Environmental Biotechnology for Efficient Utilization of Industrial Phosphite Waste

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Abstract

Phosphorus recycling is essential for the sustainable future of humanity. Phosphite (Pt), a salt of phosphorous acid (H_3PO_3), is a waste product of the chemical and automotive industries. Pt has to be oxidized to phosphate (Pi) prior to phosphorus recycling. Pt dehydrogenase (PtxD), catalyzing oxidation of Pt to Pi with concomitant reduction of NAD^+ to NADH, could have a number of applications in the efficient utilization of Pt waste. The originally isolated PtxD, however, showed both thermosensitivity and mostly insoluble expression in an *Escherichia coli* recombinant, limiting the practical application of this enzyme. To overcome this problem, we obtained a stable and solubly expressed PtxD from a thermotolerant Pt-oxidizing bacterium. Here we describe three emerging applications of PtxD in (i) an NADH regeneration system, (ii) a dominant selection system for recombinant microorganisms, and (iii) Pt fertilization in plants. Firstly, an NADH regeneration system is necessary for the production of industrially important chemicals by oxidoreductive enzymes. Stable PtxD with Pt as a reducing reagent could be used practically as an NADH regeneration system. We demonstrated production of a chiral compound using a PtxD-driven NADH regeneration system. Secondly, selective cultivation of microorganisms is important to the production of medical and chemical compounds and renewable biofuels. Transfection with *ptxD* (recombinant PtxD trait) allows selective growth of microorganisms on a medium containing Pt as its sole phosphorus source. Pt could be used as an alternative to antibiotics in large-scale cultivation of *ptxD*-recombinant microorganisms. Finally, direct utilization of Pt as a fertilizer would be the most cost-effective method for the recycling of Pt waste. Pi, however, is the only chemical form of phosphorus that can be assimilated by plants. We and another group demonstrated that *ptxD*-recombinant plants can directly utilize Pt fertilizer. These new environmental biotechnologies could contribute to efficient utilization of Pt waste.

Key words: DNA recombinant, environmental biotechnology, fertilizer, phosphite, phosphite dehydrogenase, phosphorus recycling, selection system, weed-control

1. Introduction

Phosphorus is an essential plant nutrient that has contributed to the 20th century's green revolution. In 2008, price spikes of phosphorus fertilizer increased concerns regarding the future depletion of phosphate rock reserves, the main source of phosphorus. While estimates range from 30 to 300 years, there is a general consensus that the quality and accessibility of remaining reserves are decreasing and costs will increase (Cordell & White, 2011). Although other critical global resources, such as oil, can be replaced with renewable energy sources, no other element can replace phosphorus in food production. Ensuring long-term availability and accessibility of phosphorus resources is critical to the future of humanity (Cordell & White, 2011). The solutions to these problems lie in recapturing and recycling phosphorus, as well as developing ways to utilize it more efficiently. In this report, we focus on efficient utilization of industrial phosphorus waste.

Unutilized phosphorus resources exist mainly in agricultural and municipal wastes, but also in industrial ones. For instance, a large amount of phosphite (Pt) waste is produced by the chemical industry where phosphorus trichloride (PCl_3) is used for the synthesis of acid chlorides, which are building materials for pharmaceuticals. The total volume of PCl_3 manufactured worldwide is approximately 0.7 million tons per year (Clements *et al*., 2010). On the other hand, the growth of the electroless plating in the automotive sector has led to increased consumption of hypophosphite, resulting in a marked increase in the amount of Pt as a by-product. The Pt in the electroless plating waste is usually precipitated with lime and discarded in a secure landfill. In Japan, thirty thou-

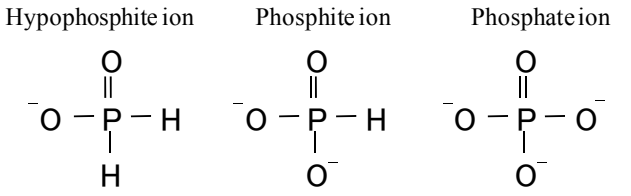

Fig. 1 Structure of hypophosphite, phosphite (Pt), and phosphate (Pi) ions.

sand tons of wastes containing high concentrations of Pt are dumped annually (Hashizume *et al.*, 2007). From the standpoint of global phosphorus security, it is important to develop technologies to reuse Pt waste.

Pt is a salt of phosphorous acid in which phosphorus has an oxidation state of +3 (Fig. 1). On earth virtually all known phosphorus exists in the +5 oxidation state. This is true for both phosphate (Pi) and Pi-esters that play important roles in many biological reactions. Therefore, Pt has to be oxidized to Pi prior to phosphorus recycling. Recently, a chemical oxidation process of Pt to Pi has been developed using H_2O_2 under ultraviolet light in a lab-scale batch reactor (Liu *et al.*, 2013). A large energy investment is necessary, however, to oxidize Pt to Pi in this process.

Use of Pt dehydrogenase (PtxD) to catalyze oxidation of Pt to Pi with concomitant reduction of NAD^+ to NADH, is a promising candidate for the efficient utilization of industrial Pt waste. Reduction of NAD^+ to NADH (NADH regeneration) is critical to the economic viability of industrial-scale biotransformations using reductase enzymes. Pt functions as a reducing agent in the PtxD reaction. The development of a PtxD-driven NADH regeneration system would combine recycling of Pt waste with production of valuable biochemicals. On the other hand, most wild-type microorganisms cannot assimilate Pt, or they grow very poorly on media containing Pt as the sole phosphorus source. Introduction of *ptxD* allows these microorganisms to grow on Pt media, indicating that Pt functions not only as a phosphorus source but also as a selective nutrient for *ptxD*-recombinant microorganisms. Similarly, Pt could be used as a selective fertilizer for *ptxD*-recombinant plants. Direct utilization of Pt as a fertilizer would be more beneficial for the recycling of Pt waste. Here we describe these potential applications of PtxD to utilize Pt waste more efficiently.

2. Stable and Soluble Pt Dehydrogenase: a Potential Biocatalyst that Oxidizes Pt to Pi

2.1 Isolation of a Pt-oxidizing thermotolerant bacterium

The primary source of phosphorus for living cells is either Pi or readily hydrolysable Pi-esters. Bacterial Pt oxidation was first reported by Adams and Conrad (1953). The mechanisms for biological oxidation of Pt, however, had been unclear for a long time until the Metcalf group demonstrated that PtxD oxidizes Pt to Pi in *Pseudomonas stutzeri* WM88 (Costas *et al.*, 2001).

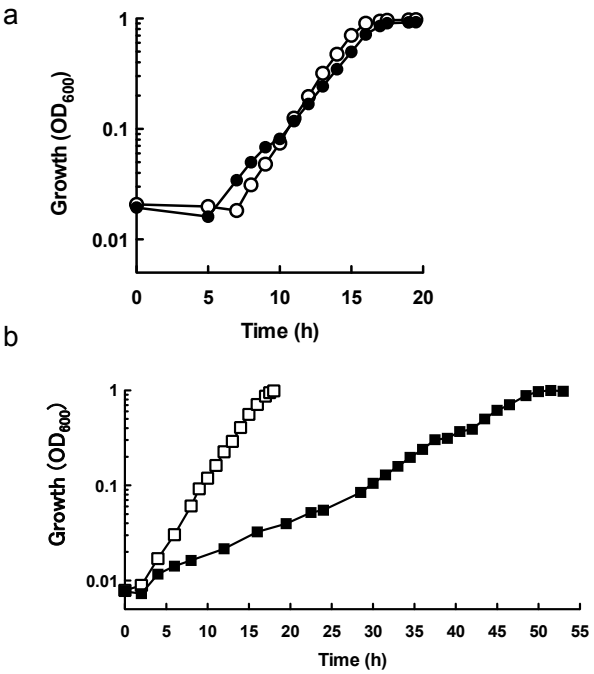

Fig. 2 Growth of *Ralstonia* sp. 4506 (a) and *P. stutzeri* WM536 (b) on a Pi-medium (open symbols) and Pt-medium (closed symbols). *Ralstonia* sp. and *P. stutzeri* were incubated at 45°C and 30°C, respectively. Growth was monitored by measuring the optical densities of the cultures at 600 nm (OD_{600}).

More recent studies have shown that *Alcaligenes faecalis* WM2072 also carries PtxD for the assimilation of Pt (Wilson & Metcalf 2005). Interestingly, Schink and Friedrich (2000) reported that *Desulfotignum phosphitoxidans* can use Pt as an energy source. All these bacteria contain PtxD for the assimilation of Pt.

The originally identified PtxD of *P. stutzeri* (designated as $PtxD_{PS}$) showed both thermosensitivity and mostly insoluble expression when produced in *Escherichia coli*, limiting the practical application of this enzyme. The half-life of $PtxD_{PS}$ at 45°C was about 1.5 min. To overcome this problem, Woodyer *et al*. (2006) improved stability, soluble expression and turnover rate of the enzyme by random mutations. On the other hand, we attempted to isolate stable PtxD from thermotolerant bacteria that can assimilate Pt as a sole source of phosphorus (Pt medium). Pt-assimilating thermotolerant bacteria were successfully enriched from many soil samples at 45°C, but not at 60°C. Since bacterial colonies on a Pt agar plate appear morphologically very similar, we expected that one predominant bacterium arose during enrichment. Indeed, most of the isolated bacteria were classified in the genus *Ralstonia* based on a 16S rRNA sequence analysis. We designated one of the Pt-assimilating thermotolerant bacteria as *Ralstonia* sp. 4506 (Hirota *et al*., 2012). Interestingly, *Ralstonia* sp. 4506 could grow as fast on the Pt medium as on a Pi-medium, while previously isolated Pt-assimilating bacteria such as *A. faecalis* WM2072 and *P. stutzeri* WM88 showed relatively slow growth on the Pt-medium (Fig. 2).

2.2 Stable and soluble PtxD

Pt dehydrogenase of *Ralstonia* sp. 4506 (designated as $PtxD_{R4506}$) was purified from an *E. coli* recombinant. Expectedly, the optimum temperature of $PtxD_{R4506}$ activity was higher than that of $PtxD_{PS}$. The thermal inactivation half-life of $PtxD_{R4506}$ at 45°C was 73 h, which was 3,100-fold greater than that of $PtxD_{PS}$ (Hirota *et al.*, 2012). Interestingly, $PtxD_{R4506}$ showed higher solubility than $PtxD_{PS}$ when overexpressed in *E. coli* (91% and 18% of total recombinant proteins were expressed in the soluble fraction, respectively) (Hirota *et al.*, 2012). Consequently, the use of $PtxD_{R4506}$ recombinants should enable cost-effective preparation of this enzyme.

A kinetics analysis revealed that $PtxD_{R4506}$ has a lower K_m value for Pt compared to $PtxD_{PS}$, resulting in approximately 6.7-fold and 10-fold greater catalytic efficiency (V_{max}/K_m) than $PtxD_{PS}$ at 30°C and 40°C, respectively. $PtxD_{R4506}$ also showed a significantly lower K_m value for NAD^+. The greater catalytic efficiency for Pt oxidation might support the fast growth of *Ralstonia* sp. 4506 on the Pt medium.

3. Environmental Biotechnology for Efficient Utilization of Pt Waste

3.1 NADH regeneration

The number of enzymes used as biocatalysts is continuing to grow. These biocatalysts can help to make industrial manufacturing processes more environmentally friendly. Many potentially useful enzymes, however, are cofactor-dependent. Due to the high cost of these cofactors, their stoichiometric use is not acceptable in industrial bioprocesses. This problem can be overcome by using a regeneration system that recycles cofactors during the reaction (Zhao & van der Donk, 2003; Weckbecker *et al.*, 2010). Since PtxD efficiently reduces NAD^+ to NADH as it oxidizes Pt, Zhao and van der Donk (2003) originally proposed practical application of this enzyme to an NADH regeneration system.

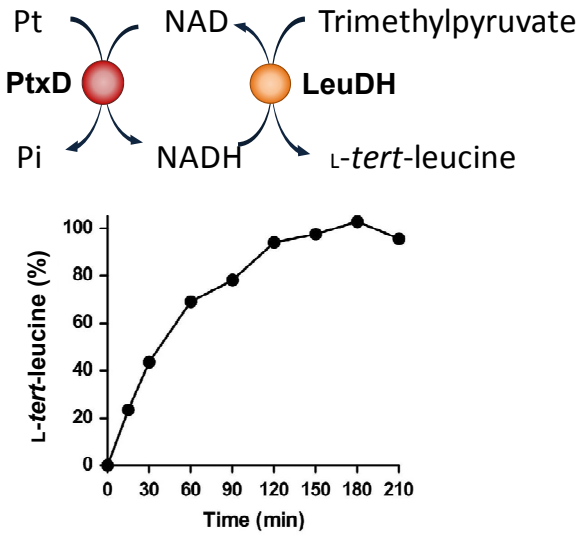

Fig. 3 Coupling of a PtxD-driving NADH regeneration system for L-*tert*-Leucine production from trimethylpyruvate. An aliquot containing 50 mM trimethylpyruvate, neutralized to pH 7.5 with ammonia, was added to a reaction mixture containing 75 mM P*t*, 0.5 mM NAD^+, 15 U/mL of leucine dehydrogenase, and 1.5 U/mL of $PtxD_{R4506}$, and incubated at 45°C. Conversion rates of L-*tert*-Leucine were measured at various times.

To test the application of $PtxD_{R4506}$ to an NADH regeneration system, the PtxD reaction was coupled with the production of L-*tert*-leucine, an important chiral building block used in the pharmaceutical industry (Hirota *et al.*, 2012). Leucine dehydrogenase (LeuDH) of *Bacillus* sp. catalyzes the reductive amination of trimethylpyruvate to L-*tert*-leucine with the concomitant oxidation of NADH to NAD^+ (Bommarius *et al.*, 1995). As shown in Fig. 3, 50 mM trimethylpyruvate was almost fully converted to L-*tert*-leucine in the presence of 0.5 mM NAD^+, indicating that $PtxD_{R4506}$ regenerated NADH approximately 100 times during the reaction (Hirota *et al.*, 2012). The use of Pt waste would further reduce the cost of NADH regeneration in cofactor-dependent biocatalytic processes.

3.2 Dominant selection system for large-scale cultivation of recombinant microorganisms

Genetically modified microorganisms play a vital role in the chemical, pharmaceutical and food industries. Selectable gene markers, which confer the ability to grow on a selective medium, are not only required for the transformation process but could also be used to avoid contamination by other microorganisms in the subsequent culturing process. Antibiotics and their resistance genes have been widely used for this purpose in laboratory-scale experiments. Their industrial use, however, is costly and has to be strictly regulated from an environmental health perspective since the release of antibiotics and genetically modified microorganisms carrying antibiotic resistance genes increases the risk of emergence of new drug-resistant microorganisms (Droge *et al.*, 1998; Heinemann, 1999; Zhang *et al.*, 2009).

The commercial use of yeasts has expanded from the food industry to the production of medicinal compounds, chemicals and renewable biofuels (Nielsen, 2013). Bacterial contamination is a major problem in commercial fermentation cultures, particularly in fuel ethanol fermentations that are not performed under sterile, pure culture conditions (Connolly, 1997). Despite efforts to prevent contamination with extensive cleaning and disinfecting procedures, saccharification tanks, continuous yeast propagation systems and notoriously resistant biofilms can act as reservoirs of bacteria that continually reintroduce contaminants (Skinner & Leathers, 2004). A variety of Gram-positive and -negative bacteria have been isolated from fuel ethanol fermentations. A major culprit, *Lactobacillus fermentum*, has been shown to reduce ethanol production in yeast fermentation cultures by as much as 27% (Bischoff *et al.*, 2009). Since no *ptxD*-homologs have been reported in *Lactobacillus* species, the development of *ptxD* and Pt-based selection systems for yeast would greatly reduce bacterial contamination risks in fuel ethanol fermentations.

Yeast *Schizosaccharomyces pombe* cannot assimilate Pt. In order to investigate whether bacterial *ptxD* can confer Pt-oxidizing ability on *Sz. pombe*, *ptxD* was introduced into the yeast genome under three promoters of different strengths (*nmt1* > *nmt41* > *nmt81*) (Kanda *et al*., 2014). A recombinant, carrying *ptxD* under the strongest *nmt1* promoter, grew quickly on a yeast Pt medium (Fig. 4a, open circle). Recombinants carrying *ptxD* under *nmt41* and *nmt81* promoters (approximately 15% and 1.3% of *nmt1* promoter activity, respectively (Basi *et al*., 1993)) grew on the Pt medium after prolonged lags (Fig. 4a, open triangle and square). A recombinant carrying only the *nmt41* promoter (without *ptxD*) did not start growing on the Pt medium within 132 hours (Fig. 4a, asterisk). These results indicate that the growth of *Sz. pombe* on the Pt medium depended on the PtxD activity. The recombinant carrying *ptxD* under the strongest *nmt1* promoter grew on the Pt medium as fast as on the Pi medium (Fig. 4a, b), indicating that a certain level of PtxD expression could fully support the growth of *Sz. pombe* using Pt as the sole source of phosphorus. We also demonstrated that a codon-adapted *ptxD* could be used as a dominant selection marker in *Saccharomyces cerevisiae* (Kanda *et al*., 2014).

Most wild-type microorganisms cannot assimilate Pt, or grow very poorly on a Pt medium. Introduction of *ptxD* into microorganisms confers the ability to grow on a Pt-medium, suggesting that *ptxD* could be used widely as a dominant selection marker of recombinants. Pt is a safe chemical and it costs far less than antibiotics. Furthermore, since Pt is virtually absent in nature, the expression of *ptxD* would not be advantageous for the survival of the recombinants outside of the Pt culture. An accidental escape of *ptxD*-recombinants would not create hazards for human society in contrast to that of recombinants carrying antibiotic resistance genes. The *ptxD* and Pt-based selection system would enable cost-effective, non-sterilized and selective cultivation of recombinants on a large scale with a lower environmental risk.

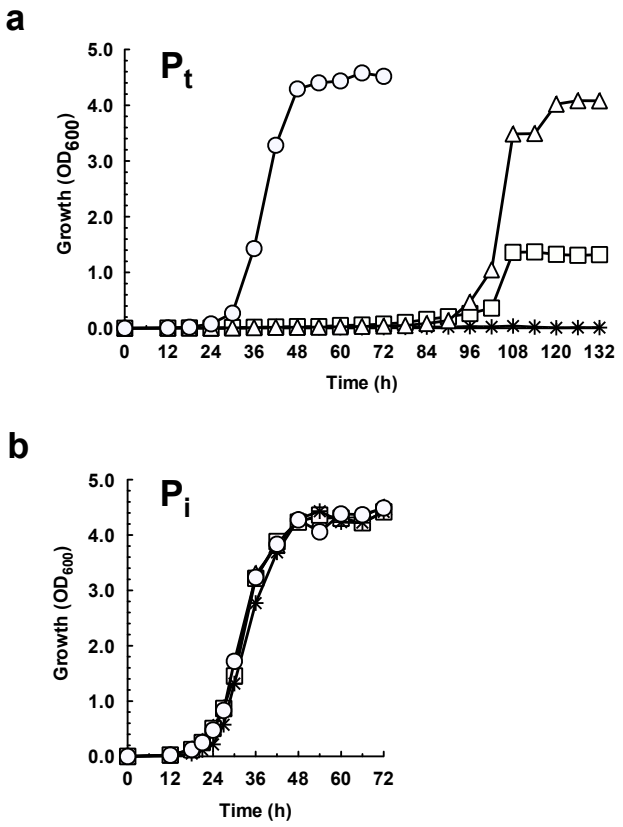

Fig. 4 Growth of *Sz. pombe* strains expressing PtxD from the genome-integrated single-copy gene on the Pt medium (a) and Pi medium (b). Circles, triangles, and squares indicate the growth of recombinants whose *ptxD* genes were driven by *nmt1, nmt41*, and *nmt81* promoters, respectively. Asterisks indicate the growth of a recombinant carrying only *nmt41*.

3.3 Pt fertilization

Pt shows higher solubility and lower reactivity with soil components compared with Pi (Morton, *et al*. 2005; Pasek, 2008). Thus, direct utilization of Pt as a fertilizer would be very beneficial for the recycling of the Pt waste. Pi, however, is the only chemical form of phosphorus that can be assimilated by plants. Expectedly, the wild type of *Arabidopsis thaliana* is unable to assimilate Pt and its growth is completely arrested when it is transferred to a Murashige and Skoog (MS) medium containing Pt instead of Pi (Fig. 5, left). To test whether *ptxD* enables plants to assimilate Pt, we introduced $ptxD_{R4506}$ under a CaMV 35S promoter into the *Arabidopsis* genome. Germinated seeds from transgenic lines (T2 seeds) were transferred to the Pt medium. The *Arabidopsis* recombinant could not grow on the Pt medium, but an *Arabidopsis* recombinant carrying $ptxD_{PS}$ could grow on the Pt medium (Fig. 5, right). The reason only $ptxD_{PS}$ could function in *Arabidopsis* is currently unclear.

During the preparation of a manuscript related to this result, López-Arredondo and Herrera-Estrella (2012, 2013) reported *ptxD*-transgenic plants that grew and germinated directly on a Pt medium. Furthermore, they carried out greenhouse growth competition experiments using both a *ptxD* recombinant tobacco plant and a grass weed (*Brachypodium distachyon*). Both the *ptxD* recombinant tobacco plant and the *B. distachyon* weed grew poorly in unfertilized nonsterile alkaline soil

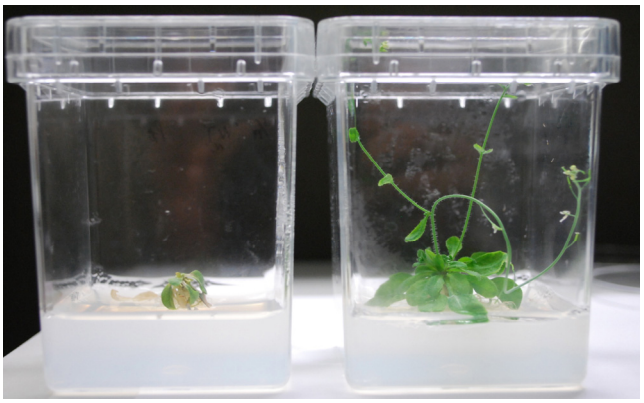

Fig. 5 Growth of a wild-type (left) and a *ptxD*-transgenic Arabidopsis plant (right) on MS medium containing 0.3 mM Pt instead of Pi (Pt medium).

obtained directly from an agricultural field in Mexico. Supplementation with Pi resulted in faster growth of the *B. distachyon* weed than the *ptxD*-recombinant tobacco plant. In contrast, fertilization with Pt resulted in limited growth of the *B. distachyon* weed and vigorous growth of the *ptxD*-recombinant tobacco plant. In addition to the inability of wild-type plants to assimilate Pt, Pt may also directly inhibit the germination of weeds by interfering with Pi-starvation signaling pathways in plants (Ticconi *et al*., 2001; Varadarajan, *et al.*, 2002). Therefore, cultivation of *ptxD*-recombinant plants while using Pt as a phosphorus fertilizer could serve as a weed control system in low-Pi soils.

In 2010, 81% of the global soybean crop was genetically modified (GM) with herbicide resistance genes (Murphy, 2012). A wide range of weeds can be controlled with the use of herbicides. The continual large-scale use of herbicides, however, increases environmental pollution and could lead to the emergence of super-weeds that have herbicide resistance. The US Environmental Protection Agency has classified Pt as a biopesticide whose use would not pose unreasonable risks of harm to human health or the environment. Pt has no carcinogenic potential after long-term human oral exposure (Mayton *et al*., 2008). We and another group (López-Arredondo & Herrera-Estrella, 2012, 2013) demonstrated that only *ptxD*-recombinant plants grew on a Pt medium as well as on soil supplemented with Pt. Introduction of *ptxD* to GM crops does not seem impracticable. Application of Pt fertilizer to GM crops could decrease the use of herbicides and reduce the related environmental risks. From the viewpoint of phosphorus security, strong expansion of agricultural production for bio-energy purposes would accelerate depletion of phosphorus resources. Use of *ptxD*-recombinant plants in bio-energy production could also contribute to the efficient utilization of Pt waste.

4. Conclusions

Phosphorus is a non-substitutable element essential for life. Since phosphorus is produced from phosphate rock that is a non-renewable and limited natural resource, efficient utilization of phosphorus resources is essential for the sustainable future of humanity. Pt is a phosphorus waste generated by the chemical and automotive industries. Reuse or recycling of Pt, however, is presently not cost-effective. We obtained stable and soluble PtxD to utilize Pt as a reducing reagent in an NADH regeneration system that could contribute to production of industrially important chemicals by the use of oxidoreductive enzymes. Pt could also become an alternative to antibiotics when used in a selection system for *ptxD*-recombinant microorganisms. We also demonstrated that Pt could be directly used as a fertilizer for *ptxD*-recombinant plants. These emerging environmental biotechnologies should contribute to the efficient utilization of Pt waste.

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