Extracellular oxidases of *Cerrena* sp. complementarily functioning in artificial dye decolorization including laccase, manganese peroxidase, and novel versatile peroxidases

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ABSTRACT

Extracellular ligninolytic oxidoreductases produced by *Cerrena* sp. strain Ra, a soil-isolated basidiomycete with high artificial dye-decolorizing activity, were purified and characterized. One thermostable laccase, one typical manganese peroxidase, and two versatile peroxidases (VPI and VPII) were found in the culture of this fungus. The different characteristics of each enzyme enable the strain express wide-range of oxidizing activity under various conditions. VPI decolorizing activity was observed toward various kinds of dye compounds, and the activity and specificity varied depending on the oxidizing mediators added to the reaction mixture. Optimized VPI/mediator-coupling decolorizing will be widely used for industrial wastewater treatment.

Keywords: versatile peroxidase, dye decolorization, *Cerrena* sp., extracellular oxidoreductase, ligninolytic enzyme

1. Introduction

Over ten thousand kinds of synthetic dyes are used for textile, leather, paper, and food dyeing or other industrial applications. Total dye production in the world is estimated at about 800,000 tons per year, and up to 15% of the used dyes are released into wastewater [28]. Although many synthetic dyes are mutagenic and carcinogenic [2, 7], most of them are not removed by conventional biological treatment using microbes. In contrast, chemical treatments are effective for dye degradation, but they are very costly.

Some kinds of basidiomycetes, known as white-rot fungi, are efficient at breaking down synthetic dyes. The fungi secrete ligninolytic enzymes in order to depolymerize lignin structures of woody plants. These extracellular ligninolytic enzymes contain two types of oxidoreductases**:** laccase and peroxidase. Laccase and peroxidase are glycosylated proteins that catalyze the oxidation of many substrates by reducing oxygen to water or hydrogen peroxide to water, respectively [30]. Ligninolytic peroxidases belong to the class II heme peroxidase superfamily and are classified into three types of enzymes: lignin peroxidase, manganese peroxidase, and versatile peroxidase.

Lignin peroxidase directly oxidizes a variety of phenolic and non-phenolic aromatic compounds including synthetic dyes [21]. Veratryl alcohol (VA) is a typical non-phenolic substrate for lignin peroxidase and is oxidized to its cation radical [9]. This cation radical can act as a diffusible redox mediator for the degradation of dyes with high redox potentials, which are not directly oxidized by lignin peroxidase [5, 6]. Alternatively, VA can reduce the Compound II form of lignin peroxidase, as a result of dye oxidation, to its native form [23].

Manganese peroxidase directly oxidizes Mn^{2+} to Mn^{3+} [4]. The complex of Mn^{3+}

and an organic acid acts as a diffusible oxidizer of phenolic compounds and some dyes [6, 10, 14].

Versatile peroxidase is a hybrid enzyme of lignin peroxidase and manganese peroxidase found in some species of *Pleurotus* [8, 15], *Bjerkandera* [16, 22], *Lepista* [31], and so on. This enzyme is able to oxidize a variety of phenolic and non-phenolic substrates including Mn^{2+}, VA and different types of dyes [27]. Moreover, it directly oxidizes some high redox-potential dyes that can be oxidized by lignin peroxidase only in the presence of VA [6, 8, 16].

Recently, a novel peroxidase family with DyP from *Thanatephorus cucumeris* Dec 1 as a representative one was proposed. DyP has several characteristics that distinguish it from all other peroxidases, including a particularly wide substrate specificity especially toward hydroxyl-free anthraquinone dyes, a lack of homology to most other peroxidases, and the ability to function well under much lower pH conditions [25].

We screened various kinds of laccase- and peroxidase-prodcuing microorganisms decolorizing microorganisms from various natural sources with dye-decolorizing activity as an indicator, and found an alkaline laccase [26], a thermostable laccase [18], and two bacterial peroxidases [11, 12, 20]. In this study, we found that a trametoid basidiomycete, *Cerrena* sp. strain Ra, could efficiently degrade the synthetic dye Remazol Brilliant Blue R (RBBR). From *Cerrena* sp. strain Ra, one laccase, one manganese peroxidase, and two isoforms of versatile peroxidase were isolated and characterized. Each enzyme complementarily represents the oxidative activity of the strain under different conditions. In particular, one of the versatile peroxidase isoforms was analyzed in detail, specifically focusing on its synthetic dye decomposition ability.

2. Materials and Methods

2.1. Isolation of dye-decolorizing microorganisms

Dye decolorizing microorganisms were isolated from soil samples by spreading soil suspended in water onto 1.5 % agar plates containing 1 % potato extract, 2 % glucose, 0.05 % RBBR (Remazol Brilliant Blue R), and 0.003 % chloramphenicol (pH 6.0). These plates were incubated at 28°C for several days, and RBBR-decolorizing colonies were collected for the following test of laccase and peroxidase activity.

2.2. Identification of strain Ra

Strain Ra underwent molecular phylogenetic analysis based on the nucleotide sequence of 18S and 28S rDNA. Sequencing of 18S rDNA was carried out using NS1 and NS8 primers as described by White et al [29], and sequencing of 28S rDNA was carried out using NL1 and NL4 primers as described by O'Donnell et al [19].

2.3. Preparation of culture supernatants for the assay of extracellular oxidoreductase activity

Selected strains were cultivated in a liquid medium containing 1 % glucose, 0.5 % polypeptone (Nihon Pharmaceutical), 0.1 % yeast extract, 0.1 % KH_2PO_4, 0.1 % K_2HPO_4, and 0.02 % $MgSO_4 \cdot 7H_2O$ at pH 6.0 and 28°C for 4 to 7 days with shaking

(300 rpm). The culture supernatants were filtered with 1.2 μm MF-Millipore membrane filter and were used for the laccase and peroxidase activity assays described below.

2.4. Enzyme assays

During microbial screening and enzyme purification, laccase and peroxidase activities were assayed with dye decolorizing assays. For the laccase assay, the reaction mixture consisted of 0.25 mM RBBR in 100 mM malonate buffer (pH 4.5). Twenty micro-liters of enzyme were added to the reaction mixture (180 μl) in a 96-well microplate, and decreases in the absorbance at 592 nm were measured using a microplate photometer, the SpectraMax 210 (Molecular Device), at 30°C. For the peroxidase assay, the reaction mixture consisted of 0.25 mM RBBR, 5 mM $MnSO_4$, and with or without 3 mM hydrogen peroxide in 100 mM malonate buffer (pH 4.5). Measurement of absorbance was carried out in the same way as in the laccase assay. Differences between absorbance values of reaction mixtures with and without hydrogen peroxide were used for calculation of peroxidase activity. In both cases, the concentration of the dye was calculated using the molar extinction coefficient (ε_{592}) = 6,170/M·cm. One unit of enzymatic activity (U) is equivalent to one μmol of the dye decomposed per minute.

2.5. Purification of enzymes

Strain Ra was cultivated in 2-liter flasks containing 300 ml of liquid medium. The liquid medium was composed of 5 % malt extract, 0.3 % yeast extract, 0.2 % KH_2PO_4,

0.2 % K_2HPO_4, and 0.001 % $MnSO_4 \cdot 5H_2O$ (pH 6.6). Cultivation was carried out aerobically at 28°C for 3 to 7 days with shaking at 120 rpm. The culture supernatant was separated from the mycelia by filtration with 1.2 μm MF-Millipore membrane filter. All of the following purification steps were carried out at 0 to 4°C and the buffer used was 20 mM Tris-HCl (pH 7.4), unless otherwise stated. The clear supernatant was concentrated with an AIP-1010 ultra filtration module (MW 10,000 cut; Asahi Chemical Industry), dialyzed against the buffer, and used for the purification of extracellular oxidoreductases.

Enzyme production was determined by using the RBBR decolorizing assays described above. The dialyzed concentrated protein solution was applied to a DEAE-Sepharose FF column (50 × 100 mm; GE Healthcare) equilibrated with the buffer. After the column was washed with the buffer, the enzymes were eluted with a linear gradient of 0 to 1 M NaCl. Depending on the activity of each oxidoreductase, the elution fractions were combined into three active pools: a pool with laccase activity and two pools with peroxidase activity. The active pools were respectively dialyzed against the buffer, and loaded to a MonoQ HR 10/10 column (GE Healthcare) equilibrated with buffer and eluted with a linear gradient of 0 to 0.5 M NaCl. The active fractions with laccase or peroxidase activity were respectively applied to a Superose 12 column (GE Healthcare) equilibrated with buffer containing 0.2 M NaCl and separated with the same buffer. The standard proteins used were glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa), and cytochrome c (12.4 kDa). The purified enzymes were used for characterization.

2.6. Enzyme characterization

Protein concentration was determined with a Protein Assay Kit (Bio-Rad). The molecular weight of native protein was determined by HPLC on a TSKgel G3000SW column (7.5 × 600 mm; Tosoh). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the oxidoreductases was performed on 12.5 % polyacrylamide gels with SDS using the Tris-glycine buffer system. The NH_2-terminal amino acid sequences of the purified enzymes were determined by automated Edman degradation with a model 476A sequencer (Applied Biosystems).

3. Results

3.1. Screening of microorganisms producing extracellular oxidoreductases

Many microorganisms in soil samples formed colonies on culture plates containing RBBR dye as an indicator of oxidoreductase secretion. The microorganisms decolorizing RBBR around their colonies were collected as potential producers of extracellular oxidoreductases. For each isolate, the laccase and peroxidase activity of the culture supernatant was assayed with RBBR-decolorizing assay. An isolate, labeled as Ra, showed strong RBBR-decolorizing activity and also produced oxidoreductases in its culture. Strain Ra was identified as a basidiomycete belonging to Polyporales based on the following microscopic observations (mycelia: smooth, thin-walled and branching; conidium: arthric, smooth or slightly rough and cylindroidal or ellipsoidal; extracellular crystal structure). Its 18S ribosomal DNA sequence corresponded to that of *Cerrena unicolor* with 99 % identity, and the 28S ribosomal DNA sequence corresponded to that of *Cerrena consors* with 98 % identity. Hence this strain was named *Cerrena* sp. strain Ra.

3.2. Purification and characterization of extracellular oxidoreductases of Cerrena *sp. strain Ra*

Several kinds of nutrient medium were examined for ligninolytic enzyme production by the strain and found that the nutrient medium containing 1 % glucose, 0.5 % polypeptone, 0.1 % yeast extract, 0.1 % KH_2PO_4, 0.1 % K_2HPO_4, and 0.02 % $MgSO_4 \cdot 7H_2O$ was the best medium for enzyme production. Furthermore, induction effects of lignin and RBBR were examined, however, both compounds at the concentration of 0.2% (w/v) slightly inhibited the growth of the strain resulting lower production of the ligninolytic enzymes.

During cultivation of *Cerrena* sp. strain Ra, the extracellular activities of laccase, Mn^{2+}-independent peroxidase and Mn^{2+}-dependent peroxidase reached the highest level at about 6 - 7 days after inoculation of mycelia (Fig. 1). Therefore, supernatants of 6-day cultures were used for enzyme purification. One laccase (LacI) and three peroxidases (MnPI, VPI, and VPII) were purified from the culture of *Cerrena* sp. strain Ra. The specific activities of LacI, MnPI, VPI, and VPII were 11.2, 9.7, 5.6, and 20.4 U/mg, respectively. Purity of the purified enzyme preparations was confirmed by SDS-PAGE observation, and the molecular masses of LacI, MnPI, VPI, and VPII were estimated to be 63.0, 42.5, 41.0, and 40.0 kDa, respectively (Fig. 2). Because their molecular weights determined by gel filtration chromatography also showed the similar values, all of these enzymes were monomeric enzymes. The NH_2-terminal amino acid sequences of the four purified extracellular oxidoreductases were analyzed, and the first 20 amino acid residues of VPI and VPII were identical. Multiple sequence alignments of the amino acid sequences of LacI, VPI, and MnPI with homologous enzymes are

shown in Fig. 3.

3.3. Effects of pH and temperature on activities and stabilities of LacI, VPI, and MnPI of Cerrena *sp. strain Ra*

Dye-decolorizing activities of purified LacI, VPI, and MnPI of *Cerrena* sp. strain Ra were examined. The optimal reaction pH values for LacI, VPI, and MnPI were estimated to be approximately 4.5, 4.6, and 4.6, respectively. LacI, VPI, and MnPI were stable, showing more than 50 % of their original activities, after 3 h incubation at pH 5.0 - 11.0, 5.0 - 9.0, and 3.0 - 9.0, respectively, at 30°C. The optimal reaction temperatures for LacI, VPI, and MnPI were 70 - 80°C, 50°C, and 55°C, respectively. LacI, VPI, and MnPI activities were stable, showing more than 50 % of their original activities, after 1 h incubation at 60, 30, and 40°C, respectively, at pH 7.5.

3.4. Kinetic parameters of MnPI and VPI from Cerrena *sp. strain Ra*

In order to fully characterize the potential dye-decomposing peroxidases, MnPI and VPI of *Cerrena* sp. strain Ra, their kinetic constants were measured toward Mn^{2+}, hydrogen peroxide, and VA (Table 1). Both MnPI and VPI oxidized Mn^{2+} in the presence of hydrogen peroxide. MnPI showed an apparent K_m of 0.059 mM for hydrogen peroxide and 0.061 mM for Mn^{2+}; the V_{max} for Mn^{2+} oxidation was 2.74 μmol/min/mg. VPI showed an apparent K_m of 0.21 mM for hydrogen peroxide and 0.21 mM for Mn^{2+}; the V_{max} for Mn^{2+} oxidation was 1.04 μmol/min/mg.

In the absence of Mn^{2+}, only VPI showed the ability to oxidize VA; MnPI had no detectable activity. Under these conditions, VPI had an apparent K_m of 2.6 mM for VA, and the V_{max} for VA oxidation was 1.96 μmol/min/mg. In the presence of Mn^{2+},

VPI oxidized VA, and had an apparent K_m of 0.39 mM for VA; the V_{max} for VA oxidation was 2.10 μmol/min/mg. Thus the affinity of VPI for VA was weaker in the absence of Mn^{2+}.

3.5. *Substrate specificity of VPI from* Cerrena *sp. strain Ra*

Substrate specificity of VPI toward various phenolic and aniline compounds was determined by the color development assay (Table 2). Relative enzyme activities toward 23 kinds of compounds were determined in the presence and absence of Mn^{2+}. VPI oxidized various kinds of substrates even in the absence of Mn^{2+}. With Mn^{2+} as an oxidizing mediator, VPI oxidized TOOS (*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline), hydroquinone, phenol, hydrocaffeic acid, *o*-chlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol, 2,4,6-trichlorophenol, and 2,6-dimethoxyphenol more effectively than without Mn^{2+}. In contrast, oxidizing activity of VPI toward *N,N*-dimethyl-*p*-phenylenediamine, guaiacol, pyrogallol, *p*-hydroxybenzoic acid, *o*-cresol, *m*-chlorophenol, and *p*-chlorophenol was stronger in the absence of Mn^{2+}.

3.6. *Dye-decolorizing ability of VPI of* Cerrena *sp. strain Ra*

Decolorizing activity of VPI toward various stained compounds is shown in Table 3. Twenty-eight compounds including commercial synthetic dyes, lignin, melanoidin, and waste from an alcohol distillery (WAD) were tested using the decolorizing assay. The activities were examined under four conditions: without oxidizing mediator; with Mn^{2+}; with VA; and with Mn^{2+} and VA. Most of the compounds were directly decolorized by VPI without oxidizing mediators. Effects of the mediators in decolorizing activity

were different depending on the compound used. In some combinations with compound and mediator, decolorizing activity was enhanced. For instance, combinations of Reactive Blue 5 with Mn^{2+}, Acid Red 52 with Mn^{2+} or VA, and Phenol Red with Mn^{2+} or VA were more suitable for the decolorizing reaction by VPI.

4. Discussion

There are some laccases reported from members of the genus *Cerrena* such as *C. unicolor* and *C. maxima* [13, 17]. Among *Cerrena* laccases, a thermostable metal-tolerant laccase, Lac IId, from *C. unicolor* MTCC 5159 [3] had the highest homology with LacI of *Cerrena* sp. strain Ra found in this study in the first 10 amino acid residues at the NH_2-terminus (Fig. 3). In addition to Lac IId, LacI of *Cerrena* sp. strain Ra is also a thermostable laccase compared with common laccases [1]. On the other hand, there has been little research about peroxidases from *Cerrena* species. We found three peroxidases, MnPI, VPI and VPII from the culture supernatant of *Cerrena* sp. strain Ra. From the results of enzymatic characterization and sequence analysis, it is clear that MnPI is a typical manganese peroxidase [28], and VPI acts as a versatile peroxidase [15, 16]. Because the first 20 amino acid residues of VPII were identical to those of VPI, VPII could be also a versatile peroxidase isoform with a slightly larger molecular mass than VPI (Fig. 2). Although VPI showed wide range of dye-decolorizing activity, the enzyme was different from DyP-type peroxidase in its NH_2-terminal amino acid sequence and in that VPI preferably acted on azo dyes such as Direct Blue 53 than anthraquinone dyes, while VPI showed high activity toward an anthraquinone dye, RBBR.

It was reported that VA does not increase oxidation of other substrates by versatile

peroxidase [24]. On the contrary, VPI from *Cerrena* sp. strain Ra showed enhanced activity in the presence of VA in some dye-decolorization such as Acid Red 52 and Phenol Red. Together with its significant low affinities toward hydrogen peroxide and Mn^{2+} compared to common versatile peroxidases, VPI might be a novel type of versatile peroxidase with unique ability to oxidize a wide range of chemical compounds depending on oxidizing mediators (Table 2). Thus, VPI is a promising catalyst for treatment of industrial dye waste. As shown in Table 3, various kinds of synthetic dyes and colored industrial wastes were decolorized. With many dyes, the strongest decolorizing activity was observed in VPI reaction mixtures without mediator. This result demonstrates that VPI is a good catalyst for mediator-independent industrial applications. In some cases, addition of mediators was effective in enhancing dye-decolorization by VPI. Moreover, indigo, phenol red, and WAD were never decolorized in the absence of appropriate mediators. Depending on the dye composition, including dyes in industrial waste waters, the reaction conditions for VPI could be optimized by adding oxidizing mediators, suggesting that VPI will be widely useful in synthetic dye decomposition.

The dye-decolorizing activity of *Cerrena* sp. strain Ra was found to be complementarily supported by VPI and MnPI. As shown in Fig. 4a, without Mn^{2+}, only VPI showed RBBR-decolorizing activity. Both VPI and MnPI showed RBBR-decolorizing activity in the presence of Mn^{2+}, however, MnPI showed the activity only with low concentrations of hydrogen peroxide in the presence of Mn^{2+}, while VPI prefer high hydrogen peroxide concentration in the presence of Mn^{2+} (Fig. 4b). These peroxidases also showed different sensitivities toward Mn^{2+}concentration. The activity of MnPI increased with increasing concentration of Mn^{2+}, while that of

VPI was rather inhibited by high concentration of Mn^{2+} (Fig. 5). These different characteristics of VPI and MnPI indicated that the combined action of these peroxidases supported the high decolorizing activity of *Cerrena* sp. strain Ra under various reaction conditions.

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Figure legends

Fig. 1 Time course of the production of extracellular oxidoreductases in *Cerrena* sp. strain Ra. Laccase (○), Mn^{2+}-independent peroxidase (□), and Mn^{2+}-dependent peroxidase (■) activities are shown. Values and error bars represent average and standard deviation of three independent experiments.

Fig. 2 SDS-PAGE analysis of purified oxidoreductases from *Cerrena* sp. strain Ra. All purified enzyme preparations were verified to be homogeneous and their molecular weights were estimated. lane 1; VPI, lane 2; VPII, lane 3; MnPI, lane 4; LacI, and lane 5; molecular weight marker.

Fig. 3 Multiple alignments of NH_2-terminal amino acid sequences of LacI, VPI, and MnPI with homologous oxidoreductases from other basidiomycetes. The NH_2-terminal 20 amino acids of LacI are shown with *Panus rudis* laccase (GenBank ID: AAR13230), *Serpula lacrymans* var. lacrymans S7.3 laccase (EGN96947), *Spongipellis*

sp. FERM P-18171 laccase 1 precursor (BAE79811), *Volvariella volvacea a* laccase 3 (AAR03582), and *Cerrena unicolor* MTCC 5159 Laccase-2d (P85430). The NH_2-terminal 19 amino acid residues of MnPI are shown with *Pleurotus eryngii* putative versatile peroxidase precursor (CAD56164), *Pleurotus sapidus* putative versatile peroxidase (CAJ01576), *Agaricus bisporus* manganese peroxidase enzyme (CAG27835), *Ceriporiopsis rivulosa* manganese peroxidase precursor (ABB83813), and *Trametes versicolor* manganese peroxidase isozyme precursor (AAT90351). The NH_2-terminal 20 amino acid residues of VPI are shown with *Heterobasidion annosum* manganese-dependent peroxidase-like protein (ACB69799), *Bjerkandera adusta* versatile peroxidase (AAY89586), *Phlebia albomellea* manganese peroxidase-like protein (ABT17238), *Trametes versicolor* manganese peroxidase isozyme precursor (AAT90349), and *Spongipellis* sp. FERM P-18171 manganese peroxidase 1 precursor (BAE79812). The amino acid residues that are the same as those of *Cerrena* sp. strain Ra are shown with a black background.

Fig. 4 Effect of hydrogen peroxide concentration on RBBR decolorization by VPI (♦)

and MnPI (■) without Mn^{2+} (A) and with 0.5 mM Mn^{2+} (B).

Fig. 5 Effect of Mn^{2+} concentration on RBBR decolorization by VPI (♦) and MnPI (■) with 0.1 mM hydrogen peroxide.

Figure1

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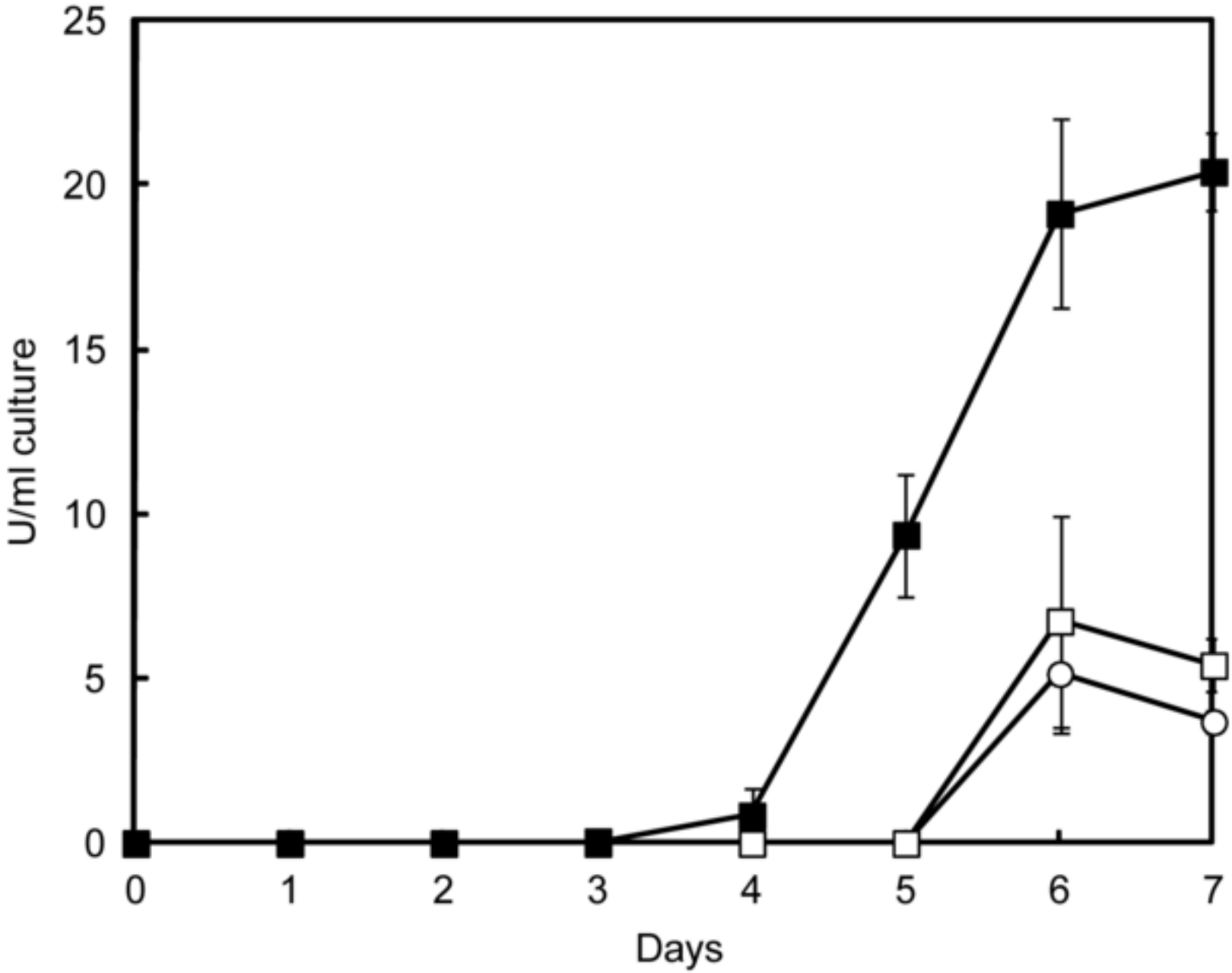

Figure2

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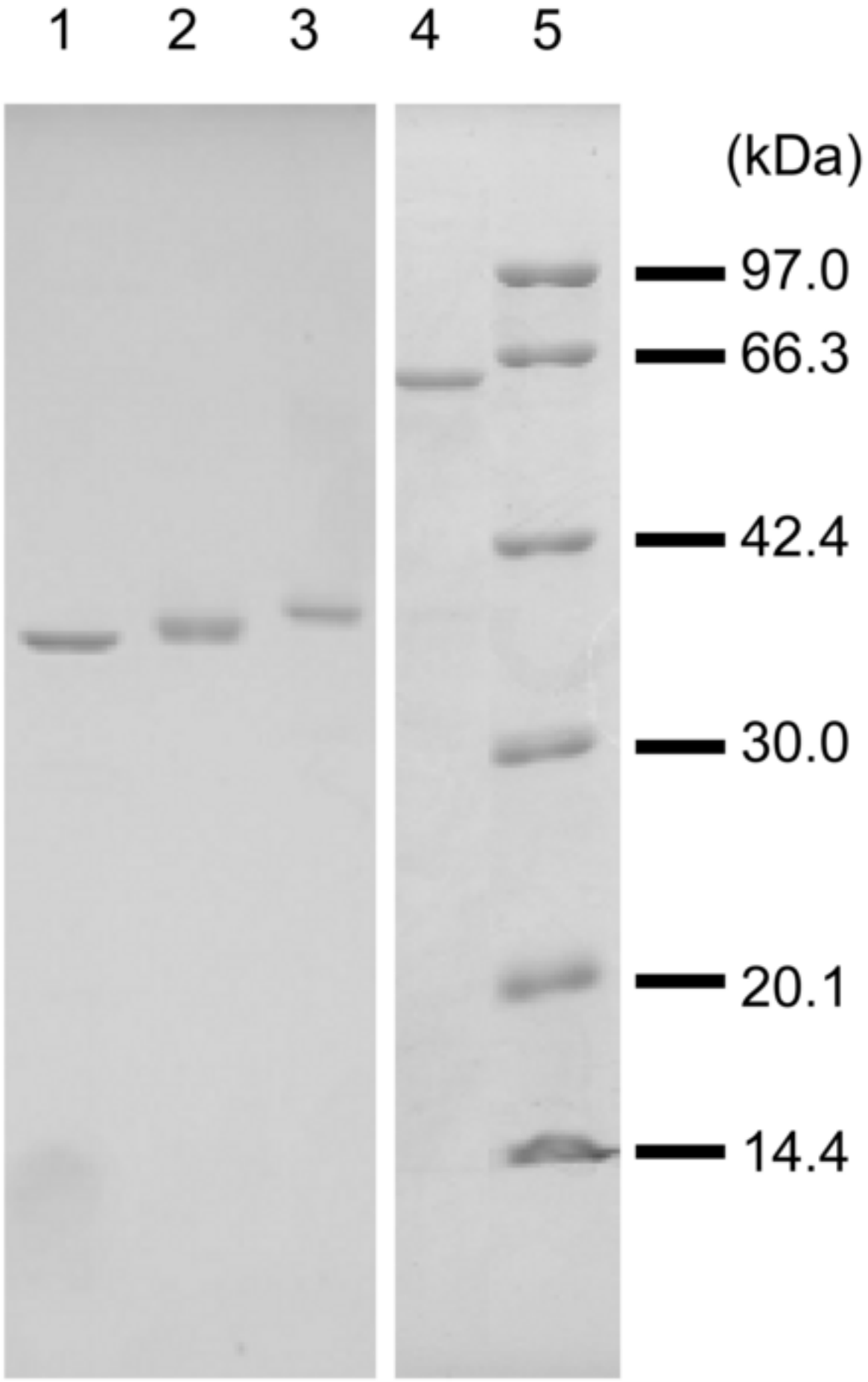

Figure3

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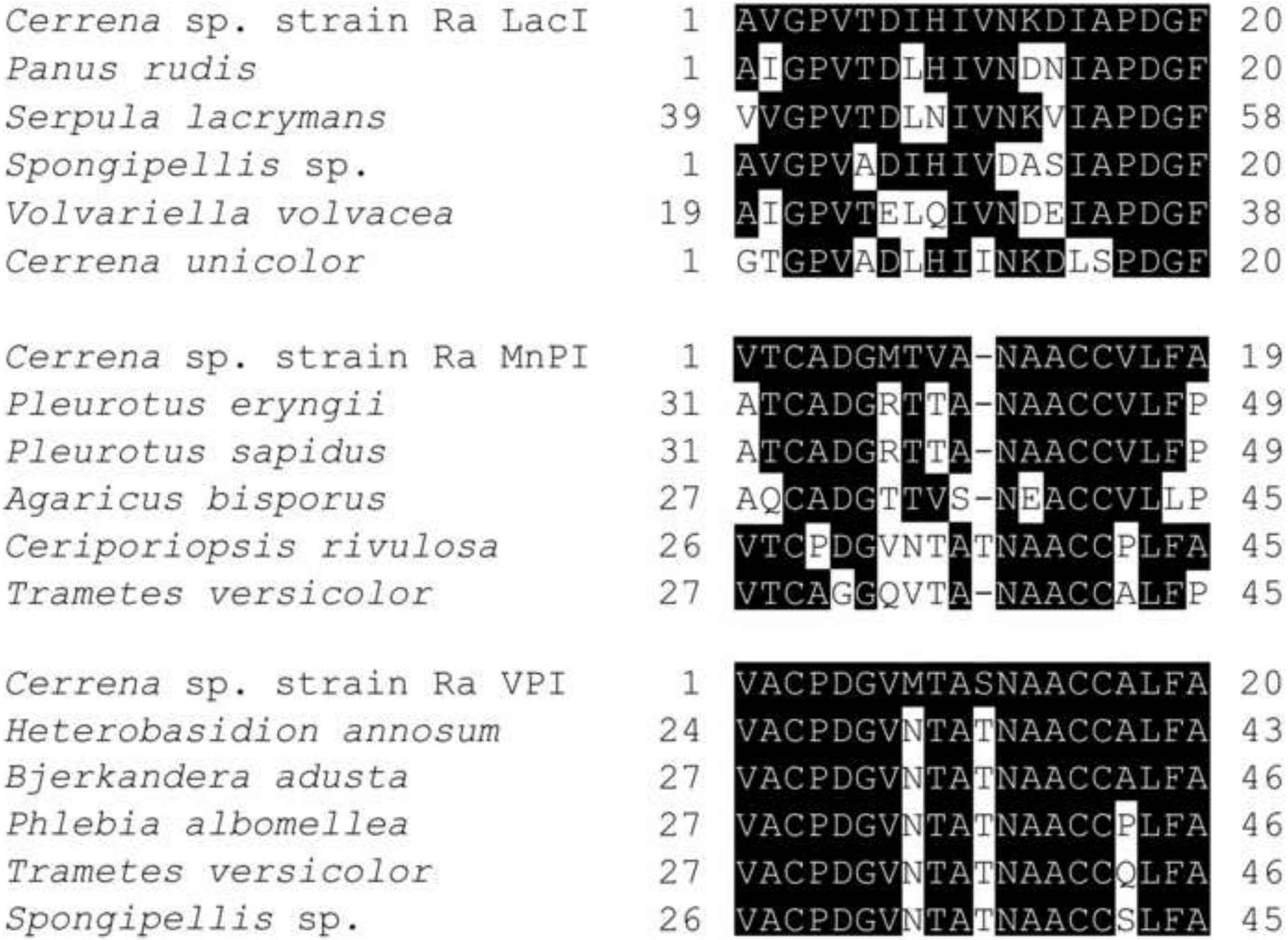

Figure4a

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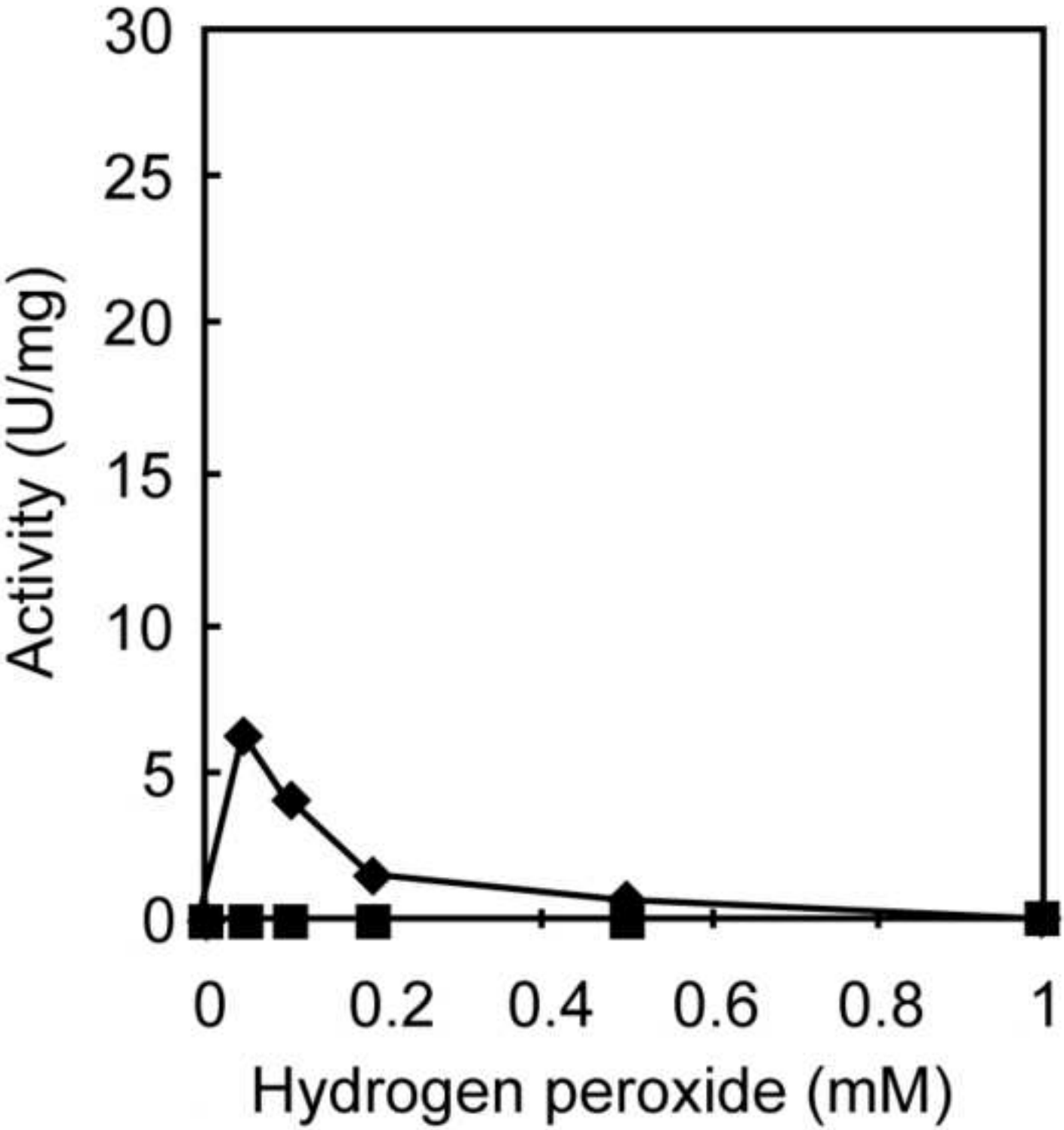

Figure4b

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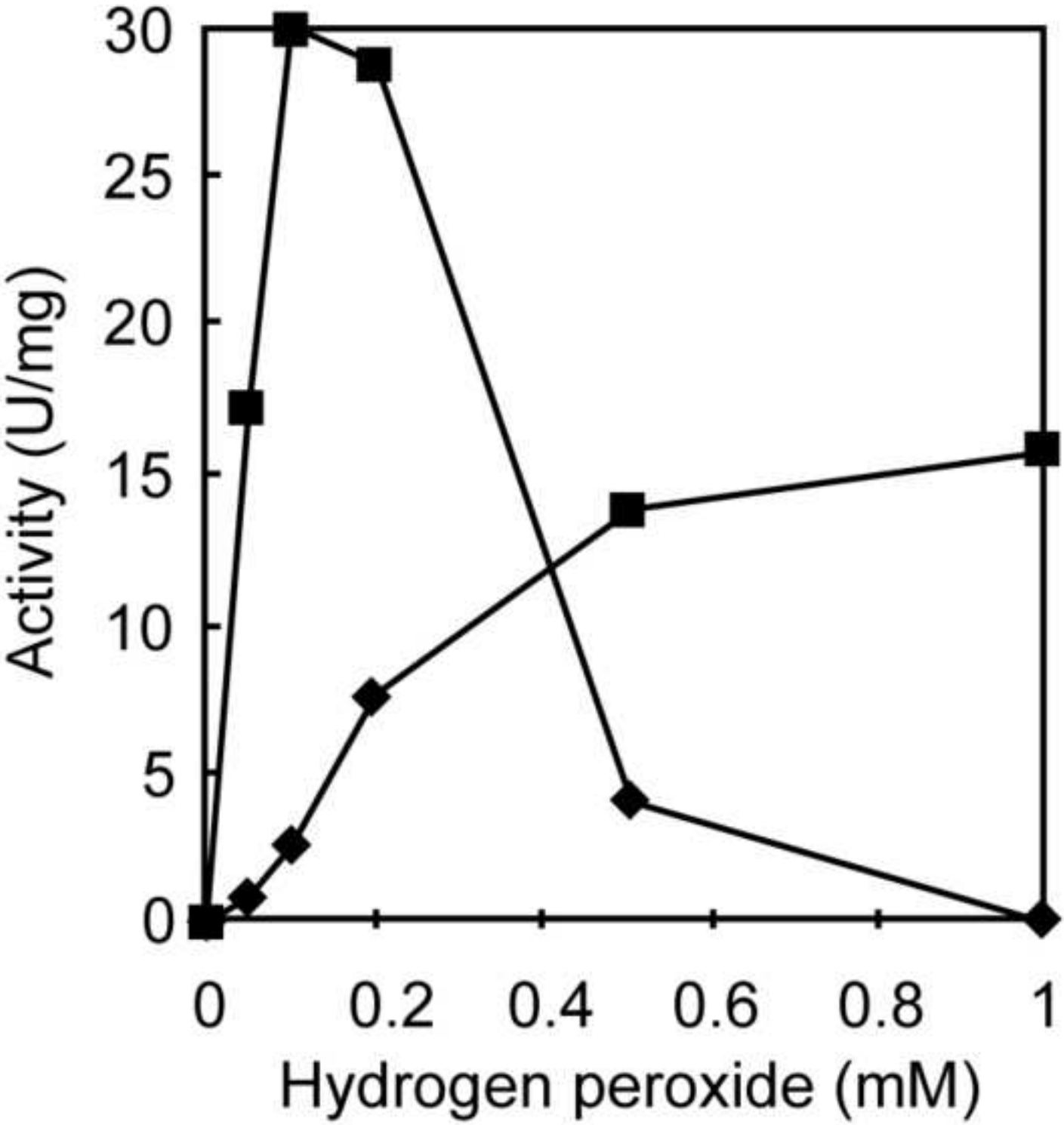

Figure5

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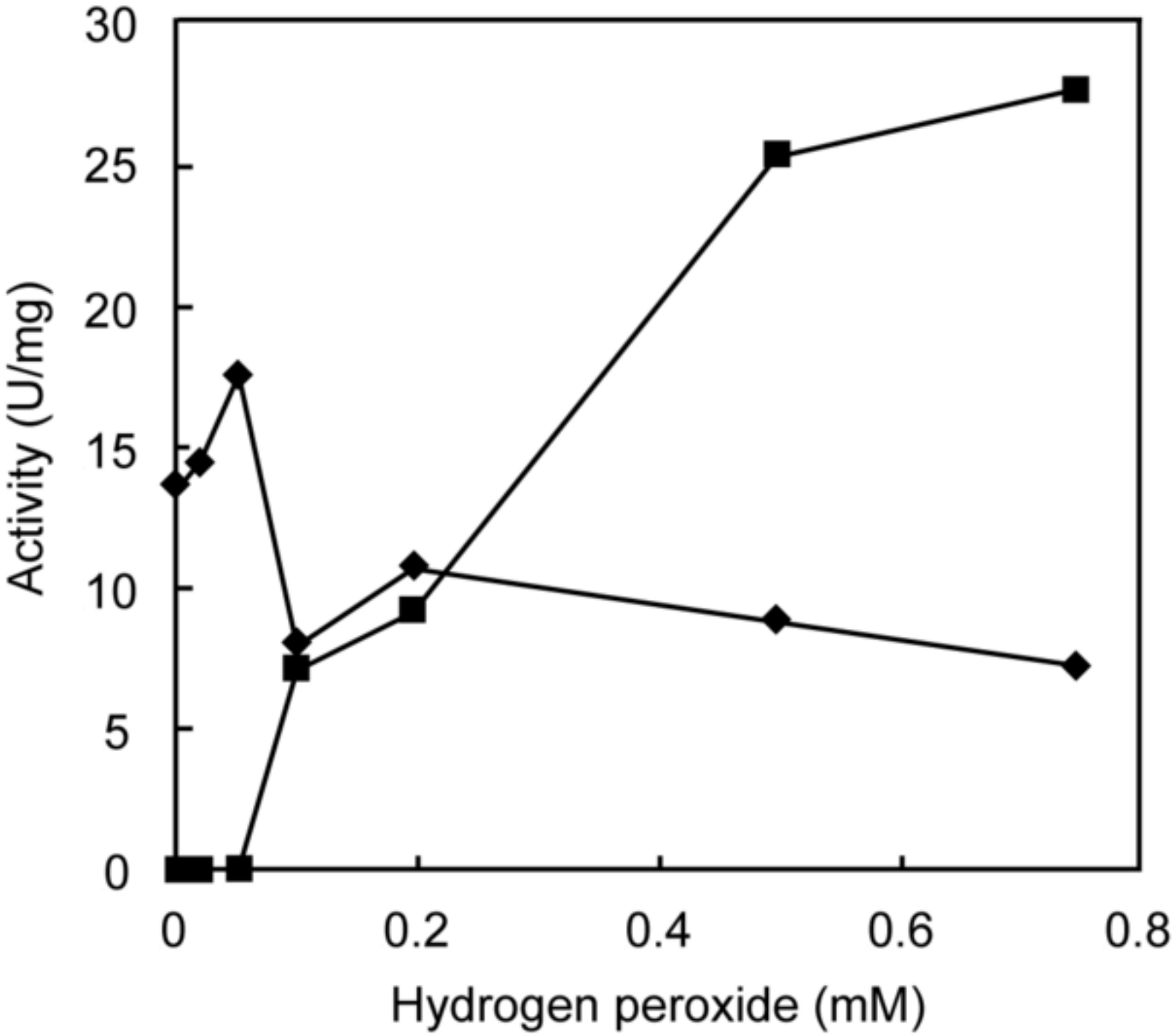

Table1

Table 1. Kinetic constants of VPI and MnPI from *Cerrena* sp. strain Ra.

Substrate	MnPI		VPI	
	K_m (mM)	V_{max} (μmol/min/mg)	K_m (mM)	V_{max} (μmol/min/mg)
Hydrogen peroxide (with Mn^{2+})	0.059	2.74[a]	0.21	1.04[a]
Mn^{2+}	0.061		0.21	
VA (without Mn^{2+})	-	-	2.6	1.96[b]
VA (with Mn^{2+})	-	-	0.39	2.10[b]

To determine kinetic constants for Mn^{2+} and hydrogen peroxide, reaction mixtures consisted of 0.1 mM hydrogen peroxide and 0.5 mM $MnSO_4 \cdot 5\ H_2O$ in 100 mM malonate buffer (pH 4.5). Increases in the absorbance at 238 nm indicating Mn^{2+} oxidation were measured at 30°C. To determine kinetic constants for VA, reaction mixtures consisted of 1.5 mM VA and 0.1 mM hydrogen peroxide with or without 0.5 mM $MnSO_4$ in 100 mM malonate buffer (pH 4.5). Increases in the absorbance at 310 nm indicating VA oxidation were measured at 30°C. Activities of these reactions were calculated from the linear phase of the reaction using molar extinction coefficients for the reaction products of Mn^{2+} (ε_{238} = 6,500/M·cm), and VA (ε_{310} = 9,300/M·cm). [a] in Mn^{2+} oxidation, [b] in VA oxidation.

Table2

Table 2. Substrate specificity of VPI from *Cerrena* sp. strain Ra.

Substrate	Absorbance (nm)	Relative activity (%)	
		without Mn^{2+}	with Mn^{2+}
TOOS	555	100 (56.8)	219
Dimethylaniline	555	68.1	51.1
N,N-Dimethyl-*p*-phenylenediamine	555	376	110
Catechol	500	174	180
Resorcinol	500	9.2	7.1
Hydroquinone	500	36.2	63.8
Phenol	500	34.0	93.6
Guaiacol	500	99.4	38.7
Pyrogallol	500	5.5	0.6
p-Hydroxybenzoic acid	500	19.1	1.3
Caffeic acid	500	13.4	19.1
Hydrocaffeic acid	500	87.2	170
o-Cresol	500	61.1	25.3
p-Toluidine	500	12.7	19.1
o-Chlorophenol	500	108	225
m-Chlorophenol	500	108	27.2
p-Chlorophenol	500	74.9	9.4
2,4-Dichlorophenol	500	61.7	142
2,6-Dichlorophenol	500	17.0	123.7
2,4,6-Trichlorophenol	500	63.8	95.7
2,6-Dimethoxyphenol	500	57.4	127

The reaction mixture for the color development assay consisted of 2 mM substrate, 2 mM 4-amino-antipyrine, and 3 mM hydrogen peroxide in 100 mM malonate buffer (pH 4.5). Twenty micro-liters of the enzyme sample were added to the 180 µl reaction mixture in a 96-well microplate, and then the increase in absorbance at the indicated

wavelength was measured at 30°C. Relative activity was defined as the percent increase in absorbance that was obtained with N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS) being taken as 100%. The values in parentheses are specific activity (μmol/min/mg) for TOOS.

Table3

Table 3. Decolorization activity of VPI from *Cerrena* sp. strain Ra.

Substrate	Concentration (w/v %)	Absorbance (nm)	Relative activity with co-substrate (%)			
			-	$+Mn^{2+}$	+VA	$+Mn^{2+}$ and VA
RBBR	0.1	592	100	18.8	13.1	15
Reactive Blue 2	0.1	600	20	15	11.3	18.1
Reactive Blue 5	0.1	600	1.3	20	0.5	18.8
Reactive Blue 15	0.1	600	60	15	53.1	10
Reactive Black 5	0.02	600	33.1	23.8	41.9	28.1
Reactive Violet	0.1	500	26.3	18.8	14.4	17.5
Naphthol Green	2	600	53.1	45	27.5	36.9
Reactive Green	0.05	600	108	53.1	108	55
Reactive Brown 10	0.1	500	41.3	23.1	62.5	9.4
Direct Blue 53	0.02	600	234	143	215	148
Direct Red 80	0.02	500	9.4	14.4	14.4	15.6
Acid Orange	0.02	500	58.8	30	63.1	23.8
Acid Blue 80	0.05	600	50	66.3	44.4	70.6
Poly R 478	0.02	430	10	n.d.	13.1	n.d.
Poly S 119	0.02	500	17.5	10	18.8	10
Guinia Green B	0.02	600	6.1	2.3	4.1	3
Lisamine Green	0.002	600	7.5	0.6	5.6	n.d.
Fast Green	0.02	600	11.9	11.3	4.4	13.1
Acid Violet 17	0.02	600	0.6	4.4	2.5	0.4
Azure A	0.002	600	4.1	1.3	2.8	n.d.
Azure B	0.02	600	1.3	n.d.	n.d.	n.d.
Azure C	0.02	600	2.5	n.d.	0.6	n.d.
Acid Red 2	0.02	430	2.7	2.1	2.3	1.8
Acid Red 52	2	430	20	131	47.5	47.5
Natural Orange 6	2	430	3.8	9.4	4.4	10.6
Indigo	2	600	n.d.	31.0	n.d.	26.3
Phenol Blue	2	600	n.d.	n.d.	n.d.	n.d.

Phenol Red	0.2	500	n.d.	52.5	25.6	58.8
Lignin	1	500	10.6	17.5	11.3	16.3
Melanoidin	0.2	600	0.1	1.2	0.3	2.1
WAD	10% v/v	600	n.d.	n.d.	0.1	0.1

The reactions were performed the same way as the substrate specificity assay. Relative activity was defined as the percent decrease in absorbance at the indicated wavelength with that obtained with RBBR i**n** the absence of co-substrate being taken as 100%**.**

n.d.; not detected