Environmental Microbiology Research Section

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1. Introduction

The relationship between energy resource consumption and environmental protection is crucial to developing a sustainable society. Despite our heavy reliance on fossil energy, there is concern that greenhouse gas emissions are disrupting the harmony of the global environment. Additionally, environmental pollution continues to be the shadow of civilization's progress due to the energy consumption of fossil fuels. One of the solutions is to develop a practical method that uses 'enzymes' derived from environmental microorganisms with high energy utilization efficiency in catabolism. Meanwhile, we are also working on sustainable agricultural techniques, which are the source of life energy. We are collaborating with academics, biotechs, and university start-ups globally to network research towards the social implementation of our technologies.

2-1. Two-compositely microbial catalysts efficiently degraded polychlorinated biphenyls.

We have developed a composite microbial catalyst that can efficiently degrade polychlorinated biphenyls (PCBs), well-known pollutants found widely in the environment. Biphenyl dioxygenase (BDO) plays a crucial role in the degradation of PCBs. It incorporates two oxygen atoms into the PCB's aromatic ring, which then induces aromatic ring cleavage. In more technological detail, our laboratory has designed a composite catalytic enzyme consisting of two BDOs with different substrate specificities. We have also forged a bioreactor that generates oxygen microbubbles to enhance the enzymatic activities of BDOs. With these innovations, we have constructed a practical system that degrades 99.3% of 40 mg L^{-1} of domestic major commercial PCBs (Kanechrol KC-300 and KC-400 of KANEKA CORP.) in 24 hours. This result meets the waste disposal measure defined by the Ministry of the Environment Government of Japan (Figure 2).

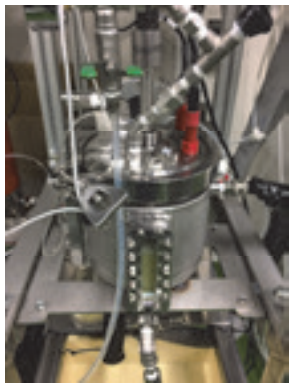

Figure 1: The composite BDOs-microbial catalyst was tested in a dedicated experimental bioreactor with an oxygen microbubble generation device.

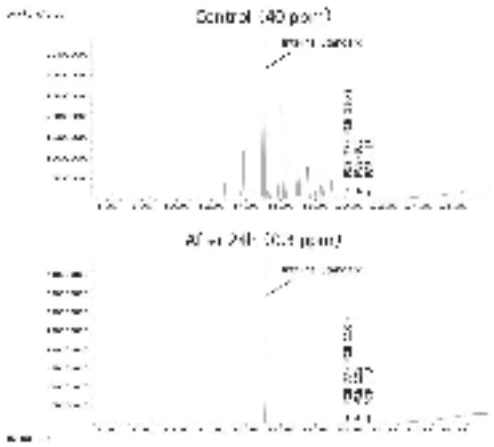

Figure 2: Gas chromatography-quadrupole mass spectrometer data show the degradation of PCBs by the composite BDOs-microbial catalyst.

2-2. Several bacterial species associated with PCB dechlorination were genetically identified at PCB-contaminated sites.

We have been developing an artificial enzyme that can dechlorinate PCBs by two-electron reduction. To do this, we collected sediments from freshwater pit pools contaminated with PCBs in the Yodogawa riverside in the Osaka area and investigated whether bacteria associated with PCB dechlorination exist.

According to 16S rRNA gene phylogenetic analysis, it has been observed that *Dehalobacter* sp. and *Desulfitobacterium* sp. are present in that specific location. Wang and He (Environ Sci Technol, 2013) have reported that "*Deharobacter*" can dechlorinate penta- and hexa-chlorinated biphenyls while "*Desulfitobacterium*" can dechlorinate tetra-chlorinated

biphenyls that are hydroxylated at the para position.

We successfully prepared the medium for growing two specific bacterial species and developed a suitable cultivation method. In addition, we observed that these bacterial species can reduce PCBs in an artificial model of a polluted environment. Even today, after a decade of starting this investigation, we continue to observe their long-term effects to confirm the accuracy of our results.

3-1. A new protein secreted by *Rhizoctonia solani* suppresses filamentous fungi growth.

Rhizoctonia solani is a filamentous fungus belonging to the phylum Basidiomycota. This fungus is well-known for its ability to infect and cause severe diseases in many crops, such as rice sheath blight, which significantly impacts paddy-rice production. The exact mechanism by which this phytopathogenic fungus infects plants has yet to be fully understood. However, studies have shown that when wheat bran is added to the growth medium, this fungus secretes glycosidases that digest plants and fungi cell walls. Our team has discovered a new protein secreted from *R. solani* when cultured with wheat bran as a solid medium. This protein has a molecular weight of approximately 10 kDa and exhibits anti-fungal properties against filamentous fungi. We reported this finding for the first time at the 2023 Annual Meeting of the Japan Society for Bioscience, Biotechnology, and Agrochemistry[1]. This protein inhibited the growth of *Fusarium fujikuroi*, another phytopathogenic filamentous fungus belonging to the phylum Ascomycota. It was suggested that this protein also inhibited conidium formation and germination of *F. fujikuroi*.

After conducting an amino acid sequence analysis, it was found that the partial amino acid sequence of this protein suggests that it has an unknown function. Additionally, the full-length amino acid sequence of the protein was deduced after investigating the whole genome sequence of the *R. solani* strain that produces it. The BLAST search results on this sequence also suggested that the protein has an unknown function.

According to the process and sequence analysis of the cDNA that encodes this protein, the complete amino acid sequence of the protein was found to be 122 residues. The mature protein's amino acid sequence, which excludes the assumed signal peptide sequence, was 88 residues in length. Based on this information, the estimated molecular weight of this protein was 9648.24. Furthermore, this particular antifungal protein has made us aware of unique structural features in its primary amino acid sequence that have never been reported. The protein consists of 88 residues and has a double-repeat structure of 41 residues, with a high homology of 92% across the central 6 amino acid residues. The functional role of such repetitive sequences still needs to be better understood. We have named this antifungal protein "**Double-Repeating Homologous Sequence Anti-Fungal Polypeptide (DRHS-AFP)**."

3-2. The completion of the genetic recombination of DRHS-AFP and investigation of its antifungal spectrum.

We have designed a system for expressing DRHS-AFP as genetically recombinant proteins. The DRHS-AFP gene was amplified by PCR and encoded the mature protein's 88 residues with an additional initiation of methionine. Then, we inserted this DRHS-AFP gene into the pET-15b, *E. coli* expression vector, to create the DRHS-AFP expression plasmid, pEp10. Finally, we transformed the *E. coli* host strain BL21(DE3) with pEp10 for the protein expression.

The efficacy of recombinant DRHS-AFP in confronting filamentous fungi that typically infect paddy rice was tested. The pathogens tested included *Fusarium fujikuroi* (Figure 3), *Trichoderma viride*, *Pyricularia oryzae*, *Pythium* sp., *Rhizoctonia solani*, and *Rhizopus microsporus*. The results indicated that the genetically modified DRHS-AFP had a growth-suppressive effect on *F. fujikuroi* and *T. viride*. Likewise, it was suggested that the anti-filamentous fungal activity of the genetically modified DRHS-AFP was equivalent to that of the native DRHS-AFP.

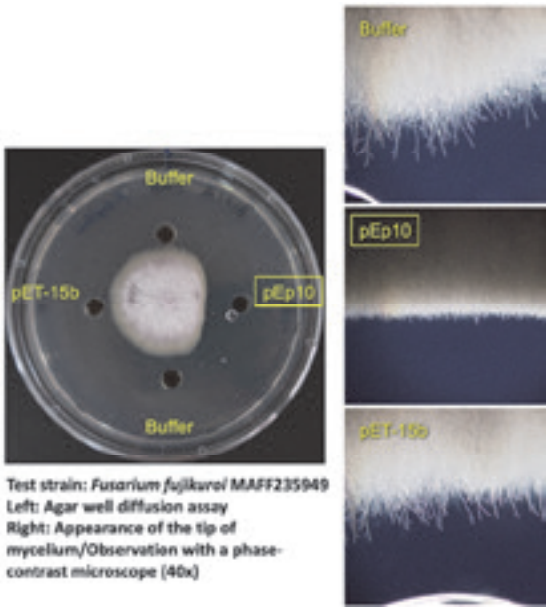

Figure 3. The anti-filamentous fungal activity of genetically recombinant DRHS-AFP.

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1. Kawamura S., Arima K., Takatsuka Y., Yamagishi J., Hara T., Isolation of a novel 10 kDa anti-fungal protein that specifically acts on filamentous fungi of Ascomycota., the 2023 annual meeting of the Japan Society for Bioscience, Biotechnology, and Agrochemistry., March 15, 2023, webinar.

Collaboration Works

原富次郎, 髙塚由美子, Lamont Doherty Earth Observatory-Columbia University（アメリカ）, ポリ塩化ビフェニル類を分解する微生物とその由来酵素

原富次郎, 髙塚由美子, Faculty of Engineering, National University of Laos（ラオス）, ラオス国コーヒー伝染病観測所の設置と病害対策

髙塚由美子, 原富次郎, Department of Civil and Environmental Engineering-National University of Singapore（シンガポール）, ポリ塩化ビフェニルを脱塩素化する細菌

Financial Support

1. Grant-in-Aid for Scientific Research
原富次郎, 基盤研究(C), 高塩素置換型ポリ塩化ビフェニル類の還元的脱塩素化を大気下で実現させる（分担金）

髙塚由美子, 基盤研究(C), 高塩素置換型ポリ塩化ビフェニル類の還元的脱塩素化を大気下で実現させる

2. Others
原富次郎, 東洋ガラス（株）, 環境微生物の探索と機能解明の研究のため

原富次郎, （株）竹中工務店, 環境微生物の探索と機能解明の研究のため

Publications

T.T. Nguyen, A.T.P. Bui, N.T.H. Le, H.T.N. Vo, A.H. Nguyen, T.D. Pham, T. Hara, K. Yokota, M. Matsutani, Y. Takatsuka, A.T.V. Nguyen, Heat-stable spores of carotenoid-producing *Bacillus marisflavi* and non-pigmented *Bacillus subtilis* cooperatively promote growth, quality, and gut microbiota of white-leg shrimp, Beneficial Microbes, 14, 623-640, 2023

Presentations

Y. Shiwa, T. Hara, Y. Takatsuka, K. Yokota, Elucidation of the novel competitive function between microorganisms of genus *Rhizoctonia* by genomic approach, The 14th International Symposium of Advanced Energy Science, 京都大学宇治キャンパス, 2023.8.30

中西梨奈、髙塚由美子、山田麗、原富次郎, 遺伝子組換え型 DRHS-AFP の抗真菌活性の検討評価, 日本農芸化学会 2024 年度大会, 東京農業大学, 2024.3.25

山田麗、髙塚由美子、中西梨奈、原富次郎, *Rhizoctonia solani* 菌糸融合群・亜群間における DRHS-AFP 分泌性の違い, 日本農芸化学会 2024 年度大会, 東京農業大学, 2024.3.25