ACS APPLIED MATERIALS & INTERFACES

www.acsami.org

Research Article

Cascade/Parallel Biocatalysis via Multi-enzyme Encapsulation on Metal–Organic Materials for Rapid and Sustainable Biomass Degradation

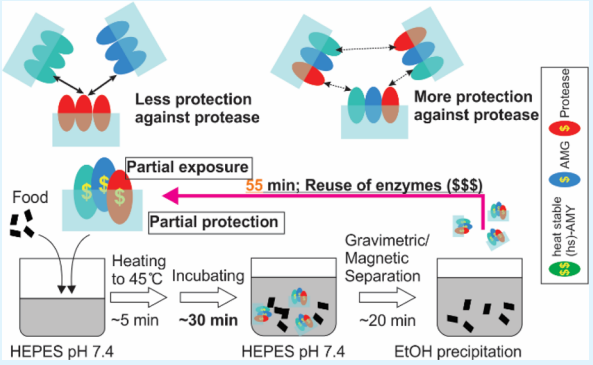
Qiaobin Li, Yanxiong Pan,* Hui Li, Mary Lenertz, Kailyn Reed, Drew Jordahl, Taylor Bjerke, Angel Ugrinov, Bingcan Chen,* and Zhongyu Yang*

Cite This: *ACS Appl. Mater. Interfaces* 2021, 13, 43085–43093

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Multiple-enzyme cooperation simultaneously is an effective approach to biomass conversion and biodegradation. The challenge, however, lies in the interference of the involved enzymes with each other, especially when a protease is needed, and thus, the difficulty in reusing the enzymes; while extracting/synthesizing new enzymes costs energy and negative impact on the environment. Here, we present a unique approach to immobilize multiple enzymes, including a protease, on a metal–organic material (MOM) via co-precipitation in order to enhance the reusability and sustainability. We prove our strategy on the degradation of starch-containing polysaccharides (require two enzymes to degrade) and food proteins (require a protease to digest) before the quantification of total dietary fiber. As compared to the widely adopted "official" method, which requires the sequential addition of three enzymes under different conditions (pH/temperature), the three enzymes can be simultaneously immobilized on the surface of our MOM crystals to allow for contact with the large substrates (starch), while MOMs offer sufficient protection to the enzymes so that the reusability and long-term storage are improved. Furthermore, the same biodegradation can be carried out without adjusting the reaction condition, further reducing the reaction time. Remarkably, the simultaneous presence of all enzymes enhances the reaction efficiency by a factor of ∼3 as compared to the official method. To our best knowledge, this is the first experimental demonstration of using aqueous-phase co-precipitation to immobilize multiple enzymes for large-substrate biocatalysis. The significantly enhanced efficiency can potentially impact the food industry by reducing the labor requirement and enhancing enzyme cost efficiency, leading to reduced food cost. The reduced energy cost of extracting enzymes and adjusting reaction conditions minimize the negative impact on the environment. The strategy to prevent protease damage in a multi-enzyme system can be adapted to other biocatalytic reactions involving proteases.



KEYWORDS: *cascade biocatalysis, enzyme immobilization, one-pot synthesis, metal–organic frameworks, metal–organic materials, protease protection, total dietary fiber quantification*

INTRODUCTION

Cascade biocatalysis in parallel with other biocatalytic reactions via multiple enzymes is an effective approach to degrade or convert large biomass substrates, given the high selectivity, biocompatibility, and reaction efficiency,[1−8] resulting in valuable chemical/biochemical components and new biofuel resources. However, the cost of the involved enzymes and the difficulty in isolating/reusing the enzymes after a reaction place a major hurdle in the cost efficiency of this strategy. Because the involved enzymes are extracted from nature or synthesized in a biochemistry laboratory, energy from non-sustainable sources is often needed, negatively impacting the environment. Furthermore, in case a protease is needed, which is common in reactions involving food-protein digestion,[9,10] the enzymes cannot work in parallel but sequentially under different conditions, increasing the reaction time and operation complexity, the latter of which often leads to high uncertainties. Immobilizing all needed enzymes, either together or individually, on large solid surfaces may improve the reusability, but the surface-to-volume ratio is not ideal.[11−15] Nanoparticles can improve such ratios but would result in protease contact with other enzymes on nearby particles.[11,16] Porous materials (including zeolites[17−20] and

Received: June 29, 2021
Published: September 3, 2021

MOFs), microporous materials, as well as enzyme aggregates may improve the protection but will block the access of the large biomass substrates.[15,16,21−25] Thus, how to simultaneously protect the involved multiple enzymes while allowing for large substrate contact, or, at least, finding a compromise of the two aspects, is essential for broadening the application of this strategy in biomass degradation.[26,27]

Here, we present a strategy that offers such a compromise and demonstrate it on an important biomass degradation reaction, the degradation of polysaccharides and food proteins, a prerequisite of quantification of total dietary fiber (TDF) in foods.[9] Due to the importance of TDF to human health, U.S. Food and Drug Administration (FDA) requires precise TDF contents for all commercial food products.[28−31] Before quantifying TDF, the polysaccharides and food proteins in a food sample need to be digested. In the widely adapted official method, three enzymes, heat-stable α-amylase (heat-stable AMY), protease, and amyloglucosidase (AMG), are applied sequentially (but not together; see Figure 1) under specific

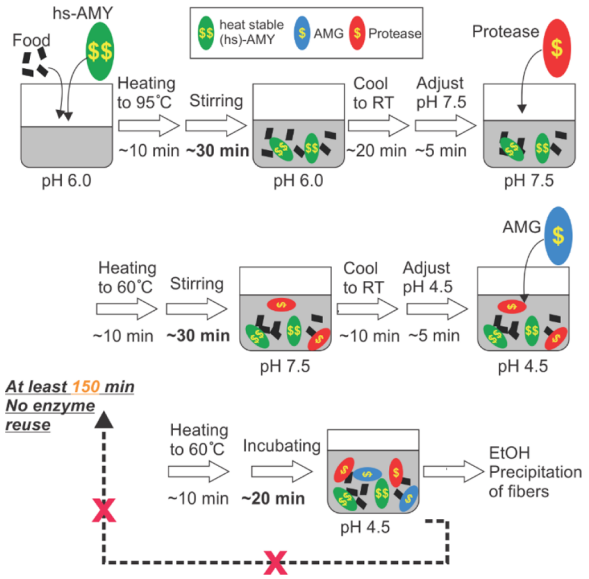


Figure 1. Schematic illustration of the complex and time-consuming procedure for TDF quantification using the "official" method. The involved enzymes are color-coded as indicated in the figure. The time and operation of each step are highlighted above and below the white arrows connecting the steps. RT = room temperature. The involved enzymes cannot be recycled (dotted arrow), while the reaction time is at least 150 min for an experienced operator.

conditions in three steps:[32] (1) at 95 °C, pH 6.0, heat-stable AMY is added for ~30 min to degrade starch to maltose,[32,33] (2) at 60 °C, pH 7.5, protease is added for 30 min to degrade food proteins and reduce viscosity, and (3) at 60 °C, pH 4.5, AMG is added for 20 min to degrade maltose to glucose (Figure 1).[34] The total time, including adjusting the reaction conditions (pH, temperature) for each step, is at least 150 min for an experienced operator with sufficient training. Poor enzyme reusability, the long/complex operation procedures, and the energy to adjust reaction conditions make the official method non-sustainable, expensive, time-/energy-consuming, and with high chances of error (due to the complicated procedure).

In our recent discovery, we found that an enzyme can be partially exposed above the surface of metal−organic frameworks/materials (MOFs/MOMs) upon co-crystallization while being partially buried (and thus, protected) in the scaffolds;[35−40] here, MOMs are usually referred to as 1D or 2D structures, while MOFs are 3D frameworks according to a strict definition.[41] This strategy can offer enhanced enzyme protection to a satisfying extent so that a reasonable number of reuse cycles can be achieved. We, therefore, apply this strategy to immobilize multiple enzymes including a protease simultaneously (designated as the "3-in-1" biocomposites) as well as individually (which can later be combined together in the same system) with a Ca-based MOM, Ca-BPDC (biphenyl-4,4′-dicarboxylic acid).[35] The reaction medium is water, which minimizes enzyme damage during co-crystallization. We proved the presence of the involved enzymes, either together or individually in each MOM, structurally (via confocal fluorescence imaging on fluoro-labeled enzymes) and functionally (via standard activity assays). The reusability was then assessed against the standard commercial starch samples, the results of which indicated comparable catalytic efficiency and reusability between the 3-in-1 composite and the mixture of three single composites in the short term (2−3 days). However, upon long-term storage, the mixture of composites showed reduced reusability likely caused by the enhanced chance of protease contact with other enzymes in the adjacent co-crystals. Remarkably, we found that both the 3-in-1 and the mixture of single composites showed a reduced reaction time (~55 min total) as compared to ~150 min in the official method, enhancing the efficiency of single digestion by a factor of ~3. Here, we define "cascade/parallel" biocatalysis because AMY and AMG work in a cascade fashion, while the protease can digest food protein in parallel with polysaccharide degradation.

To our best knowledge, this is the first experimental demonstration of using aqueous-phase co-precipitation to encapsulate multiple enzymes including a protease to carry out multiple biocatalytic reactions simultaneously. As compared to most works on enzyme encapsulation in MOFs,[15,42−51] our strategy allows for immobilization of enzymes that are larger than MOF pores. Different from the existing co-precipitation-based enzyme-immobilization works,[52−57] our method allows for partial exposure of all three enzymes. Different from the "official" method of food digestion, our method significantly enhances the digestion rate and can potentially change the way to quantify TDF in commercial food samples; together with the high reusability of the expensive enzymes, our strategy will lead to a broad impact on the food industry such as enhanced accuracy in TDF content measurements and reduced food cost. The strategy to prevent protease damage to other enzymes in a multi-enzyme system can be generalized to other systems/problems involving protease or other enzymes that degrade proteins.

RESULTS AND DISCUSSION

Experimental Design. Our goal is to co-immobilize three enzymes involved in food/polysaccharide digestion, AMY, a protease, and AMG. A popular enzyme immobilization platform is the zeolitic-imidazolate framework (ZIF-8), which requires the organic phase (MeOH) and, thus, enzyme protection via polymers during the reaction.[53] We have tested this strategy but found it difficult to retain enzymes' activities in the resultant ZIF-8 crystals (the activity data are nearly negligible as compared to our Ca-MOM and therefore, not shown) likely due to the incomplete protection of the relatively

https://doi.org/10.1021/acsami.1c12209
ACS Appl. Mater. Interfaces 2021, 13, 43085−43093

large enzymes involved in our system as well as the interference of polymer wrapping that suppresses enzyme function. Note that AMY and AMG had been immobilized on ZIF-8 in an aqueous phase;[58] however, no protease was included therein. We also attempted aqueous-phase biomineralization of ZIF with our three enzymes and found low-enzyme-loading capacity and low-quality co-crystals. We, therefore, seek an alternative co-precipitation platform. Inspired by our recent Ca-MOM work,[35] we found it possible to encapsulate enzymes via the co-precipitation of Ca^{2+} and some carboxylate ligands in water at room temperature. The co-precipitation of enzymes and Ca-MOM composites was relatively rapid with relatively large (tens of μm), round-shape crystals (see the next section), while the resultant enzymes were functional. Among the available carboxylate ligands, we found that BPDC (Figure 2A) offers a good balance of co-crystal stability and enzyme activity.[35] Thus, Ca-BPDC MOM becomes the choice of platform in this work. Because MOMs can potentially enhance the thermal stability of enzymes as well, in this work, we replaced the expensive heat-stable AMG in the official method with the regular AMY to further reduce the cost. The schematic illustration of our synthesis and resultant composite is shown in Figure 2.

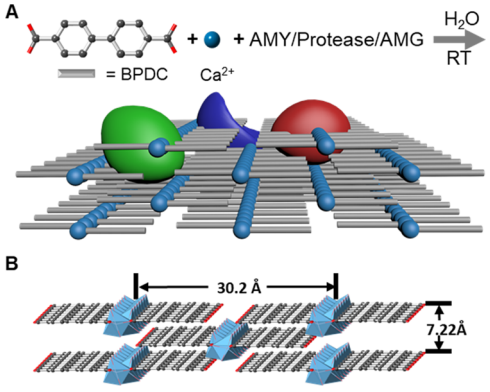


Figure 2. (A) Reaction scheme of co-crystallization of Ca^{2+}, BPDC, and three enzymes in water to generate the biocomposites with the three enzymes co-encapsulated on the surface of the Ca-BPDC MOM. (B) Structure of Ca-BPDC MOM was derived based on the PXRD pattern, which is consistent with our recent finding.[31]

Synthesis and Characterization of the Single-Enzyme@Ca-BPDC Composites. We first prepared a single-enzyme@Ca-BPDC composite to confirm that the Ca-BPDC can host and retain the function of each enzyme. We individually mixed ~1.0, 3.0, and 25.0 mg of AMY, protease, and AMG, respectively, with ~25 mM $CaCl_2$ and ~20 mM BPDC-Na_2 in ~1.0 mL of water and incubated at room temperature under gentle nutation overnight (also see the Supporting Information). The enzyme amounts were selected so that excess enzymes were present in the reaction medium to ensure a high enzyme loading, while the relative ratio was derived based on the official method.[32] Upon the removal of unreacted species via centrifugation and resuspension (with water) 3 times, each composite was subjected for characterization. The presence of each enzyme was confirmed via confocal fluorescent imaging on FITC-labeled AMY, protease, and AMG co-precipitated with Ca^{2+} and BPDC in water, one at a time. The resultant images shown in Figure 3A–C (color was tuned to show the difference) indicate the presence of each enzyme in Ca-BPDC as well as the overall shape of the MOMs. The scanning electron microscopy (SEM) images also confirmed the shape (see Figure 4A). Fourier transformation infrared was also carried out to confirm the successful immobilization of enzymes in Ca-BPDC (Figure S4). As shown in Figure 3D, the powder X-ray diffraction (PXRD) images of AMY@Ca-BPDC, protease@Ca-BPDC, and AMG@Ca-BPDC composites are consistent with that of the pure Ca-BPDC MOM and the simulated pattern as reported before,[59] which indicates that a layer-by-layer structure was formed

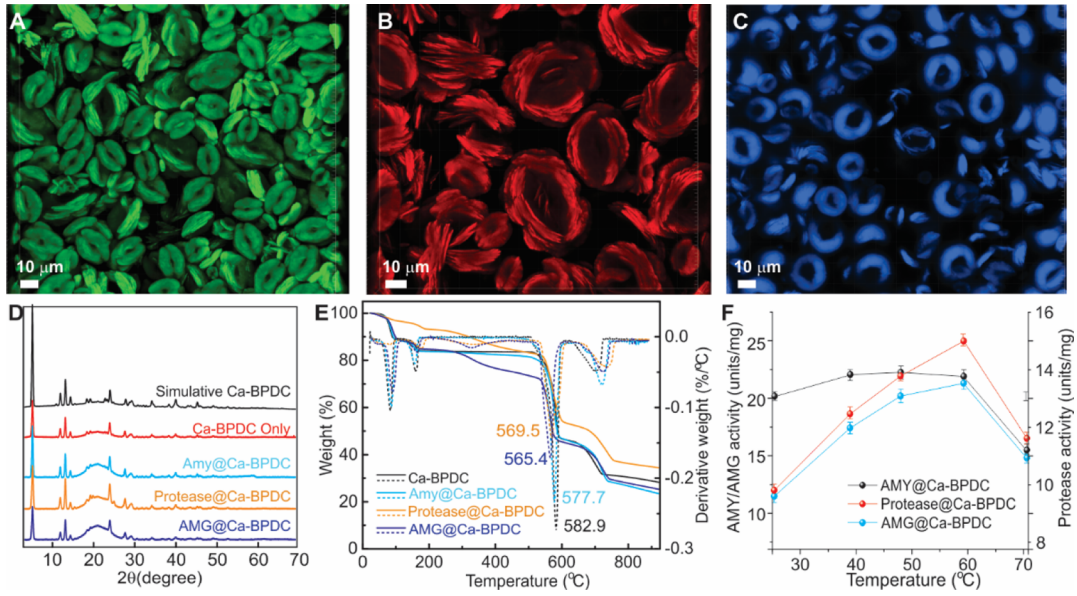


Figure 3. Confocal fluorescent images of the FITC-labeled (A) AMY, (B) protease, and (C) AMG upon encapsulation in Ca-BPDC. (D) PXRD patterns of Ca-BPDC (red) and its simulation (black) as well as AMY (cyan), protease (orange), and AMG (blue) encapsulated in Ca-BPDC. (E) TGA data of Ca-BPDC and each enzyme encapsulated in Ca-BPDC. (F) Temperature dependence of the relative activity of each enzyme@Ca-BPDC under pH 7.4. The "unit" of activity is defined by the commercial resource and/or the activity kit of each enzyme (see the Supporting Information).

https://doi.org/10.1021/acsami.1c12209
ACS Appl. Mater. Interfaces 2021, 13, 43085–43093

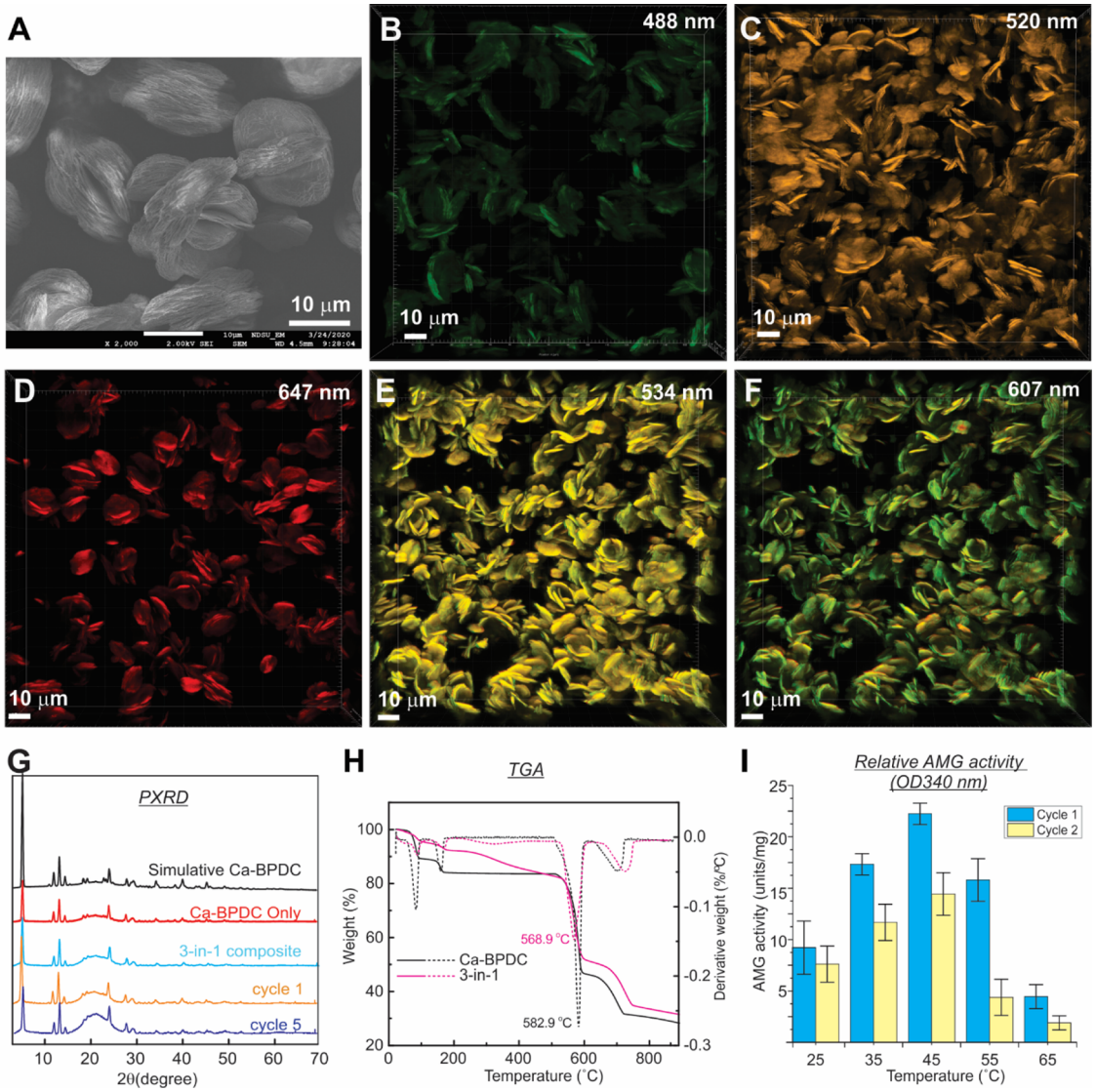


Figure 4. (A) SEM image of the 3-in-1 composite. (B–F) Confocal fluorescent images of the fluorescent-labeled mixture of (B) AMY, (C) protease, and (D) AMG upon encapsulation in Ca-BPDC with fluorescent excitation at 488, 520, 647, 534, and 607 nm. (G) PXRD patterns of Ca-BPDC (red) and its simulation (black), as well as the 3-in-1 composite encapsulated in Ca-BPDC (cyan), the 3-in-1 composite after one (orange), and five cycles (blue). (H) TGA data of Ca-BPDC and the 3-in-1 composite. (I) Temperature dependence of the relative activity of the AMG in the 3-in-1 composite measured under pH 7.4.

(because the data have been reported, we did not carry out a full analysis of the PXRD pattern). The PXRD data also confirmed that the encapsulation of the enzyme does not affect crystallinity significantly. The thermal gravimetric analysis (TGA) data (Figure 3E) suggested the gradual weight loss of enzymes in the Ca-BPDC MOMs. In particular, from 100 to 170 °C is the weight loss of water and from 240 to 500 °C is the loss of enzymes. The TGA data also indicated an ∼2.0% w/w of enzyme encapsulation (Figure 3E) for each enzyme, which was further confirmed by protein UV absorption measurements after disassembling Ca-BPDC via the PBS buffer (due to the strong interaction between PO_4^{3-} and metal which breaks the Ca-BPDC network). The enzyme loading efficiency (amount of loaded enzyme over the total added enzyme) was estimated as ∼5%. This yield is not higher than other immobilization approaches such as physical adsorption or grafting. However, our MOMs approach is more advantageous in terms of preventing leaching (compared to adsorption) and avoiding chemical changes in the enzyme (compared to grafting).

Catalytic Activity of Each Enzyme in the Composites. Commercial AMY, protease, and AMG and the associated activity kits[60] were employed to test the activity of each enzyme (see the Supporting Information). A commercial starch from potato (Sigma-Aldrich cat. no. S2004-1KG) was selected to test the activities of AMY and AMG based on the formation of coloring complexes upon the production of maltose and glucose over time, respectively.[32,34] The activity of the protease was assessed based on the enhanced fluorescence over time due to the cleavage of a fluorescent-labeled peptide provided by the kit (see the Supporting Information). For all activity assays, we are monitoring the generation of the coloring complexes over time, and thus, the activity data comprise the increased optical absorption intensity as a function of time. Because all enzymes were purchased from commercial sources, the enzyme activity was evaluated by unit

https://doi.org/10.1021/acsami.1c12209
ACS Appl. Mater. Interfaces 2021, 13, 43085−43093

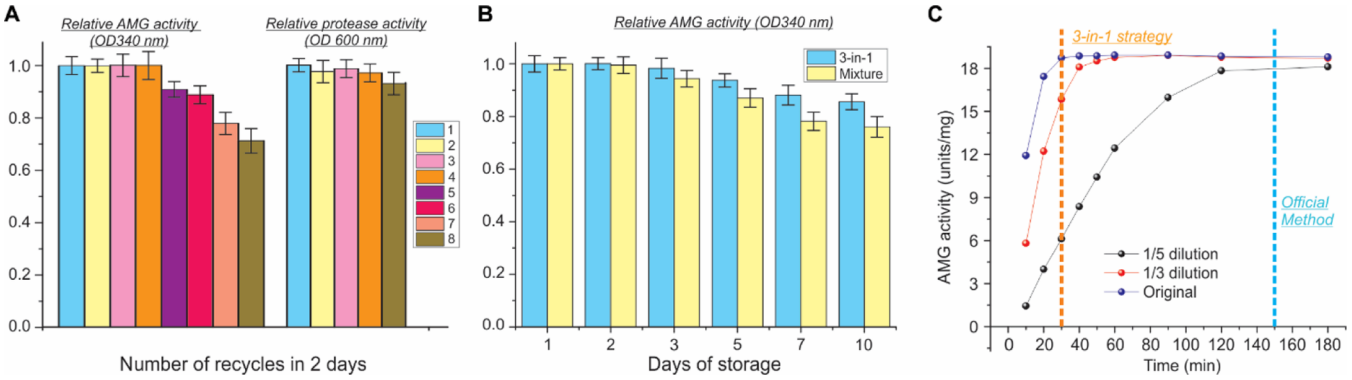


Figure 5. (A) Short-term reusability of the 3-in-1 composite assessed based on AMG activity (left) and protease activity (right). (B) Long-term reusability of the 3-in-1 composite as compared to the mixture of three single enzyme@Ca-BPDC composites. (C) Enhancement in catalytic efficiency as reflected by the reduced reaction time using the 3-in-1 composite as compared to the official method. The reaction is completed when the AMG activity reaches the plateau.

per gram/volume of the enzyme depending on/according to the definition of units from the commercial source of the enzyme (see the Supporting Information). As a positive control, the activity of each enzyme in the solution was determined. The pH dependence of each enzyme's activity at room temperature (Figure S1A−C) indicates that AMY and AMG have optimal pH at 5.5−7.0, while protease optimal pH is 7.4, close to expectations suggested by each activity assay/kit. Also, the temperature dependence of the activity test (based on total product generation) indicates that both AMY and AMG have optimal activity at 37 °C, while the protease optimal activity is at ∼60 °C (pH 7.4; see Figure S1D). Interestingly, upon encapsulation in Ca-BPDC, the temperature dependence of the relative activity of each composite (based on total product generation of each enzyme) indicates that the optimal temperature of AMY and AMG has been increased to 50−60 °C, suggesting that Ca-BPDC enhances the thermal stability of encapsulated enzymes (Figure 3F). As negative controls, Ca^{2+}, BPDC, or Ca-BPDC did not show any activity using the commercial kits (data not shown). Also, the SEM and PXRD of each composite upon treatment under high temperatures (up to 70 °C) were reasonably close to those before the treatment, suggesting that the enzyme@Ca-BPDC composites were reasonably stable and had an intact structure after the high-temperature treatment.

Synthesis and Characterization of the 3-in-1 Composite. To prepare a "3-in-1" composite, in 1 mL water we co-crystallized Ca^{2+}, BPDC-Na_2, and various relative molar ratios of AMY/protease/AMG. Note that due to the high efficiency of most proteases, the AMY/protease ratio of close to 1:1 in the official method[32] is anticipated to offer more than sufficient degradation of food proteins in most cereal samples. Furthermore, protease is not part of the AMG/AMY cascade but only works in parallel with them. Thus, the precise amount/ratio of protease is less important, and in this work, for simplicity, we kept the AMY/protease ratio at 1:1. Upon removing the unreacted species, we quickly tested the activity of AMY and AMG using the corresponding commercial kits and found that the molar ratio of 1:1:5 offered the best activity (see Figure S2). Therefore, in this work, we kept this ratio for further characterization and catalytic activity studies. The morphology of the 3-in-1 composite (Figure 4A) is close to the shape shown in Figure 3A−C. Next, we labeled AMY, protease, and AMG with FITC, ATTO-520, and ATTO-647 and acquired confocal fluorescent images at 488, 520, and 647 nm, respectively (Figure 4B−D). To confirm the co-existence of all three enzymes in the "3-in-1" composite, images were also acquired at 534 and 607 nm, which excited the fluorescence of multiple wavelengths and resulted in multiple colors in the images (Figure 4E,F). The PXRD patterns of the "3-in-1" composites were found to be similar to that of the pure Ca-BPDC (Figure 4G), indicating that the enzymes did not affect Ca-BPDC crystallinity. TGA (Figure 4H) and UV data indicated a similar loading capacity (∼2−3%) for the three enzymes together. At the current moment, it is difficult to distinguish the loading capacity of each individual enzyme in the "3-in-1" composites. Note that the fluorescent intensity in the confocal images may not reflect the relative molar ratio of the three enzymes in the 3-in-1 composites precisely due to the uncertainty in fluoro-labeling efficiency and the potential errors in acquiring the images.

Our activity assay of each enzyme in the "3-in-1" composite (Figure S3A), using the kits (see the above section "Catalytic Activity of Each Enzyme in the Composites"), suggests that co-localization of the three enzymes in one MOM does not affect the functionality of AMG and protease (AMY activity cannot be measured since maltose was degraded by AMG). Note that the activity of each enzyme can only be measured separately using the commercial kit of each enzyme (but not simultaneously), although the overall activity of the cascade reaction can be measured by the final product of the starch digestion (glucose; see the section below). The temperature effect on the catalytic performance of the "3-in-1" composite was also examined based on AMG (Figure 4I) and protease activity assays (Figure S3B); the optimal temperature becomes 45−55 °C, which will be kept for further studies. We did not directly compare the catalytic performance of the "3-in-1" approach to the physical mixture of the three free enzymes in solution because the protease would degrade the function of AMY and AMG in a physical mixture without MOM protection.

Reusability and Enhanced Catalytic Performance. To assess the reusability, we assessed the activity of the "3-in-1" composite under continuous catalytic cycles in a short-term (within 2 days) as well as long-term storage using the same activity kits. In the short term, we assessed eight cycles taking place in 2 days (Figure 5A) based on AMG and protease activities (determining the total product generation of each enzyme), which show 75−90% of activity after eight reuse cycles. Note that after five cycles, the PXRD pattern was not

https://doi.org/10.1021/acsami.1c12209
ACS Appl. Mater. Interfaces 2021, 13, 43085−43093

altered substantially (Figure 4G), indicating that the crystallinity of our composite was mostly retained after reuse. During the long-term storage (one catalytic cycle on each day; Figure 5B), we found that the "3-in-1" composite showed ~87 ± 2% activity at Day 10 as compared to that on Day 1 (uncertainty was obtained on three separately prepared samples monitored for 10 days separately). However, the mixture of three composites of single enzyme@Ca-BPDC (under the same final enzyme molar ratio) started showing reduced activity at Day 3 as compared to the "3-in-1" composite and 77 ± 3% at Day 10 (Figure 5B). The composites were believed to be stable as judged by the unchanged PXRD pattern (data similar to Figure 4G cycle five and are not shown). We rationalized this finding as a higher chance of protease to contact and degrade AMY or AMG@Ca-BPDC in the mixture, while in the "3-in-1" composites, this chance was reduced since the three enzymes are on the same surface (which at least reduces the chance of protease degrading other enzymes in the same composite; Figure 6A,B). These data indicate the effectiveness of the "3-in-1"

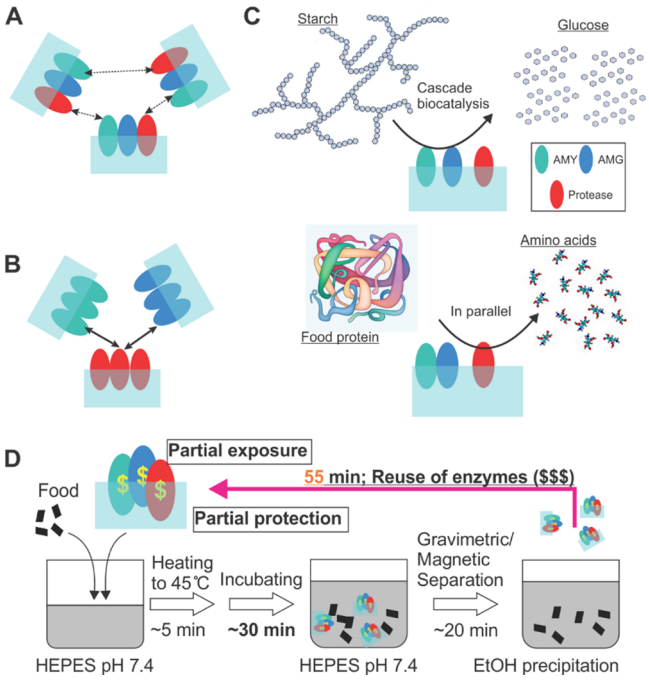


Figure 6. (A,B) Schematic illustration of using the 3-in-1 composite to reduce the long-term protease damage during storage. (C) Illustration of the parallel biocatalysis of polysaccharide degradation (by AMY and AMG) and food protein digestion (by protease). (D) Schematic illustration of the enhanced catalytic performance using our 3-in-1 composite.

composite in biocatalysis and the enhanced reusability as compared to the mixture of the three single-enzyme@Ca-BPDC composites. It has to be pointed out that the "3-in-1" approach is not perfect in preventing protease damage. We observed serious AMY/AMG activity loss (>50%) after a very long term (2 months) of storage at room temperature. However, the protease damage was much reduced (~25%) if the composites were stored at 4 °C for the same period. Thus, the "3-in-1" approach can offer some advantages for biocatalysis. We do not believe that there is noticeable enzyme leaching during the recycled catalytic experiments because of the relatively high reusability of the composites as well as the near-zero protein concentration (probed via UV−vis spectroscopy) in the supernatant of the catalytic mixture after a reaction cycle.

We also compared the catalytic performance, or, more specifically, reaction time, of our 3-in-1 composite to the "official" method. Our comparison was based on monitoring the generation of glucose using the same potato starch. Following Figure 1, using the heat-stable AMY, protease, and AMG, we found that it took slightly more than 150 min to complete the reaction. Meanwhile, using 1 mL of our "3-in-1" composites and the same substrate at 45 °C and pH 7.4 (Figure 4I), we found that it took 0.5−2 h to reach the same glucose production depending on dilution ratios (Figure 5C). Such an exciting finding suggests that the "3-in-1" strategy can be effective in boosting cascade biocatalysis under a single temperature/pH. The reproducibility of the data was also found to be excellent likely due to the simplified experimental procedure.

It has to be noted that, to our best knowledge, we cannot precisely determine the relative ratio of the three enzymes in the 3-in-1 composites using the current protein technology without breaking down the composites/crystals. Because we can only control the initial loading amount of each enzyme before forming the 3-in-1 composites, it may not be a 100% fair comparison between the 3-in-1 composite and the official method. On the other hand, if one considers Ca-BPDC as a platform or method to improve biocatalysis and compare this method to the official method, then some reasonable comparison can still be made. For example, for the same batch of enzyme mixture with the same initial ratio of the three enzymes, it is fair to compare the catalytic efficiency between the three enzymes directly applied for catalysis and three enzymes immobilized on Ca-BPDC first and then applied for catalysis. In fact, introducing Ca-BPDC as a multi-enzyme immobilization platform/method is the key goal of this work. Meanwhile, we are striving our best to develop ways to precisely determine enzyme ratios in the 3-in-1 composites (without breaking down the crystals), but in this work, we would like to present the possibility of simultaneously immobilizing three enzymes on one Ca-BPDC crystal to enhance the catalytic efficiency and reusability.

Advantages of the 3-in-1 Composite, Future Direction, and Broader Impact. Here, for proof-of-principle, we demonstrated the possibility of co-immobilizing three enzymes on the same surface of a Ca-based MOM, which can be synthesized under enzyme-friendly environments, and its application in enhancing food digestion for rapid TDF quantification. The close proximity of AMG and AMY offers an opportunity to improve the catalytic efficiency of polysaccharide degradation via cascade biocatalysis. In parallel, food proteins can be degraded by the protease (Figure 6C). All of the above relies on the partial exposure of the three enzymes on the surface of the MOM, which also offers partial protection against the reaction medium so that a certain number of reuse cycles are possible. More importantly, this allows for the use of one reaction condition (temperature, pH) to complete the food digestion, which significantly enhances the efficiency (Figure 6D). Also, all enzymes can be reused, further reducing the cost of TDF quantification. This effort can potentially change how the food industry quantifies TDF and lead to a decrease in the labor requirement for food production/quantification, eventually reducing food cost/price. Being

https://doi.org/10.1021/acsami.1c12209
ACS Appl. Mater. Interfaces 2021, 13, 43085−43093

able to reuse enzymes and minimizing operation conditions/operations also saves energy, placing a positive impact on the environment. Further improvement may be needed to improve the portion of exposed enzymes (as compared to those completely buried under the MOM surface) as well as enhance the separation/recycling efficiency after each reaction, both of which are our ongoing research directions. Nevertheless, the current data generates excitement for our unique strategy for immobilizing multiple enzymes, especially those involving proteases.

CONCLUSIONS

We found an effective approach to simultaneously immobilize multiple enzymes including a protease on the surface of micrometer-scale co-crystals for cascade/parallel biocatalysis. The synthetic reaction condition is "green", mild, and enzyme-friendly, which assists in retaining the enzyme catalytic activity and minimizes enzyme damage during the immobilization. We proved this strategy on three digestive enzymes that are frequently employed in food quantification, AMY, protease, and AMG, which were separately as well as simultaneously immobilized on a Ca-MOM, Ca-BPDC. While all enzymes showed the expected catalytic behavior, Ca-BPDC protection did enhance the thermal stability of the resultant composite, indicating the possibility of replacing the expensive heat-stable AMY with the regular AMY. The resultant composites are stable enough for multiple catalytic reaction cycles, further improving the cost-efficiency of the immobilized enzymes. The 3-in-1 approach showed advanced reusability over long-term storage as compared to that of the mixture of the three single-enzyme@Ca-BPDC composites. Most importantly, using the 3-in-1 composite, we found it possible to reduce the time required for digesting a commercial starch sample by a factor of ~3 as compared to the widely adopted official method for food digestion, indicating the possibility of boosting TDF quantification in the food industry. To our best knowledge, this is the first experimental report of using aqueous-phase co-crystallization to encapsulate multiple enzymes for multiple biocatalytic reactions in parallel. The significantly enhanced digestion efficiency can potentially change the way to quantify TDF in all commercial food samples, leading to enhanced accuracy in TDF content measurements and reduced food cost. The strategy to prevent protease damage to other enzymes in a multiple-enzyme system can be adapted to other systems/problems involving protease or other damaging enzymes.

EXPERIMENTAL SECTION

Materials and Measurements. All chemicals and biochemical supplies were purchased from commercial sources in high purity; the involved experiments were carried out without purification. All characterization, including PXRD, single-crystal X-ray diffraction, SEM, TGA, and confocal fluorescence spectroscopy of the involved materials follows the published procedures using the equipment described in our recent works.[35,50] Details are provided in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.1c12209.

Details on materials and supplies, procedures to synthesize the needed MOMs and composites, characterization of the composites via SEM, PXRD, confocal fluorescence imaging, and TGA, and activity assays for each enzyme (PDF)

AUTHOR INFORMATION

Corresponding Authors

Yanxiong Pan − *Department of Chemistry and Biochemistry, North Dakota State University, Fargo, North Dakota 58102, United States*; Email: yanxiong.pan@ndsu.edu

Bingcan Chen − *Department of Plant Sciences, North Dakota State University, Fargo, North Dakota 58102, United States*; orcid.org/0000-0002-4989-547X; Email: Bingcan.chen@ndsu.edu

Zhongyu Yang − *Department of Chemistry and Biochemistry, North Dakota State University, Fargo, North Dakota 58102, United States*; orcid.org/0000-0002-3018-3608; Email: zhongyu.yang@ndsu.edu

Authors

Qiaobin Li − *Department of Chemistry and Biochemistry, North Dakota State University, Fargo, North Dakota 58102, United States*; orcid.org/0000-0002-0206-1515

Hui Li − *Department of Plant Sciences, North Dakota State University, Fargo, North Dakota 58102, United States*

Mary Lenertz − *Department of Chemistry and Biochemistry, North Dakota State University, Fargo, North Dakota 58102, United States*

Kailyn Reed − *Department of Chemistry and Biochemistry, North Dakota State University, Fargo, North Dakota 58102, United States*

Drew Jordahl − *Department of Chemistry and Biochemistry, North Dakota State University, Fargo, North Dakota 58102, United States*

Taylor Bjerke − *Sheyenne High School, West Fargo, North Dakota 58078, United States*

Angel Ugrinov − *Department of Chemistry and Biochemistry, North Dakota State University, Fargo, North Dakota 58102, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsami.1c12209

Author Contributions

Z.Y. and Y.P. designed the project. Y.P., Q.L., H.L., and A. U. carried out the experiments and performed the data analysis. H.L. and A.U. assisted in data acquisition and data analysis. Z.Y., Y.P., and B.C. wrote the paper.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work is supported by NSF (#1942596 to Z.Y.) and NDSU New Faculty Startup Funds as well as USDA-NIFA Grant 2021-67021-34002 (to B.C.). We appreciate Prof. Hubbell for generously providing the EPR spectral simulation package.

REFERENCES

(1) Schrittwieser, J. H.; Velikogne, S.; Hall, M.; Kroutil, W. Artificial Biocatalytic Linear Cascades for Preparation of Organic Molecules. *Chem. Rev.* **2018**, *118*, 270−348.

(2) Quinlan, R. J.; Sweeney, M. D.; Lo Leggio, L.; Otten, H.; Poulsen, J.-C. N.; Johansen, K. S.; Krogh, K. B. R. M.; Jorgensen, C. I.; Tovborg, M.; Anthonsen, A.; Tryfona, T.; Walter, C. P.; Dupree, P.;

Xu, F.; Davies, G. J.; Walton, P. H. Insights into the Oxidative Degradation of Cellulose by A Copper Metalloenzyme That Exploits Biomass Components. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 15079−15084.
(3) Chen, C.-C.; Dai, L.; Ma, L.; Guo, R.-T. Enzymatic Degradation of Plant Biomass and Synthetic Polymers. *Nat. Rev. Chem.* **2020**, *4*, 114−126.
(4) Banerjee, A.; Chatterjee, K.; Madras, G. Enzymatic Degradation of Polymers: A Brief Review. *Mater. Sci. Technol.* **2013**, *30*, 567−573.
(5) Dutta, S.; Wu, K. C.-W. Enzymatic Breakdown of Biomass: Enzyme Active Sites, Immobilization, and Biofuel Production. *Green Chem.* **2014**, *16*, 4615−4626.
(6) Himmel, M. E.; Ding, S.-Y.; Johnson, D. K.; Adney, W. S.; Nimlos, M. R.; Brady, J. W.; Foust, T. D. Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production. *Science* **2007**, *315*, 804.
(7) Wang, Y.; Ren, H.; Zhao, H. Expanding the Boundary of Biocatalysis: Design and Optimization of In Vitro Tandem Catalytic Reactions for Biochemical Production. *Crit. Rev. Biochem. Mol. Biol.* **2018**, *53*, 115−129.
(8) Muschiol, J.; Peters, C.; Oberleitner, N.; Mihovilovic, M. D.; Bornscheuer, U. T.; Rudroff, F. Cascade Catalysis: Strategies and Challenges En Route to Preparative Synthetic Biology. *Chem. Commun.* **2015**, *51*, 5798−5811.
(9) Tobaruela, E. d. C.; Santos, A. d. O.; Almeida-Muradian, L. B. d.; Araujo, E. d. S.; Lajolo, F. M.; Menezes, E. W. Application of Dietary Fiber Method AOAC 2011.25 in Fruit and Comparison with AOAC 991.43 Method. *Food Chem.* **2018**, *238*, 87−93.
(10) Dhital, S.; Warren, F. J.; Butterworth, P. J.; Ellis, P. R.; Gidley, M. J. Mechanisms of Starch Digestion by α-Amylase—Structural Basis for Kinetic Properties. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 875−892.
(11) Zakharchenko, A.; Guz, N.; Laradji, A. M.; Katz, E.; Minko, S. Magnetic Field Remotely Controlled Selective Biocatalysis. *Nat. Catal.* **2018**, *1*, 73−81.
(12) Wong, L. S.; Khan, F.; Micklefield, J. Selective Covalent Protein Immobilization: Strategies and Applications. *Chem. Rev.* **2009**, *109*, 4025−4053.
(13) Bommarius, A. S.; Paye, M. F. Stabilizing Biocatalysts. *Chem. Soc. Rev.* **2013**, *42*, 6534−6565.
(14) Secundo, F. Conformational Changes of Enzymes upon Immobilisation. *Chem. Soc. Rev.* **2013**, *42*, 6250−6261.
(15) Howarth, A. J.; Liu, Y.; Li, P.; Li, Z.; Wang, T. C.; Hupp, J. T.; Farha, O. K. Chemical, Thermal and Mechanical Stabilities of Metal-Organic Frameworks. *Nat. Rev. Mater.* **2016**, *1*, 15018.
(16) Zhang, Y.; Ge, J.; Liu, Z. Enhanced Activity of Immobilized or Chemically Modified Enzymes. *ACS Catal.* **2015**, *5*, 4503−4513.
(17) Mitchell, S.; Pérez-Ramírez, J. Mesoporous Zeolites as Enzyme Carriers: Synthesis, Characterization, and Application in Biocatalysis. *Catal. Today* **2011**, *168*, 28−37.
(18) Lee, S. Y.; Show, P. L.; Ko, C.-M.; Chang, Y.-K. A Simple Method for Cell Disruption by Immobilization of Lysozyme on the Extrudate-Shaped Na-Y Zeolite: Recirculating Packed Bed Disruption Process. *Biochem. Eng. J.* **2019**, *141*, 210−216.
(19) Taghizadeh, T.; Talebian-Kiakalaieh, A.; Jahandar, H.; Amin, M.; Tarighi, S.; Faramarzi, M. A. Biodegradation of Bisphenol A by the Immobilized Laccase on Some Synthesized and Modified Forms of Zeolite Y. *J. Hazard. Mater.* **2020**, *386*, 121950.
(20) Liu, B.; Hu, R.; Deng, J. Characterization of Immobilization of an Enzyme in a Modified Y Zeolite Matrix and Its Application to an Amperometric Glucose Biosensor. *Anal. Chem.* **1997**, *69*, 2343−2348.
(21) Küchler, A.; Yoshimoto, M.; Luginbühl, S.; Mavelli, F.; Walde, P. Enzymatic Reactions in Confined Environments. *Nat. Nanotechnol.* **2016**, *11*, 409−420.
(22) Popat, A.; Hartono, S. B.; Stahr, F.; Liu, J.; Qiao, S. Z.; Qing Lu, G. Mesoporous Silica Nanoparticles for Bioadsorption, Enzyme Immobilisation, and Delivery Carriers. *Nanoscale* **2011**, *3*, 2801−2818.
(23) Tu, J.; Boyle, A. L.; Friedrich, H.; Bomans, P. H. H.; Bussmann, J.; Sommerdijk, N. A. J. M.; Jiskoot, W.; Kros, A. Mesoporous Silica Nanoparticles with Large Pores for the Encapsulation and Release of Proteins. *ACS Appl. Mater. Interfaces* **2016**, *8*, 32211−32219.
(24) Lee, C.-H.; Lin, T.-S.; Mou, C.-Y. Mesoporous Materials for Encapsulating Enzymes. *Nano Today* **2009**, *4*, 165−179.
(25) Yang, L.; Gomez-Casado, A.; Young, J. F.; Nguyen, H. D.; Cabanas-Danés, J.; Huskens, J.; Brunsveld, L.; Jonkheijm, P. Reversible and Oriented Immobilization of Ferrocene-Modified Proteins. *J. Am. Chem. Soc.* **2012**, *134*, 19199−19206.
(26) Ding, S.; Cargill, A. A.; Medintz, I. L.; Claussen, J. C. Increasing the Activity of Immobilized Enzymes with Nanoparticle Conjugation. *Curr. Opin. Biotechnol.* **2015**, *34*, 242−250.
(27) Rodrigues, R. C.; Ortiz, C.; Berenguer-Murcia, Á.; Torres, R.; Fernández-Lafuente, R. Modifying Enzyme Activity and Selectivity By Immobilization. *Chem. Soc. Rev.* **2013**, *42*, 6290−6307.
(28) Salmas, G.; DeVries, J. W.; Plank, D. Challenges for Dietary Fiber: Benefits and Costs of New U.S. Regulations. *Cereal Foods World* **2017**, *62*, 88−94.
(29) Verspreet, J.; Damen, B.; Broekaert, W. F.; Verbeke, K.; Delcour, J. A.; Courtin, C. M. A Critical Look at Prebiotics Within the Dietary Fiber Concept. *Ann. Rev. Food Sci. Technol.* **2016**, *7*, 167−190.
(30) Dahl, W. J.; Stewart, M. L. Position of the Academy of Nutrition and Dietetics: Health Implications of Dietary Fiber. *J. Acad. Nutr. Diet.* **2015**, *115*, 1861−1870.
(31) Koh, A.; De Vadder, F.; Kovatcheva-Datchary, P.; Bäckhed, F. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. *Cell* **2016**, *165*, 1332−1345.
(32) Butterworth, P. J.; Warren, F. J.; Ellis, P. R. Human Alpha-Amylase and Starch Digestion: An Interesting Marriage. *Starch/Stärke* **2011**, *63*, 395−405.
(33) Stanley, D.; Farnden, J. F. K.; Macrae, E. Plant Alpha-Amylases: Functions and Roles in Carbohydrate Metabolism. *Biologia* **2005**, *16*, 65−71.
(34) Barrera, G. N.; Tadini, C. C.; León, A. E.; Ribotta, P. D. Use of Alpha-Amylase and Amyloglucosidase Combinations to Minimize the Bread Quality Problems Caused by High Levels of Damaged Starch. *J. Food Sci. Technol.* **2016**, *53*, 3675−3684.
(35) Pan, Y.; Li, Q.; Li, H.; Farmakes, J.; Ugrinov, A.; Zhu, X.; Lai, Z.; Chen, B.; Yang, Z. A General Ca-MOM Platform with Enhanced Acid/Base Stability for Enzyme Biocatalysis. *Chem. Catalysis* **2021**, *1*, 146−161.
(36) Li, Q.; Pan, Y.; Li, H.; Alhalhooly, L.; Li, Y.; Chen, B.; Choi, Y.; Yang, Z. Size-tunable Metal-Organic Framework Coated Magnetic Nanoparticles for Enzyme Encapsulation and Large-Substrate Biocatalysis. *ACS Appl. Mater. Interfaces* **2020**, *12*, 41794−41801.
(37) Farmakes, J.; Schuster, I.; Overby, A.; Alhalhooly, L.; Lenertz, M.; Li, Q.; Ugrinov, A.; Choi, Y.; Pan, Y.; Yang, Z. Enzyme Immobilization On Graphene Oxide (GO) Surface Via One-Pot Synthesis Of GO/Metal-Organic Framework Composites for Large-Substrate Biocatalysis. *ACS Appl. Mater. Interfaces* **2020**, *12*, 23119−23126.
(38) Neupane, S.; Patnode, K.; Li, H.; Baryeh, K.; Liu, G.; Hu, J.; Chen, B.; Pan, Y.; Yang, Z. Enhancing Enzyme Immobilization on Carbon Nanotubes via Metal-Organic Frameworks for Large-Substrate Biocatalysis. *ACS Appl. Mater. Interfaces* **2019**, *11*, 12133−12141.
(39) Pan, Y.; Li, H.; Farmakes, J.; Xiao, F.; Chen, B.; Ma, S.; Yang, Z. How Do Enzymes Orient on Metal-Organic Framework (MOF) Surfaces? *J. Am. Chem. Soc.* **2018**, *140*, 16032−16036.
(40) Pan, Y.; Li, H.; Lenertz, M.; Han, Y.; Ugrinov, A.; Kilin, D.; Chen, B.; Yang, Z. One-pot Synthesis of Enzyme@Metal-Organic Materials (MOM) Biocomposites for Enzyme Biocatalysis. *Green Chem.* **2021**, *23*, 4466−4476.
(41) Seth, S.; Matzger, A. J. Metal-Organic Frameworks: Examples, Counterexamples, and an Actionable Definition. *Cryst. Growth Des.* **2017**, *17*, 4043−4048.
(42) Li, P.; Chen, Q.; Wang, T. C.; Vermeulen, N. A.; Mehdi, B. L.; Dohnalkova, A.; Browning, N. D.; Shen, D.; Anderson, R.; Gómez-Gualdrón, D. A.; Cetin, F. M.; Jagiello, J.; Asiri, A. M.; Stoddart, J. F.; Farha, O. K. Hierarchically Engineered Mesoporous Metal-Organic

https://doi.org/10.1021/acsami.1c12209
ACS Appl. Mater. Interfaces 2021, 13, 43085−43093

Frameworks toward Cell-free Immobilized Enzyme Systems. *Chem* **2018**, *4*, 1022−1034.
(43) Majewski, M. B.; Howarth, A. J.; Li, P.; Wasielewski, M. R.; Hupp, J. T.; Farha, O. K. Enzyme Encapsulation in Metal-Organic Frameworks for Applications in Catalysis. *CrystEngComm* **2017**, *19*, 4082−4091.
(44) Drout, R. J.; Robison, L.; Farha, O. K. Catalytic Applications of Enzymes Encapsulated in Metal-Organic Frameworks. *Coord. Chem. Rev.* **2019**, *381*, 151−160.
(45) Fang, Y.; Powell, J. A.; Li, E.; Wang, Q.; Perry, Z.; Kirchon, A.; Yang, X.; Xiao, Z.; Zhu, C.; Zhang, L.; Huang, F.; Zhou, H.-C. Catalytic Reactions within the Cavity of Coordination Cages. *Chem. Soc. Rev.* **2019**, *48*, 4707−4730.
(46) Lian, X.; Fang, Y.; Joseph, E.; Wang, Q.; Li, J.; Banerjee, S.; Lollar, C.; Wang, X.; Zhou, H.-C. Enzyme-MOF (Metal-Organic Framework) Composites. *Chem. Soc. Rev.* **2017**, *46*, 3386−3401.
(47) Doonan, C.; Riccò, R.; Liang, K.; Bradshaw, D.; Falcaro, P. Metal-Organic Frameworks at the Biointerface: Synthetic Strategies and Applications. *Acc. Chem. Res.* **2017**, *50*, 1423−1432.
(48) Wang, X.; Lan, P. C.; Ma, S. Metal-Organic Frameworks for Enzyme Immobilization: Beyond Host Matrix Materials. *ACS Cent. Sci.* **2020**, *6*, 1497−1506.
(49) Gkaniatsou, E.; Sicard, C.; Ricoux, R.; Mahy, J.-P.; Steunou, N.; Serre, C. Metal-Organic Frameworks: A Novel Host Platform for Enzymatic Catalysis and Detection. *Mater. Horiz.* **2017**, *4*, 55−63.
(50) Sun, Q.; Pan, Y.; Wang, X.; Li, H.; Farmakes, J.; Aguila, B.; Yang, Z.; Ma, S. Mapping out the Degree of Freedom of Hosted Enzymes in Confined Spatial Environments. *Chem* **2019**, *5*, 3184−3195.
(51) Song, Y.; Sun, Q.; Aguila, B.; Ma, S. Opportunities of Covalent Organic Frameworks for Advanced Applications. *Adv. Sci.* **2019**, *6*, 1801410.
(52) Liang, K.; Ricco, R.; Doherty, C. M.; Styles, M. J.; Bell, S.; Kirby, N.; Mudie, S.; Haylock, D.; Hill, A. J.; Doonan, C. J.; Falcaro, P. Biomimetic Mineralization of Metal-Organic Frameworks as Protective Coatings for Biomacromolecules. *Nat. Commun.* **2015**, *6*, 7240.
(53) Lyu, F.; Zhang, Y.; Zare, R. N.; Ge, J.; Liu, Z. One-Pot Synthesis of Protein-Embedded-Supporting Information. *Nano Lett.* **2014**, *14*, 5761−5765.
(54) Wei, T.-H.; Wu, S.-H.; Huang, Y.-D.; Lo, W.-S.; Williams, B. P.; Chen, S.-Y.; Yang, H.-C.; Hsu, Y.-S.; Lin, Z.-Y.; Chen, X.-H.; Kuo, P.-E.; Chou, L.-Y.; Tsung, C.-K.; Shieh, F.-K. Rapid Mechanochemical Encapsulation of Biocatalysts into Robust Metal-Organic Frameworks. *Nat. Commun.* **2019**, *10*, 5002.
(55) Chen, G.; Kou, X.; Huang, S.; Tong, L.; Shen, Y.; Zhu, W.; Zhu, F.; Ouyang, G. Modulating the Biofunctionality of Metal-Organic-Framework-Encapsulated Enzymes through Controllable Embedding Patterns. *Angew. Chem., Int. Ed.* **2020**, *59*, 2867−2874.
(56) Wang, Q.; Zhang, X.; Huang, L.; Zhang, Z.; Dong, S. GOx@ZIF-8(NiPd) Nanoflower: An Artificial Enzyme System for Tandem Catalysis. *Angew. Chem., Int. Ed.* **2017**, *56*, 16082−16085.
(57) Liang, K.; Coghlan, C. J.; Bell, S. G.; Doonan, C.; Falcaro, P. Enzyme Encapsulation in Zeolitic Imidazolate Frameworks: A Comparison between Controlled Co-Precipitation and Biomimetic Mineralisation. *Chem. Commun.* **2016**, *52*, 473−476.
(58) Salgaonkar, M.; Nadar, S. S.; Rathod, V. K. Combi-Metal Organic Framework (Combi-MOF) of α-Amylase and Glucoamylase for One Pot Starch Hydrolysis. *Int. J. Biol. Macromol.* **2018**, *113*, 464−475.
(59) Volkringer, C.; Marrot, J.; Férey, G.; Loiseau, T. Hydrothermal Crystallization of Three Calcium-Based Hybrid Solids with 2,6-Naphthalene- or 4,4′-Biphenyl-Dicarboxylates. *Cryst. Growth Des.* **2008**, *8*, 685−689.
(60) Bernfeld, P. [17] Amylases, α and β. *Methods in Enzymology*; Academic Press, 1955; Vol. *1*, pp 149−158.

https://doi.org/10.1021/acsami.1c12209
ACS Appl. Mater. Interfaces 2021, 13, 43085−43093