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ORIGINAL PAPER

Evaluation of the white-rot fungi *Ganoderma australe* and *Ceriporiopsis subvermispora* in biotechnological applications

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Abstract *Ganoderma australe* is a white-rot fungus that causes a selective wood biodelignification in some hardwoods found in the Chilean rainforest. *Ceriporiopsis subvermispora* is also a lignin-degrading fungus used in several biopulping studies. The enzymatic system responsible for lignin degradation in wood can also be used to degrade recalcitrant organic pollutants in liquid effluents. In this work, two strains of *G. australe* and one strain of *C. subvermipora* were comparatively evaluated in the biodegradation of ABTS and the dye Poly R-478 in liquid medium, and in the pretreatment of *Eucalyptus globulus* wood chips for further kraft biopulping. Laccase was detected in liquid and wood cultures with *G. australe*. *Ceriporiopsis subvermispora* produce laccase and manganese peroxidase when grown in liquid medium and only manganese peroxidase was detected during wood decay. ABTS was totally depleted by all strains after 8 days of incubation while Poly R-478 was degraded up to 40% with *G. australe* strains and up to 62% by *C. subvermispora* after 22 days of incubation. *Eucalyptus globulus* wood chips decayed for 15 days presented 1–6% of lignin loss and less than 2% of glucan loss. Kraft pulps with kappa number 15 were produced from biotreated wood chips with 2% less active alkali, with up to 3% increase in pulp yield and up to 20% less hexenuronic acids than pulps from undecayed control. Results showed that *G. australe* strains evaluated were not as efficient as *C. subvermispora* for dye and wood biodegradation, but could be used as a feasible alternative in biotechnological processes such as bioremediation and biopulping.

Keywords *Ganoderma australe* · *Ceriporiopsis subvermispora* · *Eucalyptus globulus* · Dye biodegradation · Biopulping

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Introduction

Fungi are the main organisms responsible for wood biodegradation and some species of basidiomycetes, the white-rot fungi, are able to efficiently degrade lignin and carbohydrates. In some cases, these fungi presented a preferential attack of lignin in the initial period of wood decay leaving cellulose relatively intact [1, 2]. In order to degrade lignin, these fungi produced extracellular oxidative enzymes such as laccase, lignin peroxidase (LiP), manganese peroxidase (MnP) and also low molecular mass compounds that mediate the action of these enzymes [3–5]. This non-specific oxidative system makes white-rot fungi useful for a wide range of biotechnological applications, for instance, in the pulp and paper industry for pitch control, biopulping or biobleaching [6–9] and to degrade recalcitrant compounds such as aromatic hydrocarbons, aromatic dyes and other pollutants from cellulose and textile industries [10–12]. In this work, we focused the use of white-rot fungi on two of these applications, biopulping and dye biodegradation.

Biopulping is the wood pretreatment with selected white-rot fungi to modify wood and lignin, with the aim to facilitate fiber separation and lignin removal in mechanical or chemical pulping process, respectively. The list of benefits reported using this biotreatment includes energy savings during defibration/refining steps, increase in delignification rate, decrease of alkali consumption, decrease in hexenuronic acids content in kraft pulps, and improvement of pulp strength properties [6, 9, 13–17]. A 50-ton pilot-plant built in Madison (WI, USA) in the middle 90s evaluated the technical and economical feasible of the technology in a termomechanical pulping (TMP) trial with Loblolly pine wood chips [6, 18]. Recently, a pulp mill in Brazil also evaluated biopulping in a 50-ton pilot-plant for *Eucalyptus grandis* biotreatment and TMP and CTMP (chemithermomechanical pulping) processing of decayed wood using the industrial facilities [17]. Results obtained in this trial regarding energy savings were similar to the previous laboratory-scale studies and accounted for 18 and 27% in biologically-assisted TMP and CTMP, respectively. In both of the cited trials, the white-rot fungus used was *Ceriporiopsis subvermispora*.

On the other hand, several studies on biodegradation of compounds with varied chemical complexity such as azo, anthraquinone, heterocyclic, triphenylmethane and phthalocyanine dyes, showed the ability of the white-rot fungi and their oxidative enzymes to act as important agents in breakdown of colored pollutants [19–23]. In these cases, several fungi were selected and evaluated in different culture conditions for dye degradation and effluent decoloration.

Using the approach to select and evaluate fungal strains that present ligninolytic activity and could be useful for some biotechnological application, this work reports our efforts to perform a basic and comparative study among native Chilean strains of *Ganoderma australe*, a fungus that was previously reported as being the main agent responsible for a natural phenomenon of selective delignification of some hardwoods trees, known as "palo podrido" (rotted log) [24–26] and the well-known lignin-degrading fungus, *C. subvermispora*. To achieve this goal, two strains of *G. australe* and one of *C. subvermispora* were evaluated in the biodegradation of the dye Poly R-478 and in kraft biopulping of *Eucalyptus globulus*.

Material and methods

White-rot fungi

Ganoderma australe (Fr.) Pat. (strains A-464 and A-466) and *C. subvermispora* (Pilát) Gilb. & Ryv. (strain CS-1) were from the culture collection of the Laboratory of Renewable Resources, Biotechnology Center, Universidad de Concepcion, Chile. *Ganoderma australe* strains were originally isolated by Dr. Aldo González (CIB-CSIC, Madrid, Spain) from fruiting bodies found in decayed hardwoods in the Southern Chilean rainforest and *C. subvermispora* was kindly donated by Dr. André Ferraz (EEL-USP, Lorena, Brazil). Strains were maintained at 4 °C on solid medium containing 2% malt extract (Merck KGaA), 0.5% soy peptone (Sigma-Aldrich Co.) and 2% agar (Difco Laboratories).

Dye biodegradation and determination of oxidative enzymes in static liquid cultures

Dye biodegradation and enzymatic assays were carried out in 100-mL Erlenmeyers flasks with 40 mL of MEPG (malt extract, peptone and glucose) liquid medium composed of 5 g L^{-1} malt extract (Merck KGaA), 5 g L^{-1} soy peptone (Sigma-Aldrich Co.), 10 g L^{-1} glucose (Merck KGaA), 1 g L^{-1} KH_2PO_4 (Merck KgaA) and 0.5 g L^{-1} $MgSO_4$ (Merck KGaA) (MEPG medium). The Erlenmeyer flasks with the medium were sterilized by autoclaving at 121 °C for 15 min prior to fungal inoculation or dye addition. After cooling, the flasks were inoculated with two 4-mm diameter mycelium plugs of solid medium pre-cultured with the fungi and with 1% (w v^{-1}) ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] (Sigma-Aldrich Co.) or 0.4% (w v^{-1}) Poly R-478 (anthrapyridone chromophore) (Sigma-Aldrich Co.). The flasks were incubated without agitation at 25 °C for up to 22 days (experiments running in triplicate for each treatment and period of time). Sampling was performed at regular periods by withdrawing 1 mL of liquid medium (in duplicate) from each of the three flasks prepared with the cultures. UV absorbances of the centrifuged solutions (5 min at 13,500 rpm) were measured at 420 and 520 nm for ABTS and Poly R-478, respectively [27]. After 8 days of biodegradation of ABTS and 22 days of biodegradation of Poly R-478 (periods after which no significant change in UV absorbance of liquid medium were observed), the culture was filtered and final pH of the solution was determined. Fungal biomass was washed with water and dried at 55 °C for 24 h for gravimetric determination of biomass. Liquid cultures without addition of the dye, but inoculated with the fungi were prepared for enzymes [laccase and manganese-dependent peroxidase (MnP)] quantification in the same time-course of biotreatment. Laccase was determined by ABTS (Sigma-Aldrich Co.) oxidation according to Souza-Cruz et al. [28] and MnP was determined by 2,6-DMP (2,6-dimethylphenol) (Sigma-Aldrich Co.) oxidation according to Heinzkill et al. [29].

Wood biodegradation and determination of enzymes

Wood chips from 10 to 12-year-old *E. globulus* Labbil. were obtained from a local pulp mill, air-dried until 10%

moisture and stored in dry conditions. Before fungal inoculation, wood chips were immersed in water for a 16-h period. Residual water was drained and 60 g (dry basis) of moist wood chips (55% moisture) were sterilized in 20-L bioreactors at 121 °C for 20 min. Sterilized liquid-medium (200 mL) composed of 2% (w v^{-1}) malt extract (Merck KGaA) and 0.5% (w v^{-1}) soybean peptone (Sigma-Aldrich Co.) was inoculated with 20 discs (4 mm in diameter) of *G. australe* or *C. subvermispora* pre-cultured solid medium. These liquid cultures were incubated under stationary conditions for 10 days at 27 °C. The grown mycelium mat was filtered and washed with 300 mL of sterilized water. Washed mycelium obtained from several cultures was blended in laboratory blender with sterilized water in three cycles of 15 s. The mycelial suspension was used to inoculate the sterilized wood chips with a volume corresponding to 700 mg of fungal mycelium per kilogram of dry wood. The inoculated wood chips were incubated at 27 °C and 55% of relative humidity in an acclimatized room and maintained unshaken for 15, 30, 45 and 60 days. Bioreactors with sterilized but non-inoculated wood were also prepared for use as control samples. Three bioreactors were prepared for each period of time evaluated. After each biodegradation period, bioreactors were opened and the wood chips were transferred to a 500-mL Erlenmeyer. Wood chips were extracted three times with 200 mL of sodium acetate buffer (50 mM, pH 5.5), supplemented with 0.01 g L^{-1} Tween 60 (Sigma-Aldrich Co.). Each extraction was performed in a shaker for 6 h at 120 rpm and at 4 °C according to Souza-Cruz et al. [28]. After each extraction, the liquid fraction was drained from the wood chips and aliquots were removed, centrifuged and used for enzymatic assays. Laccase and MnP were measured by the same procedure described earlier [28, 29]. Cellulases and xylanases were measured according to procedures described by Mandels et al. [30] and Bailey et al. [31], respectively.

Wood characterization and kraft pulping

Buffer-extracted wood chips were air-dried until 10–12% moisture. The initial and final dry weights were used to determine the weight loss due to fungal biotreatment. Milled wood samples (40/60 mesh) were extracted with ethanol/toluene (Sigma-Aldrich Co.) according to TAPPI standard method 204 cm-97. Wood and pulp samples were characterized for total lignin, glucan and xylan according to methodology described by Ferraz et al. [32]. A sample (300 mg) was weighed in a test tube and 3 mL of 72% (w w^{-1}) H_2SO_4 was added. The hydrolysis was carried out in a water bath at 30 °C for 1 h with stirring every 10 min. Later, the acid was diluted to 3% (w w^{-1}) with 79 mL of distilled water and the mixture was transferred to a 250-mL Erlenmeyer flask and autoclaved for 1 h at 121 °C as a post-hydrolysis treatment. The residual material was cooled and filtered through a number 3 sintered-glass filter. Solids were dried to constant weight at 105 °C and determined as insoluble lignin. Soluble lignin was determined by measuring the absorbance of the solution at 205 nm. The concentration of monomeric sugars in the soluble fraction was determined in an HPLC equipment (Merck Hitachi) with an Aminex HTX-87H column at 45 °C, eluted at 0.6 mL min^{-1} with 5 mM H_2SO_4 and using a refractive index detector. The standard deviation from the average values was lesser than 3%. Component losses were calculated on basis of wood composition from undecayed and decayed wood chips.

Eucalyptus globulus kraft pulps were produced by cooking the wood chips of untreated and 15-day biotreated wood chips by *C. subvermispora* and *G. australe* strains. Each pulping was carried out inside an oil bath and in an 80-mL reactor with 10 g of wood chips (dry basis), 6:1 liquor wood ratio, 165 °C pulping temperature and 120 min reaction time at the temperature. White liquor composition was 14, 17 or 20% active alkali and 25% sulfidity in each case. Pulps were disintegrated in a TAPPI laboratory blender, thoroughly washed with tap water and centrifuged. Total pulp yield was determined based on the weight of the pulp divided by the weight of the wood chips (both on a dry basis). Kappa number was determined following the standard procedure described in the TAPPI test method T 236 om-99. Hexenuronic acids were quantified by an UV-spectrophotometric method [33].

Pulps produced at kappa number 15 ± 1 were bleached using an elemental chlorine free sequence, D_1ED_2 (where D represents the chlorine dioxide oxidation stages and E the alkaline extraction with aqueous NaOH). Each of chlorine dioxide step (D_1 and D_2) was performed in sealed plastic bags with pulp at 10% consistency, 1% ClO_2 (pulp basis), 3 mL H_2SO_4 98%, at a temperature of 60 °C for 1 h. Between the D_1 and D_2 stages, pulps were extracted with 100 mL of 2% NaOH at 60 °C for 1 h. Kappa number, pulp brightness and viscosity were measured by following TAPPI standard procedures T 236 om-99, T 525 om-86 and T 230 om-04, respectively.

Results and discussion

Dye biodegradation

The effect of the extracellular oxidative system of the white-rot fungi was evaluated in the biodegradation of ABTS and Poly R-478. Figure 1a, b shows the results obtained for the degradation of ABTS and Poly R-478, respectively, in MEPG liquid medium, incubated in stationary conditions. *Ganoderma australe* A-464 and A-466 strains were comparatively evaluated with *C. subvermispora*

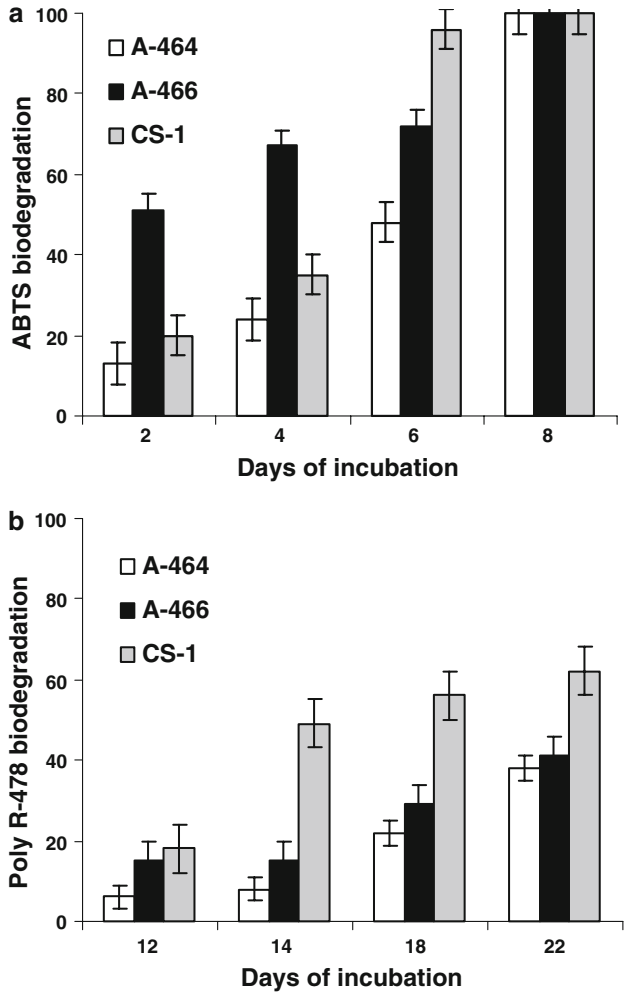

Fig. 1 **a** ABTS and **b** Poly R-478 biodegradation by *G. australe* (*A-464* and *A-466*) and *C. subvermispora* (*CS-1*) strains in liquid MEPG medium. *Errors bars* denote the standard deviation of three replicates for each experimental point

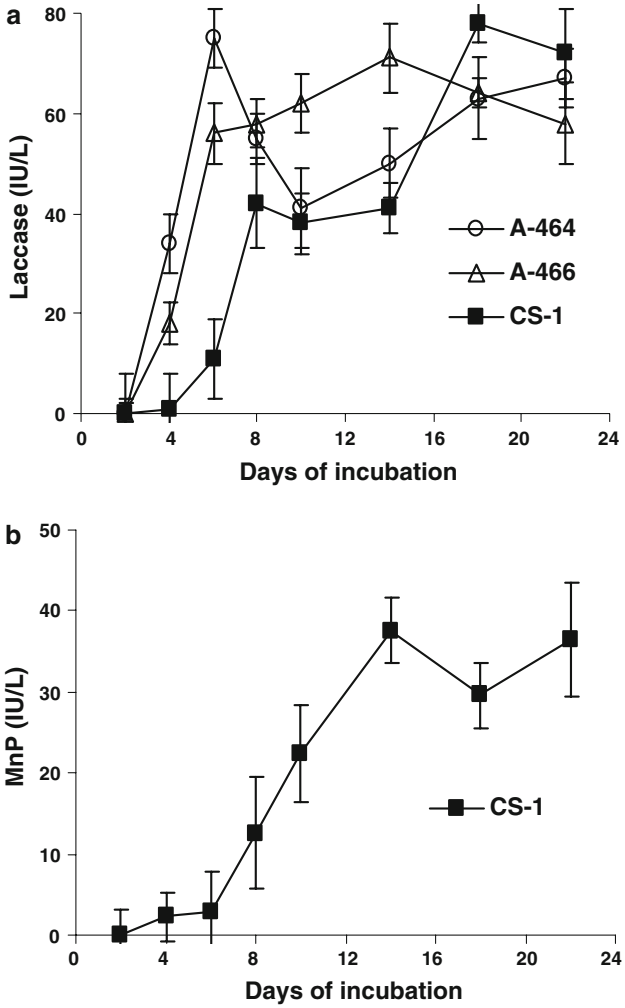

Fig. 2 **a** Laccase and **b** manganese-dependent peroxidase production by *G. australe* (*A-464* and *A-466*) and *C. subvermispora* (*CS-1*) strains in liquid MEPG medium. *Errors bars* denote the standard deviation of three replicates for each experimental point

CS-1 strain. ABTS was totally depleted by the three strains after 8 days of incubation. Poly R-478 was a more recalcitrant compound and Fig. 1b shows that after 22 days of incubation, 62% of Poly R-478 degradation was achieved with the *C. subvermispora* strain. *Ganoderma australe* strains were able to degrade only 40% of Poly R-478 in the same period.

The quantification of enzymes produced in parallel assays during the same incubation period showed that *G. australe* produced only laccase while *C. subvermispora* strains produced both laccase and manganese-dependent peroxidase (Fig. 2). *Ceriporiopsis subvermispora* is well-known for producing MnP as main ligninolytic enzyme [34, 35] with laccase only detected when the fungus was grown in presence of easily assimilable carbon and nitrogen sources [36, 37], such as in the case of the liquid medium used in the present work. Apparently, the amount of laccase produced by both the fungi was enough for an efficient degradation of ABTS, while the MnP produced by *C. subvemispora* provided additional oxidative potential that increased Poly R-478 degradation with this fungus over *G. australe* strains.

Fungal biomass produced after ABTS biodegradation was approximately 570 and 950 mg in cultures with *G. australe* and *C. subvermispora*, respectively. After the biodegradation period of Poly R-478, the fungal biomass of *G. australe* and *C. subvermispora* was up to 640 and 500 mg, respectively. The final pH of the liquid cultures was 4.1–4.5 indicating that both the strains acidified the medium during dye biodegradation (the initial pH of the MEPG medium was 5.5).

Biopulping of *Eucalyptus globulus*

Chemical composition of undecayed wood chips of *E. globulus* was: 54 ± 1% glucan, 14.5 ± 0.3% xylan,

21.9 ± 0.2% lignin, 4.6 ± 0.2% methylglucuronic acid, 1.9 ± 0.3% acetyl groups and 1.2 ± 0.1% extractives. Figure 3 shows the weight and component losses after different biotreatment periods with *G. australe* and *C. subvermispora* strains. The profile of biodegradation of *E. globulus* by *G. australe* A-464 and A-466 shows a classic white-rot decay pattern where all components are decayed at almost the same rate with no preferential degradation of lignin over glucan. Elissetche et al. [26, 38], using *G. australe* A-464 strain in biodegradation experiments of *Drymis winteri* (a hardwood), also observed a simultaneous degradation pattern of wood components with no preferential lignin decay. In the case of the biotreatment of *E. globulus* with *C. subvermispora*, higher degradation of lignin than glucan was observed over all the periods of biodegradation evaluated, indicating a more selective pattern of biodelignification. Maximum lignin mineralization was 10–13% for *G. australe* strains and 16% for *C. subvermispora* after 60 days of incubation. However, previous studies [16, 39] had demonstrated that lignin modifications caused by white-rot fungi (particularly by *C. subvermispora*), such as decrease of molar mass and cleavage of aryl-ether linkages, are more important than lignin mineralization for the benefits observed in kraft biopulping of pine wood chips.

Figure 4 shows hydrolytic (cellulases and xylanases) and oxidative (laccase and MnP) enzymes detected in the buffer solution used for extraction of the decayed wood chips. Cellulase activity was in the range of 30–40 IU kg^{-1} wood decayed by *G. australe* and it was less than 20 IU kg^{-1} wood decayed by *C. subvermispora*. Xylanase activity was detected only after 15 days of *E. globulus* biodegradation and values were similar for *G. australe* and *C. subvermispora* strains, 50 IU kg^{-1} wood with 15 days and up to 550 IU kg^{-1} wood after 60 days of biotreatment.

Ganoderma australe strains produced only laccase during biodegradation of *E. globulus* with values reaching 60 IU kg^{-1} of wood within 15 days of biodegradation. With the increase of the biodegradation period, the amount of laccase produced by *G. australe* decreased to 20–30 IU kg^{-1} wood (after 45–60 days of incubation). Only MnP was detected in *E. globulus* decayed by *C. subvermispora* with values of 500 IU kg^{-1} wood after 15 days of biodegradation up to 1,000 IU kg^{-1} wood after 60 days of incubation. As previously reported, *C. subvermispora* did not show laccase activity during wood biodegradation of hardwood or softwood when there was no addition of carbon and nitrogen sources to the culture medium [28, 40].

Undecayed control and wood chips, biotreated for 15 days, were submitted to kraft delignification under the conditions previously described. Short biotreatment period was chosen based on the previous work that showed that an extensive removal of lignin is not needed to observe the significant benefits of biopulping process [9, 15]. Different active alkali concentration in the cooking liquor were evaluated and adjusted to produce bleachable grade kraft pulps

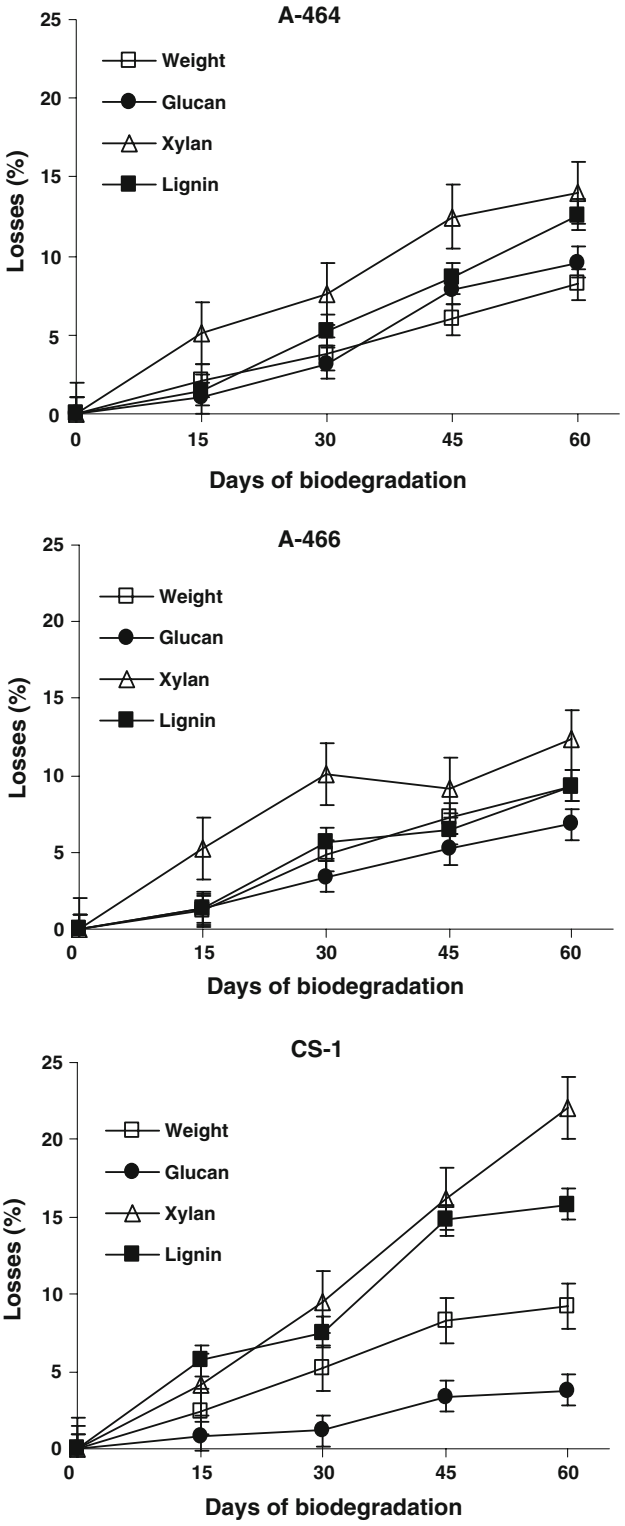

Fig. 3 Weight and component losses during biodegradation of *E. globulus* by *G. australe* (*A-464* and *A-466*) and *C. subvermispora* (*CS-1*) strains under biopulping conditions. *Errors bars* denote the standard deviation of three replicates for each experimental point

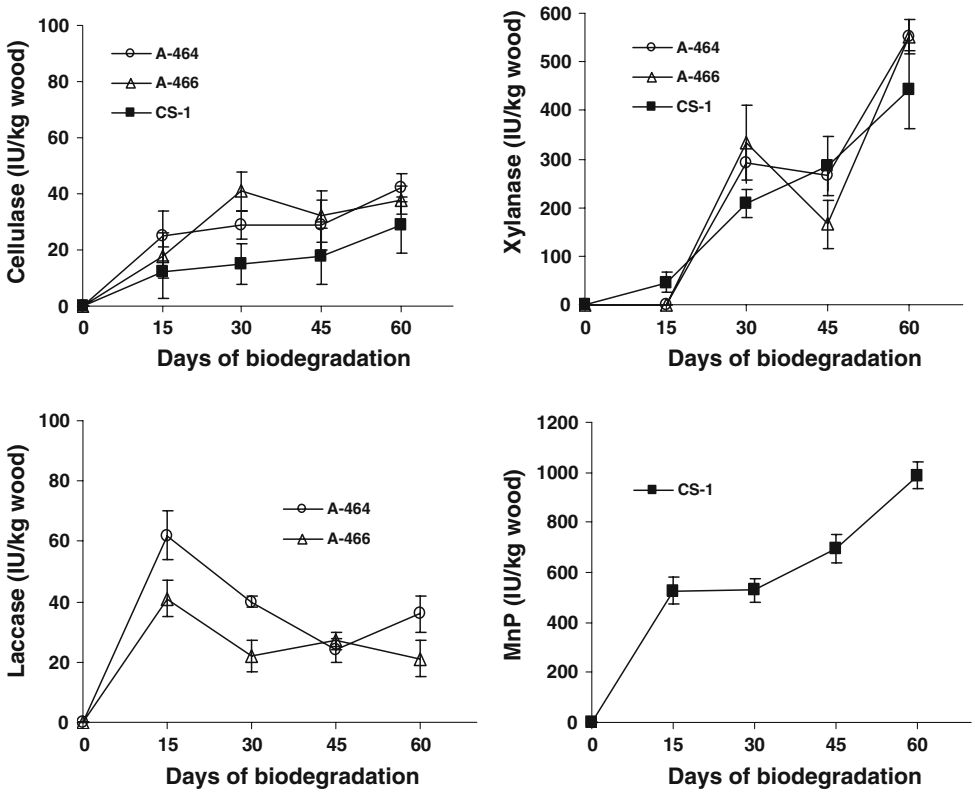

Fig. 4 Hydrolytic and oxidative enzymes produced during biodegradation of *E. globulus* by *G. australe* (*A-464* and *A-466*) and *C. subvermispora* (*CS-1*) strains under biopulping conditions. *Error bars* denote the standard deviation of three replicates for each experimental point

Table 1 Kraft biopulping of *E. globulus* decayed for 15 days by *G. australe* (A-464 and A-466) and *C. subvermispora* (CS-1) strains

Strains	Active alkali (%)[a]	Pulp yield (%)[b]	HexA (mmol/kg)[b]	Brightness (% ISO)[c]	Viscosity (mPa.s)[c]
Control	17[d]	53[d]	32[d]	80[d]	18[d]
A-464	15	53[d]	30[d]	80[d]	19[d]
A-466	15	56	29[d]	81[d]	18[d]
CS-1	15	56	25	84	24

[a] Alkali charge needed to produce pulps with kappa number 15

[b] Pulp characteristics at kappa number 15

[c] After D_1ED_2 bleaching sequence

[d] Values in a column were similar to those of control at 95% confidence level (Dunnet test)

with a kappa number of 15. Table 1 shows the results obtained for kraft control and bio-pulps produced from wood chips decayed by *G. australe* and *C. subvermispora* strains. As a result of the fungal treatment, bio-pulps were produced with 2% less active alkali in kraft white liquor and pulp yield was similar or up to 3% higher than that observed for control pulps. The increase in pulp yield could be a result of the reduced active alkali charge used to process biotreated wood. Hexenuronic acids were also lower or similar in biopulps than in control pulps, possibly due to some biodegradation of methylglucuronic acid attached to the xylan backbone, as previously published by Franco et al. [15] and Mendonça et al. [16]. Values for brightness and viscosity of bleached *C. subvermispora*-biopulps were significantly higher than that observed for control and *G. australe* pulps. Despite the effects observed from the use of biotreated wood chips for production of low-kappa pulps, the results were not as impressive as that obtained when biopulping was directed toward the production of high yield or thermomechanical pulps [6, 9, 14, 17].

As a conclusion, we found that the Chilean *G. australe* strains evaluated in this work were not as efficient as *C. subvermispora* for Poly R-478 or lignin biodegradation and some process optimization is needed to use this fungus as an alternative for some biotechnological applications such as biopulping and dye biodegradation.

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