are well-studied genera that secrete high levels of lignocellulolytic enzymes in SSF and SF. These enzymes are of great interest for a wide range of industrial and biotechnological applications (Elisashvili et al. 2008; Mtu 2012).

SSF has gained considerable attention in the use of inexpensive agro-industrial residues as substrates for fungal growth and the simultaneous production of lignocellulolytic enzymes. SSF offers additional advantages, including a high volumetric productivity, recovering enzymes with higher temperature or pH stabilities and lower operating costs (de Castro and Sato 2015). The culture temperature is a critical factor in SSF due to the difficulty of removing the metabolic heat generated by microbial growth, even after implementing strategies for temperature control (Shojaosadati et al. 2007; Figueroa-Montero et al. 2011). Low thermal conductivity of the substrates (0.1 W m⁻¹ K⁻¹) limits heat transfer. Fungal growth and enzyme production are severely affected in large-scale SSF by the non-isothermal conditions creating sharp gradients from 5 °C to 10 °C cm⁻¹ bed (Shojaosadati et al. 2007). These high temperatures in large-scale SSF are unfavorable for the optimal growth of white-rot fungi (Elisashvili et al. 2008; Mtu 2012). Thermotolerant white-rot fungi could be used in SSF to reduce the negative effect of heat on mycelial growth and enzyme production. It has been reported that various thermotolerant strains can produce thermostable enzymes in SSF and SF under heat shock treatment or elevated temperatures (Wang et al. 2012; Yan et al. 2015). Additionally, some thermotolerant strains have been capable of producing high levels of laccase on agro-industrial wastes (Chawachart et al. 2004; Khanongnuch et al. 2004; Yan et al. 2015).

White-rot fungi of the genus *Fomes* have been studied in SSF for the production of lignocellulolytic enzymes (Papinutti and Forchiassin 2003, 2007; Neifar et al. 2011); however, thermotolerant strains have not been described for this genus. In a previous study, a thermostolerant white-rot fungus was identified as *Fomes* sp. EUM1, which exhibited optimal growth at 30 °C and was able to grow at unusual temperatures for this genus such as 40 °C (Ordaz et al. 2012).

The purpose of the current study was to evaluate the potential and advantages of using the thermostolerant *Fomes* sp. EUM1 strain in SSF with a long-term goal of large-scale applications. This strain was analyzed for the effects of adverse temperature on growth and lignocellulolytic enzyme production, as well as on morphological changes of mycelia and hyphae.

MATERIALS AND METHODS

Microorganism and propagation

The *Fomes* sp. EUM1 strain was propagated on Petri dishes every 7 days at 30 °C. The culture medium MYA consisted of (g L⁻¹): 40, malt extract; 3, yeast extract; and 18, bacteriological agar.

Morphometric analysis

The invasiveness U (μm h⁻¹) and length of the leading hyphae L (μm) were measured at 30 °C, 35 °C, 40 °C and 45 °C on surface cultures. The culture medium reported by Ordaz et al. (2012) was modified to contain only 1.8% (w/v) of bacteriological agar in corn stover extract as the only nutrient source, which was obtained by filtering a suspension of ground corn stover at 15% (250 rpm, 3 h at 70 °C). The culture medium was used to resemble as closely as possible the medium composition in SSF. The morphometric measurements were obtained by using the Motic Images Plus 2.0 software.

Enzyme production in SSF

Inoculum

Five agar disks with superficial mycelium (Ø 0.6 cm and MYA medium), 4 mL of sterile distilled water and 50 glass beads were mixed for 3 min in a vortex to obtain the mycelial suspension. Fungal biomass was determined by dry weight at 65 °C for 24 h.

Substrate

Corn stover was used as the only source of nutrients for growth; particle size was between 0.61 and 4.06 mm (Membrillo et al. 2011). The experiments were carried out in Erlenmeyer flasks (250 mL) containing 3.5 g of corn stover and 7 mL of distilled water. The sterilized substrate (15 min, 121 °C) was inoculated with 4 mL of mycelial suspension and thoroughly mixed. The mycelial suspension was used to reduce the moisture variations and to improve the mycelial distribution over the substrate.

Solid state fermentation

The fungus grew on corn stover at temperatures of 30 °C, 35 °C, 40 °C and 45 °C under the following conditions: substrate 80% moisture, 0.092 mg dry biomass (g dry corn stover⁻¹)⁻¹ inoculum size, 2 cm bed height and 3 g initial dry corn stover. The cultures were maintained for 12 days in conditions of controlled humidity. All the experiments were performed in triplicates.

Enzyme extraction

Distilled water (pH 6.5) and citrate buffer (50 mM, pH 5.0) were evaluated at 15 and 30 min for enzyme recovery from corn stover. A solid-to-solvent ratio of 1:20 was the optimal condition for extraction at 200 rpm and 10 °C. The enzymatic extract was filtered and centrifuged at 10 000 × g for 15 min and 4 °C in order to obtain a clear liquid. The enzymatic extract was assayed for pH, enzymatic activities and zymogram analysis.

pH and moisture

pH changes were directly measured in enzymatic extracts (Conductronic 120, México), and moisture changes of the substrate were quantified by a moisture analyzer (OHAUS MB45, Japan).

Enzymatic assays

Xylanase and cellulase activity were assayed by using the DNS reagent (3, 5-dinitrosalicylic acid) (Miller et al. 1960). The reaction contained 200 μL of enzymatic extract and 800 μL of substrate, 0.5% soluble birchwood xylan in citrate buffer (50 mM and pH 6) for xylanase activity and 0.5% carboxymethyl cellulose sodium salt (CMC) in citrate buffer (50 mM and pH 5.5) for cellulose activity. The enzymatic reaction was carried out at 50 °C for 15 min, and stopped by adding 1.5 mL of DNS reagent. The samples were boiled in water for 10 min, and the absorbance was measured at 640 nm to quantify the reducing sugars liberated. One international unit (IU) was defined as the amount of enzyme that liberated 1 μmol of reducing sugars under the assay conditions described above.
Laccase activity was determined by measuring the oxidation of ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) (Yan et al. 2015). The reaction mixture contained 500 μL of 50 mM ABTS in citrate buffer (pH 5.0) and 500 μL of appropriately diluted enzyme extract. The absorbance was measured at 420 nm for 2 min at 40 °C (DU 640 Beckman). One international unit was defined as amount of enzyme that oxidized 1 μmol of ABTS per minute in the reaction conditions assayed.

Protease activity was determined with casein as a substrate (Hernández-Martínez et al. 2011). Briefly, the reaction mixture contained 800 μL of 1% Hammberstein casein in phosphate buffer (50 mM and pH 7.0) and 200 μL of diluted enzymatic extract. The reaction was maintained at 40 °C for 15 min and stopped by the addition of 3 mL of 10% trichloroacetic acid. The samples were filtered and soluble aromatic amino acids were measured in the supernatant at 280 nm. One international unit of protease activity was defined as the amount of enzyme liberating 1 μg tyrosine per minute under assay conditions. All enzymatic activities were determined in triplicates and reported in units per gram of initial dry corn stover, IU (g dry wt)^−1.

**Enzyme concentration**

The enzymatic extract was concentrated with ammonium sulfate precipitation in order to obtain zymogram samples (Hernández-Martínez et al. 2011). The enzymatic extract (200 mL) was firstly saturated with ammonium sulfate up to 20% (2 h and 4 °C) and subsequently saturated up to 80% (12 h and 4 °C). In each step, the samples were centrifuged at 10 000 × g for 30 min and 4 °C. The precipitated proteins were mixed with a minimum volume of citrate buffer (50 mM and pH 6.0) and protease inhibitor cocktail (Sigma). The samples were dialyzed (10 kDa cutoff membrane) four times at 4 °C for 24 h against 500 mL of citrate buffer (50 mM and pH 6.0) under continuous agitation.

**Zymogram analysis**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 10% polyacrylamide gel copolymerized with 0.3% (w/v) CMC or soluble oat spelt xylan, respectively (Breccia et al. 1995). The samples were previously heated at 50 °C for 20 min, and gels were loaded with 0.5 μL of enzymatic activity. Running conditions were 150 V at 4 °C. The gels were treated two times with 0.1% Triton X-100 for 15 min to remove SDS, rinsed briefly with distilled water, incubated at 50 °C for 40 min in 200 mL of citrate buffer (pH 6.0 and 5.0 for xylanase and cellulase activity, respectively), and then stained with 0.1% Congo red for 15 min. Excess dye was discarded and the gels were newly incubated at 50 °C in a solution of 1 mM NaCl in citrate buffer. This solution was replaced every 15 min until the enzyme bands became clearly visible. To enhance contrast, the gels were treated with 0.5% acetic acid for 5 min.

Laccase zymograms were subjected to electrophoresis on 12% polyacrylamide gels (Karp et al. 2012) at 120 V and 4 °C. The gels were treated two times with 200 mL of 0.1% Triton X-100 for 15 min, rinsed with distilled water and incubated at 40 °C in 200 mL of 0.5 mM ABTS in 50 mM citrate buffer (pH 5.0). The gels were incubated until the bands were detected.

**Statistical analysis**

The statistical analyses were performed using the program SPSS ver. 17.0 (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) was carried out and mean comparisons were run using Duncan’s multiple range tests.

**RESULT AND DISCUSSION**

Thermotolerant strains have been considered as an alternative to overcome the overheating problems in SSF; nevertheless, enzymatic profiles and morphological adaptation have not been described under adverse temperatures. The thermotolerant fungus *Fomes* sp. EUM1 was able to modify mycelial morphology at temperatures above the optimum growth of 30 °C (Table 1). The invasiveness of the mycelium and hyphal length exhibited the most significant changes, with these parameters simultaneously increasing up to 26% and 38% at 40 °C, respectively. A strong positive correlation between these variables was calculated (R² = 0.87), indicating a greater invasiveness with the formation of longer hyphae under adverse temperature conditions. Both events could be important to rapidly assimilate nutrients with a minimal energy expenditure for the formation of biomass, in order to generate enough energy for cellular metabolism at high temperatures (Maheshwari, Bharadwaj and Bhat 2000; Balmant et al. 2015). A high invasiveness of solid substrates is also a desirable feature in SSF to reduce the fermentation times and risks of contamination due to the slow growth of some fungi (de Castro and Sato 2015). Likewise, the formation of long hyphae improves the supply of nutrients for fungal growth in SSF, since the hyphae penetrate to deeper regions of solid substrates to release hydrolytic enzymes that degrade the high content of hemicellulose and cellulose (Anusha et al. 2012; Balmant et al. 2015). Normally, a high degree of hyphal branching has been associated with improved colonization of the growth medium at temperatures close to optimal (Harris 2008). Nevertheless, *Fomes* sp. EUM1 significantly reduced the hyphal branching by 46% as a response to the adverse thermal conditions of 45 °C. It has not been reported whether hyphal branching in mesophilic fungi varies with temperature shifts (Watters and Griffiths 2001). The results observed in surface cultures can be extrapolated to SSF due to good correlations within the process parameters required for fungal growth (Favela-Torres et al. 1998; Florencio, Couri and Farinás 2012). Therefore, thermal adaptation of growth by *Fomes* sp. EUM1 is relevant in a SSF process where high temperature gradients progress (Figueroa-Montero et al. 2011).

The thermotolerance of *Fomes* sp. EUM1 was explored in SSF for the production of lignocellulosic enzymes at temperatures exceeding those required for optimum growth. In preliminary studies, distilled water was more efficient than citrate buffer (10 °C, 200 rpm, 30 min) to recover the enzymes in SSF. In this way, the enzymes were recovered from corn stover using an inexpensive method based merely on mechanical agitation and filtration (Kapilan 2015). A shift in growth

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>U (μm h⁻¹)</th>
<th>L (μm)</th>
<th>U/L (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>247.2 ± 3.7b</td>
<td>402.5 ± 19.5b</td>
<td>0.62 ± 0.03b</td>
</tr>
<tr>
<td>35</td>
<td>392.3 ± 12.1b</td>
<td>499.9 ± 30.7b</td>
<td>0.65 ± 0.03b</td>
</tr>
<tr>
<td>40</td>
<td>312.3 ± 6.7b</td>
<td>558.5 ± 43.7b</td>
<td>0.56 ± 0.05b</td>
</tr>
<tr>
<td>45</td>
<td>202.4 ± 3.2b</td>
<td>313.4 ± 32.9b</td>
<td>0.33 ± 0.03b</td>
</tr>
</tbody>
</table>

Values are means ± SD. One-way ANOVA was used to analyze data using Duncan range test. Values are statistically significant at P < 0.05 (a-c). Each value is the result of 30 measurements made in triplicates. Invasiveness, U (μm h⁻¹); hyphal length, L (μm) and hyphal branching, U/L (h⁻¹).
temperature from 30°C to 40°C improved the production of cellulase and xylanase up to 1.6-fold and 2.8-fold, respectively; while laccase production increased 2.7-fold from 30°C to 35°C (Fig. 1). The maximal production levels reached for cellulase, xylanases and laccases were 65, 161 and 12.6 IU (g corn stover)$^{-1}$, respectively. Fermentation times to attain maximum enzyme production were shorter (up to 2 days) as a result of the high invasive-ness over corn stover. During the culture, a high proteolytic activity was detected (Fig. 2), which led to an abrupt drop in the maximum enzyme production, mainly at elevated temperatures (Fig. 1). Initial pH increased from 6.5 to 7.5; while the substrate moisture remained constant (80%). To date, a few reports have described the genus Fomes for producing lignocellulolytic enzymes in SSF (Papinutti and Forchiassin 2003, 2007), while its laccases have been reported to decolorize dyes (Neifar et al. 2010). Most white-rot fungi, including the genus Fomes, have potential in SSF at temperatures close to their optimum growth (28°C–32°C) (Elisashvili et al. 2008; Mtiu 2012). Nevertheless, higher temperatures reached in the bed in SSF severely affect fungal
growth and enzyme production. In this sense, the thermotoler-ant fungus Fomes has the advantage of producing comparable levels of lignocellulolytic enzymes in shorter times than most white-rot fungi, including Pleurotus, Lentinus, Trametes, in addition to the fact that Fomes commonly produce the highest en-zyme levels after 12 days (Elisashvili et al. 2008). Other thermo-tolerant strains have shown potential in SSF for enzyme produc-tion, even some Trametes species improved laccase production at elevated temperatures (Chawachart et al. 2004; Khanongnuch et al. 2004; Yan et al. 2015). Proteolysis has been undesirable for lignocellulolytic enzyme production and can be decreased by adding a nitrogen source to the production medium (Shah and Madamwar 2005). However, white-rot fungi produce lignocellul-olytic enzymes with different susceptibility against proteolytic attack and also proteases that can regulate the laccase activity by degrading and/or activating different isoenzymes (Palmieri et al. 2001; Hilden, Hakala and Lundell 2009). This could explain the differences of laccase activity shown by Fomes sp. EUM1 at elevated temperatures.

Zymogram analysis showed the secretion of new isoen-zymes of xylanase and cellulase as a response to thermic stress (>30°C) (Figs 3 and 4). In preliminary studies, the same isoenzyme pattern was found between 30°C and 35°C. Therefore, studies focused on the isoenzyme pattern at elevated tempera-tures. In fact, four bands with xylanase activity were de-tected (Fig. 3): XyI (130 kDa), XyII (120 kDa), XyIII (95 kDa) and XyIV (120 kDa). XyII and XyIII isozymes were secreted at all growth temperatures, while XyI appeared only at 30°C and XyIV only at 45°C. Multiple cellulases were secreted (Fig. 4): CI (197 kDa), CII (80.5 kDa), CIII (27.5 kDa), CIV (24 kDa), CV (21.3 kDa) and CVI (152 kDa). CV and CVI isoenzymes appeared at growth temperatures above 30°C. Zymograms of laccase activity showed only one isoenzyme (46 kDa) (Fig. 5). Normally, white-rot fungi expressed xylanases with molecular weights of <100 kDa and cellulases ranging from 30 to 100 kDa (Quiroz-Castañeda et al. 2009; Gutiérrez-Soto et al. 2015). The apparent molecular weights for isoenzymes secreted by Fomes sp. EUM1 were higher and also multiple isoenzymes were observed. This ther-motolerant strain expressed multiple isoenzymes in response to thermic stress, although post-transcriptional modifications
Figure 3. Zymogram analysis for xylanase activity. Gels were loaded with 0.5 mU of xylanase activity and stained with Congo red. Four bands with xylanase activity XyI (130 KDa), XyII (120 KDa), XyIII (95 KDa) and XyIV (120 KDa) were detected during the growth of Fomes sp. EUM1 in solid culture at several temperatures. Xylanase bands were detected at 50°C.

Figure 4. Zymogram analysis for cellulase activity. Gels were loaded with 0.5 mU of cellulase activity and stained with Congo red. Six bands with cellulase activity CI (197 KDa), CII (80.5 KDa), CIII (27.5 KDa), CIV (24 KDa), CV (21.3 KDa) and CVI (152 KDa) were detected during the growth of Fomes sp. EUM1 in solid culture at several temperatures. Cellulase bands were detected at 50°C.

Figure 5. Zymogram analysis of laccase enzyme. A single isoenzyme of 46 kDa was detected during the growth of Fomes sp. EUM1 in solid culture at several temperatures. Laccase bands were detected at 40°C.

could be present in those isoforms (Hernández-Martínez et al. 2011). Most ligninolytic fungi produce at least one laccase in SSF cultures; in fact Fomes sclerodermus secretes three laccase isoenzymes (Papinutti, Dimitriu and Forchiassin 2008). Most white-rot fungi express several laccases with molecular weights between 30 and 70 kDa (Baldrian 2006), and culture conditions play a key role in laccase patterns (Viswanath et al. 2014). The reports describing the laccase isoenzymes of the genus Fomes are scarce.

Thermostolerance of Fomes sp. EUM1 has important implications in large-scale SSF, for instance, in tray reactors where fungal growth and enzyme production are severely affected by the formation of high temperature gradients. The thermostolerant strain modified the mycelial morphology for achieving the maximum assimilation of nutrients at elevated temperatures. In addition, significant levels of enzymes were secreted in shorter times under adverse thermal conditions in which the strain secreted multiple isoenzymes.
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Conflict of interest. None declared.

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