ACTA MICROBIOLOGICA BULGARICA Volume 35 / 3 (2019)

Fungi Useful for the Purification of the Lignin Fraction from Residues of Bioethanol Production

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Abstract

Lignocellulosic residues, deriving from the production of biofuels, represent a precious source and being able to purify and exploit them is crucial for a more sustainable supply of lignin. The aim of this work was to increase the efficiency of the biological treatment of lignocellulosic residues to obtain purified lignin which can be used in biotechnological applications. The study evaluated the activity of two *Ascomycota* fungal strains on a bioethanol production waste material rich in lignocellulosic residues (LRR). The tested fungi were *Trichoderma asperellum* 1020, isolated from a hydroponic plant culture and *Paecilomyces variotii* 1030, isolated from a sample of lignin purified by chemical methods. The degradation ability of the selected fungi was verified by measuring their consumption of reducing sugars over 3 months, while the purification was assessed by measuring the degradation of reducing sugars and the activity of the secretome (endo-β-1,4-glucanase, β-glucosidase, β-1,3-glucanase, endo-1,4-β-xylanase, pectinase). Both strains appear to use almost all the available reducing sugars in the first 4 days after inoculation and a decrease of the reducing sugars up to 70% was obtained in the samples treated with *T. asperellum* 1020. The quantification of enzymes secreted revealed that *P. variotii* 1030 has greater potential in the degradation of cellulose and pectin, while *T. asperellum* 1020 degrades preferably pectin and carbohydrate residues. These strains act on the components of LRR and are particularly promising to processes lignocellulosic residues.

Keywords: Fungi, *Ascomycetes*, lignin-rich residues, enzymes, biodegradation, bioethanol

Резюме

Лигноцелулозните остатъци, получени от производството на биогорива, представляват скъпоценен източник и възможността за тяхното пречистване и експлоатация е от решаващо значение за по-устойчивото производство на лигнин. Целта на тази работа е да се повиши ефективността на биологичното третиране на лигноцелулозни остатъци за получаване на пречистен лигнин, който може да се използва в биотехнологични производства. Проучена е активността на два щама гъби от отдел *Ascomycota* върху отпадъчен материал, богат на лигноцелулозни остатъци (LRR) за производство на биоетанол. Изследваните щамове са *Trichoderma asperellum* 1020, изолиран от хидропонна растителна култура и *Paecilomyces variotii* 1030, изолиран от проба лигнин, пречистен по химически метод. Способността на избраните щамове да разграждат лигно-целулозни материали се установява чрез определяне консумация на редуциращи захари в продължение на 3 месеца, а степента на пречистване се оценява чрез измерване разграждането на редуциращите захари и активността на секретома (ендо-β-1,4-глюканаза, β -глюкозидаза, β-1,3-глюканаза, ендо-1,4-β-ксиланаза, пектиназа). Двата щама използват почти всички налични редуциращи захари през първите 4 дни след инокулацията, а в пробите, третирани с *T. asperellum* 1020 се установява понижаване количеството на редуциращите захари до 70%. Количественото определяне на секретираните ензими показа, че *P. variotii* 1030 има по-голям потенциал при разграждането на целулозата и пектина, докато *T. asperellum* 1020 разгражда предимно пектиновите и въглехидратните остатъци. Тези щамове действат върху компонентите на LRR и са особено обещаващи за процесите на лигноцелулозни остатъци.

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Introduction

Bioethanol is a biofuel that has received most interest because of the possibility to use it as a replacement of gasoline in the transport fuel market (Lewis, 1996). It can be derived from lignocellulosic materials which are renewable, low cost and abundantly available (Lang *et al*., 2002; Bjerre *et al*., 2006). These materials include crop residues, grasses, sawdust, wood chips, but also rice straw, wheat straw, corn straw and sugarcane bagasse which are the major agricultural wastes in terms of quantity of biomass available (Kim and Dale, 2004). However, the production of bioethanol from lignocellulosic wastes is not able to consume the whole raw material, leaving behind a large amount of residual lignocellulosic waste, whose further exploitation could be improved from the energetic and chemical-industrial point of view, especially for the lignin supply. Lignin is an extremely versatile, non-toxic and renewable material and it can be used, for example, as rubber intensifier, rubber packing, in composite materials, as a food additive, as a sequestering agent, blend, dispersants, binder and in other applications (Agrawal *et al*., 2014). Lignocellulosic residues from bioethanol production are mainly composed by cellulose, hemicellulose, pectin and lignin (Taiz and Zeiger, 2008). Cellulose and hemicellulose are macromolecules formed from different sugars, while lignin is an aromatic polymer synthesized from phenylpropanoid precursors. Cellulose is a linear polymer that is composed of D-glucose subunits linked by β-1,4 glycosidic bonds forming the dimer cellobiose; linear cellulose molecules are organised in parallel joined by hydrogen bonds (Taiz and Zeiger, 2008). Hemicellulose contributes to forming the cell wall by acting as an essential element to the concatenation of cellulose fibrils. It is formed from D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl-glucuronic, D-galacturonic and D-glucuronic acids. Sugars are linked together by β-1,4- and sometimes by β-1,3-glycosidic bonds (Kuhad *et al*. 1997, Ling-Ping *et al*., 2013). Pectins are heteropolymers present in the plant cell wall with not yet fully known structures and functions and they are formed by the main skeleton of α-1,4-D-galacturonic acid residues (Ochoa-Villarreal *et al*., 2012).

Many microorganisms are capable of degrading and utilizing cellulose, hemicellulose and pectins as carbon and energy sources, but fungi play an important role in the biotreatment of lignocellulosic wastes because of their typical mycelial growth that allows the fungus to transport scarce nutrients such as nitrogen and iron to a distant nutrient-poor lignocellulosic substrate that constitutes its carbon source (Hammel, 1997). The fungal strains mostly known for lignocellulose degradation belong to the basidiomycetes (Bennett *et al*., 2002; Rabinovich *et al*., 2004). Little is known about the degradation mechanisms of lignocellulose by ascomycetes; most of the data are related to *Trichoderma reesei* and its mutants used for the commercial production of hemicellulases and cellulases (Esterbauer *et al*., 1991; Nieves *et al*., 1998; Jørgensen *et al*., 2003). Fungi can grow successfully on a wide variety of lignocellulosic residues such as cereal straws, soybean, cotton stalk, and almost any lignocellulosic substrate that has a substantial cellulose component (Quintero *et al*., 2006; Rani *et al*., 2008). In addition, fungi are able to degrade also different persistent environmental pollutants, such as chlorinated aromatic compounds, aliphatic and aromatic hydrocarbons and synthetic high polymers (Bennett *et al*., 2002). In fact, fungi possess a variety of extracellular enzymes with different specificities, useful to attack cellulose and lignin, but also to degrade recalcitrant compounds.

Cellulases, the enzymes responsible for the hydrolysis of cellulose, are composed of a complex mixture of enzymes with different specificities to hydrolyze β-1,4-glycosidic linkages bonds. Cellulases can be divided into three major enzyme activity classes: endoglucanases or endo-1-4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Goyal *et al*., 1991; Rabinovich *et al*., 2002a, b). Endoglucanases, often called also carboxymethylcelluloses, are thought to initiate attack randomly at multiple internal sites in the amorphous regions of the cellulose fibre which opens sites for a subsequent attack by the cellobiohydrolases (Lynd *et al*., 1991; Deobald and Crawford, 1997). Cellobiohydrolases remove monomers and dimers from the end of the glucan chain. β- glucosidase hydrolyses glucose dimers and, in some cases, cellulose oligosaccharides to glucose. Similar enzymes are involved in cellulose and hemicellulose biodegradation. Xylan is the main carbohydrate found in hemicellulose. Its complete degradation requires the cooperative action of a variety of hydrolytic enzymes, of which the most important are the endo-1,4-b-xylanases (EC 3.2.1.8) and 1,4-β-xylosidase (EC 3.2.1.37). These enzymes degrade xylan to a short chain of xylo-oligosaccharides and then, for the complete

hydrolysis to xylose monomers, xylanases cooperate with b-xylosidases (Jeffries, 1994; Krengel and Dijkstra, 1996). The degradation of pectins requires a series of hydrolytic enzymes to completely degrade the polymer. Pectinases are an extensive family of enzymes able to attack the various and complicated polygalacturonic structures. Pectinases are classified according to the type of binding they hydrolyze in the pectin structure: polygalacturonase (EC 3.2.1.15) that hydrolyzed the α-1,4 bonds between the units of galacturonic acid, pectinesterase (EC 3.1.1.11) that remove the methyl group of pectins by hydrolyzing the bond carboxyl ester of galacturone and pectinase (EC 4.2.2.10) that hydrolyze the α-1,4 bonds between the galacturonic acid producing units unsaturated galatturonates or methyl galatturonates (Rodrigo de Souza, 2013). Pectinases are most commonly extracted from *Aspergillus niger*, an ascomycete (Singh Jayani *et al*, 2005). One of the most used methods to analyse the degradative potential of fungal strains on lignocellulosic substrates is the study of the concentration of reducing sugars performing 3,5-dinitrosalicylic acid (DNS) test. The concentration of reducing sugars is an index of the fungal degradation of the cellulosic components of lignocellulosic residues. DNS test measures the reduction of the reducing sugars concentration in a supernatant. DNS is an aromatic compound that reacts with reducing sugars and other molecules to create 3-amino-5-nitrosalicylic acid, a reduced form of DNS. This acid effectively absorbs light at 540nm (Miller, 1959).

The aim of this work was to evaluate the activity of two fungal strains (*Trichoderma asperellum* 1020 and *Paecilomyces variotii* 1030) on a bioethanol production waste material rich in lignocellulosic residues (LRR) in order to obtain purified lignin that could be used in biotechnological applications. The degradation and purification abilities of the selected fungi were verified by the degradation of reducing sugars and the activity of the different enzymes involved.

Materials and Methods

Lignin rich residue substrate

The substrate used in this study was a lignin-rich residue (LRR), which is a waste material derived from the industrial production of bioethanol. In Table 1 is reported its composition made by the University Politecnico di Milano (Italy). It can be noted that, despite the material deriving from the bioethanol extraction processes, polysaccharides such as glucans and xylans are also present in large quantities. Before being used, LRR was manually fragmented to be more easily accessible to the fungal species and then sterilized for 2 h in the autoclave at 121°C.

Table 1. Lignin rich residue composition

Water content	49,26 %
Initial sample composition (dry wight)	
Glucose	0,05%
Xylose	0,06%
Glucose oligomers	0,10%
Xylose oligomers	0,14%
Insoluble glucans	20,74%
Insoluble xylans	3,11%
Klason lignin	45,51%
Acetic acid	0,22%
Other insoluble	21,87%

Strains and inoculum preparation

The tested strains used in this study are *T. asperellum* 1020 and *P. variotii 1030* and were obtained from the Collection of the Mycology Laboratory of the University of Pavia (Italy) (Table 2) and used in this work for their known abilities to grow on recalcitrant substrates like petroleum and oils.

Table 2. Isolation substrates of the tasted strains

Species	Origin
T. asperellum 1020	Plant hydroponic culture
P. variotii 1030	Pure lignin purified by chemical methods

The fungi were preserved in water supplemented with 15% glycerol at -80°C and revitalized from frozen stocks by cultivation on Malt Extract Agar for microbiology (MEA) (Sigma-Aldrich, St. Louis, USA) for 7 days at 25°C. After this time, all the mycelium grown on the plate was collected in a tube containing water-agar 0,15% to prepare the fungal suspension. To evaluate the degradative capacity of the fungal strains, 100 mL flasks were prepared containing 50 mL sterile distilled water and 5 g of LRR. One mL of fungal suspension was added in the flasks except for the ones used as a control. To ensure statistical significance, the test was conducted in a triple copy. For all the test period (2 months) the flasks were maintained at 25°C. For the DNS test, the flasks were monitored every month and subsequently, the DNS test was repeated to evaluate the trend of

reducing sugars in the first 9 days of fungal activity. The enzymatic tests were performed extracting the supernatant, after one week from the inoculum, from the same flasks.

DNS test for measuring the concentration of reducing sugars

DNS was prepared in laboratory following the protocol reported by Miller (1959): distilled water – 1.416 L; 3,5-Dinitrosalicylic acid – 10.6 g; NaOH – 19.8. The components are dissolved and then added: Rochelle salt - 306 g; phenol – 7.6 g; sodium metabisulfite – 8.3 g (Ghose, 1987). To perform the test, 40 µl of supernatant was taken from each flask and insert in a 0.5 mL tube with 40 µl of DNS. The mix was heated at 95°C for 5 minutes in a thermocycler. The concentration of reducing sugars was evaluated with the use of a spectrophotometer, measuring the absorbance at two different wavelengths: 540 nm, which measures the absorbance of the reduced form of the DNS, and 700 nm, which measures the absorbance of the turbidity of the sample. To obtain a more precise measurement, the value from the reading at 540 nm was subtracted to the one from 700 nm reading.

Study of lignocellulolytic enzyme activity

The study of enzymatic activities was carried out in collaboration with the Laboratory of Plant Molecular Biology of the University of Pavia. After 1 week from the inoculum, 5 mL of the supernatant was transferred in 10 mL tubes and centrifuged at 3000 rpm for 5 min to divide the liquid part, in which the enzymes should be, from possible residues of LRR or spores and mycelium. The liquid part was transferred into new tubes and the supernatant was diluted before being tested.

Endo-β-1,4-glucanase (EC 3.2.1.4) assay

Endo-β-1,4-glucanase was performed using a modified protocol of Islam and Roy (2018). 40 µl of the diluted sample (1:2) were placed in 0.5 mL microtubes with 40 µl of 1% CMC (Sigma-Aldrich, St. Louis, USA) in 1N citrate buffer (pH 5.0) as substrate. The mix was incubated at 45°C for 30 minutes in a thermocycler. 80 µl of DNS was added and the mix is reinserted into thermocycler for 5 min at 95°C. The CMCase activity was assessed measuring the absorbance at 540 nm and 700 nm with a spectrophotometer. Two replicates of the same sample were analyzed. One unit of CMCase activity in 1 mL of culture broth was defined as the amount of enzyme that catalyzed the release of one micromole of CMC per minute.

β-glucosidase (EC 3.2.1.21) assay.

β-Glucosidase was performed using a modified protocol of Zhang *et al*. (2018). 40 µl of the diluted sample (1:2) were placed in 0.5 mL microtubes with 75 µl of 1% para-nitrophenyl-β-D-glucopyranoside (p-NP-β-gluc) in water as substrate. The mix was incubated at 25°C for 30 minutes in a thermocycler. 100 µl of NaOH 0.1 M were added. The β-glucosidase activity was assessed measuring the absorbance at 405 nm with a spectrophotometer. Two replicates of the same sample were analyzed. One unit of β-glucosidase activity in 1 mL of culture broth was defined as the amount of enzyme that catalyzed the release of one micromole of p-NP-β-gluc per minute.

β-1,3-glucanase (EC 3.2.1.58) assay.

β-1,3-Glucanase was performed using the DNS method by El-Katatny *et al*. (2001). Forty µl of the diluted sample (1:2) were placed in 0.5 mL microtubes with 40 µl of 0.75% laminarin (Sigma-Aldrich, St. Louis, USA) in sodium acetate (NaAc) (pH 5.0), as substrate. The mix was incubated at 45°C for 30 minutes in a thermocycler. 80 µl of DNS were added and the mix was reinserted into thermocycler for 5 min at 95°C. The β-1,3-glucanase activity was assessed measuring the absorbance at 540 nm and 700 nm with a spectrophotometer. Two replicates of the same sample were analyzed. One unit of β-1,3-glucanase activity in 1 mL of culture broth was defined as the amount of enzyme that catalyzed the release of one micromole of laminarin per minute.

Endo-1,4-β-xylanase (EC 3.2.1.8.) assay.

Endo-1,4-β-xylanase was performed using the DNS method by Royer and Naka (1989). Forty µl of the diluted sample (1:2) were placed in 0.5 mL microtubes with 40 mL of 1% xylan in 0,05M citrate-HCl buffer (pH 4.8) as substrate. The mix was incubated at 50°C for 30 minutes in a thermocycler. Eighty µl of DNS were added and the mix was reinserted into thermocycler for 5 minutes at 95°C. The xylanase activity was defined as the measure of the absorbance at 540 nm and 700 nm with a spectrophotometer. Two replicates of the same sample were analyzed. One unit of xylanase activity in 1 mL of culture broth was defined as the amount of enzyme that catalyzed the release of one micromole of xylose per minute.

Pectinase (EC 3.2.1.8) assay

Pectinase was performed using DNS method by Oumer and Abate (2018). Forty µl of the diluted sample (1:2) were placed in 0.5 mL microtubes with

40 µl of 1% pectin (Sigma-Aldrich, St. Louis, USA) in 0.1 M phosphate buffer (pH 7.5) as the substrate. The mix was incubated at 50°C for 10 minutes in a thermocycler. Eighty µl of DNS were added and the mix was reinserted into thermocycler for 10 min at 95°C. The pectinase activity was assessed measuring the absorbance at 540 nm and 700 nm with a spectrophotometer. Two replicates of the same sample were analyzed. One unit of pectinase activity in 1 mL of culture broth was defined as the amount of enzyme that catalyzed the release of one micromole of pectin per minute.

Statistical analysis

Statistical analyses were performed using Microsoft Excel software (Microsoft Office 2013) and SigmaStat 4.0 (Systat Software, Inc.). The means and the standard deviation of the mean were calculated., and then the Analysis of Variance (ANOVA) an, in some cases, the Student t-test were applied to the data. A P-value of <0.05 was considered statistically significant.

Results and Discussion

Biological treatment of LRR: DNS test

The DNS test allowed to observe the degradative activity of T. *asperellum* 1020 and *P. variotii* 1030. The concentration of reducing sugars decreased considerably between the time of the inoculation and the following 2 months, in all the samples (Fig. 1 and 2).

This is an indication that the sugars were used by fungi to carry out their metabolic activities. *T. asperellum* 1020 was the most efficient strain, with a reduction of reducing sugars of about 70% in the first month, while *P. variotii* 1030 caused a 55% reduction. The increased concentration of reducing sugars in the control (just water and LRR with no fungal strain) after 1 and 2 months is a normal

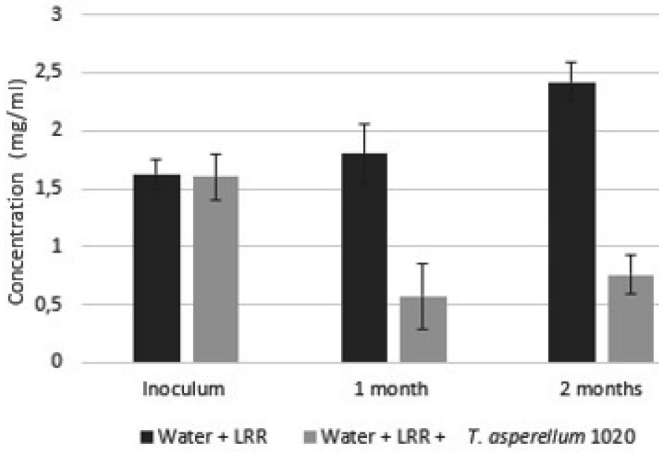

Fig. 1. Concentration of the reducing sugar in two months: *T. asperellum* 1020 in water and LRR

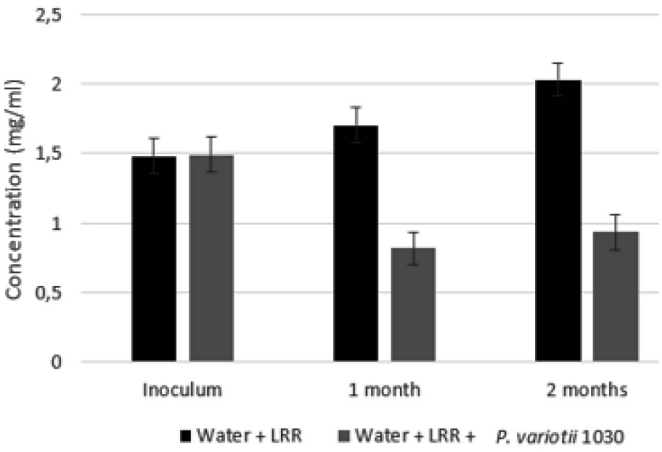

Fig. 2. Concentration of the reducing sugar in two months: *P. variotii* 1030 in water and LRR

consequence of their release in the supernatant by the lignocellulosic biomass. The tested strains reduced the concentration of sugars by 53% compared to the control, in the first 4 days from the inoculum, until reaching a value close to 0 (Fig. 3).

On day 2 the curve of *T. asperellum* 1020 showed a significantly different trend compared to the curves of *P. variotii* 1030 (P<0.05). This behaviour could indicate a greater efficiency of this strain related to the growth condition on a liquid substrate, coherently with the isolation conditions (hydroponic condition). The maximum activity of these fungi in the reduction of reducing sugars took place in the first 4 days. Looking at the figures (Fig. 1; Fig. 2; Fig. 3) in all the samples treated the concentration of reducing sugars approaches a value close to 0 without ever reaching it. This result could be due to the difficulty of the tested strains to completely degrade the cellulosic compounds contained in the LRR biomass or for the release in the supernatant of compounds containing an aldehyde or ketone group detectable by the DNS test.

Study of the lignocellulosic enzymatic activity of the strains

In all the samples, there was a significant (P<0,05) increase, up to the 600%, in enzyme activity in the presence of LRR compared to the control (Table 3). This result indicates that fungi were stimulated in producing lignocellulosic enzymes in presence of LRR. β-Glucosidase and xylanase are the most produced enzymes by the two strains. in *P. variotii* 1030, the β-glucosidase activity increased of the 421% and the xylanase of 608%; in *T. asperellum* 1020 the β-glucosidase activity increased of the 267% and the xylanase of 609%.

Pectinase also showed significant results (P<0.05), although its activity was lower than the

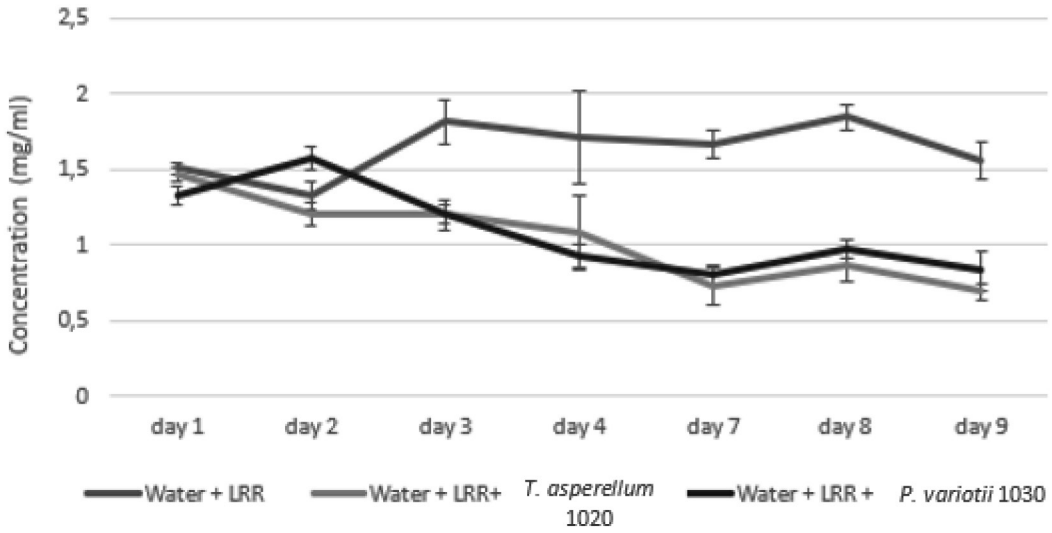

Fig. 3. Concentration of the reducing sugar in 9 days: *T. asperellum* 1020 and *P. variotii* 1030 in water and LRR

two above mentioned enzymes. In fact, pectinase activity increased of the 375% in and of the 175% *T. asperellum* 1020.

that S4F8 had significantly higher hemicellulase and β-glucosidase enzyme activities. *Paecilomyces* spp. are indicated as good producers of cellulase

Table 3. Concentrations of the tested enzymes determined with spectrophotometric protocols

	β-1,3-glucanase		endo-β-1,4-glucanase		β-glucosidase		Pectinase (U/ml)		Xilanase (U/ml)	
	Water	Water + LRR	Water	Water + LRR	Water	Water + LRR	Water	Water + LRR	Water	Water + LRR
T. asperellum 1020	Traces†	Traces	0,015 ± 0,002	0,026 ± 0,001	1,00 ± 0,02	* 2,67 ± 0,22	0,04 ± 0,005	* 0,07 ± 0,003	0,044 ± 0,011	* 0,268 ± 0,055
P. variotii 1030	Traces	Traces	Traces	0,029 ± 0,001	1,46 ± 0,11	* 6,16 ± 0,02	0,024 ± 0,01	* 0,09 ± 0,004	0,035 ± 0,002	* 0,213 ± 0,078

†Activities lower than 0.02 U/ml, The strains grew in water only and in water + LRR.

Our results are in accordance with the data reported in literature. Several studies showed that *Trichoderma* and *Paecilomyces* genera are excellent producers of cellulolytic enzymes (Sarkar *et al*., 2012; Tosi *et al*., 2014). *Trichoderma* species can degrade cellulose by a synergistic combination of different cellulase activities (Reese, 1956; Mandels er at., 1971). *T. viride* MMS 3, *T. reesei*, *T. virens* (Ang *et al*., 2015), *T. asperellum* MR1, *T. virens* UKM1, and *T. tubingensis* NKBP-55 (Prajapati *et al*., 2018) have been studied for their production of cellulases and xylanases. *T. viride* VKF3 achieved a high level of xylanase by utilizing coconut oil cake as a substrate. *T. reesei* Rut C-30 is the most well-known *Trichoderma* strain producing several xylanases and cellulases (Peterson and Nevalainen, 2012) but Marx *et al*. (2013), in a comparison study between the lignocellulolytic enzyme profiles of *T. asperellum* S4F8 and *T. reesei* Rut C30, showed

enzymes (Paganini Marques *et al*., 2018; Ingle, 2019). In their work Hussain *et al*. (2012) showed that *P. variotii* has the ability to produce high activities of all three main components of cellulase in low cost substrate and how physical and chemical factors, influenced their production.

Conclusions

In conclusion, measuring the concentration of reducing sugars in the LRR samples, *T. asperellum* 1020 showed the best performance, with reducing sugars rate decreasing by about 70%. The two strains, however, seem to use almost all the available material in the first 4 days from the inoculum. The quantification of enzymes secreted confirmed that the two strains act on the specific components of LRR. The obtained results indicate that LRR biotransformation by *Trichoderma* and *Paecilomyces* has interesting potential and that lignin purification by this method, with the

appropriate improvements, could reach the same purification percentage obtained with traditional chemical methods.

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