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1 **Ca<sup>2+</sup> based metal-organic framework as enzyme preparation to**  
2 **promote the catalytic activity of amylase**

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18

## 19 **Abstract**

20 Enzyme preparation can protect the enzyme conformation in storage, transportation, and  
21 operation process, but excessive additives can affect enzyme performance. In this work, a  $\text{Ca}^{2+}$   
22 based metal-organic framework (UTSA-280) was prepared to encapsulate amylases ( $\alpha$ -  
23 amylase, pullulanase, and glucoamylase) in situ. After release from UTSA-280, the relative  
24 activity of  $\alpha$ -amylase and pullulanase increased to 177.8 and 201.0 %, respectively, as  
25 compared to pure enzymes. Mechanism explorations indicate that amylase released from  
26 UTSA-280 has more  $\text{Ca}^{2+}$  on its surface with stronger combination, which probably leads to  
27 the activation effect to metal ion activating enzyme. Besides, UTSA-280 could protect enzymes  
28 from heat, organic solvent, and trypsin, which is beneficial for the storage, transportation, and  
29 operation of enzyme. Therefore, it is concluded that MOFs could be a promising metal ion  
30 activating enzyme preparation material with activity promoting and protective effects.

31

32 **Keywords:** Metal-organic frameworks; enzyme preparation; amylase; catalysis.

33

34

## 35 1. Introduction

36 Traditional enzyme preparations usually suffer from a number of disadvantages such as  
37 excessive additives, redundant purification operations, and reduced activity, which could  
38 restrain their application [1, 2]. Enzyme preparations often possess a small portion of active  
39 enzyme molecules, while a large amount of additives such as inactive proteins, preservatives,  
40 salts, and polymers are added to maintain the enzyme conformation on storage conditions [3-  
41 6]. However, excessive additives could affect the enzyme performance [7]. On the other hand,  
42 addition of enzyme activators such as metal ions is often ineffective and costly because high  
43 concentrations of activators are needed due to their low accessibility to and weak interactions  
44 with enzyme molecules in the catalytic process [8]. For example, a large amount of ammonium  
45 sulfate usually used in the salting-out process, as well as high operating temperature of spray  
46 drying, can significantly affect the enzyme performance [9]. Therefore, it is of crucial  
47 importance to develop advanced materials for uses in enzyme preparation.

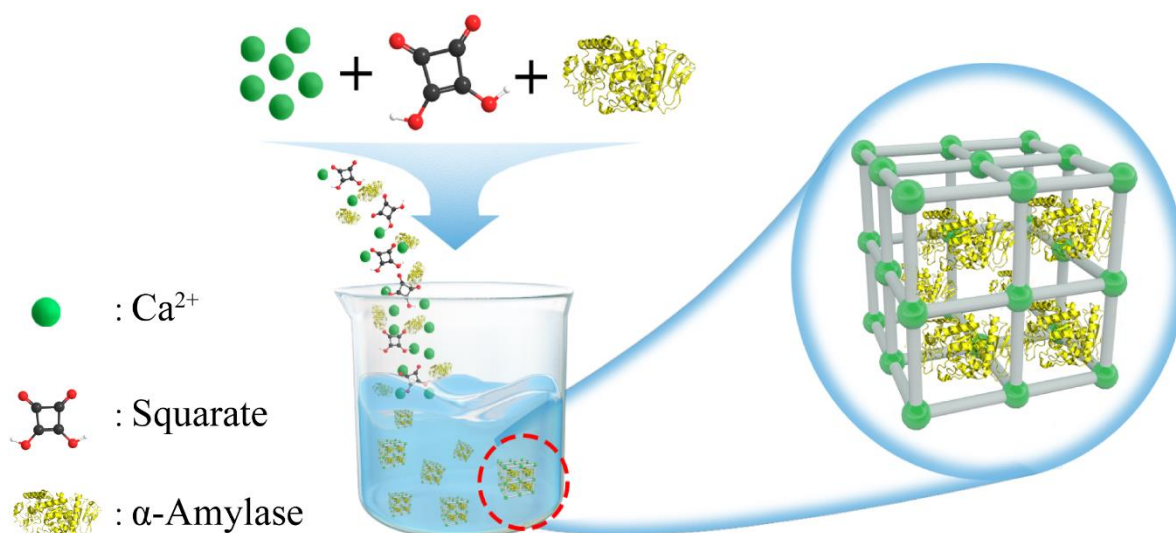
48 In enzyme applications, metal ions ( $Mn^{2+}$ ,  $Zn^{2+}$ ,  $Ca^{2+}$ ,  $Fe^{2+/3+}$ ) are often required to  
49 monitor the activity of enzymes [10]. For example,  $Ca^{2+}$  ions are considered to have crucial  
50 roles for maintaining  $\alpha$ -amylase structures in their correct conformations and improving the  
51 thermal resistance [11-13]. Mao and Kinsella found that banana  $\alpha$ -amylase activity increased  
52 significantly when calcium was incorporated into the assay medium [14].  $Mg^{2+}$  and  $K^+$  are  
53 typical activators of methionine adenosyltransferase (MAT) from all sources which can  
54 enhance its activity and reaction rate [15]. Therefore, metal ions are excellent stabilizer and  
55 activator of enzymes, and incorporating metal ions as a component is a promising approach for  
56 metal ion activating enzyme preparations.

57 Metal-organic frameworks (MOFs) are an emerging class of crystalline materials  
58 assembled by organic links and metal ions or clusters. Owing to their unique properties such  
59 as high surface areas, ultra high porosity, high thermal stability, and good surface chemistry,

60 MOFs have become an ideal material for the preparation of enzyme/MOF biocomposites [16].  
61 In fact, MOFs present several advantages for enzyme encapsulation, i.e. mild preparation  
62 environments (room temperature, aqueous solution), high loading efficiency and good  
63 protection for enzymes [17, 18]. In the past decade, great efforts have been made to develop  
64 enzyme@MOF composites for enzyme immobilization. However, these heterogeneous  
65 biocatalytic systems suffer from the decrease of enzyme activity caused by the immobilization  
66 and diffusion processes. Interestingly, unstable MOFs could degrade under mild conditions,  
67 releasing metal ions which could become enzyme activator. Therefore, enzyme-MOF  
68 composites could be promising as high-performance enzyme preparations.

69 Amylases are a class of industrial enzymes representing approximately 30% of the world  
70 enzyme production [19]. Amylases are used in many industrial processes such as food,  
71 fermentation, paper, sugar and pharmaceutical industries. Calcium ions play an important role  
72 in monitoring the structure, activity, and thermal stability of some amylases [11, 12], thus  
73 affecting their performance [20, 21]. However, there is no report on the use of  $\text{Ca}^{2+}$  based MOF  
74 for amylase preparation in spite of its potential in activating amylases.

75 In this study, a  $\text{Ca}^{2+}$  based MOF, namely UTSA-280, was synthesized as  $\text{Ca}^{2+}$  activating  
76 enzyme preparation for  $\alpha$ -amylase and pullulanase as shown in Scheme 1. UTSA-280 could  
77 efficiently encapsulate enzymes in-situ under mild synthesis conditions. The release  
78 performance of enzyme@UTSA-280 and the activity of released enzymes were evaluated.  
79 Mechanism experiments were performed to understand the strong increase in enzyme activity.  
80 The enzyme protection performance and size control of enzyme@UTSA-280 were also  
81 explored to further evaluate the potential of UTSA-280 as enzyme preparation material.



82

83 **Scheme 1.** Schematic description of enzyme@UTSA-280 preparation by in-situ method.

84

## 85 2. Materials and methods

### 86 2.1 Materials and reagents

87 Squaric acid (98%), calcium nitrate tetrahydrate ( $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 99%), starch  
 88 (analytical grade) and pullulan (analytical grade) were purchased from Mucklin (Shanghai,  
 89 China).  $\alpha$ -Amylase (from *Bacillus subtilis*, 4000  $\text{U g}^{-1}$ ) was obtained from Coolaber (Beijing,  
 90 China). Glucoamylase (from *Aspergillus niger* variant strain,  $>100,000 \text{ U g}^{-1}$ ) was purchased  
 91 from Meilunbio (Dalian, China). Pullulanase (from *Klebsiella*,  $>1000 \text{ U g}^{-1}$ ) was obtained from  
 92 Mucklin (Shanghai, China). Citric acid ( $\geq 99.5\%$ ), sodium citrate dihydrate (99%), fluorescein  
 93 isothiocyanate (FITC,  $\geq 90\%$ ), HEPES buffer solution (pH 7.0), MES monohydrate (99%),  
 94 polyvinylpyrrolidone (PVP, MW: 10 kDa) were supplied by Mucklin (Shanghai, China). All  
 95 enzymes were further purified before use (Supplementary data). All solvents were of analytical  
 96 grade and used as received.

97

### 98 2.2 Synthesis of materials

#### 99 2.2.1 Synthesis of UTSA-280

100 UTSA-280 was synthesized as follows. Aqueous solutions of  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  (2 mL,  
101 26 mg mL<sup>-1</sup>) and sodium squarate (2 mL, 15 mg mL<sup>-1</sup>) were mixed under stirring. The reaction  
102 then proceeded for 1 h at room temperature. The resulted white crystals were washed 3 times  
103 with water, and collected by centrifugation.

104

### 105 2.2.2 Synthesis of enzyme@UTSA-280

106 Enzyme@UTSA-280 was prepared by using in situ method. A solution of  $\text{Ca}(\text{NO}_3)_2 \cdot$   
107  $4\text{H}_2\text{O}$  (2 mL, 26 mg mL<sup>-1</sup>) was mixed with a solution of sodium squarate (2 mL, 15 mg mL<sup>-1</sup>)  
108 containing enzymes at various concentrations, and reaction proceeded for 1 h at room  
109 temperature. The resulted enzyme@UTSA-280 crystals were washed 3 times with water, and  
110 collected by centrifugation.

111

### 112 2.3 Calculation of encapsulation efficiency and loading content

113 The encapsulation efficiency and loading content of enzymes was determined via standard  
114 Bradford assay. Typically, the enzyme encapsulation efficiency of the preparations was  
115 calculated via determining the enzyme concentration of the supernatant solution before and  
116 after encapsulation by Bradford assay. The encapsulation efficiency and loading content were  
117 calculated from the following formulae:

$$\text{Encapsulation efficiency \%} = \frac{C_0 - C_s}{C_0} \times 100\% \quad (1)$$

118 Where  $C_0$  is the initial enzyme concentration before encapsulation, and  $C_s$  is the enzyme  
119 concentration of supernatant after encapsulation.

$$\text{Loading content (mg mg}^{-1}\text{)} = \frac{W_0 - W_s}{W_M - (W_0 - W_s)} \quad (2)$$

120 Where  $W_0$  is the weight of enzyme before encapsulation,  $W_s$  is the enzyme weight in  
121 supernatant after encapsulation, and  $W_M$  is the weight of enzyme@UTSA-280.

122

## 123 2.4 Characterization

124 X-ray diffraction (XRD) patterns were recorded in the  $2\theta$  range from 5 to  $50^\circ$  on a Rigaku  
125  $d_{\max}$  2500 diffractometer using Cu  $K\alpha$  ( $\lambda = 1.5418 \text{ \AA}$ ) radiation, with a scan speed of 1 sec/step,  
126 and a step size of  $0.02^\circ$ . Fourier transform infrared (FT-IR) spectra were registered using a  
127 Thermo Fisher spectrometer in ATR mode. 128 scans were made for each measurement in the  
128 wavenumber range from 500 to  $4000 \text{ cm}^{-1}$  at a resolution of  $2 \text{ cm}^{-1}$ . Circular dichroism (CD)  
129 measurements were performed using a spectropolarimeter BioLogic MOS-450. The spectra  
130 were collected at a rate of 60 nm per minute and a response time of 16 s. Scanning electron  
131 microscopy (SEM) was realized using JSM-7610F field-emission microscope. Freeze-dried  
132 crystals were gold-coated for 90 s and observed using an electron beam at accelerating voltage  
133 of 10.0 kV.

134

## 135 2.5 Activity test of $\alpha$ -amylase

136 The activity of  $\alpha$ -amylase was determined according to the 3, 5-dinitrosalicylic acid  
137 (DNS) method [12]. 2 mg of  $\alpha$ -amylase@UTSA-280 was placed in 1 mL of citrate buffer (50  
138 mM, pH 6.0), and released  $\alpha$ -amylase was determined by using Bradford method. The  
139 concentration of original and released  $\alpha$ -amylase was adjusted to  $0.1 \text{ mg mL}^{-1}$ .  $50 \mu\text{L}$  of original  
140  $\alpha$ -amylase or released  $\alpha$ -amylase ( $50 \mu\text{g mL}^{-1}$ ) were added in 5 mL of HEPES buffer (50 mM,  
141 pH 7.0) containing 1.0 wt% soluble starch. The relative activity (%) was the ratio between the  
142 activities of released and original  $\alpha$ -amylases. All measurements were repeated three times.  
143 The activity of pullulanase and glucoamylase was determined using the same method, and the  
144 detailed steps are described in Supplementary data.

145

## 146 2.6 Enzymatic kinetic parameters determination



147 Enzymatic kinetic parameters were determined according to the literature [22]. The  
148 kinetic constants ( $V_{max}$ ,  $K_m$ ,  $K_{cat}$ ) were derived from Lineweaver Burke plot by using eq. 3 and  
149 eq. 4.

$$1/V = (K_m/V_{max}) * 1/[S] + 1/V_{max} \quad (3)$$

$$K_{cat} = V_{max}/[e] \quad (4)$$

150 Where [S] is the substrate concentration, and [e] is the molar concentration of  $\alpha$ -amylase.

151 To determine the kinetic parameters of released and pure  $\alpha$ -amylase, experiments were  
152 performed at 37 °C with different concentrations of soluble starch of 0.1 to 2.0 wt% in HEPES  
153 buffer solution (pH 7.0), at an initial enzyme concentration of 10  $\mu\text{g mL}^{-1}$  and a reaction time  
154 of 1 min. The amount of produced glucose was determined by DNS method.

155

## 156 2.7 Enzyme protection assay

157 Pure  $\alpha$ -amylase powder (10 mg) and  $\alpha$ -amylase@UTSA-280 composite (10 mg) were  
158 treated by exposure to heat (80 °C), trypsin solution (1 mg  $\text{mL}^{-1}$ ), and acetone for 1 h. After  
159 treatment,  $\alpha$ -amylase was released from UTSA-280 in citrate buffer (pH 6.0), and the enzyme  
160 concentration was determined by using Bradford method. Then, the activity of pure and  
161 released  $\alpha$ -amylases was measured by using DNS method and compared to untreated enzymes.

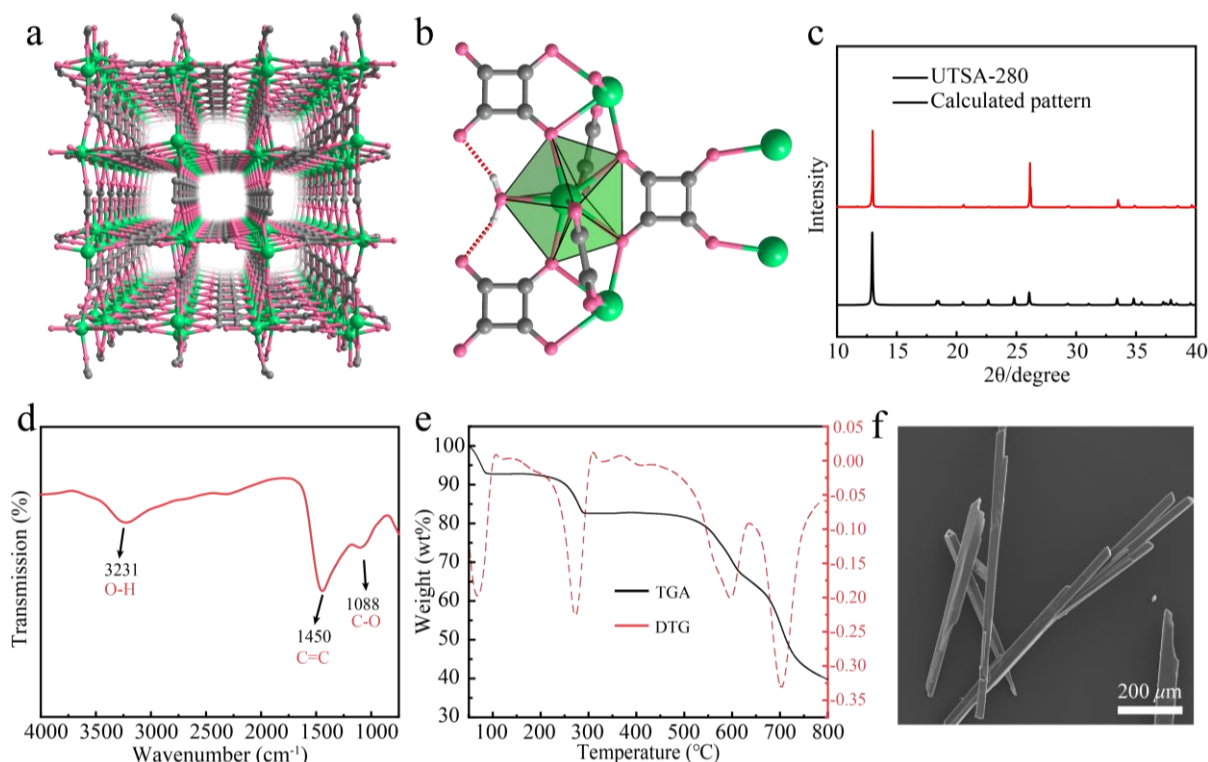
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## 163 3. Results and discussion

### 164 3.1 Characterization of UTSA-280

165 UTSA-280 was synthesized by reaction between calcium nitrate hexahydrate and sodium  
166 squarate under mild conditions in water [23]. Sodium squarate was previously obtained by  
167 neutralization of squaric acid using NaOH, as shown in Supplementary data [24]. In as-  
168 synthesized UTSA-280, the Ca atom (pentagonal bipyramidal) is coordinated by seven O atoms  
169 from five squarate linkers and one water molecule, resulting in one-dimensional channels (Fig.

170 1a). The hydrogen bonding between coordinated H<sub>2</sub>O molecules and O atoms of squarate  
 171 linkers could further stabilize the framework by restrain the rotation of the organic linkers (Fig.  
 172 1b). The XRD pattern of UTSA-280 exhibits high crystallinity in comparison with the  
 173 calculated pattern, as shown in Fig. 1c. The FT-IR spectrum of UTSA-280 shows various  
 174 characteristic signals (Fig. 1d), especially those at 1088, 1450, and 3231 cm<sup>-1</sup>, corresponding  
 175 to C-O, C=C, and O-H bonds, respectively. TGA data reveal the good thermal stability of  
 176 UTSA-280 (Fig. 1e). A weight loss of 7.2 % is observed from 50 to 96 °C, corresponding to  
 177 the loss of H<sub>2</sub>O molecules in the channels. Weight loss of 10.2 % between 156 to 303 °C  
 178 corresponds to the loss of H<sub>2</sub>O coordinated with Ca<sup>2+</sup> ions. Weight loss beyond 392 °C results  
 179 from the framework decomposition. UTSA-280 exhibits a cylindrical crystal structure as  
 180 shown in Fig. 1f, in agreement with high crystallinity. All these data demonstrate the successful  
 181 synthesis of uniform UTSA-280 crystals.



182  
 183 **Fig. 1** Structure and characterization of UTSA-280: (a) Crystal structure of UTSA-280,  
 184 showing one-dimensional channels viewed along the [001] direction. Green, red, and grey balls

185 represent Ca, O and C atoms, respectively. (b) Local coordination environment of the squarate  
186 linkers and Ca atoms. (c) XRD pattern of UTSA-280. (d) Infrared spectrum of UTSA-280. (e)  
187 TGA and DTG patterns of UTSA-280. (f) SEM image of UTSA-280.

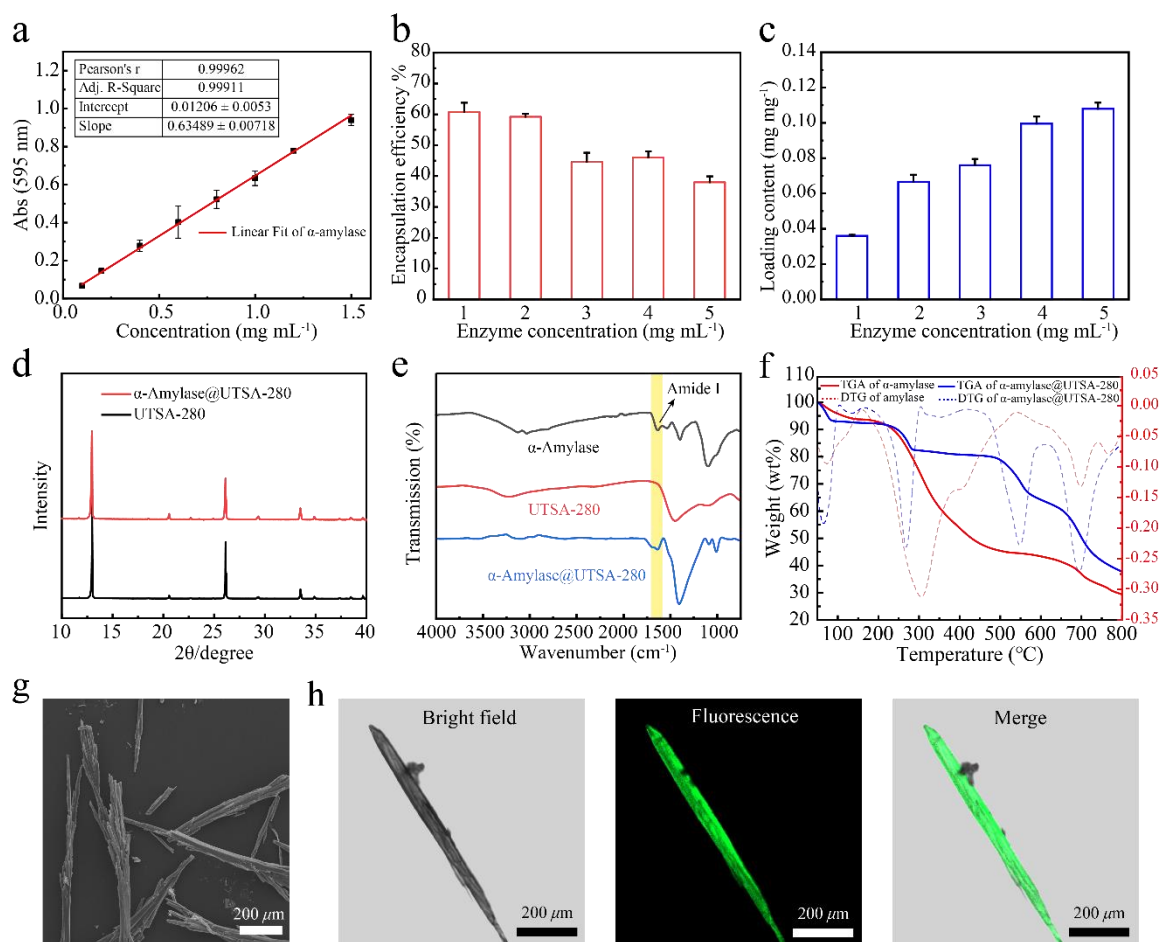
188

### 189 3.2 Characterization of $\alpha$ -amylase@UTSA-280

190  $\alpha$ -amylase@UTSA-280 was synthesized in-situ under mild conditions. In this reaction,  $\alpha$ -  
191 amylase is added together with metal ions and ligand, and  $\alpha$ -amylase is loaded during the  
192 crystallization process of MOFs. Bradford method was employed to determine the enzyme  
193 concentration in  $\alpha$ -amylase@UTSA-280. A calibration curve was first established using  $\alpha$ -  
194 amylase as the standard. A good linearity was obtained from the regression line in the 0.1-1.5  
195 mg mL<sup>-1</sup> range (Fig. 2a). The encapsulation efficiency and loading content of  $\alpha$ -  
196 amylase@UTSA-280 were then calculated (Fig. 2b-c). With increasing the initial enzyme  
197 concentration from 1.0 to 5.0 mg mL<sup>-1</sup>, the encapsulation efficiency decreases from 61% to  
198 38%, and the loading content increases from 0.036 to 0.109 mg mg<sup>-1</sup>.  $\alpha$ -Amylase@UTSA-280  
199 at enzyme concentration of 2 mg mL<sup>-1</sup> was selected for subsequent experiments as it has  
200 suitable encapsulation efficiency ( $59.2 \pm 1.0$  %) and loading content ( $0.070 \pm 0.004$  mg mg<sup>-1</sup>).

201 Fig. 2d shows the XRD patterns of  $\alpha$ -amylase@UTSA-280 and UTSA-280. Both present  
202 the same diffraction peaks, indicating that enzyme encapsulation didn't affect the crystalline  
203 structure and crystallinity of UTSA-280, even at an enzyme concentration of 5 mg mL<sup>-1</sup> in the  
204 synthesis process (Fig. S1). In the FT-IR spectra (Fig. 2e), a band assigned to the bending  
205 vibration of the N-H group is detected at 1646 cm<sup>-1</sup> for  $\alpha$ -amylase and  $\alpha$ -amylase@UTSA-280,  
206 but not for UTSA-280, thus demonstrating the successful synthesis of  $\alpha$ -amylase@UTSA-280.  
207 The thermal stability of  $\alpha$ -amylase@UTSA-280 was evaluated by TGA under nitrogen  
208 atmosphere in comparison with  $\alpha$ -amylase (Fig. 2f). In the TGA thermogram of  $\alpha$ -  
209 amylase@UTSA-280, a weight loss of 6.9 % is observed from 50 to 96 °C, corresponding to

210 the loss of H<sub>2</sub>O molecules. Weight loss of 9.9% between 168 to 287 °C belongs to  $\alpha$ -amylase  
 211 decomposition and the loss of H<sub>2</sub>O coordinated with Ca ions in UTSA-280. Weight loss beyond  
 212 430 °C mainly results from the MOF framework. In contrast, an almost continuous weight loss  
 213 is observed for  $\alpha$ -amylase beyond 200°C due to its decomposition. In SEM image of  $\alpha$ -  
 214 amylase@UTSA-280 (Fig. 2g), rod-like aggregates of crystals are observed.  $\alpha$ -Amylase was  
 215 labeled by FITC (Supplementary data). Confocal laser scanning microscopy (CLSM) shows a  
 216 uniform distribution of FITC-labeled  $\alpha$ -amylase in UTSA-280 (Fig. 2h). All these data prove  
 217 that  $\alpha$ -amylase is successfully encapsulated in UTSA-280.



218  
 219 **Fig. 2.** Characterization of  $\alpha$ -amylase@UTSA-280: (a) linear fitting of  $\alpha$ -amylase by Bradford  
 220 method; (b-c) the encapsulation efficiency and loading content of  $\alpha$ -amylase@UTSA-280; (d)  
 221 XRD patterns of UTSA-280 and  $\alpha$ -amylase@UTSA-280; (e) FT-IR spectra of  $\alpha$ -amylase,  
 222 UTSA-280, and  $\alpha$ -amylase@UTSA-280; (f) TGA and DTG patterns of  $\alpha$ -amylase and  $\alpha$ -

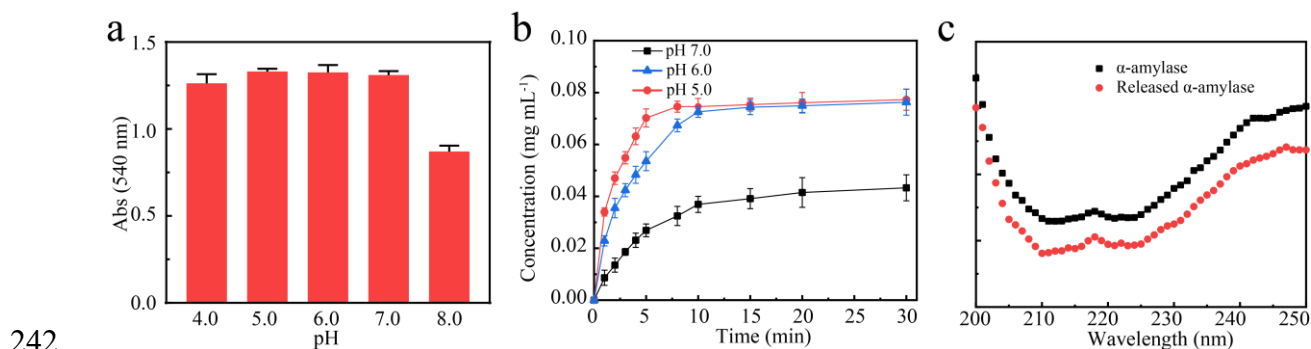
223 amylase@UTSA-280; (g) SEM image of  $\alpha$ -amylase@UTSA-280; (h) CLSM images  $\alpha$ -  
224 amylase@UTSA-280.

225

### 226 3.3 Enzyme release behavior of $\alpha$ -amylase@UTSA-280

227 The absorbance of  $\alpha$ -amylase was determined at 540 nm in MES buffers at various pH in  
228 order to determine the suitable releasing pH value. As shown in Fig. 3a,  $\alpha$ -amylase exhibits  
229 high activity between pH 5.0-7.0. Therefore, the enzyme release behavior from  $\alpha$ -  
230 amylase@UTSA-280 was evaluated in this pH range (Fig. 3b). Biphasic release profiles are  
231 observed in all cases with initial faster release followed by slower release. The release rate  
232 increases with pH decrease, which could be attributed to the accelerated degradation of MOFs  
233 under acidic conditions. The degradability and enzyme loading properties of UTSA-280 make  
234 it a potential enzyme preparation material.

235 Maintaining enzyme conformation during the storage and operation process is crucial for  
236 the application of enzymes. Thus, the secondary structure of  $\alpha$ -amylase was determined using  
237 circular dichroism (CD) to confirm the protective effect of MOFs. Released  $\alpha$ -amylase was  
238 collected via ultrafiltration with 8 kDa MWCO device to remove impurities (digested ligands  
239 and metal salts). Notably, both pristine  $\alpha$ -amylase and released  $\alpha$ -amylase exhibit a typical  $\alpha$ -  
240 helix structure without any difference [25], suggesting that the in-situ encapsulation process  
241 did not affect the conformation of enzyme (Fig. 3c).

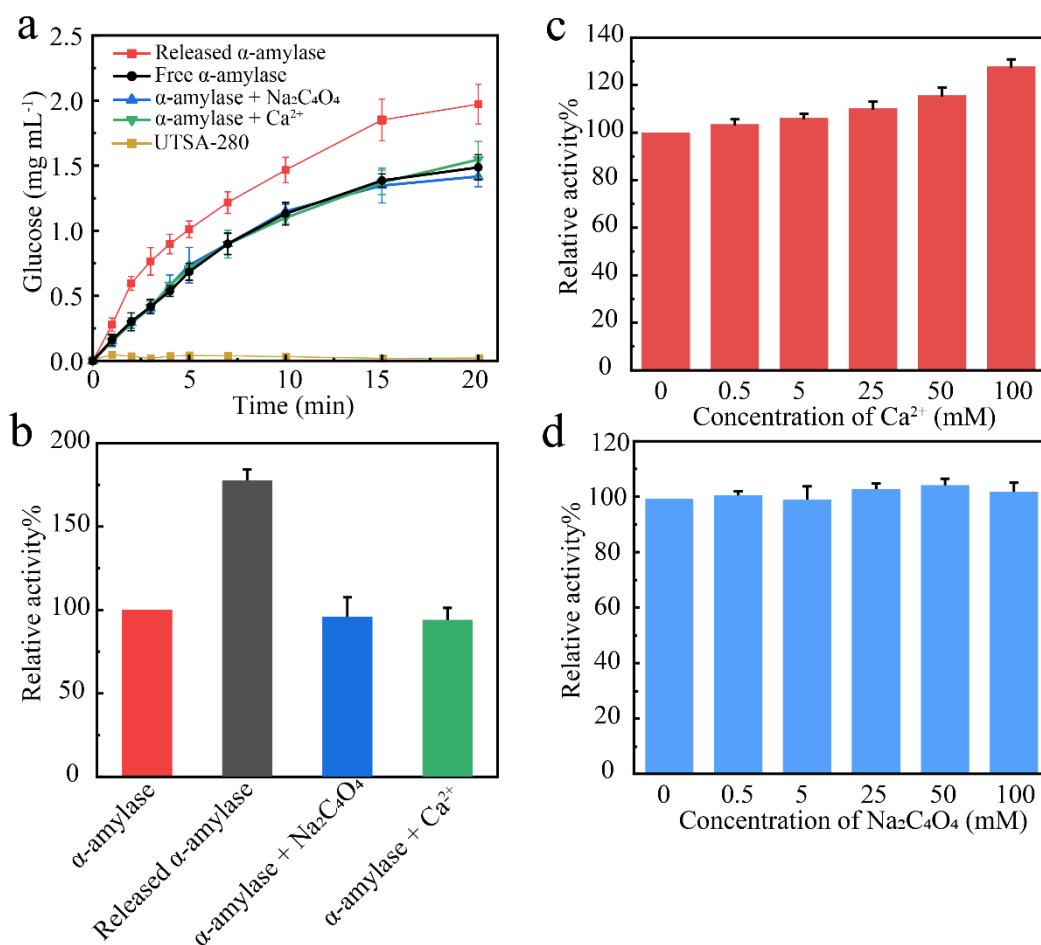


243 **Fig. 3** (a) Absorbance of  $\alpha$ -amylase at different pH values; (b) enzyme release profiles in citrate  
244 buffer (50 mM, pH 5.0-7.0); (c) CD spectra of pristine  $\alpha$ -amylase and  $\alpha$ -amylase released from  
245 UTSA-280.

246

#### 247 *3.4 Catalytic activity test*

248 The catalytic activity of released  $\alpha$ -amylase was determined using soluble starch as the  
249 substrate in HEPES buffer (50 mM, pH 7.0). The concentration of produced glucose was  
250 measured using a previously established calibration curve (Fig. S2) [26]. Fig. 4a shows the  
251 glucose concentration changes as a function of reaction time in the presence of pristine  $\alpha$ -  
252 amylase, released  $\alpha$ -amylase,  $\alpha$ -amylase +  $\text{Na}_2\text{C}_4\text{O}_4$ ,  $\alpha$ -amylase +  $\text{Ca}^{2+}$ , and UTSA-280. The  
253 added concentrations of  $\text{Ca}^{2+}$  and  $\text{Na}_2\text{C}_4\text{O}_4$  were 11.75 mM, *i.e.* equal to that of released  $\alpha$ -  
254 amylase. Higher glucose concentration is obtained for released  $\alpha$ -amylase during the whole  
255 reaction period up to 20 min. The relative activity of released  $\alpha$ -amylase is 177.8% compared  
256 to pristine  $\alpha$ -amylase. In contrast, direct addition of  $\text{Ca}^{2+}$  or squaric acid has no effect on the  
257 enzyme activity, and pure UTSA-280 material has no catalytic effect. Further investigation  
258 suggests that a relatively high concentration (100 mM) of free  $\text{Ca}^{2+}$  is required to achieve a  
259 relative activity of 127.0 % at an incubation enzyme concentration of 2 mg mL<sup>-1</sup> (Fig. 4c), and  
260 the  $\text{Ca}^{2+}$  concentration during synthesis (79.2 mM) does not have such a promoting effect. Fig.  
261 4d shows that sodium squarate has little influence on the activity of  $\alpha$ -amylase. Therefore, the  
262 enhanced activity is likely related to the release process. These results suggest that metal ion  
263 activators from the enzyme/MOF matrix could strongly ameliorate the enzymatic activity,  
264 which might be attributed to the enhanced interactions between calcium ions and enzyme  
265 molecules during the release process.



266

267 **Fig. 4** (a) Glucose concentration changes as a function of reaction time in the presence of  
 268 pristine  $\alpha$ -amylase, released  $\alpha$ -amylase,  $\alpha$ -amylase + Na<sub>2</sub>C<sub>4</sub>O<sub>4</sub>,  $\alpha$ -amylase + Ca<sup>2+</sup>, and UTSA-  
 269 280; (b) relative activity of  $\alpha$ -amylase, released  $\alpha$ -amylase,  $\alpha$ -amylase + Na<sub>2</sub>C<sub>4</sub>O<sub>4</sub>, and  $\alpha$ -  
 270 amylase + Ca<sup>2+</sup>; (c) relative activity of  $\alpha$ -amylase in the presence of Ca<sup>2+</sup> at different  
 271 concentrations; (d) relative activity of  $\alpha$ -amylase in the presence of Na<sub>2</sub>C<sub>4</sub>O<sub>4</sub> at different  
 272 concentrations. Test conditions: 1 wt% soluble starch solution in HEPES buffer (50 mM, pH  
 273 7.0) at 37 °C.

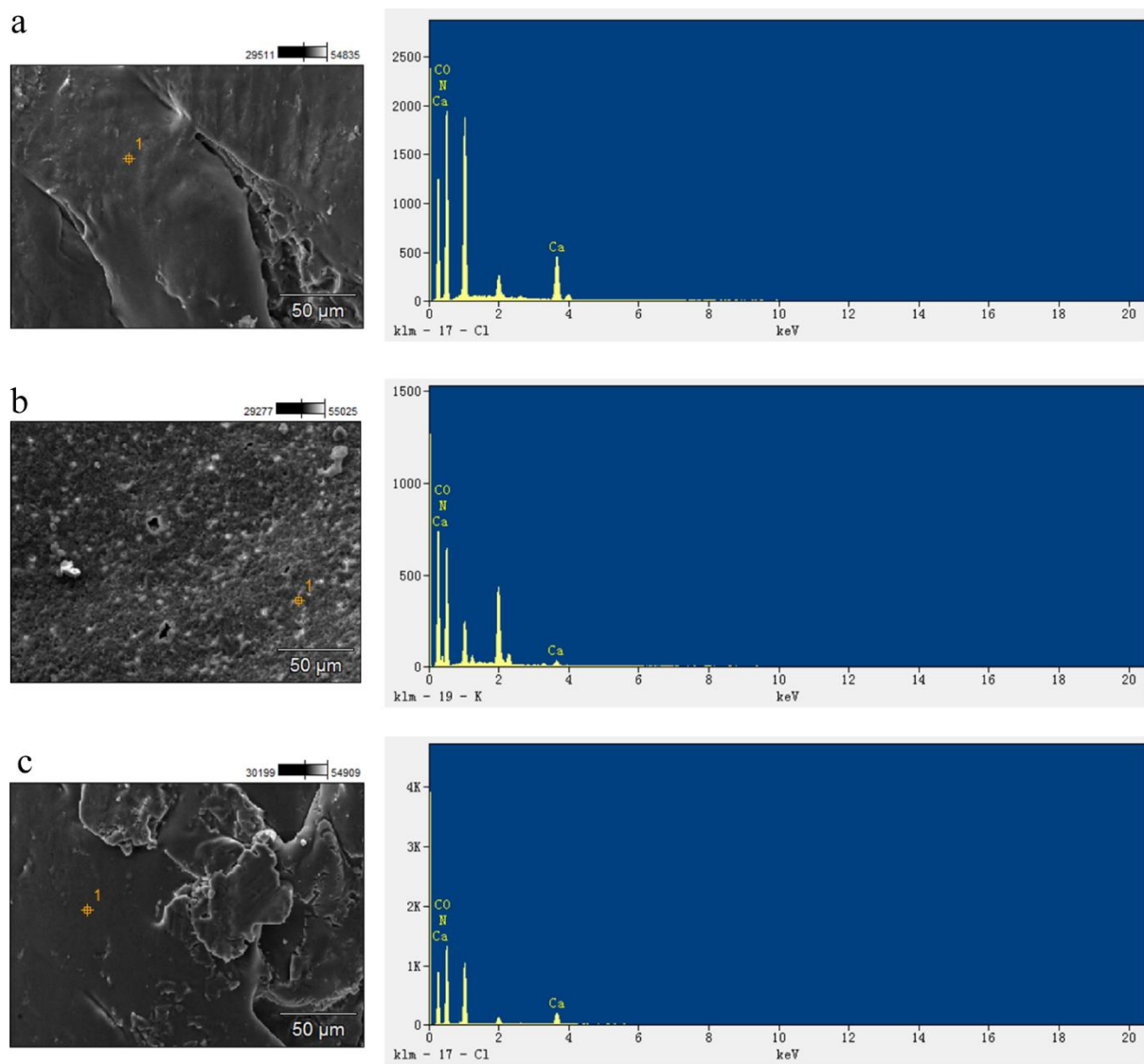
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### 275 3.5 Exploration of mechanism for the increase of enzymatic activity

276 Energy dispersive X-ray spectroscopy (EDX) was used to characterize the released  $\alpha$ -  
 277 amylase in comparison with purchased one so as to figure out the mechanism for the increase

278 of enzymatic activity. Released  $\alpha$ -amylase and  $\alpha$ -amylase incubated with  $\text{Ca}^{2+}$  were collected  
279 via ultrafiltration, 3 times washing with deionized water to remove free  $\text{Ca}^{2+}$ , and freeze-  
280 drying. As shown in Fig. 5a-c and Table 1, released  $\alpha$ -amylase has higher  $\text{Ca}^{2+}$  content ( $10.7$   
281  $\pm 2.3$  % of weight,  $4.3 \pm 1.1$  % of atom) than purchased  $\alpha$ -amylase ( $2.8 \pm 0.4$  % of weight,  $0.7$   
282  $\pm 0.1$  % of atom) and  $\alpha$ -amylase incubated with  $\text{Ca}^{2+}$  ( $4.7 \pm 0.6$  % of weight,  $1.6 \pm 0.2$  % of  
283 atom). Inductively coupled plasma-optical emission spectrometry (ICP-OES) was also used to  
284 quantify the  $\text{Ca}^{2+}$  content of these three groups, as shown in Fig. S3. The  $\text{Ca}^{2+}$  content of  
285 released  $\alpha$ -amylase, pristine  $\alpha$ -amylase, and  $\alpha$ -amylase incubated with  $\text{Ca}^{2+}$  are  $99.7 \pm 3.3$ ,  
286  $4.7 \pm 1.1$ , and  $29.2 \pm 4.7 \mu\text{g mg}^{-1}$ , respectively. ICP-OES data are consistent with those of EDX,  
287 which revealed that MOF encapsulation enhances enzyme activity more effectively than direct  
288 addition of metal ions. These results suggest that the improved enzymatic activity could result  
289 from more interaction between  $\text{Ca}^{2+}$  ions and  $\alpha$ -amylase. The enzymatic kinetic parameters of  
290  $\alpha$ -amylase released from UTSA-280 were determined by using Lineweaver Burke plot (Fig.  
291 S4) and Michaelis-Menten model [22, 27]. The results revealed that released  $\alpha$ -amylase has a  
292  $V_{max}$  of  $0.25 \text{ mM s}^{-1}$ ,  $K_m$  of  $0.27 \text{ mM}$ , and  $K_{cat}$  of  $1.25 \times 10^3 \text{ s}^{-1}$ , while untreated  $\alpha$ -amylase has  
293  $V_{max}$ ,  $K_m$  and  $k_{cat}$  of  $0.14 \text{ mM s}^{-1}$ ,  $0.46 \text{ mM}$  and  $0.69 \times 10^3 \text{ s}^{-1}$ , respectively (Table 2). The lower  
294  $K_m$  value of released  $\alpha$ -amylase implies higher affinity towards substrates while the higher  $K_{cat}$   
295 of released  $\alpha$ -amylase could be attributed to the activation effect of  $\text{Ca}^{2+}$  from UTSA-280,  
296 which is consistent with literature data [28].





297

298 **Fig. 5.** Energy dispersive X-ray spectroscopy (EDX) of (a) released  $\alpha$ -amylase, (b) pristine  $\alpha$ -  
 299 amylase, and (c)  $\alpha$ -amylase incubated with  $\text{Ca}^{2+}$  (11.75 mM) for 1 h. (n=3)

300

301 **Table 1.** Element contents of released  $\alpha$ -amylase, pristine  $\alpha$ -amylase, and  $\alpha$ -amylase incubated  
 302 with  $\text{Ca}^{2+}$  (11.75 mM) for 1 h determined by EDX (n=3)

	Element	Weight %	Atom %
Released $\alpha$ -amylase	C	$15.8 \pm 1.8$	$20.9 \pm 1.9$
	O	$65.9 \pm 0.8$	$65.9 \pm 0.4$

	N	$7.8 \pm 0.2$	$8.9 \pm 0.4$
	Ca	$10.7 \pm 2.3$	$4.3 \pm 1.1$
	C	$22.3 \pm 0.5$	$29.3 \pm 2.1$
	O	$60.1 \pm 3.6$	$53.0 \pm 4.8$
Pristine $\alpha$ -amylase	N	$14.8 \pm 0.1$	$17.3 \pm 2.7$
	Ca	$2.8 \pm 0.4$	$0.7 \pm 0.1$
	C	$12.7 \pm 0.5$	$14.6 \pm 3.1$
	O	$74.2 \pm 1.3$	$72.7 \pm 2.1$
$\alpha$ -amylase incubated with $\text{Ca}^{2+}$	N	$8.5 \pm 1.2$	$11.2 \pm 1.3$
	Ca	$4.7 \pm 0.6$	$1.6 \pm 0.2$

303

304 Two other enzymes, pullulanase ( $\text{Ca}^{2+}$  activating enzyme) and glucoamylase (non  $\text{Ca}^{2+}$   
305 activating enzyme) were also encapsulated in UTSA-280 to better understand the activation  
306 effect of UTSA-280. The enzyme concentration was determined by Bradford method using  
307 enzyme standard calibration curves (Fig. S5-6). And the encapsulation efficiency and loading  
308 content of pullulanase ( $64.1 \pm 2.4\%$ ,  $0.150 \pm 0.011 \text{ mg mg}^{-1}$ ) and glucoamylase ( $44.2 \pm 3.2\%$ ,  
309  $0.110 \pm 0.004 \text{ mg mg}^{-1}$ ) were then obtained (Fig. S7). FT-IR spectra and CLSM data prove the  
310 successful encapsulation of the two enzymes in UTSA-280 (Fig. S8-10). In addition, XRD and  
311 SEM data demonstrate the high crystallinity of UTSA-280 after enzyme encapsulation (Fig.  
312 S11-12). Then, pullulanase and glucoamylase were released from UTSA-280 and their catalytic  
313 activity was evaluated in the pH range from pH 4.0 to 8.0 (Fig. S13-14). As shown in Fig. S15,  
314 the activity of released pullulanase shows a dramatic increase ( $201 \pm 28 \%$ ), which may be  
315 attributed to the fact that pullulanase is a  $\text{Ca}^{2+}$  activated enzyme. Meanwhile, glucoamylase,  
316 which is not a  $\text{Ca}^{2+}$  activated enzyme, shows no activity increase. These findings prove that

317 this strategy can be applied to other  $\text{Ca}^{2+}$  activated amylases, suggesting that MOFs could serve  
318 as metal ion activated enzyme preparation. Thus MOFs materials with different metal ions can  
319 be selected according to metal ions-activated enzymes.

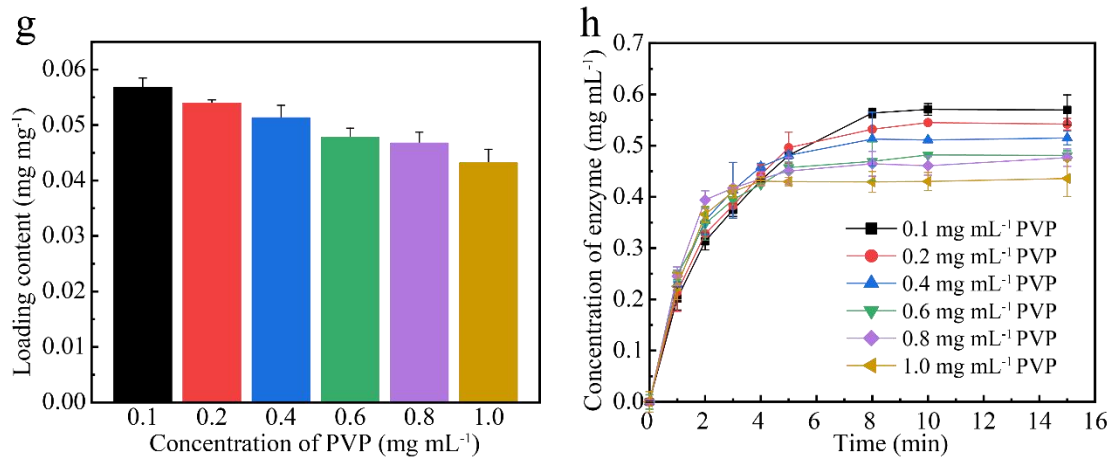
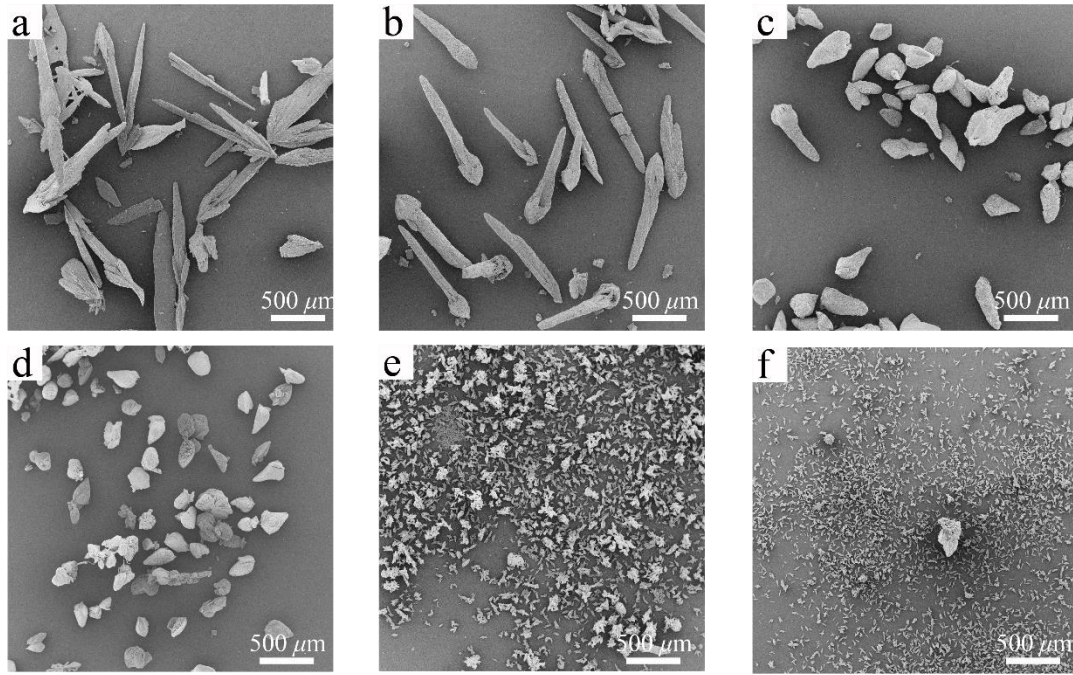
320 **Table 2** Kinetic parameters for released  $\alpha$ -amylase and pristine  $\alpha$ -amylase.

	$V_{max}$ [ $\text{mM s}^{-1}$ ]	$K_m$ [ $\text{mM}$ ]	$K_{cat}$ [ $10^3 \text{ s}^{-1}$ ]
Released $\alpha$ -amylase	0.25	0.27	1.25
Pristine $\alpha$ -amylase	0.14	0.46	0.69

321

### 322 3.6 Size control of UTSA-280

323 The size control of MOFs is important as it affects the release performance of enzymes.  
324 Polyvinylpyrrolidone (PVP) is a water soluble polymer with low toxicity and high thermal  
325 stability [29]. PVP is widely used as a capping agent to prevent colloidal aggregation, thus  
326 allowing to control the size of nanoparticles, especially MOFs [30]. The size of UTSA-280 was  
327 adjusted by using PVP to further modulate the release performance of  $\alpha$ -amylase from UTSA-  
328 280. As shown in Fig. 7a-f and Fig. S16, the crystal size of UTSA-280 decreases from above  
329  $1000 \mu\text{m}$  to below  $100 \mu\text{m}$  with increase of PVP concentration from 0.1 to  $1.0 \text{ mg mL}^{-1}$ . It is  
330 also noteworthy that larger crystal size of UTSA-280 leads to higher loading content (Fig. 6g),  
331 and thus leading to higher final concentration of released enzyme (Fig. 6h). XRD patterns show  
332 that  $\alpha$ -amylase@UTSA-280 synthesized using PVP as capping agent presents the same  
333 crystalline structure as that synthesized without PVP (Fig. S17).



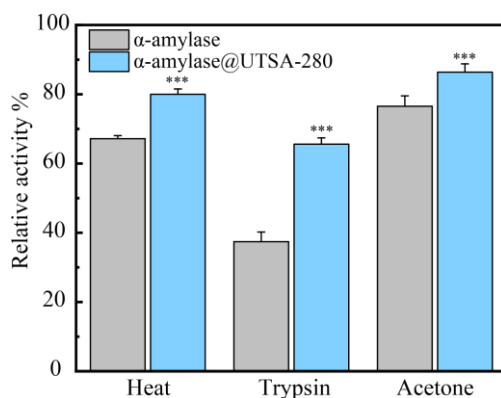
334  
 335 **Fig. 6** (a-f) SEM images (30 ×) of, (g) loading content of, and (h) enzyme release profiles from  
 336  $\alpha$ -amylase@UTSA-280 synthesized using PVP capping agent at concentrations of 0.1, 0.2, 0.4,  
 337 0.6, 0.8, 1.0 mg mL<sup>-1</sup>. Test conditions: 10 mg material in 1 mL citrate buffer (50 mM, pH 6.0)  
 338 at room temperature.

339

### 340 3.7 Protection assay of $\alpha$ -amylase@UTSA-280

341 It is of major importance to preserve enzyme activity during the storage and operation  
 342 process. The confined space created by MOFs should provide excellent protection to enzymes  
 343 [30]. The protection performance of UTSA-280 to  $\alpha$ -amylase was evaluated by exposure to

344 heat (80 °C), trypsin (1 mg mL<sup>-1</sup>), and organic solvent (acetone) for 1 h. As shown in Fig. 7,  
345 the relative activity of  $\alpha$ -amylase decreases to 67.2, 37.4, and 76.5% after treatment by heat,  
346 trypsin, and acetone, respectively. In contrast, the relative activity of enzyme in  $\alpha$ -  
347 amylase@UTSA-280 is 80.1, 65.6, and 86.4% after the same treatments. These findings  
348 indicate that UTSA-280 could efficiently protect  $\alpha$ -amylase against harsh conditions.  
349 Moreover, sodium squarate presents good biocompatibility according to MTT and live-dead  
350 staining experiments. The cell viability is above 81% for all groups (Fig. S18), and no apparent  
351 apoptosis is observed (Fig. S19). Therefore, UTSA-280 could facilitate the storage, transport,  
352 and operation of enzyme preparations.



353  
354 **Fig. 7** Relative activity of nude  $\alpha$ -amylase and  $\alpha$ -amylase@UTSA-280 after 1 h exposure to  
355 heat (80 °C), trypsin (1 mg mL<sup>-1</sup>), organic solvent (acetone). The enzyme activity was measured  
356 in HEPES buffer (50 mM, pH 7.0). (n=3, \*\*\* p<0.001)

357

#### 358 4. Conclusions

359 MOFs are able to function as metal ion activator and protector, and is thus a material of  
360 choice for enzyme preparations, especially metal-activated enzyme preparations. However,  
361 there is no research reported on MOFs as a metal ion activated enzyme preparation, so far. In  
362 this work,  $\alpha$ -amylase and pullulanase were successfully encapsulated in situ in a Ca<sup>2+</sup> based  
363 MOF (UTSA-280) under mild conditions. The enzyme@UTSA-280 composite crystals could

364 degrade rapidly in acidic environment and release enzyme. Importantly, the structural integrity  
365 of released enzyme was preserved as evidenced by CD spectra. The relative activity of released  
366  $\alpha$ -amylase and pullulanase increased to 177.8 % and 201.0 % as compared to original enzyme.  
367 Mechanistic investigation proves that the increase of enzyme activity could be attributed to the  
368 activation by  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$  could activate  $\alpha$ -amylase and pullulanase, but has no effect on  
369 glucoamylase which is not  $\text{Ca}^{2+}$  activated enzyme. Enzyme activity tests after exposure to heat,  
370 trypsin, and acetone showed that  $\alpha$ -amylase@UTSA-280 provides good protection to enzyme  
371 molecules. In addition, the size and enzyme release rate of  $\alpha$ -amylase@UTSA-280 can be  
372 tailored by using PVP capping agent at different concentrations. Compared with current  
373 enzyme preparations, this MOFs based platform present several advantages. Firstly, MOFs  
374 could combine the advantages of metal ion activation and enzyme protection, especially for  
375 metal ion activating enzymes. Secondly, the preparation conditions are milder than those used  
376 in traditional methods such as salting out or spray drying. Thirdly, this platform could avoid  
377 the use of excessive additives to maintain the enzyme conformation on storage conditions. Last  
378 but not least, this platform can efficiently activate enzymes by providing small amount of  
379 activator. Compared to direct addition of activator, this platform is more friendly to  
380 applications which require post-processing and purification. Therefore, enzyme@MOFs  
381 preparations are most promising for applications in the field of enzyme production, biocatalytic  
382 production, and food processing.

383

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387

## 388 **Author Contributions**

389 J. Song did most experiments, data analysis, and wrote the manuscript; X. Shen, and F. Liu  
390 prepared the specimens and made FT-IR and CD measurements, X. Zhao, Y. Wang, and S.  
391 Wang performed kinetic studies of enzymes, P. Wang made FITC-labeling of enzyme; J. Wang,  
392 F. Su and S. Li co-initiated the work, and revised the manuscript. All authors discussed and  
393 contributed the discussion and analysis of the results regarding the manuscript. All authors  
394 have given approval for the final version of the manuscript.

395

### 396 **Declaration of competing interest**

397 The authors declare that they have no known competing financial interests or personal  
398 relationships that could have appeared to influence the work reported in this paper.

399

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