One-Step Preparation of Antibacterial Chitin/Zn Composite from Shrimp Shells by Urea-$Zn(OAc)_2$•$2H_2O$ Aqueous Solution

Supporting Information

Mi Feng [a,b], Xingmei Lu [a,b*], Kun Jiang [a,b], Jie Zhang [b], Jiayu Xin [a], Chunyan Shi [a], Kefeng Wang [a,b], Suojiang Zhang [a,b*]

[a] *Key Laboratory of Green Process Engineering, State Key Laboratory of Multiphase Complex Systems, Institute of Process Engineering, Chinese Academy of Sciences, Beijing, 100190, China*

[b] *College of Chemical and Engineering, University of Chinese Academy of Sciences, Beijing, 100049, China*

Materials and methods

Materials

Red shrimp (*Solenocera crassicornis*) was purchased from Wal- Mart Supermarket. The shells of head and back were peeled off and washed with distilled water. The clean shrimp shells were dried by freeze drying, then them were ground with Universal Grinding Mill and sieved to the fine powder with the particle size below 125 μm. Urea and zinc acetate dihydrate were purchased from Xi Long Chemical Co. Ltd and used without further purification. Chitin was from Aladdin Industrial Corporation. *Escherichia Coli* (CCTCC AB 2012883) and *Bacillus Subtilis* (CCTCC AB 130001) were from China Center for Type Culture Collection. Luria Bertani Broth was bought from Thermo Fisher Oxoid.

Synthesis of Urea/$Zn(OAc)_2$·$2H_2O$ and U-Zn AS

*Corresponding author. Tel./Fax: +86-010-82544800.

E-mail addresses: xmlu@ipe.ac.cn（X. M. Lu）； sjzhang@ipe.ac.cn. (S. J. Zhang)

The deep eutectic solvent (DES), urea/$Zn(OAc)_2 \cdot 2H_2O$, was prepared as follows: urea was added to zinc acetate dihydrate aqueous solution in a mole ratio 4:1 under stirring at 70 °C for 4 h. The white precipitate was formed during the reaction. The supernatant was collected by filtration. Then remove most of the water by evaporation under reduced pressure. The viscous liquid was obtained and stand overnight at 4°C to form white needle-like crystals, which were collected by filtration and freeze-dried to constant to give pure urea/$Zn(OAc)_2 \cdot 2H_2O$ (yield % 90 %). The urea/$Zn(OAc)_2 \cdot 2H_2O$ was stored at 4°C before usage. The U-Zn AS was obtained by mixing the DES and water with fixed ratio at home temperature.

Synthesis chitin/Zn composite from shrimp shells with U-Zn AS

U-Zn AS was used to synthesize chitin/Zn composite material from shrimp shell. A typical procedure for the preparation of chitin/Zn composite from shrimp shells was conducted as follows. The shrimp shells powder (0.25 g) were treated by 5 wt % U-Zn AS (10 g) at room temperature for 24 h. Then the mixture was subjected to centrifugation to collect the insoluble product, which was washed repeatedly with water and ethanol and dried in vacuum oven at 80 °C until to constant weight. Finally, the white powder, namely chitin/Zn composite, was obtained and stored in bottles before analysis. The experimental parameters of temperature, time, ratio and concentration of U-Zn AS were controlled to study. All procedures were carried out three times.

Antibacterial activity test *in vitro*

Antibacterial activity test of chitin/Zn composite material was evaluated against *Escherichia Coli* (CCTCC AB 2012883) and *Bacillus subtilis* (CCTCC AB 130001)

using liquid culture method, because chitin/Zn composite was insoluble in water. *Escherichia Coli* and *Bacillus subtilis* were pre-cultured in LB liquid medium at 37 °C with 180 rpm for 12 h. Then the bacterial concentration of suspension was detected by UV-1800 spectrophotometer (Aoe Instruments) at 600 nm, then it was diluted with sterile saline to prepare bacterial suspensions with 10^3, 10^4 and 10^5 colony forming unit (CFU)/ml. Then chitin/Zn composite powder (0.025 g) was added to the three different bacterial suspensions (20 ml), which were incubated on a HYG-A double-deck TAT rotary shaker at 180 rpm, 37 °C for 12 h. In addition, chitin and $Zn(OAc)_2 \cdot 2H_2O$ were tested under the same conditions as the control group. Bacterial suspension without adding any sample as the blank group was incubated at same conditions.

The final bacteria concentration of sample, control and blank group were determined. The inhibition rate (IR %) was calculated as follows:

$$IR\ \% = \frac{A_{blank} - A_{test}}{A_{blank}} \times 100\%$$

Where, A_{blank} and A_{test} represented the absorbance of blank group and sample/control group, respectively.

Analysis content of $CaCO_3$, Zn and protein in samples

The content of $CaCO_3$ and Zn in samples were determined by inductively coupled plasma atomic emission spectrometer (ICPE-9000). The pretreated process of samples as follows: samples (0.01 g) were added to the 10 M HCl aqueous solution and heated at 85 °C until all samples were dissolved. The transparent was diluted by deionized water to adjust the pH to the ICPE-9000 demand. Five calibration standards of Ca and Zn were prepared including concentration at 0 mg/L, 0.1 mg/L, 1 mg/L, 10 mg/L and

100 mg/l.

Calculate the amount of $CaCO_3$ and Zn in the samples as follow:

$$CaCO_3\ \% = \frac{C_{Ca} \times V \times Dilution}{conversion\ factor \times ODW_{sample}} \times 100\ \%$$

$$Zn\ \% = \frac{C_{Zn} \times V \times Dilution}{ODW_{sample}} \times 100\ \%$$

Where: C_{Ca}=conc. of Ca as determined by ICPE-9000, mg/L; C_{Zn}=conc. of Zn as determined by ICPE-9000, mg/L; V= volume of tested solution, 0.01 L; Dilution=10; ODW_{sample}= the oven dry weight of the sample, mg; Conversion factor= the mole fraction of Ca in $CaCO_3$, 0.4.

The protein content of the samples was measured by the Kieldahl method.[1,2] Prior to analysis, 10 mg of sample was dissolved in 5 ml of 5 wt% NaOH for 1 h at 95 °C, followed by filtration to collect liquid, which was subjected to protein determination. Calculate the amount of protein of the samples as follow:

$$P\ \% = \frac{1.401 \times C \times (V_{titration} - V_{blank})}{ODW_{sample}} \times 6.25$$

Where, C= the concentration of titrated acid. 0.02 mol/L; $V_{titration}$= titration volume of sample, ml; V_{blank}= titration volume of blank sample, ml; ODW_{sample}= the oven dry weight of the sample, g.

Characterization

Fourier transform-infrared (FT-IR) spectra were recorded on a Nicolet 380 Spectrometer (Thermo Fisher Scientific, America) using KBr as blank in the range 4000-400 cm^{-1}. The powder X-ray diffraction spectra (XRD) were measured on SmartLab X-ray Diffractometer (Rigaku Corporation, Japan) with Ni-filtered CuKα

radiation (λ= 0.15418 nm) in the range 90-5°at a scanning rate of 10 °/min. Solid-state ^{13}C nuclear magnetic resonance (NMR)was performed in Avance Ⅲ HD 500 (Bruker, Switzerland). The morphology of chitin/Zn composite material was observed with scanning electron microscope (SEM, Hitachi, SUB020, Japan). The morphology of sample was carried out in confocal laser scanning microscope (CLSM, Carl Zeiss, Zeiss LSM 710, Germany). The size distribution was analyzed by dynamic light scattering (DLS, Malvern, NANO ZS, England). XPS measurements were conducted with a ESCALAB 250Xi (Thermo Fisher Scientific), using an Al K Alpha source (1486.6eV) X-ray source under a pressure of 3×10^{-7} mbar in the analysis chamber during the measurement. All spectra were calibrated with the binding energy of the C1s peak at 284.8eV. Survey and high-resolution spectra were acquired in CAE: Pass Energy mode with 100.0 and 20 eV, respectively. The assignments of each peals of the XPS spectrum were resulted from the decomposition method by the Thermo Avantange software based on the binding energies (BE) and atomic concentrations (AC, %).

Characterization of urea/$Zn(OAc)_2$·$2H_2O$

The urea/$Zn(OAc)_2$·$2H_2O$ was characterized by FT-IR and TGA. In addition, urea/$Zn(OAc)_2$·$2H_2O$ holds 13.77 wt% Zn. Aaccoridng to the Fig. S1, the characteristic peak of C=O shiftes from 1679.34 cm^{-1} in urea to 1667.44cm^{-1} in urea/$Zn(OAc)_2$·$2H_2O$, which indicates that the Zn is coordinated with O of C=O. The new peak at 1396.69 cm-1 of urea/$Zn(OAc)_2$·$2H_2O$ demonstrates that urea/$Zn(OAc)_2$·$2H_2O$ is new substance.

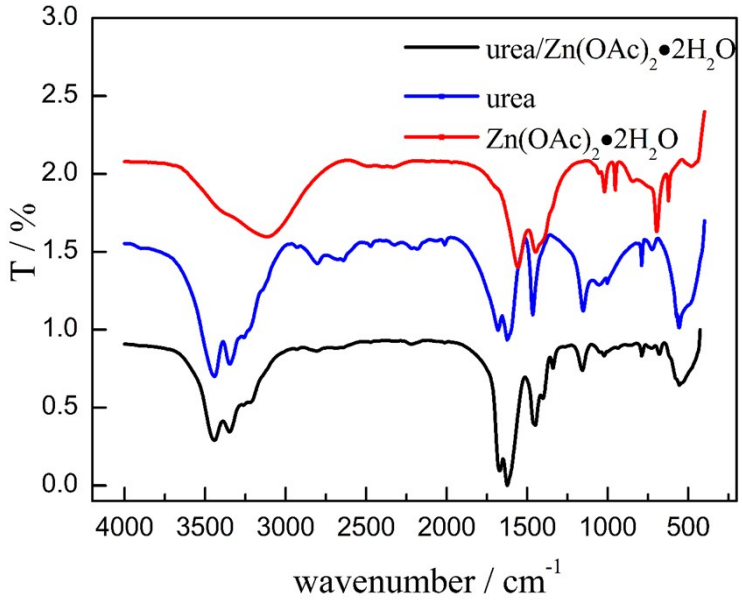

Fig. S1 FT-IR of urea/$Zn(OAc)_2$ᔓ$2H_2O$ (black), urea (blue) and $Zn(OAc)_2$ᔓ$2H_2O$ (red)

According to the TGA and DTA of the urea/$Zn(OAc)_2$ᔓ$2H_2O$ (As shown in Fig. S2), the decomposition temperature is about 148 °C, which is higher than 130 °C, the highest experiment temperature in the manuscript. As shown in the DTA, the melting point was 110 °C with lower than that of urea and $Zn(OAc)_2$ᔓ$2H_2O$, indicating that the urea/$Zn(OAc)_2$ᔓ$2H_2O$ was deep eutectic solvent according to the definition of the DES.

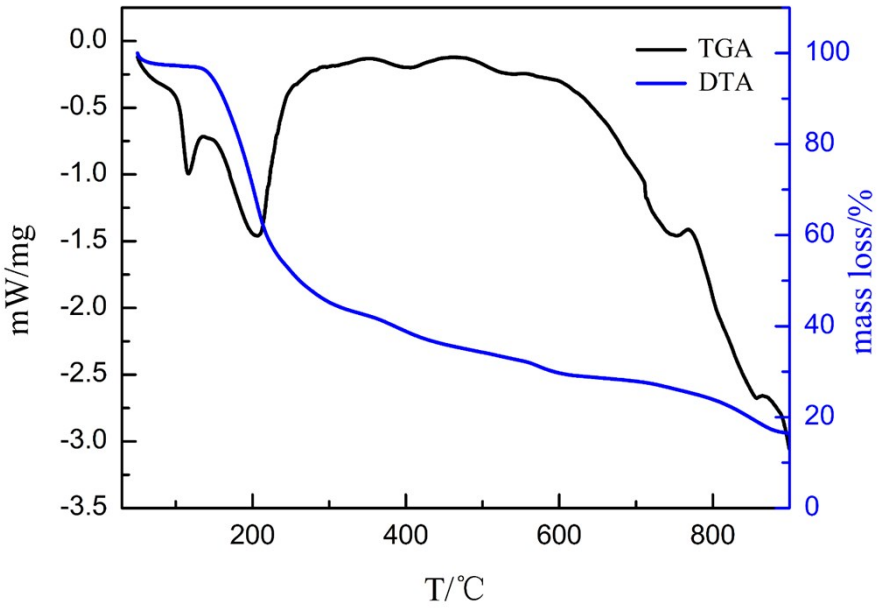

Fig. S2 The TGA (black) and DTA (blue) of urea/$Zn(OAc)_2$ᔓ$2H_2O$

Characterization of U-Zn AS and urea-$Zn(OAc)_2$ᔓ$2H_2O$-water mixture

The FT-IR, ^{13}C NMR, and ^{1}H NMR spectrum were employed to analysis the U-Zn AS and urea-$Zn(OAc)_2$ᔓ$2H_2O$-water mixture structure. The mole ratio of urea, $Zn(OAc)_2 \cdot 2H_2O$ and water was 4:1:98 in U-Zn AS and urea-$Zn(OAc)_2$ᔓ$2H_2O$-water mixture. As shown in Fig. S3, the FT-IR of the U-Zn AS and urea-$Zn(OAc)_2$ᔓ$2H_2O$-water mixture was almost same. It indicated that the main functional groups were almost same in U-Zn AS and urea-$Zn(OAc)_2$ᔓ$2H_2O$-water mixture.

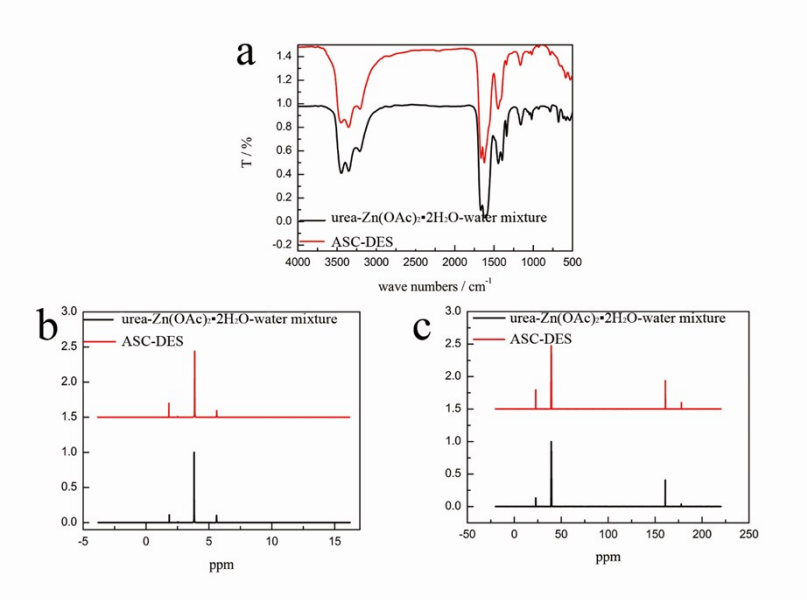

Fig. S3 The spectra of the U-Zn AS (black) and urea-$Zn(OAc)_2$ᔓ$2H_2O$-water mixture (red) (a) FT-IR (b) ^{1}H NMR (c) ^{13}C NMR

The shrimp shells powder (0.25g) was treated with urea, $Zn(OAc)_2 \cdot 2H_2O$ and water (10 g) solution at 100 °C for 24 h. The mole ratio of urea, $Zn(OAc)_2 \cdot 2H_2O$ and water was 4:1:485 (95 wt% water), 4:1:229 (90 wt% water), 4:1:144 (85 wt% water), 4:1:102 (80 wt% water), 4:1:76 (75 wt% water) and 4:1:59 (70wt% water), which was identical with mole ratio of the U-Zn AS used. However, the hydrolysis of

$Zn(OAc)_2 \cdot 2H_2O$ in urea-$Zn(OAc)_2$ら$2H_2O$-water mixture including mole ratio of 4:1:102, 4:1:76 and 4:1:59 was very obvious because of stronger alkaline condition. Meanwhile, hydrolysis of $Zn(OAc)_2 \cdot 2H_2O$ in the U-Zn AS with same mole ratio of the urea-$Zn(OAc)_2 \cdot 2H_2O$-water mixture cannot be observed. It indicated that the molecular ionic clusters still existed in the U-Zn AS (mole ratio, 4:1:102, 4:1:76 and 4:1:59), which prevented the hydrolysis of $Zn(OAc)_2 \cdot 2H_2O$. The products treated with urea-$Zn(OAc)_2$ら$2H_2O$-water mixture (mole ratio, 4:1:485, 4:1:229 and 4:1:144) were analyzed to confirm the calcium carbonate, zinc and protein content. As shown in Table S1, urea-$Zn(OAc)_2$ら$2H_2O$-water mixture showed calcium carbonate removal ability, indicating that the Zn ion was exchanged with Ca ion. The $CaCO_3$ % of product increased with mole ratio of 4:1:144 and the Zn % of product was up to 95.6 wt%, which showed that the hydrolysis of $Zn(OAc)_2 \cdot 2H_2O$. Nevertheless, the urea-$Zn(OAc)_2 \cdot 2H_2O$-water mixture showed poor protein removal ability.

Table S1. Calcium Carbonate ($CaCO_3$ %) , znic (Zn %) and Protein content (P %) of Products Treated with urea-$Zn(OAc)_2$ら$2H_2O$-water mixture

Urea-$Zn(OAc)_2 \cdot 2H_2O$-water mixture/n: n: n:	$CaCO_3$%/ wt%	Zn%/ wt%	Protein%/ wt%
4:1:485	7.1	40.9	22.9
4:1:229	6.5	47.7	24.0
4:1:144	12.3	95.6	22.2

Characterization of chitin/Zn composite

Viscosity

According to the literature,[3] the chitin and chitin/Zn composite LiCl/DMAc solutions (0.01-0.05 g/dl) were prepared with LiCl/ DMAc (5 %, w/w, 10 ml) solution at 30 °C for 5 days. The residue of the chitin/Zn composite solutions was removed by filter before measurement. Then the dynamic viscosity was determined by Lovis 2000 M/ME Rolling-ball viscometer (Anton Paar GmbH, Germany). The Mw method was according to the reported literature. [3] Calculate the Mw with the equation as follows: $[\eta]= 7.6\times10^{-5} Mw^{0.95}$.[3]

SEM

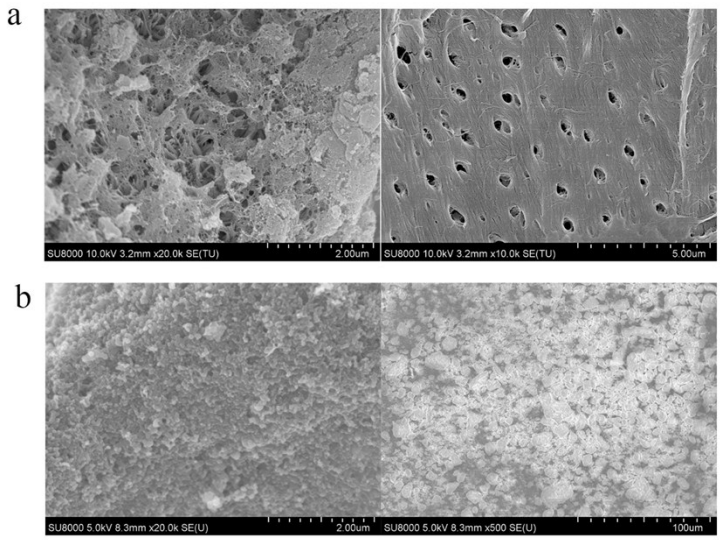

Fig. S4 SEM picture of a) shrimp shells and b) chitin/Zn composite

Antibacterial Test

Table S2. Antibacterial Activity of $Zn(OAc)_2•2H_2O$ against *Escherichia Coli* and *Bacillus subtilis*

Conc. of Bacteria/cell ml^{-1}[a]	*Escherichia Coli* /IR %[b]	*Bacillus subtilis* /IR%
10^3	-43.0 ± 0.4	-15.13 ± 1.1

[a] The the concentration of bacterial suspension was detected by UV-1800 spectrophotometer (Aoe Instruments) at 600 nm. The details of antibacterial expriments are listed in SI. [b]

$$inhibition\ rate\ (IR)\ \% = \frac{A_{blank} - A_{test}}{A_{blank}} \times 100\%$$, where, A_{blank} and A_{test} represent the absorbance of blank group and sample/control group, respectively.

Mechanism of calcium and protein removal

The shrimp shells was treated with 20 wt% $Co(OAc)_2 \cdot 2H_2O$, $Ni(OAc)_2 \cdot 4H_2O$ and $Zn(OAc)_2 \cdot 2H_2O$ aqueous solution at 100 °C for 24 h, respectively. The calcium carbonate ($CaCO_3$ %) and protein content (P %) of products were listed in the table S3.

Table S3. The Calcium Carbonate and Protein Content of Products

Type of Salt Aqueous Solution	$CaCO_3$ %/wt %	P %/wt %
$Co(OAc)_2 \cdot 2H_2O$	0.6	24.9 ± 1.3
$Ni(OAc)_2 \cdot 4H_2O$	0.8	23.6 ± 0.9
$Zn(OAc)_2 \cdot 2H_2O$	0.4	22.8 ± 1.1

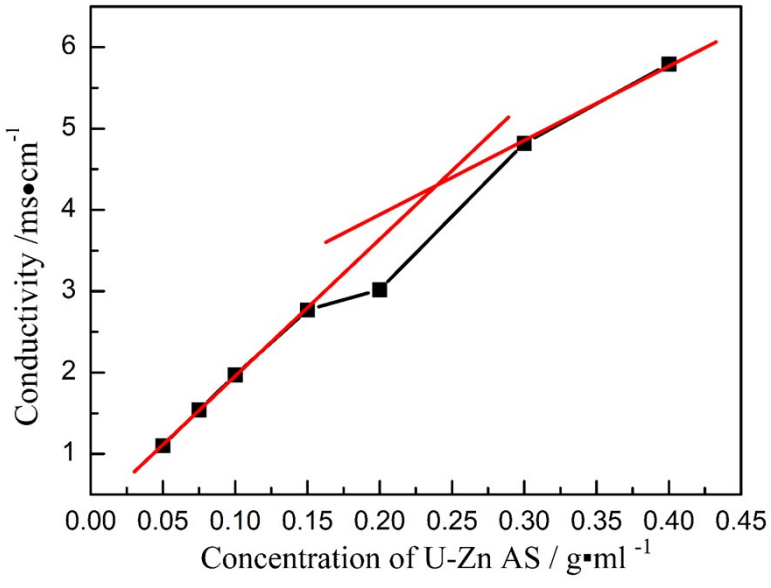

Fig. S5 Critical aggregation concentration (CAC) of U-Zn AS

The U-Zn AS (30 wt%, 5 g) and $CaCO_3$ (0.25 g) was mixed and reacted at 100 °C

for 24 h. Then the mixture was separated with centrifuge. The white solid was collected and dried in the oven. The white solid was analyzed with the ICP and FT-IR.

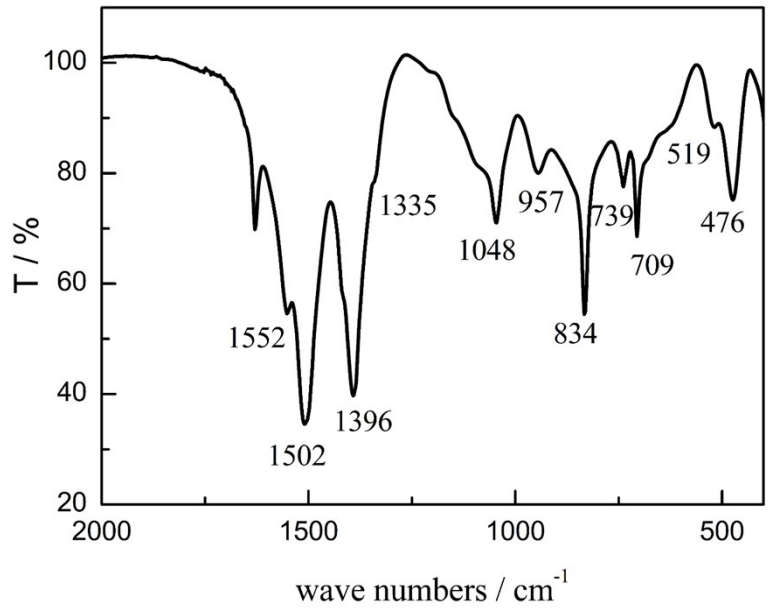

Fig. S6 FT-IR of the white solid obtained in the blank experiment

Gaussian simulation

Full geometry optimizations were run to locate all of the stationary points, using the M06-2x method,5 with the 6-311++G(d, p) basis set for C, H, O and N atoms and the def2-TZVP [6,7] basis set for Zn/Ca atoms.

Table S4 Wavenumbers (cm−1) of the internal modes of carbonates ions and OH librations of $Zn_5(OH)_6(CO_3)_2$ [4]

Wave numbers / cm^{-1}	Assignments
1552	$\nu_{as}CO_3^{2-}$
1502	
1396	
1335	
1049	OH librations
957	
834	out-of-plane CO_3^{2-} bending
739	(in-plane CO_3^{2-} bending
709	
519	
476	

Reference

1. A. Sila, M. Nasri and A. Bougatef, *Int J Biol Macromol*, 2012, **51**, 953-959

2. J. Kjeldahl, *Fresenius' J. Anal. Chem.*1883, **22**, 366–382.

3. M. Poirier and G. Charlet, *Carbohydrate polymers*, 2002, **50**, 363-370.

4 D. Stoilova, V. Koleva and V. Vassileva, *Spectrochimica Acta Part A: Molecular*

and Biomolecular Spectroscopy, 2002, **58**, 2051-2059

5. Y. Zhao and D. G, *Truhlar, Theor. Chem. Acc.*, 2008, **120**, 215–241.

6. K. Eichkorn, F. Weigend, O. Treutler and R. Ahlrichs, *Theor. Chem. Acc.*, 1997, **97**, 119–124

7. F. Weigend and R. Ahlrichs, *Phys. Chem. Chem. Phys.*, 2005, **7**, 3297–3305.