Enzymatic Cleanup of Formaldehyde in Aqueous Solutions

Zhenyu Zhai[ab], Yunjie Li[b], Zhilei Li[b], Jingting Wang[b], Yuan Li[b], and Yi-Heng P. Job Zhang[abc*]

[a]University of Chinese Academy of Sciences, 19 A Yuquan Road, Shijingshan District, Beijing 100049, China

[b]In vitro Synthetic Biology Center, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, 32 West 7th Avenue, Tianjin 300308, China

[c]Key Laboratory of Engineering Biology for Low-Carbon Manufacturing, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, 32 West 7th Avenue, Tianjin 300308, China

[*]Email: zhang_xw@tib.cas.cn

Abstract

Numerous methods have been developed to address gaseous formaldehyde pollution, but most of them cannot be applied directly to eliminate the pollution of formaldehyde in aqueous solutions. Formaldehyde in aqueous solutions can be leached from formaldehyde-containing solid materials (e.g., food, wood, clothes, resins) and absorbed from gaseous formaldehyde by water. Here we developed an enzymatic cleanup technique — the reconstitution of an enzyme cocktail consisting of three coenzyme-free oxidoreductases (i.e., formaldehyde dismutase, methanol oxidase, and formate oxidase) and catalase for the complete oxidation of formaldehyde. This enzyme cocktail catalyzed the reaction of formaldehyde and dissolved dioxygen into carbon dioxide (CO_2) and water, which was demonstrated by the stable isotope tracer technique. Significant levels of formaldehyde were detected from aqueous solutions leached from the squid, pomfret, fabric, and curtain in the market. When this enzyme cocktail was applied to treat the leachates of contaminated samples above, formaldehyde was eliminated with degradation ratios of up to 100%. This enzymatic cleanup technique, featuring excellent biosafety (for example, degradable catalysts and non-immunogenicity), independence of light, high degradation ratios, and no special equipment required, could be widely used to treat contaminated food, drinking water, and formaldehyde-containing leachate.

Keywords: Biocatalysis, Environmental chemistry, Enzyme cocktails, Formaldehyde, Biodegradation

Introduction

Formaldehyde, a feedstock chemical which is synthesized from methanol in industry and produced endogenously by organisms, is ubiquitous.[1] This volatile organic compound is widely used in chemical, pharmaceutical, textile, and wood industries.[2] However, formaldehyde poses a severe risk to human health. The International Agency for Research on Cancer has classified formaldehyde as a Group 1 carcinogen.[3] Inhalation is the primary route of formaldehyde exposure for human. Formaldehyde can induce coughing and rhinitis with a concentration as low as 0.1 ppm.[4] In addition, formaldehyde has strong neurotoxicity and can induce central-nervous-system disorders, such as headaches, sleep disturbances, and depression.[5] What's worse, it can cause DNA damage, increasing the risk of cellular transformation to cancer.[6]

The ubiquity of volatile formaldehyde has caused gaseous pollution concern of living environments, such as residences, schools, vehicles, hospitals, and shopping malls.[7] The predominant methods to eliminate gaseous formaldehyde can be categorized into three main types: adsorption, catalytic oxidation, and biological removal (**Table S1**). Adsorption is a widely-implemented method for the removal of gaseous formaldehyde. It typically employs large surface-area porous materials, such as activated carbon, ceramic honeycomb, and hydroxyapatite layer. [8] However, this method does not degrade formaldehyde but sequesters it within the adsorbents, resulting in the possible secondary pollution. Chemical oxidation of formaldehyde can be conducted by noble-metal catalysts,[9] plasma,[10] and photocatalysts.[11] These chemical methods suffer from the consumption of special chemicals and energy, poor biosafety, low efficacy, and/or

special equipment required. (3) Biological removal is of interests because some plants and microorganisms can tolerate the toxicity of formaldehyde by assimilating or decomposing it through complex metabolism.[12] However, organisms require specific environmental conditions, special equipment such as the trickling biofilter bed, and nutrients to support their lives.[13] In addition, the removal rates of most organisms are too slow to efficiently eliminate formaldehyde within a short time.

The pollution of formaldehyde in aqueous solutions is ubiquitous, too, because it is leached from formaldehyde-containing solid materials (e.g., resins, clothes, seafood, wood), is absorbed from gaseous formaldehyde by water, and is released from formaldehyde-saturated solid absorbents.[14] In the chemical industry, formaldehyde is used to make phenolic resins, urea-formaldehyde (UF), urea–formaldehyde foam insulation (UFFI), melamine-formaldehyde (MF) resins, and so on.[1] In the food industry, some foods could generate formaldehyde during their storage and transportation.[15] In addition, formaldehyde may be illegally added to prevent food from spoilage.[16] Formaldehyde is also used in textile dying and anti-wrinkle treatments.[17] Therefore, it is needed to address the pollution of formaldehyde in aqueous solutions but current methods aiming at gaseous pollution cannot be applied directly.

Enzymatic cleanup technology is receiving more interests as a cost-effective solution to the remediation of toxic pollutants in water,[18] especially when the production cost of recombinant enzymes have been decreased greatly to a range of $10-30/kg of enzyme dry weight.[19] Here we designed and developed a novel enzyme cocktail to oxidize

formaldehyde into CO_2 and water (**Figure 1A and 1B**). This environment-friendly and biosafety technique could have wide applications in the cleanup of formaldehyde in aqueous solutions.

Results and Discussion

Design of the *in vitro* Enzymatic Pathway

The straightforward enzymatic method to degrade formaldehyde could be based on formaldehyde dehydrogenase (EC 1.2.1.46), glutathione-dependent formaldehyde dehydrogenase (EC 1.2.1.1), and aldehyde oxidase (EC 1.2.3.1) (**Table 1**). These two dehydrogenases consume costly NAD^+ so that they cannot be widely used for addressing environmental pollution.[20] Aldehyde oxidase is a molybdo-flavoenzyme that strictly relies on the molybdenum cofactor, [2Fe-2S] cluster, and FAD.[21] Because this enzyme's catalytic activity for formaldehyde was not characterized and it was different to be expressed efficiently in *Escherichia coli*, it cannot be widely used for pollution control, too.

Formaldehyde dismutase (FDM, EC 1.2.98.1), a less-known oxidordeuctase containing tightly bound NAD(H) as the prosthetic group, catalyzes the dismutation reaction of two molecules of formaldehyde into formate and methanol.[22] Compared with the well-known formaldehyde dehydrogenase, the reaction catalyzed by FDM is more thermodynamically favorable and FDMs have very high specific activities of up to thousands of units per milligram according to the records in BRENDA enzyme database

(**Table 1**). Furthermore, recombinant FDM that can be expressed well in *E. coli* is less costly and lasts longer because it does not require exogenously-added NAD(H).[23]

To completely oxidize formaldehyde, we designed a novel *in vitro* enzymatic pathway consisting of FDM, methanol oxidase (MOX), formate oxidase (FOX), and catalase (CAT) (**Figure 1A**). The dual products of formaldehyde catalyzed by FDM were formate and methanol. Subsequently, formate was oxidized into CO_2 and hydrogen peroxide (H_2O_2) in the presence of dioxygen, catalyzed by a flavoenzyme, FOX. Also, we chose another flavoenzyme, MOX to oxidize methanol into formaldehyde and H_2O_2. CAT was supplemented to decompose H_2O_2 into water and dioxygen, mitigating the toxicity of H_2O_2. Through the iterative cycle of the four-step process, all formaldehyde can be degraded into CO_2 and water finally. This artificial enzymatic pathway has several appealing features: (1) complete oxidation of formaldehyde with all irreversible enzymatic reactions, (2) mild reaction conditions, (3) no costly exogenous-coenzymes required, (4) neither energy (e.g., light, electricity, heat) nor special equipment required.

Mining and Characterization of Enzymes

FDM, MOX, and FOX from different organisms were heterologously expressed in *E. coli* and CAT from bovine liver was purchased (**Figure 1B**). Molecular chaperones, GroEL and GroES, were co-expressed with FDM from *Pseudomonas putida* to enhance the soluble expression. SDS-PAGE and absorption spectra showed that the soluble FDM was successfully expressed and purified (**Figure S1**).

To mine alcohol oxidase that catalyze the oxidation of methanol, we collated all alcohol oxidase (EC 1.1.3.13) entries recorded in UniProt database and constructed a phylogenetic tree after removing sequences with more than 90% identity. The majority of alcohol oxidases exhibited high sequence similarity to those from methylotrophic yeasts, such as *Komagataella phaffii* (**Figure S2**). We selected ten alcohol oxidases from class A in the phylogenetic tree for further study and found that the one from *Phanerochaete chrysosporium* can be expressed solubly in *E. coli* and possessed desired catalytic activity (**Figure S3**).

FOX is a unique enzyme containing unusual autocatalytic-formation 8-formyl-FAD as the prosthetic group.[24] In BRENDA enzyme database, it is listed under the aldehyde oxidase (EC 1.2.3.1) entry but possesses the distinctive catalytic activity of formate oxidation.[26] Well-characterized FOXs were from *Debaryomyces vanrijiae* and *Aspergillus oryzae*.[27] We selected FOX from *A. oryzae* for following study and it was expressed well in *E. coli* (**Figure S4**).

The relative activities of FDM, MOX, and FOX from 20 to 60 °C showed that all three mesophilic enzymes demonstrated optimal catalytic activities between 20-30 °C (**Figure 1C**). Subsequently, the optimal pHs of FDM, MOX, and FOX from 2 to 12 were studied (**Figure 1D**). FDM had an optimal pH of 7 and retained significant activities at a broad pH spectrum. MOX was an alkaliphilic enzyme with an optimal pH of 9, and lost all activity below pH 5. FOX was a strictly acidophilic enzyme with an optimal pH of 5, exhibiting 1.11% of its maximum activity at pH 7. Although there was a large difference

in the optimal pHs for the three enzymes, all of them had catalytic activities at pH 5-8 and the trade-off pH range of the three-enzyme cocktail was determined to be around pH 7 (**Figure 1E**). Information of the enzymes that made up the cocktail and their specific activities at pH 7 were summarized at **Table 2**. The specific activity of recombinant FDM was 1.42 U/mg at 25 °C and pH 7, and was increased to 15.86 U/mg in the presence of 5 mM Mg^{2+}. The specific activity of recombinant MOX was 1.35 U/mg at 25 °C and pH 7. The specific activity of recombinant FOX was determined to be 0.28 U/mg at 25 °C and pH 7. K^+ should be avoided during the purification of FOX and imidazole should be removed as clean as possible through ultrafiltration, because both imidazole and K^+ had significant inhibitions to FOX (**Figure S5**).

Proof-of-Concept Experiments: ^{13}C Tracing and Time Profiles

The complete oxidation of formaldehyde was demonstrated by the stable isotope tracer technique. ^{13}C-labeled formaldehyde was used as the substrate and the enzymatic products was analyzed by gas chromatography-mass spectrometry (GC-MS). The abundance of $^{13}CO_2$ relative to normal $^{12}CO_2$ in the products was 5.04% (**Figure 2A**), significantly higher than the abundance of 1.17% in the ambient air (**Figure S6**). These results demonstrated that ^{13}C-labeled formaldehyde was successfully oxidated into $^{13}CO_2$ catalyzed by the four-enzyme cocktail.

Subsequently, we determined the time profiles of the formaldehyde degradation by high performance liquid chromatography (HPLC). In case of the four-enzyme cocktail, there was a rapid decrease in the concentration of formaldehyde within the initial 10 minutes

of the reaction, followed by the slight increases in the concentrations of formate and methanol. As the reaction proceeded, the concentration of formaldehyde continued to decline and the concentrations of methanol and formate gradually decreased. Lastly, the concentrations of formaldehyde, methanol and formate reduced to zero (**Figure 2B**). As for the three-enzyme cocktail of FDM, FOX, and CAT, formate was effectively converted to CO_2, but methanol accumulated in the reaction system (**Figure 2C**, **Figure S7**). Interestingly, we observed a very slow decrease in methanol, implying that FOX could catalyze the oxidation of methanol due to its substrate promiscuity. In case of only FDM, the trend of formaldehyde degradation was consistence with the formations of methanol and formate (**Figure 2D**). Clearly, the complete oxidation of formaldehyde into CO_2 and water was implemented by the concerted action of the four enzymes.

Degradation Ratios of the Enzyme Cocktail

The concentration ratio of FDM, MOX, FOX, and CAT was optimized to be 1:1:6:5 in the enzyme cocktail based on their specific activities. We tested three concentrations of the enzyme cocktail for the degradation of 10, 100, and 1000 mg/L formaldehyde. At a low enzyme loading (0.01 mg/mL FDM, 0.01 mg/mL MOX, 0.06 mg/mL FOX, and 0.05 mg/mL CAT), the formaldehyde degradation ratios for the concentrations of 10, 100, and 1000 mg/L reached 93.8%, 98.6%, and 89.4%, respectively, after four hours (**Figure 3A**). When the enzyme loading was increased by 10-fold, the degradation ratios reached more than 90% after one hour and 100% after four hours regardless formaldehyde concentrations (**Figure 3B**). At the highest enzyme loading (increased by 5-fold further), one-hour reaction was sufficient to reach degradation ratios of 95% for

the formaldehyde concentrations from 10 to 1000 mg/L, and the degradation ratios reached 100% after two or four hours (**Figure 3C**). These results demonstrated that the enzyme cocktail was capable of degrading formaldehyde of high (1000 mg/L) and low (10 mg/L) concentrations with the degradation ratios of up to 100% within four hours.

Applications in the Leachates of Everyday Samples

The enzyme cocktail can be applied to everyday samples, such as seafood and textiles (**Figure 4A**). Formaldehyde was detected in two aquatic products (i.e., squid and pomfret) and two textile products (i.e., fabric and curtain) among the seven kinds of samples purchased from the online market (**Figure 4B**), suggesting that formaldehyde pollution was almost everywhere. Significant formaldehyde was not detected from the latex paint here. This could be possibly attributed to the presence of some constituents in the paint that can inhibit the colorimetric reaction of the acetylacetone assay.

The concentrations of formaldehyde in the leachates of the four formaldehyde-containing samples were approximately one mg/L (**Figure 4C**). Formaldehyde in the squid and pomfret might be added by the merchants to prevent spoilage or spontaneously generated during their storage and transportation. Formaldehyde in the fabric and curtain came from various industrial agents used in textile production. As for the curtain, 4.4 g of it was cut out to extract the formaldehyde in 1.5 L of water. The formaldehyde concentration of the leachate was 1.02 mg/L, which meant the formaldehyde content of the curtain was as high as 0.35 mg/g. This alarmingly-high

content of formaldehyde likely had a significant health hazard to humans without special treatment.

The enzyme cocktail (i.e., 0.1 mg/mL FDM, 0.1 mg/mL MOX, 0.6 mg/mL FOX, and 0.5 mg/mL CAT) was added to the leachate of formaldehyde-containing samples. After four-hour reaction, formaldehyde concentrations of the pomfret and fabric were undetectable (below 0.01 mg/L), while the formaldehyde concentrations of the squid and curtain dropped to 0.02 and 0.10 mg/L, with the degradation ratios of 98.2% and 90.5%, respectively (**Figure 4C**). These results demonstrated that the enzyme cocktail can effectively degrade formaldehyde in the leachate of seafood and textiles, implying its great potential to eliminate formaldehyde pollution in aqueous solutions.

Environmental Implications and Outlooks

To clean up ubiquitous pollution of formaldehyde in aqueous solutions, such as contaminated food that was illegally-added formaldehyde and spontaneously generated formaldehyde, formaldehyde-containing leachate from textiles and plastic-resin containers, drinking water, and so on, we designed and constructed an enzyme cocktail consisting of three coenzyme-free oxidoreductases (i.e., formaldehyde dismutase, methanol oxidase, and formate oxidase) and catalase for the complete oxidation of formaldehyde (**Figure1**). The stable isotope tracer technique and time profiles demonstrated that formaldehyde can be successfully oxidized into CO_2 and water by the enzyme cocktail (**Figure 2**). Within a short time (one to four hours), the enzyme cocktail enabled to efficiently eliminate formaldehyde of different concentrations (from

10 to 1000 mg/L) and formaldehyde in the leachate of contaminated seafood and textiles (approximately one mg/L) (**Figure 3 and 4**).

As compared to microbial remediation, the enzymatic cleanup technology received less attention possibly because of costly enzyme preparation. Recent advances in low-cost high-density fermentation,[28] recombinant protein expression,[29] and large-scale commercialization of enzymatic biocatalysis for 10,000-ton production of inositol,[30] suggest that enzyme costs (e.g., $10-30/kg dry weight) are not a show-stopper for enzymatic pollution cleanup anymore.[19] As compared to one-enzyme-based cleanup techniques that cannot catalyze complicated biological reactions,[31] this cascade-enzyme technique enabled to completely oxidize formaldehyde into CO_2 and water. It was notable that FDM contained tightly bound NAD(H) as a stable prosthetic group, different from dehydrogenases that used dissociated NAD^+ as a coenzyme. Furthermore, all the enzymes in this cocktail did not require costly and labile exogenously-added coenzymes, implying that this enzymatic cleanup technique could be widely applied to clean up formaldehyde in contaminated food, drinking water, and formaldehyde-containing leachate.

This study demonstrated the technological feasibility and environmental cleanup effectiveness of this enzyme cocktail to eliminate formaldehyde in aqueous solutions. In the future, this enzyme cocktail could be formulated into a food detoxication agent for *in situ* degradation of formaldehyde that was spontaneously generated during the storage and transportation of them. It could also be developed into a formaldehyde-degrading

spray, specifically for the dry clothing and curtain treatment. Compared to current methods, this enzyme cocktail possesses unique advantages, such as excellent biosafety, independence from light or other energy sources, and no special equipment required. Future endeavors could be conducted to improve this technique through enzyme engineering and immobilization.

Conclusion

Formaldehyde in aqueous solutions poses a severe risk to human health but current methods aiming at gaseous pollution cannot be applied directly to treat it. Significant formaldehyde was detected from the leachates of seafood and textiles purchased from the market. A novel coenzyme-free enzyme cocktail, consisting of FDM, MOX, FOX, and CAT, was designed and developed in this study. This enzyme cocktail can catalyze the complete oxidation of dissolved formaldehyde in the presence of dioxygen, which naturally exists in the surroundings. Therefore, this enzymatic cleanup technique could have wide applications in eliminating formaldehyde in aqueous solutions.

Experimental Section

Chemicals and Materials

Unless mentioned otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Sinopharm (Shanghai, China) in the highest purity available. Formaldehyde solutions, the ^{13}C-labled formaldehyde solution, and catalase were procured from Aladdin (Shanghai, China). 4-Amino-3-hydrazino-1,2,4-triazol-5-thiol (AHMT, CAS number: 1750-12-5) was procured from Macklin (Shanghai, China).

Formaldehyde assay kit was procured from OKA (Beijing, China). Horseradish peroxidase (HRP) was procured from Solarbio (Beijing, China). Ni-NTA resin was purchased from GenScript (Nanjing, China).

HRP-ABTS Assay for H_2O_2

ABTS (CAS number: 30931-67-0) can react with H_2O_2 under the catalysis of HRP, and turn into the green radical (**Figure S8**). 5 mM ABTS and 10 U/mL HRP were added to the samples. Then the absorbance at 405 nm was measured by spectrophotometer of GENESYS 150 (Thermo Fisher Scientific, Waltham, MA, USA) or microplate reader of Infinite M Nano (Tecan, Switzerland). The concentration of H_2O_2 was calculated based on the millimolar extinction coefficient at 405 nm of ABTS radical (35.2 mM^{-1} cm^{-1}).

AHMT Assay and Acetylacetone Assay for Formaldehyde

AHMT can react with formaldehyde and generate purple product finally (**Figure S9**). 24.5 g/L AHMT was dissolved in 2 M NaOH solution to make the assay reagent. 50 μL of the assay reagent was added to each 200 μL of samples in the 96-well plate. The plate was shaken for 20 minutes at room temperature, and the absorbance at 550 nm was measured by the microplate reader of Infinite M Nano (Tecan, Switzerland).

Acetylacetone assay was implemented following the instruction of the formaldehyde assay kit of OKA (Beijing, China). Acetylacetone can react with formaldehyde and generate yellow product in the presence of NH_4^+ (**Figure S10**). 200 μL of the assay reagent, containing acetylacetone and NH_4^+, was added to each 200 μL of the samples

and shaken for 30 minutes at 40 °C. The samples were centrifuged if there were turbidity after the reaction. The absorbance was measured at 412 nm by the microplate reader of Infinite M Nano (Tecan, Switzerland). The background absorbance of the sample and CAT was taken into account and subtracted.

HPLC Analysis

HPLC analysis was performed on the LC-20 system equipped with differential refractive index detector (Shimadzu, Japan) and the column of Aminex HPX-87H (Bio-Rad, Hercules, CA, USA). Formaldehyde, methanol, and formic acid were isolated and quantified (**Figure S11**). The mobile phase was sulfuric acid aqueous solution of 5 mM, the column temperature was 30 °C, and the flow rate was 0.5 mL/min. The column temperature of 35 °C and higher flow rates (below 0.7 mL/min) were also feasible. Each sample was measured for 25 minutes. The background of impurities was taken into account and subtracted.

Expression and Purification of Enzymes

The gene encoding formaldehyde dismutase (FDM) from *P. putida* was synthesized by Genewiz (Suzhou, China), integrated into pET-20b(+) vector, and transformed into *E. coli* BL21(DE3)-GroES/L, which is capable of expressing the molecular chaperones GroES and GroEL.[23] The gene encoding methanol oxidase (MOX) from *P. chrysosporium* was synthesized by SiBio (Suzhou, China), integrated into pET SUMO vector, and transformed into *E. coli* BL21(DE3).[32] The gene encoding formate oxidase (FOX) from *A. oryzae* was synthesized by Genewiz (Suzhou, China), integrated into

pET-28a(+) vector, and transformed into *E. coli* BL21(DE3).[33] The amino acid sequences of recombinant enzymes (**Table S2**) and optimized DNA sequences encoding them (**Table S3**) can be found at supporting information.

The recombinant *E. coli* strain expressing FDM was cultured in TB medium supplemented with ampicillin (50 μg/mL) and chloramphenicol (10 μg/mL) at 37 °C and 260 rpm. When the OD_{600} reached 0.8, 0.1 mM IPTG was added to it. The culture was continued at 20 °C and 200 rpm for 12 h, and then harvested by centrifugation. The obtained cell pellet was washed by using normal saline, and then suspended in NaPi buffer (20 mM, pH 7.2, with 100 mM NaCl). Cell lysate was obtained by using high pressure homogenizer of AH-1500 (ATS, Suzhou, China) and centrifuged for 30 minutes at 4 °C and 20,000 g. The supernatant was loaded to a Ni-NTA column pre-equilibrated by NaPi buffer. Proteins were eluted in sequential steps by NaPi buffer (20 mM, pH 7.2, with 100 mM NaCl) containing 50 mM, 200 mM, and 500 mM imidazole, respectively. FDM in the eluted fraction was concentrated and desalted by ultrafiltration of Millipore (10 kDa cut-off). Protein concentration was determined via Bradford assay.

The preparation procedures of MOX and FOX were similar to that of FDM and were not reiterated here. The concentration of kanamycin in the medium was 50 μg/mL.

Enzyme Characterization Methods

The specific activity of FDM was determined via AHMT assay. The reaction mixture was prepared in glass tubes, containing buffer, 0.03 mg/mL FDM, and 1.5 mM

355 formaldehyde. The mixture was shaken for 10 minutes and the concentration of
356 formaldehyde was determined. The specific activity of MOX was determined via HRP-
357 ABTS assay. The reaction mixture was prepared in quartz cuvettes, containing buffer,
358 10 U/mL HRP, 5 mM ABTS, 10 μg/mL MOX, and 20 mM methanol. The change of
359 absorbance at 405 nm within 1 min was determined. The specific activity of FOX was
360 determined via HRP-ABTS assay, too. The reaction mixture was prepared in quartz
361 cuvettes, containing buffer, 10 U/mL HRP, 5 mM ABTS, 10 μg/mL FOX, and 20 mM
362 sodium formate. Then the change of absorbance at 405 nm within 1 min was
363 determined.
364
365 The specific activities of FDM, MOX, and FOX were determined at temperatures of 20,
366 25, 30, 35, 40, 45, 50, 55, and 60 °C to find the optimal temperatures. The method to
367 determine the optimal temperature of MOX was modified following the procedure
368 described above. Here the reagent of ABTS assay consisted of NaPi buffer (100 mM,
369 pH 7), 20 U/mL HRP, and 10 mM ABTS. The reaction mixtures contained glycine buffer
370 (50 mM, pH 9), 1 μg/mL MOX, and 20 mM methanol. The mixtures were shaken for 30
371 minutes in the metal bath at temperatures of 20, 25, 30, 35, 40, 45, 50, 55, and 60 °C,
372 respectively. The reactions were terminated by placing on the ice. 100 μL of the assay
373 reagent was added to each 100 μL of the mixtures in the 96-well plate. The plate was
374 shaken for one minute and the absorbance was measured at 405 nm by the microplate
375 reader of Infinite M Nano (Tecan, Switzerland). The method to determine the optimal
376 temperature of FOX was similar to that of MOX via modified HRP-ABTS assay. The

reaction mixtures contained acetate buffer (50 mM, pH 5), 0.2 μg/mL FOX, and 20 mM sodium formate.

The optimal pHs of FDM, MOX, and FOX was determined by using Britton-Robinson buffer.[34] Britton-Robinson buffer contained phosphoric acid, boric acid, and acetic acid of 40 mM and was adjusted to various pHs (2 to 12) by NaOH. The specific activities of FDM, MOX, and FOX were determined in Britton-Robinson buffer of pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 to find the optimal pHs.

The trade-off pH of FDM, MOX, and FOX was determined within pH 5 to 8. The reaction mixtures were prepared in glass tubes, containing 50 mM NaPi buffer of various pHs (i.e., 5.5, 6.0, 6.5, 7.0, and 7.5), 0.1 U/mL FDM, 0.1 U/mL MOX, 0.1 U/mL FOX, and 1.3 mM formaldehyde. The specific activities of enzymes here referred to the activities at their optimal pHs. The reaction was performed at 25 °C and 800 rpm for one hour. The concentration of H_2O_2 in the mixture was determined via HRP-ABTS assay.

Stable Isotope Tracer Method

The reaction mixture was prepared in a sealed glass vial equipped with a rubber stopper, containing NaPi buffer (50 mM, pH 7), 10 mM ^{13}C-labeled formaldehyde, 0.04 mg/mL FDM, 3.6 mg/mL FOX, 0.6 mg/mL MOX, 0.1 mg/mL CAT. Then it was shaken for three hours at room temperature. NaOH solution was added by a syringe to terminate the reaction and absorb CO_2. Barium hydroxide solution was added to obtain the precipitate of barium carbonate. Then hydrochloric acid was added to the precipitate

after it was transferred to another sealed container, and the generated CO_2 was collected in gas container. The enzymatic CO_2 was analyzed by GC-MS system of TRACE 1300 and ISQ 7000 equipped with electron impact ion source (Thermo Fisher Scientific, Waltham, MA, USA). CO_2 in the ambient air was also analyzed by GC-MS as a control.

Determination Methods of Time Profiles

The formaldehyde solutions were prepared by diluting commercial formaldehyde solution, and the amount of methanol in the commercial solution, which was added to prevent the oligomerization of formaldehyde, was determined to be 30%(mol/mol) by HPLC. All experiments were performed in the closed glass tube in order to prevent the volatilization of formaldehyde.

The reaction mixture for determining the time profile catalyzed by the four-enzyme cocktail was prepared in glass tubes, containing NaPi buffer (50 mM, pH 7), 10 mM formaldehyde, 0.04 mg/mL FDM, 3.6 mg/mL FOX, 0.6 mg/mL MOX, and 0.1 mg/mL CAT. Then it was shaken at 25 °C and 800 rpm. The mixture was sampled after 10, 20, 40, 80, and 120 minutes, respectively. The concentrations of formaldehyde, methanol, and formate in the samples were determined by HPLC.

The determination procedure of the time profile catalyzed by the there-enzyme cocktail (i.e., FDM, FOX, and CAT) was similar to that of the four-enzyme cocktail but the reaction mixture did not contain MOX.

The reaction mixture for determining the time profile catalyzed by only FDM was prepared in glass tubes, containing NaPi buffer (50 mM, pH 7), 10 mM formaldehyde, and 0.04 mg/mL FDM. Then it was shaken at 25 °C and 800 rpm. The mixture was sampled after 10, 20, 40, 80, and 120 min, respectively. The concentrations of formaldehyde in the samples were determined via acetylacetone assay. The concentrations of formate and methanol were equal to half of the reduction of formaldehyde concentration.

Determination Methods of Degradation Ratios

The formaldehyde degradation ratios under various conditions were determined under the catalysis of the four-enzyme cocktail. Three enzyme loadings were tested. The low enzyme loading was 0.01 mg/mL FDM, 0.01 mg/mL MOX, 0.06 mg/mL FOX, and 0.05 mg/mL CAT; the middle loading was 0.1 mg/mL FDM, 0.1 mg/mL MOX, 0.6 mg/mL FOX, and 0.5 mg/mL CAT; and the high loading was 0.5 mg/mL FDM, 0.5 mg/mL MOX, 3.0 mg/mL FOX, and 2.5 mg/mL CAT.

All experiments were performed in the closed glass tube in order to prevent the volatilization of formaldehyde. The reaction mixtures were prepared in glass tubes containing NaPi buffer (50 mM, pH 7), enzymes of different loadings, and various concentrations of formaldehyde (i.e., 10, 100, and 1000 mg/L). Then the mixtures were shaken at 25 °C and 800 rpm and were terminated after one hour, two hours, and four hours, respectively. The residual concentrations of formaldehyde were determined via

446 acetylacetone assay. The formaldehyde degradation ratio was defined as the ratio of
447 the concentration reduction relative to the initial concentration.
448
449 **Effect Tests on Everyday Samples**
450 Seven kinds of seafood, textiles, and paint were randomly purchased from the market.
451 The seafood samples were thawed and extruded to collect the leachate. The textile
452 samples were soaked in water at the ratio of 3 to 110 g/L for one hour to collect the
453 leachate. The latex paint was diluted 50 times with water and then centrifuged to collect
454 the leachate. The concentrations of formaldehyde in these leachates were determined
455 via acetylacetone assay.
456
457 Treatments were performed in the closed glass tube in order to prevent the volatilization
458 of formaldehyde. The enzyme cocktail, comprising 0.1 mg/mL FDM, 0.1 mg/mL MOX,
459 0.6 mg/mL FOX, and 0.5 mg/mL CAT, was added to the leachates above in glass tubes.
460 The mixtures were shaken at 25 °C and 800 rpm for four hours. The concentrations of
461 formaldehyde were determined via acetylacetone assay before and after reactions.

Supporting Information

The authors have cited additional references within the Supporting Information.[35]

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Figures

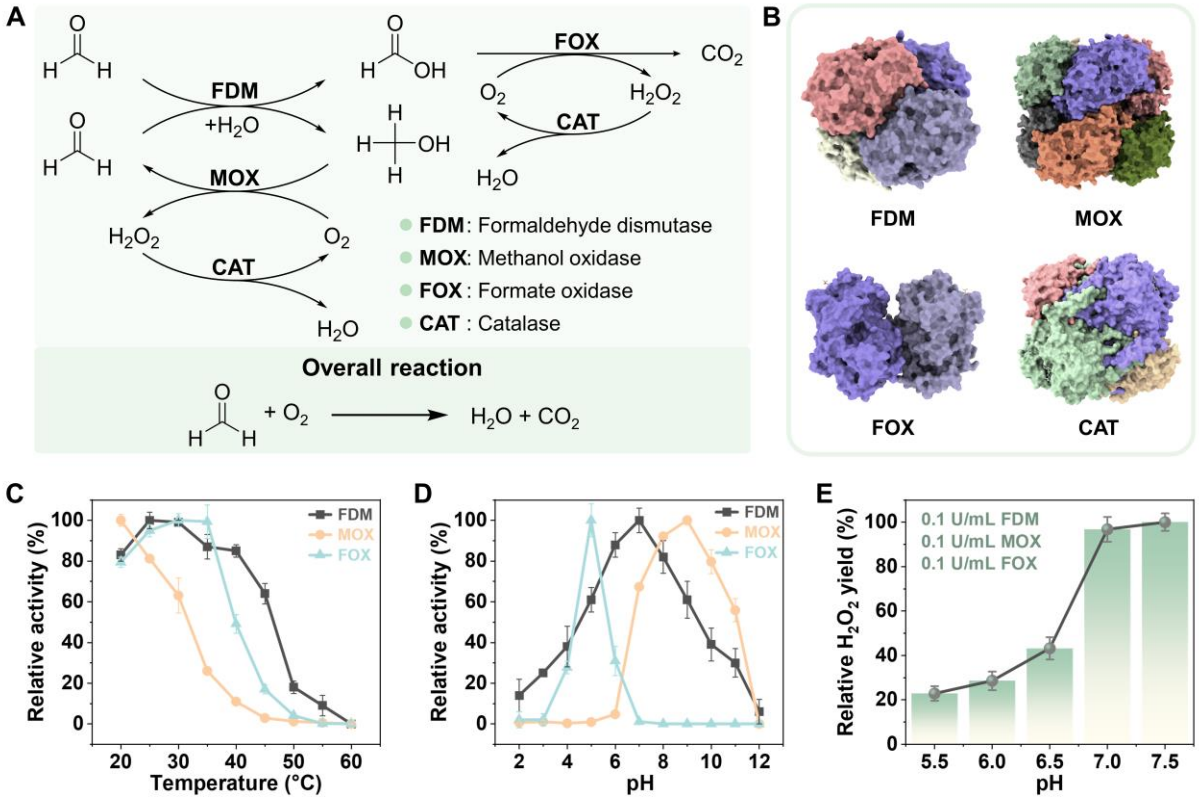
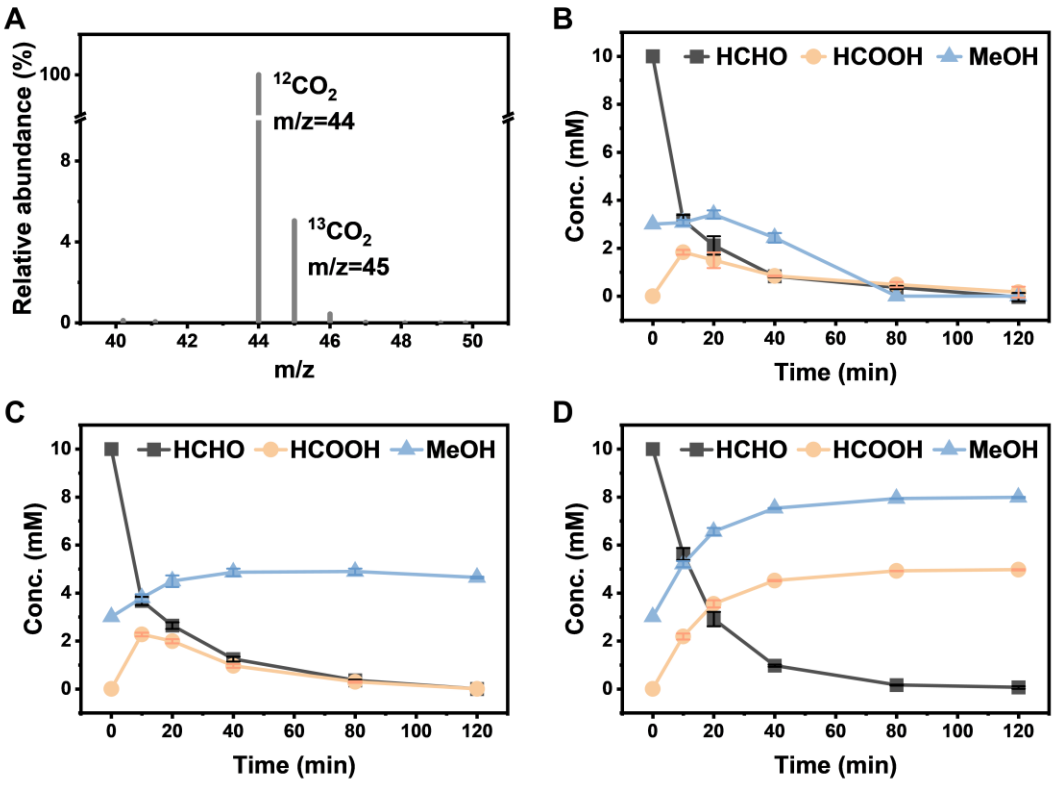


Figure 1. (A) The artificial enzymatic pathway that converts formaldehyde and dioxygen into CO_2 and water. (B) The molecular structures of FDM from *P. putida* (PDB 2DPH), which is a homotetramer; of MOX from *P. chrysosporium* (PDB 6H3G), which is a homooctamer; of FOX from *A. oryzae* (PDB 3Q9T), which is a homodimer; and of CAT from bovine liver (PDB 3J7B), which is a homotetramer. (C) The optimal temperatures of FDM, MOX, and FOX. (D) The optimal pHs of FDM, MOX, and FOX. (E) The trade-off pH of FDM, MOX, and FOX, based on the yield of H_2O_2. Data were presented as the mean of three independent replicates with error bars of one SD.



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576 **Figure 2.** (A) The mass spectrogram of products generated from ^{13}C-labeled

577 formaldehyde. The molecular ion peak of $^{13}CO_2$ was observed at a mass-to-charge ratio

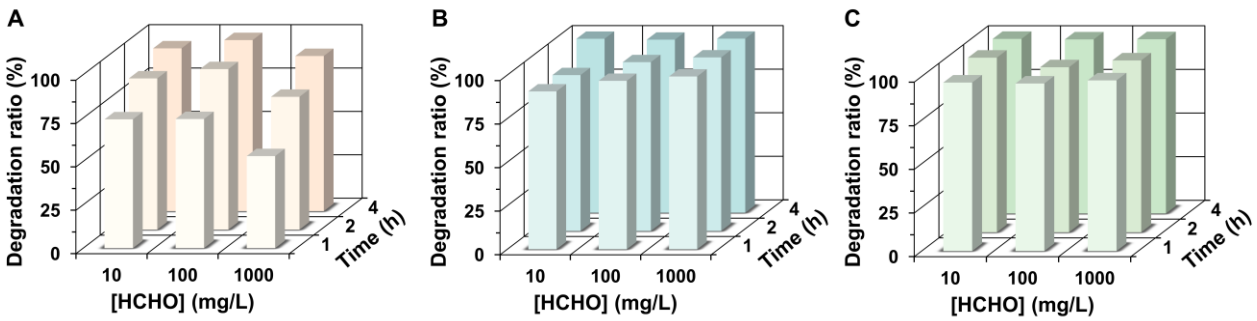
578 (m/z) of 45. (B) Time profile of formaldehyde degradation catalyzed by the four-enzyme

579 cocktail. HCHO, formaldehyde; HCOOH, formic acid; and MeOH, methanol. (C) Time

580 profile catalyzed by the three-enzyme cocktail (i.e., FDM, FOX and CAT). (D) Time

581 profile catalyzed by only FDM. Concentrations were presented as the mean of triplicate

582 experiments with error bars of one SD.



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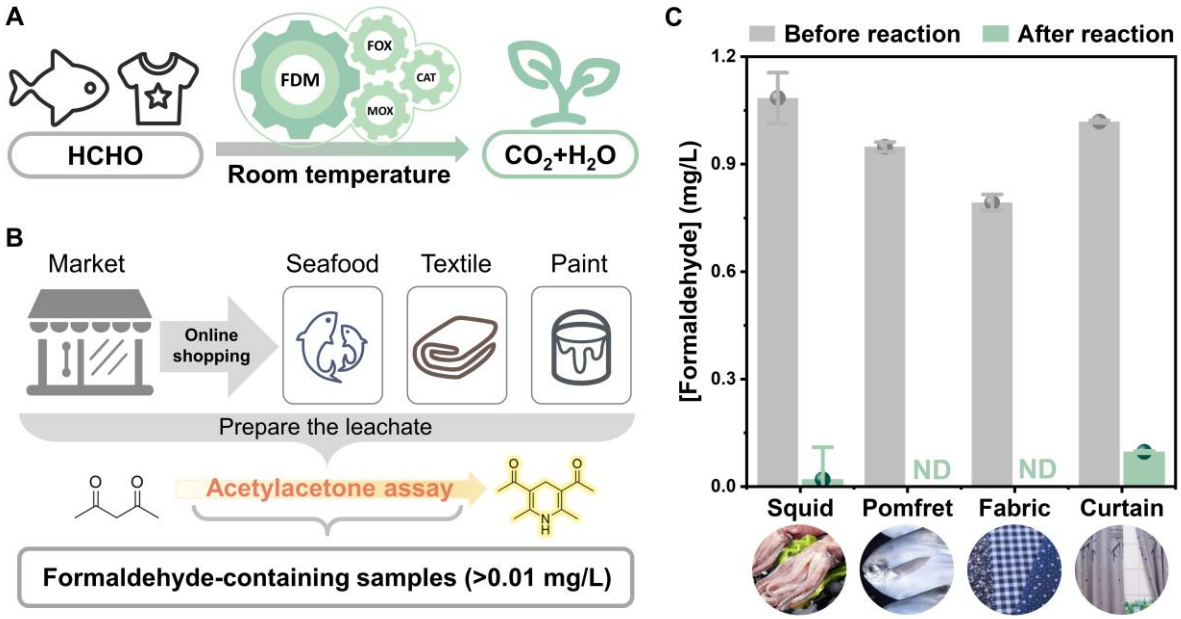
585 **Figure 3.** Degradation ratios of formaldehyde in aqueous solutions catalyzed by

586 different enzyme loadings over time. (A) A low enzyme loading, (B) a middle enzyme

587 loading, and (C) a high enzyme loading. Degradation ratios were presented as the

588 mean of triplicate experiments.



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591 **Figure 4.** (A) Schematic illustration of the enzyme cocktail applications. (B) The
592 preparation of the formaldehyde-containing samples, whose leachate contained
593 formaldehyde of more than 0.01 mg/L detected by acetylacetone assay. (C)
594 Formaldehyde concentrations in the leachate of contaminated samples (i.e., squid,
595 pomfret, fabric, and curtain) before and after four-hour reaction catalyzed by the
596 enzyme cocktail. Concentrations were presented as the mean of triplicate experiments
597 with error bars of one SD. ND, not detected.

Tables

Table 1. Four kinds of formaldehyde-oxidizing enzymes.

EC number	Reaction	$\Delta_rG'^\circ$ (kJ/mol)[a]	Cofactor
1.2.1.1	Formaldehyde + Glutathione + NAD^+ = S-Formylglutathione + NADH + H^+	-40.1	Glutathione, NAD(H)
1.2.1.46	Formaldehyde + NAD^+ + H_2O = Formate + NADH + H^+	-45.8	NAD(H)
1.2.3.1	Aldehyde + H_2O + O_2 = Carboxylate + H_2O_2	\	Molybdenum, [2Fe-2S], FAD
1.2.98.1	2 Formaldehyde + H_2O = Formate + Methanol	-79.4	Tightly bound NAD(H)

[a] The $\Delta_rG'^\circ$ was calculated by eQuilibrator.[25]

Table 2. Enzymes that made up the cocktail.

Abbr.	Full name	UniProt ID	Reaction	S.a. (U/mg)[a]
FDM	Formaldehyde dismutase	Q52078	2 HCHO + H_2O = HCOOH + MeOH	1.42[b]
MOX	Methanol oxidase	T2M2J4	MeOH + O_2 = HCHO + H_2O_2	1.35
FOX	Formate oxidase	Q2UD26	HCOOH + O_2 = CO_2 + H_2O_2	0.28
CAT	Catalase	P00432	2 H_2O_2 = 2 H_2O + O_2	\

[a] The specific activity was determined at 25 °C, in NaPi buffer of pH 7.

[b] One unit of FDM was defined as producing one μmol of formic acid per minute.