

# Properties of *Thermobifida fusca* Peroxidase Tfu-1649 and its Combined Synergistic Effects with Xylanase on Lignocellulose Degradation

Wan-Yu Liao,<sup>a</sup> Yu-Chun Huang,<sup>a,b</sup> Wei-Lin Chen,<sup>a,b</sup> Cheng-Yu Chen,<sup>c</sup> and Chao-Hsun Yang<sup>a,b,\*</sup>

Lignocelluloses are comprised of cellulose, hemicellulose, and lignins, which constitute plant biomass. Since peroxidases can degrade lignins, the authors examined peroxidase Tfu-1649, which is secreted from the thermophilic actinomycetes, *Thermobifida fusca* BCRC 19214. After cultivating for 48 h, the culture broth accumulated 43.66 U/mL of peroxidase activity. The treatment of four types of lignocellulolytic byproducts, *i.e.*, bagasse, corncob, pin sawdust, and *Zizania latifolia* Turcz husk, with Tfu-1649 alone increased the total phenolic compounds, with limited reducing sugars, but treatment with xylanase, Tfu-11, and peroxidase Tfu-1649 showed synergistic effects. Hence, the co-operative degradation of lignocelluloses by both peroxidase and xylanase could contribute to biomass decomposition and further applications in the agricultural and environmental industries.

*Keywords:* Lignocellulose; Peroxidase; Xylanase; Thermophilic actinomycetes; *Thermobifida fusca*

*Contact information:* a: Department of Cosmetic Science, Providence University, Taichung, 43301 Taiwan; b: Cosmetic Industry Research & Development Center, Providence University, 43301 Taiwan; c: Xtremes Pure Company, Taipei, 10652 Taiwan; \*Corresponding author: chyang@pu.edu.tw

## INTRODUCTION

Lignocellulose is a major part of plant biomass, which includes three major constituents, cellulose, hemicellulose, and lignins. Cellulose is the primary component of the lignocellulose cell wall and is a natural linear polysaccharide that is comprised of D-glucose units with  $\beta$ -1,4 glycosidic bonds (Kim *et al.* 2019). Hemicelluloses are another group of polysaccharides in lignocelluloses that are composed of xylans, mannans, xyloglucans, glucomannans, and  $\beta$ -glucans (Scheller and Ulvskov 2010). Lignins are composed of p-coumaryl, coniferyl, and sinapyl alcohol units *via* various inter-linkages of  $\beta$ -O-4,  $\beta$ -5, and  $\beta$ - $\beta$  (Gonzalo *et al.* 2010; Vanholme *et al.* 2010) and is involved in hemicellulose (xylan) cross-linking and xylan linkage to other polysaccharides. Lignins provide strength and rigidity to plants and is resilient to degradation.

To complete the hydrolysis of lignocelluloses, a complex enzymatic system is required. Some enzymes hydrolyze the internal glycosidic bonds at random internal positions (endo-acting enzymes), while others cleave terminal linkages from non-reducing ends (exo-acting enzymes) (Andlar *et al.* 2018). Cellulose can be degraded by cellulase, which cleaves the  $\beta$ -1,4-glycosidic linkages of glucose units in the cellulose and consist of three main classes: endo- $\beta$ -1-4-glucanase (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and exo- $\beta$ -1,4-glucosidase (EC 3.2.1.21). Hemicellulose hydrolysis demands the co-operative action of endo- $\beta$ -1-4-xylanase (EC 3.2.1.8), exo- $\beta$ -1,4-xylosidase (EC 3.2.1.37), endo- $\beta$ -1-4-mannanase (EC 3.2.1.78), and exo- $\beta$ -1,4-mannosidase (EC 3.2.1.25). Peroxidases (EC

1.11.1.7), laccases (EC 1.10.3.2), lignin peroxidases (LiP; EC 1.11.1.14), dye-decolorizing peroxidase (DyP; EC 1.11.1.19) (Catucci *et al.*, 2020), and manganese-dependent peroxidases (MnP; EC 1.11.1.3) have been reported as the primary groups of lignin degrading enzymes (Woolridge 2014; Janusz *et al.* 2017; Andlar *et al.* 2018). Acetylxyylan esterase (EC 3.1.1.72), feruloyl esterase (EC 3.1.1.73), p-coumaroyl esterase (EC 3.1.1.B10),  $\alpha$ -l-arabinofuranosidase (EC 3.2.1.55), xylan  $\alpha$ -1,2-glucuronosidase (EC 3.2.1.131), and  $\alpha$ -glucuronidase (EC 3.2.1.139) all increase the efficiency of lignocellulose degradation (Vries *et al.* 2000; Andlar *et al.* 2018). As previously reported, there are synergistic actions between an endoxylanase and a laccase against the natural lignocellulosic substrate (Fonseca-Maldonado *et al.* 2014).

Lignocellulose-degrading enzymes are produced by a variety of fungal and bacterial genera, in which most enzymes have been found in mesophilic microorganisms. Thermophilic microorganisms are considered to be a good source of novel and thermostable enzymes with potential industrial applications. The thermophilic actinomycete, *Thermobifida fusca*, was reported to be able to produce abundant extracellular lignocellulose-degrading enzymes (Chen *et al.* 2013a, 2016). However, the interaction between xylanase and peroxidase is not fully understood, especially with regards to lignocellulose products, *e.g.*, bagasse, corncob, pin sawdust, and *Zizania latifolia* Turcz husk, which are common agricultural and environmental lignocellulose byproducts in Taiwan. Hence, in this study, the authors attempted to purify and examine the properties of the extracellular peroxidase secreted by *T. fusca*. The co-operative degradation of lignocellulose *via* peroxidase and xylanase was also evaluated.

## EXPERIMENTAL

### Microorganisms

*Thermobifida fusca* BCRC 19214 was isolated and routinely maintained in the author's laboratory (Chen *et al.* 2013b) and was stored in the Bioresource Collection and Research Center (Taiwan).

### Materials

The Czapek-Dox powder, yeast extract, peptone, and agar were purchased from BD (Sparks, MD). The diethylaminoethyl cellulose (DEAE)-Sephacel CL-6B and Sephacyl S-200 columns were purchased from GE Healthcare (Little Chalfont, UK), while the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), acrylamide, buffer, reagents, molecular weight standards, and protein assay kit were purchased from Bio-Rad Laboratories (Hercules, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), while the xylanase-Tfu11 was obtained from the *E. coli* transformant pXYL11 from the author's laboratory (Huang *et al.* 2010).

### Lignocellulose Sample Preparation

The bagasse, corncobs, pin sawdust and *Zizania latifolia* Turcz husk samples were collected from a local market, which were thoroughly washed with tap water to remove dust, and then air-dried. The dried samples were chopped and milled to a 100-mesh size using an ultrafine pulverizer (MacroGreat, Yunlin, Taiwan) and stored at room temperature.

## Cultivation and Purification of the Enzyme

*Thermobifida fusca* BCRC 19214 was cultivated in a 500 mL Hinton flask containing 100 mL of an enzyme-producing medium consisting of 10.0 g of bagasse, 0.5 g of yeast extract, 4.0 g of  $\text{NH}_4\text{NO}_3$ , and 6.8 g of  $\text{KH}_2\text{PO}_4$  L<sup>-1</sup> of distilled water (with a pH of 9.0). The culture was inoculated at 50 °C and shaken (at 150 rpm) for 96 h. The culture broth was centrifuged at 10000 × gravity for 30 min to remove the cellular pellet, and the supernatant was used as the crude enzyme solution. Purification was performed using a 30 kDa Pellicon XL ultrafiltration system (Merck KGaA, Billerica, MA) as well as a DEAE-Sephacryl CL-6B and Sephacryl S-200 chromatography columns. The steps were performed according to the instructions provided by the manufacturer (Chen *et al.* 2013b).

## Enzyme Activity

The peroxidase activity was determined by monitoring 455 nm in the reaction mixture containing 200 µL of the enzyme, 20 µL of catechol (0.5 M), 20 µL of hydrogen peroxide (50 mM), and 760 µL of 50 mM Tris-HCl buffer (with a pH of 8.8) (Doğan *et al.* 2007). The enzymatic assay was performed at 50 °C. The blanks contained all compounds except for the enzyme solution, and one unit of peroxidase activity was defined as the amount of enzyme required to oxidize 1 µmol of the substrate (1 mM) in 1 min under assay conditions. The xylanase activity was determined by measuring the release of reducing sugars from oat-spelt xylan (Huang *et al.* 2000). One unit of xylanase activity was defined as the amount of enzyme that released 1 µmol of D-xylose (1 mM) per min at 50 °C.

## Analysis Methods

The total phenolics of the lignocellulolytic hydrolysate was determined using a modified Folin-Ciocalteu method, using gallic acid as the standard (Kujala *et al.* 2000). The reducing sugars liberated *via* the hydrolysis of lignocellulose substrates in the mixture was determined using the dinitrosalicylic acid (DNS) method, where D-glucose was used to construct a standard curve.

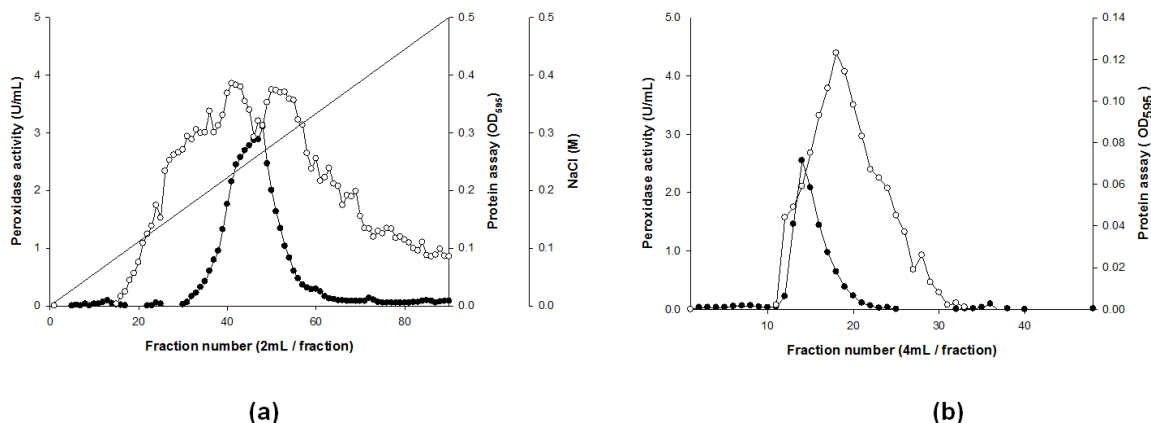
## Statistical Analysis

The data were analyzed in triplicates (or higher), which was expressed as the mean ± standard error and compared to the appropriate control using the Student's *t*-test. A *p*-value of less than 0.05 indicated statistically significant differences.

## RESULTS AND DISCUSSION

### Cultivation and Purification of *T. fusca* BCRC 19214 Peroxidase

*Thermobifida fusca* BCRC 19214 was cultured as described in the Experimental section for 48 h. A total of 43.66 U/mL peroxidase activities had accumulated in the culture broth. The results of the DEAE-Sephacryl CL-6B ion-exchange as well as the Sephacryl S-200 gel-filtration chromatography are shown in Fig. 1, while the results of the total purification process are summarized in Table 1. The obtained purified peroxidase exhibited 2.91% of the total initial activity with a 15.12-fold increase in specific activities when compared to the culture filtrate solution. The purified peroxidase was named Tfu-1649.



**Fig. 1.** Column chromatography of peroxidase Tfu-1649 purified from *Thermobifida fusca* BCRC19214. (a) diethylaminoethyl cellulose (DEAE)-Sepharose CL-6B column chromatography as well as (b) Sephacryl S-200 column chromatography. Symbol: (●) peroxidase activity, (○) protein assay, (–) NaCl

**Table 1.** Summarization of the Purification of Peroxidase Tfu-1649

Purification step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (fold)	Yield (%)
Crude supernatant	31.14	43.66	1.40	1	100
Ultrafiltration	9.35	5.95	0.64	0.45	13.63
DEAE-Sepharose CL-6B	1.35	4.35	3.22	2.3	9.96
Sephacryl S-200	0.06	1.27	21.17	15.12	2.91

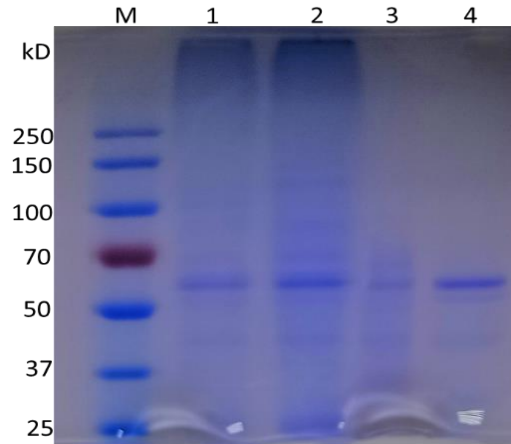
In addition, *T. fusca* is able to produce multiple types of peroxidases. Ball and Trigo (1995) used anion exchange chromatography to purify the *T. fusca* peroxidase, where two extracellular peroxidases were measured on non-denaturing gel electrophoresis. Their isoelectric points were 8.8 and 3.6, respectively. The molecular weight of the acidic peroxidase was 40 kDa. Moreover, a newly purified dye decolorizing peroxidase (DyP) was also found in *T. fusca* using a genome-mining approach with a molecular weight of 46 kDa (van Bloois *et al.* 2010); these peroxidases are all different from peroxidase Tfu-1649. Hence, to the best of the authors' knowledge, this was the first instance of identifying and purifying peroxidase Tfu-1649 from a culture broth of *T. fusca*.

### Properties of the *T. fusca* BCRC 19214 peroxidase

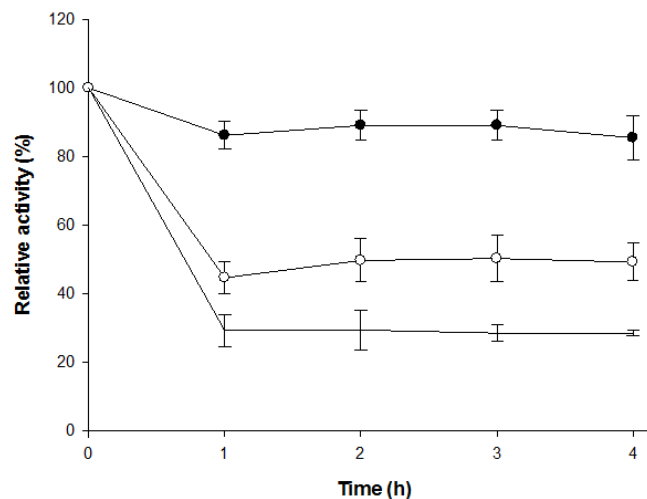
As shown in Fig. 2, the purified peroxidase Tfu-1649 sample displayed a single protein band on the 10% SDS-PAGE, which was estimated to be approximately 60 kDa based on its mobility compared to the standard proteins on the SDS-PAGE.

The optimum pH and temperature of the purified peroxidase Tfu-1649 sample were 9.0 and 60 °C, respectively. Approximately 80% of the original activity still remained at 60 °C after 4 h (Fig. 3). The optimum temperature of the peroxidase, Tfu-1649 was similar to other peroxidases found in actinomycetes (Table 2). The range of optimum temperatures

for *Streptomyces* spp. peroxidase is between 30 °C to 80 °C, with the strain AD001 showing the lowest optimum temperature. In addition, the optimum pH of peroxidase Tfu-1649 was 9.0, while in the case of the *Streptomyces* spp. peroxidase, it was lower than 8.5 (Rekik *et al.* 2015).



**Fig. 2.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of peroxidase Tfu-1649 from *Thermobifida fusca* BCRC19214. Lane M: molecular weight marker; Lane 1: culture filtrate; Lane 2: Tfu-1649 purified from concentration; Lane 3: Tfu-1649 purified via diethylaminoethyl cellulose (DEAE)-Sepharose CL-6B chromatography; and Lane 4: Tfu-1649 purified via Sephacyl S-200 chromatography.



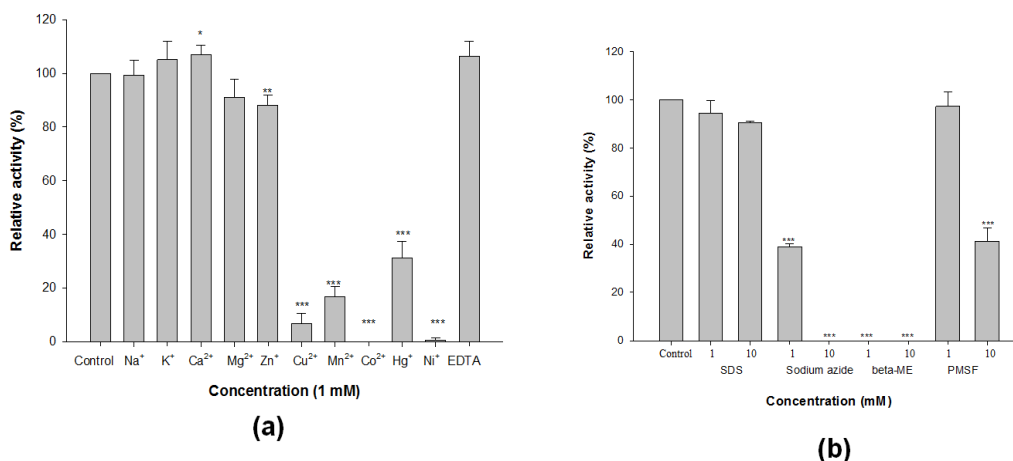
**Fig. 3.** Thermal stability of peroxidase Tfu-1649 purified from *Thermobifida fusca* BCRC19214, which was incubated at various temperatures for 1 h to 4 h, after which the residual enzyme activity was determined. Symbol: (●) 60 °C, (○) 70 °C, (◻) 80 °C

**Table 2.** Biochemical Properties of the Selected Purified Peroxidases

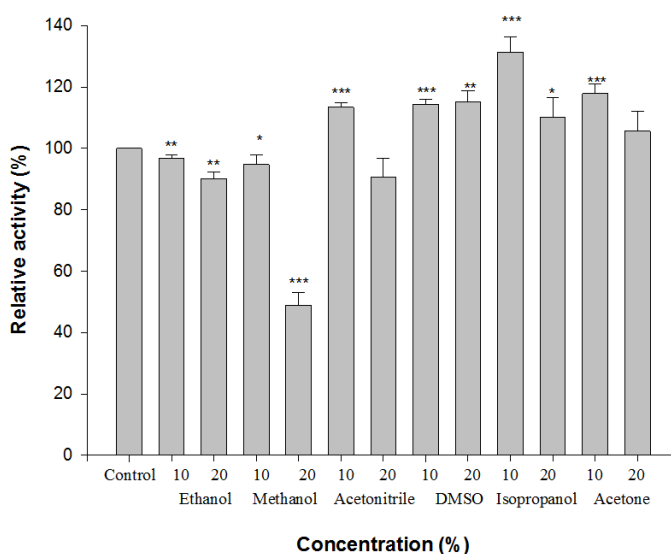
Strain Enzyme	Optimal Temperature (°C)	Optimal pH	Metal Ion (Relative activity %) Inhibitor	Source
<i>Streptomyces</i> sp. AD001	30	7.5	Hg <sup>2+</sup> (0%), Mg <sup>2+</sup> (56.5%), Zn <sup>2+</sup> (47%), Cu <sup>2+</sup> (32%), Co <sup>2+</sup> (27%) (1 mM) NaN <sub>3</sub> (11%), EDTA (36%)	Jeon <i>et al.</i> 2002
<i>Streptomyces</i> sp. strain AH4	80	5	Mn <sup>2+</sup> (225%), Mg <sup>2+</sup> (90%), Ca <sup>2+</sup> (145%), Cu <sup>2+</sup> (111%), Zn <sup>2+</sup> (25%), Co <sup>2+</sup> (85%), Ag <sup>2+</sup> (56%), Hg <sup>2+</sup> (10%), K <sup>+</sup> (165%) (5mM) NaN <sub>3</sub> (40%), Acetonitrile, DMSO (20%), Isopropyl alcohol, ethanol, methanol (increased 125%)	Fodil <i>et al.</i> 2012
<i>Streptomyces albidoflavus</i> TN644	75	4	Mn <sup>2+</sup> (290%), Mg <sup>2+</sup> (180%), Ca <sup>2+</sup> (112%), Cu <sup>2+</sup> (108%), Zn <sup>2+</sup> (75%), Co <sup>2+</sup> (65%), Ag <sup>2+</sup> (51%), Hg <sup>2+</sup> (6%), K <sup>+</sup> (145%) (5 mM), NaN <sub>3</sub> (5%)	Jaouadi <i>et al.</i> 2014
<i>Streptomyces</i> sp. strain AM2 HaP1	55	6	-	Fodil <i>et al.</i> 2011
<i>Streptomyces</i> sp. strain AM2 HaP2	55	7.5	-	Fodil <i>et al.</i> 2011
<i>Streptomyces griseosporus</i> SN9	60	8.5	1% SDS (72%)	Rekik <i>et al.</i> 2015
<i>Rhodococcus</i> sp. T1	35	4	Mg <sup>2+</sup> (100%), Ca <sup>2+</sup> (70%), Mn <sup>2+</sup> (40%), Zn <sup>2+</sup> (40%), Cu <sup>2+</sup> (60%), Co <sup>2+</sup> (60%), K <sup>+</sup> (55%) (1 mM) β-Me (30%), EDTA (95%)	Sahinkaya <i>et al.</i> 2019
<i>Thermobifida fusca</i> DyP-type peroxidase	45 to 60	5.0 to 5.5		Rahmanpour <i>et al.</i> 2016
<i>Thermomonospora fusca</i> BD25	60	7.0 to 8.0	-	Ball and Trigo 1995
<i>Thermobifida fusca</i> BCRC 19214 Tfu-1649	60	9.0	Na <sup>+</sup> (99%), K <sup>+</sup> (105%), Ca <sup>2+</sup> (107%), Mg <sup>2+</sup> (91%), Zn <sup>2+</sup> (88%), Cu <sup>2+</sup> (7%), Mn <sup>2+</sup> (17%), Co <sup>2+</sup> (0%), Hg <sup>+</sup> (31%), Ni <sup>+</sup> (1%). (1 mM) SDS (90%), NaN <sub>3</sub> (0%), β-ME (0%), PMSF (41%) (10 mM)	Measured in this study
<i>Pseudomonas aeruginosa</i> PKE117	-	3.5	Cu <sup>2+</sup> (106%), Zn <sup>2+</sup> (103%), Ag <sup>2+</sup> (92%), Co <sup>2+</sup> (92%), Mn <sup>2+</sup> (98%), Ca <sup>2+</sup> (97%), Mg <sup>2+</sup> (92%) (1 mM) SDS (91%), EDTA (100%)	Li <i>et al.</i> 2012

According to the metal ions and the chemical reagents tested, it was found that the activity of purified peroxidase Tfu-1649 was strongly inhibited by 1 mM of Cu<sup>2+</sup>, Mn<sup>2+</sup>, Co<sup>2+</sup>, Hg<sup>+</sup>, and Ni<sup>+</sup> and only partially inhibited by 1 mM of Zn<sup>2+</sup>. The 1 mM NaN<sub>3</sub>, β-

mercaptoethanol, and 10 mM phenyl-methyl-sulfonyl fluoride (PMSF) strongly inhibited the enzymatic activity of peroxidase Tfu-1649 (as shown in Fig. 4). The effects of the organic solvents on the activity of purified peroxidase Tfu-1649 are shown in Fig. 5. With 10% water miscible solvents, *e.g.*, acetonitrile, dimethyl sulfoxide (DMSO), isopropanol, and acetone, in the reaction system, the enzymatic activity increased by 115% to 135%, while the enzymatic activity of peroxidase Tfu-1649 was inhibited by 20% methanol and 10% ethanol. Isopropanol in the incubation mixture may produce a great increase of the enzyme activity. In the previous reports, it also increased the activities of starch synthetase, and thrombin (Judewicz *et al.* 1972; Shvachko and Kibirev 1988).



**Fig. 4.** Effect of various (a) ions and (b) chemical reagents on peroxidase Tfu-1649 purified from *Thermobifida fusca* BCRC19214, including ethylenediaminetetraacetic acid (EDTA), sodium lauryl sulfate (SDS),  $\beta$ -mercaptoethanol (beta-ME), and phenyl-methyl-sulfonyl fluoride (PMSF)



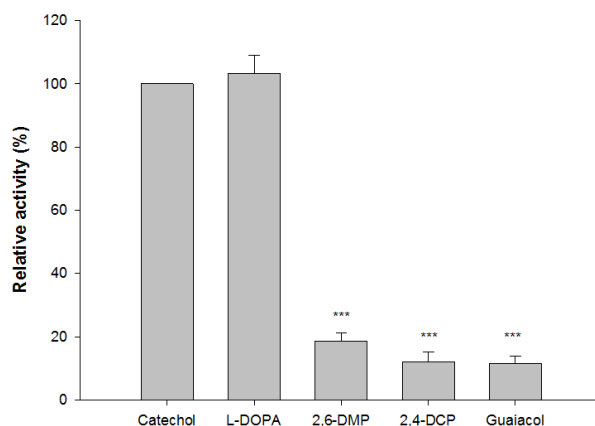
**Fig. 5.** Effect of various organic solvents, including dimethyl sulfoxide (DMSO), on peroxidase Tfu-1649 purified from *Thermobifida fusca* BCRC19214

Peroxidase Tfu-1649 was strongly inhibited by 1 mM of Cu<sup>2+</sup>, Mn<sup>2+</sup>, Co<sup>2+</sup>, Hg<sup>+</sup>, and Ni<sup>+</sup>. In addition, Cu<sup>2+</sup> also inhibited *Streptomyces* sp. AD001 (Jeon *et al.* 2002) and *Rhodococcus* sp. T1 (Sahinkaya *et al.* 2019) peroxidase, but it acted as an activator for *S.*

*albidoflavus* TN644 (Jaouadi *et al.* 2014) and *P. aeruginosa* PKE117 (Li *et al.* 2012). The peroxidase activity of *Rhodococcus* sp. T1 was inhibited by  $Mn^{2+}$  (Sahinkaya *et al.* 2019), but  $Mn^{2+}$  activated the enzymatic activities of *S. sp.* AH4 (Fodil *et al.* 2012) and *S. albidoflavus* TN644 (Jaouadi *et al.* 2014). Moreover, the inhibitory effects of  $Co^{2+}$  and  $Hg^{+}$  on peroxidase Tfu-1649 were similar to the other peroxidases summarized in Table 2. In addition, the inhibitory effect of  $Ni^{+}$  on peroxidases was first demonstrated in the author's studies, to the best of the author's knowledge. Furthermore, the enzymatic activity of peroxidase Tfu-1649 was also inhibited by  $NaN_3$ , which was similar to the strains AD001 (Jeon *et al.* 2002), AH4 (Fodil *et al.* 2012), and TN644 (Jaouadi *et al.* 2014). Phenylmethylsulfonyl fluoride (PMSF) is a serine protease inhibitor, while  $\beta$ -mercaptoethanol is used to reduce disulfide bonds. Thus, the fact that peroxidase Tfu-1649 was inhibited by PMSF and  $\beta$ -mercaptoethanol revealed that serine and cysteine may act as important amino acids in the active center of the enzyme.

### Enzymatic Activity on Lignin Model Compounds

The enzyme activities on five compounds were tested in this experiment. As shown in Fig. 6, purified peroxidase Tfu-1649 exhibited high activities for catechol and L-DOPA, while 2,6-DMP, 2,4-DCP, and guaiacol were not good candidates for the oxidation reaction. Different enzymes have different substrate specificities. The fungal peroxidase (Pspd) found in *Perenniporia subacida* had different substrate specificities; it had the highest peroxidase activity against n-propanol, while 2,6-DMP, guaiacol, and catechol had relative activities of 82.14%, 79.34%, and 74.34%, respectively (Si and Cui 2013).



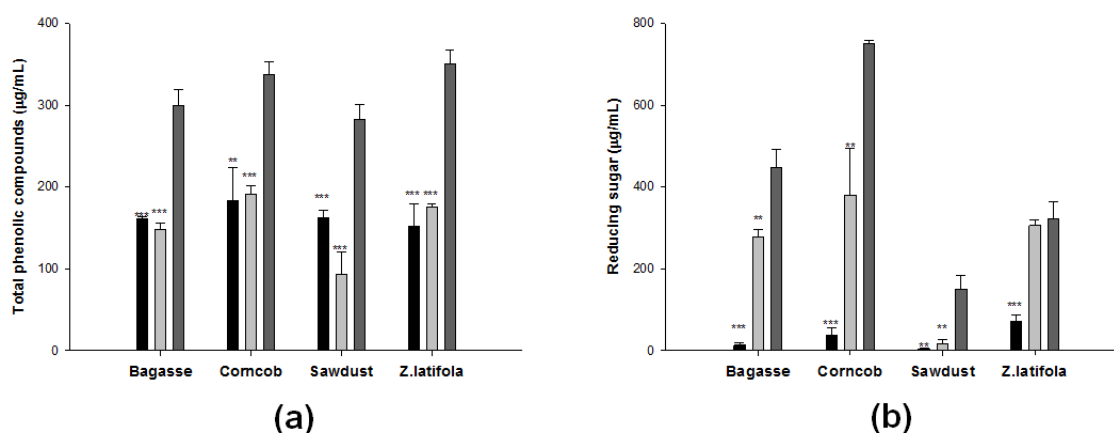
**Fig. 6.** Effect of the enzyme activities on five compounds on peroxidase Tfu-1649 purified from *Thermobifida fusca* BCRC19214

### Co-operative Enzymatic Degradation of Lignocellulose

In this study, the authors investigated the synergistic effects of xylanase and peroxidase in terms of lignocellulose degradation. Four lignocellulose substrates were treated with only xylanase to produce the reducing sugars in the reaction solution (as shown in Fig. 7a), while total phenolic compounds accumulated in the reaction solution across all four tests (as shown in Fig. 7b). When the substrates were treated with only peroxidase Tfu-1649, the total phenolic compounds all accumulated in the culture broth, with a limited amount of reducing sugars produced by the peroxidase. However, when the substrates were treated with both xylanase Tfu-11 and peroxidase Tfu-1649, synergistic effects were observed. In addition, corncob was the best candidate for degradation *via* xylanase Tfu-11



and peroxidase Tfu-1649. After incubating xylanase Tfu-11 and peroxidase Tfu-1649 for 24 h, 760  $\mu\text{g/ml}$  reducing sugars were accumulated in the reaction solution. However, after incubation with xylanase Tfu-11 or peroxidase Tfu-1649 alone for 24 h, only 380  $\mu\text{g/mL}$  or 30  $\mu\text{g/mL}$  reducing sugar was accumulated in the reaction solution. Furthermore, 336  $\mu\text{g/mL}$  of total phenolic compounds were released from the lignocelluloses and accumulated in the reaction solution after incubation with xylanase Tfu-11 and peroxidase Tfu-1649 for 24 h. There were only 190  $\mu\text{g/mL}$  or 180  $\mu\text{g/mL}$  of total phenolic compounds accumulated in the reaction solution after incubated 24 h with xylanase Tfu-11 or peroxidase Tfu-1649, respectively. Hence, xylanase Tfu-11 and peroxidase Tfu-1649 displayed significant synergistic effects when applied to corncob degradation, and these effects were also observed in the bagasse and sawdust reactions. However, the synergistic effect of xylanase Tfu-11 and peroxidase Tfu-1649 on *Zizania latifolia* Turcz husk was different relative to the other three substrates. The xylanase Tfu-11 hydrolyzed the *Zizania latifolia* Turcz husk and the reducing sugars accumulated in the broth, but the co-operation effect with peroxidase Tfu-1649 did not cause a synergistic increase in terms of the accumulated reducing sugars.



**Fig. 7.** Effects of peroxidase Tfu-1649 and xylanase Tfu-11 on the lignocellulolytic by-products concentrations of the (a) total phenolic compounds and (b) the reducing sugar.  : peroxidase, Tfu-1649;  : xylanase, Tfu-11;  : peroxidase, Tfu-1649 and xylanase, Tfu-11. Data was reported as the mean  $\pm$  standard error in triplicate (Student's *t*-test *p* values: \* = *p*-value less than 0.05; \*\* = *p*-value less than 0.01; and \*\*\* = *p*-value less than 0.001)

## CONCLUSIONS

1. *Thermobifida fusca* BCRC 19214 had a peroxidase activity of 43.66 U/mL when subjected to a bagasse substrate.
2. Synergistic effects were observed when the substrates were treated with both xylanase Tfu-11 and peroxidase Tfu-1649.
3. The results prove that the lignocellulose lignin barrier was disrupted by peroxidase Tfu-1649, thus rendering the lignocellulose structure more susceptible to degradation by xylanase Tfu-11. These findings may be helpful in granting a better understanding of the catalysis of lignocellulolytic substrates *via* complex hydrolytic enzyme systems.

## ACKNOWLEDGMENTS

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