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Full Length Research Paper

Chips and sawdust substrates application for lignocellulolytic enzymes production by solid state fermentation

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White rot fungi have an enzymatic system producing oxidative and hydrolytic enzymes that act on the degradation of the cell wall components. The abundance of sawdust and other forestry wastes in our region, due to an important forestry activity, represents an interesting ecological option. These surplus needs to be ecologically disposed. The purpose of this study was to compare wood chips and sawdust as substrates for the production of hydrolytic (endo-β-1,4-glucanase and 1,4-β-glucosidase) and oxidative (laccases and manganese peroxidase) enzymes secreted by five white rot fungi native from Misiones (Argentina) by solid state fermentation (SSF) We also evaluated the effect of the extraction method on enzyme recovery yield. Sawdust-SSF was proved to be the most suitable substrate for enzymes production. Endo-β-1,4-glucanase and laccase were the major enzymes secreted with both substrates. *Peniophora* sp. BAFC 633, *T. villosa* BAFC 2755 and *P. sanguineus* BAFC 2126 were the most efficient producers of both types of enzymes. *G. applanatum* BAFC 1168 strain F and *C. versicolor* f. *antarcticus* BAFC 266 produced mostly hydrolytic enzymes. Enzyme extraction was conducted in two steps to assure optimal recovery yield. A second extraction step was necessary to sawdust SSF samples.

Keywords: lignocellulolytic substrate, solid state fermentation, enzyme production, white rot fungi.

INTRODUCTION

Bioprocess technology involving the combination of living matter (whole organism or enzymes) supplemented with nutrients under laboratory conditions to achieve a particular biological product is one of the main significance in modern industry. In the last years many innovative technologies towards the generation of ecological energy were developed. Lignocellulolytic enzymes have important applications to alternative bioprocess; nevertheless it is necessary to optimize enzymatic production systems from new microorganisms providing low-cost bioproducts for bioremediation and bioethanol production.

White rot fungi (WRF) have an enzyme system producing hydrolytic and oxidative enzymes that act on cell wall component degradation such as lignin, cellulose and hemicelluloses (Baldrian and Gabriel, 2003). Cellulases can be classified into three types: endoglucanases (endo-β-1, 4-glucanase, EC 3.2.1.4), exoglucanases (exo-β-1,4-glucanase, EC 3.2.1.91) and 1,4-β-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) (Gielkens et al., 1999). Lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) are the most widely distributed ligninolytic enzymes (Eggert et al., 1996; Lobos et al., 1994; Lundell and Hatakka, 1994). These enzymes may be regulated by various factors such as heavy metals which have a significant influence on enzyme production (Baldrian and Gabriel, 2003). Recently, we described the ability of laccase secretion of

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five native white rot fungi strains: *Ganoderma applanatum* BAFC 1168 strain F, *Coriolus versicolor* f. *antarcticus* BAFC 266, *Peniophora* sp. BAFC 633, *Trametes villosa* BAFC 2755 and *Pycnoporus sanguineus* BAFC 2126 (Fonseca et al., 2010; Preussler et al., 2009).

It has been reported that solid state fermentation (SSF) is a better system than submerged fermentation (SMF) for lignocellulolytic enzymes production (Dinis, 2009; Elisashvili, 2001; Revankar, 2007; Levin et al., 2010). In SSF systems, the type of substrate is an important component; it has a critical effect on enzyme yield and titers, rate of hydrolytic and oxidative enzymes, enzyme extraction systems and fungal growth inhibition. There are several different alternatives of substrates, depending on the availability, composition and subsequent processing technology that will be applied to prepare them for fermentation process. Low-cost substrates such as sawdust and other forestry wastes can be used for enzyme production representing an interesting ecological alternative.

The purpose of this study was to compare wood chips and sawdust as substrates for the production of hydrolytic (endo-β-1,4-glucanase and 1,4-β-glucosidase) and oxidative (laccases and manganese peroxidase) enzymes secreted by five white rot fungi native from Misiones, Argentina. We also evaluated the effect of the extraction method on enzyme recovery yield.

MATERIALS AND METHODS

Fungal strains and culture conditions

Ganoderma applanatum BAFC 1168 strain F was provided by the Culture Collection of the Faculty of Forestry, National University of Misiones, Argentina. *Coriolus versicolor* f. *antarcticus* BAFC 266, *Peniophora* sp. BAFC 633, *Trametes villosa* BAFC 2755 and *Pycnoporus sanguineus* BAFC 2126 were provided by the Mycological Culture Collection of the Department of Biodiversity and Experimental Biology, Faculty of Exact and Natural Sciences, University of Buenos Aires, Argentina.

Cultures of white rot fungi were maintained on 12.7 g/L malt extract and 20 g/L agar (MEA) plates at 4 °C. MEA was prepared by dissolving the components in distilled water, which resulted in a culture broth with a final pH of 5.5.

Six agar plugs (6 mm diameter) from each fungus growing on 5 days malt extract agar plates were used to inoculate liquid cultures (12.7 g/L malt extract and 5 g/L corn steep liquor).

For SSF experiments, fungi were grown in 20 mL of this medium for 3 days under agitation at 50 rpm and finally total transferred to 250 mL Erlenmeyer flasks containing 23 g of Loblolly pine chips (23x13x3 mm) or sawdust (*Pinus taeda,* 80 mesh) previously sterilized at 121 ºC for 15 min.

The whole material (solid material and liquid culture) was incubated at 60% moisture content and 29 ºC in static conditions for 32 days. All cultures were grown in triplicate.

Enzymes extraction

Enzymes were extracted at 25 ºC in static conditions in two extraction cycles (5h and 3h) with 50 mM sodium acetate buffer pH 5.5 supplemented with Tween 20 (0.1 g/L). Wood chips were first soaked with 50 mL extraction buffer for 5 h. The second extraction was performed with 25 mL extraction buffer for 3h. Sawdust was first soaked with 75 mL extraction buffer for 5 h. The second extraction was performed with 110 mL extraction buffer for 3h. The supernatants collected from all extractions were filtered, centrifuged at 5.000 rpm for 15 min and used to test enzyme activity.

Determination of reducing sugar

Reducing sugar was determined with the 3,5-dinitrosalicylic acid (DNS) method. Absorbance was measured at 540 nm in a Shimadzu UV-3600 spectrophotometer. The amount of sugar liberated was calculated using a glucose standard curve (Miller, 1959).

Enzyme activity assay

Laccase (EC 1.10.3.2) activity was measured at 30 ºC using 5 mM of 2,6-dimethoxyphenol (DMP) in 0.1 M sodium acetate buffer pH 3.6. Absorbance was monitored at 469 nm (E_{469} = 27.5 $mM^{-1}cm^{-1}$) in a Shimadzu UV-3600 spectrophotometer. One laccase activity unit was defined as the amount of enzyme required to oxidize 1 µmol of DMP per min at 30 ºC (Field et al. 1993).

Manganese peroxidase (EC 1.11.1.13) activity was measured at 30 ºC using 0.1 M phenol red in sodium dimethylsuccinate buffer pH 4.5. Absorbance was monitored at 610 nm (E_{610} = 22 mM^{-1} cm^{-1}) in a Shimadzu UV-3600 spectrophotometer. One MnP activity unit was defined as the amount of enzyme required to oxidize 1 µmol of phenol red per min at 30 ºC (Kuwahara et al., 1984).

Endo-β-1,4-glucanase (EC 3.2.1.4) activity was determined by measuring the liberation of reducing sugar with the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) using 0.5% carboxymethylcellulose (CMC) as substrate in 0.05 M sodium citrate buffer pH 5. Reactions were incubated at 50 ºC for 30 min. Absorbance was measured at 540 nm in a Shimadzu UV-3600 spectrophotometer. The carbohydrate fraction was

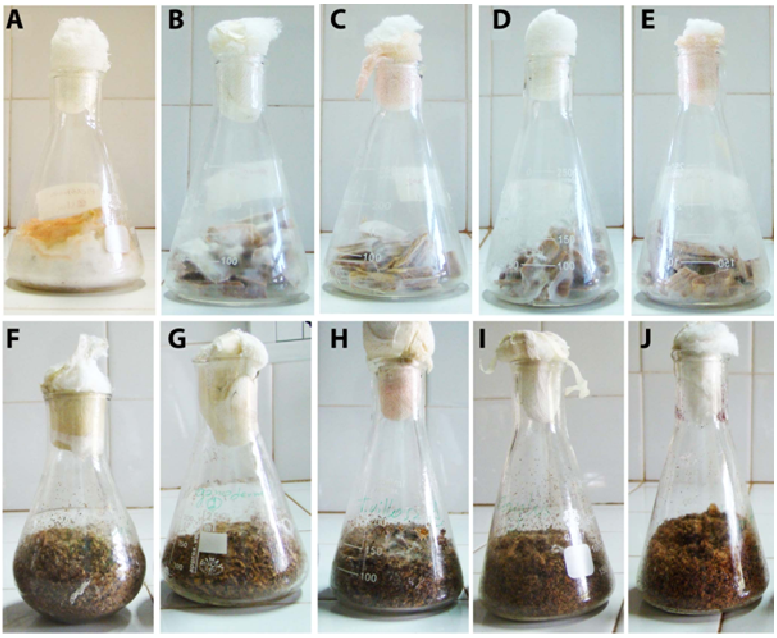

Figure 1. Fungal growth on chips SSF (upper) and sawdust SSF (lower)

extracted from the culture supernatant and the amount of sugar liberated was calculated using a glucose standard curve. One endo-β-1,4-glucanase activity unit was defined as the amount of enzyme that releases 1 µmol of reducing sugar per min at 50 ºC.

1,4-β-Glucosidase (EC 3.2.1.21) activity was measured using 4-nitrophenyl-β-glucoside (Sigma) as substrate for 4-nitrophenol determination. Absorbance was measured at 430 nm in a Shimadzu UV-3600 spectrophotometer. The incubation time was 48 min and the temperature 50 ºC. The amount of 4-nitrophenol liberated was calculated using a standard curve. One β-glucosidase activity unit was defined as the amount of enzyme that releases 1 µmol of 4-nitrophenol per min at 50 ºC (Enari and Niku-Paavola, 1987).

Statistics analysis

Two-way ANOVA statistics analysis was carried out with GraphPad Prism Program 4.00 for Windows version, GraphPad Software, San Diego California USA, www.graphpad.com.

RESULTS

Effect of type of substrate on fungal growth and enzyme production

Two forms of lignocellulosic substrates were utilized for solid state fermentation: wood chips and sawdust. Figure 1 shows the differential growth on the two types of substrates. On wood chips, *P. sanguineus* BAFC 2126 showed the densest mycelia growth covering the whole mass of the substrates, while the rest of the fungi covered the surface of the substrates in small extent.

Chips or sawdust were inoculated by liquid culture and incubated at 60% moisture content and 29ºC in static conditions for 32 days. A and F) *Pycnoporus sanguineus* BAFC 2126. B and G) *Ganoderma applanatum* strain F. C and H) *Trametes villosa* BAFC 2755. D and I) *Coriolus versicolor* f. *antarticus* BAFC 266. E and J) *Peniophora* sp. BAFC 633.

Table 1 shows the total enzymatic activity detected for each strain on both substrates. Endo-β-1,4-glucanase and 1,4-β-glucosidase activities were detected on both substrates for all fungi. On chips-SSF samples, *P. sanguineus* BAFC 2126, *Peniophora* sp. BAFC 633 and *C. versicolor* f. *antarcticus* BAFC 266 produced the greatest amounts of endo-β-1,4-glucanase (average 0.62 U/g, $p>0.05$). However, on sawdust-SSF samples, endo-β-1,4-glucanase secretion was greater than chips-SSF with the maximal levels for *P. sanguineus* BAFC 2126, *Peniophora* sp. BAFC 633 and *T. villosa* BAFC 2755 (average 4.352U/g).

Regarding 1,4-β-glucosidase, *P. sanguineus* BAFC 2126 secreted the greatest amounts on chips-SSF (0.846 U/g, $p> 0.001$), while on sawdust-SSF, the amounts of this enzyme where similar for all fungi. When comparing both substrates, the greatest differences were found with *G. applanatum* BACF 1168, with increases of 19-fold for endo-β-1,4-glucanase and 7.4-fold for 1,4-β-glucosidase, and T. *villosa* (16.3 fold for endo-β-1,4-glucanase and

Table 1. Total enzymes production on sawdust and chips SSF

Fungi	Enzyme	Chips SSF U/g substrate[a]	Sawdust SSF U/g substrate[a]	Enzyme production ratio (Sw[c]/Ch[d])
C. versicolor f. *antarcticus* BAFC 266	ENDO[b]	0.616 ± 0.120	2.437 ± 0.181	3.9
	1,4-β-glucosidase	0.256 ± 0.075	0.515 ± 0.075	2.1
	Laccase	Undetectable	0.121 ± 0.032	-
	MnP	Undetectable	Undetectable	-
G. applanatum BAFC 1168 strain F	ENDO[b]	0.135 ± 0.004	2.817 ± 0.505	19.2
	1,4-β-glucosidase	0.057 ± 0.004	0.420 ± 0.039	7.4
	Laccase	Undetectable	Undetectable	-
	MnP	Undetectable	Undetectable	-
P. sanguineus BAFC2126	ENDO[b]	0.625 ± 0.166	4.815 ± 0.621	7.6
	1,4-β-glucosidase	0.846 ± 0.033	0.443 ± 0.064	0.5
	Laccase	0.042 ± 0.003	0.207 ± 0.025	4.9
	MnP	0.018 ± 0.002	Undetectable	-
Peniophora sp. BAFC 633	ENDO[b]	0.622 ± 0.187	4.244 ± 2.118	6.8
	1,4-β-glucosidase	0. 157 ± 0.059	0.513 ± 0.000	3.2
	Laccase	0.051 ± 0.004	0.259 ± 0.069	5.1
	MnP	0.020 ± 0.001	Undetectable	-
T. villosa BAFC 2755	ENDO[b]	0.245 ± 0.020	3.998 ± 0.884	16.3
	1,4-β-glucosidase	0.098 ± 0.023	0.481 ± 0.031	4.9
	Laccase	0.143 ± 0.004	1.417 ± 0.153	9.9
	MnP	0.105 ± 0.007	6.637 ± 0.229	63.2

a). Data were shown as media ± SD; b) endo-β-1,4-glucanase; c) Sawdust; d) Chips

Table 2. Hydrolytic enzymes activities and extraction coefficients

Fungi	Enzyme	Extraction steps	Chips		Sawdust	
			Total Enzymatic activity[a] (U)	2º / 1º extraction	Total Enzymatic activity[a] (U)	2º / 1º extraction
C. versicolor f. *antarcticus* BAFC 266	ENDO[b]	1º (5 h)	11.236 ± 2.791	0.28	31.290 ± 1.454	0.80
		2º (3 h)	3.182 ± 0.209		25.020 ± 2.969	
	1,4-β-glucosidase	1º (5 h)	4.535 ± 1.381	0.30	4.877 ± 0.952	1.43
		2º (3 h)	1.348 ± 0.338		6.972 ± 0.769	
G. applanatum BAFC 1168 strain F	ENDO[b]	1º (5 h)	2.166 ± 0.337	0.56	36.595 ± 5.521	0.78
		2º (3 h)	1.200 ± 0.011		28.448 ± 6.349	
	1,4-β-glucosidase	1º (5 h)	0.935 ± 0.062	0.39	3.726 ± 0.748	1.60
		2º (3 h)	0.369 ± 0.029		5.931 ± 0.167	
P. sanguineus BAFC2126	ENDO[b]	1º (5 h)	11.811 ± 2.767	0.24	73.169 ± 6.749	0.52
		2º (3 h)	2.826 ± 1.301		37.815 ± 7.779	
	1,4-β-glucosidase	1º (5 h)	18.00 ± 0.433	0.08	5.706 ± 1.202	0.76
		2º (3 h)	1.455 ± 0.334		4.487 ± 0.268	
Peniophora sp. BAFC 633	ENDO[b]	1º (5 h)	10.734 ± 3.616	0.36	62.665 ± 26.259	0.73
		2º (3 h)	3.815 ± 0.930		41.382 ± 22.717	

Table 2. Continue

T. villosa BAFC 2755	1,4-β-glucosidase	1º (5 h)	3.252 ± 1.293	0.11	4.576 ± 0	1.58
		2º (3 h)	0.364 ± 0.070		7.233 ± 0	
	ENDO[b]	1º (5 h)	3.794 ± 0.732	0.55	59.867 ± 18.858	0.57
		2º (3 h)	2.088 ± 0		33.347 ± 2.000	
	1,4-β-glucosidase	1º (5 h)	1.808 ± 0.269	0.24	4.910 ± 0.385	1.24
		2º (3 h)	0.448 ± 0.270		6.144 ± 0.335	

a) Data were shown as media ± SD; b) endo-β-1,4-glucanase

4.9-fold for 1,4-β-glucosidase).

Regarding oxidative enzymes, *Peniophora* sp. BAFC 633, *P. sanguineus* BAFC 2126 and *T. villosa* BAFC 2755 secreted detectable levels of laccase on both substrates. *Peniophora* sp. BAFC 633 and *P. sanguineus* BAFC 2126 produced similar levels of laccases for both substrate ($p>0.005$).

C. versicolor f. *antarticus* BAFC 266 secreted detectable laccases levels only on sawdust cultures. Both oxidative enzymes were undetectable in *G. applanatum* BAFC 1168.

When comparing both substrates sawdust-SSF samples rendered superior levels of laccase for all fungi ($p<0.001$).

Regarding MnP, only *T. villosa* BAFC 2755 showed detectable amounts on both substrates.

T. villosa BAFC 2755 secreted the greatest amount of laccase (Sw: 1.417 ± 0.153 U/g; Ch: 0.143 ± 0.004 U/g) and MnP (Sw: 6.637 ± 0.229 U/g; Ch: 0.105 ± 0.007 U/g).

Influence of extraction conditions on enzyme recovery

Due to the different aggregation form of the substrates under study, appropriate experimental procedure was necessary to evaluate the amount of each enzyme recovery on SSF production in wood chips and sawdust. Bearing in mind that part of the extracellular enzymes produced during SSF incubation are absorbed on lignocellulosic surfaces and other portion can be trapped in the fibrous structure, it was decided to conduct two successive extractions with acetate buffer plus Tween 20 at 25ºC: first extraction of 5 h and second extraction of 3 h.

To evaluate the effect of the extraction process on the enzyme recovery yield, both hydrolytic (Table 2) and oxidative enzyme activities (Table 3) after the 1st and 2nd extraction step were determined and $2^{nd}/1^{st}$ extraction ratio was calculated.

Regarding hydrolytic enzymes recovery, lesser values of $2^{nd}/1^{st}$ extraction ratio were found on chips SSF samples, although the 2nd extraction step contributed with 35% of the total endo-β-1,4-glucanase extracted from *T. villosa* BAFC 2755 and *G. applanatum* BAFC 1168 strain F.

On sawdust SSF samples greater values of $2^{nd}/1^{st}$ extraction ratio were found. The second extraction step provides 43 to 62% of the total 1,4-β-glucosidase recovery yield, and 34 to 45% of endo-β-1,4-glucanase enzyme recovery. This finding confirms the necessity of a second extraction step to improve the yield of enzyme recovery.

Table 2

Regarding laccase recovery on chips-SSF, only *T. villosa* BAFC 2755 yielded greater amounts of laccase in the second extraction step (52%). *Peniophora* sp. BAFC 633 and *P. sanguineus* BAFC 2126 showed lesser values of the $2^{nd}/1^{st}$ extraction ratio, with 16% and 25% of total enzyme extracted respectively. On the other hand, laccase secreted on sawdust SSF samples showed high $2^{nd}/1^{st}$ extraction ratio for all fungi. This is a demonstration that a 2nd extraction must be considered to increase enzyme recovery.

Influence of the type of substrate on reducing sugars generation

Substrates bioconversion by hydrolytic enzymes should be evaluated by the amount of reducing sugar generated. Theoretical reducing sugar recovery from the complete hydrolysis of lignocellulosic material is approximately 40% of wood weight (Berrocal Jimenez et al., 2004). The highest reducing sugar yield produced *C. versicolor* f. *antarcticus* BAFC 266 and *Peniophora* sp. BAFC 633 ($p<0.01$) grown on sawdust with 38% and 35% respectively. Sawdust samples provide more specific area available for fungal attack resulting in a higher cellulose conversion than chips samples (Table 4).

The effect of particle size has typically been related to the available surface area for reactions (e.g., water binding, solubilization, heat transfer, and swelling) and enzymatic action (Al-Rabadi *et al*., 2009; Mahasukhonthachat *et al*., 2010). The results showed

Table 3. Oxidative enzymes activities and extraction coefficients

Fungi	Enzyme	Extraction steps	Chips-SSF Total Enzymatic activity[a] (U)	Chips-SSF 2º / 1º extraction	Sawdust-SSF Total Enzymatic activity[a] (U)	Sawdust-SSF 2º / 1º extraction
C. versicolor f. *antarcticus* BAFC 266	Laccase	1º (5 h)	Undetectable	-	1.394 ± 0.487	1
		2º (3 h)	Undetectable		1.394 ± 0.238	
	MnP[b]	1º (5 h)	Undetectable	-	Undetectable	-
		2º (3 h)	Undetectable		Undetectable	
P. sanguineus BAFC2126	Laccase	1º (5 h)	0.713 ± 0.029	0.34	2.718 ± 0.376	0.75
		2º (3 h)	0.242 ± 0.037		2.045 ± 0.208	
	MnP[b]	1º (5 h)	0.420 ± 0.047	-	Undetectable	-
		2º (3 h)	Undetectable		Undetectable	
Peniophora sp. BAFC 633	Laccase	1º (5 h)	0.989 ± 0.086	0.19	3.199 ± 0.555	0.86
		2º (3 h)	0.186 ± 0.004		2.759 ± 1.028	
	MnP[b]	1º (5 h)	0.455 ± 0.034	-	Undetectable	-
		2º (3 h)	Undetectable		Undetectable	
T. villosa BAFC 2755	Laccase	1º (5 h)	1.583 ± 0.078	1.07	4.685 ± 0.898	6
		2º (3 h)	1.700 ± 0.011		27.910 ± 2.626	
	MnP[b]	1º (5 h)	1.780 ± 0.150	0.35	12.641 ± 2.844	1.11
		2º (3 h)	0.631 ± 0.022		14.007 ± 2.420	

a) Data were shown as media ± SD; b) Manganese peroxidase

Table 4. Reducing sugar generated on SSF

Fungi	Chips-SSF mg/g[a]	Sawdust-SSF mg/g[a]	Reducing sugar Sw-SSF/Ch-SSF[b]
C. versicolor f. *antarcticus* BAFC 266	21.44 ± 9.25	155.45 ± 25.48	7
G. applanatum BAFC 1168 strain F	12.75 ± 0.73	103.96 ± 4.73	8
P. sanguineus BAFC2126	17.41 ± 7.26	103.68 ± 4.57	6
Peniophora sp. BAFC 633	33.44 ± 14.33	140.83 ± 2.42	4
T. villosa BAFC 2755	undetectable	13.73 ± 6.21	-

a)Data were shown as media ± SD; b) Sawdust-SSF/Chips-SSF

that the greater the particle size (chips), the lowest the reducing sugar concentration.

The higher available surface area of lower particle size samples improves diffusion processes rendering better access of the enzymes to the macromolecules and leads to higher sugar yields compared to higher particle sizes.

DISCUSSION

Many fungal species have the ability to degrade cellulose by producing extracellular fungal cellulose degrading enzymes. The extracellular enzymes production by SSF is greatly affected by the type of substrate used. Our study focuses on measuring two hydrolytic (endo-β-1,4-glucanase and 1,4-β-glucosidase) and two oxidative enzymes (laccases and MnP) secreted by five different white rot fungi strains from Misiones (Argentina), using wood chips and sawdust as substrates without the addition of external activators. The amounts of endo-β-1,4-glucanase and 1,4-β-glucosidase activities are comparable with other described in the literature for other fungi species such as *Trichoderma* spp. (Singhania et al., 2007) and mutant fungi strain (Yuntao et al., 2011).

Laccase production on wood chips is compared to other data reported in literature for engineering purposes (D'Souza-Ticlo et al., 2009). They are even greater than other values (2.5 mg/L - 0.23 U/mL), achieved by a heterologous expression of a laccase secreted by the white rot fungus *Trametes versicolor* in the yeast

Yarrowia lipolytica (Jolivalt et al., 2005).

Manganese peroxidase is significantly affected by natural co-oxidants of constituents present in the wood. We determined MnP activities only in basal conditions without exogenous Mn^{2+} addition. Under these conditions only *T. villosa* BAFC 2755 showed significant amounts of MnP.

T. villosa BAFC 2755 grown on sawdust-SSF was the only fungus that secreted significant amounts of hydrolytic and oxidatives enzymes. This could be a good model to study the combined effect of all enzymes in biotechnological process.

With respect to the extraction conditions, data reported by Vikineswary et al. (2006) were similar to our findings on *P. sanguineus* BAFC 2126 grown on sawdust. They found the highest laccase yield (50%) when the extraction was conducted at 25 ºC compared to 4ºC at pH 5.0. Our data suggests that it may be necessary to carry out two extraction steps for optimal recovery yield, especially in sawdust systems. This an important issue when planning an enzyme production system at large scales to assure optimal enzyme recovery. The cost of additional extraction steps must be weighed against optimal recovery yield.

Considering substrate-enzyme interactions, many authors proposed that cellulases can be deeply absorbed on cellulose substrates. Since part of the extracellular enzymes produced during wood biodegradation are adsorbed on wood cell walls, successive extractions may be necessary to recover most of the enzymes produced. The strength of these interactions bears an inverse correlation with enzyme recovery extent (Tu et al., 2009). The $2^{nd}/1^{st}$ extraction ratio is related to the adsorption capacity of the enzymes on wood substrate and also defines the requirement of additional soaking for complete enzyme extraction. Our results showed that size particle is a significant factor regarding enzyme extraction, indicating that the quantitative contribution of a second enzyme extraction is more important in substrates with small size particle. With 5 h of extraction time we recovered more than 50% of secreted enzymes on chips. Additional time involves extra amounts of enzymatic units recovered in a much diluted solution. Data reported by de D'Souza-Cruz et al. (2004) showed recovery yields of 50% after 4 h of extraction. Our data are in accordance with D'Souza-Cruz's reports, showing hydrolytic enzymes recovery of 35 – 90% after 5 h of extraction for chips SSF. However, second extraction is required on sawdust SSF due to lower enzyme extraction achieved on the first extraction step (38 – 57% of total enzyme). These observations demonstrate that a second soaking step may be required and provide indirect evidence of the recycling properties. *T. villosa* BAFC 2755 showed the greater extraction ratio indicating probably the best recycling properties. As a result, a second extraction was necessary for optimal enzyme recovery on sawdust SSF samples.

CONCLUSIONS

In conclusion, the form of substrate (sawdust or chips) has a significant effect on the yield of enzyme extracted. Sawdust, with a finer particle size is more easily colonized than wood chips and to some extent, the fungal mycelium becomes overly bound up to the substrate, requiring a second extraction step to recover the most part of the enzyme. Sawdust-SSF was the most efficient substrate for hydrolytic and oxidative enzymes production. *Peniophora* sp. BAFC 633, *T. villosa* BAFC 2755 and *P. sanguineus* BAFC 2126 produced both types of enzymes on chips and sawdust-SSF. Endo-β-1,4-glucanase and laccase were the principal enzymes obtained with both substrates. On the other hand, *G. applanatum* BAFC 1168 strain F produced only hydrolytic enzymes and *C. versicolor* f. *antarcticus* BAFC 266 only produced laccase when using sawdust as substrate.

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