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RESEARCH PAPER

Cellulolytic and ligninolytic potential of new strains of fungi for the conversion of fibrous substrates

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Abstract This research explored the lignocellulolytic potential of native strains (*Hipoxylon* sp, *Curvularia kusanoi*, *Trichoderma sp* and *Aspergillus fumigatus*) isolated from natural substrates. Their sequences were deposited in the GenBank, and it's lignocellulosic potentialities were determined quantitatively from kinetics of cellulase and ligninase production in wheat straw, and grass hay. All evaluated strains showed high cellulolytic potential and laccase activity where only found in the *C. kusanoi L7* strain. The new strains where co-cultivated with a reference strains of *Trichoderma viride* to determinate the capacity of joint growth, to know if they can be used together for a better degradation of the cell wall. A three-phase purification system was used to isolate the *C. kusanoi* laccase where a preparation 10 times purer than the initial crude was obtained, with an enzymatic activity of 2800 U/L, a specific activity of 544.74 U/g and a yield higher than 100%, which showed a more stable behavior at alkaline pH than at acid pH. The purify enzymes showed an optimum range of activity between 30 and 40 °C, and a high thermal stability. It is concluded that the isolated strains have a high degradation capacity and the *C. kusanoi* L7 strain stands out as the one with the greatest potential when expressing in addition to the cellulolytic complex the enzymes involved in the degradation of lignin.

Introduction

Lignocellulose is the main and most abundant component of the renewable biomass produced by photosynthesis; it is estimated that 200 billion tons are produced annually in the

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world. It consists of three main biopolymers that form the cell wall of plants: cellulose, hemicellulose and lignin. These biopolymers have varied and highly complex structures, generally very resistant to degradation (Reeta, Singhania, Adsul, Pandey, & P, 2017). However, in nature there are microorganisms that have different enzymes such as cellulases, laccases and peroxygenases, which are responsible for its degradation (Brijwani, Rigdon, & Vadlani, 2010; Taravillaa, Pejó, Demueza, González, & Ballesteros, 2016).

Currently many microorganisms are the main source of these enzymes, which have a wide field of industrial application, as they have numerous technical and economic advantages. They have great potential, taking into account high enzyme yields, high selectivity, low energy costs and slight handling conditions than in chemical processes (Kuhad, Gupta, & Singh, 2011; Oliva et al., 2015).

Within the cellulolytic organisms are bacteria, actinomycetes and fungi, both aerobic and anaerobic, mesophiles or thermophilic. Each group of microorganisms according to their physiological characteristics adapts to some particular materials and it is necessary to determine, according to the biomass characteristics, the most suitable species for their management.

Among the most reported of fungi species are ascomycetes (e.g. *Trichoderma ressei* and *Aspergillus niger*), basidiomycetes which includes white rot fungi (e.g. *Panaerochete chrysosporium*) and brown rot fungi (e.g. *Fomitopsis palustris*) and some anaerobic species such as *Orpinomyces* sp (Sindhu, Binod, & Pandey, 2016). However, another wide variety of fungi that also has a high lignocellulolytic potential belong to the genera *Curvularia* and *Penicillium* (Banerjee & Vohra, 1991; Rodríguez et al., 1996).

This continuous search for new strains with high degradation potential is decisive for increasing the amount of microorganisms with high potential to develop, which will also allow an efficient enzymatic technology that generates powerful biotechnological tools for different applications, aspects that constitute the fundamental objective of our investigation.

Materials and methods

Isolation

A total of 4 fungi were isolates from different natural substrates: lemon tree (*Citrus aurantifolia*), güira (*Crescentia cujete*) and orange (*Citrus aurantium*) that showed infestation by imperfect fungi and basidiomycetes. These fungi where isolated by a surface scraping and culture in plates with malt agar medium, with addition of 100 mg/L of chloramphenicol (SIGMA) to inhibit bacterial growth. The plates were deposited in an incubator (VWR) for 7 days at 30 °C. The differential colonies were transferred to new plates with malt extract agar, until the pure colonies were obtained, which were passed to wedges and preserved at 4 °C for further study.

Identification

The isolation of genomic DNA was conducted according to Kuhad et al. (2004). The mycelium from the strains cultured for 10 days on PDA agar plates was collected. For identification, ITS region primers where used as molecular markers previously described as informative for filamentous fungi (Martins & Rygiewicz, 2005). Sequencing reactions were carried out in the Sequencing Unit of the Biotechnology Institute of the National Autonomous University of Mexico; the species were identified as described in Table 1, according to BLAST results and further phylogenetic inferences (data not shown).

Degradative capacity on filter paper

The lignocellulosic potentialities of these microorganisms were qualitatively determined by their growth on Whatman No. 1 filter paper. The isolated strains were cultured in tubes with Mandel pH 5.5 medium (20 ml) and a strip of Whatman No. 1 filter paper was added as a carbon source (Mandels, Andreotti, & Roche, 1976). The cultures were incubated for 21 days at 30 °C and the degradation of the filter paper was recorded every 5 days. According to Mandels et al. (1976) and Ghorban, Karimi, Biria, Kariminia, and Jeihanipour (2015) this test is considered positive for cellulolytic activity once the paper is broken with a little agitation.

Zimografy

A zymogram of cellulolytic activity was performed in a polyacrylamide gel at 12.5% with cellulose included as substrate to be degraded. The enzyme extracts were mixed in a 1:1 ratio with the 2× sample buffer (Tris/HCl 125 mM, 20% (v/v) glycerol, 4% (p/v) SDS, phenol bromine blue 0.005% (p/v), pH 6.8). After electrophoresis, the gel was incubated with 1% (v/v) Triton X-100 for 15 min under slow stirring. Then, it was allowed to incubate overnight at 4 °C in a suitable buffer for cellulolytic activity (Tris/HCl 50 mM, $CaCl_2$ 2 mM, pH 8.0). Finally, to visualize the bands, a staining with Congo red at 0.2% (p/v) and non-staining with NaCl 1 M was performed.

Cultures in fibrous substrates

To determine the enzymatic potential expressed by the strains, wheat straw and grass hay were used as fibrous substrates to be degraded.

Fermentation of wheat straw

From the pure cultures of the isolated strains, 3 cm of each culture were taken and inoculated in a flask containing 3 g of wheat straw and 100 ml of citrate buffer (50 mM, pH 5, 0) and incubated in an orbital shed at 120 rpm for a period of 10 days at 30 °C in complete darkness. Fermentation samples were taken every 24 h, the contents of each flask were

Table 1 Molecular identification by sequencing the ITS rDNA gene region of the new fungi isolated from natural substrates.

Genbank submission ID	Genbank submission ID	Genbanksubmission ID	Genbank submission ID
BankIt1982924	KY486926	*Trichoderma sp*	N7
BankIt1982918	KY486927	*Hypoxylon sp*	Basidio
BankIt1982938	KY486929	*Aspergillus fumigatus*	Güira
BankIt 2510779	KY795957	*Curvularia kusanoi*	L7

filtered, the resulting liquid was centrifuged (4 °C, 10,000 rpm, 30 min), and the supernatant (crude extract) was stored in falcon tubes at −20 °C for further determination.

Fermentation of grass hay

The fresh fungi were grown in flasks containing 4 g of grass hay, inoculated with 5 ml of minimal medium, which were incubated for a period of 10 days at 30 °C in complete darkness. Samples were taken every 24 h. After each incubation period, the contents of the flask were suspended in 50 ml of citrate buffer (50 mM, pH 5.0) for 30 min in an ice bath. The solids were separated by filtration, and the resulting liquid was centrifuged (4 °C, 10,000 rpm, 30 min). The supernatant (crude extract) was stored in falcon tubes at −20 °C for further determination.

Determination of enzymatic activity

The endo β 1,4-glucanase activity (CMCase) was determined on the carboxymethylcellulose substrate and the exo β 1,4-glucanase activity (PFase) on crystalline cellulose. The content of reducing sugars released was determined by the 3, 5-dinitrosalicylic acid (DNS) method (Adney & Baker, 2008).

Laccase activity was spectrophotometrically determined by the oxidation of syringaldazine under aerobic conditions. The violet color produced was measured at 530 nm. The analytical conditions where 5 mM of syringaldazine, 50 mM citrate buffer pH 4.5, 30 °C, and 1 min reaction time ($\varepsilon = 64{,}000\ M^{-1}\ cm^{-1}$). One laccase unit (U) is the amount of enzyme that catalyses the conversion of 1.0 μmol syringaldazin per minute at these conditions.

Laccase purification by three phase partition (TPP)

The laccases were purified by three phase partition according to the methodology proposed by Gagaoua and Hafid (2016). An ammonium sulphate – tert-butanol ratio of 1.0:1.1, a saturation of 78% ammonium sulfate and a partition temperature of 38 °C were used.

Methodology

The ammonium sulfate and ter-butanol where added to the crude enzymatic extract. The system was then homogenized by moderate agitation in vortex. The homogeneous system was incubated for 1 h in a water bath at 38 °C where 3 phases were formed. After incubation, it was centrifuged at 4000 rpm for 10 min at 25 °C in a High Speed Refrigerated Centrifuge (Neofuge 15R), in order to make the precipitate more compact in the interface and easy to separate. Afterwards, the phases were separated with a separator funnel, the upper and lower phases were discarded and the intermediate phase was preserved and deposited in phosphate buffer at pH 7. The yield of the process and the purification factor were calculated.

Enzyme characterization

Effect of pH and type of buffer on enzymatic activity

Laccase activity determinations were carried out on the purified extract, using 0.5 mM of siringaldazine (4-hydroxy-3,5-dimethoxybenzaldehydazine) as substrate in different buffer systems (acetate buffer, phosphate buffer and citrate buffer, 50 mM each) at different pH each (3.7, 4.5, 6.5, and 8). The activity was monitored at a wavelength of 530 nm and was estimated with an extinction coefficient of $64{,}000\ M^{-1}\ cm^{-1}$. One unit of activity (U) was defined as the amount of enzyme that catalyzes the transformation of 1 μmol of substrate per minute.

Effect of temperature on activity and enzyme stability

To determine the effect of temperature on the activity of the enzyme, the laccase activity was measured with 0.5 mM of syringaldazine in 50 mM of acetate buffer, pH 6.5 at 30, 40, 50 and 70 °C. The reaction mixture without the substrate was pre-incubated for 1 min at the different temperatures mentioned. To determine the effect of temperature on enzyme stability, the product was incubated in a BANDELIN Sonorex, Germany thermostatic bath at 30, 40, 50 and 70 °C for 1 h, and 100 μl aliquots were taken every 10 min, the laccase activity was measured with 0.5 mM of syringaldazine in 50 mM of acetate buffer, pH 6.5 at 40 °C (optimum temperature)

Microbial confrontation tests

The ability to grow in co-cultures was determined as a measure of the antagonism of the strains. The selected strains were cultured in PDA. The plate was halved and in each half a microorganism was inoculated. The plate was incubated at 30 °C in complete darkness for a period of 7 days to monitor if there was any overlap between the cultures or if there was inhibition.

Statistic

For the analysis of all the data of the study, the statistical package INFOSTAD, version 1.0 (2008) of the National

University of Córdova, Argentina, was used. The differences between mediums were established, according to Duncan (1955).

For the determination of laccase activity against different buffers systems and different ph, a completely randomized statistical design with factorial arrangement (3×4) was used, arranged in three buffers systems (phosphate, citrate and acetate) and four pH values (3.5, 4.5, 6.5 and 8).

Results and discussion

Isolation and characterization

The incidence of species capable of using native cellulose is high when starting from soil isolates because this ecosystem constitutes a promising source of cellulolytic organisms (Ghorban et al., 2015). However, isolates were directly made from biodegraded lignocellulosic substrates to obtain specificity of action of fungi on the substrate and not to remove the natural habitat conditions of the species that live in them, so that, all the isolation work was considered in drastic conditions to obtain strains of greater hydrolytic power, which was checked qualitatively by the filter paper test, where all the strains showed complete degradation of the substrate after 10 days, which shows that the microorganisms evaluated express the complex of enzymes necessary for the exhaustive degradation of cellulose. The zymogram as well showed the presence of cellulolytic enzymes in all evaluated strains (data not show).

Fermentative kinetics of wheat straw

As presented in Tables 2 and 3, the determinations of endo and exo β 1, 4-glucanase activity showed that all fungi produce the cellulase complex and the maximum activity of these enzymes is mostly achieved during the first few days of fermentation, except for the *Trichoderma* sp that reach its maximum activity al 120 h with the established conditions. It is worth noting that the *Curvularia kusanoi* and *Aspergillus fumigatus* strains reach their highest values during the first 24 h of fermentation. According to Valiño et al. (2015) the candidate strains for enzymatic production fermentation processes are those capable of expressing their maximum capacity during the first hours of fermentation, reducing working time and obtaining optimization of the process. However, none of the strains tested exceeds the endoglucanase activity range of *Trichoderma viride* M5-2 as a comparative strain of cellulase production in highly lignocellulosic media such as hydrolyzed cane bagasse and legumes as fermentative substrates (Valiño, Elías, Torres, Carrasco, & Albelo, 2004).

On the other hand all strains tested maintained their enzymatic activity during the 186 h of fermentation.

Solid state fermentation of grass hay

In the solid state fermentation of the grass hay also the evaluated strains presented cellulolitic activity although in smaller levels compared with those obtained in the liquid fermentation of the wheat (data not show).

In the specific case of the enzymatic degradation of grass hay by *C. kusanoi* L7 (Table 4), an important endoglucanase cellulolytic activity was observed reaching the maximum production at 24 h, aspect that coincides with the results found in the liquid fermentation of wheat. As for the production of exoglucanase, it reached a maximum 48 h after the fermentation process started, although in a lesser proportion.

It is known that in the submerged fermentation, the culture medium where the fungus is inoculated and the fermentation process occurs, presents a determined quantity of mineral salts and/or water in a greater proportion than in the solid substrate, which results in greater contact with the environment. On the other hand, solid state fermentation implies the growth of the microorganism in a solid substrate in the absence of free water, a limiting aspect that affects the synthesis and excretion of enzymes.

Laccase activity in wheat straw and grass hay

The lacasse activity can be evidenced in lignin bioassays as the only carbon source, although the analytical tests for lignin determination make them expensive. However, other organic compounds such as ABTS or siringaldazine (used in this work) are also substrates of the lignin-modifying enzymes and the activity is easily revealed by a change in coloration of the culture medium (Winquist, Moilanen, Mettälä, Leisola, & Hatakka, 2008).

In wheat straw fermentation only the *C. kusanoi* L7 showed laccase activity, and express it maximum capacity at 168 h of fermentation (Fig. 1). This strain followed a similar pattern to most lignolytic strains, which express their highest activity at 7 days of fermentation in liquid medium

Table 2 Cellulolytic activity CMCase (endo β 1,4 glucanase) of identified strains.

	CMCase Activity (UI/ml)							ES±
Strains	24 h	48 h	72 h	96 h	120 h	144 h	168 h	
Curvularia kusanoi L7	**0.535**	0.197	0.298	0.185	0.081	0.035	0.029	0.022
Hipoxylon sp,	0.251	0.176	0.404	**0.557**	0.330	0.075	0.052	0.025
Aspergillus fumigatus	**0.692**	0.301	0.517	0.546	0.293	0.094	0.065	0.030
Trichoderma sp	0.032	0.061	0.064	0.082	**0.126**	0.014	0.002	0.001

All measurements were performed in triplicate ($p < 0.05$). Within the table, bold letters indicate the maximum value reached for each species.

Table 3 Cellulolytic activity PFase (exo β 1,4 glucanase) of identified strains.

	PFase activity (UI/ml)							ES
Cepas	24 h	48 h	72 h	96 h	120 h	144 h	168 h	
Curvularia kusanoi L7	**0.34**	0.19	0.13	0.08	0.08	0.08	0.05	0.011
Hipoxylon sp	0.13	0.21	0.25	**0.26**	0.24	0.15	0.09	0.002
Aspergillus fumigatus	**0.47**	0.36	0.31	0.21	0.20	0.15	0.10	0.040
Trichoderma sp	0.001	0.01	0.01	0.02	**0.10**	0.09	0.06	0.001

All measurements were performed in triplicate ($p < 0.05$). Within the table, bold letters indicate the maximum value reached for each species.

Table 4 Cellulolytic activity of endo and exoglucanase in solid state fermentation of grass hay with *C. kusanoi* L7 strain.

Celulolitic activity (UI/ml)	Fermentation time (h)							
	24	48	72	96	120	144	168	EE
CMCase	**2.73**	2.06	2.36	1.85	1.14	1.04	0.934	0.002
PFase	0.26	**0.80**	0.51	0.007	0.007	0.003	0.0006	0.03

All measurements were performed in triplicate ($p < 0.05$). Within the table, bold letters indicate the maximum value reached for each species.

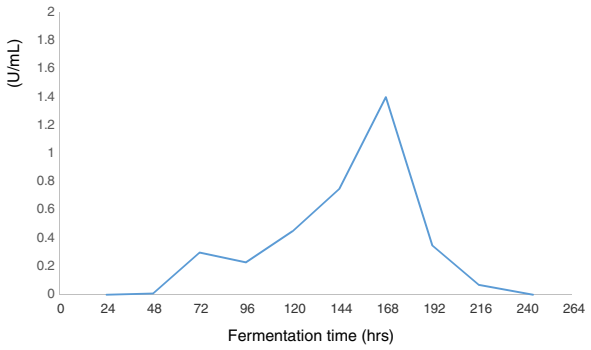

Figure 1 Kinetics of laccase production by *C. kusanoi* L7 in wheat straw liquid cultures.

(Janusz et al., 2015). Sumathi, Sri Lakshmi, Viswanath, and Sai Gopal (2016) found similar values of laccase activity in *Cochliobolus* (The teleomorphic state of different types species of *Curvularia*), which are a measure of the potential of these species for the degradation of recalcitrant compounds.

As for the solid-state fermentation of grass hay, none of the strains produce laccase activity, not even *C. kusanoi* L7, which may be associated with the substrate complexity and the low content of sugars in the medium.

In the other hand, is well know that submerged fermentation and solid state fermentation are the two forms of cultivation used for the production of laccases. Nevertheless, some authors have found a greater production of laccase under submerged fermentation conditions (Dong, Zhang, Zhang, Huang, & Zhang, 2005).

The practical applications of laccases for both biotechnological and industrial purposes is increasingly broad. It is important to study new strains of microorganisms that are capable of producing this enzyme, which must be purified and characterized in order to manipulate its different physicochemical and catalytic properties to obtain new and powerful biotechnological tools.

Laccase purification by three-phase partition

Several methods are used for the separation and purification of the laccases from crude extracts of fungi, such as chromatography, ultracentrifugation, phase formation, precipitation and filtration. The selection of one or the other is dependent on the purpose pursued and the characteristics of each biomolecule.

In this work, phase formation was chosen for the purification of the *C. kusanoi L7* laccase, specifically the Three Phase Partition (TPP). This method allows the separation of compounds obtaining an intermediate phase where the laccase is found (Gagaoua & Hafid, 2016).

Generally, laccase produced from fungi is accompanied by isoforms, proteases, cellulases and other compounds derived from the production of the crude extract (Janson, 2012)

In the present study, the crude extract had a laccase activity of 750.03 U/L and 14.45 g/L of protein. In comparison with the extracts used in other studies of three-phase partition (Kumar and Srikumar, 2012; Liu et al., 2015), the levels of enzymatic activity and the amount of protein are low, aspects that do not significantly affect the yield, but they can decrease the enzyme's purification factor.

After the purification by TPP was finished, a preparation 10 times purer than the initial crude was obtained, with an enzymatic activity of 2800 U/L, a specific activity of 544.74 U/g and a yield higher than 100% which indicates that the purification process increased the enzymatic activity. However, the purification factor obtained is low compared to reports of Kumar and Srikumar (2012) who used this type of system in the purification of the *Pleurotus ostreatus* laccase, and obtained a purification factor of 27.8, Rajeeva and

Table 5 Effect of buffer type and pH on laccase enzymatic activity.

Variable	pH	Buffers				SE ± sign
		3.5	4.5	6.5	8	
Enzimatic activity (U/ml)	Citrate	1.15[b]	1.31[c]	4.06[e]	4.41[f]	0.02 $p < 0.0001$
	Phosfate	1.11[b]	4.51[f]	4.14[e]	4.78[g]	0.02 $p < 0.0001$
	Acetate	1.09[ab]	0.99[a]	4.15[e]	3.48[d]	0.02 $p < 0.0001$

[abcdefg]Different letters indicate significant differences to $p < 0.05$ (Duncan, 1955).

Lele (2011) on the other hand obtained a purification factor of 13.2 in species of *Ganoderma*, and Liu et al. (2015) obtained yields of 70% with purification factors greater than 20 in the species *Coriolopsis trogii*.

The present study coincides with the results obtained by Gagaoua and Hafid (2016) who obtained yields above 100% with low purification factor. These low purification factor may be associated with the presence of laccase intermediate precipitate as the main protein in the crude extract compared to others crude extracts used in the studies previously reported, where there are more amounts of other compounds to be separated.

In general, the purification by three-phase partition used to isolate the laccase of the fungus *C. kusanoi L7* allows purifying the extract without affect the enzyme yield. This is an effective method for future studies of scaling up of this protein.

Enzyme characterization

Effect of pH and type of buffer on the enzymatic activity
The behavior of the *C.kusanoi* L7 purified enzyme was evaluated against different buffer systems and different pH, which is summarized in Table 5.

As shown, there is interaction between the factors (pH and buffer systems) and these significantly affect the enzymatic activity. It was found for almost all cases that the laccases of the fungus *C. kusanoi* L7 present better activity at alkaline pH than at acid pH, except in the case of the acetates buffer where the highest activity was reached at pH 6.5.

Every buffer solution works in a pH range approximately one unit over the pKa. Since acetate, phosphate and citrate are weak electrolytes; their buffering capacity is practically the same. In the case of acetates buffer, it works in the pH range of 3.75–5.75 and the phosphate buffer works in the range of 6.2–8.2. These aspects explain the differential behavior of the enzyme in different buffer systems, it depends on which species can be ionized depending on the pH, and in turn, these can vary the conformation of proteins and their electrical charges. These changes can alter the conformation suitable for the catalytic activity. The species that may be affected as a function of pH are mainly the buffer system, the substrate, the cofactor (s), and the essential ionizable groups of the active center of the enzyme (Murray et al., 2013).

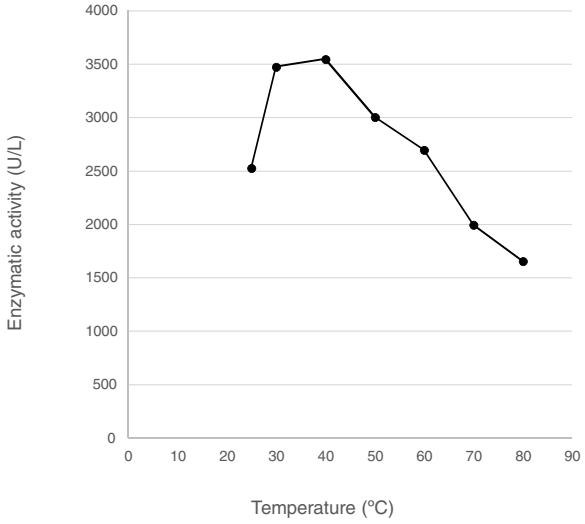

Figure 2 Effect of temperature on the enzymatic activity of *C. kusanoi L7* laccase.

It has been studied that laccase enzymes are more stable at alkaline pH than at acid pH, which is probably due to the inhibition of the hydroxide group on the auto-oxidation process (Gianfreda, Xu, & Bollag, 1999). On the other hand several authors refer optimal pH for laccase activity depending on the substrate that is being oxidized and these can vary between 3.5 and 8 (Gayosso, Leal, & Rodriguez, 2004). Nevertheless it is also known that the pH of greatest activity for ligninolytic enzymes in general varies greatly from one genus to another, finding laccases with extremely acid optimal pH (pH 2) for *Trametes trogii* and up to 5.3 for *Cerrena unicolor* (Zouari et al., 2006).

Effect of temperature on activity and enzyme stability
As for the effect of temperature on laccase activity, Fig. 2 shows the optimal range of activity for the *C. kusanoi* L7 laccase.

As shown in the figure, the interval from 30 to 40 °C is the optimum for laccase activity, a result that coincides with studies by Ramírez and Coha (2003) who found similar optimal temperatures of 40 and 45 °C for *P. ostreatus* laccase.

Concerning to thermal stability of laccases, it is known that a large part of these enzymes retain their activity in a temperature range of 5–55 °C. In this study, it was observed

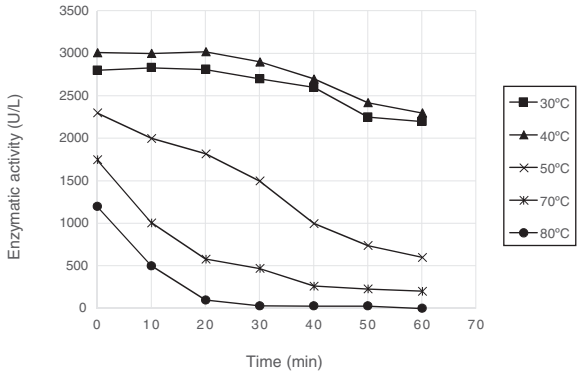

Figure 3 Effect of temperature on enzymatic stability.

that the laccases of the fungus *C. kusanoi* L7 maintain their activity in the range of 30–40 °C. At 50 and 70 °C a decrease in the enzymatic activity is observed as the incubation time progresses, more accentuated at 70 °C of temperature, and in the case of 80 °C, a rapid loss of activity is observed (Fig. 3).

It has been reported that the laccase produced by the fungus *P. ostreatus* maintains its activity unchanged at 40 °C for more than 4 h (Shraddha,Shekher, Sehgal, Kamthania, & Kumar, 2011). It has also been described that the laccases of *Pleurotus eryngii* are more stable at 25 °C for 24 h, with the 50% reduction of its activity at 55 °C for 1 h. Liu et al. (2009) tested laccase stability during 1 h at 30 and 40 °C and the maintained its residual activity between 90 and 100% of its and was rapidly reduced to less than 10% at temperatures above 60 °C.

The effect of temperature on enzymatic activity may be related to the number of disulfide bridges presented in the molecule, dissociation of laccase dimers has also been observed with the increase in temperature which can lead to modifications in the catalytic activity of these enzymes (Moreno, Ibarra, Ballesteros, González, & Ballesteros, 2013).

It is evident that there is no consensus in the bibliography about the pH and the temperatures at which the laccases are more stable, since in spite of coming from microorganisms of the same genus they present completely different and particular behaviors in each study. It is very probable that this variety of behaviors is because many microorganisms that produce laccase secrete different isoforms of the same enzyme and have been found to come from the same or different gene that encodes them. The number of isoenzymes depends on the microorganism and they differ markedly for their stability, optimal reaction conditions and affinity for different substrates. For these reason in the same fermentation can be found several types of isoenzymes produced by the same fungus (Moreno et al., 2013).

Microbiological confrontation test

To achieve a better degradation of the cellular wall of the plants, the integrated action of different enzymes is needed (Quiroz & Folch, 2011), not only the enzymes of the cellulase complex, but also those implied in the biodegradation of the lignin (Van Dyk & Pletschke, 2012). This process is extremely complex, and it implies the initial degradation of the lignin polymer, as well as its degradation products.

As mentioned before, all the microorganisms tested had a high production of the cellulase enzymatic complex; but only the *C. kusanoi L7* strain produced laccase as part of the ligninolytic enzyme system. Therefore, the co-cultures where made between these strain and the rest in order to identify the variants of co-culturing that may allow an integrated growth of these microorganisms.

In addition to these determinations, it was decided to include a new co-culture with a mutant strain of the lignocellulolytic fungus *T. viride* M5-2, belonging to the strain bank of the Institute of Animal Science of the Republic of Cuba because this strain produces phenoloxidase and cellulase enzymes, and has a high hydrolytic activity in highly fibrous substrates, evaluated by solid-state fermentation (Valiño et al., 2004).

As shown in Fig. 4, the confrontations 2, 3 and 4 present simultaneous growth of both strains, the plates show that there is no antagonism between these strains and there is evidence of a mixture between the mycelia of both fungi so that these combinations are candidates for fungi co-cultivation that can be used for biodegradation of lignocellulosic substrates.

In the specific case of the confrontations between *C. kusanoi* L7 vs. *Trichoderma* (confrontations 2 and 4) it should be noted that they are combinations of great potentialities, as for the particular case of confrontation 4 where a hyper productive cellulase strain is combined with another strain that besides expressing the highest cellulolytic activity during the first 24 h of fermentation is also capable of secreting laccase as part of the enzymes from the ligninoltic complex, aspects that constitute an interesting variant for further studies of biodegradation.

Besides it is known that *Trichoderma* species are natural parasites of some entophytic fungus like *Curvularia* and several reports have shown increased laccase production during interspecific interactions with *Trichoderma spp*. and/or its metabolites (Baldrian, 2006; Flores, Vidal, Trejo-Hernández, Galindo, & Serrano-Carreón, 2009; Hatvani & Mécs, 2002; Savoie & Mata, 1999; Zhang et al., 2006).

According to Singh, Singh, Kaur, Bansal, and Kaur (2011) co-cultures of lignocellulolytic fungi can accomplish a better and more complete degradation of plant cell wall. In the livestock sector, there are few works in which fungi are used for these purposes, as well as being limited to a few genera (Ghorai et al., 2009; Chander et al., 2016) however, they are an interesting alternative in the improvement of the nutritional quality of pastures and forages used in animal feed, which requires a greater degradation of lignin, hemicellulose and cellulose to allow better bioavailability of nutrients (Ribeiro et al., 2012).

Although not only in the agricultural sector co-cultures of microorganisms are used, they have a whole field of application, excelling the most innovative ones, such as their use in obtaining biofuels, regarding to this Taha et al. (2015) reported enhanced straw saccharification through co-culturing of lignocellulose degrading microorganisms. The results indicate that enzyme activities of fungal isolates were two-fold higher than those from bacteria. Co-culturing resulted in seven-fold increase in saccharification rate. Co-culturing significantly increases saccharification, which

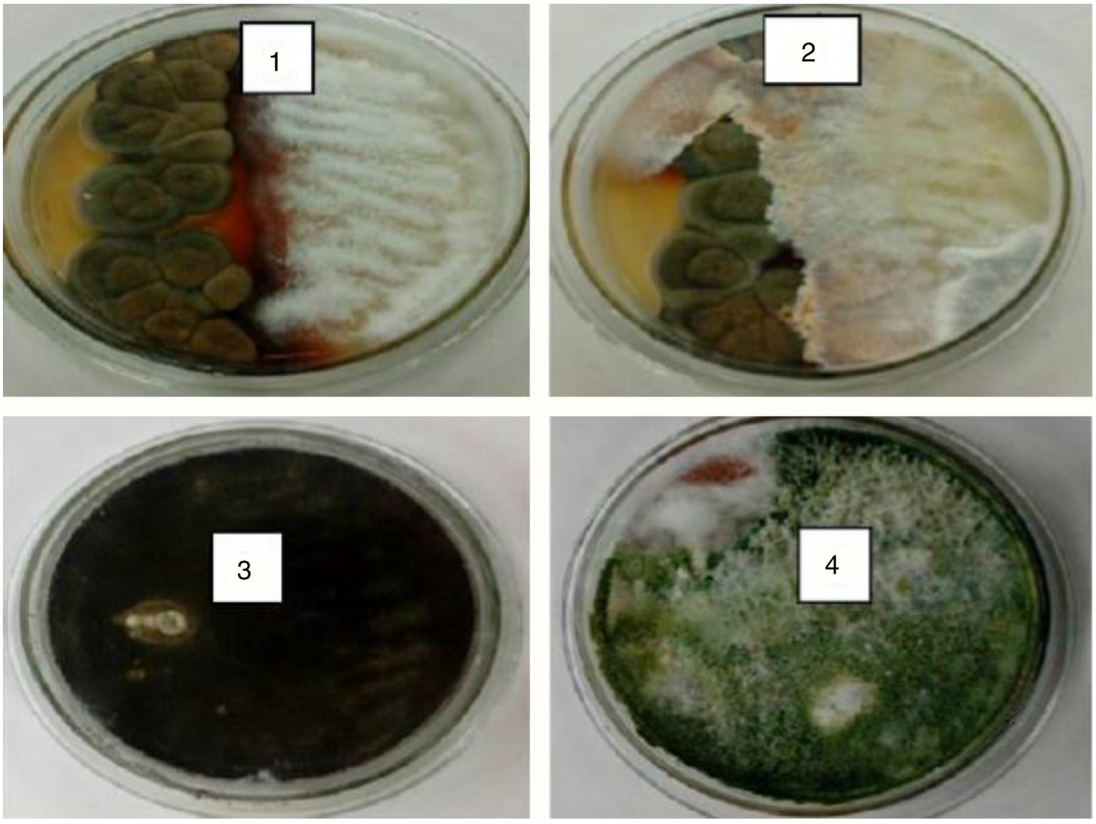

Figure 4 Microbial confrontation tests in PDA medium: 1. *C. kusanoi* L7 (left) vs. *Hypoxylon* sp (right), 2 *C. kusanoi* L7 (left) vs. *Trichoderma* sp (right), 3 *C. kusanoi* L7 left) vs. *Aspergillus fumigatus* (right), 4 *C. kusanoi* L7 (left) vs. *Trichoderma viride* M5-2 (right).

leads to increased commercial potential for the use of microbial consortia.

Conclusions

Through this research, the high degradation potential of the isolated strains was verified. Both, the isolation process and the culture conditions allowed these microorganisms to develop and express their activity. Although all strains have high productions of cellulases, the fungus *C. kusanoi* L7 stands out as the best and most complete enzyme battery. Their laccases present thermal stability and are active in a wide range of pH. Its combination with the rest of the strains evaluated and especially with the T. viride M5-2 is the starting point for future studies of microbial synergism.

Conflicts of interest

The authors declare no conflicts of interest.

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