Biotechnology Letters (2005) 27: 955–959
DOI 10.1007/s10529-005-7662-x

Carbon and nitrogen sources influence the ligninolytic enzyme activity of *Trametes versicolor*

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Received 4 February 2005; Revisions requested 25 February 2005 and 14 April 2005; Revisions received 6 April 2005 and 6 May 2005; Accepted 11 May 2005

Key words: carbon and nitrogen sources, laccase, manganese peroxidase, *Trametes versicolor*

Abstract

Among carbon sources studied, cellobiose and mannitol provided the highest laccase (Lac) activity (648 and 742 U l^{-1}, respectively) of *Trametes versicolor* 775 while glucose gave maximum manganese peroxidase (MnP) and peroxidase activities (44 and 114 U l^{-1}, respectively). Citrus fruit peel as growth substrate enhanced Lac activity 7-fold when compared to the medium with cellobiose, whereas grape vine sawdust increased MnP and peroxidase activity up to 148 and 677 U l^{-1}, respectively.

Introduction

White rot basidiomycetes are the most potent lignin degraders of all known microorganisms. Key extracellular enzymes of the fungal ligninolytic system are laccase (Lac) and two heme-containing peroxidases: lignin peroxidase and manganese peroxidase (MnP). A wide array of white rot basidiomycetes has been examined to find hyper-producers of ligninolytic enzymes for industrial and technological applications. Appropriate biotechnological exploitation of white rot fungi requires a sound knowledge of mechanisms regulating the synthesis of enzymes involved in lignocellulose degradation. Recent work has shown that these fungi grow well on media containing lignocellulose producing both MnP and Lac under both liquid and solid culture conditions (Elisashvili *et al.* 2001, 2002, Moldes *et al.* 2004). Furthermore, basidiomycetes cultivation in the presence of some lignocellulosic residues significantly stimulated ligninolytic enzyme secretion without supplementation of culture medium with specific inducers (Rosales *et al.* 2005). There is, however, no systematic research aiming to establish the reason by which these residues stimulate enzyme production. Only a few studies have been conducted on the effect of carbon sources on ligninolytic enzyme production (Elisashvili *et al.* 2002, Galhaup *et al.* 2002). Thus, supplementation of the medium by fructose instead of glucose resulted in a 100-fold increase in specific Lac activity of *Basidiomycete* sp. I-62 (Mansur *et al.* 1997). Nitrogen sources appear to be another powerful factor affecting ligninolytic enzyme production of white rot fungi (Elisashvili *et al.* 2001, Galhaup *et al.* 2002).

The aim of this study was to evaluate, for the first time, the significance of various carbon sources for oxidative enzyme production by new isolates of *Trametes versicolor*.

Materials and methods

Microorganisms and cultivation

Trametes versicolor 145 and *T. versicolor* 775 were from the Basidiomycetes Culture Collection of Haifa University, Israel (Wasser *et al.* 2002). The inoculum was prepared by fungal cultivation on a rotary shaker at 180 rpm in 500 ml flasks containing 100 ml basal synthetic medium (per liter): 10 g glucose, 0.8 g KH_2PO_4, 2 g NH_4NO_3, 0.4 g Na_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 2 g yeast extract. The following microelements were added to a basal medium (per liter): 0.001 g $ZnSO_4{\cdot}7H_2O$, 0.005 g $FeSO_4{\cdot}7H_2O$, 0.06 g $CaCl_2{\cdot}2H_2O$, 0.005 g $CuSO_4{\cdot}7H_2O$, 0.005 g $MnSO_4{\cdot}H_2O$. Medium was adjusted to pH 6.0 before sterilization. The resulting mycelium was homogenized in a Waring laboratory blender and the homogenate was used to inoculate 20 ml nutrient medium with various carbon sources (see Table 1) in 100 ml Erlenmeyer flasks and shaken at 180 rpm at room temperature. After 5 and 8 days, the mycelia were separated by filtration and dried to constant weight at 60 °C, while culture filtrates were used to measure the enzyme activity. All experiments were performed at least two times using three replicates. The data presented in the tables correspond to mean values with a standard error less than 12%.

Enzyme assays

Laccase (Lac) activity was determined by following the oxidation of syringaldazine as a substrate at 525 nm (ϵ_{525} = 65000 M^{-1} cm^{-1}) for 60 s (Leonowicz & Grzywnowicz 1981). The reaction mixture in 1 ml contained 100 mM acetate buffer (pH 5.0), 1 mM syringaldazine and 100 μl appropriately diluted culture filtrate.

Manganese peroxidase activity was assayed by the oxidation of Phenol Red (Orth *et al.* 1993). The reaction mixture in 1 ml contained 50 mM sodium lactate succinate buffer (pH 4.5), 0.1 mM

Table 1. Oxidative enzyme activity of *T. versicolor* 775 and *T. versicolor* 145 depending on the carbon source in the medium.

Carbon source*	Days	*Trametes versicolor* 775			*Trametes versicolor* 145		
		Laccase (U l^{-1})	MnP (U l^{-1})	Peroxidase (U l^{-1})	Laccase (U l^{-1})	MnP (U l^{-1})	Peroxfidase (U l^{-1})
Glucose	5	137 ± 12.3	12 ± 0.5	13 ± 1.5	66 ± 7.5	Trace	1 ± 0.2
	8	133 ± 7.8	44 ± 4.5	15 ± 7.2	62 ± 9.0	40 ± 6.0	80 ± 4.3
Mannitol	5	648 ± 9.0	8 ± 0.1	33 ± 3.8	26 ± 0.9	Trace	1 ± 0.1
	8	204 ± 16.4	40 ± 1.4	37 ± 4.7	16 ± 0.4	4 ± 0.4	14 ± 1.3
Cellobiose	5	663 ± 22.2	14 ± 2.7	7 ± 0.9	34 ± 4.1	Trace	1 ± 0.1
	8	742 ± 29.8	26 ± 2.0	16 ± 1.1	26 ± 3.4	7 ± 0.5	12 ± 0.2
Maltose	5	178 ± 3.4	8 ± 0.3	11 ± 1.4	69 ± 10.3	Trace	1 ± 0.1
	8	95 ± 3.6	10 ± 0.4	29 ± 2.4	48 ± 3.5	10 ± 0.9	22 ± 1.0
Lactose	5	290 ± 14.8	10 ± 0.6	17 ± 3.4	30 ± 3.2	4 ± 0.3	14 ± 1.0
	8	323 ± 18.1	9 ± 1.3	12 ± 2.0	49 ± 1.9	12 ± 0.7	22 ± 3.1
Sodium gluconate	5	293 ± 12.7	2 ± 0.2	4 ± 0.4	10 ± 0.7	Trace	Trace
	8	360 ± 19.2	2 ± 0.3	5 ± 0.3	29 ± 1.0	Trace	1 ± 0.1
Xylan	5	131 ± 2.7	12 ± 0.2	16 ± 0.7	22 ± 2.4	8 ± 0.7	17 ± 1.1
	8	136 ± 9.5	18 ± 1.4	39 ± 9.0	31 ± 1.7	24 ± 1.9	53 ± 3.7
Avicel	5	48 ± 2.7	10 ± 0.1	25 ± 1.9	15 ± 0.1	1 ± 0.1	2 ± 0.1
	8	30 ± 2.5	19 ± 4.3	34 ± 3.2	11 ± 0.5	36 ± 3.7	47 ± 2.9
CMC	5	131 ± 3.7	5 ± 0.4	11 ± 0.2	27 ± 2.7	Trace	Trace
	8	136 ± 12.9	1 ± 0.1	4 ± 0.6	35 ± 2.8	Trace	1 ± 0.1
Grape vine sawdust	5	261 ± 15.1	144 ± 10.2	177 ± 19.0	43 ± 6.2	43 ± 5.8	69 ± 3.4
	8	27 ± 1.4	148 ± 9.7	677 ± 13.7	24 ± 4.1	54 ± 2.9	238 ± 14.3
Mandarin peels	5	5243 ± 113	28 ± 3.7	61 ± 4.3	428 ± 19.5	16 ± 1.2	43 ± 3.1
	8	3434 ± 80.9	54 ± 1.7	95 ± 2.5	79 ± 2.2	18 ± 0.7	75 ± 2.2

*All carbon sources were added at 10 g l^{-1} except for grape vine sawdust and ground mandarin peels, which were used at 40 g l^{-1}.

$MnSO_4$, 0.1 mM H_2O_2, 3 mM Phenol Red, and 100 μl enzyme filtrate. In addition, peroxidase activity was measured under the same conditions but without manganese. The reaction was terminated by the addition of 40 μl of 2 M NaOH. Absorbance was recorded at 610 nm (ϵ_{610} = 22,000 M^{-1} cm^{-1}). All enzyme assays were carried out at 20 °C. One unit of Lac, MnP, and peroxidase activity was defined as an amount of enzyme that transformed 1 μmol substrate min^{-1}.

Results and discussion

Effect of carbon sources on enzyme production

Trametes versicolor is an excellent producer of both extracellular Lac and MnP in fermentation of mandarin peels (Mikiashvili *et al.* 2004). Since this material contains 11–13% (w/w) reducing sugars and 32–34% (w/w) soluble carbohydrates, we supposed that the extractable carbohydrates from mandarin peels might enhance the ligninolytic enzyme production. In connection with this, we studied the role of different carbon sources in *T. versicolor* ligninolytic enzyme production for the first time. All tested compounds ensured good growth of fungi providing biomass accumulation from 6 to 11 g l^{-1}, however, fungi enzyme activity significantly depended on the carbon source in the medium (Table 1). The polysaccharides appeared to be poor substrates for Lac production while easily metabolizable compounds ensured comparatively high Lac activity. However, none of the carbohydrates tested produced as high Lac titers as did mandarin peels in the presence of which the Lac activity of *T. versicolor* 775 reached 5250 U l^{-1}, i.e., it was 7-fold higher than that in fungus cultivation in the presence of cellobiose.

The data are in agreement with the results of Galhaup *et al.* (2002) who found that glucose and cellobiose were efficiently and rapidly utilized by *Trametes pubescens* resulting in high Lac activity. Similarly, the replacement of crystalline cellulose or xylan by cellobiose increased Lac activity of *Cerrena unicolor* by 21- and 70-fold, respectively (Elisashvili *et al.* 2002). Furthermore, in *T. versicolor* lignocellulosic material (barley bran) increased almost 50-fold laccase activity (up to 3200 U l^{-1}) compared to the control culture with glucose (Moldes *et al.* 2004).

Lac activity secreted by both strains during growth in the presence of sawdust of grape vine cuttings appeared to be significantly low in this study (Table 1), which can be attributed to its low content of reducing sugars (1.6%) and high content of lignin (23–27%). In contrast, this lignified material seems to be a convenient growth substrate followed by mandarin peels for MnP and peroxidase production by both fungi (Table 1), while supplementation of the medium with different carbon sources caused the variation in MnP activity of *T. versicolor* 775 and *T. versicolor* 145, respectively, from 5.3 and 0.2 U l^{-1} (medium with CMC) to 43.8 and 40 U l^{-1} (medium with glucose). Schlosser *et al.* (1997) indicated that MnP activity was lacking during cultivation of *T. versicolor* on glucose and appeared only during growth on lignocellulosic substrates.

In this study, peroxidase activity of *T. versicolor* was analyzed for the first time. The highest peroxidase titer was revealed when *T. versicolor* 145 was grown on medium containing glucose (Table 1). Xylan and crystalline cellulose also ensured high peroxidase activity of both fungi. In contrast, sodium gluconate and CMC were rather poor carbon sources for the production of this enzyme.

Maximum Lac activity was recorded after 5 days of fungal growth while the highest peroxidase activity, in general, was observed after 8 days of growth. Of the two strains, *T. versicolor* 775 gave the higher activities of the enzymes.

Effect of mandarin peel concentration on enzyme production

The data of some authors indicate that the effect of carbon sources significantly depends on its concentration in the nutrition medium (Galhaup *et al.* 2002). In this study, increasing substrate concentration in the medium from 20 to 40 g l^{-1} increased the extracellular laccase activity of *T. versicolor* 775 more than 5-fold (Figure 1) presumably by increasing the content of fermentable carbohydrates. In contrast, the activity of MnP and peroxidase varied to a small extent on the changes in concentration of milled mandarin peels, and no correlation was revealed for these enzymes.

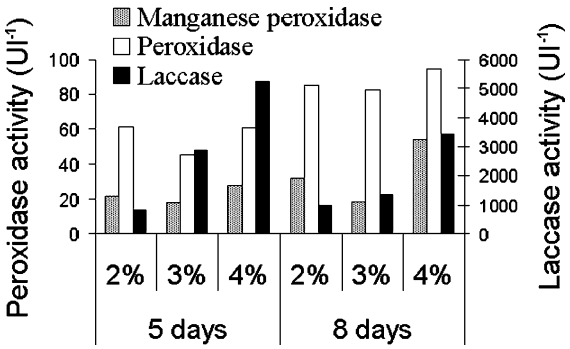

Fig. 1. Effect of mandarin peels concentration on laccase, manganese peroxidase and peroxidase activity of *T. versicolor* 775 in submerged cultivation on basal medium.

Effect of nitrogen source on enzyme production

Many previous studies have proved that both the nature and concentration of nitrogen sources are powerful nutrition factors regulating ligninolytic enzyme production by wood-rotting basidiomycetes (Buswell *et al.* 1995, Elisashvili *et al.* 2001, Galhaup *et al.* 2002). In our study, all nitrogen sources were added to the medium with glucose at 20 mM nitrogen. None of them significantly changed the final pH of medium. Organic nitrogen sources positively affected *T. versicolor* 775 Lac accumulation in the culture liquid (Table 2). Among them, peptone and corn steep liquor exhibited significant influence on Lac secretion giving a 7-fold increase compared with the control medium containing only yeast extract as a nitrogen source. Utilization of ammonium nitrate seems to sharply (44-fold) accelerates the secretion of MnP, while medium supplementation with $NaNO_3$, $NH_4H_2PO_4$, and $(NH_4)_2SO_4$ inhibit this enzyme accumulation. Concerning peroxidase activity, ammonium nitrate and ammonium chloride increased enzyme production by 101- to 104-fold compared with the control medium. In addition, it is worth noting that all organic nitrogen sources tested favored both comparatively high MnP and peroxidase accumulation by *T. versicolor* 775.

The submerged cultivation of *T. versicolor* 145 in the presence of ammonium phosphate followed by casein hydrolysate and corn steep liquor supported maximum Lac production.

Table 2. Oxidative enzyme activity of *T. versicolor* 775 and *T. versicolor* 145 depending on the nitrogen source in the medium.

Nitrogen source*	Days	*Trametes versicolor* 775			*Trametes versicolor* 145		
		Laccase (U l^{-1})	MnP (U l^{-1})	Peroxidase (U l^{-1})	Laccase (U l^{-1})	MnP (U l^{-1})	Peroxidase (U l^{-1})
Control	5	73 ± 3.5	6 ± 0.3	4 ± 0.3	73 ± 5.3	24 ± 2.4	18 ± 2.1
	8	29 ± 2.8	1 ± 0.1	2 ± 0.1	64 ± 2.8	24 ± 2.3	15 ± 0.1
NH_4NO_3	5	122 ± 12.9	3 ± 0.1	7 ± 0.1	95 ± 3.5	8 ± 0.1	21 ± 0.7
	8	125 ± 6.0	240 ± 27.1	364 ± 25.7	161 ± 4.0	34 ± 3.1	41 ± 1.9
$NaNO_3$	5	40 ± 3.0	4 ± 0.2	6 ± 0.3	47 ± 5.0	34 ± 1.5	28 ± 1.2
	8	26 ± 0.9	3 ± 0.2	6 ± 0.3	48 ± 7.0	29 ± 1.3	27 ± 1.1
NH_4Cl	5	95 ± 2.7	1 ± 0.1	2 ± 0.2	116 ± 2.6	3 ± 0.1	5 ± 0.3
	8	331 ± 24.6	84 ± 3.8	355 ± 17.3	142 ± 2.8	24 ± 1.4	31 ± 2.6
$NH_4H_2PO_4$	5	103 ± 4.4	Trace	1 ± 0.1	198 ± 5.9	37 ± 2.1	55 ± 2.1
	8	413 ± 30.6	3 ± 0.3	16 ± 0.8	375 ± 6.7	21 ± 0.3	30 ± 1.4
$(NH_4)_2SO_4$	5	206 ± 6.6	1 ± 0.1	2 ± 0.1	53 ± 4.6	1 ± 0.1	1 ± 0.1
	8	226 ± 8.2	3 ± 0.3	6 ± 0.3	195 ± 8.2	1 ± 0.1	2 ± 0.2
Peptone	5	287 ± 26.4	24 ± 2.2	58 ± 6.3	170 ± 12.6	46 ± 1.9	58 ± 1.5
	8	521 ± 47.8	33 ± 4.0	69 ± 6.2	188 ± 14.5	48 ± 2.3	81 ± 2.4
Urea	5	257 ± 18.0	50 ± 4.3	89 ± 8.0	94 ± 7.3	13 ± 0.6	41 ± 2.9
	8	219 ± 20.1	11 ± 0.6	19 ± 1.0	95 ± 3.5	20 ± 0.2	52 ± 2.6
Casein hydrolysate	5	173 ± 11.7	32 ± 3.1	59 ± 3.0	202 ± 8.8	41 ± 1.7	45 ± 1.4
	8	264 ± 14.8	10 ± 0.7	27 ± 1.6	255 ± 11.5	58 ± 1.4	98 ± 2.8
Corn steep liquor	5	171 ± 7.7	28 ± 1.1	39 ± 1.1	200 ± 8.1	60 ± 2.3	131 ± 3.9
	8	526 ± 7.1	23 ± 0.6	36 ± 1.7	250 ± 5.9	50 ± 2.5	64 ± 2.7

*All nitrogen sources used to give 20 mM N.

Supplementation of casein hydrolysate and corn steep liquor enhanced the MnP and peroxidase production 2- to 7-fold, while fungus cultivation in the presence of ammonium sulfate sharply inhibited enzyme secretion by *T. versicolor* 145.

Conclusion

The results obtained in this paper showed that the secretion of ligninolytic enzymes by *T. versicolor* to a great extent is regulated by carbon and nitrogen sources in the medium. The proportion of ligninolytic enzymes also can be changed by variation of these compounds in nutrition medium. *T. versicolor* 775 could be a very efficient producer of Lac, MnP, and peroxidase in cultivation on a simple medium containing lignocellulosic substrate. Elucidation of the role of other compounds extractable from mandarin peels in stimulation of ligninolytic enzyme production by *T. versicolor* is under investigation.

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