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Production of Mn-Dependent Peroxidase from *Bjerkandera fumosa* and Its Enzyme Characterization*1

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ABSTRACT

Manganese dependent peroxidase (MnP) is the most ubiquitous enzyme produced by white-rot fungi. MnP is known to be involved in lignin degradation, biobleaching and oxidation of hazardous organo-pollutants. *Bjerkandera fumosa* is a nitrogen-unregulated white-rot fungus, which produces high amounts of MnP in the excess of N-nutrients due to increased biomass yield. The objective of this study was to optimize the MnP production in N-sufficient cultures by varying different physiological factors such as Mn concentration, culture pH, and incubation temperature. The growth of fungus was optimal in pH 4.5 at 30°C. N_2-unregulated white-rot fungus produces high amounts of MnP in the excess N-nutrients. The fungus produced the highest level of MnP (up to 1000 U/ℓ) with 0.25 g/ℓ asparagine and 1 g/ℓ NH_4Cl as N source at 1.5 mM $MnCl_2$ concentration, pH value of 4.5 at 30°C. Purification of MnP revealed the existence of two isoforms: MnP1 and MnP2. The molecular masses of the purified MnP1 and MnP2 were in the same range of 42~45 kDa. These isoforms of *B. fumosa* strictly require Mn to oxidize phenolic substrates. Concerned to kinetic constants of *B. fumosa* MnPs, *B. fumosa* has similar Km value and Vmax compared to the other white-rot fungi.

Keywords : white-rot fungi, manganese-dependent peroxidase, *Bjerkandera fumosa*, Mn concentration, pH, incubation temperature

1. INTRODUCTION

Lignin biodegradation is a key step for carbon recycling in terrestrial ecosystems, where white-rot basidiomycetes degrade this recalcitrant wood polymer enabling cellulose utilization by microbial populations (Eriksson *et al.* 1990). The unique ability of these fungi to degrade lignin has been a matter of high scientific interest with the aim of developing environmentally-sound biotechnical alternatives to reduce the cost of energy and chemicals or lowering the environmental impact in paper pulp manufacturing (the first non-food industrial uti-

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lization of plant biomass) (Young and Akhtar, 1998). Their non-specific extracellular lignin degrading system has been shown to degrade a wide array of persistent environmental pollutants. These organisms have been investigated for environmental friendly removal of lignin and lignin-derived compounds in the manufacture of paper pulp (Samejima and Eriksson, 1991).

The extracellular ligninolytic system from white rot fungi consists mainly of oxidative enzymes: laccases (Lac; EC 1.10.3.2), lignin peroxidase (LiP; EC 1.11.1.14) and manganese peroxidase (MnP; EC 1.11.1.13). Those enzymes are involved in degradation of lignin by white-rot fungi together with laccase and H_2O_2-producing oxidases (such as EC 1.1.3.7 or EC 1.1.3.10) (Kirk and Farrell, 1987; Shimada and Higuchi, 1991). The degradation mechanism involves extracellular oxidoreductases (Leonowicz *et al.* 2001; Conesa *et al.* 2002), among which MnP is thought to play a crucial role. MnP is capable of oxidizing and depolymerizing natural and synthetic lignins as well as entire lignocellulosic substrate (Bao *et al.* 1994; de la Rubia *et al.* 2002; Giardina *et al.* 2000; Steffen *et al.* 2002; Steffen *et al.* 2003). MnP preferentially oxidizes manganese (II) Mn^{2+} ions into highly reactive Mn^{3+} ions, which in turn chelated by carboxylic acids oxidize various phenolic and certain non-phenolic aromatic substances (Muheim *et al.* 1990a; Muheim *et al.* 1990b; Heinfling *et al.* 1998a; Heinfling *et al.* 1998b). The degradative potential of MnP makes this enzyme attractive for biotechnological applications, *e.g.* in pulping and bleaching of cellulose, in removing of hazardous wastes or in certain organic syntheses (Bao *et al.* 1994; Forrester *et al.* 1988; Vares *et al.* 1995; Heinfling *et al.* 1998a; Heinfling *et al.* 1998b; Hofrichter, 1999a; Hofrichter, 1999b; Young *et al.* 2000; Conesa *et al.* 2002). Since individual species of white-rot fungi differ in the composition of their sets of ligninolytic enzymes (Galliano *et al.* 1991; Vares *et al.* 1995), it is necessary to study the production of these enzymes in species with different ecological backgrounds.

LiP is characterized by its ability to oxidize high redox-potential aromatic compounds and MnP requires Mn(II) to complete its catalytic cycle and forms Mn(III) chelators acting as diffusing oxidizers (Martínez, 2002). However, during last years, a novel class of ligninolytic peroxidase has been described. This peroxidase efficiently oxidizes both Mn(II) to Mn(III) (like MnP) and carries out Mn(II)-independent activity on aromatic substrates (like LiP). Peroxidase was described only in various strains of two fungal species—*Pleurotus* (Heinfling *et al.* 1998 b; Ruiz-Dueñas *et al.* 2001) and *Bjerkandera* (Heinfling *et al.* 1998b; Heinfling *et al.* 1998c; Mester and Field, 1998; Moreira *et al.* 2005).

In the case of *Bjerkandera* sp. BOS55, a versatile peroxidase is Mn - lignin peroxidase hybrid enzyme capable of oxidizing various phenolic and non-phenolic substrates, such as veratryl alcohol, in the absence of Mn(II) ions (Mester and Field, 1998). Peroxidase from *Bjerkandera adusta* described by Pogni *et al.* (2005) looks like a structural hybrid between LiP and MnP, and this hybrid combines the catalytic properties of both peroxidases, being able to oxidize typical LiP and MnP substrates. The catalytic mechanism of peroxidase is that of classical peroxidases, where the substrate oxidation is carried out by a two-electron multistep reaction at the expense of hydrogen peroxide (Pogni *et al.* 2005; Ortiz de Montellano, 1992). Peroxidase oxidizes Mn(II) to Mn(III), degrades the non-phenolic lignin model dimers, and oxidizes veratryl alcohol and *p*-dimethoxybenzene (Heinfling *et al.* 1998c; Camarero, 1999). Moreover, it can directly oxidize hydroquinones and substituted phenols that are not efficiently oxidized

by LiP and MnP in the absence of mediator or Mn(II), respectively (Heinfling *et al.* 1998b; Ruiz-Dueñas *et al.* 1999; Martínez, 2002). *Bjerkandera fumosa* is one of the most frequently occurring white-rot fungal species. It occurs as a saprophytic (and sometimes weakly parasitic) strain on stubs and stems of deciduous trees. Although the fungus grows well under laboratory conditions, its ligninolytic system has not yet fully studied.

This study was carried out to optimize extracellular enzymes, mainly MnP production from white-rot fungus, *Bjerkandera fumosa*, by various different culture conditions, such as Mn concentration, culture pH, and incubation temperature. The characteristics were described after MnP purified by anion exchange chromatography on a DEAE-Sepharose column, ultrafiltration and Sephadex G-100 column chromatography were described.

2. MATERIALS and METHODS

2.1. Microorganism

Bjerkandera fumosa (Pers.: Fr.) Karst. was obtained from the culture collection of the Department of Biochemistry, Maria Curie-Sklodowska University, Lublin, Poland. Stock cultures were maintained on 2% (w/v) malt extract agar slants at 4°C. *B. fumosa* was precultured on malt extract agar plates at 30°C for 7 days, from which five 6 mm agar plugs were transferred to Erlenmeyer flasks, and used for the further experiments.

2.2. Culture Conditions

The inoculum was prepared by homogenizing 7 day cultures growing in 250 mℓ Erlenmeyer flasks with 100 mℓ of the basic Kirk's medium (BKM) (Kirk *et al.* 1978). BKM consisted of (per liter) 10 g glucose, 2 g KH_2PO_4, 0.5 g $MgSO_4x7H_2O$, 0.1 g $CaCl_2$, 0.5 g ammonium tartrate, and 1.8 g 2,2-dimethyl succinate. The cultures were incubated in an orbital shaker at 30°C and 150 rpm. Every day, 3 flasks were taken for enzymatic, analytical and biomass determination.

2.3. Media Compositions

All experiments were cultured on the BKM in 250 mℓ Erlenmeyer flasks with 100 mℓ medium. Different cultures were assayed to reach nutritional conditions of different C (glucose), N (asparagine and NH_4Cl), Mn^{2+} ($MnCl_2x4H_2O$) concentrations (Forrester *et al.* 1988; Youngs *et al.* 2000) and different pH (adjusted with 0.1% HCl and 0.1% NaOH). Glucose concentration was varied from 5~20 g/ℓ in an N-sufficient medium (asparagine and NH_4Cl).

2.4. Enzyme Assays

Extracellular ligninolytic enzymatic activities were determined as follows; MnP activity was measured at 270 nm by following the formation of Mn^{3+}-malonate-complexes (Wariishi *et al.* 1991; Wariishi *et al.* 1992), Laccase (Lac) activity was determined by following oxidation of syringaldazine at 525 nm (Leonowicz and Grzywnowicz, 1981), and Lignin peroxidase (LiP) activity was measured at 310 nm using veratryl alcohol as the substrate (Kirk *et al.* 1978; Kirk and Farrell, 1987). All activities were expressed in units (U) defined 1 mol of substrate oxidized per one minute.

2.5. MnP Purification

Culture fluid was harvested after 5 days of incubation, separated from the mycelium by filtration and concentrated using 10 kDa cut-off Millipore system. Extracellular proteins were precipitated by addition of $(NH_4)_2SO_4$ (0~

Table 1. Different concentration (g/ℓ) of asparagine and NH_4Cl in BKM with 10 g/ℓ of glucose as the main C source

Nutrients	Variation of culture medium							
	A	B	C	D	E	F	G	H
Asparagine, g/ℓ	0	0	0	0.25	0.25	0.25	0.5	1
NH_4^+Cl, g/ℓ	0.2	0.5	1	0.2	0.5	1	0.5	0.5

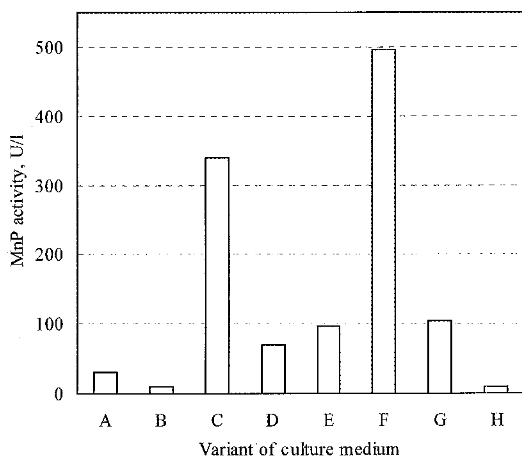

Fig. 1. Mn peroxidase activity in 10th day of *B. fumosa* growing on basal mineral Kirk medium (BKM) with different concentration of asparagine and NH_4Cl (see Table 1).

100% saturation) and dialyzed against 5 mM Tris-HCl buffer pH 6.3. Purified proteins were separated by anion exchange chromatography on a DEAE-Sepharose (Pharmacia, Sweden) column with 0～0.5 M NaCl gradient in 5 mM Tris-HCl buffer (pH 6.3). The elution of absorbing material from the column was monitored at 280 and 405 nm, respectively. Fractions of 6 ml were collected at a flow rate 1 mℓ min^{-1}. Fractions containing MnP were pooled, concentrated, and filtered using 10-kDa cut-off Amicon cell. MnP1 and MnP2 active fractions were chromatographed in Sephadex G-100 (Pharmacia, Sweden) column equilibrated with 30 mM Tris-HCl buffer of pH 6.3. Protein concentrations were determined using Bradford method with bovine serum albumin as a standard (Bradford, 1976).

2.6. MnP Characterization

The Km values of MnP1 and MnP2 for H_2O_2 and Mn^{2+} oxidation in 50 mM sodium malonate buffer (pH 4.5) at 30°C, were determined in the range micromole (μM). All kinetic studies were performed three times. Lineweaver-Burke plots were made from the initial rates obtained at varying substrate conditions. The rate of aromatic substrate oxidation was determined by spectrophotometric methods.

3. RESULTS and DISCUSSION

3.1. MnP Production at Different N and C Concentrations

Eight different concentrations of nitrogen source (N) were investigated to determine optimal nutritional conditions, which could maximize MnP production by *B. fumosa*. We have evaluated the influence of the organic N source as asparagine, versus inorganic N source as ammonium chloride (NH_4Cl) (Table 1 and Fig. 1).

The best MnP levels were obtained with 0.25 g/ℓ asparagine and 1 g/ℓ NH_4Cl as N source. We observed that the high concentration of inorganic N-source (C, F) produced 4 folds higher MnP activity compared to that of organic N-source (G, H). *B. fumosa* is a nitrogen-unregulated white-rot fungus, which produces high amounts of MnP in the excess of N-nutrients due to increased biomass yield.

Additional experiments were conducted to determine the effect of carbon concentration on

MnP production and fungal growth. For this purpose, different C concentrations added as glucose were assayed. The culture medium selected corresponded to F medium with 0.25 g/ℓ asparagine and 1 g/ℓ NH_4Cl as N source. Results indicated that MnP activity reached maximum levels around 400～500 U/ℓ. The growth and ligninolytic enzymes production by white-rot fungi depend strongly on the mode of oxygenation (Dosoretz *et al.* 1990a; Dosoretz *et al.* 1990b), the medium composition (Jimenez-Tobon *et al.* 1997; Moreira *et al.* 2001), mode of operation, and on the type of strain. The ligninolytic peroxidase activities in cultures of *Bjerkandera* are stimulated by high N medium (Mester *et al.* 1996; Mester and Field, 1998). In this study, the development of MnP activity in the cultures of *B. fumosa* is the result of a nitrogen-based regulatory mechanism rather than due to carbon limitation, but the maximum MnP production rate corresponds to excess glucose and nitrogen (asparagine and NH_4Cl) concentration.

3.2. Optimization of MnP Production

Cultures were incubated in N- and C-optimal medium supplemented with different concentrations of Mn^{2+} (0 to 2 mM) in order to investigate the effect of Mn level on the MnP production. Fig. 2 shows the peak of MnP activity at different levels of Mn^{2+}. The highest MnP activities were obtained at 1.5 mM $MnCl_2$ concentration when Mn^{2+} was added simultaneously with inoculation. *B. fumosa* produces MnP in high quantities under different culture conditions and this fungus produces MnP in the presence of manganese ions.

The production of MnP under manganese deficiency was reported for two *Pleurotus* species, *P. eryngii* and *P. ostreatus,* and the isozymes were characterized (Martinez *et al.* 1996; Sarkar

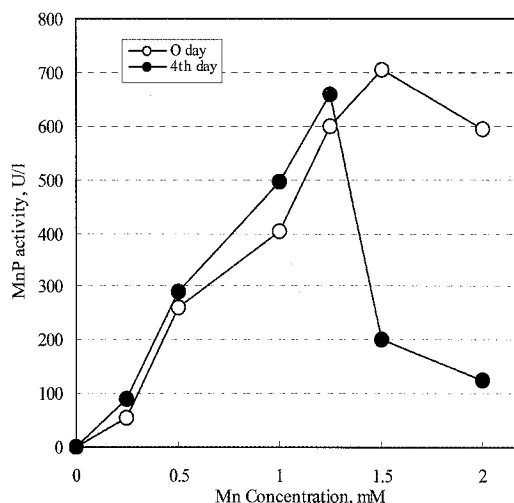

Fig. 2. Influence of $MnCl_2$ on the peak MnP activity during the cultures of *B. fumosa*.

et al. 1997). In the *Pleurotus* strains, MnP production was inhibited by even trace amounts of manganese. In contrast, *B. fumosa* MnP production is stimulated by manganese nutrients.

Fig. 3 represents the peak titres of MnP as a function of medium pH and temperatures. In this experiment the Mn concentration was 1 mM. The optimal pH value for MnP form *B. fumosa* was 4.5 resulting in 1000±120 U/l activity. Only cultures in pH 2 did not produce MnP because this pH completely inhibited the fungal growth. The growth of fungus was optimal in the pH range of 4.5 to 6.5 indicated by mycelial dry weight measurements. The fungal cultures were incubated at different temperatures in the range of 20～35°C and the temperature optimum was determined for MnP production (Fig. 3). The temperature from the range of 27～32°C resulted in similar MnP titres. However, the peak activity was reached 5 day later at 32°C compared to those of cultures incubated at 27 and 30°C. The growth of *B. fumosa* and the production of MnP slowed down at 20°C. A noteworthy finding was the production of MnP and the growth of *B. fumosa* at 30°C.

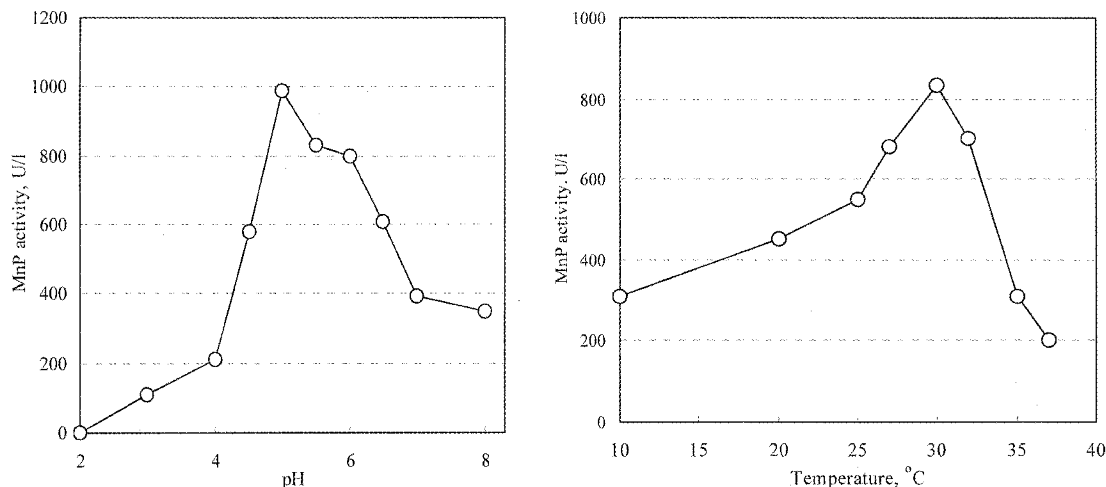

Fig. 3. Influence of pH and temperature on the peak MnP activity during the culture of *B. fumosa.*

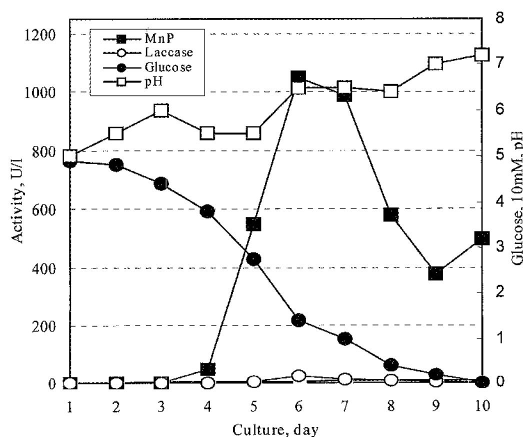

Fig. 4. Fermentation profile in medium basic Kirk's medium (BKM) with optimal concentration of glucose (16 g/ℓ), asparagine (0.25 g/ℓ), NH_4Cl (1 g/ℓ) and $MnCl_2$ (1.6 mM).

3.3. Production of MnP in Fermentor

MnP was the main oxidoreductase produced in liquid cultures of *B. fumosa* that were supplemented with Mn^{2+}. We used basic Kirk's medium (BKM) with optimal concentration of glucose (16 g/ℓ), asparagine (0.25 g/ℓ), NH_4Cl (1 g/ℓ) and $MnCl_2$ (1.5 mM). In this condition this strain additionally produced low level of laccase, but no LiP activity was observed (Kirk *et al.* 1986). MnP activity *B. fumosa* cultures reached its maximum of 1000 U/ℓ after 5 days growing in 5-l fermentor (Fig. 4). This 5-day old culture medium was used for MnP purification.

3.4. Purification of MnP

The cultures were harvested at 5th day after fermentor inoculation. To separate MnP from the concentrated culture filtrate, the saturation of NH_4Cl, fractionation by chromatography on DEAE-Sepharose and Sephadex G-100 were employed. Separation of extracellular proteins from the concentrated culture liquid demonstrated that two isoforms of Mn-dependent peroxidase (MnP1 and MnP2) are secreted by *B. fumosa* (Fig. 5). The physical characteristics in terms of molecular mass and heme absorbance of the purified MnP1 and MnP2 do not differ from those of other MnP isozymes described from other white rot fungi. Molecular masses (42~45 kDa, data not show) were in the same range as those of other white rot fungi, *e.g.*, *Bjerkandera adusta* (Heinfling *et al.* 1998a; Heinfling *et al.* 1998b), *Phlebia radiata* (Hatakka, 1994) 15). *Nematoloma frowardii* (Hofrichter *et*

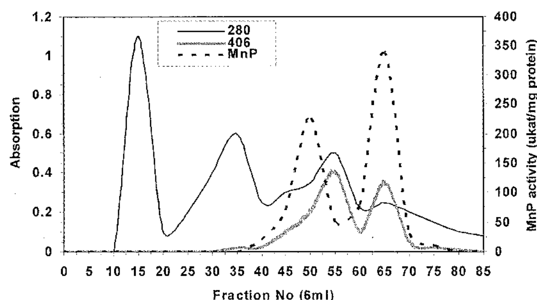

Fig. 5. Purification of MnP from *B. fumosa* by anion-exchange chromatography on DEAE-Sepharose.

al. 1999a; Hofrichter *et al.* 1999b), *Clitocybula dusenii* (Ziegenhagen and Hofrichter, 2000). MnP isoenzymes were separated on DEAE-Sepharose and rechromatographed on Sephadex G-100 column (Table 2).

3.5. Catalytic Properties of MnP Isoforms

The MnP isoenzymes exhibited both Mn^{2+}-oxidizing and Mn^{2+}-independent activities on the various substrates. Different substrates were used to evaluate the manganese dependence of their oxidation at pH 4.5 in 50 mM malonate buffer. The results are summarized in Table 3. MnP isoforms of *B. fumosa* and *Phanerochaete chrysosporium* MnPs, strictly require Mn^{2+} to oxidize phenolic substrates (de la Rubia *et al.* 2002; Giardina *et al.* 2000). The main substrate ABTS, was oxidized by both MnP1 and MnP2 in the presence and absence of manganese. This trend was observed in the case of other substrates such as *o*-dianizidine, 2,6-dimethoxyphenol, *o*-phenyldiamine in presence of both MnP1 and MnP2, and additional for syringaldazine in the case of MnP2. In the complete reaction mixture the higher specific activity was obtained for the oxidation of ABTS and syringaldazine in the case of MnP1, and for ABTS, syringaldazine, *o*-dianizidine and 2,6-dimethoxyphenol in the case of MnP2. We have also found some catalytic common properties between *B. fumosa* MnP1 and MnP2 and manganese peroxidase from *B. adusta* (Dzedzyulya and Becker, 2000). Both MnPs oxidized ABTS in the absence of Mn^{2+}. *B. adusta* was not able to oxidize veratryl alcohol (Muheim *et al.* 1990a; Muheim *et al.* 1990b) and we found that *B. fumosa* did not oxidize this substrate in pH 3.0 and in pH 4.5 this oxidation was very weak. We found that manganese independent peroxidase activity in the case of *o*-phenyldiamine and ABTS for *B. fumosa* MnPs was similar to *Phanerochaete flavido-alba* and *Pleurotus os-*

Table 2. Purification of two isoforms of MnP (MnP1, MnP2) from *B. fumosa*

Purification step	Total protein (mg)	Activity		Purification (fold)	Yield (%)
		Total (U)	Specific (U/mg protein)		
Culture filtrate	470.0	476.5	1.01	1.00	100
Ultrafiltration	112.5	267.9	2.38	2.35	56.2
$(NH_4)_2SO_4$					
0～100% sat.	35.7	127.9	3.58	3.35	26.8
DEAE-Sepharose					
MnP1	4.05	54.4	13.6	13.2	11.4
MnP2	2.09	40.3	19.2	18.9	8.45
Sephadex G-100					
MnP1	0.38	12.0	31.6	31.1	2.51
MnP2	0.3	7.4	25.9	25.5	1.50

Table 3. Influence of Mn^{2+} ions on the oxidation of various substances by MnP1 and MnP2

Substrate	λ (nm)	MnP1 activity (ΔAbs/μg protein/min)		MnP2 activity (ΔAbs/μg protein/min)	
		$+Mn^{2+}$	$-Mn^{2+}$	$+Mn^{2+}$	$-Mn^{2+}$
ABTS	420	1.32	0.51	2.83	1.03
o-dianizidine	460	0.53	0.17	1.16	0.31
2,6-dimethoxyphenol	469	0.49	0.09	1.34	0.28
Guaiacol	465	0.07	0.02	0.29	0.07
Syringaldazine	525	1.28	0.04	2.49	0.49
o-phenyldiamine	440	0.39	0.36	0.96	0.81
Veratryl alcohol pH 4.5	310	0.07	0	0.18	0.01
Veratryl alcohol pH 3.5	310	0	0	0	0

Table 4. Kinetic parameters of two isoforms of MnP from *B. fumosa*

Substrate	Km (μM)		Vmax (nkat/mg)	
	MnP1	MnP2	MnP1	MnP2
$MnCl_2$	20.9±37.0	54.1±27.9	3.59±0.58	47.1±5.24
H_2O_2	4.91±1.15	6.76±1.36	36.2±4.65	92.7±11.2

treatus (de la Rubia *et al.* 2002; Giardina *et al.* 2000). When compared kinetic constants with other fungal peroxidases we found that *B. fumosa* has similar activity with MnP peroxidases from the other white rot fungi (Table 4). The results provide baseline data for further research on large-scale production of MnP by *B. fumosa* and its biodegradation and biotechnological application.

4. CONCLUSIONS

This study was performed to investigate the optimization of MnP production by various factors, such as Mn concentration, culture pH, and incubation temperature. *Bjerkandera fumosa* was selected as a good MnP producer. The growth of fungus was optimal in the pH of 4.5 at 30°C. This white-rot fungus is a nitrogen-unregulated, and produces high amounts of MnP in the excess condition of N-nutrients. The highest MnP levels were obtained with 0.25 g/ℓ asparagine and 1 g/ℓ NH_4Cl as N source at 1.5 mM $MnCl_2$ concentration.

In the BKM medium with optimal concentrations of carbon, nitrogen, $MnCl_2$ condition, this strain produced very low level of laccase, and MnP activity reached to its maximum of 1000 U/ℓ after 5 days cultures. Separation of extracellular enzymes demonstrated two isoforms of Mn-dependent peroxidase (MnP1 and MnP2) was secreted by *B. fumosa.* The molecular masses of the purified MnP1 and MnP2 were in the same range of 42~45 kDa. MnP isoforms of *B. fumosa* strictly require Mn to oxidize phenolic substrates. In the complete reaction mixture the higher specific activity was obtained for the oxidation of ABTS and syringaldazine in the case of MnP1, and for ABTS, syringaldazine, *o*-dianizidine and 2,6-dimethoxyphenol in the case of MnP2. Concerned to kinetic constants of *B. fumosa* MnPs, *B. fumosa* has similar Km value and Vmax compared to the other white-rot fungi.

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