Makoto Shoda

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Decolorization by Thanatephorus Cucumeris Dec 1

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PREFACE

This book summarizes the results of more than 20 years of research on a fungus, Dec 1, which was isolated in my laboratory, as a candidate to decolorize colored substances that are visible xenobiotic pollutants and cause serious environmental problems.

Various studies have attempted to improve or change environments using biological activity, but practical examples of this technique are rare. This is mainly because of the lack of basic information and the lack of optimization in reactor systems. In this book, one possibility of using a single microorganism to resolve environmental issues related to colored substances is demonstrated.

This book is composed of six chapters, and various aspects of isolated Dec 1, including its scientific analysis and the optimization of the engineered production of enzymes responsible for decolorization, are provided in it.

The features and characteristics of Dec 1 included in this book are as follows:

1. Dec 1 has been isolated as a fungus for dye decolorization. It has been finally identified as the non-white rot fungus, *Thanatephorus cucmerins* Dec 1.

2. The decolorization spectrum of Dec 1 is vast, indicating that Dec 1 produces multiple enzymes that are responsible for decolorization.

3. New peroxidase DyP, Aryl Alcohol Oxidase (AAO), Manganese Peroxidase (MnP), and Versatile Peroxidase (TcVP1) have been purified from Dec 1 and characterized.

4. The gene, dyp, has been cloned from Dec 1 and expressed in the fungus, Aspergillus oryzae.

5. Recombinant DyP (rDyP) has been shown to be almost identical to DyP.

6. The unique characteristics of DyP have been verified using crystallization and X-ray analysis.

7. The immobilization of rDyP has been found to be successful only when using a new mesoporous material as a carrier.

8. The enhanced production of DyP has been attempted using liquid culture, Solid-state Culture (SSC), repeated-batch culture, and fed-batch culture.

9. To overcome the problems involved in these culture methods, an Air Membrane Surface (AMS) reactor has been introduced, and increased rDyP production has been confirmed.

10. The final level of rDyP production has been found to be more than a half million-fold higher than the original level of DyP production by *T. cucumeris* Dec 1.

11. Dec 1 has demonstrated efficient decolorization of molasses waste, kraft pulp bleaching effluent, and oxygen-delignified bleaching effluent.

12. As substrates for Dec 1 growth, complex media, including rice bran powder, wheat bran

powder, and molasses, have been used, and the advantages of each medium have been described.

I believe that this book will provide researchers in this field with a useful resource to support current knowledge on biological decolorization and will provide students with a logical and practical scheme for approaching colored substance treatment. During my time as the Director of the Resources Recycling Process Laboratory, at the Chemical Resources Laboratory, Tokyo Institute of Technology, many researchers and graduate and undergraduate students at the campus and in private companies have helped to accomplish these results. I am indebted to the following researchers for giving me the opportunity to study Dec 1 and for offering many valuable suggestions: Drs. Y. Sugano, M. Hirai, T. Sato, M. Iwamoto, T.H. Lee, N. Uematsu, and J. Sugiura. l am also grateful to the following graduate and undergraduate students: S.J. Kim, M. Shakeri, N. Shintani, T. Shimokawa, K. Sasaki, Y. Matsushima, A. Ichiyanagi, R. Muramatu, R. Sasaki, N. Suzuki, and C. Matsuo.

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CHAPTER 1

Introduction

Several colored effluents, including dyes, molasses, and pulp bleaching effluents, are released into environments, and efficient treatments of these colored substances are urgent because severe regulation has been established worldwide. Conventional treatment methods have several disadvantages, such as energy consumption or the risk of treated products. On the other hand, biological treatment has advantages over conventional chemical and physical treatment because the products after treatment are relatively safer and the utilization of energy is smaller than other methods. However, in biological methods, finding efficient microorganisms and utilizing them under optimal conditions are key challenges.

A fungus, strain Dec 1 was isolated in our laboratory, exhibiting a wide spectrum of degradability for colored substances [1]. This fungus was initially identified as *Geotrichum candidum* Dec 1, but finally reidentified as *Thanatephorus cucumeris* Dec 1. In this book, the characteristics of Dec 1 have been described not only from the basic biochemical points, but also from an engineering point of view associated with its application for the decolorization of dyes, molasses, and pulp bleaching effluents.

More than 5×10^4 tons of dyes are used annually in Japan [2], and 10 to 15% of these dyes are estimated to be discarded into the environment [3]. As colored effluents from major textile and dyestuff industries are recalcitrant to biodegradation, the treatment of these effluents in wastewater treatment systems is mainly based on physical and/or chemical procedures, such as adsorption, concentration, chemical transformation, and incineration. Although these treatment methods are effective, they have several shortcomings, such as high cost, formation of hazardous byproducts, and intensive energy requirements. Therefore, biological degradation methods are receiving attention as better alternatives. Several strains as dye-degrading microorganisms have been reported, such as white-rot fungi *Phanerochaete chrysosporium* [4, 5], *Pleurotus ostreatus*

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[6], *Coriolus versicolor* [7], and *Streptomyces* spp [4]. The effectiveness of microbial treatment depends on the survival, adaptability, and stable activity of the selected microorganisms in the treatment environment.

The newly isolated strain, Dec 1 decolorized various reactive dyes, including azo and anthraquinone dyes, as shown in Chapter 2. The involvement of several extracellular enzymes, such as Lignin Peroxidase (LiP) and Manganese Peroxidase (MnP), was suggested by the broad decolorization spectrum of this strain. LiP, MnP, Laccase (Lac), and Horseradish Peroxidase (HRP) have also been reported to have the ability to decolorize various dyes [8 - 14]. Among them, MnP from *P. chrysosporium* is a representative enzyme and plays a major decolorizing role in the presence of manganese ions [15, 16]. However, Mn-oxidizing peroxidases isolated from *Bjerkandera adusta* and *Pleurotus eryngii* decolorized with multiple azo dyes, regardless of the presence of manganese ions [17]. Those enzymes were able to oxidize Mn^{2+} to Mn^{3+} at pH 5 and also oxidized aromatic compounds, such as Veratryl Alcohol (VA), a typical substrate of LiP, at pH 3, regardless of the presence of Mn^{2+} [18, 19]. Therefore, these enzymes that expressed both LiP-like and MnP-like characteristics were named MnP–LiP hybrid peroxidase or manganese-independent peroxidase or Versatile Peroxidases (VPs).

The enzymes responsible for the dye-decolorizing activity of Dec 1 were purified. Their characteristics are clarified in Chapter 3. One of them is a new peroxidase, DyP that is a glycoprotein with a molecular mass of 60 kDa, showing a high ability to decolorize anthraquinone dyes [20].

The culture broth of Dec l showed the ability to oxidize Veratryl Alcohol (VA), but DyP did not degrade VA [21], suggesting Dec 1 to produce Aryl Alcohol Oxidase (AAO) [22]. Therefore, a veratryl alcohol-oxidizing enzyme was purified from the culture broth of Dec 1, and its enzymatic characteristics and roles in dye decolorization have been characterized *in vivo*, as elucidated in Chapter 3.

Dec 1 showed complete decolorization of anthraquinone dye, Reactive Blue 5 (RB 5) *in vivo*. However, this phenomenon was not observed *in vitro* by DyP alone. DyP changed the color of RB5 from dark blue to a light reddish-brown colored substance composed of an azo complex mixture. Based on this observation, for the complete decolorization of Dec 1, the involvement of other enzymes in addition to DyP has been suggested. Then, a novel Versatile Peroxidase (VP) from Dec 1, named TcVP1, was isolated and characterized. The first complete *in vitro* decolorization of an anthraquinone dye using DyP and TcVP1 [23] is described in Chapter 3.

DyP has two specific characteristics. The first characteristic is its high ability to decolorize anthraquinone dye at around pH 3 and it lacks an important histidine residue that is involved in other fungal peroxidase tertiary structures. Instead, DyP includes aspartic acid and arginine [24], as described in Chapter 4.

The second characteristic is that DyP belongs to a novel DyP-type peroxidase family.

Peroxidases are classified into two types: animal and plant peroxidase superfamilies. The plant peroxidase superfamily is further categorized into three classes according to the origin [25]. Class I peroxidases are prokaryotic, and the representatives are Cytochrome C peroxidase (CCP) and *Escherichia coli* Peroxidase (ECP) [26, 27]. Class II peroxidases are secretory fungal peroxidases and the representatives are *Arthromyces ramosus* Peroxidase (ARP), Lignin Peroxidase (LiP), and Manganese Peroxidase (MnP) [28 - 30]. Class III peroxidases are classical, secretory plant peroxidases, and the representatives are Horseradish Peroxidase (HRP) and Turnip Peroxidase (TP) [31, 32]. According to this classification, DyP belongs to class II. However, the characteristics of DyP are different from those of class II peroxidases. Thus, to clarify the classification of DyP, the gene encoding DyP was cloned from the cDNA library of Dec 1, and the primary structure of DyP was compared with those of other peroxidases. This analysis revealed DyP as a unique peroxidase among previously reported peroxidases. The detail is provided in Chapter 4.

Cloning the cDNA of the *dyp* gene was successful, and the gene encoding DyP was transformed into the host, *Aspergillus oryzae*, under the control of the *amyB* promotor. The massive production of rDyP using recombinant *A. oryzae* is of primary interest in order to use crude rDyP directly for decolorization. This aspect is described in Chapter 4.

A. oryzae was selected as a host because this fungus is recognized as a safe host and it has a high growth rate and can secrete gram-per-liter quantities of heterologous proteins [33].

As the productivity of original peroxidase, DyP, by Dec 1 was extremely low, the enhanced productivity of rDyP was tried. First, the production of rDyP by recombinant *A. oryzae* was enhanced, but it was still not found to be satisfactory [34, 35]. Most common bioprocesses for large-scale production of chemicals use batch culture, which has the advantages of stable nongrowth-associated product formation, maintenance of genetic stability, and a relatively low risk of contamination [36]. Especially when natural substrates are chosen, complex solid particles are involved in the substrates, and continuous cultivation complicates the

CHAPTER 2

Characteristics of a New Fungus Isolated for Dye Decolorization

Abstract: As a dye-decolorizing microorganism, a fungus, identified as *Geotrichum candidum* Dec 1, was isolated from the soil. Re-identification of this fungus revealed it to be *Thanatephorus cucumeris* Dec 1 abbreviated as Dec 1. A crude extracellular enzyme solution was prepared from the culture of Dec 1, and the enzyme solution showed a broad decolorization spectrum of dyes in the presence of H_2O_2, indicating the existence of multiple enzymes, including peroxidases. Dec 1 decolorized approximately 12 g/1 of the dye, Reactive Blue 5 (RB5), without a decline in the decolorizing activity at the inhibitory concentration for most of the microorganisms, indicating the resistant property of Dec 1 to a high concentration of the dye RB5. Dec 1 expressed decolorizing activity in the presence of carbon sources and oxygen.

Keywords: *Geotrichum candidum* Dec 1, *Thanatephorus cucumeris* Dec 1, Peroxidase, Reactive Blue 5, Veratryl Alcohol, Azo dye, Anthraquinone dye.

1. ISOLATION OF A NEW DECOLORIZING FUNGUS

1.1. Methods

1.1.1. Isolation of Dye-decolorizing Microorganisms

For the isolation of dye-decolorizing bacteria and fungi, the following media were used: GPY agar for the isolation of bacteria and PDA media for the isolation of fungi. GPY agar medium consists of glucose, Polypeptone, yeast extract, KH_2PO_4, $MgSO_4.7H_2O$, and agar (pH=7). PDA medium contains potato extract, glucose, and agar (pH=5.6). Each of the following dyes, Reactive blue 114, Acid blue 324, and Dispersive blue 79, were mixed with the media shown above, and microbial colonies that appeared on the media and formed transparent dye zones on the media were purified. Each purified microorganism was used to confirm dye decolorizing ability by the method described below.

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1.1.2. Structures of the Dyes Used

Table **1** lists the dyes and simplified structures of the dyes used. The simplified structures of Reactive Blue 5 (RB5) are AQ-1[1-amino-4-(3-amino-4-sodiumsulfonoanilino)-2-sodium anthraquinone sulfonate] and AQ-2 (l-amino-4-methylamino-2-sodium anthraquinone sulfonate). The simplified compound of Reactive red 33 is AZ-1[1-hydroxy-6-methylamino-3-sodium naphthalene sulfonate-2-azo-(4'methoxy-2'sodium benzene sulfonate)]. Fig. (**1**) shows the structures of some dyes and the model compounds used. The simplified structure of AQ-2' is 1,4-diamino-2-sodium anthraquinone sulfonate.

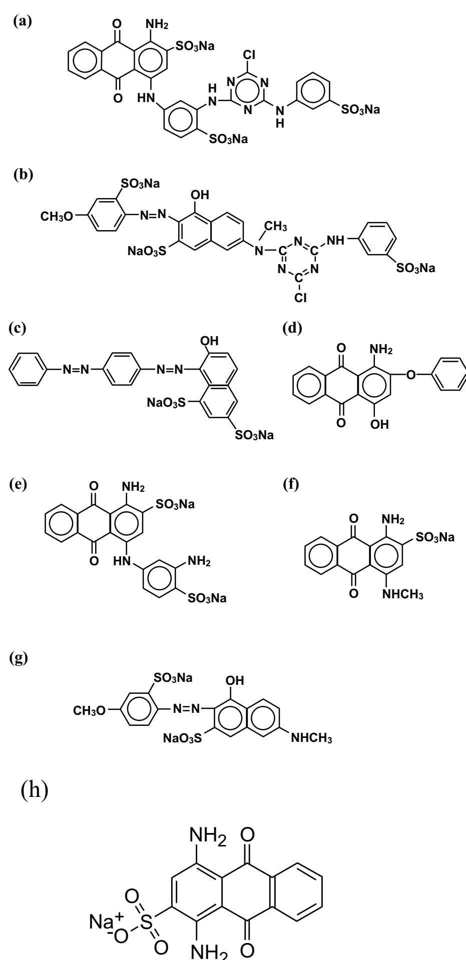

Fig. (1). Structure of representative dyes (**a-d**) and the model compounds of RB5 and Reactive red 33 (**e-h**) used. (**a**) Reactive blue 5; (**b**) Reactive red 33; (**c**) Acid red 73; (**d**) Dispersive red 60; (**e**) AQ-1; (**f**) AQ-2; (**g**) AZ-I; (**h**) AQ-2' [1].

Table 1. Degree of decolorization of various dyes by *T. cucumeris* Dec 1 on PDA medium after 4 day incubation at 28°C.

Color Index	Chromophore	Initial Concentration (ppm)	Decolorized Diameter (cm)[a]	Relative Value[b]
Reactive blue 5	Anthraquinone	100	8.5	1
Reactive blue 19	Anthraquinone	100	8	0.94
Reactive blue 114	Anthraquinone	100	7.5	0.88
Reactive blue 182	Azo	100	8	0.94
Reactive black 5	Azo	100	8	0.94
Reactive red 33	Azo	100	7	0.82
Reactive red 120	Azo	100	5.5	0.65
Reactive red 123	Azo	100	6.5	0.77
Reactive red 187	Azo	100	4	0.47
Reactive red 202	Azo	100	6.5	0.77
Reactive red 225	Azo	100	6	0.71
Reactive orange 13	Azo	100	3.5	0.41
Reactive orange 30	Azo	100	4	0.47
Reactive violet 23	Azo	100	7.5	0.88
Reactive yellow 2	Azo	100	3.5	0.41
Acid red 73	Azo	100	6.5	0.77
Acid blue 324	Azo	100	7	0.82
Disperse red 60	Anthraquinone	100	3	0.35
Disperse blue 79	Azo	100	n.d.	n.d.
AQ-1[c]	Anthraquinone	100	7.5	0.88
AQ-2[c]	Anthraquinone	100	8.5	1
AZ-1[d]	Azo	100	6	0.71

a Decolorized diameter after 4 day incubation.
b Reactive value of diamater to diameter of Reactive blue 5.
c Model chemicals of Reactive blue 5.
d Model chemicals of Reactive red 33.
n.d.Not detected.

1.1.3. Decolorization Using the Purified Dec 1 on PDA Medium

Dyes were mixed with PDA medium and the isolated Dec 1 mycelia were placed on the center of the PDA plate containing each dye; the plates were incubated until their transparent surface appeared.

CHAPTER 3

Characterization of Multi-Enzymes Produced by the Fungus, Dec 1, Responsible for Dye Decolorization

Abstract: A peroxidase, DyP, produced by the fungus Dec 1 having the ability to decolorize dyes was purified. DyP contains 17% sugar comprising GlcNAc and Man. The molecular mass of DyP was estimated to be 60 kDa and the isoelectric point (pl) was determined to be 3.8. DyP degraded various dyes, and also phenolic compounds of 2,6-dimethoxyphenol and guaiacol. However, veratryl alcohol, abbreviated VA, a substrate of lignin peroxidase, was not degraded by DyP.

Molasses was utilized by Dec 1 to produce DyP as carbon and energy source. Within 10 g/l of molasses concentration, decolorization activity of Dec 1 toward RB5 gradually increased. However, at more than 20 g/l of molasses concentration, the inhibitory effect on the decolorizin g activity of Dec 1 was observed. As the activity of purified DyP was inhibited by 10 g/l of molasses concentration, this indicates that molasses has a stimulative effect on the decolorization activity of Dec 1, but has an inhibitory effect on the DyP activity.

Dec 1 also produced Aryl Alcohol Oxidase (AAO). The role of AAO in the Dec 1 decolorization process of dyes has been observed to be as follows: the first role was that H_2O_2 was produced by AAO oxidation of Veratryl Alcohol (VA) to veratraldehyde and then utilized by the peroxidase DyP. In the cultivation of Dec l, the presence of H_2O_2 and veratraldehyde has been detected. The second role was that the polymerization of products produced by DyP oxidation of a simplified RB5, AQ-2', was prevented by AAO. This was confirmed by the result that the molecular weight of the products was reduced in the mixed decolorization of DyP and AAO.

A new versatile peroxidase named TcVP1 was purified from the Dec 1 culture. Purified TcVP1 behaved as Manganese Peroxidase (MnP) at pH 5, and the enzyme functioned as a Lignin Peroxidase (LiP) at pH 3. As TcVP1 decolorized preferentially azo dyes, co-application of TcVP1 and DyP conducted complete decolorization of anthraquinone dye, RB5, *in vitro* to colorless products.

Keywords: Peroxidase, DyP, Anthraquinone dye, Aryl alcohol oxidase, *Thanatephorus cucumeris* Dec 1, Versatile oxidase, TcVP1, Simplified RB5, AQ-2', Veratryl alcohol, VA, Veratraldehyde, Hydrogen peroxide, Molasses, Manganese peroxidase, MnP, Lignin peroxidase, LiP.

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1. NEW PEROXIDASE, DYP

1.1. DyP Purification

The purification steps for a new peroxidase, DyP, are shown in Table **3**. The total DyP activity in the supernatant derived from Dec 1 cultivation increased from 24U to 420 U after ultrafiltration with a YM 40 membrane treatment. Fig. (**8**) shows the main peak of DyP activity after the Butyl Toyopearl chromatography treatment.

Table 3. Purification steps for peroxidase, DyP, from cell-free culture of Dec 1 [20].

Purification Step	Protein (mg)	Total Activity (U)	Sp Act (U/mg)	Activity Yield (%)	Purification (fold)
Supernatant	88	24[a]	0.28[a]		1
Ultrafiltration (YM 10)	28	4.2×10^2	15	100[a]	55
Super Q chromatography	7.8	3.0×10^2	34	71	140
Butyl Toyopearl chromatography	1.5	85	57	20	210

Sp Act: specific activity.
a: The activity yield was calculated based on the value after the YM 10 treatment.

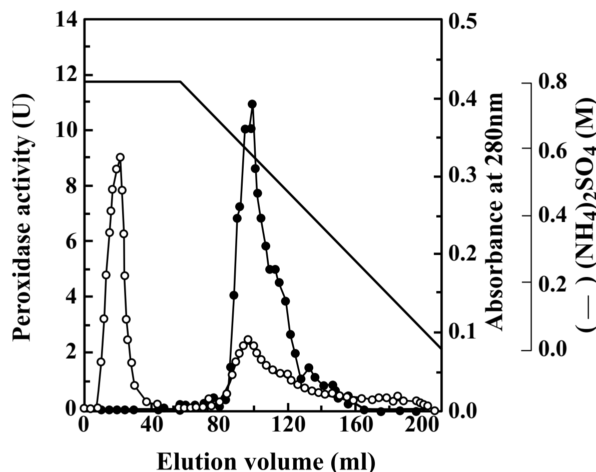

Fig. (8). Purification of peroxidase, DyP, produced by Dec 1 during Butyl Toyopearl chromatography. A solid line is a linear gradient of 0.8 to 0 M $(NH_4)_2SO_4$ in citrate buffer.
Symbols: ●, DyP activity; ○, absorbance at 280 nm [20].

Fractions 86 to 99, corresponding to the left half of the main peak, were called purified DyP having a specific activity of 57 U/mg of protein, as shown in Table **3**. SDS-polyacrylamide gel electrophoresis determined the molecular mass of DyP as 60 kDa. That value was significantly larger than those reported previously [105

- 107], ranging from 40 to 44 kDa. The Isoelectric point (*p*I) of DyP was determined as 3.8 by isoelectric point electrophoresis.

1.2. Spectral Characteristics of DyP

Fig. (**9**) shows the spectral characteristics of purified DyP. A Soret band appeared at 406 nm, representing the absorption peak of peroxidase, and the molar extinction coefficient of the band was almost the same as those of other peroxidases previously reported [108 - 110]. The A_{406}/A_{280} RZ value, which represents the purity and spectral characteristics of DyP, was 1.6 at pH 5. After H_2O_2 oxidation of the purified DyP, the peaks at 406 and 510 nm shifted to 400 nm and 530 nm, respectively. Then, $Na_2S_2O_4$ was added to reduce DyP that was oxidized by H_2O_2, and a peak at 556 nm appeared, as can be seen in the inset figure in Fig. (**9**). This confirms that DyP has a heme-pyridine complex, meaning that its prosthetic group is protoheme. DyP holds a single heme because the estimated heme content is 0.6 per mole of DyP.

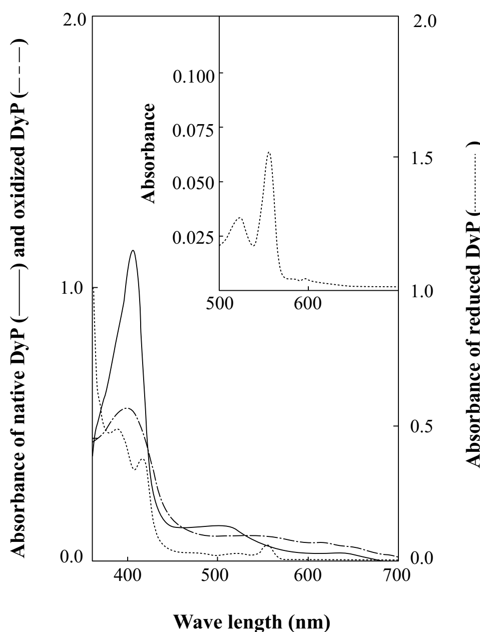

Fig. (9). Spectral characteristics of the following three DyPs: purified DyP, DyP oxidized by H_2O_2, and DyP reduced by $Na_2S_2O_4$. The inset shows reduced DyP in the form of a heme-pyridine complex observed at 556 nm [20].

1.3. Substrate Specificity of DyP

Table **4** shows the decolorization by DyP of four azo dyes, three anthraquinone dyes, and three simplified model compounds of RB5, AQ-1, AQ-2, and AQ-2'.

CHAPTER 4

Enhanced Productivity of a New Peroxidase DyP by Genetic Manipulation and by Cultivation Methods

Abstract: A novel peroxidase, DyP gene, *dyp*, was cloned from a cDNA library of a newly isolated fungus, Dec 1. The open reading frame consisting of 1494 nucleotides indicated a primary translation product of 498 amino acids, and the Molecular mass (Mr) was estimated as 53,306. X-ray diffraction data using crystallized DyP revealed DyP to have a unique tertiary structure differing from that of most other well-known peroxidases. The analyzed amino acid sequence of DyP did not share homology with any other peroxidases except that of a peroxidase derived from *Polyporaceae* sp.

Mature cDNA encoding *dyp* was fused with the *Aspergillus oryzae* α-amylase promoter, *amyB*, and recombinant DyP was produced. The total activity of the purified recombinant DyP, rDyP, was produced. The total activity of rDyP, was about 400-fold higher than that of the native DyP derived from Dec 1.

The further productivity enhancement of rDyP was carried out using the following different cultivation methods and different media. Recombinant *A. oryzae* holding a DyP gene, *dyp*, was grown using the repeated batch method. When a synthetic liquid medium containing maltose as a carbon source was used in repeated batch culture, production of high-level rDyP activity continued for 26 repeated cycles of every 1-day batch.

When the production of rDyP by *A. oryzae* was carried out using complex media containing rice bran powder both in liquid repeated-batch and in fed-batch cultures, average rDyP productivities were similar in the two batch cultures.

The Solid-state Culture (SSC) was also attempted for the production of rDyP by recombinant *A. oryzae* using wheat bran as a solid medium and the productivity of rDyP was compared to that in the liquid cultures. The maximum productivity of rDyP in SSC reached 5.3g per kg wheat bran, and this productivity value was equivalent to the productivity using a 56 kg liquid culture. When the unit of the productivity per gram carbon of the medium was introduced, the productivity in SSC was 4.1-fold higher than that in the liquid cultures.

In order to overcome the disadvantages of SSC and liquid culture, Dec 1 was grown in an Air Membrane Surface (AMS) reactor. Although the growth of Dec 1 in AMS culture was almost the same as that in liquid culture at optimum temperatures, DyP productivity, DyP activity, and Aryl Alcohol Oxidase (AAO) activity in AMS culture

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were 18-, 232-, and 108-fold higher than those in liquid culture, respectively. The advantage of AMS culture of Dec 1 1over SSC and liquid culture has been proven, particularly in the production of DyP.

A biofilm was formed in the AMS culture of Dec 1 and the relationship between biofilm formation and DyP and MnP was analyzed. Two new DyP isozymes, DyP2 and DyP3, were purified from the Dec 1 culture in the AMS reactor, and they were characterized and compared with the characteristics of rDyP.

Keywords: *Aspergillus oryzae*, Air-membrane surface reactor, Biofilm formation, Crystallization, Decolorizing peroxidase gene (*dyp*), DyP isozymes , Fed-batch culture, Recombinant DyP (rDyP), Repeated-batch culture, Rice bran powder, Solid-state culture, Wheat bran powder, X-ray diffraction, α-amylase promoter (*amyB*).

1. CLONING OF *DYP* GENE FROM DEC 1

The cloning and sequencing of gene, *dyp*, derived from Dec 1 cells are briefly described as follows [139]:

A peroxidase, DyP, derived from strain Dec 1 was partially digested by trypsin, and oligonucleotides encoding the two peptide fragments were synthesized for use as primers for Polymerase Chain Reaction (PCR). Then, the amplified DNA fragment by PCR was used as a probe for plaque hybridization using the cDNA library of Dec 1 derived from lambda phage λgt 10 [140]. The target gene was ligated to the plasmid pUC18 and sequenced.

Plaque hybridization produced eleven positive clones. The Molecular mass (Mr) of DyP was previously determined as 60,000. As this DyP contains 17%(w/w) saccharides, the Mr of the polypeptide was modified as 49,800 [20]. Therefore, an open reading frame corresponding to approximately 460 amino acid residues holding 1380 nucleotides was searched. PCR was carried out using the positive clones to estimate the size of each fragment and, one of them, clone 92 with1600 bp was digested into 2 fragments by *Eco*RI and was ligated into the *Bam*HI site of pUC18 and named pB92.

The pB92 contained an open reading frame consisting of 1494 nucleotides. The primary translation product of this open reading frame was composed of 498 amino acids and the molecular mass was estimated as 53,306. The value has been found to be reasonable with respect to the molecular mass of DyP. As all predicted amino acid sequences of the 5 oligopeptides obtained from DyP by trypsin digestion were found in this frame, the nucleotide sequence of this frame was judged to be the *dyp* gene encoding DyP in Dec 1.

2. CHARACTERISTICS OF DYP AS A PEROXIDASE

So far, all peroxidases have been categorized into animal peroxidase family and plant peroxidase family. The plant peroxidase family is further classified into three classes, classes I to III, according to the origin. As fungal peroxidases belong to class II peroxidases, DyP should belong to the class II peroxidases. However, the characteristics of DyP have been clarified to be significantly different from those of other known peroxidases [20, 139].

The first characteristic of DyP is as follows: the nucleotide sequence of the gene, *dyp*, and its primary translation product has no homology with any other peroxidases. One exception is a nucleotide sequence of peroxidase, *cpop*21 from *Polyporaceae* sp., which is registered under accession no. U77073 in GenBank, EMBL, and DDBJ. However, no characteristics of *cpop*21 have been published and thus, a detailed comparison between *cpop*21 and *dyp* is impossible.

The second characteristic of DyP is that all heme-containing peroxidases have two conserved His residues and one conserved Arg residue. One His residue is named proximal histidine and functions as the axial ligand for the heme. The other His residue is called distal histidine and Arg residue is known as essential arginine residue, being responsible for charge stabilization in the reaction between heme and H_2O_2 [20, 141, 142]. As all these three residues are conserved in all peroxidases, these residues have been considered essential for peroxidase activity, and there are no exceptions to this rule. Fig. (**28**) shows amino acid sequences of some well-known class II peroxidases together with DyP. It is clear that DyP has no heme-binding region, which is common to the plant peroxidase family [139]. Furthermore, His residue, which is responsible for heme binding, and is located at residue 51 of the amino acid sequence deduced from the cDNA of *dyp*, has been lost by N-terminal processing, as shown in Fig. (**28**). Therefore, DyP should have a heme-binding site different from those of other peroxidases, and thus the heme binding of DyP should be specific for Dec 1. Therefore, the recombinant rDyP obtained from *A. oryzae* should not express enzyme activity because it lacks heme. However, both original DyP and rDyP actually work as peroxidase. This suggests that there should be a novel heme-binding region other than the characterized region in the plant peroxidase family.

CHAPTER 5

Dye Decolorization by Immobilized Recombinant rDyP and Turnover Capacity of rDyP

Abstract: For an efficient application of the soluble recombinant enzyme of rDyP, immobilization of the enzyme was caried out to enhance and stabilize catalytic efficiency of rDyP. Although several conventional immobilization methods have been attempted for rDyP, no methods have been successful. Therefore, new catalysts developed for exhaust gas removal were employed. They were silica-based mesocellular foams and two silica-based porous materials, FSM-16 and AISBA-15, which were chemically synthesized. Immobilization of rDyP on them was carried out and immobilization efficiency was assessed. The overall efficiency was defined as adsorption efficiency x activity efficiency to find the maximum efficiency. The efficiency of rDyP immobilized on FSM-16 and AISBA-15 was maximum at pH 5 and pH 4, respectively. FSM-16 showed advantages over AISBA-15 in terms of stronger affinity for rDyP due to its anionic surface and much lower leaching of rDyP from FSM-16. When the rDyP immobilized on FSM-16, an anthraquinone dye, RBBR, was decolorized in repeated-batch mode, and eight sequential batches were possible, while rDyP immobilized on AISBA-15 enabled only two batches.

For evaluation of the practical potential of rDyP, the turnover capacity of rDyP was introduced. In order to minimize H_2O_2 inactivation for rDyP activity, four H_2O_2 supply methods were attempted and the turnover capacity of each method was compared. The continuous fed-batch supply of H_2O_2 and the stepwise fed-batch supply of the dye gave the maximum turnover capacity of 20.4. At this turnover capacity, one liter of crude rDyP solution containing 5,000 U could decolorize up to 102 g dye in 10h.

Keywords: AISBA-15, Adsorption, FSM-16, Immobilization, Inactivation by H_2O_2, Leaching, Mesoporous materials, Overall efficiency, Remazol Brilliant Blue R, Recombinant DyP, rDyP, Turnover capacity.

1. IMMOBILIZATION OF RDYP ON MESOPOROUS MATERIALS

1.1. Properties of Newly Synthesized Mesoporous Materials

The synthesis and characteristics of newly synthesized mesoporous materials of FSM-16 and AISBA-15 have been described in detail previously [186, 187]. Table **26** presents the structural and chemical properties of two synthesized materials, FSM-16 and AlSBA-15.

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Table 26. Structural and chemical properties of FSM-16 and AISBA-15 [186].

	Pore Volume (cm^3/g)	Pore Diameter (nm)	Specific Surface Area (m^2/g)	External Specific Surface Area (m^2/g)	Si/Al
FSM-16	1.9	5.4	1108	66	-
AISBA-15	1.29	10.6	918	22	30

-: not measured., Si/Al: silicate/aluminum

1.2. Effect of pH on Adsorption and Activity Efficiency of rDyP Immobilized on the Mesoporous Materials

The adsorption efficiency is defined as [(Ai – Ar)/Ai] x 100

Where, Ai is the initial rDyP activity before immobilization and Ar is the last rDyP activity after immobilization.

The expected activity of rDyP is defined as (Ai – Ar)/concentration of the material.

The activity efficiency is defined as (measured rDyP activity/expected rDyP activity)x100. Thus, the overall efficiency that reflects the overall activity performance of the immobilized rDyP is defined as adsorption efficiency x activity efficiency.

The immobilization procedure have been described previously [186]. rDyP was immobilized on the mesoporous materials under different pHs. Table **27** shows the result of FSM-16. The adsorption efficiency of rDyP on FMS-16 increased when pH declined from 6 to 3, and expected efficiencies were higher at pHs 3 and 4. Table **28** shows the result of MISBA-15. Although adsorption efficiency was 98% when rDyP was immobilized at pH 3, it was only 20% at pH 6. From the two tables, it is obvious that the expected efficiency of rDyP on FMS-16 was significantly higher than that on AISBA-15. However, the activity efficiency of rDyP on AISBA-15 was higher than that on FSM-16 at pH 4, 5, and 6. Therefore, in order to evaluate the overall efficiency, adsorption efficiency x activity efficiency was defined. As a result, the maximum values of the overall efficiency were 28% at pH 5 for FSM-16 and 43% at pH 4 for AISBA-15. The actual measured activity of rDyP was the maximum, being 2800 U/g-FSM-16 at pH 5, as shown in Table **27**.

Table 27. Characterization of rDyP immobilization on 1 g FSM-16 under different pH conditions (initial rDyP activity used = 10,000U) [186].

pH	Adsorption Efficiency	Expected Activity	Measured Activity	Activity Efficiency	Overall Efficiency
	(%)	(U/g-FSM-16)	(U/g-FSM-16)	(%)	(%)
3	98	9800	933	9.5	9.3
4	97	9700	1833	19	18
5	83	8330	2800	34	28
6	66	6600	2100	32	21

Table 28. Characterization of rDyP immobilized on 1 g AISBA-15 under different pH conditions at the initial rDyP activity of 10,000U [186].

pH	Adsorption Efficiency	Expected Activity	Measured Activity	Activity Efficiency	Overall Efficiency
	(%)	(U/g-AISBA-16)	(U/g-AISBA-16)	(%)	(%)
3	98	9830	833	8.5	8.3
4	97	4360	1933	44	43
5	17	1660	1600	96	16
6	20	1960	1433	73	15

1.3. Leaching of Immobilized rDyP from Mesoporous Materials

As leaching of immobilized rDyP from mesoporous materials reduces the activity of immobilized rDyP, the leaching degree of immobilized rDyP was evaluated by washing the immobilized rDyP repeatedly in buffer solution. Fig. (**45A**) shows the change in rDyP activity immobilized on FSM-16 after leaching under different pH conditions. The rDyP activity decreased by more than 57% at pHs 5 and 6. However, no changes in activity of rDyP were observed at pHs 3 and 4. Fig. (**45B**) shows the result of AISBA-15. The decrease in the activity of rDyP on AISBA-15 was more significant compared to that on FSM-16. No change in activity of rDyP on FSM\16 was observed at pHs 3 and 4.

CHAPTER 6

Application of Dec 1 for Decolorization of Other Colored Substances

Abstract: Molasses is a primary carbon source, especially in the microbial industry; however, molasses includes many colored substances, like melanoidins, which become concentrated by the Maillard reaction after sterilization [41]. Thus, these remain in the Molasses Wastewater (MWW) after use. For effective treatment of MWW, biological methods are attracting attention. Section 1-10, in the previous Chapter 3, showed molasses to be available as a carbon source for the growth of Dec 1, and that partial color removal of molasses by Dec 1 was possible. The enhanced color removal of molasses by Dec 1 was conducted using a jar fermenter system consisting of fan-type agitators and a pressure swing adsorption oxygen generator. The oxygen-enriched air supply was effective not only in obtaining the highest decolorization degree of molasses, but also the highest activity of peroxidase, DyP for the decolorization of several dyes.

By simultaneous decolorization of molasses and an anthraquinone dye, RB5, the degree of decolorization of molasses reached 87%, and thus, the maximum decolorization rate of the dye, RB5, was achieved. However, the decolorizing activity of purufied DyP toward molasses was significantly lower than that of culture broth of Dec 1 due to the inhibitory effects of molasses on DyP, but the inhibition was reduced in the progress of degradation of molasses by growing Dec 1 concentration. Dec 1 degraded molasses containing substances with a wide range of molecular weights prepared by ultrafiltered fractions of molasses. As Dec 1 was not able to utilize sucrose, sucrose in the molasses was hydrolyzed with invertase to utilize all sugars in molasses. As a result, the decolorization of molasses and rate of decolorization of the dye, RB5, by Dec 1 reached the highest level.

A long and stable decolorization of molasses was attempted using both suspended and immobilized cells of Dec 1. In semi-batch cultivation using suspended cells of Dec 1, 80% decolorization of molasses and a stable DyP activity were maintained for approximately four weeks. When repeated batch cultivation of Dec 1 cells immobilized on polyurethane foam was applied, a longer and stable decolorization of molasses as well as stable DyP activity lasted for more than eight weeks.

Dec 1 was applied for the decolorization of kraft pulp bleaching effluent, abbreviated as E-effluent when glucose was supplemented. The color removal of E-effluent and the reduced amount of Absorbable Organic Halogens (AOX) reached 78% and 43%, respectively. The average molecular weight of colored substances in molasses was reduced to less than 3000 from the original 5600. The contribution of extracellular

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enzymes, such as Peroxidase (DyP) and Manganese Peroxidase (MnP), to the decolorization of the kraft pulp bleaching effluent was observed in the later stage of decolorization.

Dec 1 decolorized up to 72% of Oxygen-delignified Bleaching Effluent (OBE). Biobleaching of Oxygen-delignified Kraft Pulp (OKP) was conducted at 2% pulp concentration. The brightness of OKP increased by 13% and the kappa value of OKP decreased by 4 points only for 3 days. However, at 25% of pulp concentration, the brightness of OKP increased only by 4% and the kappa value decreased by 3 points during a 12-day incubation period mainly because of oxygen limitation. When the culture after OBE decolorization was used for bleaching of OKP, the brightness of OKP increased to 62.7% at 2% pulp concentration. In the decolorization and biobleaching, the involvement of DyP and MnP was confirmed. From these results, the potentiality of Dec 1 for the decolorization of kraft pulp wastewater and biobleaching of kraft pulp in paper mills can be observed.

Keywords: Absorbable organic halogens, Immobilization on polyurethane foam, Kraft pulp bleaching effluent, Kappa value, Molasses, Oxygen-delignified bleaching effluent, Oxygen-delignified kraft pulp, Pressure swing adsorption oxygen generator, Repeated-batch cultivation, Ultrafiltered fractions of molasses.

1. DECOLORIZATION OF MOLASSES BY DEC 1

1.1. Methods

1.1.1. Decolorization Methods

The crude molasses used contained 33% sucrose, 6.5% glucose, 7.5% fructose, and 0.57 g Kjeldahl nitrogen/kg (w/w) [42]. The crude molasses was diluted to 50 g/1 and 20 g of glucose, 0.5 g of ammonium tartrate, 1 g of KH_2PO_4, and 0.5g of $MgSO_4$.$7H_2O$ (per 1 liter) were supplemented. This medium was referred to as the molasses medium. The dye used for measuring the DyP activity was Reactive Blue 5 (RB5).

The decolorization degree of the molasses (%) was calculated by the methods reported previously [44, 46, 121].

A conventional jar fermenter (nominal volume=7 liter) was modified by removing some gears in the fermenter to simplify the inside and to avoid adhesion of Dec 1 to the solid parts. Three fan-type propellers were prepared to agitate the culture broth homogeneously and two top propellers and one bottom propeller were fixed for the broth to flow in opposite directions.

The sampling of the culture broth was aseptically carried out using a pump and the loss of the broth was supplemented by a fresh molasses medium, which was

fed by another pump. Oxygen-enriched air prepared by a pressure swing adsorption oxygen generator avoided oxygen limitation. By a Dissolved Oxygen (DO) sensor, DO concentration was maintained.

1.1.2. Determination of the Activity of DyP

The decolorizing activity of DyP in the supernatant prepared from the culture broth of Dec 1 was determined in a mixture of the supernatant, H_2O_2, and dye, RB5, in citrate buffer (pH3.2) at the maximum absorbance wavelength of 600 nm of the dye [1]. The decolorizing rate was expressed as a concentration difference for the dye between two different times in the culture broth per unit time, and expressed as mg /1/ min.

1.2. Results

The effect of aeration and agitation in a reactor was investigated. The air supply rate was fixed at 0.4 vvm (volume of air/volume of medium/minute), and the agitation speed was changed from 150 to 250 rpm (rotation per minute). At 150 rpm, the growth of Dec 1 was slow, which may be mainly due to the low oxygen transfer rate. When the agitation speed increased to 250 rpm to supply enough oxygen, large pellets of Dec 1 with 2 mm in diameter were formed, and the decolorization rate of the molasses reduced significantly. At 180 rpm, the small mycelial pellets of Dec 1 were homogeneously dispersed and the enhancement of the decolorizing activity and DyP activity was observed. At a fixed agitation speed of 180 rpm, different air supplies were tested to obtain efficient decolorization conditions. Fig. (**55**) shows the results. The decolorization degree of molasses (Fig. **55a**) and DyP activity (Fig. **55b**) were the highest when enriched 60% oxygen was supplied at 0.4 vvm. Under this condition, the absorbance of the cell-free culture broth at 475 nm changed from 2.5 at 0 day to 0.5 at 10 days, suggesting the steady decolorization of molasses.

The growth of Dec 1 (Fig. **55c**) at 60% oxygen supply or air supply at 0.4 vvm was similar. The apparent growth in the later days reflected the fast consumption of glucose, as shown in Fig. (**55d**). Although the activity of DyP increased in 6 days, the contribution of DyP to the decolorization of molasses was found to be small. The details are provided in the next section 6-2.

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