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An Eminent Approach towards next Generation Solvents for Sustainable Packaging and Stability of Enzyme: A Comprehensive Study of Ionic Liquid and Deep Eutectic Solvent Mixtures

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This supporting information contains instrumentation and methods, 7 figures and 4 table in 13 pages.

1.1. INSTRUMENTATION OR METHODS

1.1. Fourier Transform Infrared Spectroscopy (FT-IR) for ILs, DES and HIFs and mixtures with Lyz

FT-IR spectra of synthesized DES, ILs, HIFs and their mixture with lysozyme (Lyz) at 0.3M were studied using iS50 FTIR spectrometer (Thermo Scientific Nicolet). The spectral range for all the samples is between 400-4000 cm^{-1}.

1.2. UV-Visible Spectroscopy for Lyz in the Presence of ILs, DES, and HIFs

The UV-Visible absorption spectra of Lyz in phosphate buffer as well as in DES, ILs and HIFs were recorded from double beam UV-Visible spectrophotometer (UV-1800) acquired from Shimadzu co., Japan. The Instrument is having the wavelength accuracy of ± 0.3 nm along with automatic wavelength correction and the spectral bandwidth of 1.0 nm. Herein, aliquot from prepared sample solution was uniformly transferred into a quartz cuvette of 1.0 cm pathlength. The spectrum was recorded after averaging the values from three scan.

1.3. Steady State Fluorescence for Lyz in the Presence of ILs, DES and HIFs

Steady state emission spectra were recorded with a Peltier temperature-controlled Cary Eclipse fluorescence spectrofluorometer of Lyz in phosphate buffer, DES, ILs and HIFs at temperature T = 25 ºC. In order to monitor the tryptophan residue contribution, the spectra were obtained at excitation wavelength of 295 nm and the slit width of both excitation and emission were set at 5 nm.

1.4. Circular Dichroism (CD) Studies for Lyz in the Presence of ILs, DES, and HIFs

In order to investigate any change in the secondary or tertiary structure of Lyz in the presence of DES, ILs, and HIFs, the CD studies were performed via Jasco-185 spectrophotometer (USA). The spectrophotometer is equipped with Peltier system for controlling the temperature which had the accuracy of ± 0.1 ºC. The calibration of

spectrophotometer were executed by using (1S)-(+)-10-camphorsulfonic acid (Aldrich, Milwaukee, WI) with a molar extinction coefficient (e) of 34.5Mcm^{-1} at λ = 285 nm, while the a molar ellipticity (Y) of 2.36 M cm^{-1} at λ = 295 nm. Each measurement was done at room temperature and it was recorded by averaging three accumulations and the spectrum was obtained after subtracting appropriate blank. The composition of secondary structures for Lyz under different conditions was calculated by using an online CD analysis program, DICHROWEB, with the help of SELCON3 algorithm. The graphs of secondary structure compositions of Lyz in IL, DES and HIFs are provided in Figure S4.

1.5. Dynamic Light Scattering Measurements for Lyz in the Presence of ILs, DES and HIFs

The hydrodynamic diameter (d_H) of Lyz in phosphate buffer and in different green solvents namely, DES, ILs and HIFs were obtained via dynamic light scattering (DLS) measurement which is carried out by using a Zetasizer Nano Instrument (ZS90), UK. The instrument is having the transmission of 100% to 0.0003% with a fitted 4 mW He-Ne laser (633) which is having an automatic laser attenuator. All the measurements were carried out at 273.15 K within the fixed detection rate ranges from 0.0001 -10 µm. In order to reduce signal-to-noise ratio, each measurement was repeatedly recorded for100 runs with three concordant readings. The Stoke-Einstein expression was employed by the software of instrument which help to calculate the diffusion coefficient, which help to calculate d_H of the mixtures.

1.6. Thermodynamic Analysis for Lyz Stability in the Presence of ILs, DES and HIFs Using Fluorescence Spectroscopy

The analysis of Lyz unfolding was done by Cary Eclipse fluorescence spectrofluorometer within the temperature range of 15 ºC - 95 ºC. The excitation wavelength was fixed at 295 nm with the heating rate 2 ºC per minute. The thermal stability of the Lyz was investigated by two-state equilibrium i.e., between the folded state (*N*) and the unfolded (*U*) one as given in equation 1 and the Tm values are given in Table S1.

The fraction of unfolded protein is determined by using equation 2.

$$\text{Folded} \leftrightarrow \text{Unfolded} \tag{1}$$

$$P_U = \frac{X_F - X}{X_F - X_U} \tag{2}$$

Where, P_u is the fraction of unfolded protein, X_F, X_u, and X, is the measured fluorescence emission intensity of folded state, unfolded state and at a given temperature respectively. The equilibrium constant (K) for the transition represented in equation 1 is calculated using equation 3.

$$K = \frac{P_F}{1 - P_U} = \frac{X_F - X}{Y - X_U} \tag{3}$$

The difference in free energy between unfolded and the native state (ΔG_u) is obtained using the equation given below.

$$\Delta G_u = -RT(lnK) \tag{4}$$

Where R is the universal gas constant and T is the absolute temperature.

At equilibrium, the ΔG_u is always zero, thus, the temperature at which the ΔG_u turns out to be zero is said to be the T_m value for the protein. The value enthalpy change of unfolding (ΔH_m) at T_m can be obtained by analysis of the plot of ΔG_uversus T. The slopeof this plot at T_m gives the entropy change of unfolding (ΔS_m). The ΔH_m was calculated using the following equation-

$$\Delta H_m = T_m \Delta S_m \tag{5}$$

The value of change in heat capacity (ΔC_p) at 25 °C was calculated using Gibbs–Helmholtz equation given below-

$$\Delta G_u(T) = \Delta H_m \left[1 - \frac{T}{T_m}\right] - \Delta C_p \left[(T_m - T) + T\ln\left(\frac{T}{T_m}\right)\right] \tag{6}$$

The thermal transition curves were utilized to calculate different thermodynamic parameters such as transition temperature (T_m), free energy change of protein unfolding (ΔG_u), enthalpy change of protein unfolding (ΔH_m), and entropy change of unfolding (ΔS_m) at Tm. The detailed description of thermodynamic parameters of Lyz in IL, DES and HIFs are delineated in Tables S2.

1.7. Lyz Enzymatic Activity

The time dependent enzymatic activity of Lyz was determined by using UV-Visible spectrophotometer. This study helps to measure a decrement in the light scattering intensity of the samples which occurs due to decrease in the absorbance of the solution at 450 nm. Herein, the bacteria named, *Micrococcus Lysodeikticus* were employed as a substrate. Stock

solution of (0.3 mg/mL) above mentioned bacteria were prepared in 0.1M sodium phosphate buffer at pH 7.0. The enzymatic activity of Lyz was performed on suspended *Micrococcus Lysodeikticus* cell of varying concentration of DES, ILs and HIFs. Representative kinetic plots corresponding to hydrolysis of suspended bacterial cells in Lyz were studied according for the calculation of percent activity by using equation 1.

$$Percent\ Activity\ (\%) = (1 - A_t) \times 100$$

Where, A_t is absorbance at time 't' equals to 300s.

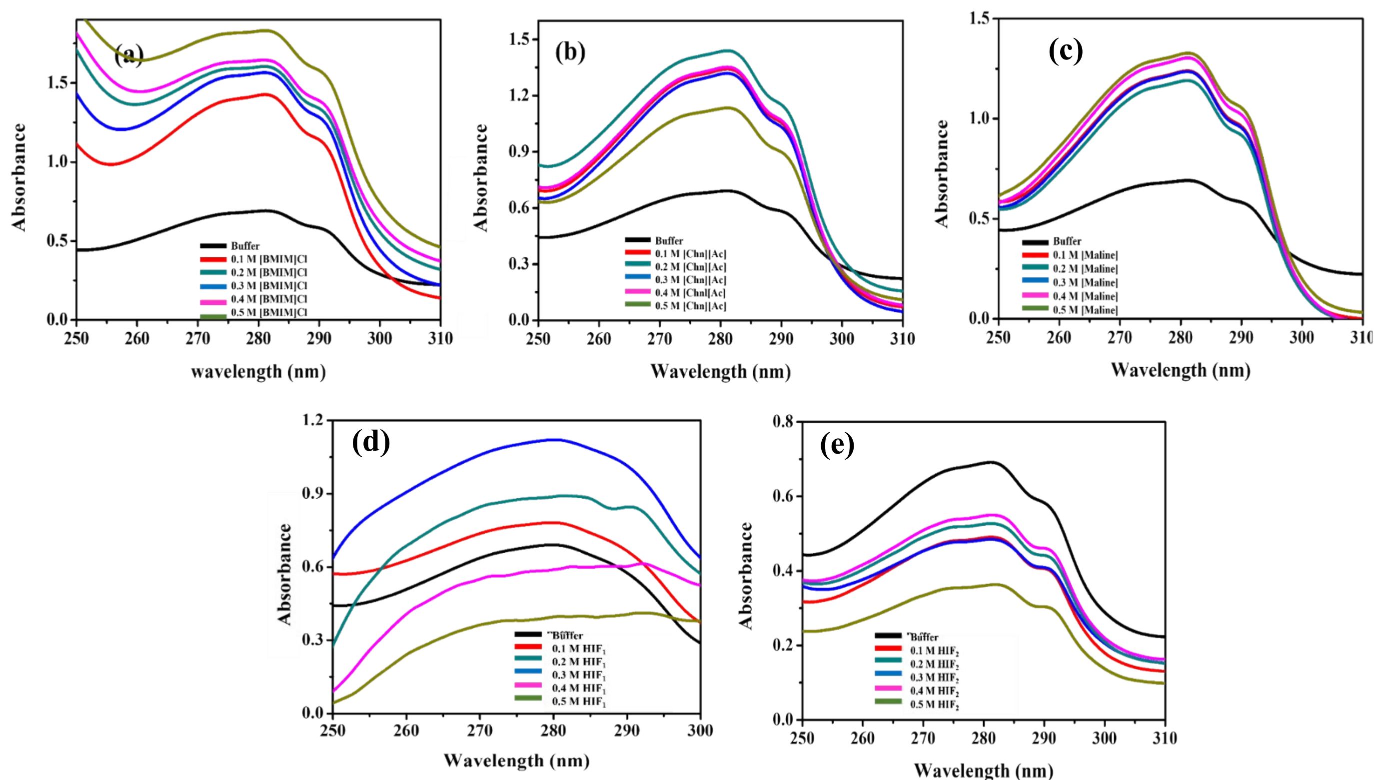

Figure S1. UV-Vis spectroscopic analysis of Lyz (0.5mg/mL) in buffer (control) in the presence of different concentrations of ILs; (a) [BMIM]Cl, (b)[Chn][Ac], (c) DES; Maline and HIFs; (d) HIF_1 (e) HIF_2.

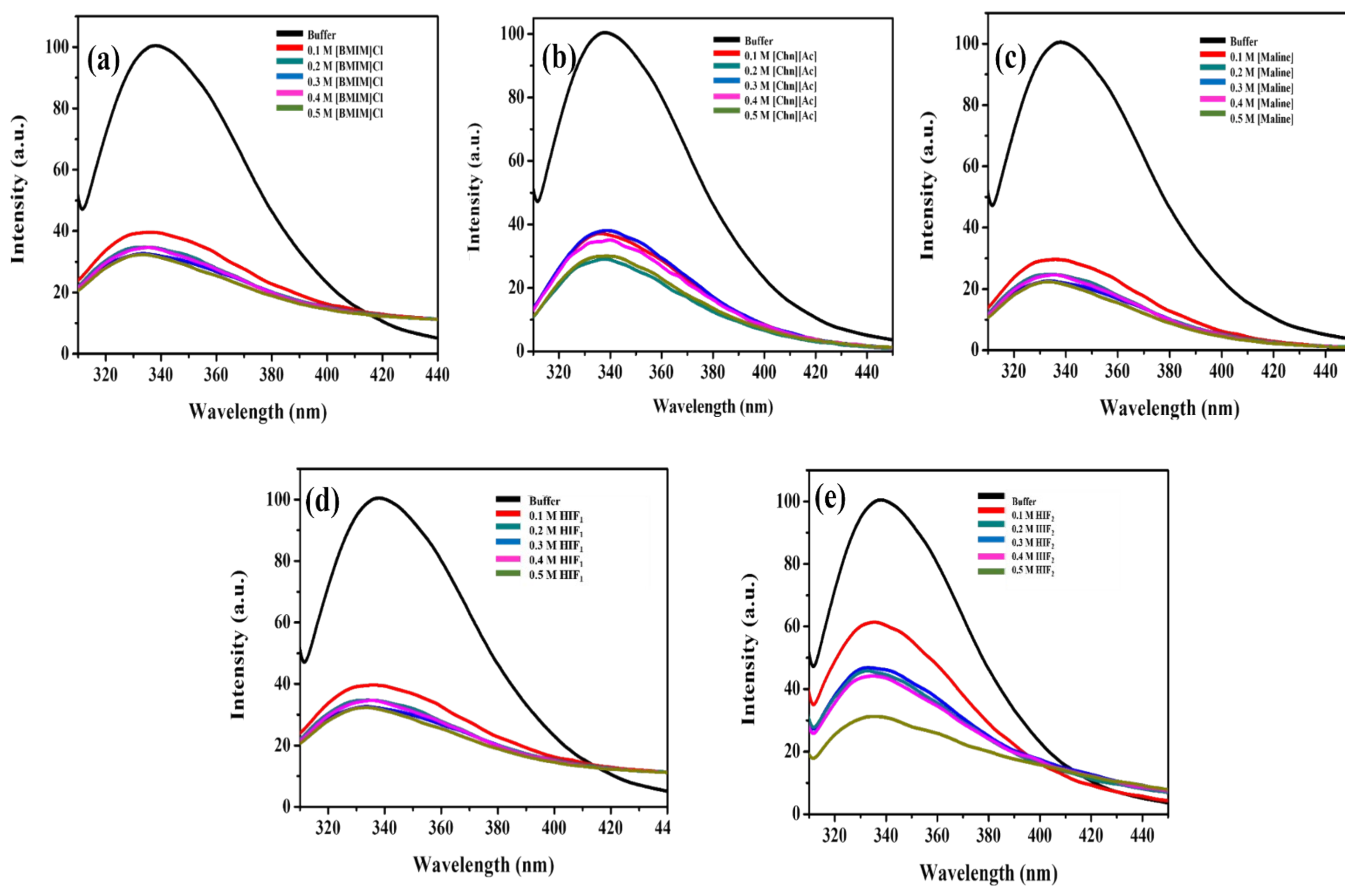

Figure S2. Steady-state fluorescence spectroscopic analysis of Lyz (0.5 mg/mL) in buffer (control), in the presence of different concentrations of ILs; (a) [BMIM]Cl, (b) [Chn][Ac], (c) DES; Maline and HIFs; (d) HIF_1 (e) HIF_2.

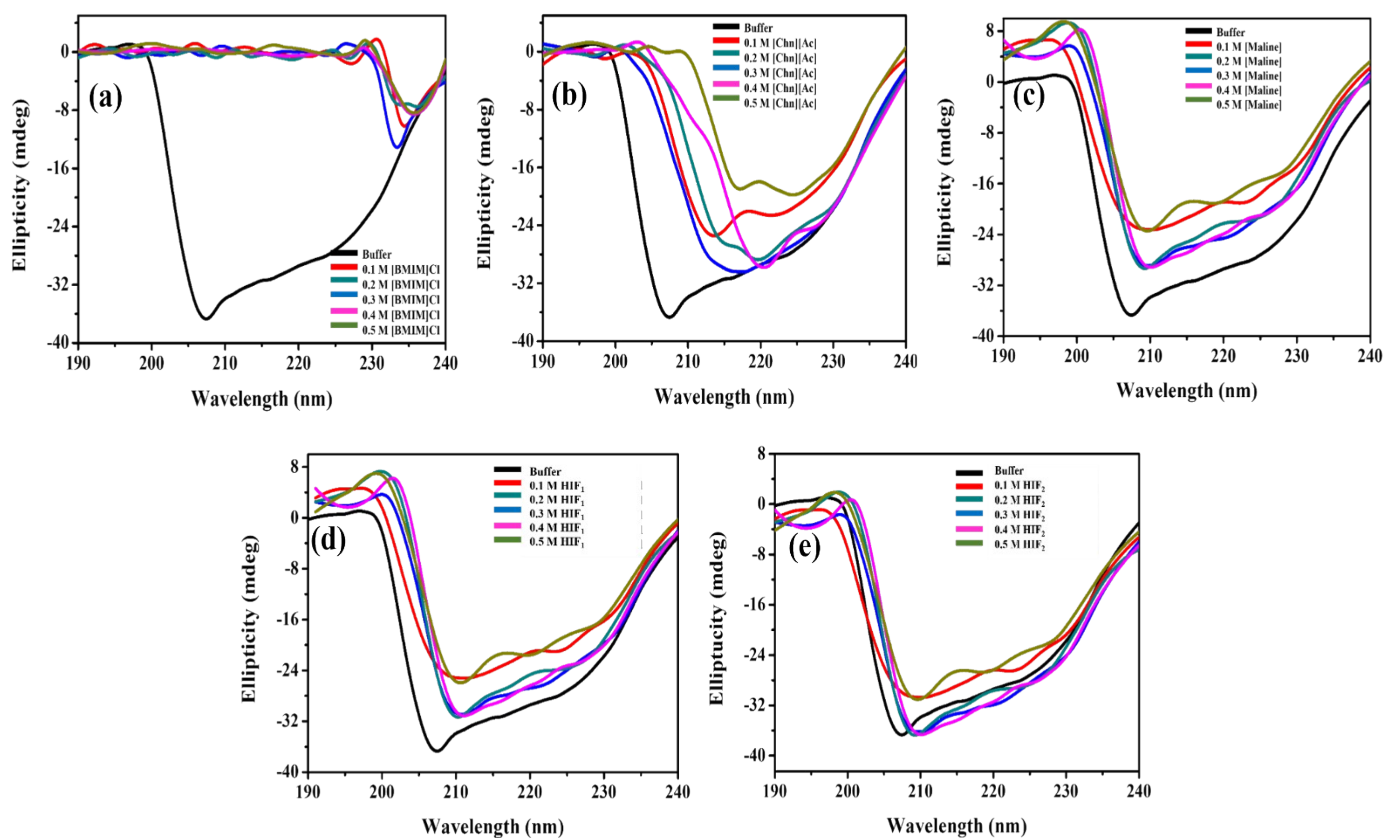

Figure S3. Far UV-CD spectroscopic analysis of Lyz (0.5 mg/mL) in buffer (control), in the presence of different concentrations of ILs; (a) [BMIM]Cl, (b) [Chn][Ac], (c) DES; Maline and HIFs; (d) HIF_1 (e) HIF_2.

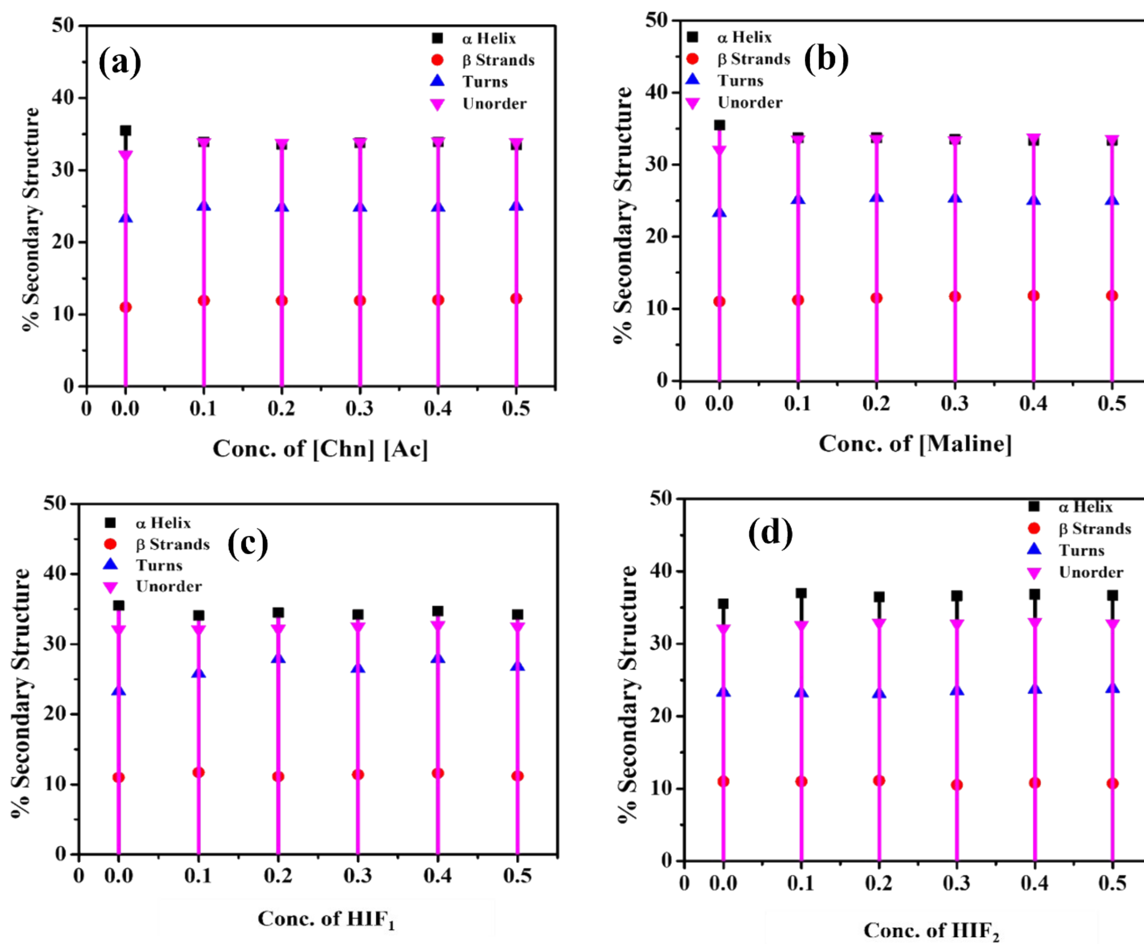

Figure S4. % Secondary Structure of Lyz in buffer (control), in the presence of different concentrations of IL; (a) [Chn][Ac], (b) DES; Maline and HIFs; (c) HIF_1 (d) HIF_2 while, secondary composition was not obtained for Lyz in [BMIM]Cl

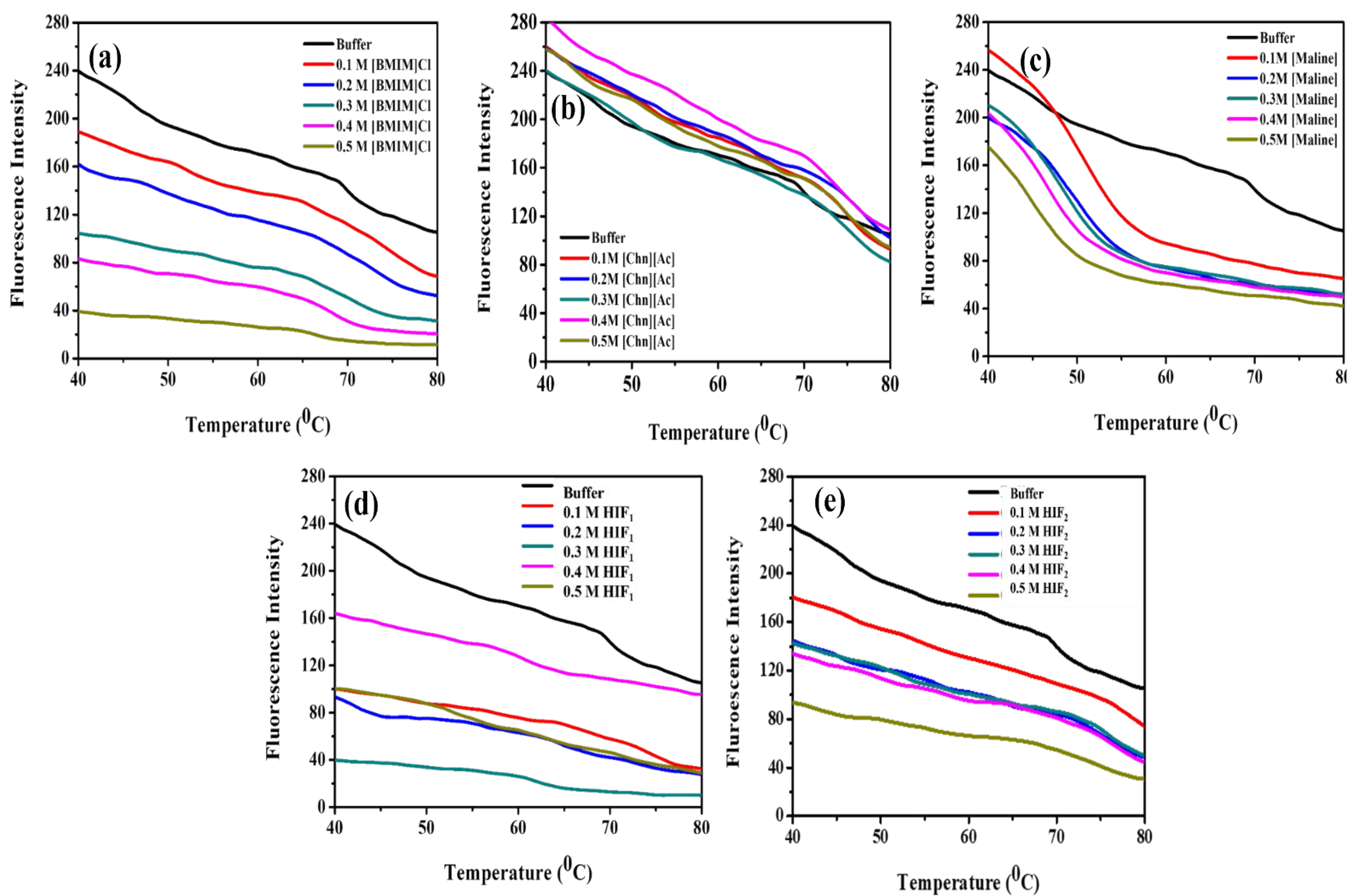

Figure S5. Thermal Transition curves of Lyz (0.5 mg/mL) in buffer (control), in the presence of different concentrations of ILs; (a) [Chn][Ac], (b) DES; Maline and HIFs; (c) HIF_1 (d)HIF_2.

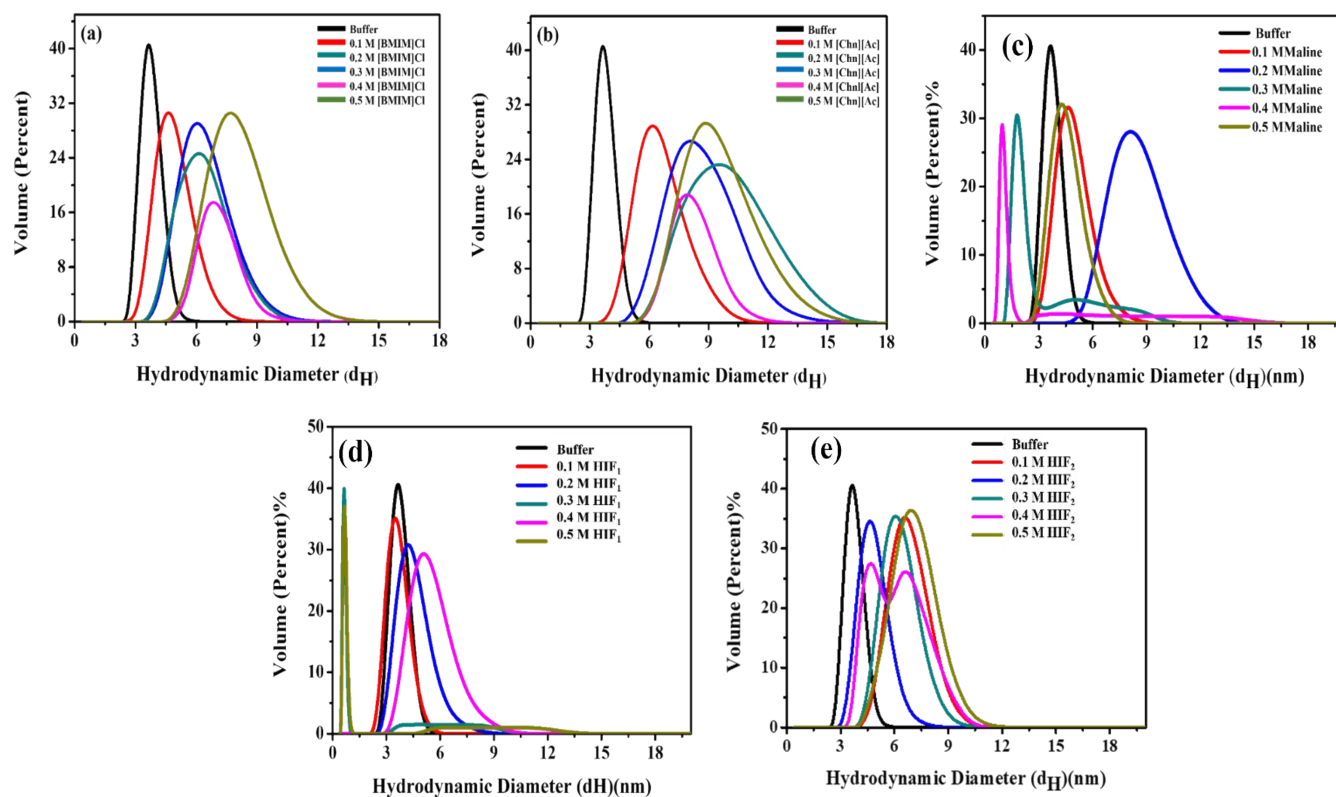

Figure S6. Volume percent graph of DLS measurement of Lyz (0.5 mg/mL) in buffer (control), in the presence of different concentrations of ILs; (a) [Chn][Ac], (b) DES; Maline and HIFs; (c) HIF_1 (d)HIF_2.

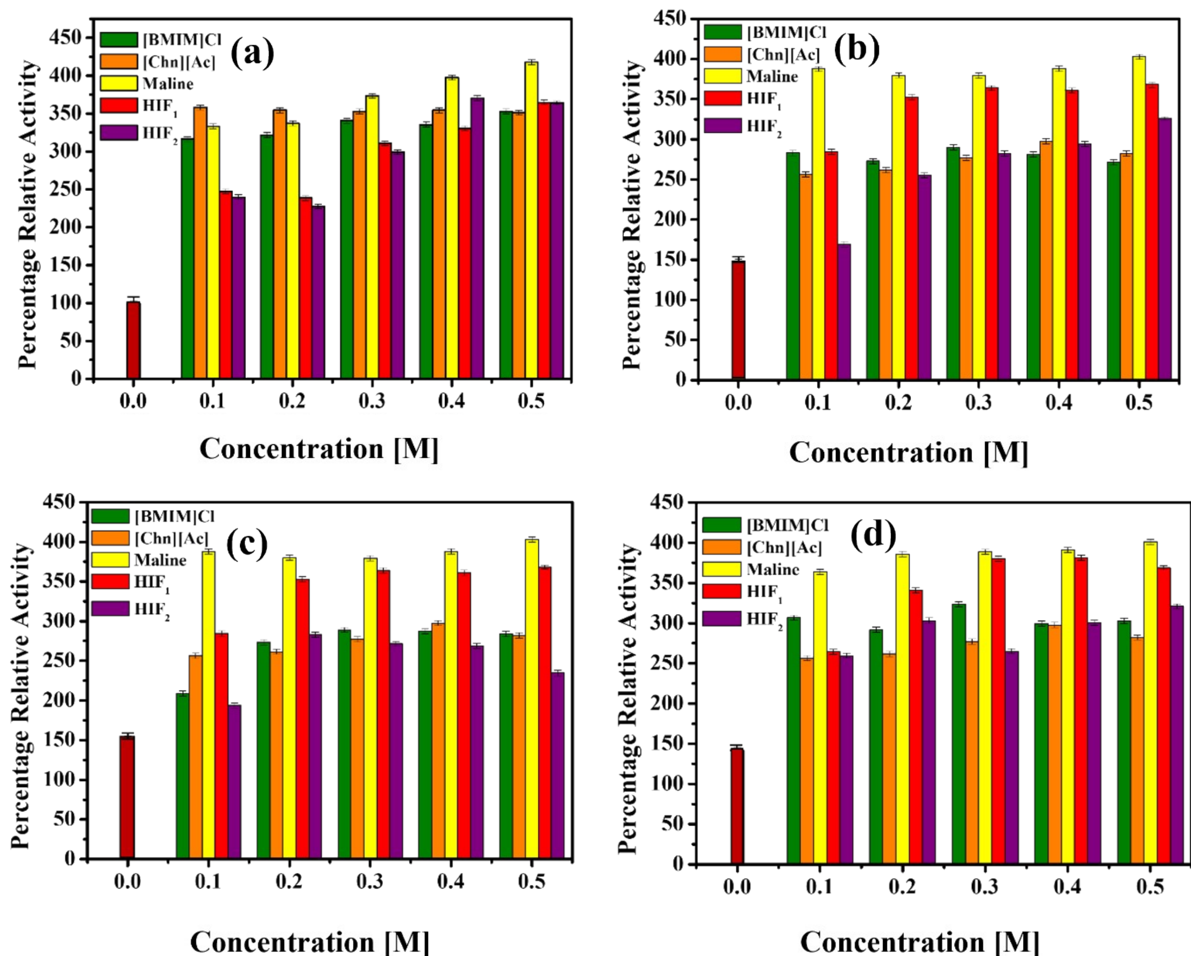

Figure S7. Percent relative enzyme activity of Lyz (0.5mg/mL) in buffer (control) at 25 ºC in the presence of varying concentrations ILs; [BMIM]Cl, [Chn][Ac], DES; Maline and HIFs; HIF_1 and HIF_2 at different time intervals (a) Week 1, (b) Week 2, (c) Week 3 and (d) Week 4.

Table S1. Thermal transition temperature (Tm) values of Lyz in buffer (control), in presence of different concentrations (0.1 to 0.5 M) of [BMIM]Cl, [Chn][Ac] ILs, Maline DES and HIFs; HIF_1, HIF_1.

Conc. (M)	**T_m/ºC**	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**
Buffer	70.87 ±0.79	**T_m/ºC**				
[BMIM]Cl		72.49±1.10	70.72±1.17	68.47±1.00	67.86 ±1.24	66.84±1.26
[Chn][Ac]		74.97±1.86	74.17±1.23	73.22±1.54	73.08±1.98	72.96±1.13
[Maline]		54.25±1.85	48.75±1.45	46.45±1.00	46.55±1.00	43.57±1.02
HIF_1		72.62±1.92	65.10±1.57	62.86±0.89	62.46±1.23	58.95±1.03
HIF_2		79.48±1.76	75.91±1.92	76.66±1.58	74.97±1.76	73.69±1.23

Table S2: Transition temperature (T_m), Gibbs free energy change of unfolding (ΔG_u), Enthalpy change, Entropy change and heat capacity change of unfolding at 25 ˚C determined by thermal fluorescence analysis of thermal denaturation of Lyz in absence and presence of varying concentration of co-solvents.

Conc.of [BMIM]Cl [M]	**T_m (K)**	**ΔG_u (kJ.mol^{-1})**	**ΔH_m (kJ.mol^{-1})**	**ΔS_m (kJ.mol^{-1}K^{-1})**	**ΔC_p (kJ.mol^{-1}K^{-1})**
0.0	344.02	3.63	65.36	0.19	10.04
0.1	345.64	3.24	162.45	0.47	23.07
0.2	343.87	4.14	130.67	0.38	18.58
0.3	341.62	4.38	119.35	0.35	16.90
0.4	341.01	4.25	170.50	0.50	19.91
0.5	339.99	3.81	200.59	0.59	26.12
Conc. of [Chn][Ac] [M]	**T_m (K)**	**ΔG_u (kJ.mol^{-1})**	**ΔH_m (kJ.mol^{-1})**	**ΔS_m (kJ.mol^{-1}K^{-1})**	**ΔC_p (kJ.mol^{-1}K^{-1})**
0.0	344.02	3.63	65.36	0.19	10.04
0.1	348.12	3.72	160.14	0.46	23.91
0.2	347.32	3.24	180.61	0.52	26.56
0.3	346.37	4.68	197.43	0.57	28.96
0.4	346.23	3.51	145.41	0.42	21.17
0.5	346.11	4.53	190.36	0.55	27.72
Conc. of [Maline] [M]	**T_m (K)**	**ΔG_u (kJ.mol^{-1})**	**ΔH_m (kJ.mol^{-1})**	**ΔS_m (kJ.mol^{-1}K^{-1})**	**ΔC_p (kJ.mol^{-1}K^{-1})**
0.0	344.02	3.63	65.36	0.19	10.04
0.1	327.4	3.38	136.00	0.42	9.74
0.2	321.9	4.22	134.94	0.42	5.28
0.3	319.6	3.14	145.05	0.45	5.10
0.4	319.7	3.83	158.29	0.50	5.56
0.5	316.72	3.98	159.12	0.50	2.16
Conc. of HIF_1 [M]	**T_m (K)**	**ΔG_u (kJ.mol^{-1})**	**ΔH_m (kJ.mol^{-1})**	**ΔS_m (kJ.mol^{-1}K^{-1})**	**ΔC_p (kJ.mol^{-1}K^{-1})**
0.0	344.02	3.63	65.36	0.19	10.04
0.1	345.77	4.50	159.87	0.46	20.71
0.2	338.25	1.45	75.99	0.22	8.42
0.3	336.01	3.16	190.49	0.57	20.03
0.4	335.69	3.19	52.92	0.16	4.46
0.5	332.10	2.44	20.63	0.06	0.75
Conc. of HIF_2 [M]	**T_m (K)**	**ΔG_u (kJ.mol^{-1})**	**ΔH_m (kJ.mol^{-1})**	**ΔS_m (kJ.mol^{-1}K^{-1})**	**ΔC_p (kJ.mol^{-1}K^{-1})**
0.0	344.02	3.63	65.36	0.19	10.04
0.1	352.63	3.42	153.92	0.44	23.01
0.2	349.81	3.47	164.43	0.47	23.42
0.3	349.06	2.08	176.05	0.50	25.17
0.4	348.12	3.34	220.97	0.63	30.83
0.5	346.84	3.07	199.87	0.58	27.20

Table S3. Hydrodynamic Diameter (d_H) values in (*nm*) of Lyz in buffer (control), in the presence of varying concentrations (0.1 to 0.5 M) of ILs; [BMIM]Cl, [Chn][Ac], DES Maline and HIFs; HIF_1 and HIF_2.

Conc. [M]	d_H (nm)	0.1	0.2	0.3	0.4	0.5
Buffer	3.8	d_H (nm)				
[BMIM]Cl		4.67	6.10	6.28	6.94	7.78
[Chn][Ac]		6.22	8.14	9.63	7.96	9.69
[Maline]		4.66	8.12	1.73	1.00	4.33
HIF_1		3.46	4.26	0.73	5.12	0.76
HIF_2		6.65	4.59	6.06	4.72	6.92

Table S4. Enzyme activity values of Lyz in buffer (control), at 25 ºC in the presence of varying concentrations (0.1 to 0.5 M) of ILs; [BMIM]Cl, [Chn][Ac], DES [Maline] and HIFs; HIF_1 and HIF_2.

Conc. of [BMIM]Cl [M]	Week#1	Week #2	Week#3	Week#4
0.0	100.00	147.32	152.02	141.77
0.1	316.49	283.11	208.53	306.67
0.2	321.73	273.00	273.61	291.65
0.3	340.77	289.89	288.99	323.49
0.4	335.60	281.46	287.13	299.18
0.5	352.85	271.28	283.87	302.94
Conc. of [Chn][Ac] [M]	**Week#1**	**Week #2**	**Week#3**	**Week#4**
0.0	100.00	147.32	152.02	141.77
0.1	358.09	256.44	256.44	256.44
0.2	354.64	261.63	261.63	261.63
0.3	352.85	277.02	277.02	277.02
0.4	354.64	297.60	297.60	297.60
0.5	351.17	282.22	282.22	282.22
Conc. of [Maline] [M]	**Week#1**	**Week #2**	**Week#3**	**Week#4**
0.0	100.00	147.32	152.02	141.77
0.1	333.42	387.88	387.88	363.82
0.2	337.22	379.85	379.85	385.91
0.3	373.50	379.45	379.45	388.67
0.4	397.45	388.10	388.10	391.00
0.5	417.96	402.90	402.90	401.08
Conc. of HIF_1 [M]	**Week#1**	**Week #2**	**Week#3**	**Week#4**
0.0	100.00	147.32	152.02	141.77
0.1	247.54	284.62	284.62	264.32
0.2	238.47	352.92	352.92	340.84

0.3	310.86	364.07	364.07	380.14
0.4	330.66	360.92	360.92	381.32
0.5	364.90	368.20	368.20	368.91
Conc. of HIF_2 [M]	**Week#1**	**Week #2**	**Week#3**	**Week#4**
0.0	100.00	147.32	152.02	141.77
0.1	239.84	169.34	193.98	259.45
0.2	227.50	255.22	283.15	303.16
0.3	299.35	282.32	271.42	265.01
0.4	370.56	294.33	268.63	300.36
0.5	364.25	326.14	234.67	320.72