Immobilization of Baeyer–Villiger monooxygenase from acetone grown *Fusarium* sp.

Michio Takagi · Kotchakorn T.sriwong · Ayaka Masuda · Nozomi Kawaguchi · Shusuke Fukui · Lan Huang Le Viet · Dai-ichiro Kato · Takashi Kitayama · Mikio Fujii · Afifa Ayu Koesoema · Tomoko Matsuda

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Abstract

**Objective** A novel biocatalyst for Baeyer–Villiger oxidations is necessary for pharmaceutical and chemical industries, so this study aims to find a Baeyer–Villiger monooxygenase (BVMO) and to improve its stability by immobilization.

**Results** Acetone, the simplest ketone, was selected as the only carbon source for the screening of microorganisms with a BVMO. A eukaryote, *Fusarium* sp. NBRC 109816, with a BVMO (FBVMO), was isolated from a soil sample. FBVMO was overexpressed in *E. coli* and successfully immobilized by the organic–inorganic nanocrystal formation method. The immobilization improved the thermostability of FBVMO. Substrate specificity investigation revealed that both free and immobilized FBVMO were found to show catalytic activities not only for Baeyer–Villiger oxidation of ketones to esters but also for oxidation of sulfides to sulfoxides. Furthermore, a preparative scale reaction using immobilized FBVMO was successfully conducted.

**Conclusions** FBVMO was discovered from an environmental sample, overexpressed in *E. coli*, and

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immobilized by the organic–inorganic nanocrystal formation method. The immobilization successfully improved its thermostability.

**Keywords**  Baeyer–Villiger monooxygenase · *Fusarium* sp. NBRC 109816 · Immobilization · Thermostability

**Introduction**

The Baeyer–Villiger oxidation (BVO), the transformation of an acyclic ketone or cyclic ketone to the corresponding ester or lactone, respectively, is an important organic reaction for pharmaceutical and chemical industries. However, commonly used oxidants for BVO, such as *meta*-chloroperbenzoic acid (mCPBA) and trifluoroperacetic acid (TFPAA) (Know 1993), are potentially explosive. Moreover, the stoichiometric amount of reagent, a peroxyacid, is converted to a carboxylic acid, producing chemical waste. Therefore, an effort to use environmentally benign oxidants such as H₂O₂ and O₂ has been made (Bryliakov 2017; Liu et al. 2020). On the other hand, biocatalysts have been considered to be sustainable catalysts (Matsuda 2017; Dong et al. 2018; Sheldon and Woodley 2018; Biroll et al. 2019; Wu et al. 2020; Koesoema et al. 2020). For biocatalytic BVO, Baeyer–Villiger monooxygenase (BVMO) can use oxygen in the air as an oxidant, producing water as a byproduct (Fig. 1), so that the reactions are not explosive (Morii et al. 1999; Kyte et al. 2004; Rehdorf et al. 2007; Franceschini et al. 2012; Leipold et al. 2012, 2013; Fürst et al. 2017, 2019; Nguyen et al. 2017; Fordwour et al. 2018; Woo et al. 2018). Approximately a hundred of BVMOs (Fürst et al. 2019) such as *Acinetobacter* cyclohexanone monooxygenase (AcCHMO) (Donoghue et al. 1976; Chen et al. 1988; Bong et al. 2018), *Pseudomonas putida* 2,5-diketocamphane monooxygenase (2,5-DKCMO) (Cassimjee et al. 2014), and *Thermocrispum municipale* cyclohexanone monooxygenase (TmCHMO) (Delgove et al. 2019) have been used for biocatalytic BVO. However, comparing other kinds of biocatalysts such as lipases and carbonyl reductases, the available BVMOs are limited, so that there is a high demand for new enzyme discovery (Fürst et al. 2019).

While many BVMOs have been discovered by genome mining (Fürst et al. 2019), the potential of catalytic BVO activity found in the environments by function-driven screening is still fully underexplored recently (Rahm and Sih 1966; Donoghue et al. 1976; Willetts et al. 2012). Therefore, this study aims to find a unique BVMO from environments. Acetone, the simplest ketone, was used as the only carbon source, expecting that the simplest substrate is the best to apply for further development. Besides being the simplest, the three acetone metabolic pathways, including BVO, carboxylation to form acetoacetate, and terminal hydroxylation to form acetol (1-hydroxy-2-propanone), have been reported (Hausinger 2007). Therefore, acetone was used as the only carbon source for screening microorganisms with a BVMO in this study, resulting in the isolation of two species of *Fusarium*, eukaryote, with a BVMO from soil samples. The BVMO from one of them, *Fusarium* sp. NBRC 109816 (FBVMO), was overexpressed in *E. coli*.

Then, FBVMO was immobilized to improve stability since one of the major obstacles to the utilization of BVMO for organic synthesis is its low stability. So far, BVMOs have not been successfully immobilized due to their low stability, except in a few cases (Cassimjee et al. 2014; Delgove et al. 2019). A thermostable cyclohexanone monooxygenase from *Thermocrispum municipale* (TmCHMO) was co-immobilized on amino-functionalized agarose-based support with a glucose dehydrogenase (GDH) (Delgove et al. 2019). 2,5-Diketocamphane monooxygenase from *Pseudomonas putida* (2,5-DKCMO) was co-immobilized on controlled porosity glass (CPG) with two cofactor-reconverting enzymes (Cassimjee et al. 2014). Among enzyme immobilization methods (Liese and Hilterhaus 2013; Mohamad et al. 2015; Basso and Serban 2019; T. sriwong et al. 2021a), the protein-inorganic nanocrystal formation method is one of the simplest and most effective methods (Ge et al. 2012; Yin et al. 2015; Zhang et al. 2020). Lipase
(Zhang et al. 2020), peroxidases (Ge et al. 2012; Yu et al. 2015; Altinkaynak et al. 2016), alcohol dehydrogenases (López-Gallego and Yate 2015; Tsriwong et al. 2020), and aldehyde dehydrogenase (Tsriwong et al. 2021b) have been immobilized by this method, achieving the improvement in the activity and/or stability. However, no BVMO has been immobilized by this method, to the best of our knowledge. Therefore, immobilization of FBVMO by the method was conducted in this study, resulting in a significant improvement in thermostability. Substrate specificity investigation revealed that both free and immobilized FBVMO were found to show catalytic activities not only for BVO of ketones to esters but also for oxidation of sulfides to sulfoxides. Furthermore, a preparative scale reaction using immobilized FBVMO was successfully conducted.

Materials and methods

Reagents, materials, and apparatus

Materials written in the Supplementary Information were used.

Screening of microorganisms using acetone as the only carbon source

Environmental samples from soil, river, and ponds were collected, and diluted by 1.0–10⁵ times with sterilized water, and cultivated at 30 °C and 250 rpm in a liquid medium (Wiegent and De Bont 1980) at pH 7.0 consisting of K₂HPO₄ (1.55 g/L), KH₂PO₄ (0.97 g/L), NH₄Cl (2.0 g/L), MgCl₂·6H₂O (0.075 g/L), (NH₄)₂SO₄ (0.10 g/L), NaCl (0.39 g/L), FeSO₄·7H₂O (0.010 g/L), ZnSO₄·7H₂O (0.010 g/L), MnSO₄·5H₂O (0.010 g/L), KHCO₃ (0.50 g/L), and acetone (50–300 mM). Out of 300 samples, two microorganisms were able to grow with acetone as the only carbon source. They were identified as Fusarium sp. and Fusarium oxysporum Schltdl based on morphological tests (Supplementary Fig. 1) and ITS-5.8S rDNA sequencing. Fusarium sp. was deposited to the National Institute of Technology and Evaluation (Tokyo, Japan) as Fusarium sp. NBRC 109816.

Isolation of the gene

Among the known genomic sequences of various Fusarium species, F. oxysporum f. sp. conglutinans race 2 54008 (accession number AGNF01000703.1) was selected by searching a genome with the highest homology with one of the most studied BVMO, CHMO from Acinetobacter sp. NCIMB9871 (Donoghue et al. 1976). To determine the sequence of FBVMO, four sets of primers (Supplementary Table 1) were constructed based on the sequence of AGNF01000703.1. for PCR using the genetic DNA of Fusarium sp. NBRC 109816 as a template. To remove the intron, two sets of primers (Supplementary Table 1) were constructed for PCR using the genetic DNA of Fusarium sp. NBRC 109816 as a template. The two PCR products were inserted into pUC19 and transformed into E. coli DH5α. Another set of primers (Supplementary Table 1) was used for PCR with pUC19-FBVMO as a template to obtain the FBVMO gene (Supplementary Figs. 2 and 3) to construct pET-21b(+) -FBVMO, which was transformed into E. coli BL21(DE3).

Overexpression

A single colony of the recombinant cells, BL21(DE3)-pET-21b (+)-FBVMO, was inoculated in LB medium (4.0 mL) with carbenicillin (125 μg/mL) at 250 rpm at 37 °C to an optical density at 600 nm (OD₆₀₀) reached 0.8–1.0. The pre-cultured cells (2.5 mL) were transferred into LB medium (250 mL) with carbenicillin (125 μg/mL) and cultivated at 250 rpm at 37 °C until OD₆₀₀ reached 0.5–0.6. Then, IPTG (0.2 mM) was added and cultivated at 250 rpm at 18 °C for 18 h. The cells were harvested by centrifugation at 10,000×g for 5 min at 4 °C, washed with 0.8% NaCl, and suspended in a sodium phosphate buffer (30 mM, pH 7.4, 20 mM) containing imidazole (5 mM), PMSF (1 mM), and DTT (1 mM). The mixture was sonicated at 100 W for 20 min at 0 °C and centrifuged at 15,000×g for 30 min at 4 °C, and the supernatant (30 mL) was used as a cell-free extract for further study.

Purification

The cell-free extract (30 mL) was loaded onto a HisTrap™ FF crude equilibrated with a sodium
phosphate buffer (pH 7.4, 20 mM) with PMSF (0.2 mM), and DTT (0.2 mM). The bound protein was eluted by the buffers with 5 mM imidazole (20 mL), 10 mM imidazole (10 mL), 20 mM imidazole (10 mL), 30 mM imidazole (5 mL), and the FBVMO was eluted by the buffer with 70 mM imidazole (5 mL) with PMSF (0.2 mM), and DTT (0.2 mM). The protein was concentrated by ultrafiltration using Amicon Ultra-4 10-K MWCO and used for further study. The protein concentration was measured by the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as a standard. SDS-PAGE was conducted on 10% polyacrylamide gel (Supplementary Fig. 4). The purification steps are summarized in Supplementary Table 2.

Activity measurement of the free enzyme

Cyclohexanone solution (10 μL, 0.1 M solution with 10% diethylene glycol (final concentration 1 mM)) and sodium phosphate buffer (968 μL, pH 7.4, 50 mM) were mixed, and then NADPH solution (12 μL, 10 mg/mL) and purified enzyme (10 μL, 1.6–2.0 U/mL) were added. Initial velocity was determined by following NADPH consumption at 340 nm for 3 min. Activity assays were done at 25 °C in duplicate in a 1.0 mL scale. One unit of enzyme is defined as μmol of NADPH produced in 1 min under the above conditions.

Immobilization

The purified FBVMO was immobilized with a similar method to our previous study (T.sriwong et al. 2020, 2021b). The phosphate-buffer saline (PBS) was prepared by dissolving NaCl (0.80 g), KCl (0.020 g), Na₂HPO₄ (0.142 g), and KH₂PO₄ (0.024 g) in distilled water (100 mL) and adjusting pH to 7.4 with HCl (aq). The metal solutions (50 mM (final concentration 5 mM), 100 mM (final concentration 10 mM), 200 mM (final concentration 20 mM), and 400 mM (final concentration 40 mM)) were prepared by dissolving ZnSO₄, MgSO₄, MnSO₄, CuSO₄, FeSO₄, NiCl₂, CoCl₂, or CaCl₂ in distilled water. PBS (350 μL), the purified FBVMO (100 μL, 1 U/mL in sodium phosphate buffer (50 mM, pH 7.4)), and metal solution (50 μL) were mixed by gently turning it upside down, and incubated at 4 °C for 8 h. The solution was centrifuged at 4 °C and 5000×g for 5 min. The precipitant was suspended in PBS and centrifuged at 4 °C and 5000×g for 5 min twice, and suspended in PBS, giving immobilized FBVMO nanocrystal solution (50 μL). The residual protein concentration in the supernatant (protein leakage) was determined by the Bradford method (Bradford 1976), and the immobilization yield was calculated using Eq. (1).

\[
\text{Immobilization yield} \ (\%) = \frac{[\text{Protein}]_I - [\text{Protein}]_R}{[\text{Protein}]_I} \times 100
\]

\([\text{Protein}]_I\) = Initial protein concentration (mg/mL).

\([\text{Protein}]_R\) = Concentration of protein in the supernatant after the FBVMO nanocrystal formation and centrifugation (mg/mL).

Scanning electron microscope (SEM) analysis of immobilized FBVMO nanocrystal

FBVMO nanocrystal was analyzed by SEM with a similar method reported in our previous study (T.sriwong et al. 2020, 2021b). The FBVMO nanocrystal was washed with distilled water several times before being dried at room temperature (Supplementary Fig. 5).

Activity measurement of the immobilized enzyme

Cyclohexanone solution (10 μL, 0.1 M solution with 10% diethylene glycol (final concentration 1 mM)) and sodium phosphate buffer (928 μL, pH 7.4, 50 mM) were mixed, and then NADPH solution (12 μL, 10 mg/mL), and immobilized FBVMO solution (50 μL) were added. Initial velocity was determined by following NADPH consumption at 340 nm for 3 min. Activity assays were done at 40 °C in duplicate in a 1.0 mL scale.

Characterization of free and immobilized FBVMO

Effects of pH and on their activities were investigated at 25 °C using 50 mM MES-NaOH buffer (pH 5, 6), 50 mM sodium phosphate buffer (pH 7, 7.5, 8), 50 mM Tris–HCl buffer (pH 8, 8.5, 9), or 50 mM Gly-NaOH buffer (pH 9, 10) with the methods described above. For pH 8 and 9, the averages of the activities in
the two buffers are shown. Effects of temperature on their activity were investigated using sodium phosphate buffer (50 mM, pH 8.0) with the methods described above. Thermostabilities of free and immobilized FBVMO were investigated by incubating at 40°C and collecting the portion of the enzymes at 0, 5, 10, 20, 30, 60, 120, and 300 min for activity measurement described above.

Preparative-scale oxidation of cyclohexanone by the immobilized FBVMO

FBVMO nanocrystal solution (1 mL) prepared from 1 U of free FBVMO, heat-treated Thermoplasma acidophilum glucose dehydrogenase solution (1 mL, 1.95 U) prepared as reported previously (Are et al. 2021), cyclohexanone solution dissolving 100 mg of cyclohexanone in 1 mL of an aqueous solution containing 10% of diethylene glycol, NADPH (50 mg), glucose solution (1 mL of 1 M solution), and sodium phosphate buffer (17 mL, 50 mM, pH 8.0) were mixed and incubated at 40°C at 150 rpm for 3 days. The reaction was repeated 5 times to convert 500 mg (5.09 mmol) of cyclohexanone in total. The reaction mixtures were combined, and the product was extracted with diethyl ether (25 mL), dried over MgSO₄, evaporated under reduced pressure, purified by silica gel column chromatography (hexane: ethyl acetate = 3:1), and characterized by ¹H-NMR analysis using CDCl₃ as a solvent. The ¹H-NMR spectrum was in agreement with that in literature (Omura et al. 2009). Yield 27% (157 mg, 1.37 mmol). ¹H-NMR (400 MHz, CDCl₃; -δ 1.65–1.87 (6H, m), 2.55–2.65 (2H, m), 4.19 (2H, t, J = 4.7 Hz) (Supplementary Fig. 6).

Result and discussion

Screening, overexpression, and purification of FBVMO

Environmental samples from soil, river, and ponds were collected and cultivated using acetone (50–300 mM) as the only carbon source. Out of 300 samples, two microorganisms were able to grow with acetone as the only carbon. They were identified as Fusarium sp. and Fusarium oxysporum Schltldl based on morphological tests (Supplementary Fig. 1) and ITS-5.8S rDNA sequencing. Encouraged by the literature search showing the whole cell catalyzed BVO of 2-methylcyclohexanone by Fusarium sp. AP-2 (Kawamoto et al. 2008) and the whole-cell catalyzed BVO of alkyl-substituted hexanones by Fusarium oxysporum and Fusarium avenaceum (Ratu et al. 2009), we examined the presence of BVMO in the newly isolated Fusarium sp. and F. oxysporum Schltldl. Cyclohexanone was used as a substrate for the whole-cell reactions. e-Caprolactone was successfully obtained in both reactions, suggesting the presence of a BVMO in both species. Fusarium sp. has higher activity than F. oxysporum Schltldl, so that Fusarium sp. was deposited to the National Institute of Technology and Evaluation (Tokyo, Japan) as Fusarium sp. NBRC 109816, and used for further study.

For the use as an efficient biocatalyst, it is necessary to express BVMO from Fusarium sp. NBRC 109816 (FBVMO), heterogeneously. Therefore, the gene encoding FBVMO was identified, amplified, and cloned in an expression vector, pET-21b (+), after the removal of the intron, leading to pET-21b (+)-FBVMO. The vector was transformed into E. coli BL21(DE3). His-targeted FBVMO was induced by IPTG and purified by Ni affinity chromatography by 17.4 fold in 41% yield (Supplementary Table 2). As shown in the SDS-PAGE (Supplementary Fig. 4), the size of a single band around 64 kDa in the purified enzyme lane is in accordance with the expected size of 64 kDa. The DNA sequence and amino acid sequence are shown in Supplementary Figs. 2 and 3, respectively. In the deduced amino acid sequences, type I BVMO fingerprint FxGxxxHTxxW[P/D] (Fraaije et al. 2002; Rebehmed et al. 2013) and [A/G]xWxxxxxF/YP[G/M]xxxD (Riebel et al. 2012) and two Rossmann fold domains with a GxGxx[G/A] motif were found.

Immobilization of FBVMO and morphology study

To improve the stability, FBVMO was immobilized by the organic–inorganic nanocrystal formation method according to our previous report (T.sriwong et al. 2020, 2021b). The purified FBVMO was mixed with a metal solution to give a catalytically active protein–inorganic nanocrystal. The kind and the concentration of the metal ion were optimized by investigating 5 mM, 10 mM, 20 mM, and 40 mM of Zn²⁺, Mg²⁺, Mn²⁺, Cu²⁺, Fe³⁺, Ni²⁺, Co²⁺, and Ca²⁺. The activity of these nanocrystals toward the
BVO of cyclohexanone is shown in Fig. 2. Nanocrystal formed using 10 mM of Ca\(^{2+}\) showed the best activity among those tested, while high activity was observed in the nanocrystals from most of the ions except Mn\(^{2+}\). The low activity of the Mn\(^{2+}\) nanocrystal may be related to the possibly low activity of free FBVMO in the presence of Mn\(^{2+}\) as BVOs from *Rhodococcus aetherivorans* (RaBVMO) and *Amycolatopsis methanolica* (AmBVMO) were significantly inhibited by 2.5 mM of Mn\(^{2+}\) (Wei et al. 2021).

Next, the stability of the nanocrystals formed under the optimum concentrations for each metal was investigated. The nanocrystals used for the activity assay were recovered by centrifugation as a precipitate, re-suspended in a buffer, and used for the activity assays (Fig. 3a). To quantify the leakage of the protein from the nanocrystal, the protein concentration in the supernatant was measured. The amount of protein retained in the nanocrystal (immobilization yield) is shown in Fig. 3b. It was found that the FBVMO nanocrystal could be recycled up to 8 times with retained activity. The remaining activity after recycling largely depends on the kind of metal ions used for immobilization. The activity of the 10 mM Ca\(^{2+}\) nanocrystal, showing the best activity for the 1st usage, decreased dramatically by recycling. Only 23% of the protein was retained, and the activity was completely lost for the 8th usage. The reasons for activity decrease could be due to both enzyme leakage and enzyme deactivation within the nanocrystal. Ca\(^{2+}\) has affinities with protein in some cases, but its bonding strength depends on the proteins (Franke et al. 2010). FBVMO might have a weak affinity with Ca\(^{2+}\).
so that Ca$^{2+}$ nanocrystal recycling was not efficient. On the other hand, while the activity of 20 mM Cu$^{2+}$ nanocrystal was moderate for the 1st usage (52%), it did not decrease significantly for the 8th usage (42%), so as for the immobilization yield being 69% for the 1st usage and 53% for the 8th usage. Therefore, the best metal ion and its optimum concentration were determined to be Cu$^{2+}$ and 20 mM. The difference between metals can be explained by the affinity of transition metal ions such as Zn$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, and Co$^{2+}$ to histidine and cysteine of enzymes (Block et al. 2009). Cu$^{2+}$ has the highest affinity to His-tag compared with others (Chaga 2001; Winzerling et al. 1992). The high affinity between Cu$^{2+}$ and his-tag, histidine, or cysteine on FBVMO could be the reason why Cu$^{2+}$ was the best metal ion for FBVMO immobilization.

The morphology study of FBVMO-Cu$^{2+}$ nanocrystal was conducted by SEM analysis (Supplementary Fig. 5). We found that the FBVMO-Cu$^{2+}$ nanocrystal forms a porous structure (Supplementary Fig. 5b), which could not be seen in the control copper (II) phosphate crystal without the enzyme (Supplementary Fig. 5a). The high porosity of the sponge-like structure may cause the high activity of the FBVMO-Cu$^{2+}$ nanocrystal.

Characterization of free FBVMO and FBVMO-Cu$^{2+}$ nanocrystal

First, the effect of pH on the activity of the free and immobilized FBVMO (FBVMO-Cu$^{2+}$ nanocrystal) toward the oxidation of cyclohexanone was investigated. As shown in Fig. 4a, similar results were obtained for the free and immobilized enzyme, showing the highest activity around pH 8.0–8.5. The effect of immobilization on pH profile depends on the kind of enzymes. Relative activities under acidic conditions for the Geotrichum candidum acetophenone reductase—Co$_3$(PO$_4$)$_2$ nanocrystal (T.sriwong et al. 2020) and Geobacillus stearothermophilus alcohol dehydrogenase—Co$_3$(PO$_4$)$_2$ sponges (López-Gallego and Yate 2015) for reductions were better than those for the corresponding free enzymes, while optimum pH for the bovine milk lactoperoxidase was changed from pH 6 for the free enzyme to pH 8 for the enzyme—Cu$_3$(PO$_4$)$_2$ hybrid nanoflowers (Altinkaynak et al. 2016). For G. candidum aldehyde dehydrogenase, there were no significant differences in pH profile for oxidation between the Mn$_3$(PO$_4$)$_2$ nanocrystal and free enzyme (unpublished data). Next, the effect of temperature on the activity of the free and immobilized FBVMO toward the oxidation of
cyclohexanone was investigated. As shown in Fig. 4b, the optimum temperature for the free and immobilized FBVMO were 30°C and 40°C, respectively. The activity at 40°C increased significantly by immobilization. The free enzyme activity at 40°C was 1.27 nmol/min/mg protein, while the immobilized enzyme activity at 40°C was 15.3 nmol/min/mg protein. The immobilization improved the activity at 40°C by 12 times. This phenomenon at 40°C might be explained by a favorable and/or stable enzyme conformation fixed by the immobilization as seen in the case of the Cu₃(PO₄)₂—papain nanoflower (Yu et al. 2018) and lipase immobilized on hydrophobic support (Mateo et al. 2007). Then, the thermostability of the free and immobilized FBVMO was investigated. As shown in Fig. 4c, the free FBVMO had only 18% of the remaining activity after incubation for 5 min at 40°C, while >90% of the activity of the immobilized FBVMO was retained after 5 h at 40°C. The thermostability of the FBVMO was significantly improved by immobilization. The nanocrystal formation method was proven as a promising approach for BVMOs immobilization. The confinement of enzymes in the nanocrystal may have fixed the unstable residue of the protein, as support materials act as a shell to protect the enzyme from harsh environments, including high temperatures in general immobilization methods (Hu et al. 2018; T.sriwong et al. 2021b). The successful result of immobilization of FBVMO, improving the thermostability, is remarkable since there are only a few examples (Cassimjee et al. 2014; Delgove et al. 2019) for the immobilization of BVMOs due to the low stability of BVMOs.

| Entry | Substrate | Relative activity (%) |
|-------|-----------|----------------------|
|       | Free FBVMO | Immobilized FBVMO |
| 1     | Cyclohexanone | 100 | 100 |
| 2     | Cycloheptanone | 16 | 18 |
| 3     | Cyclooctanone | 5.1 | 1.8 |
| 4     | 2-Pentylocyclopentan-1-one | 83 | 18 |
| 5     | Pentan-2-one | 5.1 | 4.6 |
| 6     | Heptan-2-one | 2.7 | 2.8 |
| 7     | 1-Cyclopropylethan-1-one | 3.0 | 2.2 |
| 8     | 4-Phenylbutan-2-one | 83 | 18 |
| 9     | Dimethyl sulfide | 2.8 | 3.2 |
| 10    | Diethyl sulfide | 14 | 16 |
| 11    | Dipropyl sulfide | 3.2 | 3.0 |
| 12    | Methyl phenyl sulfide | 29 | 18 |
| 13    | Diphenyl sulfide | 3.1 | 0.6 |

The activities were determined under the standard assay conditions at pH 8.0 in 50 mM sodium phosphate buffer at 25°C for the assay with the free FBVMO and 40°C for the assay with the immobilized FBVMO. The activities of free or immobilized FBVMO toward cyclohexanone were set to 100%, respectively.
Substrate specificities of the free and immobilized FBVMO were investigated using varieties of ketones and sulfides. Both of the free and immobilized FBVMO successfully oxidized a wide range of ketones (Table 1, Entries 1–8), and sulfides (Table 1, Entries 9–13). In general, it was clear that the broad substrate specificity of the free FBVMO was greatly retained after immobilization. However, the difference in the relative activities between the free and immobilized FBVMO was also seen for the bulky substrates. The free enzyme exhibited about 3–5 times higher activities for cyclooctanone, 2-pentylcyclopentan-1-one, 4-phenylbutan-2-one, and diphenyl sulfide (Table 1, Entries 3, 4, 8, 13). The change in the substrate preference upon immobilization might be caused by the subtle structural change of the enzyme by the immobilization or by the restriction in the substrate transfer to the active site of the immobilized enzyme due to the diminished flexibility of the residue at the entrance caused by the metal shell. The change in the substrate preference by the immobilization is an intriguing phenomenon since it was not observed for the case of other enzymes such as G. candidum aldehyde dehydrogenase (T.sriwong et al. 2021b).

At last, a preparative scale reaction by the immobilized FBVMO was conducted using cyclohexanone as a substrate. The reaction proceeded smoothly, and the product was successfully isolated, purified, and identified by the $^1$H NMR (Supplementary Fig. 6).

Conclusion

FBVMO was discovered from an environmental sample by the screening of microorganisms using acetone as the only carbon source. FBVMO was successfully immobilized by the organic–inorganic nanocrystal formation method, resulting in an improvement in thermostability. Both free and immobilized enzymes were characterized and found to be versatile for both BVO and oxidation of sulfide. At last, preparative scale reaction of BVO of cyclohexanone was successfully conducted.

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Supporting Information  Table 1—Primers used for PCR to construct BL21(DE3)-pET-21b(+)−FBVMO 2.
Table 2—Summary of purification steps of FBVMO overexpressed in E. coli 2.
Figure 1—Microscopic observation images of Fusarium sp. NBRC 109816 2.
Figure 2—DNA sequence of FBVMO without intron 3.
Figure 3—Amino acid sequence of FBVMO 3.
Figure 4—SDS–PAGE of FBVMO on 10% polyacrylamide gel 4.
Figure 5—SEM images of crystals formed by mixing PBS and CuSO₄ solution (a) without and (b) with FBVMO 4.
Figure 6—$^1$H NMR spectrum of $\varepsilon$-caprolactone obtained by oxidation of cyclohexanone with immobilized FBVMO.

Author contributions  MT and KT contributed equally to this work.

Declarations

Conflict of interest  The authors declare that they have no conflict of interest.

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