

**BIOREFINERY APPROACH TOWARDS VALORIZATION OF  
LIGNOCELLULOSIC AGRO-WASTES FOR PRODUCTION OF  
VALUE ADDED MICROBIAL PRODUCTS**

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INDIAN INSTITUTE OF TECHNOLOGY DELHI  
APRIL 2019**

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VALUE ADDED MICROBIAL PRODUCTS**

*by*

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*Submitted*

*in fulfillment of the requirements of the degree of Doctoral of Philosophy*

*to the*



**INDIAN INSTITUTE OF TECHNOLOGY DELHI**

**APRIL 2019**

## CERTIFICATE

This is to certify that the thesis entitled “**Biorefinery approach towards valorization of lignocellulosic agro-wastes for production of value added microbial products**” being submitted by **Ms. JASNEET GREWAL** 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. Jasneet Grewal 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.



Dr. S. K. Khare  
Professor of Biochemistry  
Department of Chemistry  
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Date: April 30, 2019  
Place: New Delhi

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# Abstract

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Currently, 90% of the chemicals and 80% of energy demands are met by fossil fuels and the consumption is further increasing incessantly. The depleting fossil fuels, energy crisis and deteriorating environmental conditions have put impetus on developing sustainable technologies derived from renewable bioresources. The biomass constitutes the feedstock of the biorefineries, which can be transformed into plethora of marketable products and energy, contributing to the transition from linear fossil-based economy to bio-based circular economy.

Agro-wastes are constituted by the residual biomass from agriculture and other related sources. India, United States and China account for 60% of the crop residues. Annually, 140 billion tons of biomass is generated from the agricultural sector and the subsequent generation of agro-wastes adds to environmental and economic burden. The present work addressed the major challenge of agro-waste management by their valorization into various value added products. The main emphasis was laid on cottonseed cake, an agro-residue generated in abundance after oil extraction, whose meaningful utilization is restricted due to presence of toxic gossypol content in it.

GABA ( $\gamma$ -aminobutyric acid), a C4 platform chemical and high value neurotransmitter was produced by *Lactobacillus brevis* mediated solid-state fermentation (SSF) of cottonseed cake. The SSF conditions were optimized, so as GABA production of 19.7 mg/g cottonseed cake was obtained on 6<sup>th</sup> day of fermentation with simultaneous reduction in toxic gossypol content. The GABA producing ability of *L. brevis* cells was also confirmed by biotransformation of monosodium glutamate (MSG) into GABA. The potential application of this bio-based GABA was demonstrated in the synthesis of 2-pyrrolidone, feedstock for the synthesis of valuable industrial products, N-methyl-pyrrolidone, polyvinylpyrrolidone (PVP) and nylon 4.

In similar approach, production of hydrolytic enzymes (cellulase and xylanase) from cottonseed cake with simultaneous degradation of toxic gossypol was achieved by *Aspergillus niger*, *Trichoderma reesei* and *Phanerochaete chrysosporium* mediated SSF. The consortium of these three fungi produced highest titre of cellulase and xylanase with almost complete reduction of gossypol content. The secretome profiling carried out by LC-ESI MS/MS, revealed presence of versatile mix of proteins comprising of hemicellulases, cellulases,

amylases, esterases, proteases, hypothetical proteins and some other proteins. The glycoside hydrolase (GH) proteins constituted major part of the secretome. The protein profiles thus, confirmed the ability of these fungi to use cottonseed cake as substrate. The efficiency of hydrolases produced from seed cake was verified by saccharification of wheat straw. The high yield of reducing sugars obtained, confirmed the suitability of these hydrolases for use in lignocellulosic biorefinery.

The lignocellulosic biorefinery is gaining colossal attention for production of energy, fuels and other value added chemicals in sustainable and eco-friendly manner. The lignocellulosic agro-waste utilization involves three inter-dependent and essential steps namely (i) pretreatment (ii) saccharification (iii) fermentation. These three steps are carried out independently due to instability of cellulase (step ii) and fermentative microorganisms (step iii) in the residual ionic liquid (IL) used for pretreatment of biomass (step i). The biorefinery processes can be viable, if cellulases and fermentative microbes are stable to ILs. The present thesis entails the various studies conducted to overcome the constraints encountered in these separate processing steps. An active, IL stable and reusable cellulase has been developed in present work by covalently immobilizing *Trichoderma reesei* cellulase onto amino-silane functionalized magnetic (iron oxide) nanoparticles. The magnetic nanoparticles (MNP) immobilized cellulase was used for one-pot *in situ* saccharification of sugarcane bagasse and wheat straw, which were pretreated with 1-ethyl-3-methylimidazolium acetate [EMIM][Ac], a most commonly used IL. The disruption of the recalcitrant lignocellulosic fibrils, enabling release of reducing sugars, was supported by SEM, XRD and FTIR studies on [EMIM][Ac] pretreated and saccharified biomass. The high hydrolysis yields obtained in this one-pot process coupled with IL stability and recycled use of immobilized cellulase, potentiates its application for biofuel and biochemical production from agro-wastes.

Further, to explore the feasibility of using *L. brevis* for fermentative production of lactic acid from IL pretreated and saccharified agro-wastes, without removing residual IL, the stability of *L. brevis* was investigated in the presence of [EMIM][Ac]. The viability and stability of cells in [EMIM][Ac] was confirmed by their growth profile, MTT assay and SEM studies. The proteomic profiling by 2-D gel electrophoresis showed matching protein spots in the unexposed (control) and [EMIM][Ac] treated *L. brevis* samples. The in-solution tryptic digestion and subsequent LC-ESI MS/MS analysis revealed that all the key proteins involved in carbohydrate metabolism including lactate dehydrogenase dominated the metabolic flux of

*L. brevis* cells even in presence of [EMIM][Ac] (2 and 4%, v/v). The detection of proteins such as aldo/keto reductases, thioredoxin, glutathione reductase, Asp 23/Gls 24 family envelope stress response protein, universal stress protein, chaperone protein DnaK and 60 kDa heat shock protein on exposure to IL, indicated their role in maintaining the cell redox balance, protection against membrane disruption and coping with oxidative stress induced by [EMIM][Ac]. The IL stability in *L. brevis* is a significant development for sustainable biorefinery. Additionally, *L. brevis* was also found to utilize both hexose and pentose sugars without carbon catabolite repression (CCR), which is highly desirable trait for fermenting microorganism in biorefinery.

In final biorefinery process, three different agro-wastes i.e. cottonseed cake, wheat straw and sugarcane bagasse were pretreated by [EMIM][Ac], saccharified by nanoimmobilized cellulase (without washing step) and simultaneously fermented by *L. brevis* to achieve high yields of lactic acid. Moreover, merging all three steps in one-pot could be beneficial for future biorefineries with sustainable production of bioproducts.

The present work highlights:

- A sustainable approach for production of petrochemicals and enzymes by bio-based processes using vastly available agro-wastes as substrates, vis-a-vis paving a way for their meaningful utilization to address their disposal problems.
- Usage of IL stable saccharifying enzyme and fermentative microorganism paves the way for one-pot biorefining process, which can be scaled up into cost effective model industrial process.
- Understanding basic biochemistry of enzymes and microbial cells stability in IL, may enable to develop large array of IL stable systems for industrial usage.

## सार

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वर्तमान में 90% रसायन और 80% ऊर्जा की पूर्ति जीवाश्म ईंधन स्रोत के द्वारा पूर्ण हो रही है और इनकी आवश्यकता तेजी से बढ़ती जा रही है। घटते संसाधन व पर्यावरण के पतन ने सतत व अक्षय स्रोतों की आवश्यकता की ओर ध्यान आकर्षित किया है।

बायोरिफाइनिंग के लिए बायोमास उपयुक्त फीडस्टॉक का काम करता है। यह विभिन्न प्रकार के उपयोगी उत्पाद, ऊर्जा स्रोत का कार्य करता है तथा जैव आधारित अर्थव्यवस्था में योगदान करता है। भारत, अमेरिका और चीन लगभग 60% फसल अवशेष के लिए उत्तरदाई है। लगभग 140 अरब टन बायोमास कृषि क्षेत्र से उत्पन्न होता है। इनकी अनुपयोगिता से पर्यावरण एवं अर्थव्यवस्था पर बुरा प्रभाव पड़ता है।

वर्तमान खोज में अपशिष्ट पदार्थों की उपयोगिता व उत्पन्न बायोमास के सही इस्तेमाल की योजना की सिफारिश की गई है। कपास के बीज का केक एक ऐसा कृषि जन्य पदार्थ है, जिसका सार्थक रूप से इस्तेमाल नहीं हो पाता है क्योंकि इसमें गॉसीपोल जैसे विषाक्त पदार्थ मौजूद हैं।

कपास के बीज के केक के साथ छह दिनों की किण्वन प्रक्रिया द्वारा 19.7 मिलीग्राम गाबा ( $\gamma$ -अमीनो ब्यूटॉयरिक एसिड) उत्पादित किया गया। यह *लैक्टोबैसिलस ब्रेविस* की सहायता से ठोस अवस्था किण्वन द्वारा प्राप्त हुआ। उत्पादित गाबा का अनुप्रयोग 2-पैरोलिडोन बनाने में सफलता पूर्वक किया गया।

कपास के बीज के केक में प्रस्तुत गॉसीपोल को ठोस अवस्था किण्वन द्वारा 90% कम किया गया। इसके लिए तीन कवकों के सम्मिश्रण (*एस्पेरगिलस नाइजर*, *ट्राइकोडर्मा रीसि* और *फनरोकेट क्राइसोजीनम*) का उपयोग किया गया। इस प्रक्रिया द्वारा सेल्यूलेज़ व जाइलेनेस एंजाइम की भी प्राप्ति हुई। सेक्रेटोम की रूपरेखा एल.सी/ ई.एस.आई/ एम-एस (LC-ESI-MS/MS) से की गयी। इस जांच में भांति भांति के प्रोटीन जैसे हेमीसेल्यूलेज़, सेल्यूलेज़, एमाइलेज़, एसटरेस, प्रोटीएज एवं अतिरिक्त आपरंपरागत प्रोटीन को पाया गया। इनमें ग्लाइकोसाइड हाइड्रोलेज़ (GH) की मात्रा सर्वाधिक पाई गई। इनका परीक्षण गेहूँ के अपशिष्ट पदार्थों का सकरीफिकेशन करने में किया गया व सही पाया गया।

पर्यावरण व सतत ऊर्जा के स्रोतों की कड़ी में लिगनोसैलूलोज बायोरिफाइनिंग का दौर चल रहा है। मुख्य तीन पदों द्वारा इन लिगनोसैलूलोजिक पदार्थों का अपघटन किया जाता है। (क) पूर्व उपचार (ख) सकरीफिकेशन (ग) किण्वन। सेल्यूलेज़ की अस्थिरता के कारण तीन पदों को बारी-बारी से लागू किया जाता है। इस खोज में पूर्व उपचार के लिए [EMIM][Ac] आयनिक लिक्विड (IL) का उपयोग किया गया है। वर्तमान खोज में बारी-बारी से पदों को

करने के दौरान आए व्यवधानों से बचाव के हेतु योजना का निर्माण किया गया है। *ट्राइकोडर्मा रीसि* द्वारा जनित सेल्यूलोज एंजाइम का स्थिरीकरण चुंबकीय लोह ऑक्साइड नैनो कणों (MNP) पर इम्मोबिलिज़ेशन की मदद से किया गया। [EMIM][Ac] द्वारा पूर्व उपचार और आयनिक लिक्विड (IL) स्थिर एंजाइम का उपयोग करके गन्ने के अपशिष्ट पदार्थों व गेहूँ के अपशिष्ट पदार्थों से शर्करा उत्पन्न की गई। यह सभी पद वर्तमान प्रयोगों की मदद से एक पात्र में संभव हो पाये। SEM, XRD, FTIR जैसी जांच से बायोमास की क्रिस्टलिनिटी कम होने की पुष्टि की गई।

*लैक्टोबैसिलस ब्रेविस* के किण्वन प्रक्रिया से लैक्टिक एसिड का उत्पादन किया गया, जिसके लिए तीन बायोमास (कपास के बीज के केक, गेहूँ और गन्ने के अपशिष्ट पदार्थ) का उपयोग किया गया। [EMIM][Ac] की उपस्थिति में *लैक्टोबैसिलस ब्रेविस* की जीवन क्षमता को जांचा गया तथा SEM के अध्ययन से कोशिका के अंतर जीविता को भी जांचा गया। विकास की जांच प्रोटियोमिक रूपरेखा (2-D जेल इलेक्ट्रोफोरेसिस, LC-ESI-MS/MS) द्वारा तुलना में लाई गई। इसमें लैक्टेट डीहाइड्रोजीनेस एंजाइम सर्वाधिक मात्रा में चयापचय प्रवाह को प्रभावित करता हुआ पाया गया। अन्य प्रोटीन जैसे अल्डोकीटो-रिडक्टेज, थिओरडोक्सिन, ग्लूटाथिओन रिडक्टेज और Asp 23/Gln 24 स्ट्रेस रिस्पॉंस प्रोटीन परिवारों के सदस्य भी इनमें पाए गए। आयनिक लिक्विड (IL) की प्रस्तुति में, हेक्सोज व पेंटोस शर्करा का उपयोग *लैक्टोबैसिलस ब्रेविस* के किण्वन द्वारा बिना कटाबॉलीट रीप्रेशन (CCR) के किया गया।

अंततः कपास के बीज के केक, गेहूँ व गन्ने के अपशिष्ट पदार्थों का पूर्व उपचार [EMIM][Ac] करने के पश्चात, चुंबकीय नैनो कणों द्वारा सैंकॅरिफ़ाइड किया गया और *लैक्टोबैसिलस ब्रेविस* के किण्वन प्रक्रिया से लैक्टिक एसिड बनाया गया। यह सब इम्मोबिलिज़ेड सेल्यूलोज एवं *लैक्टोबैसिलस ब्रेविस* की [EMIM][Ac] में स्थिरता के कारण, तीन पदों को हटाते हुए एक ही पात्र में संभव किया गया।

वर्तमान खोज के विशिष्ट बिंदु:

(क) कृषि अपशिष्ट पदार्थों की सार्थक उपयोगिता, एंजाइम व लैक्टिक एसिड जैसे प्लेटफोर्म रसायन के निर्माण में सतत व पर्यावरण संरक्षित मार्ग द्वारा संभव की गयी।

(ख) एक ही पात्र में आयनिक लिक्विड (IL) द्वारा पूर्व उपचार में स्थिर एंजाइम का उत्पादन किया गया। यह ना सिर्फ मितव्ययी मार्ग सिद्ध हुआ अपितु पर्यावरण को भी संरक्षित करता हुआ पाया गया।

(ग) औद्योगिक उत्पादन में आयनिक लिक्विड (IL) की सहभागिता व एंजाइम की सहिष्णुता का बेहतरीन वैज्ञानिक प्रमाण दर्शाया गया।

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## List of Abbreviations

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[AMIM][Cl]	1-allyl-3-methylimidazolium chloride
[BMIM][Ac]	1-butyl-3-methylimidazolium acetate
[BMIM][BF <sub>4</sub> ]	1-butyl-3-methylimidazolium tetrafluoroborate
[BMIM][Cl]	1-butyl-3-methylimidazolium chloride
[BMIM][MeSO <sub>3</sub> ]	1-butyl-3-methylimidazolium methanesulfonate
[BMIM][PF <sub>6</sub> ]	1-butyl-3-methylimidazolium hexafluorophosphate
[BMPL][OTf]	1-butyl-1-methylpyrrolidinium trifluoromethanesulfonate
[C <sub>10</sub> MIM][BF <sub>4</sub> ]	1-decyl-3-methylimidazolium tetrafluoroborate
[C <sub>10</sub> MIM][Br]	1-decyl-3-methylimidazolium bromide
[C <sub>12</sub> MIM][Br]	1-dodecyl-3-methylimidazolium bromide
[EMIM][Ac]	1-ethyl-3-methylimidazolium acetate
[EMIM][CF <sub>3</sub> SO <sub>3</sub> ]	1-ethyl-3-methylimidazolium trifluoromethanesulfonate
[EMIM][DEP]	1-ethyl-3-methylimidazolium diethylphosphate
[EMIM][DMP]	1-ethyl-3-methylimidazolium dimethylphosphate
[EMIM][EtSO <sub>4</sub> ]	1-ethyl-3-methylimidazolium ethyl sulfate
[EMIM][HSO <sub>4</sub> ]	1-ethyl-3-methylimidazolium hydrogen sulphate
[EMIM][Lys]	1-ethyl-3-methylimidazolium lysinate
[EMIM][MeO(H)PO <sub>2</sub> ]	1-ethyl-3-methylimidazolium methylphosphonate
[HMIM][PF <sub>6</sub> ]	1-hexyl-3-methylimidazolium hexafluorophosphate
[MMIM][DMP]	1,3-dimethylimidazolium dimethylphosphate
[PrMIM][BF <sub>4</sub> ]	1-propyl-3-methylimidazolium tetrafluoroborate
CBM	Carbohydrate binding module
CCR	Carbon catabolite repression
CFU	Colony forming unit

CMC	Carboxymethyl cellulose
COGs	Clusters of orthologous groups
DES	Deep eutectic solvent
FPU	Filter paper unit
FTIR	Fourier transform infrared spectroscopy
GABA	$\gamma$ -Aminobutyric acid
GAD	Glutamate decarboxylase
GH	Glycoside hydrolase
GRAS	Generally regarded as safe
ILs	Ionic liquids
LAB	Lactic acid bacteria
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
MSG	Monosodium glutamate
MNP	Magnetic nanoparticles
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
PLP	Pyridoxal-5'-phosphate
ROD	Relative optical density
SEM	Scanning electron microscopy
SHF	Separate hydrolysis and fermentation
SNP	Silica nanoparticles
SSCF	Simultaneous saccharification and co-fermentation
SSF	Solid-state fermentation
TEM	Transmission electron microscopy
XRD	X-ray diffraction