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Full Length Research Paper

Autochthonous fungal strains with high ligninolytic activities from Tunisian biotopes

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This work represents the first report on the ability of autochthonous fungi of Tunisia to produce ligninolytic enzymes. Three hundred fifteen fungal strains were isolated from different Tunisian biotopes. These fungal strains were first screened for lignin-modifying enzymes on solid media containing Poly R-478 or ABTS. Of the 315 tested strains, 49 exhibited significant ABTS-oxidation activity, expressed within the first week of incubation and only 18 strains decolourised the Poly R-478. These positive strains were further screened in liquid culture and laccase, and lignin and Mn^{2+}-oxidizing peroxidases activities were assayed. Of the 67 strains grown on liquid medium, 28 produced at least one of these 3 enzymes. The 8 highest producers of ligninolytic activities were identified by molecular techniques and 3 among them produced Lac, MnP and LiP simultaneously. New isolates reported in this work as fungi with significant ligninolytic activities includes *Oxyporus*, *Stereum* and *Trichoderma*. The isolated *Trametes trogii* CTM 10156 was the best Lac producer. Culture conditions and medium composition were optimised for this strain and resulted in high Lac production of 110 U ml^{-1} within 15 days of incubation (367 times higher than control medium).

Key words: Screening, autochthonous fungi, white rot fungi, ligninolytic enzymes, peroxidases, laccases.

INTRODUCTION

Fungi are recognized for their superior aptitudes to produce a large variety of extra-cellular enzymes. The organisms principally responsible for lignocellulose degradation are aerobic filamentous fungi, and the most rapid degraders in this group are basidiomycetes (Kirk and Farrell, 1987). Wood-rotting basidiomycetous fungi are usually divided into white-rot, brown-rot and litter-decomposing fungi (Steffen, 2003). The only organisms capable of mineralising lignin efficiently are basidiomycetous white rot fungi and related litter-decomposing fungi (Hatakka, 2001).

The white rot fungi (WRF) belonging to the basidiomycetes produce various isoforms of extracellular ligninolytic enzymes: laccases (Lac) and different peroxidases, including lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP), the latter sharing LiP and MnP catalytic properties (Martínez, 2002). These enzymes are directly involved not only in the degradation of lignin in their natural lignocellulosic substrates but also in the degradation of various xenobiotic compounds including dyes (Wesenberg et al., 2003). Some WRF produce all the three lignin modifying enzymes while others produce only one or two of them. Lignin modifying enzymes are produced by WRF during their secondary metabolism

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since lignin oxidation provides no net energy to the fungus (Eggert et al., 1996). The synthesis and secretion of these enzymes is often induced by limited carbon or nitrogen levels. MnP and LiP production is generally optimal at high oxygen tension but is repressed by agitation in submerged WRF liquid culture, while Lac production is often enhanced by agitation, aromatic compounds or organic solvent (Galhaup et al., 2002) and VP, described initially as a Mn^{2+}-oxidizing enzyme with ability to oxidize veratryl alcohol, is expressed in aerated cultures, without oxygen supplement, and shaken conditions (Martínez et al., 1996).

WRF and their enzymes are being studied for their application in the degradation of aromatic pollutants causing environmental problems like pulp and paper mills (Machii et al., 2004), olive mill wastewater (Jaouani et al., 2003), polycyclic aromatic hydrocarbons (PAHs) (Clemente et al., 2001), chlorinated phenols, polychlorinated biphenyls (Sato et al. 2002), dioxins, pesticides, explosives and dyes (Wesenberg et al., 2003; Levin 2003, 2004). Frequently, more than one isoforms of ligninolytic enzymes are expressed by different taxa and culture conditions. These features are important in the process design and optimisation of fungal treatment of effluents. Purified Lac, LiP and MnP are potential enzymes for various industrial applications (Ahuja et al., 2004; Ikehata et al., 2004).

Recently however there has been a growing interest in studying the lignin-modifying enzymes of a wide array of WRF, not only from the standpoint of comparative biology but also with the expectation of finding better lignin-degrading systems for use in various biotechnological applications. There is little information available as regards the production of extracellular oxidoreductases by autochthonous fungal strains belonging to different ecophysiological and taxonomic groups. Thus, the aim of our work is i) to isolate fungi from different Tunisian biotopes and from ecophysiological and taxonomic groups, ii) to screen these fungal strains for ligninolytic activities and iii) to select high producer strains of white-rot fungi and to optimise cultivation conditions supporting high Lac yields.

MATERIALS AND METHODS

Fungi isolation

Fungi fruiting bodies were collected essentially in the forest of the north of Tunisian. Some others were collected from central regions having semi-arid climate. Samples of wet decayed wood, litter, and soil were collected especially in zones colonized by indigenous trees as oak cork and olive. Samples of compost locally made from agriculture waste were also used. Three hundred fifteen fungus strains were isolated from these different Tunisian biotopes and were preserved as pure culture in the “Centre de Biotechnologie de Sfax” culture collection (CTM). The fungi were grown and isolated in agar-malt medium and examined by light microscopy to check the absence of bacteria and the unique fungi isolation.

Primary screening method on solid medium

As an initial screening method for detecting the ability of the fungal strains to produce lignin-modifying enzymes, we adopted the dye-decolourising method. For global lignin-modifying activities, screening was performed in Petri dishes (60 mm diameter) with 15 ml of (PMM) medium containing per litre of medium: 2 g of glucose, 2 g of ammonium tartrate, 2 g of malt extract, 0.26 g of KH_2PO_4, 0.26 g of Na_2HPO_4, 0.5 g of $MgSO_4.7H_2O$, 0.01 g of $CuSO_4.5H_2O$, 0.0066 g of $CaCl_2.2H_2O$, 0.005 g of $FeSO_4$, 0.0005 g of $ZnSO_4.7H_2O$, 0.02 mg of Na_2MoO_4, 0.09 mg of $MnCl_2.2H_2O$, 0.07 mg of H_3BO_3, 0.1 g of Poly R-478, and 20 g of agar-agar. The pH was adjusted to 5.5 before autoclaving at 121°C for 20 min. The Poly R-478 dye (Sigma) turns from purple to yellow colour when it is decolourised by lignin-degrading fungi.

The ability of the fungal strains to produce extracellular ABTS-oxidizing activity was performed in Petri dishes (60 mm diameter) with 15 ml of (AMM) medium which has the same composition as PMM medium except that the 0.1 g of Poly R-478 was replaced by 0.35 g of 2,2’-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Sigma). The chromogen ABTS is a very sensitive substrate that allows a rapid screening of fungal strains producing extracellular ABTS-oxidising enzymes by means of a colour reaction (Niku-Paavola et al., 1988).

Plates were inoculated with agar disks (0.5 mm diameter) of active mycelia previously cultured in 2% malt extract agar. Each strain was processed on AMM and on PMM media; the plates were incubated at 30°C for 1-30 days.

Any colony that turned to yellow or decolourised the Poly R-478 was considered as ligninolytic positive and was consequently selected. Colonies that showed green halo on AMM medium that exceeded the colony diameter were considered as ABTS-oxidizing activity producer.

Screening in liquid media

Decolourising Poly R-478 and ABTS-oxidising strains were screened on liquid culture for LiP, Mn^{2+}-oxidizing peroxidases (MnP or VP), and Lac activities production. For Lac activity determination, the isolated fungi were grown at 30°C for 12 days with rotary shaking (150 rpm) in 500-ml baffled-erlenmeyer flasks containing 50 ml of a (LMM) medium composed of: glucose (10 g l^{-1}), ammonium tartrate (2 g l^{-1}), KH_2PO_4 (1 g l^{-1}), $MgSO_4.7H_2O$ (0.5 g l^{-1}), KCl (0.5 g l^{-1}), yeast extract (1 g l^{-1}), Soy-tone (5 g l^{-1}), $CuSO_4.5H_2O$ (150 µM), and 10 mg l^{-1} of trace elements containing per litre of distilled water: EDTA (0.5 g), $FeSO_4$ (0.2 g), $ZnSO_4.7H_2O$ (0.01 g), $MnCl_2.4H_2O$ (0.003 g), H_3BO_4 (0.03 g), $CoCl_2.6H_2O$ (0.02 g), $CuCl_2.2H_2O$ (0.001 g), $Na_2MoO_4.2H_2O$ (0.003 g).

For LiP and Mn^{2+}-oxidizing activities determination, the isolated fungi were grown at 30°C for 12 days in static conditions in 500-ml erlenmeyer flasks containing 50 ml of a (SMM) medium composed of: glycerol (10 g l^{-1}), ammonium tartrate (1.84 g l^{-1}), sodium tartrate (2.3 g l^{-1}), KH_2PO_4 (2 g l^{-1}), $MgSO_4.7H_2O$ (0.7 g l^{-1}), $CaCl_2.2H_2O$ (0.14 g l^{-1}), $FeSO_4.7H_2O$ (0.07 g l^{-1}), $ZnSO_4.7H_2O$ (0.046 g l^{-1}), $MnSO_4.7H_2O$ (0.035 g l^{-1}), $CuSO_4.5H_2O$ (0.007 g l^{-1}), thiamine (0.0025 g l^{-1}), yeast extract (1 g l^{-1}), Veratrylic alcohol (0.067 g l^{-1}) and tween80 (0.5 g l^{-1}) (Sayadi and Ellouz, 1995).

Enzyme assay

Lac activity was determined by the rate of oxidation of 0.5 mM ABTS buffered with 50 mM sodium-tartrate buffer, pH 4.5; the increase of absorption at 420 nm was measured (Eggert et al.,

Table 1. Lac, MnP and LiP activities and identification of the highest oxidoreductase producers screened in liquid culture.

Strain-Ref.	Lac (U/l)	MnP (U/l)	LiP (U/l)	Identification
CTM 10125	19 (7)	182 (8)	-	*Phlebia Sp*
CTM 10133	-	108 (8)	4 (7)	*Oxyporus latemarginatus*
CTM 10136	-	202 (11)	6.5 (2)	*Oxyporus latemarginatus*
CTM 10154	8990 (10)	67.2 (6)	25 (5)	*Trametes trogii*
CTM 10155	7393 (10)	96.7 (2)	35 (4)	*Polyporus Sp*
CTM 10156	9956 (7)	56.5 (7)	5.5 (1)	*Trametes trogii*
CTM 10313	97 (9)	-	-	*Stereum annosum*
CTM 10476	9005 (6)	-	-	*Trichoderma atroviride*

1996). Lignin peroxidase activity was determined using to the veratryl alcohol oxidation assay (Tien and Kirk, 1984).

Manganese-dependent peroxidase was assayed according to Paszcynski et al. (1985) using vanillylacetone as substrate. One unit of enzymatic activity was defined as the amount of enzyme transforming 1 µmol of substrate per minute.

Molecular characterisation of fungal strains

Fungal DNA was extracted from freeze-dried mycelia according to Ruiz-Duenas (1999). PCR amplification of the internal transcribed spacer "ITS" region of the ribosomal DNA was performed using primers ITS1 and ITS4 (White et al., 1990). PCR products were cloned using the pGEM®-T Easy Vector Systems (Promega). Sequences generated during this work were assembled into one complete sequence using Bioedit (Hall, 1999). The consensus sequence was corrected manually for errors and the most homologous sequences were determined against the GenBank database using BLAST (Altschul et al., 1997)

RESULTS

Screening of powerful lignin-degrading strains on solid media

Three hundred fifteen pure fungal strains were tested on solid media to screen for ligninolytic activities. The solid cultures of the fungal strains grown on PMM medium containing Poly R-478 resulted in only 18 positive strains which decolourised the dye after 4 to 30 days incubation.

In the same way, the fungal strains were screened on ABTS containing AMM agar plates. The formation of the dark-green ABTS cation radical indicated a positive extracellular oxidoreductase secretion. The diameter of the halo and the colour intensity were used to monitor the level of ligninolytic enzyme production of each strain (high, medium or weak producer strain). The strain was qualified as high extracellular ABTS-oxidizing activity producer if the dark green coloration appeared in the first week of incubation and the ratio [diameter of the halo/diameter of the colony] is superior to 1.

Of the142 strains exhibiting extracellular ABTS-oxidizing activities, 49 were estimated as super producer and chosen for detailed investigations in the liquid culture.

Screening of powerful lignin-degrading strains on liquid media

The 18 strains decolourising Poly R-478 and the 49 ABTS-oxidizing strains were screened in agitated and static cultures on LMM and SMM media to determine their Laccase and peroxidase activities respectively. Eight of the 67 tested strains in liquid cultures were able to produce significant rate of extracellular Lac, MnP and/or LiP (Table 1). These 8 strains (CTM 10125, CTM 10133, CTM 10136, CTM 10154, CTM 10155, CTM 10156, CTM 10313, and CTM 10476) were identified by molecular techniques using the internal transcribed spacer (ITS) (White et al., 1990).

The highest level of Lac was obtained by the strain CTM 10156 identified as *Trametes trogii* which also produced LiP and MnP. The strain CTM 10136 *Oxyporus latemarginatus* produced the highest rate of MnP, trace of LiP but not Lac. The highest LiP activity was found after 4 days incubation of the strain CTM 10155 (*Polyporus* Sp.) which also produced an appreciate level of Lac and MnP (Table 1).

We noticed also that strains CTM 10154 and CTM 10156 were identified as *Trametes trogii* and they produced Lac broadly at the same level. However, these strains showed different colour pigmentation in old cultures. CTM 10156 gave a blackish colour whereas CTM 10154 was rather sallow. Further molecular investigations will confirm or not if these 2 strains are the same species. Strains *O. latemarginatus* and *Stereum annosum* producing MnP and strain *Trichoderma atroviride* producing Lac are new fungal genera which have not been reported as super ligninolitic enzymes producers.

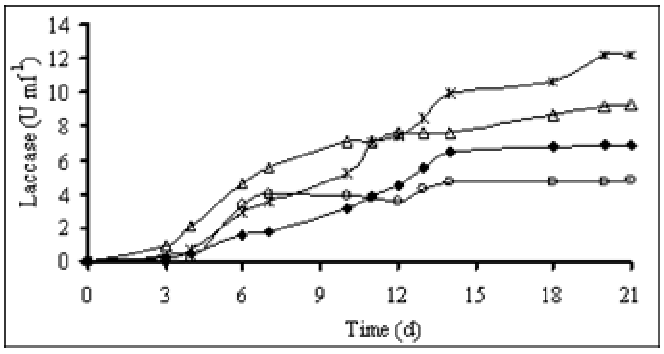

Figure 1. Laccase production by *Trametes trogii* CTM 10156 cultivated on various carbon sources (-∗- Glucose; -•- Wheat bran; -Δ- Gruel; -o- Olive Mill Wastewater) (at 10 g l^{-1} each one).

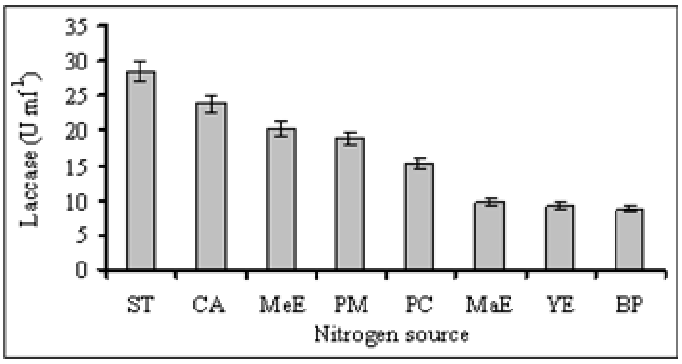

Figure 2. Effect of different nitrogen sources (at 6 g l^{-1} each one) on laccase production by *Trametes trogii* CTM 10156. (ST: Soy tone; CA: Casaminoacid; MeE: Meat extract; PM: peptone of meat; PC: peptone of casein; MaE: Malt extract; YE: yeast extract; BP: Bactopeptone).

Optimisation of laccase production by Trametes trogii CTM 10156

In the second part of this work, we investigated to optimize the culture conditions and medium composition of *T. trogii* CTM 10156 for higher Lac production. Levin et al. (2005) reported that optimal conditions for Lac production by *T. trogii* are 28°C, pH 4-6.5 and 10-20 g l^{-1} of glucose. Our results showed that *T. trogii* CTM 10156 grew better at 33°C while optimal temperature for Lac production was at 30°C. This strain produced Lac at wide spectrum of pH (5-6.5). Glucose, wheat bran, and gruel at 10 g l^{-1}, and olive mill wastewater (10 g l^{-1} of COD equivalent) were tested as carbon sources for extracellular Lac production. Figure 1 showed that *T. trogii* CTM 10156 produced Lac using all these carbon sources with the highest level (12.5 Uml^{-1}) on glucose.

The effect of different complex organic nitrogen sources (casaminoacid, malt extract, meat extract, yeast extract, bactopeptone, soy-tone, peptone of casein, and peptone of meat) (at 6 g l^{-1} each one) on Lac production by *T. trogii* CTM 10156 was assessed (Figure 2). Optimal enzyme production (28.5 Uml^{-1}) was achieved using peptone of soy "Soy-tone" as nitrogen source. On the other hand, the concentration of $CuSO_4$ for Lac production was optimised. Results showed that increased Cu^{++} concentration up to 350 mM led to an increase of Lac production.

Several aromatic compounds were used as inducers such as *p*-coumaric, ferulic, gallic, syringic, protocatechic, veratric, caffeic, and vanillic acids, 4-hydroxybenzaldehyde, catechol, 4-hydroxybenzyalcool, gaïacol, pyrogallol, and veratryl alcohol. These compounds were added to the culture of *T. trogii* CTM 10156 in the 3rd day of incubation at a final concentration of 300 µM. Ethanol (3%) and methanol (3%) and $MnCl_2$ (300 µM) were also tested as potential inducers of the Lac production. Results showed that all the tested compounds did not have any effect on Lac production except ethanol which increased it slightly (3-folds compared to the control) the enzyme production. Synergic effect of ethanol and Cu^{++} on Lac production was observed when they were added together in the *T. trogii* CTM 10156 culture.

In comparison to Lac production in control medium, the simultaneous addition of ethanol (3%) and Cu^{++} (350 µM) led to 240 folds increase of Lac production (Table 2). The production of Lac by *T. trogii* CTM 10156 in the optimized conditions described below (glucose 10 g l^{-1}, Soy-tone 6 g l^{-1}, ethanol (3%), Cu^{++} (350 µM), temperature 30°C, pH 5.5) resulted in a very high production of Lac (367 times higher than control medium). The maximum Lac activity reached after 15 days was 110 Uml^{-1} (Figure 3).

Table 2. Synergic effect of Cu++ and ethanol on laccase production by Trametes trogii CTM 10156

Parameter	Control	Cu^{++} (350 µM)	Ethanol (3%)	Ethanol + Cu^{++} (3% + 350 µM)
Laccase produced (U ml^{-1})	0.3	18	0.9	72

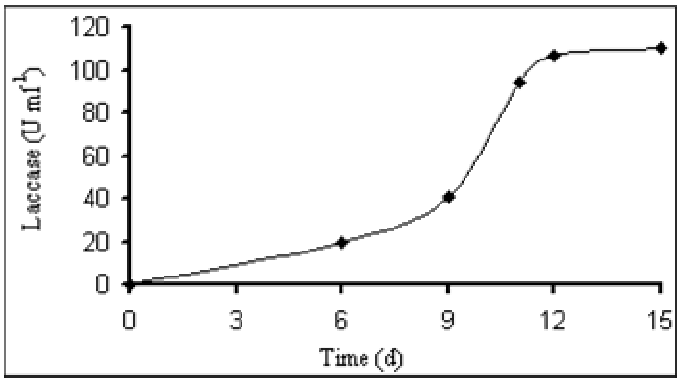

Figure 3. Laccase production by *Trametes trogii* CTM 10156 in optimised conditions.

DISCUSSION

During this study, a rapid screening technique was developed to isolate Tunisian autochthonous fungal flora able to produce oxidative enzymes. Qualitative and quantitative tests allowed us to select 8 powerful strains among 315 screened fungi. There are few published papers studying geographical and climatic repartition of autochthonous fungal flora or describing biodiversity in a given region for ligninolytic activities production. Most of screening surveys for ligninolytic enzymes producers were usually done on culture collection strains such us BCCM/MUCL (Belgium, LPSC (Argentina), INRA (France) (Jaouani et al., 2003; Sayadi and Ellouz, 1993).

Levin et al. (2004) and Saparrat et al. (2002) evaluated the ability of native Argentinean WRF for ligninolytic enzymes and found essentially Lac activity. Muzariri et al. (2001) screened 224 fungal strains from Zimbabwe for ligninolytic and cellulolytic activities. Okino et al. (2000) developed a quick screening method and isolated 116 Brazilian tropical rainforest basidiomycetes expressing respectively laccase and peroxidase activities. Peláez et al. (1995) reported that among 68 species isolated from fruit-bodies of basidiomycetes collected in the Central region of Spain, laccase and Mn^{2+}-oxidizing peroxidase are more common ligninolytic activities that LiP in the studied conditions. De Koker et al. (1998) demonstrated both enzymatic and molecular variation among the 55 *Phanerochaete chrysosporium* strains isolated in South Africa.

In this study, we report the isolation from different Tunisian biotopes novel strains belonging to different taxonomic groups as *Basidiomycota* and *Ascomycota* (Table 1) producing high titres of ligninolytic enzymes. The novel Ascomycete Lac super-producer *Trichoderma atroviride* CTM 10476 (Table 1) was also described recently by Kiiskinen et al. (2004). For the first time, two basidiomycetous strains: *Oxyporus latemarginatus* and *Stereum annosum* were presented in this study as high ligninolytic enzyme producers.

In a second part of this study, the optimization of culture conditions has been achieved for the Lac super producer *T. trogii* CTM 10156. White-rot fungi constitutively produce low concentrations of laccases. Higher production could be achieved by the addition of various aromatic compounds (Eggert et al., 1996; Collins et al.. 1997; Galhaup et al. 2002) but our strain *T. trogii* CTM 10156 appeared to be non-inducible by the addition of such compounds. However, Cu^{++} and ethanol strongly enhanced Lac secretion. The same results concerning the effect of organic solvent such as dimethylsulphoxide or methanol on Lac production was reported by Jones et al. (2001) and Lamascolo et al. (2003). Ligninolytic systems of white-rot fungi are mainly activated during the secondary metabolic phase of the fungus (Hammel, 1997) and are often triggered by nitrogen depletion (Keyser et al., 1978). The nitrogen concentrations had no effect on Lac production by *T. trogii* CTM 10156. Among several complex nitrogen sources, Soy-tone increased the Lac production by 4 folds (Figure 2). In the optimised conditions the maximum Lac activity of *T. trogii* CTM 10156 reached 110 U ml^{-1}, while Levin et al. (2005) reported only 45.32 U ml^{-1} of Lac by *T. trogii* (MYA 28-11).

Many microorganisms and their enzymes have been discovered by means of extensive screening and are now commonly used in industrial applications. The discovery of new microbial enzymes through extensive and persistent screening has brought about many new and simple routes for synthetic processes and provided one possible way to solve environmental problems. Our work contributes also to the knowledge and the determination of the biodiversity of the Tunisian fungal flora. Screening for such enzymes, in combination with the fungi identification, will offer further exciting possibilities for the discovery of new fungal strains producing such enzymes and their industrial use.

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