SRI VENKATESWARA INTERNSHIP PROGRAM FOR RESEARCH IN ACADEMICS (SRI-VIPRA)

SRI-VIPRA Project Report (Project No. SVP-2322)

"Improving the activity and stability of enzymes in the presence of green solvents"

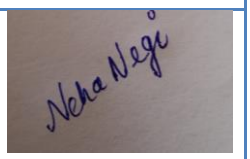
IQAC
Sri Venkateswara College
University of Delhi
Benito Juarez Road, Dhaula Kuan, New Delhi
New Delhi -110021

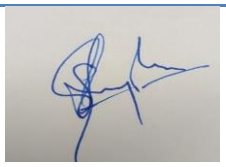
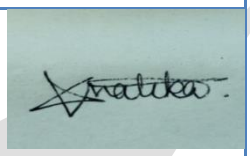
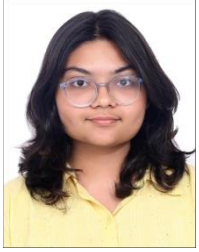
SRIVIPRA PROJECT (SVP-2322)

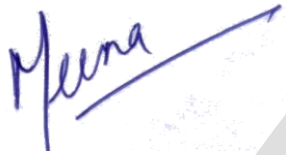
Title: Improving the activity and stability of enzymes in the presence of green solvents

Name of Mentor: Dr. Meena Bisht **Name of Department:** Chemistry **Designation:** Assistant Professor	

List of students under the SRIVIPRA Project

S.No	Photo	Name of the student	Roll number	Course	Signature
1		Urshita Sharma	1121137	B.Sc. (Prog.) Life Sciences	
2		Shikha Yadav	1121108	B.Sc. (Prog.) Life Sciences	
3		Neha Negi	1521051	B.Sc. (Hons.) Chemistry)	

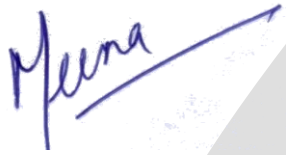
4		Shivani Yadav	1121065	B.Sc. (Prog.) Life Sciences	
5		Vratika Chandel	1121077	B.Sc. (Prog.) Life Sciences	
6		Riva Sarkar	1121063	B.Sc. (Prog.) Life Sciences	



Signature of Mentor

Certificate of Originality

This is to certify that the aforementioned students from Sri Venkateswara College have participated in the summer project SVP-2023 titled "**Improving the activity and stability of enzymes in the presence of green solvents"**. The participants have carried out the research project work under my guidance and supervision from 15 June, 2023 to 15th September 2023. The work carried out is original and carried out in an online/offline/hybrid mode.



Signature of Mentor

Acknowledgements

I would like to thank **Prof. C. Sheela Reddy** (Formal Principal) and **Prof. K. Chandramani Singh** (Acting Principal) of Sri Venkateswara College for proving me the opportunity to do this wonderful project. I wish to convey my gratefulness to all members of **SRIVIPRA** team, for their constant support, motivation and for guiding me throughout this project work. I am very much thankful to my collaborators, **Prof. P.Venkatesu,** Department of Chemistry, University of Delhi, **Prof. Siddharth Pandey,** Head & Institute Chair Professor, Department of Chemistry, Indian Institute of Technology Delhi, **Prof. K.K Pant**, Professor, Department of Chemical Engineering, Indian Institute of Technology Delhi for providing lab facilities to my students.

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Report summary

This report provides an analysis and evaluation of the experimental work developed in the project entitled "Improving the activity and stability of enzymes in the presence of green solvents." Until now, we have effectively completed the initial stage of this project. The main works we have developed under this project are (i) optimization of the concentration of various zwitterion ionic liquids (ZWILs) for improving the activity of *Cellulase* and (ii) improving the stability of *Cellulase* in the presence of aqueous solutions of ZWILs.

Introduction

The depletion of fossil fuels and the environmental concerns resulting from fossil fuel combustion have urged the development of clean and renewable energies.[1,2] In this context, lignocellulosic biomass offers the potential to be a renewable and biodegradable alternative to petroleum-based fuels and chemicals.[3,4] Lignocellulosic biomass is highly abundant, biocompatible and natural resource present in large quantities in agricultural residues (e.g., wheat straw, sugarcane bagasse, corn stover), forest products (hardwood and softwood), and dedicated crops. Therefore, it clearly represents a sustainable and low-cost resource that can be converted into fuels and chemicals on a large scale.[1-4] However, recalcitrance towards traditional chemical processes and solvents provides a significant barrier to its widespread utility.[5] In a biorefinery concept, the typical biochemical conversion process follows three major steps starting from a i) pretreatment process that alleviates the recalcitrance of biomass followed by ii) hydrolysis of polysaccharides to sugars and finally iii) biochemical conversion of sugars to ethanol or other products.[6] The main purpose of the pretreatment step is to break down the lignin structure and disrupt the crystalline structure of cellulose, so that the enzymes can easily access and hydrolyze the cellulose.[7] During hydrolysis, cellulose and hemicellulose are selectively converted into soluble sugars using lignocellulolytic enzymes. In the next step, these soluble sugars are converted into widely used platform molecules, such as polyols, furan-based chemicals, organic acids and its ester derivatives. Compare to acidic hydrolysis, enzymatic hydrolysis is thought to be less corrosive and toxic, having the potential for almost complete conversion of cellulose to sugars with minimum by-products.[8] In lignocellulosic biorefinery process, the pre-treatment and enzymatic hydrolysis steps are the most cost intensive, as it is responsible for reducing the structural complexity by depolymerising and subsequently increasing the accessibility of the biomass.[9] The traditional pretreatment methods use acidic and organic solvents which operate at temperatures above 150 °C. Moreover, the solvents or chemicals used for pretreatment are usually toxic to the enzymes.[10] Cellulases are one of the most widely used industrial enzymes which are commercially available for more than 30 years.[1] Plant cell wall degrading enzymes like cellulases are responsible for cellulose degradation by hydrolyzing the β-1,4-glycosidic bonds and converting it to simple sugars, glucose, which can be fermented into cellulosic biofuels. One of the applications of microbial enzymes is pretreatment of lignocellulosic biomass (LCB). Cellulose forms lignocellulose which is crystalline and hence hard to breakdown. Therefore, cellulose is insoluble in water and causes limitation in hydrolysis thereby making pretreatment of LCB essential as it loosens up the crystalline structure and facilitate the degradability to release fermentable sugar forms.[12] In the last few years ionic

liquids (ILs) and deep eutectic solvents have emerged as a new class of solvents for biocatalysis, either as single or co-solvents for dissolution and pretreatment of cellulose, making cellulose more accessible to enzymatic hydrolysis. ILs are salts with melting points <100°C. In 2002, Swatloski *et al*. reported dissolution of cellulose in ILs.[13] Although their ability to disrupt or fractionate LCB under mild conditions is admitted, these affect the performances of both enzymatic hydrolysis and microbial fermentation steps during LCB bioconversion process. More recently, Kuroda et al. demonstrated that zwitterion ionic liquids (ZWILs) could be an interesting alternative to ILs, particularly for LCB pretreatment.[14] In general zwitter ions are solid at room temperature. The melting point of zwitter ions are above 100°C, which is quite high compared with the melting point of ILs. In the present work, our aim was to improve the activity and stability of *cellulase* in the presence of varying concentration of ZILs by using UV-visible spectroscopy and fluorescence spectroscopy.

Experimental Section

The enzyme cellulase from *Trichoderma reesei* was purchased in its lyophilized powder form from Sigma. Zwitterion ionic liquids IL-1 (M= 218.27 g/mol) and IL-2 (M=232.30 g/mol) were synthesized. Citric acid (anhydrous, M_W= 192.12) was purchased from qualigens and tri-Sodium citrate from fisher scientific. The concentration of enzyme was 0.5 mg/mL. All samples were prepared in sodium citrate buffer (0.05 M, pH-4.8). For preparation of samples different concentrations of ZWILs were varied from 10-200 mM in buffer. All chemicals were of high purity and of analytical grade. Prior to measurements all samples were incubated for 30 min at room temperature. For the enzymatic activity samples were prepared and incubated at 50 ◦C. The thermal stability studies were performed by varying temperature from 25 to 75 ◦C.

Results and discussion

Catalytic activity of cellulase in presence of ILs at various concentrations

The functional activity of cellulase at various concentrations of choline-based DESs has been evaluated through 3,5-dinitrosalicylic acid (DNSA) test.[15] Fig. 1 shows the functional activity of the enzyme in IL-1 and IL-2 at different concentrations and different incubation time (0–48 h). Fig 1 (a) demonstrates that at 0 h incubation time, the enzymatic activity has increased for the initial concentrations of IL-1 up to 6 wt% of IL. Further, there was a slight deactivation at higher concentrations of IL but still, the activity was close to 90 % of the native cellulase. Whereas, when the enzyme was incubated for 48 h at 50 °C, surprisingly cellulase retains almost 80 % of the original activity for both ILs at all concentrations. Similarly, Fig. 9(b) displays that at 0 h incubation time the enzyme was highly active in IL-2 at all concentrations of IL. The maximum enhancement in activity was observed up to 116 % of the native enzyme.

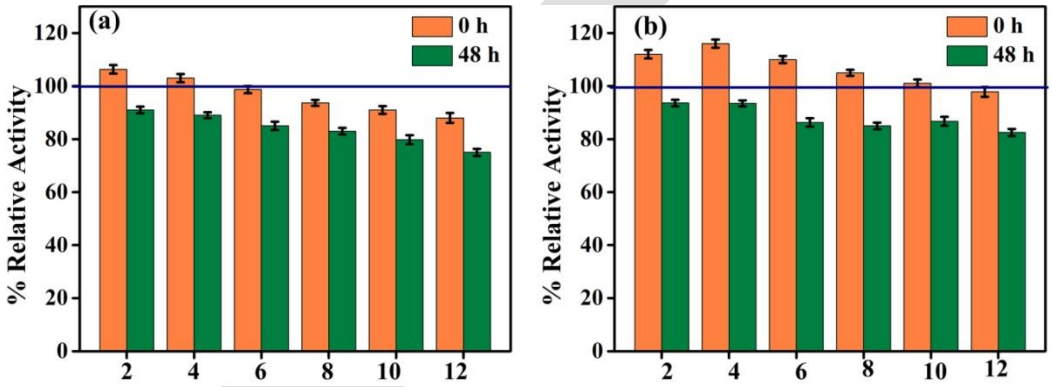

Fig.1 The variation in the % relative activity of cellulase in presence of varying concentration of (a) IIL-1 and (b) IL-2 at 50 °C.

UV–visible spectroscopic analysis of structural changes induced in cellulase by varying concentrations of ILs

UV–visible (UV–vis) spectroscopy is an efficient method for analyzing protein structural alterations and protein environment. Cellulase enzyme absorbs the ultraviolet light at 230 and 280 nm due to n-π* transition of aromatic amino acid residues and π-π* transition in protein backbone as shown in 2. These

peaks appear due to absorbance of aromatic amino acids such as tryptophan (Trp), phenylalanine (Phe) and tyrosin (Try). 2 (a) and (b) represent the absorption spectra of cellulase in buffer and three different ILs at various concentrations from 2 to 12 wt%. The alterations in absorbance and wavelength maxima (λ_{max}) in the absorption spectra signify the changes induced near the microenvironments of the aromatic amino acid residues. As evident from 2 (a) and (b) the absorbance of cellulase has increased slightly in presence of IL-1 and IL-2 , respectively without any shift in λ_{max} of cellulase. These observations clearly indicate the no structural disturbances induced in enzyme on addition of this ILs.

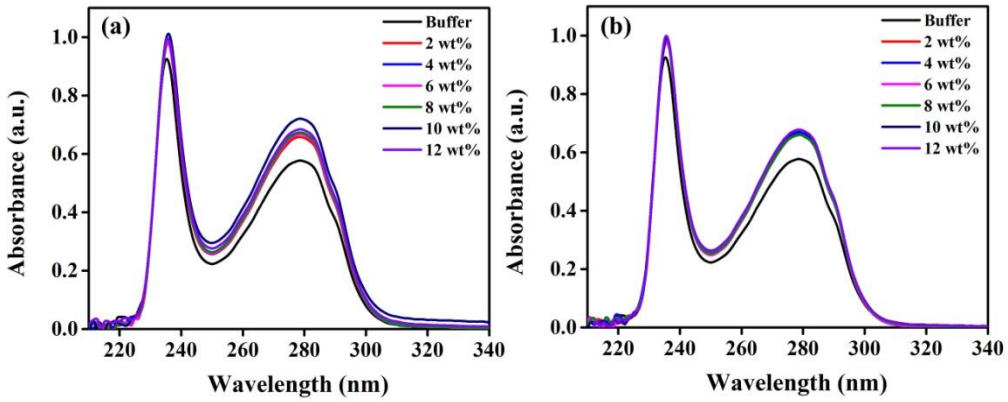

Fig. 2. UV–vis absorption spectra of cellulase in presence of varying concentrations of (a) IL-1l, (b) IL-2.

Fluorescence spectral analysis of cellulase in presence of varying concentrations of ChCl-based DESs

The fluorophore residues such as Phe, Tyr, and Trp are responsible for the intrinsic fluorescence of proteins. Cellulase has 39 fluorescent amino acids (7 Trp, 22 Tyr, and 10 Phe) in different domains. However, the major contributors of fluorescence of cellulase are 7 Trp and 22 Tyr as the quantum yield of Phe is low, its contribution is usually ignored. The modifications in the protein structure can be probed from the alterations occurring in the fluorescence maximum intensity (I_{max}) as well as from changes in wavelength maxima (λ_{max}). These fluorescence properties are highly sensitive towards the polarity variation around the microenvironment of the amino acid residues. The emission spectra of cellulase at various concentrations of the three DESs are shown in Fig.3 (a)and (b). The λ_{max} for native cellulase incubated in citrate buffer pH 4.8 is observed at ~337 nm and this value is well agreement with the literature value. The results from Fig. 2(a) and (b) indicate that the fluorescence intensity of the enzyme was increased in presence of two ILs. This amplification in I_{max} can be attributed to the movement of the

aromatic residues towards hydrophobic environment and indicates protein stabilization. This clearly justifies that enzyme is stabilized in both ILs.

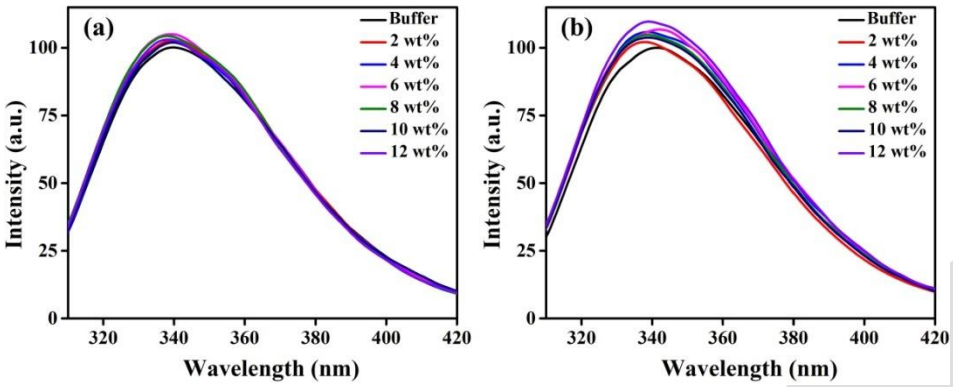

Fig. 3. Fluorescence emission spectra of cellulase in presence of varying concentrations of (a) IL-1 and (b) IL-2.

Conclusions

Cellulase is a crucial enzyme which offers huge applications in the processing of biomass bioresources. Hence, its stability and activity are of prime significance. In this context, IL is a suitable co-solvent which is environmental-friendly and which can boost the enzyme stability under extreme conditions is a hot topic. Following this, in this study, the influence of two ZWILs on the structural stability of cellulase was examined. For catalytic efficiency of cellulase under different conditions of time and concentration of ILs, cellulase was incubated under 50 °C and the enzymatic activity was evaluated. These findings may bring forward an understanding in tailoring different 'greener' ILss with desired properties aiming to accomplish high thermal stability and enhanced activity for specific enzymes. Further investigations, including long term stability studies would be needed to evaluate the enzyme's suitability as biocatalyst in ZWILs for practical use conditions.

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