SPOROTRICHUM THERMOPHILE XYLANASE: STABILITY IN IONIC LIQUIDS AND APPLICATIONS IN BIOMASS UTILISATION

AYESHA SADAF

DEPARTMENT OF CHEMISTRY
INDIAN INSTITUTE OF TECHNOLOGY DELHI
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by

AYESHA SADAF

DEPARTMENT OF CHEMISTRY

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Dedicated to the Almighty

CERTIFICATE

This is to certify that the thesis entitled "***Sporotrichum thermophile* xylanase: Stability in ionic liquids and applications in biomass utilisation**" being submitted by **Ms. AYESHA SADAF** to the Indian Institute of Technology Delhi for the award of the degree of ***Doctor of Philosophy*** in Chemistry is a record of bonafide research work carried out by her. Ms. Ayesha Sadaf has worked under my guidance and supervision, and has fulfilled the requirements for the submission of the thesis which, to my knowledge, has reached the requisite standard.

The results contained in this dissertation have not been submitted in part or full to any other University or Institute for the award of any degree or diploma.

Date:
Place:

Dr. S. K. Khare
Professor of Biochemistry
Department of Chemistry
Indian Institute of Technology Delhi

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Ayesha Sadaf

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Abstract

Ionic liquids (ILs) are salts which remain liquid below 100 °C and composed of a cation and anion moiety. They exhibit advantageous properties like low vapour pressure, high solvating ability and thermal stability. Ionic liquids are widely used for pre-treatment of lignocellulosic biomass prior to enzymatic saccharification. However, their toxicity towards saccharification enzymes and fermentative microorganisms, high costs and complexity in recycling pose major bottleneck in their large scale applications. The present thesis entails various studies conducted to investigate the stability of extremophilic enzyme systems in the presence of ionic liquids. Extremophiles and their enzymes which are generally adapted to function under extreme conditions of pH, temperature, pressure and salts provide an interesting system for the study of ionic liquid-enzyme interactions.

Sporotrichum thermophile, a thermophilic fungus was characterised for its morphological and biochemical characteristics. The strain secreted good amount of xylanase. De-oiled *Jatropha curcas* seed cake, a plentiful by-product of biodiesel industry was used as substrate for xylanase production from *S. thermophile* by solid state fermentation. Under the optimized conditions, 1025 U xylanase/g DSC (Deoiled Seed Cake) was produced. The xylanase exhibited half life of 4 h at 45 °C and 71.44 min at 50 °C respectively. It was stable in a broad pH range of 7.0–11.0. K_m and V_{max} were 12.54 mg/ml and 454.5 U/ml/min respectively.

Ionic liquid stability of thermophilic *S. thermophile* xylanase was explored. It was active and stable in [EMIM][OAc] upto 50% concentration (v/v). The pre-treatment of wheat straw (18-25%, w/w xylan) with [EMIM][OAc] followed by IL stable *S. thermophile* xylanase treatment (containing 12 U/ml xylanase activity and 2 U/ml CMCase activity) led to 281 mg reducing sugars (consisting of mainly xylose and xylo-oligosaccharides) per g wheat straw

much higher as compared to non-pre-treated. IL stable enzymes enabled simultaneous pre-treatment, saccharification and the intermediate step of washing of residual IL can be eliminated to make the process cost effective. SEM, XRD and FT-IR confirmed the loss of crystallinity by [EMIM][OAc] pre-treatment followed by hydrolytic bond cleavage and deacylation by xylanase in the subsequent step.

Furthermore the cells of *S. thermophile* were screened for their viability in the presence of various hydrophilic imidazolium based ILs. [EMIM][OAc], [BMIM][$MeSO_4$] and [BMIM][OTf] were found to be compatible and did not affect the growth and xylanase production whereas [HMIM][BF_4] and [BMIM][BF_4] were not compatible and inhibited the growth drastically. The impact of two types of ILs systems compatible, [EMIM][OAc] and non-compatible, [BMIM][BF_4] on *S. thermophile* membrane permeability, surface morphology, membrane characteristics and fatty acid composition were studied by SEM, TEM, AFM and FAME analysis. [EMIM][OAc] was tolerated by *S. thermophile* upto 4% (v/v) concentrations and the cellular response in this particular IL was comparable with that of control. The antioxidative enzyme levels and the changes in the intracellular proteome response to [EMIM][OAc] and [BMIM][BF_4] indicated global changes in the major metabolic pathways as well. In order to explore the feasibility of using *S. thermophile* cells in place of its crude xylanase, a study was conducted for *in situ* pre-treatment and saccharification of [EMIM][OAc] treated wheat straw and high amount of reducing sugars were released as compared to controls.

The last part of the thesis covers the activity levels of another important enzyme system, β-lactamase which is manifested in β-lactam mediated antimicrobial resistance. The lactamase produced from soil strain EMB20 of *Bacillus cereus* was purified using three phase partitioning and gel filtration chromatography to a 30 fold purification and 15% recovery yield. Contrary to the general trend, the lactamase was not a metalloenzyme but its activity

was enhanced in presence of Mg^{2+} and Mn^{2+}. The EMB20 lactamase exhibited improved stability against inhibitors and denaturing agents such as urea and GdmCl (Guanidinium chloride) as compared to its commercial analogue. Consequently various types of ILs and Deep Eutectic Solvents (DESs) were screened for their lactamase inhibiting ability. Maline, a DES was found to efficiently inhibit the lactamase. The effect of individual components of maline on lactamase inhibition showed that malonic acid was mainly responsible for inhibiting lactamase. Structure-function and docking studies justified the work. Further the antibacterial and cytotoxic studies also confirmed the potential of maline as a potent growth inhibitor of β-lactamase producing *B. cereus* EMB20.

सार

आयनिक तरल पदार्थ (ILs) नमक होते हैं जो 100 डिग्री सेल्सियस के नीचे तरल होते हैं और धनआयन और ऋणायन से बने होते हैं। वे कम वाष्प दबाव, उच्च घुलनशीलता और थर्मल स्थिरता जैसे लाभप्रद गुण प्रदर्शित करते हैं। आयनिक तरल पदार्थों को व्यापक रूप से एंजाइमिक सिक्रेशन के पूर्व लिगोनोसेलुलोजिक जैवभार के पूर्व उपचार के लिए उपयोग किया जाता है। हालांकि, सिक्रेशन एंजाइम और किण्वित सूक्ष्मजीवों के प्रति उनकी विषाक्तता, उच्च लागत और रीसाइक्लिंग में जटिलता उनके बड़े पैमाने पर अनुप्रयोगों में बड़ी बाधा उत्पन्न करती है। वर्तमान शोध में ईओणिक तरल पदार्थ की उपस्थिति में चरमपसंदी एंजाइम प्रणालियों की स्थिरता की जांच के लिए किए गए विभिन्न अध्ययनों पर जोर दिया गया है। चरमपसंदी सूक्ष्मजीव और उनके एंजाइम, जो आम तौर पर पीएच, तापमान, दबाव और लवण की चरम स्थितियों के तहत कार्य करने के लिए अनुकूलित होते हैं, ईओणिक तरल-एंजाइम पारस्परिक क्रिया के अध्ययन के लिए एक दिलचस्प प्रणाली प्रदान करते हैं।

स्पोरोट्रिचम थर्मोफाइल, एक थर्मोफिलिक कवक है। इसे इसकी रूपात्मक और जैव रासायनिक विशेषताओं के द्वारा चिह्नित किया गया है। यह कवक जाइलनेस अच्छे मात्रा में स्रावित करते हैं। De-oiled जेट्रोफा curcas बीज केक, बायोडीजल उद्योग का एक बहुतायत से उप-उत्पाद सब्सट्रेट है, जोकी जाइलनेस स्रावित करने के लिए इस्तेमाल किया गया था। अनुकूलित परिस्थितियों में, 1025 U जाइलनेस / ग्राम डीएससी का उत्पादन किया गया था। जाइलनेस ने क्रमशः 50 डिग्री सेल्सियस पर 45 डिग्री सेल्सियस और 71.44 मिनट के हाफ लाइफ का प्रदर्शन किया। यह 7.0-11.0 की व्यापक पीएच श्रेणी में स्थिर था। K_m और V_{max}, क्रमशः 12.54 मिलीग्राम / एमएल और 454.5 U/ एमएल / मिनट थे।

थर्मोफिलिक एस. थर्मोफाइल जाइलनेस की आयनिक तरल स्थिरता का पता लगाया गया। यह [EMIM][OAc] में 50% तक सक्रिय और स्थिर था। [EMIM][OAc] के साथ गेहूं के भूसे का पूर्व उपचार (18-25%, w/w जाइलन) के साथ ILs स्थिर एस. थर्मोफाइल जियालेनेस उपचार (12 U /

एमएल जाइलनेस गतिविधि और 2 यू / एमएल CMCase गतिविधि युक्त) के साथ गैर-पूर्व-उपचार के मुकाबले 281 मिलीग्राम कम करने वाली शर्करा (मुख्य रूप से सेलुलोजिक और जियालो-ऑलिगोसेकेराइड युक्त) प्रति ग्राम गेहूं का भूसा प्राप्त किया जोकी बहुत अधिक था। ILs स्थिर एंजाइम एक साथ पूर्व उपचार, शर्करीकरण और अवशिष्ट आईएल के धोने के मध्यवर्ती कदम को सक्षम किया जा सकता है ताकि प्रक्रिया लागत प्रभावी हो सके। SEM, XRD और FT-IR ने [EMIM][OAc] पूर्व उपचार द्वारा क्रिस्टलटाइटी के कम करने की पुष्टि की और इसके बाद के चरण में जाइलनेस द्वारा हाइड्रोलाइटिक बंधन दरार और डेआसेटयलाति की गई ।

इसके अलावा एस. थर्मोफिल की कोशिकाओं को विभिन्न हाइड्रोफिलिक इमिडाजोलियम आधारित ILs की उपस्थिति में उनकी व्यवहार्यता के लिए जांच की गई । [EMIM][OAc], [BMIM][$MeSO_4$] और [BMIM][OTf] ने कोशिकाओं की वृद्धि और जाइलनेस उत्पादन को प्रभावित नहीं किया था जबकि [HMIM][BF_4] और [BMIM][BF_4] वृद्धि संगत नहीं थेI दो प्रकार के ILs सिस्टम, संगत [EMIM][OAc] और गैर-संगत [BMIM][BF4], के द्वारा एस. थर्मोफिल की झिल्ली पारगम्यता, सतह के आकारिकी, झिल्ली विशेषताओं और फैटी एसिड संरचना पर SEM, TEM AFM और FAME विश्लेषण की गई । एस. थर्मोफिल [EMIM][OAc] की 4% (v/v) सांद्रता तक सहन किया था और इस विशेष IL में सेलुलर प्रतिक्रिया नियंत्रण के साथ तुलनीय थी। एंटीऑक्सीडेटिव एंजाइम का स्तर और [EMIM][OAc] और [BMIM][BF4] के लिए इंट्रासेल्युलर प्रोटीम प्रतिक्रिया में परिवर्तन ने प्रमुख चयापचय मार्गों में भी परिवर्तन का संकेत दिया है। अपने क्रूड जाइलनेस के स्थान पर एस. थर्मोफाइल कोशिकाओं का उपयोग करने की व्यवहार्यता का पता लगाने के लिए, [EMIM][OAc] पूर्व-उपचार और शर्करीकरण के लिए एक अध्ययन किया गया, जिसमें गेहूं का भूसा का उपयोग किया गया और शर्करा की उच्च मात्रा को विमोचित किया गया।

शोध प्रबंध के अंतिम भाग में एक और महत्वपूर्ण एंजाइम प्रणाली की गतिविधि के स्तर को शामिल किया गया है, जो β- लैक्टमैज़ है, जो β-लैक्टम मध्यस्थता वाले रोगाणुरोधी प्रतिरोध में प्रकट होता है।

मिट्टी से उत्पादित लैक्टमैज़ तीन चरण विभाजन और जेल निस्पंदन क्रोमैटोग्राफी को 30 गुना शुद्धि और 15% रिकवरी यील्ड से शुद्ध किया गया था। सामान्य प्रवृत्ति के विपरीत, लैक्टमैज़ मेटलोनोसिम नहीं था लेकिन इसकी गतिविधि को Mg^{2+} और Mn^{2+} की उपस्थिति में बढ़ाया गया था। EMB20 लैक्टमैज़ ने अपने वाणिज्यिक अनुरूप की तुलना में यूरिया और GdmCl (Guanidinium chloride) जैसे संदमक और विकृतिकरण एजेंट के खिलाफ बेहतर स्थिरता का प्रदर्शन किया। नतीजतन, विभिन्न प्रकार के ILs और Deep Eutectic Solvents (DESs) को उनके लैक्टमैज़ संदमक क्षमता के लिए जांच की गई। Maline, एक DES है, जो कुशलतापूर्वक लैक्टमैज़ को बाधित करने के लिए मिला था। लैक्टमैज़ अवरोध पर Maline के अलग-अलग घटकों का असर दिखाता है कि मलोनिक एसिड मुख्य रूप से बाधित लैक्टमैज़ के लिए जिम्मेदार था। संरचना-कार्य और डॉकिंग अध्ययन ने काम को उचित ठहराया। आगे जीवाणुरोधी और साइटोटेक्सिक अध्ययन ने भी Maline की संभावित बी. सेरेउस उत्पादक β- लैक्टमैज़ के एक प्रभावी विकास-अवरोधक के रूप में पुष्टि की।

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[3a-$C_3P(C_4)_3$][Ala]	(3-amino-propyl)-tributyl-phosphonium alanine
[3a-$C_3P(C_4)_3$][Gly]	(3-amino-propyl)-tributyl phosphonium glycine
[AMIM][Cl]	1-allyl-3-methylimidazolium chloride
[BMIM][BF_4]	1-butyl-3-methylimidazolium tetrafluoroborate
[BMIM][CF_3COO]	1-butyl-3-methylimidazolium trifluoroacetate
[BMIM][CH_3COO]	1-butyl-3-methylimidazolium acetate
[BMIM][Cl]	1-butyl-3-methylimidazolium chloride
[BMIM][HSO_4]	1-butyl-3-methyl-imidazolium hydrogen sulphate
[BMIM][$MeSO_3$]	1-butyl-3-methylimidazolium trifluoromethanesulfonate
[BMIM][$MeSO_4$]	1-butyl-3-methylimidazolium methanesulfonate
[BMIM][$MeSO_4$]	1-butyl-3-methylimidazolium methylsulfate
[BMIM][NO_3]	1-butyl-3- methylimidazolium nitrate
[BMIM][OH]	1-butyl-3-methylimidazolium hydroxide
[BMIM][OTf]	1-butyl-3-methylimidazolium trifluoromethanesulfonate
[BMIM][PF_6]	1-butyl-3-methylimidazolium hexafluorophosphate
[BMIM][SCN]	1-butyl-3-methylimidazolium thiocynate
[Bmpy][Cl]	1-butyl-1-methylpyrrolidinium chloride
[C_{10}MIM][Cl]	1-decyl-3-methylimidazolium chloride
[C_{16}MIM][Cl]	1-*n*-hexadecyl-3-methylimidazolium chloride
[C_1C_1IM][C_1PO_2OH]	1,3 dimethylimidazolium methyl phosphonate
[C_8MIM][Cl]	1-methyl-3-octylimidazolium hydrochloride
[Ch][Asp]	Cholinium aspartate
[Ch][Lys]	Cholinium lysine
[Ch][OAc]	Cholinium acetate
[C_nMIM][PF_6]	1-alkyl-3-methyl-imadazolium hexafluorophosphate
[DBNH][OAc]	1,5-diazabicyclo[4.3.0]non-5-enium acetate
[EMIM][TOS]	1-ethyl-3-methylimidazolium tosylate
[EMIM][Arg]	1-ethyl-3-methylimidazolium arginine
[EMIM][Asp]	1-ethyl-3-methylimidazolium aspartate
[EMIM][BF_4]	1-ethyl-3-methylimidazolium tetrafluoroborate

[EMIM][DMP]	1-ethyl-3-methylimidazolium dimethylphosphate
[EMIM][$EtSO_4$]	1-ethyl-3-methylimidazol-3-ium ethylsulfate
[EMIM][Glu]	1-ethyl-3-methylimidazolium glutamate
[EMIM][Lys]	1-ethyl-3-methylimidazolium lysine
[EMIM][MA]	1-ethyl-3-methylimidazolium methylammonium methylsulfate
[EMIM][$MeO(H)PO_2$]	1-ethyl-3-methylimidazolium methylphosphonate
[EMIM][$MeSO_4$]	1-ethyl-3-methylimidazolium methylsulfate
[EMIM][OAc]	1-ethyl-3-methylimidazolium acetate
[EMIM][Val]	1-ethyl-3-methylimidazolium valine
[$FurEt_2NH$][H_2PO_4]	N-ethyl-N-(furan-2-ylmethyl)ethanamine, H_3PO_4 salt
[HEMA]	tris-(2-hydroxyehtyl) methylammonium
[HMIM][BF_4]	1-hexyl 3-methyl imidazolium tertrafluoroborate
[HMIM][Cl]	1-hexyl-3-methyl-imidazolium chloride
[HPy][Cl]	Hexylpyridinium chloride
[MMIM][$MeSO_4$]	1,3-dimethylimidazolium methylsulfate
[MMIM]DMP]	1-methyl-3-methylimidazolium dimethylphosphate
[NBu_4][ac]	Tetrabutylammonium acetate
[NBu_4][$MeSO_3$]	Tetrabutylammonium methylsulfonate
[$NHMe_3$][$MeSO_3$]	Trimethylammonium methylsulfonate
[$P_{6,6,6,14}$][NTf_2]	Trihexyltetradecylphosphonium bis(trifluoromethylsulfonyl)imide
[p-$AnisEt_2NH$][H_2PO_4]	N-ethyl-N-(4-methoxybenzyl)ethanamine, H_3PO_4 salt
[PrMIM][BF_4]	1-propyl-3-methylimidazolium tetrafluoroborate
[PrMIM][PF_6]	1-propyl-3-methylimidazolium hexafluorophosphate
[PV_4][Pr]	Poly [3-butyl-1-vinylimidazolium] L-proline
[Q][Br]	1-alkylquinolinium bromide
[TBA][OH]	Tetrabutylammonium hydroxide
[TEAAc]	Triethyl Ammonium Acetate
2-HEAA	2-hydroxyl-ethylammonium acetate
AFM	Atomic Force Microscopy
BEPB	2,2-Bis(methyl *N*-ethanoate pyridium bromide)hexadecyl *N*-ethanoate pyridinium bromide
BETAB	2,2-Bis(methyl *N*-ethanoate *N*,*N*,*N*-trimethylammonium bromide)hexadecyl *N*-Ethanoate *N*,*N*,*N*-trimethylammonium bromide

BF_4	Tetrafluoroborate
CD	Circular Dichroism (CD)
CF_3SO	Trifluoromethanesulfonate
CFU	Colony Forming Units
CH_3CO_2	Acetate
ChCl/lactic acid	Choline chloride
CVA	Clavulanate
DESs	Deep Eutectic Solvents
DSC	Deoiled Seed Cake
EDTA	Ethylenediaminetetraacetic Acid
FAME	Fatty Acid Methyl Ester Analysis
FT-IR	Fourier Transform Infrared
GdmCl	Guanidinium chloride
ILs	Ionic Liquids
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, thiazolyl blue
PF_6	Hexafluorophosphate
PMSF	Phenylmethanesulphonyl Fluoride
SDS-PAGE	Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SSF	Solid State Fermentation
TAZ	Tazobactam
TEM	Transmission Electron Microscopy
TF_2N	bis(trifluoromethylsulfonyl)imide
TPP	Three Phase Partitioning
XRD	X-ray Powder Diffraction