

Bioconversion of agricultural waste biomass for ethanol production

*A thesis submitted in fulfillment of the
requirement for the award of the degree of*

**DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY**

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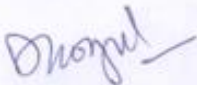


**Department of Biotechnology
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Patiala, Punjab
July, 2014**

CERTIFICATE

Certified that the thesis **“Bioconversion of agricultural waste biomass for ethanol production”** which is submitted by Mr. Nadeem Akhtar, in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology, Thapar University, Patiala, Punjab, India is a record of the candidate's own independent and original research work carried out by him under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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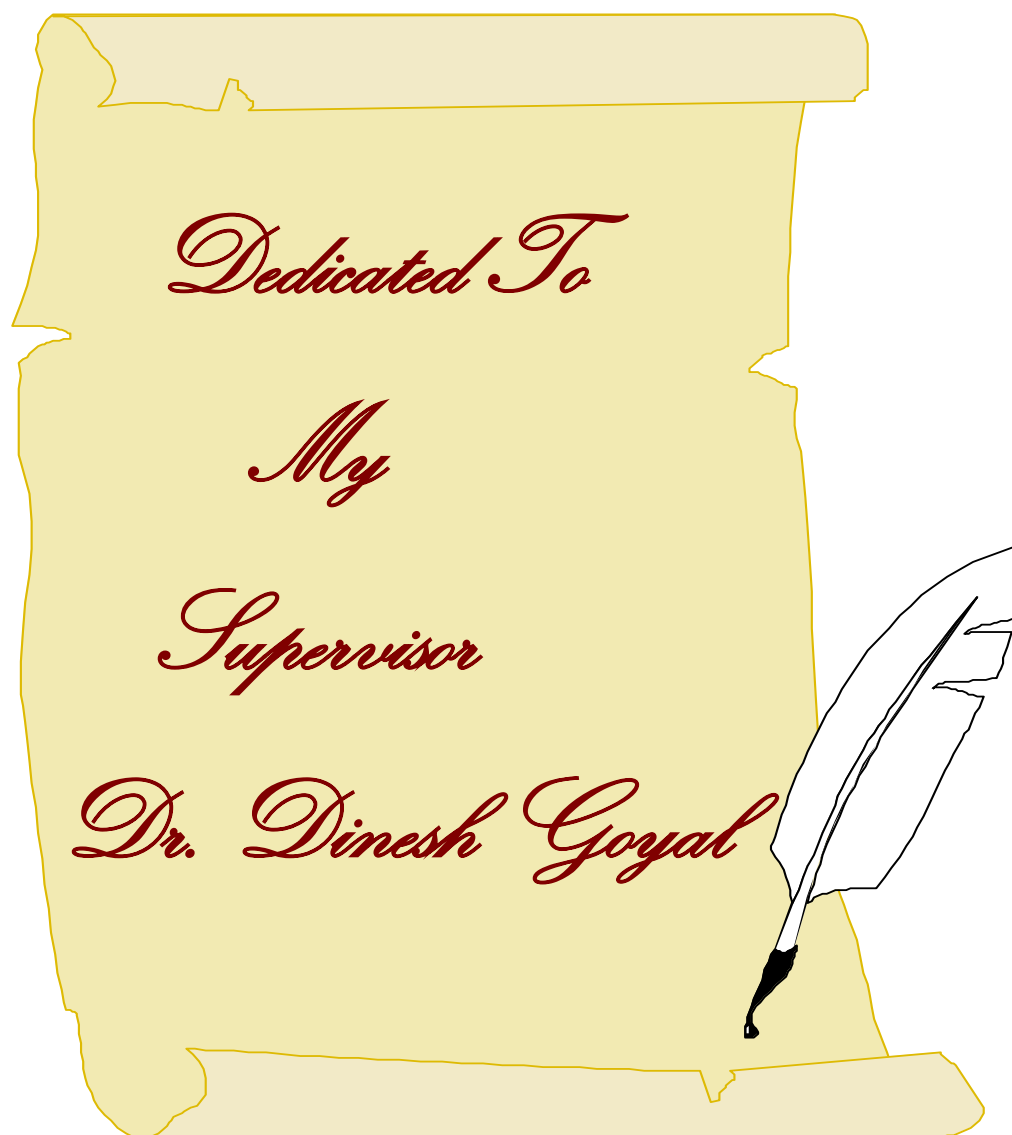

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DECLARATION

I hereby declare that the work which is being presented in this thesis "**Bioconversion of agricultural waste biomass for ethanol production**" submitted by me for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of Dr. Dinesh Goyal, Professor & Head, Department of Biotechnology, Thapar University, Patiala, Punjab, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

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Date: July 16, 2014

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ACKNOWLEDGEMENT

In pursuit of this academic endeavor, I feel that I have been singularly fortunate because inspiration, guidance, direction, cooperation, love and care - all came in my way in abundance. I feel lot of nostalgia to look back on the last four years of my PhD but it seems almost an impossible task for me to acknowledge the same in adequate term. It was a long journey that I finished, not alone!! One of the joys of completion is to look over the past and remember all people who supported me along this long, but fulfilling road. Without them, I may not have gotten to where I am today, at least not sanely.

*First and foremost I will forever be thankful to the person who made the biggest difference in my life, my supervisor, **Dr. Dinesh Goyal**, Professor and Head, Department of Biotechnology, Thapar University, Patiala. He has always been there to help me at every single step for the past four years, motivating me, guiding me and encouraging me to take up new challenges every day and tackle them with determination with optimistic attitude. It was his support which made me thrive for excellence and nothing less. His intelligent ideas thought provoking discussions and pertinent guidance helped me sail through this tedious journey. Though during the initial times, I was little apprehensive of my decision to join PhD but **Dr. Goyal** was always there with intelligent ideas, comprehensive understanding, serendipitous observation and vision of converting the ideas into scientific possibilities, which always kept me moving forward. Besides being a supportive advisor throughout, he gave me full freedom to pursue independent work to inculcate great confidence in me. Not only has he been a great scientist, but a very kind and helpful human being. He taught me another aspect of life that “goodness can never be defied and good human beings can never be denied”. I really feel privileged to be associated with a person like him during my life. His association with this endeavor of mine will remain a beacon light to me throughout my life. Though I will never find words to tell what I owe to him, and if I start doing it, I would not know where to stop...thanks for everything Sir...to me, professionally and personally, you are “perfection personified”.*

*I wish to express my thanks to **Dr. Prakash Gopalan**, **Director**, Thapar University, Patiala, for providing infrastructure and helping me towards the smooth and timely completion of my research work.*

I extend my sincere word of thanks to **Dr. Pramod K. Bajpai**, Dean (Research and Sponsored Projects), Thapar University, Patiala, for his encouragement and support during my research work in the Institute.

My special word of thanks should also go to members of my doctoral committee, **Dr. Sanjai Saxena**, **Dr. Abhijit Ganguli**, DBT, TU and **Dr. (Mrs.) Ranjana Prakash**, School of Chemistry and Biochemistry for their invaluable suggestion, constant support and encouragement during the course of my study.

A sincere token of gratitude to **Dr. B. N. Chudasama**, School of Physics and Material Science, **Dr. N. Tejo Praksh** and **Mr. Anoop Verma**, School of Environment and Energy, TU for their encouragement and support during the course of my PhD.

I wish to place on record my thanks to **Dr. Arun Goyal**, Professor, Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, Assam for his constant motivation, constructive suggestions, meticulous approach toward science and encouragement throughout.

This research was possible due to the support of the **Department of Biotechnology, Government of India** for proving me financial support to accomplish my work.

Most of the results described in this thesis would not have been obtained without a close collaboration with few laboratories. I am thankful to Sophisticated Analytical Instrument Facility at IITB, Mumbai and Panjab University, Chandigarh, Advanced Instrumentation Research Facility, Jawaharlal Nehru University, New Delhi, Sophisticated Analytical Instruments Laboratories, Thapar Technology Campus, Patiala and Centre for Advance study in Crystallography & Biophysics, University of Madras, Chennai, India for providing necessary instrumentation facilities to carry out my research work.

I would like to thank **TIFAC-CORE** and **Science & Technology Entrepreneur's Park (STEP)**, Thapar University for providing me necessary equipments and facilities to carry out work.

My acknowledgement will never be complete without the special mention of all my lab seniors **Dr. Sarabjit**, **Dr. Indu**, **Dr. Rajeev**, **Dr. Meenakshi**, **Dr. Purnima** and **Dr. Misha** who have taught me the lab culture and willingly devoted their valuable time whenever I needed their help.

*I owe my deepest gratitude to my dearest **Dr. Achal, Dr. Mukesh** and **Dr. Giri** for their continuous support and encouragement. They made available their support in infinite number of ways every time and anytime.*

*Words fail me to express my appreciation to my seniors come siblings for their moral support and motivation whenever I was low, **Dr. Pathak, Dr. Raghu, Dr. Anshu, Dr. Vijya** the list is endless..... thanks to one and all.*

*Most of the work would have been incomplete without the sincere support of Lab assistants. So, I owe a word of thanks to **Mr. Ram Newal (Babban ji)** and **Ms. Lalita**.*

*I am grateful to **Mr. Varinder Jain, Mr. Ashwani Aggarwal, Mr. Prem, Mr. Phoolchand** and all the **non-teaching staff** for helping and their ungrudging assistance.*

*I am very thankful to **Mr. Vijay** (Accountant) and **Mr. Pawan** (Helper) for their timely help and cooperation.*

*Some friends are never too busy to give us a hand whenever they are needed. I want to make a special mention for the friends who showed faith in me and my intellect even when I felt like digging hole and crawling into one because I didn't have faith in myself. I could not have asked for more than what I got from you all. I express my sincere love and affection towards all those benevolent souls and true friends **Vidya Sagar, Karan, Kunal, Rashmi, Anamika, Aditi, Sapna, Siddartha, Singla, Param, Mintu** and **Deepinder**.*

*I also welcome the opportunity to note the generous help that I received from my colleagues **Sumit, Neha Lohia, Rajinder Kaur, Vinit, Mahiti, Taran, Neha, Gurpreet, Charu** and **Prerna**. I owe a word of thanks to all the fellow research scholars for their help.*

*Many thanks to my juniors **Alok, Aanchal** and **Kanika** for propping me up and the remarkable team work.*

*I warmly thank my lovely juniors **Gourav, Vivek, Pargat** and **Sarabjot** for their consistent support.*

*I feel lacunae of words to acknowledge **Late Ms. Shipra** who left us untimely. I miss her a lot.*

I find it difficult to pen down my deepest sense of indebtedness towards my parents and all other family members who soulfully provided me their constant support and right impetus to undertake the challenge of this proportion like all other spheres of life, for what I cannot measure but treasure. They believed in me before I believed in myself. When my words ebbed

and enthusiasm waned, it was my **Mother** and **Father** who helped me getting out of it, a feat that can never be paid back in feelings or words. To them, I owe my wonderful today and dream filled future.

I highly appreciate unsolicited cooperation and cheery assistance of little champs **Abid, Asim, Urooj, Rahbar** and **Tabish**, my loving brother **Er. Azeem Akhtar** and his beloved wife, my dearest sisters **Dr. (Mrs.) Sahar** and **Advocate (Mrs.) Sumbul**, brother-in-laws **Dr. Qaiser** and **Advocate Athar Alam**. Their abundant affection, unflagging love and support were the constant source of inspiration for me.

I would also like to pay homage to my omnipresent maternal and paternal grandparents, who left me too soon. I wish you could have been with me for some more time. I consider myself the luckiest in the world to have such a supportive family, standing behind me with their love and support.

I would like to recompense my veneration to the Almighty for giving me strength and patience to work through all these years so that today I can stand proud with my head held high.

It has been a great privilege to spend several years in the **Department of Biotechnology, Thapar University, Patiala** and its members will always remain dear to me.

I love to acknowledge, **Thapar University, Patiala** for providing me an opportunity to perform the research activities and an environment for my all-round growth

Last but not the least, I wish to acknowledge all those, whose names have not figured here, but who helped me in any form during the period of my research work.

Date: July 16, 2014

Place: Patiala

(Nadeem Akhtar)

LISTS OF PUBLICATIONS

Published

1. **Akhtar, N.**, Aanchal, Goyal, D. & Goyal, A. (2014). Simplification and optimization of media ingredients for enhanced production of CMCase by newly isolated *Bacillus subtilis* NA15. *Environmental Progress and Sustainable Energy*. DOI 10.1002/ep. (SCI, Wiley-Blackwell, IF= 1.3).
2. **Akhtar, N.**, Sharma, A., Deka, D., Jawed, M., Goyal, D. & Goyal, A. (2012). Characterization of cellulase producing *Bacillus* sp. for effective degradation of leaf litter biomass. *Environmental Progress and Sustainable Energy*. 32:4, 1195-1201. (SCI, Wiley-Blackwell, IF= 1.3).
3. **Akhtar, N.**, Gupta, K., Jain, A. K., Goyal, D. & Goyal, A. (2014). Surfactant assisted microwave-acid pretreatment of leaf litter biomass for enhanced enzymatic release of sugars. *Cellulose Chemistry and Technology*. (**Accepted**) (SCI, Editura Acad Romane, IF= 0.8).
4. **Akhtar, N.**, Goyal, D. & Goyal, A. (2014). Biodegradation of leaf litter biomass by combination of *Bacillus* sp. and *Trichoderma reesei* MTCC 144. *Minerva Biotechnologica*. (**Accepted**). (SCI, Edizioni Minerva Medica, IF= 0.4)

Communicated

1. Sharma, A., **Akhtar, N.**, Goyal, D. & Goyal, A. (2014). Response surface methodology for optimization of microbial cellulase production. A Review. *Romanian Biotechnological Letters*. (**Under Revision**, Manuscript No. RBL 10079). (SCI, Ars Docendi, IF= 0.4).
2. **Akhtar, N.**, Sharma, A., Goyal, D. & Goyal, A. (2014). Biodiversity of cellulase producing bacteria and their application. *Cellulose Chemistry and Technology*. (**Under Revision** Manuscript No. 1076) (SCI, Editura Acad Romane, IF= 0.8).
3. **Akhtar, N.**, Gupta, K., Goyal, D. & Goyal, A. (2014). Recent advances in pre-treatment technologies for efficient hydrolysis of lignocellulosic biomass. A Review. *Environmental Progress and Sustainable Energy*. (**Submitted**, Manuscript No. EP-14-290). (SCI, Wiley-Blackwell, IF= 0.9).

Book Chapter (Invited)

1. **Akhtar, N.**, Goyal, D. & Goyal, A. (2014). Biodegradation of cellulose and agricultural waste material, Chapter 14, In 'Advances in Biodegradation and Bioremediation of Industrial Waste' Editor Ram Chandra. CRC Press, USA (**In Press**).

PARTICIPATION IN CONFERENCES AND SYMPOSIA

International

1. **Akhtar**, N., Goyal, D. & Goyal, A. Biodegradation of leaf litter biomass by co-inoculation of *Bacillus* sp. and *Trichoderma reesei* MTCC164. 29th International Conference on Solid waste Technology and Management (ICSW-2014). March 30- April 02, 2014. Philadelphia, USA.
2. **Akhtar**, N., Gupta, K., Goyal, D. & Goyal, A. Surfactant assisted microwave-acid pretreatment of leaf litter biomass for enhanced enzymatic release of sugars. 29th International Conference on Solid waste Technology and Management (ICSW-2014). March 30- April 02, 2014. Philadelphia, USA.

National

3. **Akhtar**, N. & Goyal, D. Characterization of leaf litter biomass for effective renewable fuel. 3rd National Conference on Advances in Metrology (AdMet-2014). February 19-21, 2014, Thapar University, Patiala, Punjab.
4. **Akhtar**, N., Goyal, D. & Goyal, A. Characterization and purification of endoglucanase from *Bacillus licheniformis*. 54th Annual Conference of Association of Microbiologist of India (AMI). November 17-20, 2013. Maharshi Dayanand University, Rohtak, Haryana, India.
5. **Akhtar**, N., Goyal, D. & Goyal, A. Characterization of cellulose degrading *Bacillus licheniformis* T8 for effective degradation of leaf litter biomass. 6th National Conference on Recent Advances in Chemical & Environmental Sciences (RACES). November 13-14, 2013. Multani Mal Modi College, Patiala, Punjab, India.
6. **Akhtar**, N., Goyal, D. & Goyal, A. Characterization and biodegradation of leaf litter biomass by *Trichoderma reesei* MTCC 164 and *Bacillus* sp. AS3. National seminar on current perspectives of fungi in health care and environment (kavaastha), March 13-14, 2013. Bangalore University, Karnataka, India.
7. **Akhtar**, N., Kaur, D., Goyal, D. & Goyal, A. Ethanol production from Agricultural waste biomass. International Conference on Industrial Biotechnology & IX Convention of the Biotech Research Society, India & Indo-Italian workshop on food biotechnology: industrial processing, safety & health. November 21-23, 2012. Punjabi University, Patiala, Punjab, India.
8. **Akhtar**, N., Goyal, D. & Goyal, A. Biodegradation of leaf litter biomass by *Bacillus* sp. AS3. 52nd Annual Conference of Association of Microbiologist of India (AMI). International Conference on Microbial biotechnology for Sustainable development. November 3-6, 2011. Punjab University, Chandigarh, India.

ABSTRACT

Conversion of lignocellulosic biomass to ethanol remains a key bottleneck at industrial scale to enhance the world energy security. Identification of promising feedstock, its pre-treatment with process integration for enhanced saccharification by microbial conversion of substrate to ethanol is important in development sustainable energy system. Cellulose degrading bacteria from different sources such as compost, paper pulp and sugarcane bagasse were isolated, screened and characterized. Out of 32, eight bacterial isolates (designated as NA8, NA9, NA11, NA14, NA15, DGA, DGB and DGC) were found to be cellulose degraders. Biolog GEN III MicroPlate™ identification and 16S rRNA gene sequence analysis revealed that NA8, NA9, NA11, NA14, DGB and DGC showed maximum similarity with *Bacillus licheniformis*, isolate NA15 had maximum similarity with *Bacillus subtilis* whereas, DGA shared maximum similarity with *Lysinibacillus fusiformis*. *Bacillus subtilis* NA15 was found to be the best cellulose degrader among all the eight screened isolates with maximum carboxymethyl cellulase (CMCase) activity of 0.06 U/mL in cell free supernatant.

Screening of 11 media ingredients using Plackett-Burman design was done to explicate the parameters that significantly influence the CMCase production. Maximum CMCase activity of 0.47 U/mL was obtained using Response surface methodology (RSM) with carboxy methyl cellulose (CMC): 18 g/L, peptone: 5 g/L, yeast extract: 5 g/L and MnCl₂: 0.5 g/L. The model predicted the maximum CMCase activity (0.45U/mL) which was in good agreement with the experimental value of 0.47 U/mL showing 7-fold increase as compared to unoptimized medium. CMCase from *Bacillus subtilis* NA15 was purified using DEAE-Sepharose ion exchange chromatography with increase in CMCase activity from 2.65 to 30.42 U/mg of protein (11.5 fold) with a yield of 43.7%. The approximate molecular mass of the purified cellulase was 45 kDa. Purified CMCase exhibited highest activity of 3.24 U/mL at pH 7.0, which was stable at pH 5-7 and thermally stable up to 60°C.

Agricultural waste such as wheat and rice straw and leaf litter from Mango, Poplar, Ashoka and Eucalyptus were collected, processed and subjected to different pre-treatments. Four different pre-treatment strategies such as fungal alkaline fractionation, fungal solvolysis, dilute acid alkali and microwave-acid-alkali treatment were employed for all six biomass samples to enhance saccharification. Microwave-acid-alkali pre-treatment was found to be the best and most

promising technique among all pre-treatments. Scanning electron microscopy (SEM), X-ray diffraction (XRD), Thermal gravimetric analysis (TGA), Fourier transform infrared (FTIR) spectroscopy analysis and Solid state ^{13}C CP/MAS NMR spectroscopy of native and microwave-alkali-acid pre-treated rice straw was done to investigate physical and chemical changes after pre-treatment. Solid state ^{13}C CP/MAS NMR spectroscopy was most reliable technique to determine biochemical changes before and after pre-treatment of biomass.

Microwave-acid-alkali pre-treated rice straw was selected as feedstock for ethanol production with maximum reducing sugar (246 mg/g of biomass) yield. The process parameters were optimized using Taguchi design to obtain greater yield of ethanol using purified cellulase from *Bacillus subtilis* NA15 and *Saccharomyces cerevisiae* via simultaneous saccharification and fermentation (SSF). Maximum ethanol concentration (18.9 g/L) was found with optimized parameters of substrate loading: 11% (w/v), pH: 4.5, temperature: 30°C, enzyme loading: 0.5% (v/v), inoculum size: 8% (v/v), after 24 h of incubation. The optimized conditions were further used for scale up of ethanol production in 3-L bioreactor using microwave-acid-alkali pre-treated rice straw and maximum ethanol concentration (25.2 g/L) corresponding to 59% yield was obtained after 24 h of SSF process.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APS	Ammonium persulfate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
^{13}C CP/MAS NMR	^{13}C cross-polarization/magic angle spinning nuclear magnetic resonance
CBU	Cellobiose unit
CFU	Colony forming units
CMC	Carboxy methyl cellulose
CO	Carbon monoxide
CO ₂	Carbon dioxide
<i>CrI</i>	Crystallinity index
DNA	Deoxyribonucleic acid
DNS	3, 5-dinitrosalicylic acid
dNTP	2'-deoxynucleoside-5'-triphosphate
EDTA	Ethylenediamine tetraacetic acid
et al	And others
etc	And other things
FeSO ₄ .7H ₂ O	Ferrous sulphate heptahydrate
FPU	Filter paper unit
FTIR	Fourier transform infrared spectroscopy
g	Gram
g/L	Gram per litre
GHGs	Green house gases
h	Hour
H ₂ O ₂	Hydrogen peroxide
H ₂ SO ₄	Sulfuric acid
HCl	Hydrochloric acid
Hz	Hertz

K_2HPO_4	Di potassium hydrogen phosphate
kb	Kilo base
kGy	Kilo-gray
KOH	Potassium hydroxide
L	Litre
M	Molar
mA	Milliampere
mg	Milligram
$MgSO_4 \cdot 5H_2O$	Magnesium sulphate pentahydrate
MHz	Mega hertz
min	Minute
Mj/kg	Mega joules per kilogram
mL	Millilitre
mm	Millimetre
mM	Millimolar
MMT	Million metric ton
MT	Metric ton
MTCC	Microbial type culture collection
N	Normal
NCBI	National centre for biotechnology information
NCIM	National collection of industrial microorganisms
ng	Nanogram
nm	Nanometre
NO _x	Nitrogen Oxides
O ₂	Oxygen
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
ppm	Parts per million
rDNA	Ribosomal deoxyribonucleic acid
rpm	Revolution per minute

rRNA	Ribosomal ribonucleic acid
s	Second
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
TEMED	Tetramethylethylenediamine
TGA	Thermo gravimetric analysis
viz.	As follows
W	Watt
XRD	X-ray diffraction
μg	Microgram
μL	Microlitre

LIST OF SYMBOLS

%	Percentage
μ	Micron
C	Carbon
Da	Dalton
H	Hydrogen
h	Hours
kDa	Kilo Dalton
L	Litre
M	Molar
Mg	Magnesium
N	Nitrogen
S	Sulfur
U	Unit
V	Volt
v/v	Volume by volume
w/v	Weight by volume
α	Alpha
β	Beta
%	Percent
°C	Degree Celsius
®	Registered
θ	Angle
™	Trademark

CHAPTER I. INTRODUCTION

Increase in global energy demand has put immense pressure on utilization of alternative energy resources. In this context, it has become indispensable to scout for alternative sources of energy which can replace conventional fossil fuels (Wan and Li, 2011). Transportation sector is one of the largest consumer of petroleum based fuels: motor gasoline, distillate fuel oil (diesel), jet fuel, residual fuel oil, lubricants, aviation gasoline and liquefied petroleum gas. Increase in consumption of these fuels has contributed to hike in fuel prices, depletion of energy resources and accumulation of greenhouse gases (GHGs) in the atmosphere. Biomass can be promising substitute for the production of clean and environmental-friendly energy and other valuable biobased products.

Ethanol has higher octane number (96-113) than conventional gasoline (82-90), is generally blended to increase the octane number, in turns reducing the need for toxic, octane enhancing additives (Mielenz, 2001). Furthermore, ethanol has great potential in formulation of solvent, as an antifreeze agent, in pharmaceuticals, dyestuffs and perfumes etc. Brazil and USA are the two major ethanol producers accounting for 62% of the world production (Himmel et al., 2009). During twentieth century, large-scale production of fuel ethanol was mainly based on energy crops, sucrose from sugarcane in Brazil and corn starch in the USA. Largest producer of bioethanol is USA with annual production of 10 billion gallons and is expected to reach 30 billion gallon by 2030 (Nesbitt et al., 2011).

The most popular blend (E85) contains 85% (v/v) anhydrous ethanol and 15% gasoline (Balat et al., 2008), commonly used in US and Europe to run flexible-fuel vehicles. Emission of GHGs were decreased with an increase in the ethanol fraction to 85% (v/v), however it causes significant air pollution in the form of nitrogen oxides (NO_x) and organic compounds (Kemppainen and Shonnard, 2005; Niven, 2005; Rass-Hansen et al., 2007). Low ethanol-gasoline blend [10% (v/v) or E10] resulted into significant reduction in CO and hydrocarbon volatile organic compounds (VOCs) with slight increase in other non-hydrocarbon VOCs; however no remarkable increase in NO_x was reported (Jia et al., 2005). Production of ethanol as a fuel from lignocellulosic biomass represents closed carbon cycle, lowers the release of fossil fuel based CO₂ to the atmosphere (Sommer et al., 2004; Kemppainen and Shonnard, 2005; Aida et al., 2007; Miyazaki et al., 2008).

Permissible bioethanol-gasoline blend in India has now taken into consideration and is being used to meet country's automotive fuel need. In November 2006, the Government of India (GoI) had ordered use of 5% ethanol blend in petrol to be sold throughout the country, except Jammu and Kashmir and the north-eastern states which was optionally raised to 10% from October 2007, and then made compulsory from October 2008 (<http://www.dancewithshadows.com/business/government-makes-5-ethanol-in-etrolmandatory>). However, oil marketing companies (OMCs) such as Bharat Petroleum, Hindustan Petroleum, Indian Oil Corporation could not ensure the mandatory blend due to scarcity of ethanol. Since October 2007, the ethanol blend programme (EBP) has been struggling to take off, GoI had directed to impose financial penalty on OMCs from November 2009, for their failure to reach targets which require 105 crore litres of ethanol annually, raising the shortage to the tune of 41% as OMCs contracted for just 62 crore litres of ethanol (http://articles.economictimes.indiatimes.com/2012-11-23/news/35300815_1_cent-ethanol_crore-litres-ebp-programme). First generation bioethanol was derived from starch and sugar crops, such as corn and sugar cane, respectively. However, the long term availability and sustainability of these crops are questionable due to competition with the world's food and animal feed supply. Thus, the second generation of bioethanol made from cellulosic feedstocks without a food use, namely cellulosic ethanol, has premise for a new industry. Ethanol from lignocellulosic biomass seems to be a promising substitute for traditional fossil fuels, which can be used to run dedicated engines or gasoline based engine or in fuel blends.

The process for converting lignocellulosic biomass such as agricultural and forest residues and herbaceous energy crops to ethanol requires (1) delignification to liberate cellulose and hemicelluloses (2) hydrolysis of the carbohydrate polymer to produce sugars and (3) fermentation of hexose and pentose to ethanol (Lee, 1997; Lin and Tanaka, 2006; Champagne, 2008; Tomas-Pejo et al., 2008). Hydrolysis mechanism is still technically problematic because the digestibility of cellulose is hindered by many physico-chemical, structural and compositional factors (Alvira et al., 2010). Selection of a suitable lignocellulosic feedstock and its pre-treatment based on physico-chemical characteristics for subsequent hydrolysis and fermentation for greater yield of ethanol is of prime concern. Crystallinity, degree of polymerization, particle size and accessible surface area limit the decrystallization of cellulose and depolymerization of hemicellulose during pre-treatment. Intelligent combinatorial pre-treatment approaches such as

microwave-acid-alkali, ultrasound-surfactant, supercritical carbon dioxide and hot water-acid have been applied to enhance the sugar yield from wide variety of biomass such as sugarcane bagasse, sugarcane tops, corn stover and switch grass. Furthermore, biological pre-treatment using white, brown and soft rot fungi and some bacteria are attractive in terms of being cost effective, low energy requirement and environment friendly.

Bioconversion of cellulose plays a key role in the global carbon cycle. Many types of organisms produce cellulase, which go through secretory pathways to reach the extracellular space, where enzymatic reactions take place (Yan and Wu, 2013). Both fungi and bacteria have been widely used for their abilities to produce a wide variety of cellulases. More emphasis had been given for use of fungi because of their capability to produce copious amounts of cellulases, which are secreted in the medium for easy extraction and purification and are less complex than bacterial glycoside hydrolases. However, due to certain advantages such as higher growth rate of bacteria, synergy of complex enzyme system, higher compatibility and feasibility towards genetic engineering, bacterial system is being preferred over fungal organisms. Thrust for efficient cellulase producing bacteria, low enzyme dose requirement and broad range of tolerance is to achieve optimum utilization of these enzymes for use in industrial bio-refineries.

Currently, there are several routes to ferment lignocellulosic materials to ethanol using ethanologenic microorganisms. This includes mainly simultaneous saccharification and fermentation (SSF), separate hydrolysis and fermentation (SHF). In addition to SSF and SHF, the available alternatives are consolidated bioprocessing (CBP) and simultaneous saccharification and co-fermentation (SSCF) (Alvira et al., 2010). In SSF, the ethanol production rate is controlled by the cellulase hydrolysis rate and not the glucose fermentation, and hence, steps to increase the rate of hydrolysis will lower the cost of ethanol production via SSF (Tomas-Pejo et al., 2008). SSF is more favored because in this process glucose released by the action of cellulase which is converted quickly to ethanol, thus minimizing end-product inhibition to enzyme caused by glucose and cellobiose accumulation.

Given such huge demand of energy sources in future, the main ethanol feedstock will be shifted from food crops to agricultural waste biomass. While sugarcane and corn are dominant sources for ethanol production at present, ethanol production from agricultural waste biomass is under developmental stage. It can be concluded that the use of this feedstock will increase dramatically in the near future and became the main resource for ethanol production. The present

investigation deals with characterization of efficient cellulolytic bacteria from different habitats and physico-chemical characterization of different types of agricultural waste biomass, their pre-treatment and using it as feedstock for ethanol production via SSF process.

Therefore the objectives of the present work are:

1. Screening and characterization of efficient cellulose degrading microorganisms
2. Biochemical characterization of cellulose degrading enzymes
3. Optimization of ethanol production by simultaneous saccharification and fermentation (SSF)

CHAPTER II. REVIEW OF LITERATURE

2.1. Agricultural waste biomass

Lignocellulosic biomass is widely available and most abundant feed-stock available on the Earth (Kim et al., 2002; Kim et al., 2005; Dawson and Boopathy, 2007). Among various agricultural waste biomass, rice straw and wheat straw is produced mainly by Asia, whereas America is the major producer of corn straw and bagasse (Kim and Dale, 2004). These feed stocks are most favorable for bioethanol production due to their availability throughout year (Sarkar et al., 2012). However there are some other lignocellulosic substrate such as barley straw (Strehler and Stutzle, 1987; Adapa et al., 2009; Naik et al., 2010), flax straw, pine wood, timothy grass (Naik et al., 2010), rye husk, oat straw (Adapa et al., 2009), sugarcane bagasse (Pereira et al., 2011; Rezende et al., 2011; Corrales et al., 2012; Chandel et al., 2014) and biomass from *Lantana camara* (Sugumaran and Seshadri, 2009; Kuila et al., 2011), *Tamarix ramosissima* (Xiao et al., 2011), *Casuarine equisetifolia* (Sugumaran and Seshadri, 2009), switch grass (Xu et al., 2010), *Miscanthus giganteus* (Brosse et al., 2009), *Poplar* sp. (Wang et al., 2012), *Eucalyptus* sp. (Yanez et al., 2013) have been exploited for bioethanol and biobased products. Rice straw, wheat straw, corn stover and sugarcane bagasse production amount reached to approximately 731, 354, 204 and 181 million tons (MT) per year, respectively (Glassner et al., 1999; Kim and Dale, 2004; Binod et al., 2010; Sarkar et al., 2012).

In India, rice straw is one of the most abundant lignocellulosic waste which is mainly used as domestic fuel and as fuel to run the boilers. In north Indian region, mainly Punjab and Haryana, post harvesting residue of rice crop is generally burnt in the field which adversely affects the nutritional quality of soil and human health. Punjab accounts for the production of 15 MT rice straw per year, 80% of them are burnt (Sidhu and Beri, 2008). It causes emission of harmful air pollutants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), threatening human health (Chen et al., 2008; Shi et al., 2008; Estrellan and Iino, 2010). Different trees such as Mango, Poplar, Ashoka and Eucalyptus are most commonly found in north India. The cellulosic and hemicellulosic

component of these leaf litters may be a potential candidate for its conversion to fermentable sugars.

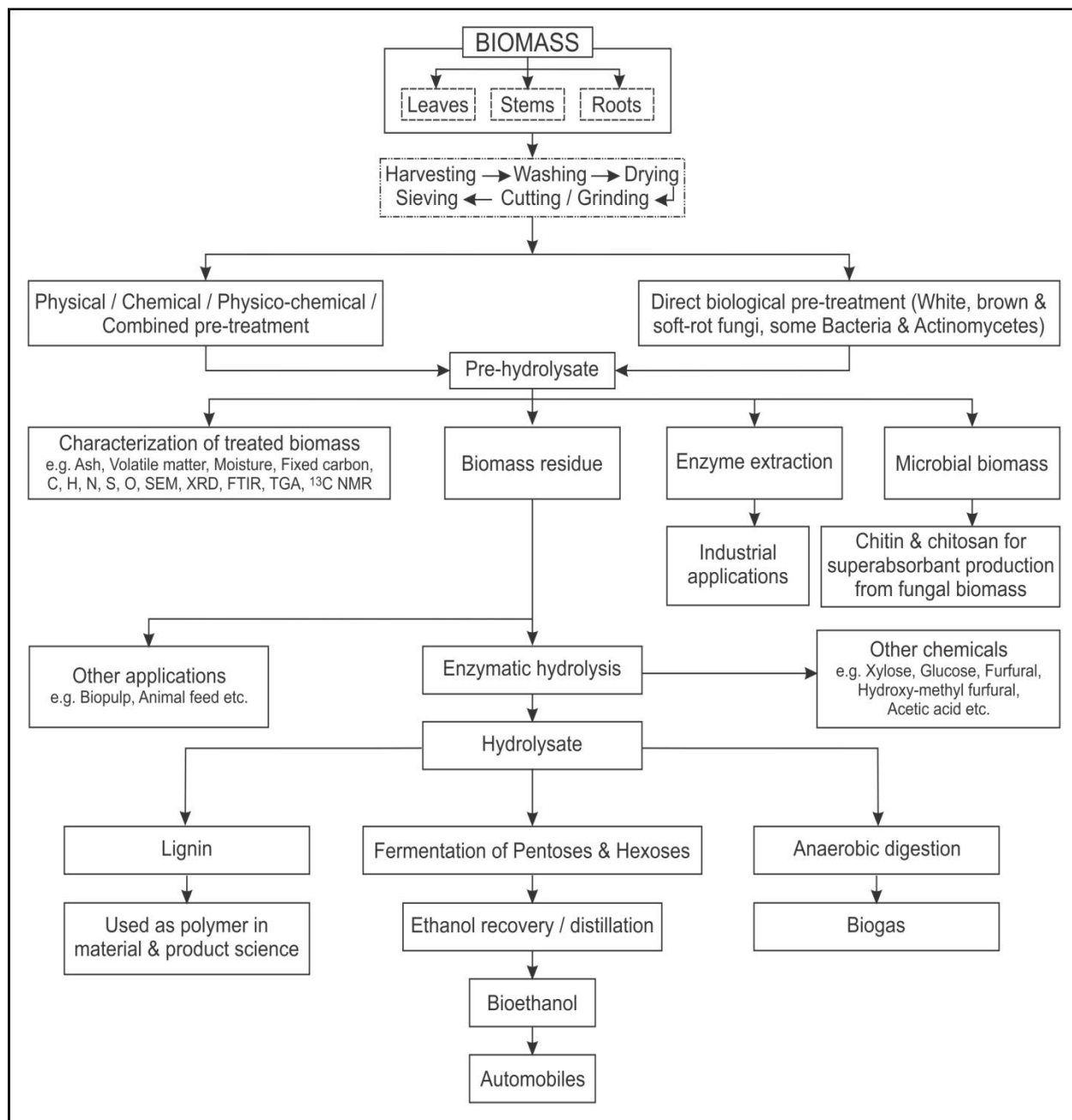


Figure 2.1. Overview of ethanol production and other biobased products from lignocellulosic waste biomass.

Lignocellulosic biomass is composed of cellulose (30-50%), hemicellulose (20-40%), lignin (15-25%), pectins, extractives, glycosylated proteins and several inorganic materials (Sjostrom,

1993; Mielenz et al., 2001; Badger, 2002; Knauf and Moniruzzaman, 2004; Kaparaju et al., 2009; Girio et al., 2010). The cellulose, hemicellulose and lignin content of the different biomasses are depicted in Table 2.1. Cellulose and hemicelluloses are tightly linked to the lignin component through covalent and hydrogen bonds that make the structure highly robust and resistant to any treatment (Mielenz et al., 2001; Knauf and Moniruzzaman, 2004; Edye et al., 2008). Recalcitrant structure and complexity remains a major economic and technical obstacle to lignocellulosic-based biofuel (Zhang et al., 1995). As a result, only 20% of its theoretical maximum yield of glucose can be obtained by enzymatic hydrolysis unless it is pretreated by physical, chemical or biological means (Taherzadeh and Karimi, 2008).

2.1.1. Cellulose

Cellulose, the most abundant organic compound on the earth consists of β ,1-4-polyacetal of cellobiose (4-O- β -D-glucopyranosyl-D-glucose). It is more commonly considered as linear chain of several hundred to ten thousands recurring D-glucose units (Figure 2.2 a), linked by β (1 $\rightarrow$ 4) glycosidic bonds (Delmer, 1995; McKendry, 2002; Zhang et al., 2004). Cellulose, having molecular weight of about 100,000, forms structural component of a primary cell wall in green plants and algae. Lignocellulosic material consists of both crystalline and amorphous forms of cellulose; tightly bounded parallel arranged bundles of cellulosic chains formed by strong inter chain hydrogen bonds forms crystalline region while this is less ordered and conspicuous in amorphous region.

2.1.2. Hemicellulose

The second most abundant natural polymer is hemicellulose (Hendriks and Zeeman, 2009; Agbor et al., 2011). Hemicellulose is the heterogenous polymer consists of pentoses (D-xylose, D-arabinose), methyl pentoses (L-rhamnose), hexoses (D-glucose, D-mannose, and D-galactose), and carboxylic acids (D-glucuronic acid, D-galacturonic acid, methyl glucuronic acid) (Figure 2.2b) which can be employed in bioconversion processes for the production of ethanol and other value glucomannans are most common (Singh et al., 2008; Hendriks and Zeeman, 2009; Agbor et al., 2011). Hemicelluloses have a random, amorphous, and branched structure with little resistance to hydrolysis, are more easily hydrolyzed by acids to their monomer components

(O'Dwyer, 1934; Mod et al., 1981; Morohoshi et al., 1991; Sjostrom, 1993; Ademark et al., 1998). Xylan, the most common type of polysaccharide in hemicellulose family consist of D-xylopyranose linked together by β -1,4-linkage with less than 30, 000 molecular weight and up to 200 degree of polymerization

Table 2.1. Percent dry weight composition of some lignocellulosic and organic waste.

Biomass	Cellulose	Hemicellulose	Lignin	Reference
Wheat straw	35	29	21	Naik et al., 2010
	34	24	14	Adapa et al., 2009
Rice straw	39	24	6	Chandra et al., 2012
Rice husk	31	24	14	Raveendran et al., 1995
Corn stove	43	24	11	Zeng et al., 2011
Sugarcane bagasse	35	24	22	Rezende et al., 2011
	45	28	23	Pereira et al., 2011
Sweet sorghum bagasse	45	25	18	Ballesteros et al., 2004
Leaves	15-20	80-85	--	Prasad et al., 2007
Barley straw	32	26	23	Naik et al., 2010
Corn stalks	43	24	17	Prasad et al., 2007
Corn cobs	45	35	15	Prasad et al., 2007
Switch grass	45	31	12	Sun et al., 2002
	36	31	6	Greenhalf et al., 2012
Flax straw	29	27	22	Naik et al., 2010
Oat straw	31-37	27-38	16-19	Sanchez, 2009
Rye straw	33-35	27-30	16-19	Sanchez, 2009
Timothy grass	34	30	18	Nanda et al., 2013
Elephant grass	22	24	24	Sanchez, 2009
Coastal Bermuda grass	25	36	6	Prasad et al., 2007
Esparto grass	33-38	27-32	17-19	Sanchez, 2009
Bamboo	26-43	15-26	21-31	Sanchez, 2009
Leaves	15-20	80-85	--	Prasad et al., 2007
Poplar wood	35	17	26	Ballesteros et al., 2004
Cotton gin waste	78	16	--	Raveendran et al., 1995
Newspaper	40-55	25-40	18-30	Prasad et al., 2007
Nut shells	25-30	25-30	30-40	Prasad et al., 2007
Poplar wood	44	32	21	Meng et al., 2012
Eucalyptus wood	45	15	26	Emmel et al., 2003
Waste papers from chemical pulps	60-70	10-20	5-10	Prasad et al., 2007
Bast fibre jute	45-53	18-21	21-26	Sanchez, 2009
Banana waste	13	15	14	Sanchez, 2009

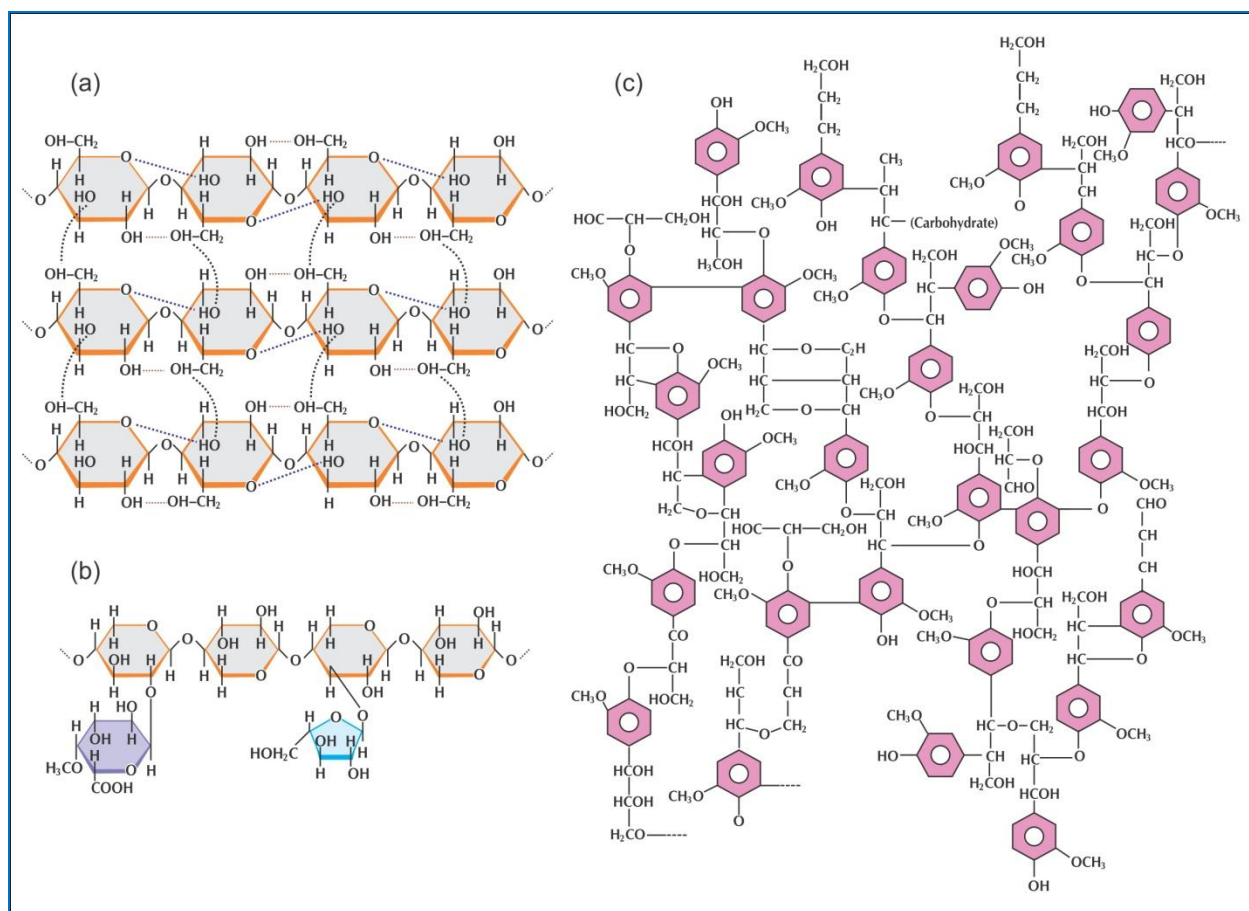


Figure 2.2. Chemical structure of (a) cellulose, (b) hemicellulose and (c) lignin

2.1.3. Lignin

Lignin, the third largest available biopolymer in nature (Hendriks and Zeeman, 2009; Agbor et al., 2011), consists of phenyl propane (p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) units linked with ester bonds that form a complex with hemicellulose to encapsulate cellulose (Figure 2.2 c), making it resistant towards chemical and enzymatic hydrolysis. Lignin, generally having a molecular weight of less than 20,000, is found more in soft wood (pine, balsam, spruce, tamarack, fir) than in hardwood (oak, walnut, maple, poplar, birch). Lignin-enriched biomass is more resistant to depolymerisation of holocellulose (cellulose and hemicellulose) to produce fermentable sugars. In addition, during the degradation process, it may form furan (furfural and hydroxymethyl-furfural) compounds that could inhibit fermentation (Zaldivar et al., 1999; Girio et al., 2010; Ran et al., 2014).

2.2. Physico-chemical characterization of lignocellulosic biomass

In last five years, various biomass such as rice straw, barley straw, timothy grass, flax straw, pine wood (Naik et al., 2010), biomasses of *Areca catechu*, *Ziziphus rugosa* and *Albizia lucida* (Sasmal et al., 2012), broom, heath, carqueisa and gorse (Viana et al., 2012) have been physico-chemically characterized for biofuel production. There is a need for selection of suitable lignocellulosic feedstock based on physico-chemical characteristics to assess biomass having potential for biofuel production. Digestibility of lignocelluloses is technically problematic since it is hindered by many physico-chemical, structural and compositional factors (Alvira et al., 2010). The primary obstacle impeding the widespread production of bioenergy from biomass feedstocks is the absence of low-cost technology for overcoming the recalcitrance of these materials mainly due to presence of lignin (Palonen et al., 2004). To make cellulose more accessible for hydrolytic enzymes, lignin component must be separated (Selvi et al., 2009), which primarily depends upon degree of polymerization, degree of crystallinity, structural composition and availability of surface area (Qi et al., 2009). The general scheme of biomass characterization is represented in Figure 2.3.

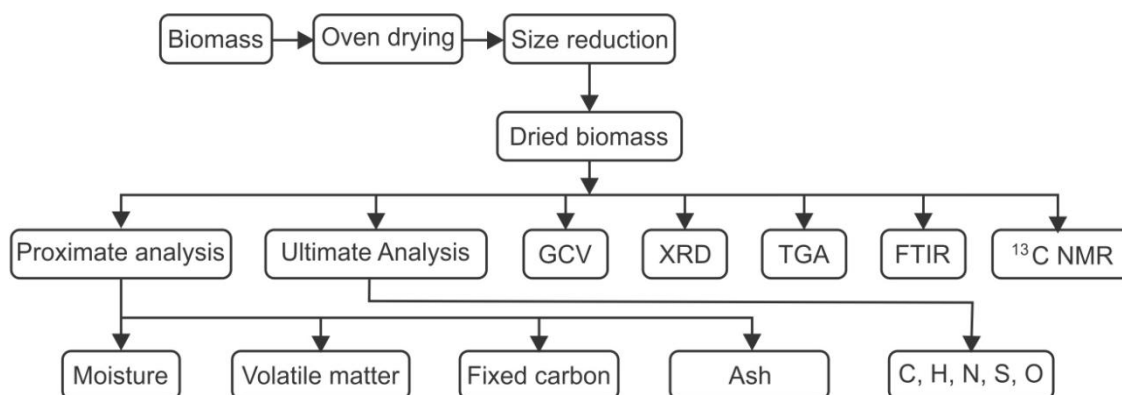


Figure 2.3. Schematic representation of physico-chemical characterization of biomass.

2.2.1. Proximate analysis

Proximate analysis is the most often employed practice for biofuel characterization. It determines the weight percent of moisture, volatile matter, fixed carbon and ash content of a biomass. Moisture content plays a key role in selection of energy conversion process technology (Cuiping et al., 2004). High moisture (>40%) is generally observed in bagasse, sludge, wood bark etc., on the other hand low moisture (~ 6 to 16%) content has been reported in biomass of juliflora, cotton, chilly stalk, rice husk and straw (Patel and Ghami, 2012). Comparative low moisture content (<15%) feedstock is preferred for thermo-chemical conversion process, while bio-conversion can utilize biomass with high or optimum moisture content (Nanda et al., 2013). Chemical, thermo-chemical and bio-chemical breakdown of biomasses produce solid residue, representing recalcitrant carbon, converted to ash when subjected to complete combustion. Ash, the incombustible solid mineral matter of the biomass, mainly contains oxides of silica (SiO), aluminium (AlO), iron (FeO), calcium (CaO) and magnesium (MgO). The distribution of chemical in ash invites operational problems such as slagging, fouling, sintering and corrosion owed to high alkali concentration. Generally, ash content varies from 1-10% in promising lignocellulosic substrate used in biorefining industry. Ignition and combustion problems for use of biomass fuels are mainly due to high moisture and ash content (Demirbas, 2004). The cations present in ash content retards the enzymatic saccharification of biomass samples, hence washing is recommended in order to remove water soluble and sticky inorganic materials before hydrolysis (Bin and Hongzhang, 2010). In biomass fuel, chemical energy is stored mainly in the form of fixed carbon and volatile matter. The components releases as a gas (including moisture) by heating to 950°C for 7 min is volatile matter, whereas the non volatile organic mass which may also contain oxygen with hydrogen, excluding the ash and moisture content is fixed carbon (Parikh et al., 2007). The fixed carbon is used to relate the heating value of the product and co-products (Capareda, 2011). The extent of biomass ignition and gasification as energy source can be estimated with volatile matter and fixed carbon content depending on biomass utilization (McKendry, 2002). Both volatile matter and fixed carbon have positive effects on the calorific value (Majumder et al., 2008). Biomass with high volatile matter and low ash content may be selected as promising substrate for biofuel production (Sanchez et al., 2012).

2.2.2. Ultimate analysis

Ultimate analysis illustrates the biomass composition in correspondence to the top five element carbon (C), hydrogen (H), nitrogen (N), sulphur (S) and oxygen (O) and total mineral matter (Shukla,1997). It is particularly important in evaluating fuel feedstock in terms of its pollution potential, higher heating value and theoretical air-fuel ratio (Mansaray, 1997). Carbon is the most principal component of biomass fuels, accumulated from atmospheric CO₂ during photosynthesis process and majorly contribute to total heating value of biomass. Lignin, hemicellulose and cellulose content of lignocellulosic biomass correspond to fuel carbon content. Nitrogen, procured from soil generates oxides as gaseous emission (NO_x) on combustion, therefore does not contribute to total heating value of biomass. Sulphur, also produce oxides on combustion and may contribute to the total heating value. In contrast to conventional fuels, biofuels demonstrate higher proportion of O and H, in comparison to C, thus deteriorating fuel energy value due to lower energy contained in C=O and C-H bonds, in comparison to C-C bonds (McKendry, 2002). Lower nitrogen and sulphur content in energy fuels is crucial for environment protection due to their gaseous emissions. High nitrogen and ash content reduce hydrocarbon yield (Sanderson, 1996), therefore composition of biomass may affect the conversion process. In response, it can be advocated that lower nitrogen content (<0.3%) of native lignocellulosic biomass suits enzymatic saccharification for ethanol production.

2.2.3. Gross calorific value

The gross calorific value (GCV) of lignocellulosic feedstocks is foremost aspect which signifies energy or amount of heat released when ignited in presence of excess air. It can also be expressed as calorific value (CV), higher heating value (HHV) and net calorific value (NCV) (McKendry, 2002). Demirbas (2001) reported HHV of 18.60 kJg⁻¹ for holocellulose (cellulose and hemicelluloses), whereas it was in the range of 23.26 to 26.58 kJg⁻¹ for lignin due to higher state of oxidation. Gasoline and diesel have heating value of about 47 and 45 MJ/kg, respectively, used as fuel for running internal combustion engines (Capareda, 2009). High calorific value of wheat straw and pinewood makes the biomass more efficient for the production of bio-energy (Naik et al., 2010).

2.2.4. X-ray diffraction (XRD)

X-ray diffraction, a central tool for polymer scientists to predict the structure of cellulose polymorphs (Segal et al., 1959; Weimer et al., 1995), has been shown to reflect the degree of polymerization (Puri et al., 1984) as well as structure (Andersson et al., 2004). Various physico-chemical characterizations of lignocellulosic biomasses have been done to determine its potential as renewable energy source (Alvira et al., 2010). Two third of cellulose contribute to its crystalline form (Chum et al., 1985) which has been expressed as variable with pronounced effect on enzymatic digestibility (Fan et al., 1980), but greater attention needs to be given to the size, alignment and packing of cellulose fibrils within the cell wall matrix (Jakob, 1996; Jung et al., 2008), as well as its co-organization with hemicelluloses and lignin (Pingali, 2010).

2.2.5. Thermal gravimetric analysis (TGA)

Different biomass shows different devolatilization behaviors which may be used to know about the chemical constituents of biomass, mainly cellulose, hemicelluloses and lignin (Hubbard et al., 1956; Wang et al., 2008). The hemicellulose and cellulose part of biomass usually start to decompose at around 206°C and 315°C, respectively, while the decomposition of lignin start above 330°C (Hornof et al., 1977; Ledakowicz and Stolerak, 2002). Plateau formation of the TGA plot in the temperature range of 250-500°C shows comparatively cellulose and hemicellulose rich biomass. A sharp decline beyond temperature of 500°C generally shows more cellulose and hemicelluloses, whereas slow rate of devolatilization beyond that temperature may primarily be due to high mannose or lignin content (Naik et al., 2010).

2.2.6. Fourier transform infra red (FT-IR) spectroscopy

Studying the biomass materials for compositional analysis using FT-IR has attracted much attention during the last decade. Quantification of data, accurate attribution of chemical functional groups and cell wall components is challenging without proper models (Foston and Ragauskas, 2012). It has been broadly used for investigating plant cell wall developmental and compositional variations among feedstocks (Kacurakova et al., 2000). The FT-IR spectrum is a complex chemical fingerprint delineating to adsorption bands due to the bond vibration

frequencies of the chemical functional groups comprising the cell wall (Foston and Ragauskas, 2012). FTIR technique was employed to estimate carbohydrate content in hardwood and softwood pulps by Bjarnestad and Dahlman (2002). Quantitative analysis of lignocellulosic components on untreated and steam exploded barley, canola, oat and wheat straw was also reported by Adapa et al. (2009) using FTIR. It can be used in biomass research including analysis of native biomass, biomass decomposition and reaction kinetics (Kalisz et al., 2008). Lignocellulose biomass most likely consists of alkene, esters, aromatics, ketone and alcohol, with different oxygen-containing functional groups.

2.2.7. Solid state ^{13}C cross polarising magic angle spinning (CP/MAS) nuclear magnetic resonance (NMR) spectroscopy

Solid state ^{13}C CP/MAS NMR spectroscopy has gained importance as tool to investigate the structural features of lignocelluloses. Different chemical shifts are accomplished for crystalline and amorphous cellulose in chemically equivalent nuclei environment due to magnetic non-equivalences in solid-state NMR (Foston and Ragauskas, 2012). The technique has been extensively applied to reveal the structural features of biomass derived from wood-derived materials (Kolodziejewski et al., 1982; Taylor et al., 1983; Haw et al., 1983), seeds (Rutar et al., 1980; O'Donnell et al., 1981; Haw and Maciel, 1983), grasses (Himmelsbach et al., 1983; Samuel et al., 2010), rice straw (Sun et al., 2002), wheat straw (Sun et al., 2004), sugarcane bagasse (Rezende et al., 2011), corn stover (Shi et al., 2011), *Poplar* sp. (Kohn et al., 2011) and *Eucalyptus* sp. (Yu et al., 2012).

2.3. Effective parameters limiting the hydrolysis of lignocelluloses

The aim of the pre-treatment is to alter physico-chemical characteristics of lignocellulosic biomass in order to provide more surface area for hydrolytic enzymes. These modifications are highly dependent on the type of pre-treatment employed and have great effect on the enzymatic hydrolysis (Kumar et al., 2009b). The enzymatic hydrolysis is limited by several factors; crystallinity of cellulose is just one of them. Other factors include degree of polymerization (DP), moisture content, available surface area and lignin content (Puri, 1984; Koullas et al., 1992; Chang and Holtzaple, 2000; Laureano-Perez et al., 2005).

2.3.1. Crystallinity and degree of polymerization

Crystallinity depends on relative amounts of crystalline and amorphous regions of cellulosic microfibrils (Taherzadeh and Karimi, 2008). Determining true crystallinity of cellulose is ambiguous, as conventional X-ray methods measure the crystallinity of the entire material including the hemicellulose and lignin in addition to amorphous cellulose (Kim and Holtzapple, 2006; Zhu et al., 2008). The degree of crystallinity and changes in the crystal structure of cellulose has been well established factors influencing enzymatic hydrolysis (Cetinkol et al., 2010). High crystallinity of biomass hinders enzymatic hydrolysis of lignocelluloses (Fan et al., 1980). Amorphous cellulose is readily accessed by cellulase as compared to crystalline microfibrils of cellulose (Taherzadah and Karimi, 2008) and it is widely accepted that decrease in crystallinity increases the digestibility of lignocelluloses (Fan et al., 1980; Chang and Holtzapple, 2000; Nanda et al., 2013). On contrary, more crystalline lignocelluloses have been reported to show more digestibility (Grethelin 1985; Wyman, 1996; Xiao et al., 2011). Several factors such as enzyme inactivation, low reactivity of the substrate and non-specific adsorption of enzymes onto lignin, are related to higher crystallinity of the substrate and contribute to reduction in digestibility of cellulose at the lower enzymatic loading (Vlasenko et al., 1997). However, due to the heterogeneous nature of celluloses, presence of hemicelluloses and lignin, it is not the only factor determining the effective enzymatic hydrolysis of biomass (Taherzadeh and Karimi, 2008).

Degree of polymerization (DP) determines the characteristic of cellulosic substrates such as strength of cellulose fibre, accessible surface area, pore size and encapsulation of cellulose and hemicellulose by lignin. It is essentially related to substrate characteristics, such as crystallinity (Alvira et al., 2010). Endoglucanases and cellobiases acts upon β , 1-4 glycosidic bond at internal sites of the cellulose chains and bonds linking two glucose units, primarily responsible for decreasing DP of cellulosic substrates. However, regardless the substrate being utilized there seems to be a “leveling off” of the cellulose DP, correlated with the enhanced recalcitrance of the residual crystalline cellulose (Mansfield et al., 1999). Pre-treatment with alkali such as NaOH, KOH, $\text{Ca}(\text{OH})_2$, hydrazine and anhydrous ammonia cause swelling of biomass, resulting into increases in internal surface area of the biomass, and decreases both the DP and cellulose crystallinity (Agbor et al., 2011).

2.3.2. Effect of accessible surface area

Size reduction by chipping, shredding, grinding, milling has been used to enhance the digestibility of lignocellulosic biomass (Palmowski and Muller, 1999), resulting into increase in available specific surface area and reduce both DP and cellulose crystallinity (Sun and Cheng, 2002). External surface area of lignocellulosic biomass is related to the size and shape of the particles, while the internal surface area depends on the capillary structure of cellulosic fibres (Taherzadah and Karimi, 2008). The interaction of enzyme with its accessible surface area in lignocellulosic material can be limiting in hydrolysis of lignocelluloses (Stone et al., 1969; Fan et al., 1980; Sun and Cheng, 2002; Taherzadah and Karimi, 2008).

2.3.3. Encapsulation by lignin

Lignin limits the rate and extent of enzymatic saccharification by acting as a shield, preventing the digestible parts of the substrate to be hydrolyzed (Chang and Holtzapple, 2000). Various cellulases differ in their inhibition by lignin, while the xylanases and glucosidase are less affected by lignin (Berlin et al., 2006). Softwoods are more recalcitrant than hardwoods, probably due to presence of guaiacyl lignin while hardwoods have a mix of guaiacyl and syringyl lignin (Taherzadah and Karimi, 2008). It has been suggested that guaiacyl lignin restricts fiber swelling and enzyme accessibility more than syringyl lignin (Ramos et al., 1992). Delignification results into biomass swelling, disruption of lignin structure, increases in internal surface area and increased accessibility of cellulolytic enzymes (Agbor et al., 2011), in turns enhanced saccharification yield can be obtained.

2.3.4. Hemicellulose content

Depletion in hemicellulose content increases the pore size of the substrate and therefore elicit the accessibility and the probability of cellulose hydrolysis (Chandra et al., 2007). On the contrary, hemicelluloses hydrolyzed into hexoses and pentose sugar, can be transformed into fuel ethanol and other value-added products (5-hydroxymethylfurfural, xylitol, ethanol, butanediol, butanol) and polymers (polyhydroxyalkanoates, polylactates). Degree of acetylation in the hemicellulose is another key factor because lignin and acetyl groups are attached to the hemicellulose matrix and may hinder breakdown of polysaccharides (Chang and Holtzapple, 2000).

2.3.5. Porosity

Studies showed that the pore size of the biomass in relation to the size of the enzymes, is the main limiting factor in the enzymatic saccharification of lignocellulosic biomass (Chandra et al., 2007). Cellulases can get trapped and enter into the pores if the internal area is much larger than the external area which is the case for many lignocellulosic materials (Zhang and Lynd, 2004). Drying of pretreated lignocellulose may cause collapse in pore structure, resulting in a decreased hydrolysability (Grous et al., 1986). Pre-treatment process increases the porosity of the substrate which significantly improves the hydrolysis (Alvira et al., 2010).

2.4. Pre-treatment technologies for lignocellulosic feedstock

Characterization of native and pre-treated biomass becomes inevitable to judge the effectiveness of the treatment employed to enhance the saccharification yield. Feasibility of specific pre-treatment process in search of promising feedstock is difficult to envision owing to the characteristics of biomasses. An effective and economical pre-treatment should produce reactive cellulosic fiber for enzymatic attack, minimizing the energy demand and by-products, avoid destruction of cellulose and hemicelluloses and possible inhibitors for hydrolytic enzymes and fermenting microorganisms (Taherzadah and Karimi, 2008; Gamage et al., 2010). Conversion of lignocellulosic biomass into biofuels after its pre-treatment and hydrolysis is shown in Figure 2.4.

Several methods have been introduced for pre-treatment of lignocellulosic materials prior to enzymatic hydrolysis or digestion. These methods are classified mainly into physical pre-treatment (Table 2.2), chemical pre-treatment, physico-chemical pre-treatment (Table 2.3), and biological pre-treatment (Table 2.4) (Fan et al., 1982; Wyman, 1996; Berlin et al., 2006). In the recent past, combinatorial pre-treatment of biomasses (Table 2.5) has been tested for enhances digestibility of cellulose. Different pretreatment required for the enzymatic hydrolysis of biomasses is detailed in Table 2.6.

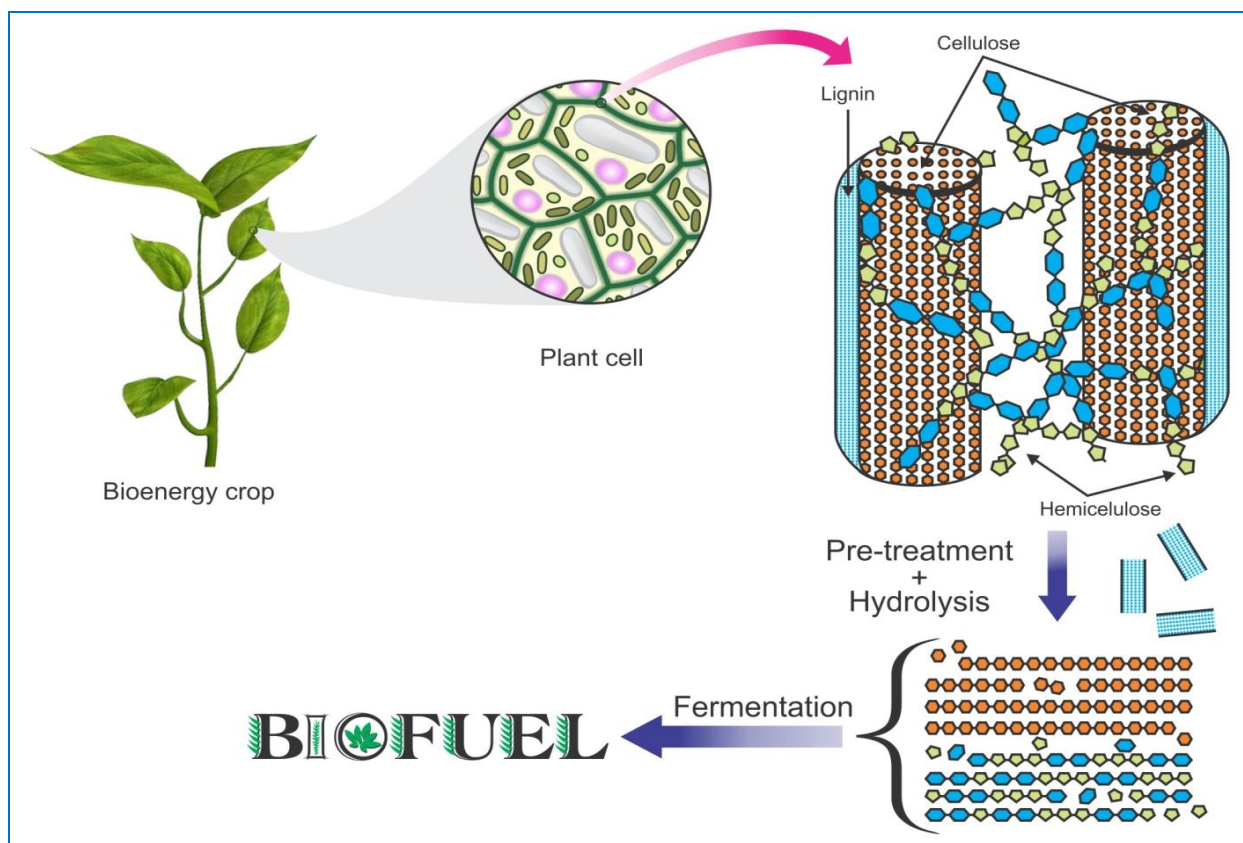


Figure 2.4. Production of second generation biofuel from bioenergy crop.

2.4.1. Physical pre-treatment

2.4.1.1. Mechanical comminution

Reduction in crystallinity, particle size and degree of polymerization is the prime motive of mechanical pre-treatment. It involves chipping, grinding, milling or their combination to a mesh size of 0.2 to 2 mm (Sun and Cheng, 2002). Different milling process such as two roll milling, ball milling, vibro energy milling, colloid milling and hammer milling, wet disk milling can be used to improve the enzymatic hydrolysis of lignocellulosic feedstock (Taherzadah and Karimi, 2008). Reduction in biomass size (Zeng et al., 2007) and crystallinity (Fan et al., 1980) using these methods are not economic owing to hike in energy price (Hendriks and Zeeman, 2009). Vibratory ball milling is more effective than ordinary ball milling in spruce and aspen chips (Millet et al., 1976) to reduce cellulose crystallinity whereas; disk milling leads over hammer milling in enhancing cellulose hydrolysis (Zhua et al., 2009). Milled corn stover (53-75 μm) is

found to be more productive than native corn stover (425-710 μm) by Zeng et al. (2007). Enzymatic hydrolysis of rice straw using disk milling and ball milling yielded glucose and xylose of 78% and 41% and 89% and 54%, respectively (Hideno et al., 2009). Maximum glucose and xylose yields for sugarcane bagasse and straw using wet disc milling were 49.3% and 36.7% and 68.0% and 44.9%, respectively (Silva et al., 2010). To enhance the efficiency of enzymatic hydrolysis, ball milling aided with different chemicals was also evaluated for corn stover (Lin et al., 2010).

2.4.1.2. Irradiation

Biomass pre-treatment using irradiation generally involves the use of gamma rays, electron beams and microwaves to improve enzymatic hydrolysis. However, very high radiation (100 MR) can lead to the decomposition of oligosaccharides and glucose ring (Kumakura et al., 1983). Enzyme hydrolysed electron beam irradiated (80 kGy at 0.12 mA and 1 MeV) rice straw yielded 52% of fermentable sugar (Bak et al., 2009). Pre-treatment of wheat straw using gamma-radiation was reported to yield 13% of glucose, after enzymatic hydrolysis (Chunping et al., 2008).

2.4.1.3. Extrusion

Extrusion is one of the novel and promising approach in which heating, mixing and shearing of materials take place to alter the physico-chemical properties while passing through extruder. The process can be modified by changing the design of the screw (Bernatoviciute and Juodeikiene, 2008), screw speed and barrel temperature which causes defibrillation, fibrillation and shortening of cellulose fibre to enhance enzymatic saccharification (Karunanithy and Muthukumarappan, 2010). The process has been shown to improve the nutritional value of animal feed stuffs (Amornthewaphat and Attamangkune, 2008). In recent studies application of enzymes during extrusion process is being considered as a promising technology for ethanol production (Alvira et al., 2010). Extrusion processing parameters (barrel temperature, in-barrel moisture, screw speed) and processing aids (starch, ethylene glycol) were studied which resulted into 95% cellulose to glucose conversion via thermo-mechanical extrusion pre-treatment of soyabean hulls by Yoo et al. (2011). The efficiency of extrusion pre-treatment was evaluated by enzymatic hydrolysis and simultaneous saccharification and fermentation (SSF) of rapeseed straw by Choi et al. (2013).

2.4.1.4. Pyrolysis

Pyrolysis is an endothermic process with applied temperature greater than 300°C to decompose cellulose, mainly to H₂ and CO and residual char. The decomposition is much slower and less volatile products are formed at lower temperatures (Sanchez et al., 2008; Prasad et al., 2007, Mtui et al., 2009). The residual char is treated by leaching with water or mild acid which contains enough carbon sources mainly glucose to support microbial growth for bioethanol production (Sarkar et al., 2012). Fan et al. (1987) showed 80-85% conversion of cellulose to reducing sugars with more than 50% glucose through mild acid leaching. Pyrolysis at different temperature changed the chemical and surface characteristics of nut shell (Bonelli et al., 2001).

2.4.1.5. Pulsed electric field pre-treatment

Using pulsed-electric field (PEF) pre-treatment, a critical electric potential is induced across the cell membrane leading to rapid electrical breakdown resulting into structural changes of lignocellulosic feedstock. Based on this phenomenon, many practical applications of high electric fields for reversible or irreversible permeabilization of various biological systems have been studied in the fields of medicine and bioscience (Chang et al., 1992; Ho et al., 1996; Lynch et al., 1996; Knorr et al., 1998; Angerbasch et al., 2000). Applying electric pulses with high field strengths, PEF can create permanent pores in the cell membrane, facilitating entry of acids or enzymes to elicit hydrolytic mechanism. Kumar et al. (2011) have designed and fabricated a PEF system for the treatment of Southern pine and switchgrass.

2.4.1.6. Thermal and hydrothermal pre-treatment

During thermal pre-treatment, lignocellulosic biomass is generally heated at a temperature range of 130-230°C. At higher temperature, firstly the hemicelluloses and shortly after that lignin start to solubilize (Bobleter, 1994; Garrote et al., 1999). The process is effective without adding catalysts (Larsen et al., 2008), as water at high temperatures (~200°C) has acidic pH, acts as a catalyst for the bio-mass disruption (Mosier et al., 2005). Liquid hot water pre-treatment (LHW) can enlarge the accessible and susceptible surface area of the cellulose and make it more accessible to hydrolytic enzymes (Zeng et al., 2007). LHW pretreatments are both helped and hindered by the cleavage of O-acetyl and uronic acid substitutions from hemicellulose to generate acetic and other organic acids (Mosier et al., 2005).

Table 2.2. Physical methods of pre-treatment of lignocellulosic biomass.

Pre-treatment method	Advantage (s)	Disadvantage (s)	Reference
Milling			
Milling can improve susceptibility to enzymatic hydrolysis, alter the inherent ultrastructure of lignocelluloses and degree of crystallinity and making it more accessible.	-Reduces size and degree of crystallinity	-High power and energy consumption	Fan et al., 1980; Zeng et al., 2007 Taherzadeh and Karimi, 2008; Alvira et al., 2010
Irradiation			
Using irradiation the cellulose component is broken down to fragile fibers and low molecular weight oligosaccharides.	-Improve enzymatic hydrolysis -Improved yield of reducing sugar	-Expensive -Difficult in industrial application	Kumakura et al., 1983; Taherzadeh and Karimi, 2008
Liquid hot-water pretreatment			
During pre-treatment process, water penetrates under high pressure, hydrate cellulosic material and remove hemicellulose and lignin.	-No need for size reduction of feedstock and corrosion-resistant container -No chemicals and toxic inhibitor production	-Average solid load -Not successful with softwood	Weil et al., 1994; Mosier et al., 2005a; Dien et al., 2006; Banerjee et al., 2009; Bunnell et al., 2010
Pulsed-Electric-Field			
The electric field results in a reasonable increase in mass permeability and mechanical breakdown of the plant tissue.	-Improve mass transfer -Can be carried out at ambient conditions -Low energy requirement	-Inactivate enzymes	Kumar et al., 2009
Pyrolysis			
Subjected to temperature (>300°C), whereby cellulose rapidly decomposes to produce gaseous H ₂ and CO and residual char.	-Less volatile products are formed	-Decomposition is much slower	Sarkar et al., 2012

These acids help to catalyze formation of oligosaccharides and sugars. However, the polysaccharides, mainly hemicellulose, may be further hydrolyzed to monomeric sugars which may subsequently convert to aldehydes in presence of an acid. These aldehydes, principally furfural from pentoses and 5-hydroxymethyl furfural from hexose, are inhibitory to microbial fermentation (Palmqvist and Hahn-Hagerdal, 2000). An optimized condition for LHW pre-treatment of corn stover was reported to be 190°C for 15 min, in which 90% of the cellulose conversion was observed by subsequent enzymatic hydrolysis (Mosier et al., 2005). Hot compressed water (HCW) pre-treatment has been successfully done for woody biomass of *T. ramosissima* and achieved a 43% dry weight yield of glucan and increase in *CrI* (Xiao et al., 2011). Co-current liquid hot water pre-treatment is being used to pre-treat corn fiber processing for the current fuel ethanol industry (Weil et al., 1998b; Beery et al., 2000; Mosier et al., 2003a,b). Hot-water pre-treatment on hybrid poplar was achieved and optimized (200°C, 18 min, 20% w/w biomass) using response surface methodology by Dai and McDonald (2013), resulted into sugar yield of 33%.

2.4.2. Chemical pre-treatment

2.4.2.1. Acid pre-treatment

The acid pre-treatment involves the use of either dilute or concentrated acid, to dissolve the lignin-hemicellulose matrix making the cellulose better accessible. Dilute acid treatment has gained importance in fermentation process, owing to less formation of inhibitors, minimum chance of equipment corrosion, high recovery rate of chemicals, high operation and maintenance cost. It can be performed at high temperature (>160°C), low substrate concentration (5-10 wt%) during a short time period; or at lower temperature (≤160°C), high substrate concentration (10-40 wt%) for longer reaction time (30-90 min).

Concentrated sulfuric acid (H₂SO₄) and hydrochloric acid (HCl) have been widely used for treating lignocellulosic materials (Sun and Cheng, 2002). Dilute acid (1-3%) hydrolysis requires a high temperature (200-240°C) to disrupt cellulose crystals (Xiang et al., 2003). It is followed by hexose and pentose degradation and formation of toxic compounds such as HMF and phenolics detrimental to an effective saccharification (Sun and Cheng, 2002). The most commonly used acid is dilute H₂SO₄, which has been commercially used to pretreat diverse

biomass such as switchgrass (Digman et al., 2010; Li et al., 2010), corn stover (Du et al., 2010; Xu et al., 2009), spruce (Shuai et al., 2010) and poplar (Wyman et al., 2009; Kumar et al., 2009), while other acids such as HCl and HNO₃ (Himmel et al., 1997; Taherzadeh et al., 2007) and H₃PO₄ (Marziales et al., 2008) are also being put to use. Softwoods were studied by Nguyen et al. (2000) to optimize sugar yield using two-stage diluted H₂SO₄ pre-treatment process. After 48 h of incubation, enzymatic hydrolysis of acid (1.5% H₂SO₄) pre-treated bermuda grass and rye straw yielded 20 and 23% total reducing sugar, respectively (Sun and Cheng, 2005). Other studies done by Saha et al. (2005) showed 56% sugar yield obtained by enzymatic saccharification of acid (0.75% H₂SO₄) treated wheat straw. Marziales et al. (2008) studied the acid hydrolysis of loblolly pine using a number of different acids (Trifluoroacetic acid, HCl, H₂SO₄, HNO₃ and H₃PO₄) and obtained soluble monosaccharides (mannose, glucose, galactose, xylose, and arabinose) with 70% yield from the hemicellulose fraction. As an alternative to inorganic acids, organic acids (e.g. maleic acid, fumaric acid) can be used for dilute acid pre-treatment of wheat straw (Kootstra et al., 2009). Furthermore, less amount of furfural was formed in the maleic and fumaric acid pre-treatments than with sulfuric acid (Kootstra et al., 2009). The effect of dilute acid pre-treatment on the supra molecular and ultrastructure of lignocellulosic biomass was evaluated by Foston and Ragauskas (2010). Ethanol yield of 47% was achieved in fermentation tests with cashew apple bagasse pre-treated with diluted H₂SO₄ at 121°C for 15 min (Rocha et al., 2009).

2.4.2.2. Alkaline pre-treatment

Alkaline pre-treatment involves the use of bases, such as NH₄OH, KOH, NaOH, Ca(OH)₂, NH₃ and (NH₄)₂SO₃. The use of an alkali causes the degradation of ester and glycosidic side chains, results into structural deformation of lignin, cellulose swelling, partial decrystallization of cellulose (Cheng et al., 2010; McIntosh et al., 2010; Ibrahim et al., 2011) and partial solvation of hemicellulose (McIntosh et al., 2010; Sills et al., 2010). Alkali pre-treatment is found to be effective for agricultural residue as compared to wood materials with less degradation of sugar (Kumar et al., 2009a). NaOH has been reported to increase the hard wood digestibility from 14% to 55% by reducing lignin content from 24-50% to 20% (Kumar et al., 2009a). Lime [Ca(OH)₂] treatment also destroys the lignin encapsulation and lignin-hemicellulose matrix to provide more accessible area of cellulose for enzymatic hydrolysis. The effectiveness of NaOH pre-treatment

for hardwoods, wheat straw, switchgrass and softwoods with less than 26% lignin content was studied by Zhao et al. (2008). Saha and Cotta (2006) reported an excellent fermentable sugars yield (97%) in alkaline peroxide pre-treatment of wheat straw. Lime has been used to pre-treat wheat straw (85°C, 3 h) (Chang et al., 1998), Poplar wood (150°C, 6 h, 14 atm) (Chang et al., 2001), switchgrass (100°C, 2 h) (Chang et al., 1997), and corn stover (100°C, 13 h) (Karr and Holtzapple, 2000).

Alkali pre-treatment (2% NaOH, 121°C, 90 min) of cotton stalks showed 65% delignification and 61% cellulose conversion (Silverstein et al., 2007), while alkali treatment (3% NaOH) of spruce in presence of urea (12%) at low temperature (15°C) gives 60% cellulose conversion (Zhao et al., 2008). Optimised condition (2% NaOH, 60°C, 90 min) for enzymatic saccharification of wheat straw was studied by McIntosh and Vancov (2010), showed 5-fold increase in total sugar yield. NaOH pre-treatment at different loading (1%, 2.5%, 5.0% and 7.5% w/w) was applied to enhance biogas production from corn stover through solid-state anaerobic digestion by Zhu et al. (2010).

2.4.2.3. Microwave and ultrasound

Pre-treatment using microwave may be considered as a physico-chemical process, since it involves both non-thermal and thermal treatment. This particular heating feature results in an explosion effect among the particles and improves the disruption of recalcitrant structures of lignocellulose (Hu et al., 2008). Microwave pre-treatment of rice straw and bagasse followed by lignin extraction has been reported to yield 43-55% sugar (Kitchaiya et al., 2003). The method has gained importance to improve enzymatic hydrolysis of lignocelluloses such as rice straw and wheat straw (Zhu et al., 2005; Ma et al., 2009). Systematic optimization of pre-treatment conditions for rice straw hydrolysis, and effects of microwave intensity, irradiation time, and substrate concentration on the hydrolytic conversion of cellulose and hemicellulose was studied by Ma et al. (2009). It was believed that thermal and non-thermal effects of microwave-based pre-treatment hydrolyzes hemicellulose and solubilize lignin to enlarge the pore size resulting into enhanced cellulose accessibility for cellulases (Banik et al., 2003). Another promising method for hydrolyzing hemicellulose and lignin is use of ultrasound pre-treatment, widely used in the extraction of hemicellulose, proteins and organic waste materials (Sun and Tomkinson, 2002; Yin et al., 2004). Enhanced saccharification was also reported from cellulose (Yachmenev

et al., 2009) and rice hull (Yu et al., 2009) hydrolysis using ultrasound. Ultrasonication is a promising method of pre-treatment to enhance sugar yield and decrease the particle size ($<4\ \mu\text{m}$) for microcrystalline cellulose such as Avicel (Zhang et al., 2013). Pre-treatment of wood wastes (Kunaver et al., 2012), sugarcane bagasse (Velmurugan and Muthukumar, 2012; Li et al., 2012) and kenaf powder (Ninomiya et al., 2012) was also studied using ultrasound.

2.4.2.4. Solvent extraction/Organosolv pre-treatment

Solvent extraction employs use of organic solvent or mixtures of organic solvents with water (ethylene glycol, swelling agent, butanol-water, benzene-water, ethanol-water) for hydrolysis of hemicellulose and lignin mineralization. The main advantage of organosolv process lies in the recovery of pure lignin as a by-product (Zhao et al., 2009a). Solvents with low boiling point (methanol and ethanol), high boiling points (ethylene glycol, glycerol, tetrahydrofurfuryl alcohol) and other classes of organic compounds (dimethylsulfoxide, ethers, ketone, phenols) have been tested for pre-treatment (Thring et al., 1990). Solvents need to be separated because they might inhibit enzymatic hydrolysis and growth of fermentative microorganisms (Sun and Cheng, 2002). Ethanol-organosolv pre-treatment of Hybrid Poplar and beetle-killed *Lodgepole pine* yielded 85 and 97% sugar, respectively (Pan et al., 2006a; 2007). Recovery of 95% cellulose with 70% lignin removal was observed using atmospheric aqueous glycerol autocatalytic organosolv pre-treatment of wheat straw (Sun and Chen, 2007). Araque et al. (2008) also reported ethanol yield of 99% using SSF of acetone-water (195°C , 5 min, pH 2.0) pre-treated *Pinus radiata* chips.

2.4.2.5. Oxidation

Delignification of lignocellulosic materials can be performed by treatment with oxidizing agents such as hydrogen peroxide, ozone, oxygen or air. Ozone pre-treatment referred to as “ozonolysis”, targeted towards the cleavage of aromatic rings structures of lignin, while hemicellulose and cellulose are hardly decomposed. It can be used to disrupt the structure of many different lignocellulosic materials, such as wheat straw, bagasse, pine, peanut, cotton straw and poplar sawdust (Sun and Cheng, 2002). Biomass such as straw, reed and residues of some cereal crop have a dense wax coating of silica and protein, removed by wet oxidation (Schmidt et al., 2002). Tannins containing grape stalk has also been shown to benefit wet oxidation with 50% cellulose conversion (Ping et al., 2011). Na_2CO_3 treated wheat straw showed 96% recovery of

cellulose with 65% conversion of glucose (Klinke et al., 2002). Similarly, wet oxidation of sugarcane bagasse resulted in 57% conversion of cellulose with comparatively more by-products than that of steam explosion (Martin et al., 2007). Ozonolysis also elicited enzymatic hydrolysis of wheat straw and rye straw (Garcia-Cubero et al., 2009).

2.4.2.6. Ionic liquid/Green solvents

Processing of lignocellulosic biomass with ionic liquids (IL) and similar solvents has gained importance in the last decade to meet the suitable, efficient and cost effective technology. Ionic liquids such as 1-*n*-butyl-3-methylimidazolium chloride ([Bmim]Cl), 1-allyl-3-methylimidazolium chloride ([Amim]Cl), N-methyl morpholine N-oxide (NMMO), 1-butyl-3-methylimidazolium acetate [BMIM][OAc], 1-ethyl-3-methylimidazolium acetate ([Emim]Ac) and 1-ethyl-3-methylimidazolium diethyl phosphate ([Emim]Dep), typically composed of a small anion and a large organic cation, liquid at room temperature with very low vapour pressure. The chemistry of the anion and cation can be tuned to generate a wide variety of liquids which can dissolve a number of biomass types such as corn stover (Cao et al., 2010), cotton (Zhao et al., 2009), bagasse (Wang et al., 2009), switchgrass (Samayamand et al., 2010), wheat straw (Li et al., 2009) and woods (pine, poplar, eucalyptus, and oak) of different hardness (Fort et al., 2007). NMMO, generally known as Lyocell solvent, used commercially to produce Tencel fiber, retains the advantages of the ionic liquids ability to dissolve a variety of lignocellulosic substrates (Kuo and Lee 2009a, 2009b; Shafiei et al., 2010) with >99% solvent recovery rate (Perepelkin, 2007). Increased rates of cellulose hydrolysis was also reported from NMMO treatment, showed the ability to disrupt the crystalline structure of cellulose (Kuo and Lee, 2009a, 2009b; Aslanzadeh et al., 2011). Shafiei et al. (2010) showed improve ethanol yield from hard and soft wood from 19% and 7% to 85% and 89%, NMMO treatment (85wt%, 130°C, 3 h) using non-isothermal SSF. Kuo and Lee (2009) also reported two times improvement enzymatic hydrolysis using NMMO treated (130°C, 1 h) sugarcane bagasse. Hydrolysis yield of switch grass was enhanced from 16.5% to 72.5 % after treatment by ([Emim]Ac) (Singh et al., 2009). Dadi and coworkers (Dadi et al., 2006; Dadi et al., 2007) showed enhanced hydrolysis kinetics of Avicel by ([Bmim]Cl) and ([Amim]Cl). Rice straw pre-treatment using NMMO and [BMIM][OAc]) at 120°C was reported to enhance saccharification and ethanol production

(Poornezad et al., 2013). Effect of ([EMIM][OAc]) pre-treatment on the chemical composition, structure and enzymatic hydrolysis of sugarcane bagasse was also studied by Qiu et al. (2012).

2.4.3. Physico-chemical pre-treatment

2.4.3.1. Explosion /Autohydrolysis

Steaming with or without explosion (autohydrolysis) has received substantial attention in pre-treatment for ethanol production. Steam explosion (uncatalysed or catalysed) is widely used pre-treatment technique due to its limited use of chemicals energy. This method employs injection of high-pressure saturated steam into a batch or continuous reactor filled with biomass. The steam-explosion process includes saturating the dry biomass with steam at elevated pressure and temperature, followed by a sudden release of pressure while forcing the biomass through a small orifice, during which the flash evaporation of water exerts a thermo-mechanical force causing the biomass to rupture. The rapid thermal expansion used to terminate the reaction opens up the particulate structure of the biomass but enhanced saccharification of cellulose in the pre-treated solid is only weakly correlated with this physical effect (Biermann et al., 1984; Brownell et al., 1986). Steam-explosion pre-treatment depends on residence time, temperature, particle size and moisture content (Sun and Cheng, 2002). Limitations of steam explosion include formation of inhibitory products during downstream processing, high energy consumption and possessing lowest energy efficiency (Garcia-Aparicio et al., 2006; Zhu et al., 2010). The method has been used frequently to pre-treat wood (Galbe et al., 2002), corn stover (Tucker et al., 2003), municipal solid waste (Liu et al., 2002; Vogt et al., 2002) to release sugars for production of ethanol, butanol, and methane. Ruiz et al. (2006) studied steam explosion (220°C, 5 min) prior to enzymatic hydrolysis for pre-treatment of sunflower stalks to achieve highest hemicellulose recovery. Steam explosion of olive tree pruning at 240°C for 5 min yielded 7% ethanol (Cara et al., 2008).

2.4.3.3. Ammonia pre-treatment

Pre-treatment of biomass using aqueous ammonia at elevated temperature disrupts lignin and removes some hemicellulose while decrystallizing cellulose. The pre-treatment includes the Ammonia fibre explosion-method (AFEX), Ammonia recycle percolation (ARP) and soaking in aqueous ammonia (SAA). Kim et al. (2008) evaluated SAA (15% aqueous ammonia, 75°C for 48

h, without agitation, solid:liquid ratio 1:12) as a pre-treatment method of biomass and obtained 66% lignin solubilisation and saccharification yields of 63 and 83% for xylan and glucan, respectively. In AFEX, biomass is exposed to liquid ammonia at relatively high temperature (90-100°C, 30 min), followed by immediate reduction in pressure. The effective parameters of AFEX process are ammonia loading, temperature, water loading and blow down pressure, time, and number of treatments (Holtzaple et al., 1991). The optimum conditions in AFEX pre-treatment of switch grass were reported to be about 100°C, 1:1 ratio of ammonia to dry biomass with retention time of 5 min (Alizadeh et al., 2005). Process optimization of AFEX to convert forage and sweet sorghum bagasse to ethanol was demonstrated by Li et al. (2010). AFEX pre-treatment followed by enzymatic hydrolysis of empty palm fruit bunch fiber yielded 90% ethanol after 72 h of fermentation (Lau et al., 2010).

2.4.3.4. Supercritical fluid pre-treatment

A supercritical fluid (SCF) is a material which can be either liquid or gas, used above critical temperature and pressure where gases and liquids can coexist. SCF has the potential to penetrate the crystalline structure of lignocellulosic biomass, overcoming the mass transfer limitations encountered in other pre-treatments (McHardy et al., 1998). Supercritical carbon dioxide (CO₂) with a critical temperature (T_c) of 31°C and a critical pressure (P_c) of 7.4 MPa, has shown excellent result in pre-treatment of biomass. Enzymatic hydrolysis of supercritical CO₂ pre-treated cellulose yielded 100% glucose (Park et al., 2001) and 32% from rice straw (Gao et al., 2010). Alinia et al. (2010) reported 15% sugar yield from supercritical CO₂ treated wheat straw. Yields of CO₂ explosion are lower than those obtained with steam or ammonia explosion, but they are higher than those reached with enzymatic hydrolysis without pre-treatment (Sun and Cheng, 2002).

2.4.4. Biological pre-treatment

Biological pre-treatment, mainly involve the use of white, brown and soft rot fungi employed to release cellulose from hemicellulose and lignin matrix. Treatment degrade lignin and hemicellulose but leave the cellulose intact (Shi et al., 2008; Kumar et al., 2009; Sanchez et al., 2009).

Table 2.3. Chemical and physico-chemical methods of pre-treatment of lignocellulosic biomass.

Pre-treatment method	Advantage (s)	Disadvantage (s)	Reference
Alkaline hydrolysis			
Pre-treatment can be performed at low temperature for a long time with high concentration of alkali.	-Effective in breaking the ester bonds between lignin, hemicellulose and cellulose without fragmentation of hemicellulose.	-High cost of alkaline catalyst -Alteration of lignin structure	Gaspar et al., 2007; Brodeur et al., 2011
Oxidizing agent			
An oxidative pre-treatment employs oxygen or air as catalyst, allows reactor operation at relatively low temperatures and short reactor times.	-Less toxic compound generation -Efficient removal of lignin -Minimizes the energy demand	-High cost of oxidizing agents used.	Palonen et al., 2004; Saha and Cota, 2006
Organosolv process			
Organic liquid and water is heated to dissolve the lignin and part of the hemicellulose, leaving reactive cellulose in the solid phase.	-Relatively pure lignin recovery -Effective for both hardwood and softwood.	-High capital investment -Formation of toxic inhibitors -Need of solvent recycling	Katzen et al., 1995; Sun and Cheng, 2002; Pan et al., 2006a;
Green Solvents/Ionic liquid			
The chemistry of the anion and cation can be tuned to generate a wide variety of liquids which can dissolve a number of biomass types.	- Enhanced recovery rate - No formation of toxic products	-Deactivate the enzymes -Requires longer pre--treatment time. -Can cause explosion effects.	Brodeur et al., 2011; Pooornejad et al., 2013
Microwave-chemical pre-treatment			
Process utilizes thermal and non-thermal effects generated by microwaves in aqueous environments as it break down lignin-hemicellulose complex and expose more accessible surface area of cellulose.	-Accelerate reactions during the pre-treatment process -Improve sugar content	-Not feasible for large scale operations. -High cost and slow process	Zhu et al., 2006; Keshwani et al., 2008; Keshwani et al., 2010; Narayanswami et al., 2013
Ultrasound pre-treatment			

Ultrasound greatly enhance the transport of enzyme macromolecules toward the substrate surface by cavitation and break down cell wall to make the surface accessible for enzymes	-Higher enzymatic hydrolysis	-Not feasible on large scale.	Sun and Tomkinson, 2002; Yachmenev et al., 2009
Acid hydrolysis			
Operated either under a very high temperature with dilute acid or under a low temperature with concentrated acid.	-Simple technique -Does not require thermal energy -Effectively hydrolyze hemicellulose with high sugar yield	-High cost of acid recycling -Generate toxic inhibitors	Taherzadeh and Karimi, 2007
Ammonia fiber explosion (AFEX)			
Biomass is exposed to liquid ammonia at relatively high temperature for a period of few minutes followed by immediate reduction of pressure.	-Increases accessible surface area -Low formation of inhibitors -Effectiveness for herbaceous material and low lignin content biomass	-High cost of ammonia and its recycling -Less hemicellulose solubilisation -Alters lignin structure	Wyman, 1996; Sun and Cheng, 2002; Brodeur et al., 2011
CO₂ explosion			
CO ₂ can penetrate the minute pores of lignocelluloses by high pressure resulting into disruption of cellulose and hemicellulose structure and makes the surface more accessible to enzymatic attack.	-Availability at relatively low cost -Non-toxic and non flammable -Easy recovery after extraction -Environmental acceptability.	-Less effect on lignin -Very high pressure requirement -Too expensive for industrial application	Zheng et al., 1995
Explosion or autohydrolysis			
Thermo-mechanical force results into disruption of biomass by saturation of biomass with sudden release of pressure generated by steam.	-Cost effective -High yield of glucose and hemicellulose in two-step process	-Incomplete destruction of lignin-carbohydrate matrix -Toxic compound generation	Brodeur et al., 2011; Menon and Rao, 2012
Super critical fluid pre-treatment			
Super critical fluid explosion opened up minute pores of the biomass which increased accessible surface area for subsequent enzymatic hydrolysis.	-Low degradation of sugars -Cost effective -Increases cellulose accessible area	-High pressure requirement -Less effect on lignin and hemicellulose	Brodeur et al., 2011

The biological pre-treatment requires low energy and mild operation conditions. Nevertheless, the rate of biological hydrolysis is usually very low, so this pre-treatment requires long residence time (Sun and Cheng, 2002; Tengerdy and Szakacs, 2003; Cardona and Sanchez, 2007). Microbial treatment of different biomass results in loss of cellulose, hemicellulose and lignin (Table 2.4).

2.4.4.1. White-rot fungi

Lignolytic basidiomycetes, a physiologically distinct group of saprophytic fungi, causes white-rot in wood is commonly known as white-rot fungi. Over the years, white-rot fungi have been used in mineralization of lignin (Vicuna, 2000; Martinez et al., 2009; Wong, 2009; Xu et al., 2010; Tuyen et al., 2012; Suzuki et al., 2012), *Phanerochaete chrysosporium* (Mishra et al., 2007; Shi et al., 2009), *Pycnoporus cinnabarinus* (Meza et al., 2006), *Phlebia* spp. (Arora and Sharma, 2009), *Echinodontium taxodii* (Yu et al., 2010b), *Irpex lacteus* (Xu et al., 2009; Dias et al., 2010; Yu et al., 2010a), and *Pycnoporus sanguineus* (Lu et al., 2010), have shown high lignin degradation specificity, while significant levels of carbohydrate degradation have been reported for *Ceriporiopsis subvermispora* (Wan and Li, 2010), *Phlebia brevispora*, *P. floridensis* and *P. radiata* (Sharma and Arora, 2010), Euc-1 (Dias et al., 2010), *Gonoderma* sp. (Bourbonnais et al., 1997; Haddadin et al., 2002; Tripathi et al., 2008), *Oxysporus* sp. (Haddadin et al., 2002), *Trametes versicolor* (Vaidya and Singh, 2012), *Pleurotus sajor-caju* (Kannan et al., 1990) and *Trichoderma reesei* (Singh et al., 2008), with 40-80% reduction in lignin content. Biodelignification has direct effect on fermentable sugar for ethanol production, significantly improves the yield of biofuel production. Biodelignification has been used prior to chemical pre-treatments, showed delignification up to 80% (Yu et al., 2010a; Yu et al., 2010b).

2.4.4.2. Brown-rot fungi

Brown-rot fungi efficiently degrade cellulose and hemicellulose as compared to lignin, resulted in brown-rotted wood due to incomplete degradation of lignin, hence named as ‘brown-rot fungi’. Brown-rot fungi preferentially degrade the cellulose and hemicellulose fraction of wood, but do not oxidize lignin (Hastrup et al., 2012; Schilling et al., 2012). Many of them such as *Serpula lacrymans*, *Coniophora puteana*, *Meruliporia incrassata*, *Laetoporeus sulphureus*, and *Gleophyllum trabeum* have been used in many biodegradation studies (Monrroy et al., 2011; Rasmussen et al., 2010).

2.4.4.3. Soft-rot fungi

There are two types of soft-rot fungi; type I, consisting of biconical or cylindrical cavities that are formed within secondary walls and type II, refers to an erosion form of degradation (Blanchette, 2000). Most efficient fungus of type II group is *Daldinia concentrica*, primarily affects hardwood (Narayanswami et al., 2013) and reported to achieve 53% loss in weight of birch wood within 2 months (Nilsson et al., 1989). *Paecilomyces* sp. (Kluczek-Turpeinen et al., 2003) and *Cadophora* spp. (Chandel et al., 2013) are also involved in rapid biodelignification of biomass. In early stages of classification, wood-rotting fungi, *Xylariaceous ascomycetes* from genera *Daldinia*, *Hypoxylon* and *Xylaria* have often been classified under white-rot fungi, but these fungi are now considered as members of soft-rot fungi as they cause type II soft-rot in many woods.

2.4.4.4. Bacteria and actinomycetes

Bacteria are of less use than white, brown and soft rot fungi in biodelignification of lignocellulose materials, as they lack or poor producer of lignolytic enzymes. *Bacillus circulans* and *Sphingomonas paucimobilis* (Kurakake et al., 2007), *Cellulomonas* and *Zymomonas* spp. have been reported for biodegradation of lignocellulosic biomass (Singh et al., 2008). Bacterial delignification may lead to 50% lignin loss, which is somewhat similar to fungi-mediated delignification. Mineralization of lignin is mainly mediated by extracellular xylanases, although a synergistic effect has been observed by the addition of MnP, pectinase or α -L-arabinofuranosidase (Bezalel et al., 1993; Kaur et al., 2010). However, high lignin removal might be accompanied with enhanced degradation of cellulose (31-51%), as observed for *Bacillus macerans*, *Cellulomonas cartae*, *C. uda* (Singh et al., 2008). Bacteria such as *Aneurinibacillus aneurinilyticus* (Raj et al., 2007) and *Paenibacillus* sp. ITRC S6 and *Bacillus* sp. ITRC S8 (Chandra et al., 2008) were found to be involved in degradation of kraft lignin. One strategy to overcome this issue is the use of cellulase free extracts (Kaur et al., 2010) or purified enzymes (Bezalel et al., 1993) that allows up to 20% delignification levels within shorter incubations times than those observed with the whole microorganisms.

Table 2.4. Effect of biological treatment on different type of lignocellulosic biomass.

Microorganism	Biomass	Cellulose loss (%)	Hemicellulose loss (%)	Lignin loss (%)	Reference
FUNGI					
<i>Dichomitus squalens</i>	<i>Fagus crenata</i> wood chips	8	--	21.7	Itoh et al., 2003
<i>Ceriporiopsis subvermispora</i>	<i>Fagus crenata</i> wood chips	5	--	6.7	Itoh et al., 2003
	Sugarcane bagasse	9.1	21.5	38.4	Sasaki et al., 2011
	Corn stover	<5	27	39.2	Wan and Li, 2010
<i>Pleurotus ostreatus</i>	<i>Fagus crenata</i> wood chips	4.1	--	10.8	Itoh et al., 2003
<i>Coriolus versicolor</i>	<i>Fagus crenata</i> wood chips	13.2	--	19.5	Itoh et al., 2003
	Rice straw	17	48	41	Taniguchi et al., 2005
<i>Phanerochaete chrysosporium</i>	Rice straw	49	--	21	Taniguchi et al., 2005
<i>Trametes versicolor</i>	Rice straw	58	--	37	Taniguchi et al., 2005
	Corn straw	47.5	--	54.6	Yu et al., 2010
	<i>Pinus radiata</i> wood chips	16.8	29	31	Ferraz et al., 2001
<i>Echinodontium taxodii</i>	<i>Salix babylonica</i> wood	27	51	45	Yu et al., 2009
	<i>Cunninghamia lanceolata</i> wood	12	31	40	Yu et al., 2009
	Corn straw	15.8	--	42.2	Yu et al., 2010.
<i>Ganoderma lucidum</i>	Corn straw	39	---	32.7	Yu et al., 2010
<i>Punctularia atropurpurascens</i>	<i>Pinus radiata</i> wood chips	0	5	9	Ferraz et al., 2001
<i>Poria medula-panis</i>	<i>Pinus radiata</i> wood chips	10	15	18	Ferraz et al., 2001
<i>Merulius tremellosus</i>	<i>Pinus radiata</i> wood chips	7	18	28	Ferraz et al., 2001
<i>Ganoderma australe</i>	<i>Pinus radiata</i> wood chips	26	31	35	Ferraz et al., 2001
<i>Ceriporia lacerate</i>	<i>Pinus densiflora</i> wood chips	--	8	13	Lee et al., 2007
<i>Stereum hirsutum</i>	<i>Pinus densiflora</i> wood chips	--	7.8	14	Lee et al., 2007
<i>Polyporus brumalis</i>	<i>Pinus densiflora</i> wood chips	--	10.6	12	Lee et al., 2007
<i>Trametes hirsute</i> YJ9	Corn stover	34.06	77.84	71.99	Sun et al., 2011

<i>Gloeophyllum trabeum</i> KU-41	Corn stover	17	43	--	Gao et al., 2012
<i>Gloeophyllum trabeum</i>	<i>Pinus radiata</i> wood chips	--	31	--	Monrroy et al., 2011
	<i>Eucalyptus globulus</i> wood chips	--	24	--	Monrroy et al., 2011
<i>Laetoporeus sulphureus</i>	<i>Eucalyptus globulus</i> wood chips	--	6	--	Monrroy et al., 2011
	<i>Pinus radiata</i> wood chips	21	47	3	Fissore et al., 2010
<i>Laetoporeus sulphureus</i>	<i>Pinus radiata</i> wood chips	--	7.5	--	Monrroy et al., 2011
<i>Wolfiporia cocos</i>	<i>Pinus radiata</i> wood chips	6	23	2.6	Ferraz et al., 2001
<i>Laetiporus sulphureus</i>	<i>Pinus radiata</i> wood chips	6	14.6	5.3	Ferraz et al., 2001
<i>Coniophora puteana</i>	<i>Pinus sylvestris</i> wood blocks	17	--	59.7	Irbe et al., 2011
BACTERIA					
<i>Bacillus</i> sp.	Rice straw	3.2	16	20	Chang et al., 2014
<i>Cellulomonas cartea</i>	Sugarcane trash	25.4	--	5.5	Singh et al., 2008
<i>Cellulomonas uda</i>	Sugarcane trash	21.8	--	5.5	Singh et al., 2008
<i>Bacillus macerans</i>	Sugarcane trash	30.4	--	5.5	Singh et al., 2008
<i>Zymomonas mobilis</i>	Sugarcane trash	26.8	--	8	Singh et al., 2008
<i>Paenibacillus</i> sp. AY952466	Kraft lignin	--	--	43	Chandra et al., 2007
<i>Aneurinibacillus</i>	Kraft lignin	--	--	56	Chandra et al., 2007
<i>Bacillus</i> sp. AY952465	Kraft lignin	--	--	37	Chandra et al., 2007

2.4.5. Combined pre-treatment

2.4.5.1. Thermal pre-treatment coupled with acid

Effect of thermal steam or LHW pre-treatment can be improved by addition of acid. Acid catalyses the solubilisation of the hemicellulose, lowers the pre-treatment temperature and provides enzyme compatible substrate for hydrolysis. (Brownell et al., 1986; Gregg and Saddler, 1996). In the work done by Jung et al. (2011), acid and thermal pre-treatment were combined to enhance H₂ production from *Laminaria japonica* using response surface methodology. Efficient treatment of *Populus deltoids* wood and bark chips were observed with dilute acid (1% H₂SO₄) and hot water pretreatment (140°C for 100 min) with high recovery of xylose (Martin et al., 2011).

2.4.5.2. SO₂-impregnated thermal pre-treatment

The lignocellulosic biomass is often impregnated (soaked) with SO₂ or H₂SO₄, prior to steam explosion to enhance digestibility of fibres and hydrolysis of hemicelluloses. Sequential SO₂-catalyzed steam pre-treatment and enzymatic hydrolysis of aspen chips yielded 65% xylose with minimum retained SO₂ (Bari et al., 2007). Ohgren et al. (2005) reported 89% glucose and 78% xylose yield from SO₂-impregnated steam exploded corn stover. Pre-treatment of softwood with SO₂ was also reported by Soderstrom et al. (2002) for highest hemicellulose recovery.

2.4.5.3. Oxidative lime pre-treatment

According to Chang et al. (2001), thermal lime pre-treatment is not capable of removing enough lignin of high-lignin biomass to enhance the enzymatic digestibility and therefore oxygen as oxidant must be included during the pre-treatment. Chang and Holtzapple (2000) described effectiveness of process as lime pre-treatment makes substrate more accessible to hydrolysis due to opening of the acetyl and lignin valve. Pre-treatment was carried out in presence of Ca(OH)₂ (0.1g/g of substrate) for switch grass, corn stover and hybrid poplar, showed 13-fold higher enzymatic digestibility, low sugar degradation with 21% lime recovery by carbon dioxide carbonation (Chang et al., 2001b). Optimized oxidative lime pre-treatment conditions (110°C, 240 min, 6.89 bar) combined with milling for switch grass *Panicum virgatum* showed glucan and xylan yield of 93 and 68%, respectively, was reported by Falls and Holtzapple (2011).

2.4.5.4. Supercritical CO₂ with steam explosion

Combination of supercritical CO₂ and steam effectively mineralize lignin and elicit the cellulose to sugar conversion. Alinia et al. (2010) investigated the effect of dry and wet wheat straw for pre-treatment using supercritical CO₂ and CO₂ coupled with steam under different operating conditions (temperature and residence time in the reactors). Work by Luterbacher et al. (2010) dealt with high pressure (200 bar) CO₂-H₂O pre-treatment at high loadings (40%) of a wide variety of biomass (switchgrass, corn stover, big bluestem, and mixed perennial grasses) and resulted into glucose yields of 73, 81 and 85% for wood, switchgrass and corn stover, respectively.

2.4.5.5. Wet oxidation coupled with steam explosion

Chemical degradation and physical rupture of the biomass is achieved by combining wet-oxidation and steam explosion in one process, resulting in wet explosion (Sorensen et al., 2008). The advantages include the ability to process larger particle sizes and to operate at higher substrate loadings (50%) (Georgieva et al., 2008). Cellulose and hemicellulose conversion of 70% and 68% was obtained from wheat straw using wet explosion (Georgieva et al., 2008).

2.4.5.6. Dilute-acid pre-soaking before organosolv pre-treatment

Recent study revealed that pre-soaking of dilute acid-pretreated sugarcane bagasse prior to an organosolv process using NaOH as a catalyst under the optimized conditions (60 min, 195 °C, 30% v/v ethanol) yielded 67% glucose in 24 h (Mesa et al., 2011). The optimized dilute acid presoaking and aqueous ethanol organosolv treatment of *Miscanthus* led to enhance digestibility and recovery of xylans (20%), with 98% cellulose to glucose conversion (Brosse et al., 2009).

2.4.5.7. Alkaline peroxide treatment coupled with steam explosion

Maximum amount of reducing sugar (568 mg/g initial dry biomass) extracted from Bamboo by enzyme saccharification with 1% (v/v) H₂O₂ and 1% (w/w) NaOH was reported by Yamashita et al. (2010). Similar sugar yield (460 mg/g initial dry sample) from Bamboo was obtained with steam explosion (20 atm) in presence of 10% (w/w) NaOH (Chen and Qiu, 2010).

By combining steam explosion (198°C, 10 min, 1.5 MPa) with alkali hydrogen peroxide pre-treatment, the cellulose content in wheat straw was enhanced with an overall ethanol yield of 81% using SSF (Chen et al., 2008). Sun et al. (2005) studied a two-stage process based on steam

explosion (200-220°C, 15-22 bar) pre-treatment followed by alkaline peroxide (2% H₂O₂, 50°C, 5 h, pH 11.5) and obtained 92-99% of lignin loss.

2.4.5.8. Superfine grinding prior to steam explosion

Steam explosion pre-treatment cannot separate the fibrous tissue from non- fibrous tissue of lignocellulosic biomass efficiently and destroys hemicellulose, results into generation of inhibitors (Li and Chen, 2008). Superfine grinding generally results into decrease in particle size, improved reactive surface area and efficiency of hydrolysis. Superfine grinding combined with low severity steam explosion was used for treating rice straw to shorten the grinding time, save the energy cost, minimize the inhibitor and obtain improved efficiency of enzymatic hydrolysis (Mansfield et al., 1999). Study indicated that the superfine ground rice straw showed highest hydrolysis with sugar yield of 61% after 24 h (Jin and Chen, 2006).

2.4.5.9. Alkali/acid assisted microwave pre-treatment

Modeling of alkali assisted microwave pre-treatment and its comparison with high pressure reactor pre-treatment was carried out by Vani et al. (2012) and maximum reducing sugar yield of 49% was obtained after hydrolysis. Comparison of microwave-acid, microwave-alkali and combined microwave-alkali-acid pre-treatment were done using sugarcane bagasse as the lignocellulosic biomass by Binod et al. (2012) and reported 83% sugar yield using microwave-alkali-acid treatment.

2.4.5.10. Surfactant assisted ultrasound pre-treatment

Ultrasound acoustic waves can break the cohesion of a liquid and can create micro cavities, led to generation of high temperatures. Ionic surfactants generally modify the surface area of biomass and interact hydrophobically with lignin to remove it for a better surface area for hydrolysis. Surfactant assisted ultrasound pre-treatment of sugarcane top gave enhanced yield (66%) of reducing sugar after enzymatic hydrolysis (Sindhu et al., 2012). Ultrasound assisted alkali and ultrasound assisted acid pre-treatment of sugarcane bagasse for ethanol production was studied by Velmurugan and Muthukumar (2011) providing lignin reduction of 75.44% and the hemicellulose and cellulose recovery was about 79% and 99%, with ultrasound alkali pre-treatment whereas, 77% of cellulose and 81% of pentose conversion was reported for ultrasound acid pre-treatment.

2.4.5.11. Other combined pre-treatment

Combined effect of ammonia and ([Emim]Ac) on rice straw was studied by Nguyen et al. (2010) and reported 97% conversion of cellulose to glucose. Treatment of ([BMIM]Cl) in addition with steam explosion resulted in complete enzymatic hydrolysis of rice straw (Liu and Chen, 2006). Ethanol (40%) extraction combined with steam explosion (180°C for 20 min) pre-treatment of wheat straw showed 82% delignification with cellulose recovery of 94% by Chen and Liu (2007). Combining hot compressed water and ball milling pre-treatment improved efficiency of the enzymatic hydrolysis of Eucalyptus wood chips and yielded 70% sugar (Inoue et al., 2008). The effect of combined pre-treatment of different biomass is represented in Table 2.5.

2.5. Enzymatic hydrolysis of lignocellulosic biomass

Enzymatic conversion of cellulose to sugars is one of the most important factors to evaluate the efficiency of pretreatment. Enzymatic hydrolysis is more preferred route than chemical (e.g., acid hydrolysis) in terms of high sugar yield, minimal by-product formation, low energy requirements and chemical disposal cost and mild operating conditions (O'Dwyer et al., 2007). Enzymatic hydrolysis without pretreatment yields less than 20% sugar, whereas enzymatic saccharification followed by pretreatment may yield up to 90% sugar yield (Ghosh and Ghose, 2003; Nguyen et al., 2010; Chen et al., 2011; Binod et al., 2011; Velmurugan and Muthukumar, 2012). Hence, the need exist for suitable pretreatment to enhance the saccharification process using hydrolytic enzymes.

There are several commercial cellulases produced around the world such as Celluclast[®] 1.5 L (Novozyme, Denmark), neutral cellulase (Zytex India Pvt. Ltd, Mumbai, India) and CodeXyme cellulase[®] (Codexis, California, USA), most of them are commercially obtained from *Trichoderma* spp. and *Apergillus* spp. ACCELERASE[®] TRIO[™] (Genecor-DuPont, Netherland), a mixture of cellulase, hemicelluase and β -glucosidase may also attain feasibility for improved enzymatic hydrolysis. The cost of these enzymes is too high; therefore it is necessary to scout cellulase from natural efficient cellulase producers to make the hydrolysis step economically feasible.

2.5.1. Cellulase: The enzyme complex

Cellulases belong to the family of glycoside hydrolase, act by cleaving β , 1-4 glycosidic bonds of the polysaccharides and oligosaccharides. Due to the potential of these enzyme systems to convert cellulosic materials into useful sugars, it holds a great potential in biofuel production. Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization (Wen et al., 2005) and it contributes, to worldwide industrial enzyme demand. The cellulase enzyme market is expected to expand dramatically with its application in hydrolysis of pretreated cellulosic materials to sugars and further to bioethanol and biobased products (van Beilen and Li, 2002). Cellulases are inducible enzymes which are synthesized by large number of microorganisms, either cell-bound or extracellular during their growth on cellulosic materials (Lee, 2001). Cellulases, historically, been divided into three major groups: endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and β -glucosidase (EC 3.2.1.21) and the synergistic actions of these enzyme is widely accepted mechanism for cellulose hydrolysis (Lynd et al., 2002; Zhang and Lynd, 2004; Bayer et al., 2004; Zhang, 2008). Endoglucanase randomly cleaves internal bonds at amorphous region, exoglucanase cleaves the exposed chain produced by endoglucanase, whereas, β -glucosidase hydrolysed the cellobiose unit produced by exoglucanases (Figure 2.5).

These enzymes can either be free in aerobic microorganisms, or grouped in a multicomponent enzyme complex, cellulosome, in anaerobic cellulolytic bacteria (Lynd et al., 2002). Enormous amounts of agricultural, industrial and municipal cellulosic wastes have been accumulating or used inefficiently due to the high cost of their utilization processes (Kim et al., 2003; Lee et al., 2008). Therefore, it has become considerable economic interest to develop processes for effective treatment and utilization of cellulosic wastes as cheap carbon source. These resources comprise of leaves, stems, and stalks from sources such as corn fiber, corn stover, sugarcane bagasse, rice, rice hulls, woody crops and forest residues.

Table 2.5. Effect of combined pre-treatment on different lignocellulosic biomass.

Biomass	Combined pre-treatment	Achievement (s)	Reference
Rice straw	Acid (3% H ₂ SO ₄) catalysed steam explosion (180°C, 20 min)	72% reducing sugar yield after 72 h of enzymatic hydrolysis	Chen et al., 2011
	Ammonia solution (10%) followed by ionic liquid ([Emim]Ac) at 130°C for 24 h	82% cellulose recovery with 97% reducing sugar yield	Nguyen et al., 2010
	Steam explosion (179.9°C, 5 min) followed by super fine grinding (feed loads 15 kg/h, rotational speed 4544 rpm, grinding time of 25 min)	61% reducing sugar yield after 24 h hydrolysis	Jin and Chen, 2006
Wheat straw	Steam explosion (198°C, 10 min, 1.5 MPa) followed by alkaline peroxidase (4.0% H ₂ O ₂) along with (1% NaOH)	Hemicellulose and lignin removal by 5 and 6%, respectively	Chen et al., 2008
	Steam explosion (198°C, 10 min, 1.5 MPa) followed by alkaline pre-treatment (1% w/v NaOH)	Hemicellulose and lignin removal by 6 and 15%, respectively	Chen et al., 2008
	Steam explosion (4.5 min, 1.5 MPa) followed by ethanol extraction (40% ethanol, fiber/liquor ratio 1:50 (w/v) at 180°C for 20 min and 0.1 % NaOH)	82% lignin removal	Chen and Liu, 2007b
Sugarcane bagasse	Microwave (600 W, 4 min) assisted with alkali-acid (1% NaOH, 1% H ₂ SO ₄)	83% reducing sugar yield	Binod et al., 2012
	Ultrasound (400 W, 24 kHz) assisted alkali treatment (2.89% NaOH, 70.15 °C, 47.42 min)	92% reducing sugar yield	Velmurugan and Muthukumar, 2012
	<i>Ceriporiopsis subvermisporea</i> treatment for 8 weeks followed by microwave hydrothermolysis (200°C, 20 min)	32% sugar recovery in insoluble fraction and 15% sugar recovery in soluble fraction	Sasaki et al., 2011

Corn cob	Dilute acid (2% H ₂ SO ₄ , 121°C, 45 min) followed alkali (2% NaOH) treatment	81% lignin removal	Zhang et al., 2010
Corn stalk	<i>Irpex lacteus</i> treatment for 15 day followed by alkaline (0.25 M NaOH, 75°C, 120 min)	80% lignin loss	Yu et al., 2010a
Rice hull	Ultrasonic treatment (250 W, 30 min) followed by <i>Pleurotus ostreatus</i> treatment for 18 days	32% reducing sugar yield	Yu et al., 2009
	H ₂ O ₂ (2%, 48 h) treatment followed by <i>Pleurotus ostreatus</i> treatment for 18 days	50% reducing sugar yield	Yu et al., 2009
Switch grass	Microwave (190°C, 30 min) assisted by alkali (10% NaOH) treatment	59% reducing sugar yield	Hu and Wen, 2008
Poplar wood chips	<i>Lenzites betulina</i> treatment at 28°C for 4 weeks followed by liquid hot water pretreatment at 200°C for 30 min	92.33 % hemicellulose removal and 2.7-fold increase of glucose yield	Wang et al., 2012
Water hyacinth	<i>Echinodontium taxodii</i> treatment for 10 days followed by acid treatment (0.25% H ₂ SO ₄ , 100°C, 60 min)	46% reducing sugar yield	Ma et al., 2010

Table 2.6. Pretreatments required for enzymatic hydrolysis of different feed-stocks and sugar yield.

Feedstock	Pre-treatment	Enzymes	Hydrolysis conditions	Achievement(s)	References
Wheat straw	0.75% (v/v) H ₂ SO ₄	Cellulase, β -glucosidase, xylanase, esterase at each enzyme dose of 2 mL/100 g of wheat straw	45°C, pH 5.0, 72 h	56.5% sugar of total biomass	Saha et al., 2005
Wheat straw	2.15% (v/v) H ₂ O ₂	Carboxymethyl cellulase, β -glucosidase, xylanase at each enzyme dose of 4 mL/100 g of wheat straw	45°C, pH 5.0, 120 h	67.2 % sugar of total biomass	Saha and Cotta, 2006
Wheat straw	Dilute H ₂ SO ₄	10 U g ⁻¹ of cellulases, 5 U g ⁻¹ of xylanases, 20 U g ⁻¹ of recombinant feruloyl esterase	50°C, pH 4.8, 24 h	51.4% sugar of total biomass	Tabka et al., 2006
5 % (w/w) cellulose	1-n-butyl-3-methylimidazolium chloride	Cellulase (50-115 FPU g ⁻¹ glucan)	50°C, pH 4.8, 12 h	Glucose yield 72%	Dadi et al., 2006
Yellow poplar	Ethanol organosolv pulping	Cellulase (0.37 g protein g ⁻¹ cellulose)	50°C, pH 5.0, 24 h	Glucose yield 92%	Berlin et al., 2006
Rice hulls	7.5 % (v/v) H ₂ O ₂	Carboxymethyl cellulase, β -glucosidase, xylanase at each enzyme dose of 4 mL/100 g of rice hulls	45°C, pH 5.0, 72 h	Sugar yield 90%	Saha and Cotta, 2007
Spruce	Steam and 3% (w/w) SO ₂	Celluclast 1.5 L (cellulose activity of 107 FPU g ⁻¹ and β -glucosidase activity of 33 IU g ⁻¹)	50°C, 48 h	Glucose yield 82%	Borjesson et al., 2007
Corn stover	Steam and 3% (w/w) SO ₂	Enzyme (Cellulase, β -glucosidase, xylanase) 15 FPU g ⁻¹ cellulose	45°C, 72 h	Glucose yield 96%, xylose yield 86%	Ohgren et al., 2007
Olive tree	1 % H ₂ SO ₄ at 190 °C	15 FPU g ⁻¹ substrate Cellulase, 15 IU g ⁻¹ substrate β -glucosidase	50°C, pH 4.8, 72 h	36.3% sugar of total biomass	Cara et al., 2008
Switch grass	0.1 g g ⁻¹ alkali at 190°C	12 FPU g ⁻¹ of substrate cellulase, 21 IU g ⁻¹ of substrate β -glucosidase	pH 4.8, 50°C	58.7% sugars of total biomass	Hu and Wen, 2008

Silvergrass, rice straw and bagasse	1-3 % (w/w) sulfuric acid at 121 °C for 10- 180 min	6.5 FPU g ⁻¹ DM Spezyme CP (commercial cellulase)	50°C for 72 h	Xylose yield 70-75%	Guo et al., 2008
Cardoon (<i>Cynara cardunculus</i> L.)	0.15% (w/w) H ₂ SO ₄ at 184.5°C	15 FPU g ⁻¹ of substrate of Celluclast 1.5 L FG and 15 15 IU g ⁻¹ dry pretreated substrate of-glucosidase Novozym 188	50°C, pH 4.8, 72 h	Glucose yield 70% and xylose yield 93 %	Ballesteros et al., 2008
Rice straw	AFEX pretreatment at 140°C for 30 min	15 FPU g ⁻¹ cellulose Spezyme® CP, 64 pNPGU g ⁻¹ cellulase Novozyme™ 188, 0-10.68 mg protein g ⁻¹ glucan Multifect® xylanase and 0- 5.21 mg protein g ⁻¹ glucan Multifect® Pectinase	50°C, pH 4.8, 168 h	12.3% of the total glucose and 37.0 % of the total xylose	Zhong et al., 2009
Sugarcane bagasse and agave bagasse	2% (w/v) NaOH treatment	20% (w/w) of each enzyme per bagasse dry weight (Viscozyme, Celluclast, Novozyme, Cellubrix and Pulpzyme)	55°C for 4 h	Reducing sugar 11-20 % for sugarcane bagasse and 12-58% for agave bagasse	Hernandez-salas et al., 2009
Switchgrass	1.2% (w/w) sulfuric acid at 160 °C for 20 min and 3.23% (w/w) [C ₂ mim][OAc] (ionic liquid)	50 mg protein g ⁻¹ glucan cellulase (NS50013) and 5 mg protein g ⁻¹ glucan β-glucosidase (NS50010)	55°C, pH 4.8	47.6% with acid pretreatment and 96% with ionic liquid pretreatment	Li et al., 2010
Corn stover	1.5 % H ₂ SO ₄ at 106-108°C for 6 h, 0.4 g g ⁻¹ Ca(OH) ₂ of substrate at 120°C for 4 h, 10% NH ₄ OH at 26°C for 24 h and 2 % NaOH at 120°C for 30 min	20 FPU cellulase g ⁻¹ substrate with cellobiase (2 FPU:1 CBU)	50°C, pH 4.8, 48 h	Hydrolysis yield 39.4% for acid-pretreated, 50% for lime pretreated, 60% for NH ₄ OH pretreated and 81.2% for NaOH pretreated	Chen et al., 2009

Rice straw	2% NaOH at 85°C for 1 h,	Cellulase from <i>Trichoderma reesei</i> ZM4-F3	pH 4.5, 35°C, 120 h, 180 rpm	68.21% of pretreated rice straw was decomposed	Zhang and Cai, 2008
Rice straw	0.5-1% (w/w) H ₂ SO ₄ with a reaction time of 125 min at 160-190°C	Celluclast 1.5L 10 FPU g ⁻¹ dry matter and Novozym 188 β-glucosidase 15 IU g ⁻¹	50°C, pH 4.8, 48 h	Glucose yield 60%	Hsu et al., 2010
Wheat and barley straw	1-5% (w/w) NaOH and steam pre-treatment	3.7 g Celluclast 1.5 L (60 FPU g ⁻¹ , 30 β-glucosidase IU g ⁻¹), 0.8 g Novozyme 188 (500 β-glucosidase IU g ⁻¹) and 0.8 g Multifect Xylanase (43 g protein mL ⁻¹)	40°C, 72 h	Xylan and cellulose conversion 83-95% for barley straw and 63-76% for wheat straw	Persson et al., 2009
Wheat and barley straw	1-5 % (w/w) NaOH and steam pre-treatment	3.7 g Celluclast 1.5 L (60 FPU g ⁻¹ , 30 β-glucosidase IU g ⁻¹), 0.8 g Novozyme 188 (500 β-glucosidase IU g ⁻¹) and 0.8 g Multifect Xylanase (43 g protein mL ⁻¹)	40°C, 72 h	Xylan and cellulose conversion 83-95 % for barley straw and 63-76% for wheat straw	Persson et al., 2009
<i>C. ladanifer</i> (rock-rose) and <i>C. striatus</i> (broom)	2.6 % (w/w) H ₂ SO ₄ for 75 min at 150°C	Cellulase complex 60 FPUg ⁻¹ dry biomass 25 FPU g ⁻¹ of cellulose celluclast 1.5L	50°C, pH 4.8, 16 h	0.313 g of glucose per g of dry biomass to rock-rose and 0.448 g of glucose per g of dry biomass to broom	Ferreira et al., 2009
Bermudagrass, reed and rapeseed	Phosphoric acid (85%) and Acetone, 120 rpm, 50°C, 1 h	25 FPU g ⁻¹ of cellulose celluclast 1.5 L	50°C, pH 4.8, 16 h	Enzymatic digestibility was 75.4% for bermudagrass, 73.1% for reed and 57 % rapeseed	Li et al., 2009

Wheat bran	Washed And boiled in water at 60°C for 30 min	150 U g ⁻¹ of <i>Thermomonospora</i> xylanase	60 °C, pH 7.0, 48 h	58% xylan conversion	Menon et al., 2010
Wheat straw	2 % H ₂ SO ₄ and 1% Tween 20,	Cellulase 20 FPU g ⁻¹ glucan and β-glucosidase 40 CBU g ⁻¹ glucan	50°C, pH 4.8, 72 h	73.3 % glucose yield and 79.9% xylose yield	Qi et al., 2010
Sugarcane bagasse	SO ₂ -catalyzed steam pretreatment at 190°C for 5 min	65 FPU g ⁻¹ Celluclast 1.5 L and 17 IU g ⁻¹ of β- glucosidase	40°C, pH 4.8, 72 h	Sugar yield 87%	Carrasco et al., 2010
<i>Pinus radiata</i>	Biological pre-treatment with <i>Trametes versicolor</i> 26°C for 6 weeks	Celluclast (20 FPU g ⁻¹ dry substrate), β-glucosidase 188 to 25 IU g ⁻¹ dry substrate	pH 4.8, 50°C, 24h	25% increase in glucose yield	Vaidya and Singh, 2012
<i>Miscanthus</i> biomass	Organosolv treatment with 80% aqueous ethanol, and 0.5% sulfuric acid at 170°C for 60 min	Celluclast from <i>T. reesei</i> with β-glucosidase from <i>Aspergillus niger</i> at a loading of 40 IFPU and 80 IU per g cellulose, respectively	pH 4.8, 50°C, 170 rpm	delignification yield of 93% cellulose-to-glucose conversion of 75%	Obama et al., 2012.

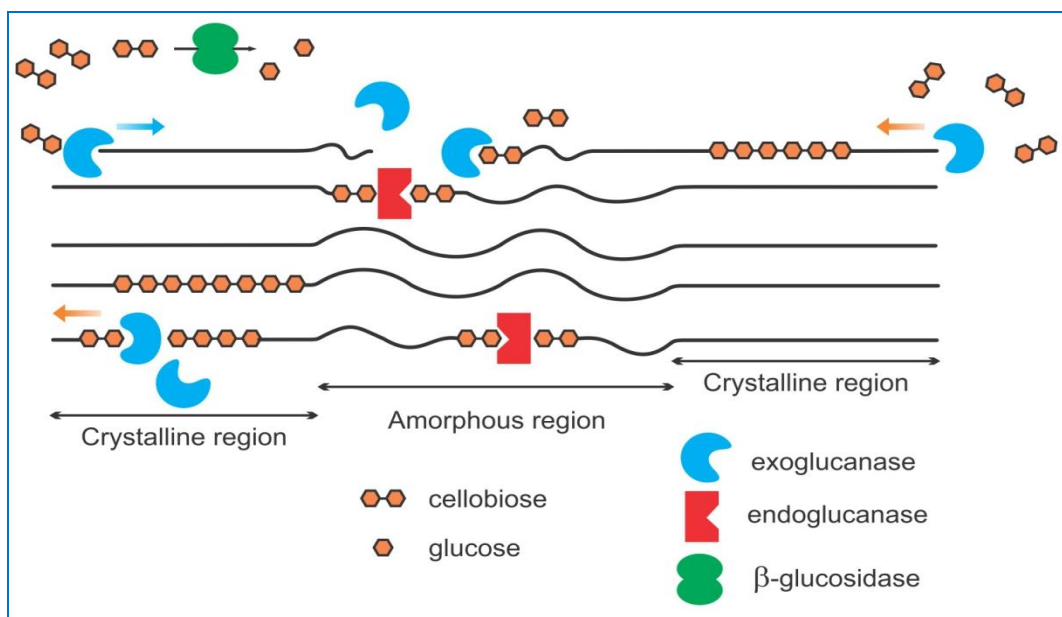


Figure 2.5. A simplified schematic representation of enzymatic action of cellulase involving exoglucanase, endoglucanase and β -glucosidase on cellulose.

In the last two decades, research has been aimed at developing new technologies and microbial strains to reduce the cost of cellulase production and improving the bioconversion of cellulose, particularly for biofuel industry. Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization (Wen et al., 2005). But currently, two significant points of these enzyme-based bioconversion technologies are reaction conditions and the production cost of the related enzyme system (Lee et al., 2008). Therefore, much research has been aimed at obtaining new microorganism producing cellulolytic enzymes with higher specific activities and greater efficiency (Pattana et al., 2000; Subramanian and Prema, 2000; Lee and Koo, 2001; Johnvesly et al., 2002). Cellulase yield appears to be dependent upon a complex relationship involving a variety of factors like inoculum size, pH, temperature, presence of inducers, medium additives, aeration, and growth time (Robson and Chambliss, 1984).

2.5.2. Bacterial diversity among cellulase producers

Many microorganisms are able to produce and secrete cellulolytic, hemicellulolytic and lignolytic enzymes. These microorganisms are found among extremely variegated taxonomic groups inhabiting diverse habitats from extreme thermophilic conditions to Polar Regions and aerobic to anaerobic systems. Already by 1976, an impressive collection of more than 14,000

fungi active against cellulose and other insoluble fibres were reported (Mandels and Sternberg, 1976; Lynd et al., 2002). Most of the cellulolytic bacteria fall within the phyla Actinobacteria, Bacteroidetes, Fibrobacteres, Firmicutes and Proteobacteria. Phyla Actinobacteria and Firmicutes constitute 80% of the isolated cellulose degrading bacteria (Bergquist et al., 1999; Lynd et al., 2002). Besides fungi, bacteria and protozoa, detached avocado fruit *Persea americana* and slime mould *Dictyostelium discoideum* (Bayer et al., 2000) have been also been reported for cellulolytic activity. An inspection of taxonomic diversity reveals that the ability to digest cellulose is widely distributed among many genera in the domain Bacteria (Table 2.7), although no cellulolytic members of domain *Archaea* have yet been identified. Within the eubacteria there is considerable concentration of cellulolytic capabilities among the predominantly aerobic order Actinomycetales (Phylum Actinobacteria) and the anaerobic order Clostridiales. The mechanisms through which microorganisms degrade cellulosic materials consist of production and secretion of enzymes, acting synergistically to release simple carbon compound to be used as source of chemical energy. In-situ composting of sugarcane residue by indigenous bacteria and fungi was reported by Boopathy et al. (2007). Recent worldwide efforts have continuously been made to increase the folds in cellulases activity through strain improvement by mutagenesis techniques, optimization of physical and chemical components and expression of desired gene into suitable vector, all with limited success. Consortium of fungi *Aspergillus nidulans* (Th4), *Scytalidium thermophilum* (Th5) and *Humicola* sp. (Th10) was used to compost paddy straw and lignin rich soybean trash to obtain nutritionally rich compost (Kumar et al., 2008). The co-cultivation of fungi (Kausar et al., 2010) have also increased desirable components of the cellulase and resulted into improved saccharification of lignocellulosic biomass.

2.5.3. Aerobic cellulase producing bacteria

Aerobic bacteria produce numerous individual, extra-cellular enzymes, most of them contains carbohydrate binding module (CBM) joined to one end of the catalytic domain for different cellulose conformations. Eubacteria are now being widely exploited for purification and production of novel various glycoside hydrolases. High growth rate than fungi, enhanced production of cellulases, simple nutritional requirement, broad range of environmental tolerance

and enormous range of different environmental and industrial habitats are the key reasons to hunt bacteria for increased function and synergy. In the current scenario, *Bacillus amyloliquefaciens*, *B. licheniformis*, *B. pumilus*, *B. subtilis*, *Paenibacillus barcinonensis*, *Pseudomonas aeruginosa* and *P. fluorescens* are the potent cellulase producers isolated from soil, compost, cowdung, goldmine, acidic water and mud (Tabel 2.7). Of these, about 70% of the isolated cellulolytic aerobic bacteria are of genus *Bacillus* and *Paenibacillus*. The best-studied species of cellulolytic aerobic bacteria belong to the genera *Cellulomonas* and *Thermobifida* (formerly *Thermomonospora*) (Lynd et al., 2002). *Cellulomonas* species are coryneform bacteria that produce at least six endoglucanases and at least one exoglucanase (Cex) (Chaudhary et al., 1997). Three endo-glucanases (E1, E2 and E5), two exo-glucanases (E3 and E6) and an unusual cellulase with both endoglucanase and exoglucanase activity (E4) have been reported from *Thermobifida fusca*, a thermophilic filamentous bacterium from soil (Lynd et al., 2002).

The latter enzyme has high activity on bacterial microcrystalline cellulose and exhibits synergism with endoglucanases and exoglucanases of *T. fusca* (Irwin et al., 1993). Rumen acts as chemostat for many cellulolytic bacteria to enhance the digestibility of cellulosic feed. Since past five years, many bacteria such as *Bacillus* sp. C1 (Sadhu et al., 2013), *Bacillus* sp. (Shanmugapriya et al., 2012) from cow dung and *B. amyloliquefaciens* SS35 (Singh et al., 2013) from rhinoceros dung has been isolated and found effective for utilizing various cellulosic substrate. Among different bacteria inhabiting the soil, potential cellulose degraders are *Paenibacillus barcinonensis* (Asha et al., 2012), *B. amyloliquefaciens* DL-3 (Lee et al., 2008), *B. subtilis* GN156 (Apiraksakorn et al., 2008), *B. pumilus* (Kotchoni et al., 2003), *Cytophaga huthincini* (Kauri et al., 1985; Li and Gao, 1997; Louime et al., 2006), *Anoxybacillus flavithermus* (Ibrahim et al., 2007) and *Pseudomonas aeruginosa* (Kurishita et al., 2010). Thermophilic and hyperthermophilic procaryotes represent a unique group of microorganisms that grow at temperatures that may generally exceed 45°C or even 100°C. Several cellulolytic hyperthermophiles have been isolated during the past decade (Bergquist et al., 1999).

Table 2.7. Aerobic cellulolytic bacteria, their characteristics and phylogenetic position.

Bacteria	Source	Phylum	Characteristics	References
<i>Bacillus</i> sp.	Cowdung	Firmicutes	Gram positive, oval shape, sporic	Shanmugapriya et al., 2012
<i>Bacillus subtilis</i> GN156	Soil	Firmicutes	Gram positive, rod, endosporic	Apiraksakorn et al., 2008
<i>Bacillus</i> sp.	Soil	Firmicutes	Gram-positive, motile, rod-shaped	Vijayaraghavan and Vincent , 2012
<i>Bacillus</i> sp. C1	Cow dung	Firmicutes	Gram-positive, motile, rod-shaped	Sadhu et al., 2013
<i>Bacillus pumilus</i>	Soil	Firmicutes	Gram positive, rod, sporic	Ariffin et al., 2006
<i>B. amyloliquefaciens</i> DL-3	Farm soil	Firmicutes	Gram positive, rod, endosporic, mesophilic	Lee et al., 2008
<i>Bacillus licheniformis</i> B-41361	--	Firmicutes	Gram positive, rod, mesophilic	Bischoff et al., 2006
<i>Geobacillus</i> sp.	Deep subsurface of goldmine	Firmicutes	Gram positive rod, sporic, mesophilic	Rastogi et al., 2009
<i>Brevibacillus</i> sp.	Deep subsurface of goldmine	Firmicutes	Gram positive, rod, sporic, mesophilic	Rastogi et al., 2009
<i>Lysinibacillus sphaericus</i>	Vermicompost	Firmicutes	Gram positive, rod shaped	Jyotsna et al., 2010
<i>Paenibacillus barcinonensis</i> MG 7	Soil	Firmicutes	Gram positive, rod, sporic	Asha et al., 2012
<i>Paenibacillus</i> sp.	Compost	Firmicutes	Gram positive, rod, endosporic, mesophilic	Wang et al., 008; Rastogi et al., 2009
<i>Cellulomonas iranensis</i> , <i>C. persica</i>	Forest humus soil	Actinobacteria	Gram positive, rod, alkalophilic	Elberson et al., 2000
<i>Cytophaga</i> sp.	Soil	Bacteroidetes	Gram negative, rod, mesophilic, gliding,	Li and Gao, 1997; Louime et al., 2006;
<i>Acidothermus cellulolyticus</i>	Acidic water, mud samples	Actinobacteria	Gram positive, rod, thermophilic	Mohagheghi et al., 1986; Baker et al., 1994, Berquist et al., 1999
<i>Sinorhizobium fredii</i>	Soil	Proteobacteria	Gram negative, rod	Chen et al., 2004
<i>Anoxybacillus flavithermus</i>	Soil	Firmicutes	Gram positive, rod, endosporic, mesophilic	Ibrahim et al., 2007
<i>Pseudomonas aeruginosa</i> CX1, <i>P. fluorescens</i> CX2	Soil	Proteobacteria	Gram negative, rod, mesophilic, flagellar	Kurishita et al., 2010

Table 2.8. Anaerobic cellulolytic bacteria, their characteristic and phylogenetic position.

Bacteria	Source	Phylum	Characteristics	References
<i>Clostridium thermocellum</i>	Sewage soil	Firmicutes	Gram positive, rod, flagellar, thermophillic	Chuvilskaya et al., 1986; Lamed et al, 1987; Sakka et al., 1994
<i>C. acetobutylicum</i>	Soil, grass	Firmicutes	Gram positive, rod, flagellar	Petitdemange et al., 1984; Sabathe et al., 2002
<i>C. stercorarium</i>	Compost	Firmicutes	Gram positive, rod, flagellar	Schwarz et al., 1995
<i>C. cellulovorans</i>	Dairy farm soil	Firmicuts	Gram positive, rod, flagellar, thermophillic	Doi et al., 2001
<i>C. hungatei</i>	Soil	Firmicutes	Gram positive, rod, flagellar, thermophillic	Monserate et al., 2001
<i>C. cellulolyticum</i>	Compost	Fermicutes	Gram positive, rod, flagellar	Belaich et al., 1997; Pages et al., 1997b
<i>C. josui</i>	Compost	Fermicutes	Gram positive, rod, flagellar	Kakiuchi et al., 1998
<i>C. herbivorans</i>	Deep soil	Firmicutes	Gram positive, rod, mesophillic	Varel et al., 1995
<i>C. papyrosolvans</i>	Paper mills	Firmicutes	Gram positive, rod, flagellar, mesophillic	Pohlschroder et al., 1994
<i>C. aldrichii</i>	Wood digester	Fermicutes	Gram positive, rod, flagellar, mesophillic	Yang et al., 1990
<i>C. cellobioparum</i>	Mud	Firmicutes	Gram positive, rod, flagellar, thermophilic	Hungate et al., 1944; Lamed et al., 1987
<i>Ruminococcus albus</i>	Rumen	Firmicutes	Gram positive, coccus, mesophillic	Bryant, 1959; Hungate, 1966; Ohara et al., 2000
<i>R. flavefaciens</i>	Rumen	Firmicutes	Gram positive, coccus, mesophillic	Bryant, 1959; Hungate 1966; Kirby et al., 1997; Aurilia et al., 2000
<i>Bacteroides cellulosolvans</i>	Sewage sludge	Firmicutes	Gram negative, non-endosporic	Lamed et al., 1991a; Ding et al., 2000
<i>Acetivibrio cellulolyticus</i>	Sewage sludge	Firmicutes	Flagellar, mesophillic	Patel et al., 1980; Ding et al., 1999
<i>Butyrivibrio fibrisolvens</i>	Rumen	Firmicutes	Gram positive, rod, mesophilic	Hungate et al., 1966; Lin et al., 1991
<i>Thermotoga neapolitana</i>	Deep soil	Firmicutes	Gram positive, rod, thermophillic	Zverlov et al., 2001
<i>Halocella cellulolytica</i>	Rumen	Firmicutes	Gram positive, rod, mesophilic	Simankova et al., 1993
<i>Fibrobacter succinogenes</i>	Rumen	Fibrobacteres	Gram positive, rod, flagellar, mesophillic	Bryant 1959; Hungate 1966; Montgomery et al., 1982; Stewart et al., 1989
<i>F. succinogenes</i>	Intestinal track	Fibrobacteres	Gram positive, rod, flagellar	Fields et al., 2000

Thermophilic bacteria, such as *Clostridium straminisolvans*, *Sporotrichum thermophile*, *Thermonospora curvata* (Hutnan, 2000), *Rhodothermus* sp. (Lynd et al., 2002), *Geobacillus pallidus* (Baharuddin et al., 2010) and an actinomycetes *Acidothermus cellulolyticus* (Lynd et al., 2002) are well-known to produce cellulase for biodegradation of cellulosic substrates. Moving forward, thermophilic organisms may be used as potent sources of thermostable cellulases for efficient hydrolysis of lignocellulosic biomass.

2.5.4. Anaerobic cellulase producing bacteria

Many anaerobic bacteria possess a unique extracellular multi-enzyme complex, called cellulosome (Schwarz et al., 2001; Wilson, 2011). Only a few of the enzymes in cellulosomes contain a CBM, but the protein to which they are attached (called scaffoldin) does contain a CBM, which binds the complex to cellulose (Wilson, 2011). Majority of the cellulolytic anaerobic bacteria of genus *Clostridium* are *Clostridium acetobutylicum*, *C. aldrichii*, *C. cellulolyticum*, *C. cellovioparum*, *C. cellulovorans*, *C. herbivorans*, *C. hungatei*, *C. josui*, *C. papyrosolvans*, *C. stercorarium* and *C. thermocellum* (Table 2.8). Although cellulolytic *Acetovibrio cellulolyticus*, *Bacteroides cellulosolvans*, *B. succinogenes*, *Butyrivibrio fivrisolvans*, *Fibrobacter succinogenes*, *Ruminococcus albus* and *R. flavefaciens* have also been reported from different niche. Cellulosomes from different *Clostridium* sp. and *Ruminococcus* sp. have been exhaustively studied during last decade. The architecture of cellulosomes is similar among these organisms, although cellulosome composition varies from species to species. The cellulosome of the thermophilic *C. thermocellum* is discussed and briefly compared to those of the mesophilic *C. cellulolyticum*, *C. cellulovorans* and *R. albus* (Schwartz et al., 2001). However, *C. stercorarium* is the only species for which no cellulosome has been observed (Schwartz et al., 2001). Ruminococcus bacteria of the genus *Ruminococcus* are phylogenetically related to, but do not fall within the family Clostridiaceae (Lynd et al., 2002). Recently, the presence of dockerin-like sequences in at least seven of the cellulase and xylanase genes of *Ruminococcus flavefaciens* have been reported and the production of 1.5-MDa cellulosome like structures on the *R. albus* cell surface in the presence of cellobiose and organic acids (phenylacetic and phenylpropionic acid), suggested that *Ruminococcus* sp. indeed produce cellulosomes (Ding et al., 2000). The structure of the *R. albus* cellulosomes differs from that of

the Clostridia, suggesting an independent evolutionary path (Ohara et al., 2000). *Fibrobacter succinogenes* S85 is another efficient cellulolytic bacterium isolated from the rumen, actively adheres to cellulose (Fields et al., 2000). Although the cellulases of *F. succinogenes* are cell associated, no cellulosome structures have been identified.

2.5.5. Characterization of bacterial cellulases

Enzymes show its own pH sensitivity, temperature optima, thermal stability, substrate specificity, kinetics and effect of different chemical towards its activity. The broad range of pH tolerance and thermal stability displayed promising characteristics of cellulases for the biofuel and biobased industry. Many workers have purified and characterized cellulases from different bacteria such as *Cellulomonas* sp. YJ5 (Yin et al, 2010), *Thermomonospora* sp. (George et al. 2001), *Melanocarpus* sp. MTCC 3922 (Kaur et al., 2007), *Pseudomonas fluorescens*, *Pyrococcus horikoshi* (Kang et al., 2007), *Bacillus* sp. C1 (Sadhu et al., 2013), *Bacillus subtilis* AS3 (Deka et al., 2013), *B. licheniformis* WBS1 and *Bacillus* sp. WBS3 (Acharya and Chaudhury, 2012), *Paenibacillus barcinonensis* MG7 (Asha et al., 2012), *Bacillus* sp. M9 (Bajaj et al. 2009), *B. licheniformis* (Bischoff et al., 2006), *Bacillus* sp. HSH-810 (Kim et al. 2005) and *Bacillus sphaericus* JS1 (Singh et al., 2004).

The molecular weight of purified cellulase has been reported in the range of 30-250 kDa from different bacterial strains and purification strategy led to enhancement of activity up to 280 folds (Table 2.9). Purified cellulases from bacteria are found stable at a pH range of 4 -7.5 and it was thermally stable up to 90°C for *Bacillus* sp. M9 (Bajaj et al., 2009) with an optimum temperature of 35-50°C for different bacteria, however optimum activity of 60°C or more has been observed for *Cellulomonas* sp. ASN2 (Irfan et al., 2012) and *Caldibacillus cellulovorans* (Huang and Monk, 2003) (Table 2.10)

Characterization of cellulases becomes inevitable for the production of recombinant cellulase or *in situ* mass production using bacterial strain for reducing the need of multiple enzymes production during hydrolysis mechanism. Multifunctional cellulase (CelAB) with two catalytic carbohydrate domain, cellobiohydrolase and β -1,4(3) endoglucanase activity responsible for degrading multiple complex polysaccharides showed an optimum pH and temperature of 6 and 42°C, respectively and also able to reduce viscosity of CMC by 40% in 25 min (Perez-Avalos et

al., 2008). A bifunctional endoglucanase/endoxylanase was isolated from *Cellulomonas flavigena* showed optimum cellulase and xylanase activity at pH 6 and 9, respectively, with optimum temperature at 50°C (Perez-Avalos, 2008), providing potential for use in different industrial processes such as biofuel production. Similarly, in 2007, a multifunctional enzyme was found to be produced by *Terendinibacter turnerae* T7902, which was a bacterial symbiont isolated from the wood-boring marine bivalve *Lydrodus pedicellatus* (Maki et al., 2009). Purification of bacterial cellulase by ion-exchange chromatography such as DEAE (diethylaminoethanol) sepharose and sepharyl chromatography has been extensively used at pilot scale. It can be also used for direct recovery of proteins and other charged molecules, as the technique is known to have high resolving power, simple to operate, highly robust, generic and economical (Abdullah, 2004). The purified cellulase from Bacilli revealed molecular mass of 37 kDa from *B. licheniformis* B-41361 (Bischoff et al., 2006), 97 kDa from *Bacillus* sp. C1 (Sadhu et al., 2013), 61-80 kDa from *Bacillus* sp. AC1 (Li et al., 2006), 80 kDa from *Bacillus* sp. HSH-810 (Kim et al., 2005), 54 kDa from *B. amyloliquefeciens* DL-3 and *Bacillus* sp. M-9 (Lee et al. 2008; Bajaj et al. 2009).

Similarly, molecular mass of purified cellulase was reported for other group of bacteria and it was 36, 38 and 43.7 kDa for *Pseudomonas fluorescens* (Bakare et al. 2005), *Thermomonospora* sp. (George et al. 2001) and *Cellulomonas* sp. YJ5 (Yin et al., 2010), respectively. More recently, modifications to bacterial cellulases through protein engineering by adopting rational design and directed evolution (Maki et al., 2009) is taking a lead in the production of efficient hydrolytic enzymes.

2.5.6. Fungal cellulase

Degradation of plant biomass by fungi is due to the production and secretion of enzymes, acting synergistically on the plant cell wall, releasing simple carbon source that is used by the microorganism as source of chemical energy. Fungal cellulose utilization is mainly distributed among Ascomycota, Basidiomycota (Table 2.11) and Neocallimastigomycota (Table 2.12). In the entire kingdom, filamentous fungi *Trichoderma reesei*, was one of the first potent cellulase producer among fungi in 1950s. Cellulolytic enzyme systems from *Trichoderma reesei* contain two exoglucanases or cellobiohydrolases (CBH1 and CBH2), four endoglucanases (EG1, EG2, EG3, EG5), and one β -glucosidase (Kumar et al., 2008).

Table 2.9. Molecular weight of purified cellulase and their activity from different cellulolytic bacteria.

Bacteria	Source	Molecular weight (kDa)	Enzyme activity in crude cellulase	Enzyme activity in purified cellulase	Purification fold	Reference
<i>Bacillus</i> sp. CH43	Hot springs	40	--	--	--	Mawadza et al., 2000
<i>Bacillus</i> sp.	Cow dung	33	4 U/mg	6.6 U/mg	--	Shanmugapriya et al., 2012
<i>B. amyloliquefaciens</i> DL-3	Soil	53	292 U/mL	6070 U/mL	21	Lee et al., 2008
<i>B. pumilus</i> EB3	--	30-65	0.57 U/mL	2 U/mL	1	Ariffin et al., 2006
<i>Bacillus</i> sp.	Soil	58	16 U/mL	246 U/mg	15	Vijayaraghavan and Vincent, 2012
<i>Bacillus</i> sp. C1	Cow dung	97	8 U/mL	68 U/mL	8	Sadhu et al., 2013
<i>Bacillus</i> sp. AC1	Fermented foods	33	24 U/mg	7001 U/mg	289	Li et al., 2006
<i>B. licheniformis</i> B-41361	--	37	3 U/mL	183 U/mg	60	Bischoff et al., 2006
<i>Paenibacillus barcinonensis</i> MG7	Soil	59	--	17 U/mg	--	Asha et al., 2012
<i>Caldibacillus cellulovorans</i>	Compost	85	0.27 U/mL	32 U/mL	117	Huang and Monk, 2004
<i>C. thermocellum</i>	--	85-210	13 U/mL	100 U/mL	15	Sakka et al., 1994
<i>Ruminococcus albus</i> F-40	Rumen	40-250	--	--	--	Ohara et al., 2000
<i>Rhizobium fredii</i> J1	Indigenous soil	97	0.42 U/mg	3.8 U/mg	10	Chen et al., 2004

U/mg: Specific activity; U/mL: Enzyme activity

Table 2.10. Physiological conditions for cellulases produced by different bacteria.

Bacteria	Optimum pH	Optimum temperature (°C)	Thermal stability (°C)	Reference
<i>Bascilus</i> sp.C1	7	50	20-70	Sadhu et. al., 2013
<i>Bacillus</i> sp.	6	50	--	Vijayaraghavan and Vincent., 2012
<i>Bacillus thuringiensis</i>	4	40	20-70	Lin et al., 2012
<i>B. subtilis</i> YJ1	6-6.5	60	<50	Yin et al., 2010
<i>B. subtilis</i> DLG	4.8	55	--	Robson and Chambliss, 1984
<i>B. pumilus</i> EB3	6	60	30-70	Ariffin et al., 2006
<i>B. coagulans</i> Co4	7.5	60	35-75	Adeleke et al., 2012
<i>Bacillus</i> sp. M-9	5	60	40-90	Bajaj et al., 2009
<i>Paenibacillus barcinonensis</i> MG7	7	35	25-50	Asha et al., 2012
<i>Caldibacillus cellulovorans</i>	6.5-7.0	80	65-89	Huang and Monk, 2003
<i>Cellulomonas</i> sp. ASN2.	7.5	60	40-80	Irfan et al., 2012
<i>Cellvibrio gilvus</i>	7.6	<40	--	Sasaki, 1988
<i>Rhizobium fredii</i> J1	7	35°C	30-45	Chen et al., 2004

Trichoderma viride and many other fungi produce cellulases and degrade cellulosic material or soluble cellulose derivatives. However, they are not very effective on crystalline cellulosic substrates. Besides *Trichoderma viride*, the other cellulase producing mesophilic strains producing cellulases are *Fusarium oxysporium*, *Piptoporus betulinus*, *Penicillium echinulatum*, *P. purpurogenum*, *Aspergillus niger* and *A. fumigatus* (Sharma, 2001; Szijarto, 2004, Valaskova, 2006). Cellulases from *Aspergillus* spp. usually have high β -glucosidase activity but lower endoglucanase levels, whereas, *Trichoderma* spp. have high endo and exoglucanase components but lower β -glucosidase levels, and hence has limited efficiency in cellulose hydrolysis (Mohanram et al., 2013). However, significant activity of β -glucosidase was observed with *Curvularia* sp. (Banerjee, 1990).

Table 2.11. Aerobic cellulases producing fungi, their sources and phylogenetic position.

Microorganisms	Source	Division	Class	References
<i>Trichoderma reesei</i>	Soil	Ascomycota	Sordariomycetes	Bergquist et al., 2002; Mach and Zeilinger 2003; Villena and Gutierrez-Correa, 2006
<i>T. reesei</i> NCIM 1052	--	Ascomycota	Sordariomycetes	Satyavarathanetal., 2013
<i>Trichoderma</i> sp. F4	Saw dust	Ascomycota	Sordariomycetes	Jaafaru, 2013
<i>T. viride</i> F1 <i>T. viride</i> F2	Decomposed rice straw, goat farms	Ascomycota	Sordariomycetes	Kausar et al., 2010
<i>Penicillium glabrum</i>	--	Ascomycota	Eurotiomycetes	Cabezas et al., 2012
<i>Chaetomium</i> sp. 2779	--	Ascomycota	Sordariomycetes	Cabezas et al., 2012
<i>Aspergillus niger</i>	Pulp and paper industry effluent	Ascomycota	Eurotiomycetes	Umbrin Ilyas et al., 2011,
<i>A. niger</i>	Soil	Ascomycota	Eurotiomycetes	Gielkens et al., 1999; Bergquist et al., 2002; Mach and Zeilinger, 2003; Villena and Gutierrez-Correa 2006
<i>A. flavus</i> NRRL5521	--	Ascomycota	Eurotiomycetes	Hussein Azzaz Murad et al., 2013
<i>Piptoporus betulinus</i>	Trees	Basidiomycota	Agaricomycetes	Valaskova et al., 2006
<i>Rhizopus stolonifer</i>	Bread	Zygomycota	Mucormycotina	Pothiraj et al., 2006
<i>Serpula lacrymans</i>	Decaying wood, organic mater	Basidiomycota	Agaricomycetes	Hastrup et al., 2006
<i>Melanocarpus albomyces</i>	Soil	Ascomycota	Sordariomycetes	Bhat et al., 2000; Miettinen-Oinonen et al., 2004
<i>Phanerochaete chrysosporium</i>	Decaying wood	Basidiomycota	Agaricomycetes	Covert et al., 1992; Ruggeri and Sassi, 2003
<i>Pycnoporus cinnabarinus</i>	Dead deciduous wood	Basidiomycota	Basidiomycetes	Sigoillot et al., 2002
<i>Sporotrichum pulverulentum</i>	Decaying organic matter	Basidiomycota	Agaricomycetes	Erikson and Petersson, 1975

Table 2.12. Anaerobic cellulase producing fungi, their source and phylogenetic position.

Microorganisms	Source	Division	Class	Reference
<i>Piromyces</i> sp. E2	Diegective tract	Neocallimastigomycota	Neocallimastigomycetes	Steenbakkers et al., 2002
<i>Piromyces equi</i>	Intestinal tract	Neocallimastigomycota	Neocallimastigomycetes	Fillingham et al., 1999
<i>Piromyces</i> sp.	Caecum	Neocallimastigomycota	Neocallimastigomycetes	Ali et al., 1995
<i>Anaeromyces mucronatus</i>	Intestinal tract	Neocallimastigomycota	Neocallimastigomycetes	Lee et al., 2001
<i>Cyllamyces aberensis</i>	Rumen	Neocallimastigomycota	Neocallimastigomycetes	Ozkose et al., 2001
<i>Orpinomyces</i> sp.	Rumen	Neocallimastigomycota	Neocallimastigomycetes	Chen et al., 1998
<i>Caecomyces communis</i>	Caecum	Neocallimastigomycota	Neocallimastigomycetes	Gerbi et al., 1996

Cellulases from *A. niger* (Knag et al., 2004; Haq et al., 2006; Immanuel et al., 2007; Acharya et al., 2008; Kausar et al., 2010; Khan et al., 2011; Devi and kumar, 2012; Sharda et al., 2012), *T. harzianum* (Haq et al., 2006), *T. reesei* (Zhao et al., 2011) and *A. fumigatus* (Immanuel et al., 2007) have been widely used in biodegradation of lignocellulosic biomass.

Thermophilic fungi such as *Sporotrichum thermophile*, *Scytalidium thermophilum*, *Clostridium straminisolvens* and *Thermonospora curvata* also produce the cellulase complex and can degrade native cellulose (Hutnan, 2000). Thermophilic organisms may be used as sources of thermostable cellulases for efficient hydrolysis of lignocellulosic biomass. Recent and worldwide efforts have continuously been made to increase the cellulases activity by many folds through strain improvement by various mutagenesis techniques, optimization of physical and chemical components and expression of desired gene into suitable vector, all with limited success.

2.6. Ethanol fermentation: Microorganisms and their abilities

The fermentation of pretreated lignocellulosic material after subsequent hydrolysis is carried out using recovered solid fractions or liquid fractions or whole hydrolysate using ethanologic microbes (Table 2.13). Ethanol production from lignocellulose biomass involves mixed sugar fermentation (both hexose and pentose) in the presence of many inhibitory compounds such as organic acids, furan derivatives, phenolics and inorganic compounds released and formed during pretreatment and/or hydrolysis of the raw material (Hahn-Hagerdal et al., 2006; Panagiotou and Olsson, 2007, Yu and Zhang, 2004). There are many wild type microbes available for fermentation process such as *Saccharomyces cerevisiae*, *Candida shehatae*, *Pichia stipitis*, *Candida brassicae*, *Mucor indicus*, *Pachysolen tannophilus* and *Zymomonas mobilis* (Moniruzzaman, 1995; Nigam et al., 2001; Balat et al., 2008; Sanchez and Cardona, 2008; Talebnia et al., 2010; Sukumaran et al., 2010; Girio et al., 2010). Currently, ethanol fermentation process is executed mostly with the yeast *Saccharomyces cerevisiae* because of its well-known characteristics such as high ethanol yield, tolerant to high ethanol and inhibitory compounds, adaptation to grow at low pH. However, this yeast has the ability to metabolize only hexose sugars and have been used in brewery industry since decades, while the pentose sugars can be fermented by other organisms such as *Pichia stipitis* and *Candida shehatae* (Moniruzzaman, 1995; Das et al., 2014). *S. cerevisiae* can be genetically engineered to ferment pentoses (Kim et

al., 2005; van Zyl et al., 2007). The enzymatic hydrolysis and fermentation can be executed via SHF, SSF, SSCF and CBP (Lynd, 1996; Lynd et al., 2002).

2.6.1. Separate hydrolysis and fermentation (SHF)

In SHF, lignocellulosic feedstocks are hydrolyzed to sugars and subsequently fermented to ethanol in separate bioreactors. The advantage of this process is that the best condition can be attained by optimization of each step individually. Mostly, the optimum activity of cellulase lies in temperature range of 40-50°C, depending upon the source of cellulase, while, optimum temperature for most of the ethanologenic microorganisms is in between 30 and 35°C. The major disadvantage of this process is that hydrolyzed cellobiose may inhibit the performance of cellulase (Reese, 1963) and β -glucosidase can be also inhibited by glucose (Holtzapple et al., 1990). Therefore, SHF requires lower solids concentrations at higher enzyme loadings to achieve greater yield of ethanol. However, low solid concentrations resulted in low ethanol yield, which may lead to increase in cost of downstream processing (Wyman, 1996). Another disadvantage is the chance of contamination even at higher temperature (40-50°C), which may be during transfer of biomass hydrolyzate from one vessel to another. A possible source of contamination could be the hydrolytic enzymes used for saccharification of biomass. In practice, it is difficult to sterilize cellulases at pilot scale, since these enzymes need membrane sterilization to avoid deactivation in an autoclave (Taherzadeh and Karimi, 2007).

2.6.2. Simultaneous saccharification and fermentation (SSF)

The fermentation of sugars released during hydrolysis is simultaneously consumed by the ethanologenic organisms, avoiding inactivation of cellulolytic enzymes. The process reduces the number of steps involved, resulting into a promising route for conversion of cellulosic materials to ethanol and other biobased products (Wyman, 1994; Lynd et al., 2005; Erdei et al., 2010), thus making the process economically feasible for large scale conversion. Whereas, the difference in optimum temperature conditions of enzyme for hydrolysis and fermentation poses some limitations (Bjerre et al., 1996; Hamelinck, 2005; Neves et al., 2007).

Table 2.13. Hydrolysis and fermentation of lignocellulosics for ethanol production.

Feedstock	Type of hydrolysis	Microorganisms	Fermentation conditions	Ethanol concentration	References
Wheat straw	Acid (H ₂ SO ₄) pre-treated, enzyme hydrolysis (cellulase, β -glucosidase, xylanase, esterase)	Recombinant <i>E. coli</i> FBR5	35°C, pH 6.0, 112 h	17 g/L	Saha et al., 2005
Wheat straw	Alkaline H ₂ O ₂ pre-treated enzyme hydrolysis (cellulase, β -glucosidase, xylanase)	Recombinant <i>E. coli</i> FBR5	37°C, pH 6.5, 48 h (SHF) 37°C, pH 6.0, 48 h (SSF)	18.9 g/L 15.1 g/L	Saha and Cotta, 2006
Rice hulls	Alkaline H ₂ O ₂ pre-treated and enzyme hydrolysis (cellulase, β -glucosidase, xylanase)	Recombinant <i>E. coli</i> FBR5	35°C, pH 6.5, 48 h	8.0 g/L	Saha and Cotta, 2007
Lodgepole pine	SO ₂ - catalyzed steam explosion, cellulase, β -glucosidase	<i>S. cerevisiae</i> T1	37°C, pH 5.0, 30 h	0.24, 231 g/Kg (SHF) 205 g/Kg (SSF)	Ewanick et al., 2007
Corn stover	SO ₂ - catalyzed steam explosion, celluclast, β -glucosidase	Baker's yeast	30°C, pH 5.5, 96 h (SSF)	26.4 g/L	Ohgren et al., 2007
Sugarcane bagasse	0.5-3.5 % (v/v) HCl	<i>Candida shehatae</i> NCIM 3501	30°C, pH 5.5, 24 h	8.67 g/L	Chandel et al., 2007
Cardoon (<i>Cynara cardunculus</i> L.)	Acid (H ₂ SO ₄) pretreatment, Celluclast 1.5 L FG, β -glucosidase Novozym188	<i>Kluyveromyces marxianus</i> CECT 10875	42°C, 72 h (SSF)	23 g/L	Ballesteros et al., 2008
Sugarcane bagasse	1.25% (w/w) H ₂ SO ₄ at 121°C for 2 h	<i>P. tannophilus</i> DW06	pH 5.0, 30°C, 30 h	221.9 g/L	Cheng et al., 2008
Sugarcane bagasse and agave bagasse	(2% (w/v) NaOH treatment and enzyme hydrolysis	<i>S. cerevisiae</i>	30°C, 48 h	12.9 g/L for sugarcane and 6.6 g/L for agave bagasse	Hernandez-Salas et al., 2009

Corn cob	Dilute acid and alkali pretreatment, 11.9 FPUg ⁻¹ Cellulase produced by <i>P. Decumbens</i> JUA10-1	<i>S. cerevisiae</i>	30°C, 141.5 h (SSF)	57.2 g/L	Liu et al., 2010
Bermudagrass, reed and rapeseed	Phosphoric acid and Acetone, 25 FPU Celluclast	<i>S. cerevisiae</i>	3°C, 96 h (SSF)	56.1 g/L from Bermuda grass, 55.0 g/L from reed, 36.6 g/L from rapeseed.	Li et al., 2009
Wheat bran	<i>Thermomonopsora</i> xylanase and Accellerase for 48 h at pH 7.0 and temperatures 40-70 °C	<i>Debaromyces hansenii</i>	Ca-alginate immobilized cells (10%) at 40°C, 36 h	6.89 g/L	Menon et al., 2010
Orange peels	Two-stage Sulfuric acid hydrolysis	<i>S. cerevisiae</i>	32.5°C, pH 5.0, 15 h	12.97 g/L	Oberoi et al., 2010
Wheat straw	Pretreatment with 3% H ₂ SO ₄ and 0.5% Tween 20, cellulase 20 FPU g ⁻¹ glucan and β-glucosidase 40 CBU g ⁻¹ glucan	<i>S. cerevisiae</i>	40°C, pH 4.8, SSF	11.2 g/L	Qi et al., 2010
Wheat straw, Sweet sorghum bagasse, <i>Brassica carinata</i>	Steam explosion	<i>K. marxianus</i> CECT 10875	(SSF)15 FPU/g Celluclast 1.5 L, 42 °C for 72-82 h.	18.1 g/L with wheat straw, 16 g/L with sorghum bagasse and 19 g/L with <i>B. carinata</i> residue	Ballestros et al., 2004
Rice straw	Dilute-acid hydrolysis, cellulase (BTXL)	<i>S. cerevisiae</i> , <i>Mucor indicus</i> , <i>Rhizopus oryzae</i>	38°C, pH 5-5.5, 7 days	10.20 g/L from <i>S. cerevisiae</i> , 11.35 g/L from <i>M. indicus</i> , 12.35 g/L from <i>Rhizopus oryzae</i>	Karimi et al., 2006

Rice straw	Aqueous-ammonia soaking treatment, cellulase (Celluclast 1.5L), β -glucosidase (Novozyme 188), xylanase from <i>Trichoderma viride</i>	<i>Saccharomyces cerevisiae</i> D ₅ A (ATCC 200062)	38°C, pH 4.8 and 150 rpm, 120 h.	12.7 g/L	Ko et al., 2009
Corn cob	Combination of acid and alkaline pre-treatments, (H ₂ SO ₄ –NaOH), 30 FPU/g cellulose	<i>S. cerevisiae</i>	37°C, pH 4.8, 96 h.	69.2 g/L	Zhang et al., 2010
wheat straw	Coupling steam explosion with alkaline peroxide	Yeast	40°C, pH 5.0, 120 h	51.5 g/L	Chen et al., 2008
Sugarcane bagasse	Alkali pre-treatment with 214 FPU/g cellulase mixed with xylanase	<i>K. marxianus</i> NCYC 587	pH 5.0, 42°C, 25% solid loading (SSF)	42.21 g/L	Gao et al., 2014
Sugarcane bagasse	Phosphoric acid-acetone pre-treatment, cellulase	<i>Z. mobilis</i> ATCC 29191	30 °C, pH 6	6.24 g/L (SHF) 5.53 g/L (SSF)	Wirawan et al., 2012
Bagasse pith hydrolysate	Steam and acid (8% w/w H ₂ SO ₄) treatment in a solid vs. liquid ratio of 1:10 at 120°C. for 90 minutes	<i>Kluyveromyces</i> sp. IPE453 (MTCC 5314)	45°C, pH 4.5, 5% inoculum, 4% substrate loading, 48 h	17.40 g/L	Dasgupta et al., 2013
Softwood	2% dilute acid (HCl) pretreatment at 170°C for 30 min	<i>Pichiastipitis</i> CBS6054 and <i>S. cerevisiae</i> Y5	30°C, pH 5, 96 h	27.40 g/L	Wan et al., 2012
Miscanthus biomass	NaOH pre-treatment and enzymatic hydrolysis (cellulase complex and β -glucosidase)	<i>S. cerevisiae</i>	32°C, 7% inoculum, 24% substrate loading, 48 h	59.2 g/L	Han et al. 2011
Tapioca stem	Dilute acid (1.25% H ₂ SO ₄) and dilute alkali NaOH	<i>Fusarium oxysporum</i>	30°C, pH 5.5, 2% inoculum,	8.64 g/L	Magesh et al. 2011

Bolivian straw material Paja Brava	Two-stage acid hydrolysis	<i>S. cerevisiae</i> , <i>P. stipitis</i> CBS 6054, <i>C. shehatae</i> CBS 4410, and <i>P. tannophilus</i> CBS 4044	30°C, pH 5.5, 140 h	5.7 g/L with <i>P. stipitis</i> CBS 6054	Sanchez et al., 2004
Kinnow waste and banana peels	Steam explosion (121°C, 1 h)	<i>Pachysolen tannophilus</i> MTCC 1077, and <i>S. cerevisiae</i> G	30°C, 48 h	26.84 g/L	Sharma et al., 2007
Sugarcane bagasse	Sodium chlorite and acetic acid pre-treatment	<i>Kluyveromyces fragilis</i>	35°C, 3% substrate loading, pH 5.5, 72 h	32.6 g/L	Sasikumar and Viruthagiri 2008

Further, some thermo-tolerant microorganisms like *Kluyveromyces marxianus* has been employed to withstand the higher temperatures needed for enzymatic hydrolysis (Bjerre et al., 1996). SSF involves use of same bioreactor for hydrolysis and fermentation, thereby keeping the concentration of glucose low (Ballesteros et al., 1993; Ballesteros et al., 2004; Fan and Lynd, 2007; Lin and Tanaka, 2006). The accumulation of ethanol does not inhibit cellulases as a high concentration of glucose does (Shonnard, 2003; Lin and Tanaka, 2006; Cardona and Sanchez, 2007). Therefore, SSF can achieve higher rates of fermentation yield and ethanol concentrations in comparison with separate hydrolysis and fermentation (SHF) (Wyman, 1992; Ballesteros et al., 2004; Lin and Tanaka, 2006; Cardona and Sanchez, 2007). SSF reduces the probability for microbial contamination, lowers the initial osmotic stress of yeast by avoiding a concentrated glucose solution, and is generally more energy-efficient (Bothast and Schlicher, 2007; O'Dwyer et al., 2007).

SSF requires compatible fermentation and saccharification conditions, with a similar pH, temperature and optimum substrate concentration (Ballesteros et al., 1991; Ballesteros et al., 1993). Highest ethanol concentration of 33.8 g/L was obtained using thermostable cellulase complex from corn stover, corresponding to 80% overall ethanol yield based on the glucose content in the feedstock (Ohgren et al., 2007).

2.6.3. Simultaneous saccharification and co-fermentation (SSCF)

The process of fermentation of hexoses and pentoses to ethanol and other chemical in a single step is generally referred as SSCF. Co-fermentation using combination of *Candida shehatae* and *Saccharomyces cerevisiae* microorganisms were found to be suitable for the SSCF process (Neves et al., 2007). Using genetically engineered microorganisms which are capable of converting hexoses and pentoses to ethanol can be a better approach for SSCF (Cardona and Sanchez, 2007).

2.6.4. Consolidated bioprocessing (CBP)

In CBP, cellulase production, hydrolysis of biomass and further fermentation to ethanol integrated in a single step using single reactor (Bjerre et al., 1996), minimizing capital cost investment and operational expenses for enzyme production. The prime objective is to develop a

single microorganism capable of performing these steps simultaneously (Lynd et al., 2005; van Zyl et al., 2007; Cardona and Sanchez, 2007). Recently, advancement of bioconversion technologies has focused on CBP due to minimum production cost with enhanced conversion efficiency. The availability, screening and characterization of suitable microorganisms capable of all required features are the bottleneck of CBP, however, some bacteria and fungi exhibiting needed features have been identified (Lynd et al., 2002; van Zyl et al., 2007). Most of the studies showed that *Clostridium thermocellum* is widely employed for enzyme production, cellulose hydrolysis, and ethanol fermentation, while co-fermentation using *Clostridium thermosaccharolyticum* allows the simultaneous conversion of hydrolyzed monomeric hemicellulose sugars to ethanol (Wyman, 1994). Thus, more research should be focused on exploitation of right microorganism with whole combination of features required for the successful execution of CBP (Cardona and Sanchez, 2007).

2.7. Response surface methodology for optimization of process parameters

Statistical based experimental designs, namely, Plackett-Burman design, Box- Behnken design, and Taguchi orthogonal array design, summarize the collection and sorting of variables to be taken for consideration, determine the variable amount, and analyze the variable at different parameters. Optimum production and effective utilization of extracellular bacterial cellulase, the medium ingredients have to be optimized, as its production in microorganisms is significantly influenced by media constituents (Deka et al., 2011; Lee et al., 2012), temperature, pH, aeration and other physical parameters (Immanuel et al., 2006). Relationship between these parameters has significant effect on the production of the cellulase in bacteria (Rajoka, 2004) and fungi (Saravanan et al., 2012). However, optimization of medium ingredients by classical method involves changing single independent variable at a time while keeping others at a constant level is time-consuming and expensive for large number of variables (Adinarayana et al., 2003) and may result in unacceptable conclusions (Oh et al., 1995). Plackett-Burman design plays a key role in screening of many variables and allows obtaining unbiased estimate of linear effects of all factors with maximum accuracy for a given number of observations. Better quality at low cost is the main aim for generation of Taguchi experimental design approach to maximize robustness of the products and processes. Taguchi design is a fast and considerable way of optimization,

conferring remarkable outcome in simultaneous study of many factors, making its mark in quality products supplemented with better process performance and rendering high yield and better stability (Taguchi, 1986). The basic principle involved is the encompassment of large experimental data as orthogonal array in determining the effect of various factors which govern the reaction happening, ensuring reduction in experimental error and improving efficiency.

2.8. Concluding remarks

Lignocellulose based biofuel necessitates development of sustainable energy system to enhance the world energy security. Pre-treatment of lignocellulosic feedstock is a pre-requisite to obtain fermentable sugar for bioethanol production. Many factors, like crystallinity, degree of polymerization, particle size and accessible surface area limit the decrystallization of cellulose and depolymerization of hemicellulose during pre-treatment. There are still several hindrances towards development of an efficient pre-treatment technology, as diverse biomass shows different cellular and molecular structure. Despite of the monopoly in producing various types cellulases by fungi and bacteria, bacterial cellulase has gained importance due to higher growth rate, broad range of pH, temperature and capability to degrade diverse kind of cellulosic substrate with great ease. Furthermore, a robust techno-economic analysis such as energy balance, low dose of enzyme loading and enhanced saccharification yield could provide insights for successful implementation of these technologies for large-scale production of ethanol. Thus, a need exists for intense coordinated research to optimize integrated biorefining processes, delineate pre-treatment chemistries and their impact on cellulosic biomass to obtain greater yield of bioethanol via simultaneous saccharification and fermentation.

CHAPTER III. MATERIALS AND METHODS

3.1. Screening and characterization of cellulose degrading microorganisms

Screening and characterization of cellulose degrading microorganisms were done from the samples collected from different sites. Samples were characterized for their physico-chemical properties and bacterial isolates were screened for their cellulose hydrolyzing properties. Screened cellulase producing bacterial isolates were further characterized for morphological, biochemical and physiological properties. Biolog GEN III MicroPlate™ identification and 16S rDNA sequence analysis were done to know the phylogenetic position of these isolates.

3.1.1. Sample collection

Samples of compost and paper pulp were collected from Thapar University (Site 1), and Vishal Paper Industries Pvt. Ltd. (Site 2), Patiala, Punjab (India), whereas, sugarcane bagasse samples were procured from K M Sugar Mills, Kanpur, Uttar Pradesh (Site 3). Samples were collected from depth of 0-50 cm using alcohol-sterilized implements. The samples were transported to the laboratory in an ice box and stored in sterile containers at 4°C until their further use.

3.1.2. Characterization of compost, paper pulp and sugarcane bagasse

3.1.2.1. Determination of pH and Electrical conductivity (EC)

Dried sample (20 g) was taken in 250 mL beaker and 100 mL of distilled water was added to it and thoroughly stirred for 2-3 min using a glass rod. Further, it was kept under shaking condition (120 rpm) for 3 h. Suspension was allowed to settle for 30 min. The instrument was checked with two buffer solutions of known pH viz. one acidic (pH 4.0) and other alkaline (pH 9.2). The electrode was rinsed with distilled water and carefully wiped with filter paper. The pH of the sample was measured by immersing the electrode in supernatant solution. The pH value was recorded when the reading was stabilized (usually after 1 min). The sample was mixed with water and stirred as described previously. KCl solution (0.01 M; EC = 1.413 mS m⁻¹) was used to calibrate the meter. The electrode was dipped in the supernatant solution and recorded the EC.

The pH and electric conductivity was determined in 1:2.5 (w/v) water extract using a Thermo Orion Model 290 pH meter and conductivity meter (Orion Model 125).

3.1.2.2. Estimation of total organic carbon (Walkley-Black method, 1934)

Dried sample (1 g) was transferred into a 150 mL conical flask and 10 mL of 1N $K_2Cr_2O_7$ was added and mixed well. The blank was prepared in which all reagents except soil were added. The conical flasks were kept on teflon / asbestos sheet and 20 mL of concentrated H_2SO_4 from the sides of the flask was added. The flask was swirled slowly during addition. The flask was allowed to stand for 30 min and there after 70 mL of water was added. These flasks were swirled thoroughly and allowed the suspended particles to settle overnight. Absorbance of the supernatant was recorded at 660 nm. For standard, 0, 10, 20, 30, 40 and 50 mg of anhydrous sucrose crystals was weighed into a 100 mL volumetric flask and repeated the same procedure.

Calculation:

The quantities of sucrose (0, 10, 20, 30, 40 and 50 mg) were multiplied by 0.4207 and found the quantities (%) of carbon present. Because 10 mg sucrose contained 4.207 mg carbon and if suppose 4.207 mg carbon was found in 1000 mg of sample, it meant 100 mg sample contained = $(4.207 \times 100) / 1000$ mg carbon.

$$\text{Organic carbon (\%)} = \text{Colorimetric reading} \times 0.0042$$

3.1.2.3. Estimation of total nitrogen (Piper, 1960)

Sample (1 g) was mixed thoroughly with sulphuric-salicylic acid (1 g salicylic acid mixed with 30 mL concentrated H_2SO_4) followed by 5 g of sodium thiosulphate. The mixture of sample was heated for 5 min and the allowed to cool at room temperature for 30 min. To this, 10 g of digestion mixture [10 g HgO , 5 g $CuSO_4$ and 100 g K_2SO_4 (2:1:20)] was added and contents were mixed well in a Kjeldahl flask. The flask was kept in the digestion chamber at $100^\circ C$ for 2 h. Further, the gradual color change was monitored from dark brown to greenish white after that the contents were cooled and 300 mL distilled water was added. Then 20 mL of the digested sample, 15-20 mL NaOH (50%, w/v) and glass beads were added to the distillation flasks to avoid bumping through the open end of the condensor attachment and were stoppered. Water flow was maintained through the condenser. The distillate was collected through a receiver tube in a beaker containing 15 mL boric acid (4%, w/v) and 2 drops of mixed indicator (0.066 g of methyl red and 0.099 g of bromocresol green dissolved in 100 mL of ethyl alcohol) till the end-

point color changes from pink to green. The distillate was titrated against 0.02 N H₂SO₄ until the endpoint color changed from green to pink.

Calculation :

$$\text{Total N\%} = [(T - B) \times 0.0014 \times 100] / \text{Weight of sample}$$

Where, T is the titre value for sample and B is for blank.

3.1.3. Isolation, screening and enrichment of cellulose degrading bacteria

All samples were first enriched for cellulose degrading bacteria by inoculating one-gram of respective sample in 50 mL nutrient broth (HiMedia, Mumbai, India) (Appendix I) containing 0.5% (w/v) carboxymethyl cellulose (CMC) and then incubated at 37°C for 24 h under shaking condition (120 rpm). For isolation and enumeration of cultivable bacteria, all the samples were serially diluted in saline (0.85%) and plated on nutrient agar supplemented with 0.5% (w/v) CMC (pH 7.0). The plates were then incubated at two different temperatures (28°C and 37°C). The grown bacterial colonies from the plates were sub-cultured several times on the same medium from where it was picked. For screening and selection of cellulolytic bacteria, the colonies were randomly picked and transferred onto nutrient agar enriched with 0.5% (w/v) CMC to check the zone of hydrolysis produced by cellulase.

3.1.4. Screening of cellulose degrading bacteria

All the bacterial isolates along with a positive control *Paenibacillus polymyxa* MTCC 3088, procured from MTCC, IMTECH, Chandigarh, India, were maintained on nutrient agar plates for further use. A loop full of pure culture from agar plate was added to 5 mL of nutrient broth (Appendix I) in a test tube and incubated at 37°C under shaking condition (120 rpm) until the inoculum turbidity reached to 0.6 (OD at 600 nm). The grown culture (5 µL) was spotted on the petri plate containing nutrient agar supplemented with 0.5% (w/v) CMC and then incubated at 37°C for 24 h. The culture plates were stained with 0.1% (w/v) Congo red for 15 min followed by destaining with 1M NaCl for 15 min (Teather and Wood, 1982). Cellulose hydrolysis was checked as the clear zone around the colonies.

3.1.5. Quantitative estimation of cellulase activity

3.1.5.1. CMCase assay

The bacterial isolates were grown in medium containing 0.5% tryptone, 0.5% yeast extract, 0.1% K_2HPO_4 , 0.25% $MgSO_4 \cdot 5H_2O$ and 0.25% $FeSO_4 \cdot 7H_2O$ supplemented with 0.5% (w/v) CMC. Culture medium (100 mL) in 250 mL flask was inoculated with 1% (v/v) overnight grown culture (0.6 OD at 600 nm) and incubated at 37°C under shaking condition (120 rpm). Cellulase activity in cell free supernatant collected after centrifugation (11,000 rpm for 10 min) was analyzed. CMCase activity was carried out by incubating cell free supernatant with substrate CMC for 30 min at 50°C. The reaction mixture (2 mL) contained 0.2 mL of cell free supernatant in 1.8 mL of 1% (w/v) CMC dissolved in phosphate buffer (50 mM, pH 7.0). The reaction was stopped by addition of 3 mL of DNS (3,5-dinitrosalicylic acid) reagent (Appendix II). The reaction mixture was then boiled in a water bath for 10 min and then allowed to cool down for 30 min; absorbance was taken at 540 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that released one μ mol of glucose per min. The amount of reducing sugar liberated was quantified by 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Calibration curve of glucose (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) was made to determine the amount of reducing sugar liberated.

3.1.5.2. FPase assay

For FPase assay, Whatman no. 1 filter paper strip (1 x 3 cm) soaked in 1.8 mL of 50 mM phosphate buffer (pH 7.0) was used as substrate. Cell free supernatant (0.2 mL) was added in pre-soaked filter paper and then incubated at 50°C for 60 min. The reaction was stopped by addition of 3 mL of DNS reagent (Appendix II). The reaction mixture was then boiled in a water bath for 10 min and allowed to cool down for 30 min; absorbance was taken at 540 nm. The amount of reducing sugar liberated was quantified by DNS method as described in section 3.1.5.1. One unit (U) of enzyme activity was defined as the amount of enzyme that released one μ mol of glucose per min. Calibration curve of glucose (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) was made to determine the amount of reducing sugar liberated by the enzymatic action.

3.1.5.3. β -glucosidase assay

β -glucosidase activity was done as per modified protocol of Nagano et al. (1992). Activity was determined by adding 0.2 mL of cell free supernatant in 1.8 mL of 5 mM of pNPG (p-

nitrophenyl- β -D-glucopyranoside) as substrate, prepared in phosphate buffer (50 mM, pH 7.0) and then incubated at 50°C for 30 min. The reaction was terminated by addition of 1 mL of NaOH-glycine buffer (0.4 M, pH 10.8) (Appendix II), allowed to cool for 30 min and then absorbance was taken at 505 nm. One unit of β -glucosidase activity was defined as 1 μ mole of p-nitrophenol liberated per min. Calibration curve of p-nitrophenol (0, 200, 400, 600, 800 and 1000 μ M) was made to determine the amount of p-nitrophenol liberated by the enzymatic action.

3.1.6. Protein estimation by Folin-Lowry method (Lowry et al., 1951)

To study the protein concentrations of bacterial isolate, 1% (v/v) overnight grown culture (0.6 OD at 600 nm) were added to 100 mL of nutrient broth supplemented with 0.5% (w/v) CMC in 250 mL flask for and incubated at 37°C under shaking condition (120 rpm) for 40h. The aliquots were taken at an interval of 4 h, centrifuged (11,000 rpm, 20 min) and culture supernatants were used for protein estimation by Folin-Lowry method (Lowry et al., 1951).

Bovine serum albumin (BSA) fraction V (HiMedia, Mumbai, India) standard solutions were prepared in the range of concentrations 0.0, 0.2, 0.4, 0.6, 0.8, 1.0 mg/mL. From these solutions, 0.2 mL protein solutions were pipetted out into different test tubes. Simultaneously bacterial samples were also taken. 2 mL of alkaline copper sulphate reagent [prepared by (a) 50 mL of 2% sodium carbonate mixed with 50 mL of 0.1 N NaOH solution (0.4 g in 100 mL distilled water) (b) 10 mL of 1.56% copper sulphate solution mixed with 10 mL of 2.37% sodium potassium tartarate solution. Analytical reagent was prepared by mixing 2 mL of (b) with 100 mL of (a)] was then added and the solutions were mixed well. The final volume in each of the test tubes was 5 mL. These solutions were incubated at room temperature for 10 min. This was followed by addition of 0.2 mL of Folin-Ciocalteu reagent solution (1 N) to each tube and incubation for 30 min. The absorbance was measured at 660 nm. The absorbance was plotted against protein concentration to get a standard calibration curve. The absorbance of sample was checked and concentration of the sample was determined from the standard curve.

3.1.7. Morphological, biochemical and physiological characterization of bacterial isolates

The characterization of all bacterial isolates was done by conventional physiological and biochemical tests described in Bergey's Manual of Systematic Bacteriology (Holt et al., 1994).

3.1.7.1. Growth kinetics of bacterial isolate

The growth profiles of bacterial isolates were obtained by growing the isolates in nutrient broth (Appendix I) supplemented with 0.5% (w/v) CMC at 37°C under shaking condition (120 rpm). Aliquots were taken at regular time intervals of 4 h till 40 h and corresponding absorbance (OD at 600 nm) and cfu/mL were counted.

3.1.7.2. Gram staining

Bacterial smear was prepared from actively growing cells by spreading on a glass slide and fixed by gentle heating. Glass slide was flooded with crystal violet for 10 s followed by gentle washing with water to remove excess crystal violet. Gram iodine was then flooded for 10 s and washed with water. Further, it was decolourised with ethyl alcohol until the moving dye front has passed the lower edge of the section and washed immediately with tap water. Safranin was used to counter stain for 15 s and washed with water to remove excessive stain. The slide was air dried and visualized under microscope at different magnifications.

3.1.7.3. Capsule staining

The capsule stain is a differential stain, which selectively stains external capsules surrounding bacterial cells. Capsules are highly ordered polymers of sugars and proteins that surround some bacterial cells, and can be easily dislodged by heat or water. The primary stain applied was 2% crystal violet for 2 min, which stains both the bacterial cell and the surrounding capsule. A 20% copper sulfate solution is then applied to rinse gently, which serves a dual function as both decolorizer and counterstain. It removes and replaces the crystal violet in the capsule only. At the end of the staining procedure, the capsule appears as a faint blue or white halo around a purple cell observed under microscope.

3.1.7.4. Catalase test

A loopful of bacterial cells was placed onto a clean glass slide. A few drops of H₂O₂ (3%) were added onto the smear. Rapid evolution of O₂ bubbles indicates the presence of catalase activity. Lack of bubbles or only a few scattered bubbles marks absence of catalase activity.

3.1.7.5. Oxidase test

Small amount of bacteria from an agar slant or plate was obtained with a sterile swab. One drop of N,N,N',N'-tetramethyl phenylenediamine dihydrochloride reagent was placed onto the culture on the swab. Positive reactions turned the bacteria violet immediately or within 10 to 30 s.

3.1.7.6. Nitrate reduction test

Nitrate Agar media (HiMedia, Mumbai, India) (Appendix I) was used to determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification of nitrate to nitrite. Nitrate broth contained potassium nitrate as a source of nitrate. Bacteria were grown on nitrate agar plate for 24 h. After sufficient growth of bacteria, 2-3 drops of sulfanilic acid and α -naphthylamine were added. If the organism has reduced nitrate to nitrite, the nitrites in the medium will form nitrous acid. Sulfanilic acid reacts with the nitrous acid to produce diazotized sulfanilic acid. This reacts with the α -naphthylamine to form a red-colored compound. Therefore, if the medium turns red after the addition of the nitrate reagent, it was considered a positive result for nitrate reduction.

3.1.7.7. DNase test

By making a single streak line on DNase Test Agar (HiMedia, Mumbai, India) (Appendix I) with methyl green containing dye on agar plate incubated at 37°C for 24 - 48 h, DNase test was performed. Methyl green forms a complex with intact (polymerized) DNA to form the green colour of the medium. DNase activity depolymerises the DNA, breaking down the methyl green-DNA complex, which results in the formation of colourless zones around colonies of the test organism. A negative test is indicated by the absence of a colourless zone around the colonies.

3.1.7.8. Citrate utilization test

Simmons Citrate Agar (HiMedia, Mumbai, India) (Appendix I) slant was streaked with a loopful of bacteria and incubated at 37°C for 18-24 h. Visible growth on slant surface and colour change to intense prussian blue, showed positive result. The alkaline carbonates and bicarbonates produced as by-products of citrate catabolism raise the pH of the medium to above 7.6, causing the bromothymol blue to change from the original green colour to blue. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium.

3.1.7.9. Bile-Esculin test

Bile-Esculin Agar (HiMedia, Mumbai, India) (Appendix I) was streaked and incubated at 37°C for 24-72 h. Organisms that metabolize esculin produce esculetin as a by-product. Esculetin reacts with ferric citrate present in media to form a dark (black) coloration which is an indication of positive result.

3.1.7.10. Voges-Proskauer (V-P) test

Overnight grown culture (0.6 OD at 600 nm) was inoculated (1%, v/v) to Methyl Red and Voges Proskauer (MR-VP) broth (HiMedia, Mumbai, India) (Appendix I) and incubated at 37°C for 24 h under shaking condition (120 rpm). After incubation, 10 drops of Barritt's A reagent [(5% (w/v) α -naphthol in absolute ethanol] and 10 drops of Barritt's B reagent [40% (wtv) KOH in deionized water] were added to the culture broth. If glucose is being broken down, it will react with α - naphthol and KOH to form a red color, indicating the presence of acetoin and thus a positive Voges-Proskauer test within 15-20 min.

3.1.7.11. Urease test

Urea Agar Base medium (HiMedia, Mumbai, India) (Appendix I) was streaked and incubated at 37°C for 48 h to check the production of urease. As the substrate urea splits into its products, the presence of ammonia creates an alkaline environment that causes the phenol red to turn to deep pink, an indication of positive result.

3.1.7.12. Alkalinity, salinity and temperature test

Different sodium chloride concentrations (0, 2, 5, 7, 10, 12 and 15%) were amended in the nutrient broth (pH 7.0) to determine the survival of the bacterial isolates in saline conditions. Growth of bacterial isolates was recorded by measuring the absorbance at 600 nm of 24 h grown cells. Seven different temperatures (25, 30, 37, 45, 50, 55 and 60°C) were chosen to test the growth pattern of bacterial isolates. Bacterial isolates were grown in liquid and solid media and incubated at different temperatures as stated above.

3.1.7.13. Antibiotic profiling of bacterial isolates

Bacterial isolates were grown in nutrient broth until the absorbance reached to 0.6 at 600 nm. The grown bacterial cells were spreaded on nutrient agar plate and antibiotic discs were placed on it. These plates were incubated at 37°C for 24-48 h and the inhibition zone was monitored. Precoated antibiotic discs (HiMedia, Mumbai, India) were used to check the sensitivity of the bacterial isolates. These were: Norfloxacin (10 μ g), Gentamicin (10 μ g), Chloramphenicol (30 μ g), Cefuroxime (30 μ g), Ciprofloxacin (5 μ g), Cefoperazone (75 μ g), Ceftazidime (30 μ g), Roxithromycin (30 μ g), Clarithromycin (15 μ g), Co-trimoxazole (25 μ g), Netillin (30 μ g), Cefaclor (30 μ g), Cephodoxil (30 μ g), Azithromycin (15 μ g),

Ampicillin/Cloxacillin (10/10 µg), Penicillin (10 units), Amikacin (30 µg), Sparfloxacin (5 µg) and Ampicillin/sulbactam (10/10 µg).

3.1.7.14. Scanning electron microscopy (SEM) of bacterial isolate

Overnight grown cultures of bacterial isolate was centrifuged at 11,000 rpm for 10 min. Cell pellets were washed with 0.85% saline and resuspended in 2.5% gluteraldehyde in 50 mM sodium phosphate buffer (pH 7.0) for fixation and incubated at 4°C for 3 h (Huber et al., 1998). Bacterial cells were then dehydrated with 75% ethanol followed by absolute alcohol and air dried for 5 h and then completely dried in a desiccator for 12 h under vacuum. The cells were then coated with gold with gold sputter and visualized under Scanning electron microscope (SEM) (Model: JEOL JSM-6510 LV, USA) under different magnifications.

3.1.7.15. Biolog GEN III MicroPlate™ identification of bacterial isolates

Microbial identification was done using Biolog GEN III MicroPlate™ (Biolog, Inc., Hayward, California, USA) as per manufacture's instruction. Biolog uses powerful carbon source utilization technology to identify environmental and pathogenic microorganisms by producing a characteristic pattern or "metabolic fingerprint". It analyzes microorganism in 96 well microtiter plates with 94 phenotypic tests: 71 carbon source utilization assays and 23 chemical sensitivity assays along with positive and negative control. All the bacterial isolates to be identified were grown on Biolog recommended agar medium (agar with 5% sheep blood) and then further suspended in a proprietary inoculating fluid (IF) at the recommended cell density (90-98% transmittance). The cell suspension was inoculated (100 µL per well) into the GEN III MicroPlate™ and then incubated at 37°C under aerobic condition. After 22 h of incubation, the phenotypic fingerprint of GEN III MicroPlate™ was analyzed using GEN III OmniLog® Combo Plus System (Biolog, Inc., Hayward, California, USA).

3.1.8. Molecular characterization

3.1.8.1. Isolation of genomic DNA

A single colony of bacterial isolate was inoculated to 25 mL of nutrient broth in a 100 mL flask and incubated overnight at 37°C under shaking condition (120 rpm). Liquid cultures (2.0 mL) were harvested by centrifugation at 8,000 rpm for 10 min. Bacterial pellets were suspended in 800 µL of saline EDTA (0.15 M NaCl and 0.1 M EDTA of pH 8.0) and lysed by crystalline

lysozyme (~100 µg) followed by incubation at 37°C for 1 h. To this suspension, 200 µL of 2.0% sodium dodecyl sulfate (SDS) was added and incubated at 65°C for 15 min. DNA was recovered by phenol: chloroform: isoamyl alcohol (25:24:1) extraction followed by precipitation with 0.7 volume isopropanol to the aqueous phase, followed by 10 min centrifugation at 12,000 rpm. The DNA pellets were washed with 750 µL EtOH (70%) and microfuged for another 10 min. DNA pellets were resuspended in 50 µL of TE buffer (10 mM Tris of pH 8; 1 mM EDTA of pH 8.0) and stored at 4°C.

3.1.8.2. DNA Purification

The DNA was purified by elution through the Wizard DNA Clean up system (Promega) according to manufacturer's instructions in order to remove contaminants, which can hamper in manipulation of DNA.

3.1.8.3. Electrophoresis of DNA on agarose gels

DNA was loaded on agarose gels (0.7% w/v) prepared in 1X TBE, pH 8.0 (Appendix II) using a 6X loading dye (Appendix II). Ethidium bromide (0.5 µg/mL) was added to stain the gel prior to pouring. The nucleic acids were then electrophoresed at 3 volts/cm for 45-60 min and visualized on a U.V. transilluminator.

3.1.8.4. Spectrophotometric analysis of DNA

The concentration of extracted DNA in suspension was estimated by spectrophotometric measurement at A_{260} . For double-stranded DNA suspensions, an OD of 1.0 at a wavelength of 260 nm and using a cuvette with 1 cm light path, is equal to a concentration of 50 µg/mL. The quality of the DNA was evaluated by measurement of the A_{260}/A_{280} and the A_{230}/A_{260} ratios. Ideally, the A_{260}/A_{280} ratio should be 1.8-2.0 while the A_{230}/A_{260} ratio should be 0.3-0.9. Ratios (A_{260}/A_{280}) less than 1.8 indicate protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA.

3.1.8.5. Ethidium bromide fluorescent DNA quantification

DNA migrated electrophoretically in an agarose gel containing ethidium bromide (0.5 µg/mL). The quantity of DNA was visually determined with reference to a known DNA concentration of lambda phage (Fermentas, USA) by comparing the intensity of fluorescence.

3.1.8.6. Amplification of 16S rDNA and purification of PCR products

The polymerase chain reaction (PCR) provides a rapid and highly sensitive method for the primer-mediated enzymatic amplification of specific target sequences in genomic DNA resulting in the exponential increase of target DNA copies. The 16S rRNA gene fragment of the DNA was amplified by PCR using 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1510R (5'-GGTTACCTTGTTACGACTT-3') universal primers (Lane, 1991) with GeneAmp[®] 2700 PCR (Applied Biosystems, USA). Reaction mixture was prepared for PCR to a final volume of 100 µL containing 1X PCR buffer (InVitrogen, USA), each deoxynucleotide triphosphate at a concentration of 200 µM, 1.5 mM MgCl₂, each primer at a concentration of 0.1 µM, 2.5U of *Taq* DNA polymerase (InVitrogen, USA) and 2 µL (~50 ng) of template DNA. The PCR was performed with an initial denaturation at 92°C for 2 min followed by 36 temperature cycles as follows: 92°C for 1 min, 48°C for 30 s, 72°C for 2 min, and a final extension at 72°C for 6 min. Amplification was verified by electrophoresis of 5 µL aliquot of PCR product on a 0.8% agarose gel. Amplified 16S rDNA was purified using the QIAquick PCR purification kit (Qiagen, USA), following the instructions of the manufacturer. Purified PCR products were eluted from the purification columns by the addition of 50 µL 10 mM Tris buffer (pH 8.0).

3.1.8.7. 16S rRNA gene sequencing

The purified PCR products were sequenced using automated DNA sequencer AB3500 xL (Applied Biosystem, USA). Sequences were generated by chain termination method (Sanger et al., 1977).

3.1.8.7.1. Analysis of sequence data

Sequences were submitted to the CHECK-CHIMERA program of the RDP II (Maidak et al., 2001), in order to detect the presence of possible chimeric artefacts generated by PCR. Similarities were calculated for nearly complete 16S rDNA sequences using only unambiguously determined nucleotide positions. The gene sequence was compared with Ribosomal Database Project- II (RDP-II) (Cole et al., 2003) and with those from GenBank (<http://www.ncbi.nlm.nih.gov/>) using the BLASTN program (Altschul et al., 1997). The sequences were aligned using multiple alignments CLUSTALW program (Thompson et al., 1997). The evolutionary distance was calculated by Kimura 2 parameter, phylogenetic dendrogram were constructed by neighbor-joining method by the use of MEGA 5 package

(Tamura et al., 2007). For analysis, 1,000 bootstrap replicates were performed to assess the statistical support for the tree.

3.2. Biochemical characterization of cellulose degrading enzymes

Optimization of process parameters for maximum production cellulase from selected bacterial isolate *B. subtilis* NA15 was done using response surface methodology followed by biochemical characterization of cellulose degrading enzymes.

3.2.1. Statistical optimization of media ingredients for enhanced production of CMCase

In order to enhance CMCase production from most efficient bacterial isolate NA15, media ingredients were optimized using response surface methodology (RSM). It was accomplished in two consecutive steps: first, a Plackett-Burman (PB) design was executed to determine the significant parameters affecting CMCase production. Whereas, in the second stage, a five-level RSM design (CCD) with eight axial points and six replicates at the centre point was performed to estimate linear and quadratic effects and the corresponding response surface.

3.2.1.1. Plackett-Burman design

Plackett Burman design is a powerful and efficient mathematical approach to determine the effect of medium constituents on enzyme activity (Kaur and Satyanarayana, 2005). Experimental runs were performed according to the design and response (enzyme activity) was recorded. The effect of variables was investigated and experiments were carried out at 37°C and 120 rpm for 24 h. Each variable was set at two levels, high level and low level. The experimental design is given in Table 3.1. Experimental runs were performed according to the design and response (enzyme activity) was recorded. CMCase activity after 24 h was recorded as per methodology given in section 3.1.5.1. Significant parameters were selected from the data and studied further by response surface method. A pre-selection of the medium constituents that might affect CMCase activity was carried out using a two level screening Plackett-Burman design for 11 different medium constituents such as CMC, peptone, yeast extract, K_2HPO_4 , $MgSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, NaCl, KCl, NaH_2PO_4 and Na_2HPO_4 using Design-Expert (Version 8.0.7.1, Stat-Ease, Minneapolis, MN). Experimental runs were performed according to the design and response (CMCase activity) was recorded. The effect of variables was investigated in terms of CMCase activity (Section 3.1.5.1) after 24 h of incubation at 37°C and 120 rpm.

Effect of each factor was assessed in terms of the difference between the average of the measurements made at the high level of factor and the average of the measurements made at the low level of that factor, which was determined using the following equation:

$$E_{(Xi)} = 2 (\sum P_{i+} - \sum P_{i-}) / N$$

Where $E_{(Xi)}$ is the effect of the tested variable, P_{i+} and P_{i-} are the activities from the trials whereas the variable (Xi) measured were present at high and low level, respectively, and N is the number of trials (experiments) presented in Table 3.1. Standard error (SE) of the concentration effect was the square root of the variance of an effect, and the significance level (P-value) of the effect of each concentration was determined using student's t-test as given by the equation:

$$t_{(Xi)} = E_{(Xi)} / SE$$

3.2.1.2. Designing of optimal medium using central composite design (CCD)

To derive the optimum level of screened variables, a CCD with five coded levels (Table 3.2) and four variables was used to study the combined influence of CMC (A), peptone (B), yeast extract (C) and $MnCl_2 \cdot 4H_2O$ (D). For the four factors, the design was made up of a full 2^2 factorial design with its four points augmented with six replications of the centre points (all factors at level 0) and the four star points, that is, points having for one factor an axial distance to the centre of $\pm a$, whereas the other two factors are at level 0. The axial distance was chosen to be 2.0 to make this design orthogonal. Thirty experiments were employed in the present study (Table 3.3). Using least squares, the regression model was fit to evaluate the linear interaction and the quadratic effects of screened variables. The second order polynomial model was described as follows:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j$$

The model behaviour was explained by above quadratic equation. Where Y is predicted response (CMase activity), β_0 is the model constant, β_i is the linear coefficient, β_{ij} is the coefficient for interaction effect, and X_i is the dimensionless coded value if x_i (independent variable).

Table 3.1. Coded experimental design layout of Plackett-Burman design for the screening of media constituents.

Runs		Coded values (g/L)										
Std	Run	A: CMC	B: Peptone	C:Yeast Extract	D: K ₂ HPO ₄	E: MgSO ₄	F: FeSO ₄	G: MnCl ₂ ·4H ₂ O	H: NaCl	J: KCl	K: NaH ₂ SO ₄	L: Na ₂ HPO ₄
8	1	18	8	1	0.5	0.05	0.45	0.01	0.5	1	0.01	0.2
6	2	2	2	1	2	0.05	0.45	1	0.02	1	0.1	0.2
3	3	18	2	9	2	0.05	0.45	1	0.5	0.01	0.01	0.05
1	4	18	8	1	2	0.45	0.45	0.01	0.02	0.01	0.1	0.05
11	5	18	2	9	2	0.45	0.05	0.01	0.02	1	0.01	0.2
4	6	2	8	1	2	0.45	0.05	1	0.5	1	0.01	0.05
5	7	2	2	9	0.5	0.45	0.45	0.01	0.5	1	0.1	0.05
7	8	18	2	1	0.5	0.45	0.05	1	0.5	0.01	0.1	0.2
2	9	2	8	9	0.5	0.45	0.45	1	0.02	0.01	0.01	0.2
9	10	18	8	9	0.5	0.05	0.05	1	0.02	1	0.1	0.05
10	11	2	8	9	2	0.05	0.05	0.01	0.5	0.01	0.1	0.2
12	12	2	2	1	0.5	0.05	0.05	0.01	0.02	0.01	0.01	0.05

Table 3.2. Range of values for response surface method.

Independent variables		Coded levels				
	(g/L)	$-\alpha$	-1	0	1	$+\alpha$
A	CMC	2	6	10	14	18
B	Peptone	2	3.5	5	6.5	8
C	Yeast Extract	1	3	5	7	9
D	MnCl ₂ .4H ₂ O	0.01	0.2575	0.505	0.75	1

The results were analysed by the experimental design model and 2-D contour, plots using the statistical software package Design-Expert (Version 8.0.7.1, Stat-Ease, Minneapolis, MN). The fit quality of the model was evaluated by R^2 and analysis of variance (ANOVA). The robustness of the model and optimal values of parameters was assessed by the determination coefficient, F-test (Kim et al., 2006).

Table 3.3. Coded experimental design for the response surface to obtain maximum CMCase activity of *B. subtilis* NA15 as a function of CMC, Peptone, Yeast Extract, and $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$.

Runs		Coded values (g/L)			
Number	Order	A:CMC	B:Peptone	C:Yeast	D: $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$
29	1	10	5	5	0.505
13	2	6	3.5	7	0.752
15	3	6	6.5	7	0.752
8	4	14	6.5	7	0.257
26	5	10	5	5	0.505
5	6	6	3.5	7	0.257
14	7	14	3.5	7	0.752
23	8	10	5	5	0.01
30	9	10	5	5	0.505
10	10	14	3.5	3	0.752
25	11	10	5	5	0.505
28	12	10	5	5	0.505
18	13	18	5	5	0.505
6	14	14	3.5	7	0.257
24	15	10	5	5	1
4	16	14	6.5	3	0.257
7	17	6	6.5	7	0.257
19	18	10	2	5	0.505
16	19	14	6.5	7	0.752
12	20	14	6.5	3	0.752
2	21	14	3.5	3	0.257
17	22	2	5	5	0.505
3	23	6	6.5	3	0.257
9	24	6	3.5	3	0.752
11	25	6	6.5	3	0.752
27	26	10	5	5	0.505
20	27	10	8	5	0.505
1	28	6	3.5	3	0.257
21	29	10	5	1	0.505
22	30	10	5	9	0.505

3.2.2. Purification of CMCase

3.2.2.1. Ammonium sulfate precipitation and dialysis

In order to study the protein profile of the most efficient CMCase producing bacterium (isolate NA15), it was grown in an optimized medium as revealed by RSM. Culture medium (100 mL) in 250 mL flask was inoculated with 1% overnight grown culture (0.6 OD at 600 nm) and incubated at 37°C under shaking condition (120 rpm) for 24 h. For partial purification, ammonium sulfate precipitation (up to 80% saturation) was done using cell free supernatant obtained after centrifugation (11, 000 rpm for 10 min) at 4°C for 1 h with gentle stirring and then left overnight at 4°C. Precipitated protein was recovered by centrifugation (11, 000 rpm for 10 min) at 4°C and redissolved to a final volume of 15 mL in potassium phosphate buffer (50 mM, pH 7.0). Precipitated proteins were subjected to dialysis using 12-14 kDa cut off size dialysis membrane (HiMedia, Mumbai, India) at 4°C against the same buffer for 12 h with three consecutive changes of buffer.

3.2.2.2. DEAE-Sepharose ion exchange chromatography

After dialysis, the enzyme was further purified using ion exchange chromatography. DEAE SepharoseTM fast flow (GE Healthcare, Uppsala, Sweden) was packed into a vertically mounted column (1.5 x 10 cm) and equilibrated with three bed volume of potassium phosphate (50 mM, pH 7.0) buffer. Dialyzed proteins were layered onto prepared column eluted in potassium phosphate buffer (50 mM, pH 7.0) with linear gradient of 0 - 0.6 M NaCl gradient. The flow rate was maintained (1 mL/min) using Peristaltic Pump P-1 (GE Healthcare, Uppsala, Sweden). Each fraction of 3 mL was collected and tested for CMCase activity (U/mL) and protein concentration (mg/mL).

3.2.2.3. SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to check proteins in the cell free supernatant, partially purified protein and DEAE-Sepharose ion exchange chromatography using a 4% stacking and a 12.5% separating gels as described by Laemmli (1970). Samples were heated at 100°C for 5 min before electrophoresis. A standard protein molecular weight marker (Fermentas) containing β -galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), REase Bsp981

(25 kDa), β -lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa) was used for reference. The compositions of reagents used in SDS-PAGE are provided in Appendix II.

3.2.2.4. Zymogram analysis (*In gel detection of CMCase activity*)

To detect cellulolytic activity, Zymogram was performed as described elsewhere (Schwarz et al., 1987), with minor modifications. Acrylamide gel (12.5%) was prepared by including 0.5 mL of 2% (w/v) CMC before polymerization in the SDS-PAGE resolving gel mixture. CMC was added slowly while mixing and then heated to 30°C to prevent aggregation. Prior to loading, purified protein (40 μ L) was partially denatured at 70°C for 20 min to decrease activity band smearing. After electrophoresis, gels were washed five times with potassium phosphate buffer (50 mM, pH 7.0) at room temperature; each wash for 30 min. The gel was then stained with 0.1% (w/v) Congo red for 15 min at room temperature followed by destaining with 1 M NaCl until a zone of clearance was visualized within the gel, which confirmed cellulase activity. The zymogram gel was counter stain with 1M HCl. To improve the band visualization, glacial acetic acid (100 μ L) was added to the gel wash (Waeonukul et al., 2007).

3.2.3. Optimization of assay conditions for cellulase activity

The next aim of the work was to optimize the assay conditions to obtain maximum CMCase activity of the enzyme produced from best cellulase producing isolates NA15. With the objective of optimizing its activity, various assay conditions such as pH, temperature, and thermal stability were studied. CMCase activity was also observed in presence of various ions and reagents to see the response of additives. The bacterial isolates were grown in an optimized media (CMC: 18 g/L, peptone: 5 g/L, yeast extract: 5 g/L and MnCl_2 : 0.5 g/L) as worked out by RSM. Culture medium (100 mL) in 250 mL flask was inoculated with 1% (v/v) overnight grown culture (0.6 OD at 600 nm) and incubated at 37°C under shaking condition (120 rpm) for 24 h. The cell free extract was collected by centrifugation (11, 000 rpm for 10 min) at 4°C and analyzed for CMCase activity as described in section 3.1.5.1.

3.2.3.1. Effect of pH and temperature and stability of CMCase

The CMCase activity of purified enzyme was further studied for optimum pH, temperature and stability over a temperature tolerance range. The optimum pH for CMCase was determined by incubating the cell free supernatant (0.2 mL) in 1.8 mL of CMC in the presence of appropriate

buffers for different pH like 50 mM sodium acetate (pH 4.0 and 5.0), 50 mM potassium phosphate (pH 6.0 and 7.0) and 50 mM glycine-NaOH buffer (pH 8.0, 9.0 and 10.0). The reaction mixture of various pH buffers were incubated for 30 min at 50°C. For stability of CMCase at different pH (4.0-10.0), CMCase was incubated in different buffers at 30°C over a time period of 72 h. Aliquots were withdrawn at a regular interval of 4 h and CMCase activity was monitored as per the protocol described in section 3.1.5.1.

The optimum temperature for the purified cellulase was determined by assaying the enzyme at various temperatures ranging between 30°C to 80°C in 2 mL reaction mixture containing 1% (w/v) CMC dissolved in phosphate buffer (50 mM, pH 7.0) for 30 min. Thermal stability for CMCase was determined by incubating the purified cellulase at various temperatures ranging from 30°C to 80°C for 72 h. The aliquots of enzyme were withdrawn at a regular interval of 4 h and assayed at 50°C using 1% (w/v) CMC dissolved in 50 mM phosphate buffer (pH 7.0). The CMCase activity were carried out by determining the amount of reducing sugars released as described in section 3.1.5.1.

3.2.3.2. Effect of various chemicals on CMCase activity

The effect of various additives on the purified cellulase activity was determined by the presence of metal ions and other reagents. The additives used in this study were AgNO₃, CaCl₂, HgCl₂, PbCl₂, CuSO₄, CdCl₂, BaCl₂, KCl, NaCl, MgSO₄, MnCl₂, FeSO₄, CoCl₂, ZnCl₂, EDTA, SDS and Tween-80. The reaction mixtures (2 mL) containing 0.2 ml of purified enzyme and 1% (w/v) CMC (1.8 ml) dissolved in potassium phosphate buffer (50 mM, pH 6.0) with various additives ((1, 5 and 10 mM each) were incubated separately at 50°C for 30 min and CMCase activity were carried out by determining the amount of reducing sugars released as described in 3.1.5.1.

3.2.3.3. Substrate specificity of purified enzyme

The substrate specificity of the purified enzyme was determined by performing the assay with different substrates. The enzyme was incubated with CMC, β -D-Glucan (barley), lichenan, hydroxyethylcellulose, avicel, starch, pNPG, birch wood xylan, beechwood xylan and oat spelt xylan for 30 min at 50°C. The reaction mixture (2 mL) contained 0.2 mL of enzyme and 1.8 mL of 1.0% (w/v) concentration each of different substrates dissolved in phosphate buffer (50 mM, pH 7.0). The hydrolysis of different substrates was measured with estimation of the liberated reducing sugar following DNS method as described in section 3.1.5.1. For determination

of pNPG hydrolysis the amount of p-nitrophenol liberated from pNPG was measured as per protocol discussed in section 3.1.5.3.

3.2.4. Determination of kinetic parameters for CMCase

The enzyme kinetics (K_m and V_{max}) of purified enzyme were determined by measuring the CMCase activity in the presence of varying CMC substrate (0.1-1 mg/mL) concentration and CMCase activity were carried out by determining the amount of reducing sugars released as described in 3.1.5.1. Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) was determined using the Lineweaver-Burk double reciprocal plot (Lineweaver and Burk, 1934).

3.3. Optimization of ethanol production by simultaneous saccharification and fermentation (SSF)

3.3.1. Sample collection and processing of agricultural waste and leaf litter biomass

Agricultural waste biomass from wheat and rice straw and leaf litter biomass from different trees viz. *Mangifera indica*, *Populus deltoides*, *Polyalthia longifolia* and *Eucalyptus globulus* (Table 3.4) were collected from Thapar University campus and nearby villages of Patiala, Punjab (India), located at 30°19'48"N, 76°24'0"E and 310 m above the sea level. Samples were washed to remove adhered debris (Figure 3.1), dried, grounded and sieved to particle size of 0.5 mm (Figure 3.2). Sieved samples were stored in air tight containers at room temperature for further use.

Table 3.4. Botanical nomenclature of biomass sample sources.

Common name	Family	Genus	Scientific name
Wheat straw	Poaceae	<i>Triticum</i>	<i>Triticum aestivum</i>
Rice straw	Poaceae	<i>Oryza</i>	<i>Oryza sativa</i>
Mango leaf litter	Anacardiaceae	<i>Mangifera</i>	<i>Mangifera indica</i>
Poplar leaf litter	Salicaceae	<i>Populus</i>	<i>Populus deltoides</i>
Ashoka leaf litter	Annonaceae	<i>Polyalthia</i>	<i>Polyalthia longifolia</i>
Eucalyptus leaf litter	Myrtaceae	<i>Eucalyptus</i>	<i>Eucalyptus globulus</i>

3.3.2. Physico-chemical characterization of agricultural waste and leaf litter biomass

3.3.2.1. Moisture content

The moisture content of the biomass was determined using oven drying test method given in ASTM 3173-87. Thoroughly mixed biomass sample (5.0 g) was dried at 105 ± 5°C in a hot air oven till a consistency in weight was observed. Following drying, the sample was moved to a dessicator for cooling, without absorbing moisture.

The percent moisture was calculated as follows:

$$\text{Moisture (\%)} = [(W_2 - W_f) / (W_f - W_1)] \times 100$$

Where W_1 is the weight of empty crucible, W_2 is the weight of the crucible and sample, and W_f is the constant weight of crucible and sample after drying.

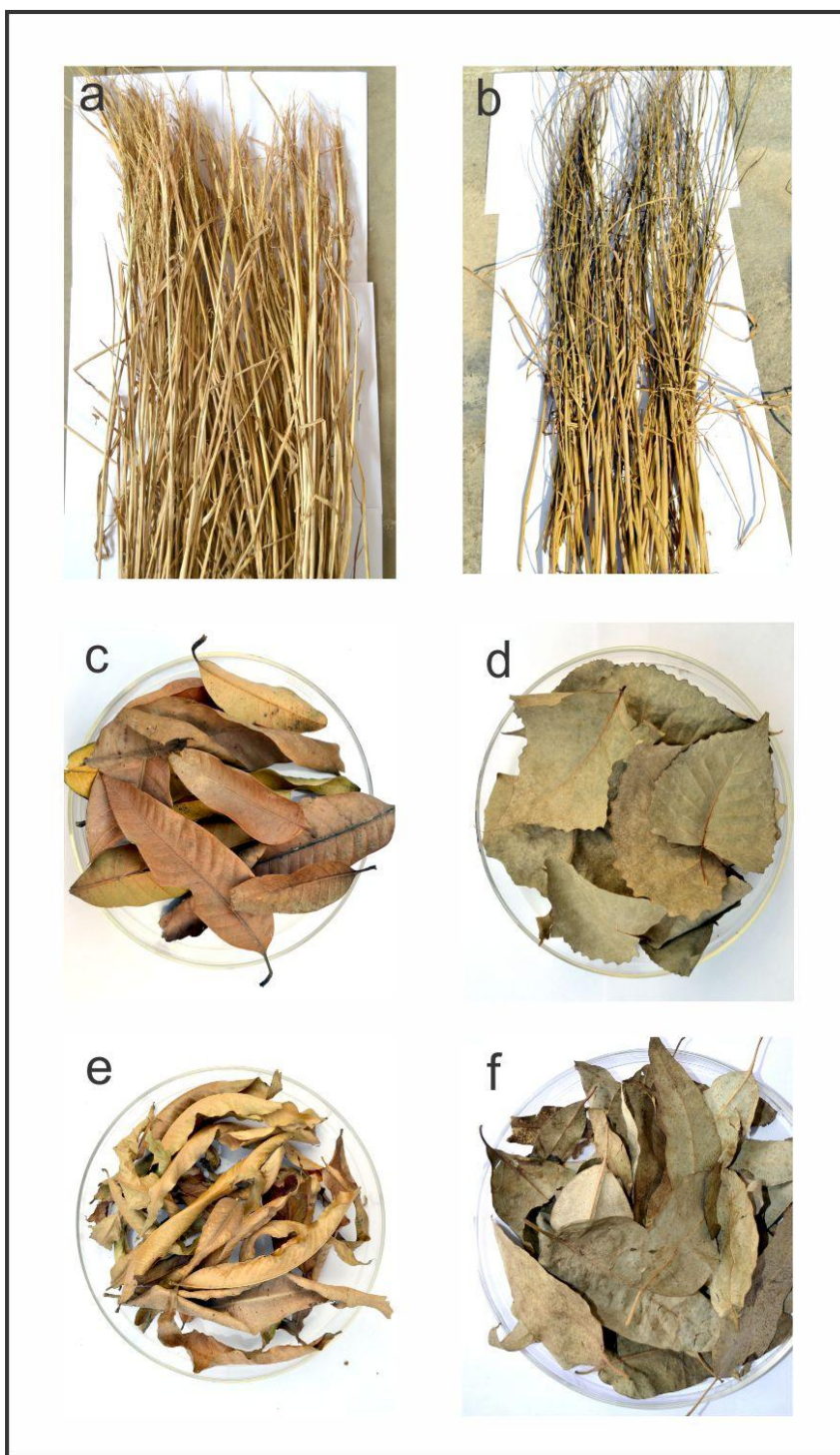


Figure 3.1. Different agricultural waste and leaf litter biomass samples.

a: Wheat straw; b: Rice straw; c: Mango leaf litter;

d: Poplar leaf litter; e: Ashoka leaf litter; f: Eucalyptus leaf litter.

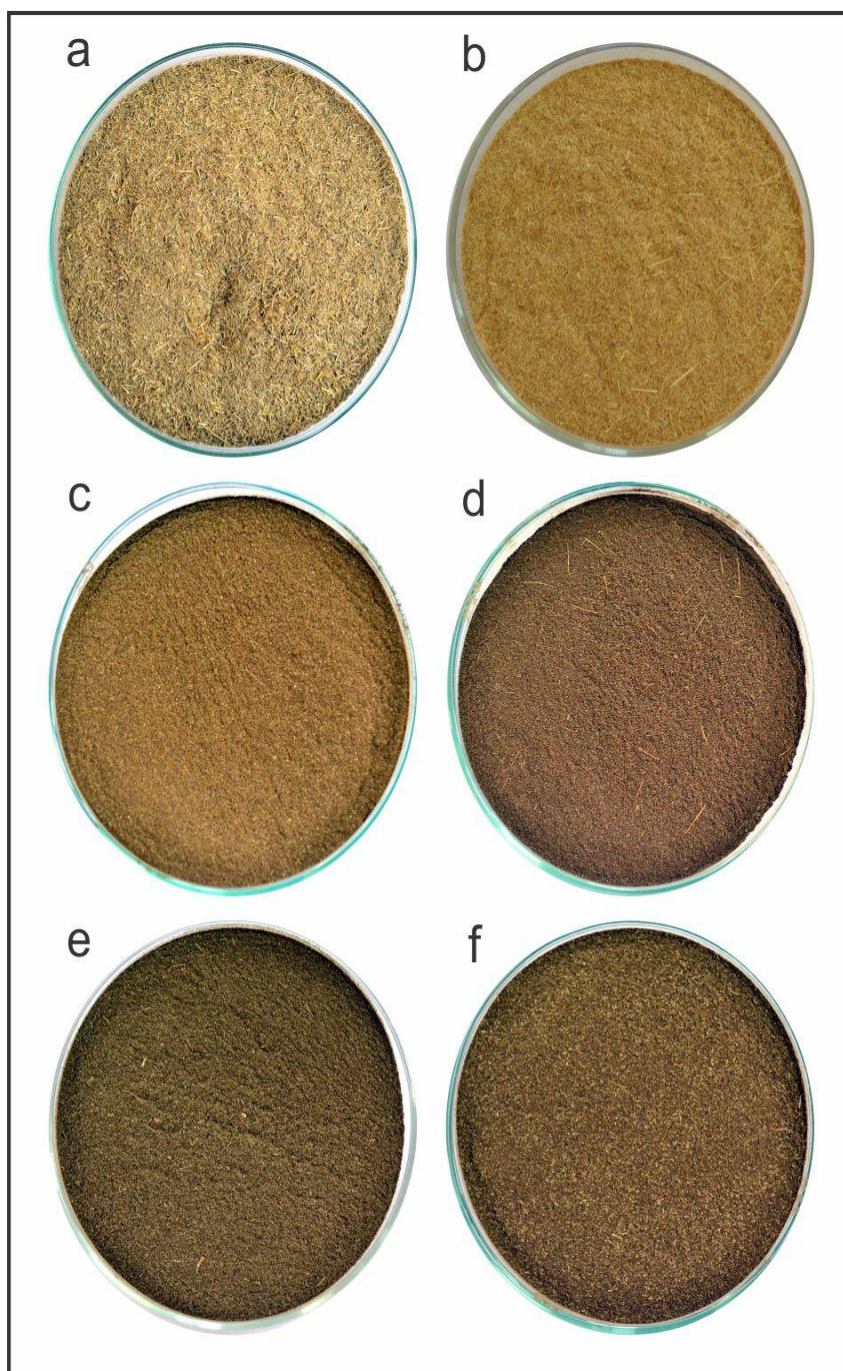


Figure 3.2. Pulverized (0.5 mm) agricultural waste and leaf litter biomass samples.

a: Wheat straw; b: Rice straw; c: Mango leaf litter;
d: Poplar leaf litter; e: Ashoka leaf litter; f: Eucalyptus leaf litter.

3.3.2.2. Ash content

Determination of ash content in the biomass was adopted as per the protocol described in ASTM D 3174-04. For determination of ash content, oven dried biomass sample (1.0 g) was taken in crucible and mass of crucible along with sample was determined. Crucible was placed in muffle furnace maintained at $575 \pm 10^\circ\text{C}$ for 4 h. Crucible was removed from the furnace and placed in a dessicator. The above process of heating and cooling was repeated until constant weight was obtained. This process allows removal of the volatiles and unburnt carbon.

$$\text{Ash (\%)} = [(W_f - W_1) / (W_2 - W_1)] \times 100$$

Where W_1 is the weight of the silica crucible, W_2 is the weight of the crucible and the oven-dried sample, and W_f is the constant weight of the crucible and the sample after combustion.

3.3.2.3. Volatile matter

The volatile matter in the leaf litter biomass was determined by the procedure given in ASTM D 3175-07. Biomass sample (1.0 g) was taken in crucible with cover and initial weight was determined. The covered crucible was placed in muffle furnace regulated at $950 \pm 10^\circ\text{C}$ for 7 min to obtain rapid heating. Then the covered crucible was removed from the furnace and cooled to room temperature in a dessicator and weight was recorded. The percentage weight loss was regarded as the percentage of volatile matter.

$$\text{Volatile matter (\%)} = [(W_f - W_1) / (W_2 - W_1)] \times 100$$

Where W_1 is the weight of the silica crucible, W_2 is the weight of the crucible and the oven-dried sample, and W_f is the constant weight of the crucible and the sample after heating.

3.3.2.4. Fixed carbon

Fixed carbon was the resultant of the summation of percentage moisture, ash, and volatile matter subtracted from 100. All percentages were on the same moisture reference base.

$$\text{Fixed carbon (\%)} = 100 - (\text{moisture, \%} + \text{ash, \%} + \text{volatile matter, \%})$$

3.3.2.5. Calorific value

The calorific values of all the biomass samples were determined in a static bomb calorimeter; BCM 211059, using the procedure described in ASTM E711-87. Each biomass sample was mixed thoroughly in the sample bottle and was made into pellet, taking care that the sample is well distributed. Samples (1.0 g) in the form of pellet was measured out and put into pre-weighed crucibles. A cotton thread was attached to a platinum ignition wire and placed in contact with the pellet. Distilled water (1 mL) was poured into the bomb and then filled with oxygen to a

consistent pressure between 20 and 30 atm (2.03 and 3.04 MPa). The calorimeter was placed in an isothermal jacket with an air gap separation of 10 mm between all surfaces. The electrical energy for ignition, discharged through a platinum wire of ~ 40 V, was calculated using the change in potential across a 1256 or 2900 mF capacitor. The bomb calorimeter was submerged in a calorimeter can filled with distilled water. The calorimeter jacket was maintained at constant temperature by circulating water at 25°C.

3.3.2.6. CHNSO analysis

The basic elements of any biomass are carbon (C), hydrogen (H), nitrogen (N), sulphur (S) and oxygen (O). These elements were analyzed in PerkinElmer Elementar CHNSO analyzer. The sample (1.0 mg) was used in a tin boat assortment at 900°C in an oxygen atmosphere to determine the percentage composition of C, H, N and S analysis. The percentage O was calculated by means of difference.

$$\text{Oxygen (\%)} = 100 - (\text{C, \%} + \text{H, \%} + \text{N, \%} + \text{S, \%})$$

3.3.2.7. Determination of extractives

Biomass sample (5 g) was extracted using Soxhlet extraction apparatus through cotton cellulose extraction thimbles (external length 94 mm and inner diameter 33 mm). About 190 mL of n-hexane was added to the tared receiving flask and then receiving flask was placed on the Soxhlet apparatus. Heating mantle was adjusted to provide a minimum of 6-10 siphon cycles per hour. Samples were refluxed for 6 h. After refluxing and cooling, thimble was removed and extracted solid was transferred onto cellulose filter paper in a Buchner funnel. Solid portion was washed with approximately 100 mL of fresh n-hexane, dried and weighed. n-hexane extracted biomass sample was again extracted with ethanol and then with water (HPLC grade). Same procedure was followed for each biomass samples.

3.3.2.8. Estimation of cellulose

Estimation of cellulose in biomass samples were done by Anthrone Assay (Updegraff, 1969). For this, 10 mL acetic acid/nitric acid reagent (150 mL of 80% acetic acid mixed with 15 mL of concentrated nitric acid) was added to 0.1 g of biomass and the mixture was kept in a water bath (100°C) for 30 min. After cooling the sample was centrifuged at 8,000 rpm for 10 min and supernatant was discarded. The residue was washed twice with distilled water and 10 mL sulphuric acid (67%, v/v) was added and resulting suspension was allowed to stand for 1 h. The suspension was diluted to 5 times (50 mL volume), and 4 mL of Anthrone reagent was added to

1 mL of diluted mixture. The mixture was incubated in a boiling water bath for 15 min for colour development. After cooling the tubes, the optical density was read at 620 nm against the reagent blank (1 mL distilled water and 4 mL Anthrone reagent). To calculate the amount of cellulose present in the sample, standard graph was plotted using 0, 40, 80, 120, 160 and 200 µg/L cellulose.

3.3.2.9. Estimation of hemicellulose

Estimation of hemicellulose in the biomass samples were done using the method given by Georging and van Soest (1991).

Determination of neutral detergent fiber (NDF):

Biomass (1.0 g) was mixed with 100 mL of cold neutral detergent solution (solution A) (18.61g of disodium ethylenediamine tetraacetate and 6.81g of sodium borate decahydrate) were added to 200 mL of distilled water and dissolved by intermittent heating. To solution A, 30 g of sodium lauryl sulphate and 10 mL of 2-ethoxy ethanol was added. To this 4.5 g of disodium hydrogen phosphate was added and volume was made up to 1 L and pH was adjusted to 7.0. To this biomass detergent solution, 2 mL of decalin (decahydronaphthalene) and 0.5 g of sodium sulphite were added. Above mixture was heated to boiling and refluxed for 1 h. After cooling, sample was filtered and residue was collected. Residue was washed with hot water followed by acetone, collected in pre-weighed crucible and then placed in an oven at $100 \pm 5^\circ\text{C}$ for 8 h. After drying, crucibles were weighed and percentage weight difference was expressed as NDF.

$$\text{NDF (\%)} = [(W_f - W_1) / (W_2)] \times 100$$

Where W_1 is the weight of the silica crucible, W_2 is the weight biomass sample and W_f is the weight of crucible and NDF.

Determination of acid detergent fiber (ADF):

Biomass sample (1 g) was transferred to refluxing flask and 100 mL of cold acid detergent solution (2 g of CTAB in 100 mL of 1N sulphuric acid) was added. Sample was refluxed as detailed for neutral detergent fiber (NDF) and percentage weight difference was expressed as ADF. Hemicellulose content in the biomass samples were estimated as difference between NDF (%) and ADF (%).

$$\text{ADF (\%)} = [(W_f - W_1) / (W_2)] \times 100$$

Where W_1 is the weight of the silica crucible, W_2 is the weight biomass sample and W_f is the weight of crucible and ADF.

$$\text{Hemicellulose (\%)} = \text{NDF (\%)} - \text{ADF (\%)}$$

3.3.2.10. Estimation of lignin

Acid insoluble lignin (AIL) was determined using TAPPI T 222 om-02 and acid soluble lignin (ASL) was estimated using TAPPI Useful method UM 250 (1985). For lignin estimation, 5 g of extractive free biomass sample was prepared by n-hexane, ethanol and water extraction as discussed earlier. Sample was dried thoroughly in oven at 60°C till constant weight was achieved and then 1 g of dried sample was weighed. 15 mL of 72% (v/v) sulphuric acid was added to 1 g of dried biomass sample in a beaker and were thoroughly mixed using glass rod. Beakers were kept in water bath at 30°C for 1 h with constant stirring after every 5-10 min using stirring rod. Upon completion of the 60 min of hydrolysis, beakers were removed from the water bath. Sample was diluted to 4% concentration by adding 420 mL of distilled water. The preparation was then autoclaved for 1 h at 121°C. After cooling, the samples were filtered. The residue and filtrate were both used for AIL and ASL, respectively. The residue after filtration was weighed, to determine AIL. The optical density of the filtrate was measured at 320 nm for determination of ASL. The samples were diluted to bring the absorbance to 0.7–1.0 and dilution factors were recorded. Sulphuric acid (4%, v/v) was used to dilute the samples and as blank.

Acid insoluble lignin (AIL) concentration was calculated as follows:

$$\text{AIL (\%)} = (A/W) \times 100$$

Where, A is weight of lignin in g, W is oven dried weight of sample

$$\text{ASL (\%)} = [(\text{UVabs} \times \text{vol. of filtrate} \times \text{dilution factor}) / \epsilon \times B \times a] \times 100$$

Where UVabs is the absorbance of sample at 320 nm, volume of filtrate is 440 mL, dilution is the volume of sample of diluting solvent/volume of sample, ϵ is the absorptivity of biomass at specific wavelength (113 L/g-cm) and B is the oven-dried weight of sample and a corresponds to path length.

$$\text{Total lignin (\%)} = \text{AIL (\%)} + \text{ASL (\%)}$$

3.3.2.11. Estimation of reducing sugar in biomass samples

Reducing sugars in the biomass samples were estimated using DNS method as per protocol given by Miller (1959).

Biomass sample (0.1 g) was taken in a test tube containing 500 μ L potassium phosphate buffer (50 mM, pH 7.0) and volume was made to 1 mL using distilled water. To this mixture, 3 mL of DNS reagent (Appendix II) was added and placed in boiling water bath for 10 min. Solution was

cooled down to room temperature, centrifuged (11,000 rpm for 10 min at room temperature) and absorbance was recorded at 540 nm. To calculate the amount of reducing sugar present in the sample, standard graph was plotted using 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL glucose.

3.3.2.12. Scanning electron microscopy (SEM) of biomass samples

Physical appearance of biomass samples were observed by SEM. The samples were coated to provide conductivity with gold using gold sputter at a voltage of 10-15 kV. Images were taken using SEM (Model: JEOL JSM-6510 LV, USA) at different magnifications.

3.3.2.13. X-ray diffraction (XRD) analysis

Crystallinity of biomass samples were analyzed at room temperature in the scanning angle of 5-50° at the scan speed of 5° min⁻¹ using a PANalytical X'Pert PRO diffractometer (Netherlands) with Ni-filter, operated at 45 kV and 40 mA with λ (Cu K α) = 1.5406 Å. The crystallinity index (*CrI*) of the biomass samples were determined as described by Segal et al. (1959) as follows:

$$CrI = [(I_{002} - I_{am}) / I_{002}] \times 100$$

Where I_{002} is the intensity for the crystalline portion of biomass (i.e. cellulose) at about 2θ of 21-23°, and I_{am} is the peak for the amorphous portion (i.e. cellulose, hemicellulose, and lignin) at about 2θ of 16-18° in most literatures (Harris et al., 2008; Naik et al., 2010; Kuila et al., 2011; Xiao et al., 2011).

3.3.2.14. Thermal gravimetric analysis (TGA)

To understand the devolatilization characteristics, TGA of biomass samples were performed using Mettler Toledo instrument (Model: TGA/SDTA 851e). The devolatilization of biomasses were studied the from 20 to 700°C with a heating rate of 10°C min⁻¹ with purge gas (Nitrogen) flow rate of 60 mL min⁻¹.

3.3.2.15. Fourier transform infrared (FTIR) spectroscopy

Accurate predictions of the chemical composition of biomass samples were obtained using Nicolet Instrument (Model: MAGNA 550, USA). The biomass sample (10 mg) was well mixed with 200 mg of KBr and the mixture was compressed for preparation of pellets. Each spectrum was the average of 64 scans with a total scan time of 15 s in the IR range of 400 - 4000 cm⁻¹.

3.3.2.16. Solid state ¹³C CP/MAS NMR spectroscopy

Solid state ¹³C CP/MAS NMR spectroscopy is an important tool to analyze the structural features of lignocellulosic biomass. Solid state ¹³C CP/MAS NMR spectra of biomass samples were obtained at 75.475 MHz using a Bruker AV-300 FT NMR spectrometer (Germany). Dried

samples were spun throughout at a rate of 10 kHz using 4 mm zirconia (ZnO₂) rotor with CP pulse programme. Typically 18,000 transients were accumulated in the time domain with contact time of 1 ms and a recycle delay of 3 s. Tetramethylsilane (TMS) was used as an external standard for the calibration of the ¹³C chemical shift.

3.3.3. Pre-treatment of agricultural waste and leaf litter biomass

After physico-chemical characterization of agricultural waste and leaf litter biomass in search for promising substrate for biofuel production, these biomasses were subjected to different combined pre-treatment technique to enhance enzymatic hydrolysis which can be further converted to ethanol production using SSF.

3.3.3.1. Fungal pre-treatment followed by alkali fractionation

Inoculum preparation

Fungal pre-treatment of biomass samples were done using *P. chrysosporium* MTCC 787, procured from MTCC, IMTECH, Chandigarh, India. Sporulated slants grown on PDA (Appendix I) slant were obtained after 4 days of incubation at 28°C. 10 mL of sterile distilled water supplemented with 0.1% (v/v) Tween-80 was aseptically added to each sporulated slants and scraped gently to release the spores. Spore counts were determined using hemocytometer and an inoculum with a final spore concentration of 2.2×10^6 spores/mL was prepared.

Biological pre-treatment

Biological pre-treatment was carried out in a 250 mL flask with 5 g of native biomass sample in 100 mL of distilled water. The samples were sterilized in an autoclave for 20 min (12°C, 15 lbs), cooled and then 5 mL of fungal inoculum was added. The samples were incubated at 28°C for 14 days with agitation at 120 rpm. At the end of incubation, biomass was thoroughly washed with distilled water to remove mycelia and dried at 60°C in an oven for 12 h.

Alkaline fractionation

Native and biologically pre-treated biomass samples were further subjected to alkaline fractionation in 250 mL flask by immersing 10 g of sample in 100 mL of 70% (v/v) ethanol aqueous solution containing 1% (w/v) NaOH. Sample mixture was incubated at 75°C for 3 h (Yang et al., 2013). The insoluble fractions were collected by filtration, thoroughly rinsed using distilled water till neutral pH obtained. Sample was dried overnight in an oven (75°C) and then kept in air tight containers for SSF.

3.3.3.2. Fungal pre-treatment followed by solvolysis

Biologically pre-treated samples (as described in section 3.3.2.1) were subjected to solvolysis. Mixed solvent system was prepared for solvolysis using ethanol: acetic acid: water in ratio of 40:10:50. Native and untreated biomasses were added to this mixed solvent system at liquid to solid ratio of 6:1 (v/w) to ensure biomass is fully submerged in liquid (Baba et al., 2011). Pre-treatment reaction was carried out at 200°C for 60 min in an autoclave. Samples were allowed to cool and then subjected to filtration and solid fraction obtained was washed with 80% (v/v) acetone three times and then two times with 100 mL of distilled water. After washing, samples were dried overnight at 60°C in an oven and stored in air tight containers for SSF.

3.3.3.3. Dilute sulphuric acid-sodium hydroxide (H₂SO₄-NaOH) pre-treatment

Biomass sample (10 g) was treated with 100 mL of 2% (v/v) H₂SO₄ and heated at 121°C for 45 min. After cooling, solid fractions were separated by filtration, washed with distilled water until neutral pH was achieved and then 1 g of biomass was treated with 10 mL of 2% (w/v) NaOH at 80°C for 12 h. The solid fractions obtained were again separated by filtration and washed with distilled water until neutral pH. The solid residues obtained were dried overnight in an oven at 60°C and then stored in air tight containers for SSF.

3.3.3.4. Microwave-alkali-acid pre-treatment

Biomass sample (0.5 g) was placed in proprietary express vessel and 5 mL of 1% (w/v) NaOH was added and subjected to microwave pre-treatment using Microwave Accelerated Reaction System (MARS6[®], CEM, Buckingham, United Kingdom) with an operating frequency of 2450 MHz and the power range of 100-1800 W. Microwave pre-treatment was carried out for 3 min at 850 W and 150°C (Binod et al., 2012). After treatment, biomass samples were neutralized using distilled water with two consecutive wash and then dried at room temperature. After drying, microwave-alkali pre-treated biomass samples were subjected to 1% (v/v) H₂SO₄ at 10% solid loading ratio for 1 h. Following acid treatment samples were washed thoroughly, air dried and used as substrate for ethanol production using SSF.

After completion of pre-treatment of biomass using four different strategies as described above, samples were analysed for cellulose, hemicellulose, lignin and reducing sugar content to determine pre-treatment efficiency. The best pre-treated biomass feedstock obtained was further characterized for SEM, XRD, TGA, FTIR and solid state ¹³C CP/MAS NMR spectroscopy to know about the changes in physico-chemical properties which took place in treated biomass.

3.3.4. Optimization of ethanol production via SSF

After completion of pre-treatment of biomass samples using four different strategies as described in section 3.3.3, the best pre-treated biomass sample was used as substrate for ethanol production using SSF.

3.3.4.1. Inoculum preparation

The ethanologenic microorganism, *Saccharomyces cerevisiae* NCIM 3215, was maintained at 4°C on 5 mL of Malt extract glucose yeast extract peptone (MGYP) slants (Appendix I). Starter culture was prepared by inoculation of one loopful of *S. cerevisiae* NCIM 3215 from agar slant to 50 mL of glucose yeast extract (GYE) medium (Appendix I) in two separate 250 mL flask. Flasks were incubated at 30°C and 120 rpm for 48 h prior to inoculation into fermentation media. The cell count of actively growing *S. cerevisiae* NCIM 3215 was measured using haemocytometer, corresponding to 3.2×10^8 cells/mL.

3.3.4.2. Hydrolytic enzyme preparation

Partially purified cellulase from *B. subtilis* NA15 after ammonium sulphate precipitation and dialysis (described in section 3.2.2.1) was lyophilized (SCANVAC, LaboGene, Denmark) and then redissolved in sterile potassium phosphate buffer (50 mM, pH 7.0). Bacterial cellulase was highly stable up to 12 h at pH 5.0 (Figure 4.2.7) and 30°C (Figure 4.2.9) by retaining its activity to 82 and 87%, respectively, suggesting that the enzyme will work efficiently at 30°C and pH of 5.0 required for ethanol fermentation. The activity of the enzyme used in SSF had CMCase, FPase and β -glucosidase activity of 1.46, 0.43 and 0.12 U/mL, respectively (described in section 3.1.5).

3.3.4.3. Screening of substrate for ethanol production using SSF

After pre-treatment of the biomass samples such as wheat straw, rice straw, Mango, Poplar, Ashoka and Eucalyptus leaf litter. Each substrate (10%, w/v) were taken independently in a 250 mL flask in a total volume of 100 mL supplemented with nutrient solution composed of 100 g/L each of yeast extract, peptone and MgSO_4 so as to have final concentration of 2 g/L each of yeast extract, peptone and MgSO_4 in the fermentation media. pH of the SSF mix was adjusted to 5.0 using 50 mM citrate buffer (Appendix II). After autoclaving (121°C, 15 lbs) for 15 min, it was allowed to cool for 30 min. Then 1% (v/v) partially purified cellulase (1.46 U/mL CMCase, 0.43 U/mL FPase and 0.12 U/mL β -glucosidase) from *B. subtilis* NA15 and 10% (v/v) yeast inoculum

was added aseptically to it. The SSF experiments were carried out at 30°C for 48 h under shaking condition (120 rpm). Although, optimum temperature requirements for hydrolytic enzymes and fermenting yeasts are different, a compromise was made in selection of temperature (Wilkins et al., 2007). Samples were withdrawn and estimated for ethanol concentration using Gas chromatography (GC).

3.3.4.4. Ethanol estimation using Gas chromatography (GC)

Gas chromatography was used to measure the ethanol produced during SSF. The Gas chromatograph (NUCON SERIES 5700) utilizing Porapak-Q column as the stationary phase. Sample (1 µL) was injected at inlet temperature of 175°C, oven temperature of 153°C and flame ionization detector (FID) temperature of 250°C using nitrogen gas at flow rate of 30 mL/min as a carrier. Hydrogen and air flow rate were maintained at 30 and 300 mL/min, respectively. Ethanol (100%, v/v) was used as standard (Appendix II) and the eluted peak was noted using NUCHROM software.

The ethanol yield was calculated as follows:

$$Y (\%) = [(E \times 0.9) / (S \times 0.51)] \times 100$$

Where E is the ethanol concentration in g/L and S is carbohydrate (cellulose and hemicellulose) concentration in substrate in g/L.

3.3.4.5. Optimization of process parameters of SSF using Taguchi design

Taguchi experimental design matrix (Taguchi, 1986), a standard orthogonal array L₂₅ (6₅), was used to examine six factors, namely, as substrate loading (w/v), pH, temperature (°C), enzyme loading (v/v), time (h) and inoculum size (v/v), in five coded levels (Table 3.5). The L and the subscript (25) represent the latin square and the number of experimental runs, respectively. The layout of the L₂₅ Taguchi's orthogonal array is represented in Table 3.6. The lower and upper levels of optimized factors were selected on the basis of the suitable conditions for the desired growth of the fermentative microbes for efficient bioethanol production. Each of the twenty-five simultaneous saccharification and fermentation (SSF) experiments denoted by “runs” were carried out as per the defined values of six different parameters.

Table 3.5. Factors and levels in Taguchi experimental design for SSF process.

Factors	Coded levels				
	L ₁	L ₂	L ₃	L ₄	L ₅
Substrate loading (w/v %)	3	5	7	9	11
pH	3.5	4	4.5	5	5.5
Temperature (°C)	25	30	35	40	45
Enzyme loading (v/v %)	0.5	1	1.5	2	2.5
Time (h)	24	36	48	60	72
Inoculum size (v/v %)	2	4	6	8	10

3.3.4.6. Experimental setup of SSF at bench scale

Microwave-alkali-acid pre-treated rice straw in varying proportion (w/v) was autoclave-sterilized at 121°C for 15 min in 250 mL flask encompassing 100 mL working volume and initial pH which was adjusted to a given value with 50 mM citrate buffer (Appendix II). SSF reaction mix (biomass immersed in buffer) was prepared by adding pre-treated biomass (w/v), enzyme (v/v) and inoculum (v/v), aseptically to ensure final volume of 100 mL supplemented with sterile nutrient solution composed of 100 g/L each of yeast extract, peptone and MgSO₄ so as to have final concentration of 2 g/L each of yeast extract, peptone and MgSO₄ in the fermentation media. The SSF experiments were executed in 25 independent runs as per Taguchi design (Table 3.5) under shaking condition (120 rpm). Fermented samples were centrifuged (11,000 rpm for 10 min) and then filtered using a 0.45 µm membrane filter for ethanol estimation using Gas chromatography.

3.3.4.7. Analysis of the Taguchi Orthogonal Array Experiments (Runs)

The results were analysed by experimental design model and three dimensional (3D) contour plot using Design Expert software (Version 8.0.7.1, Stat-Ease, Minneapolis, MN), a statistical software package. The controlling factors were identified, with the magnitude of the effects qualified and the statistically significant effects were determined. Accordingly, the optimal conditions were determined by combining the levels of factors that had the highest main effect value. The analysis of variance for the response of ethanol production was carried out according to the factors contribution by Taguchi method. The factors in the experimental design, considered to be statistically significant at 95% confidence limit were used to determine the ratio (*F*) and the *p*-value.

Table 3.6. Taguchi design layout for optimization of SSF.

Runs		Factors					
Std	Run	A: Substrate loading (w/v)	B: pH	C: Temperature (°C)	D: Enzyme loading (v/v)	E: Time (h)	F: Inoculum Size (v/v)
2	1	3	4	30	1	36	4
23	2	11	4.5	30	0.5	72	8
6	3	5	3.5	30	1.5	60	10
22	4	11	4	25	2.5	60	6
1	5	3	3.5	25	0.5	24	2
24	6	11	5	35	1	24	10
8	7	5	4.5	40	2.5	24	4
7	8	5	4	35	2	72	2
17	9	9	4	45	1.5	24	8
20	10	9	5.5	35	0.5	60	4
16	11	9	3.5	40	1	72	6
19	12	9	5	30	2.5	48	2
4	13	3	5	40	2	60	8
10	14	5	5.5	25	1	48	8
9	15	5	5	45	0.5	36	6
25	16	11	5.5	40	1.5	36	2
15	17	7	5.5	30	2	24	6
5	18	3	5.5	45	2.5	72	10
11	19	7	3.5	35	2.5	36	8
3	20	3	4.5	35	1.5	48	6
18	21	9	4.5	25	2	36	10
13	22	7	4.5	45	1	60	2
21	23	11	3.5	45	2	48	4
12	24	7	4	40	0.5	48	10
14	25	7	5	25	1.5	72	4

3.3.4.8. Validation of the experimental model

In order to determine the accuracy of the model, response was numerically optimized using Design Expert software (Version 8.0.7.1, Stat-Ease, Minneapolis, MN). The model was validated by performing the SSF trial runs with optimized fermentation process parameters in 100 mL of fermentation medium as described in section 3.3.4.6. The fermentation was carried out at 120 rpm for 24 h and ethanol concentration was observed as per protocol discussed in section 3.3.4.4.

3.3.4.9. Ethanol production in 3-L bioreactor using SSF

The SSF experiments were conducted using microwave-alkali-acid pre-treated rice straw as substrate in a 3-L thermostatically controlled glass reactor (Model: BioSpin-03A, Bio-Age

Equipments Pvt. Ltd, Mohali, India). Glass reactor had provisions for temperature, agitation, and pH control in addition to facilities like condensate removal and exhaust valve. SSF experiments were conducted with optimized conditions [substrate loading (11%, w/v), pH (4.5), temperature (30°C), enzyme loading (0.5%, v/v), inoculum size (8%, v/v)] as revealed by Taguchi design for 72 h. The glass reactor containing microwave-alkali-acid pre-treated rice straw (11%, w/v) supplemented with nutrient solution composed of 100 g/L each of yeast extract, peptone and MgSO₄ so as to have final concentration of 2 g/L each of yeast extract, peptone and MgSO₄ in a total working volume of 2 L. It was autoclave sterilized (121°C, 15 lbs) for 15 min prior to addition of partially purified cellulase and fermentative yeast. The cellulase enzyme (1.46 U/mL CMCase, 0.43 U/mL FPase and 0.12 U/mL β -glucosidase activity) used for hydrolysis remained same as mentioned in section 3.3.4.2. The agitation speed was maintained at 120 rpm, while pH of the medium was maintained at 5.0 using sterilized 5 N NaOH solution. Temperature of 30°C can accommodate both the enzymes and *S. cerevisiae* NCIM 3215 in the same vessel at the same time. Samples were drawn at 12-h intervals until 72 h and analyzed for concentrations of reducing sugars and ethanol as described in section 3.3.2.11 and 3.3.4.4, respectively. Parallel SSF experiments were conducted to compare the reducing sugar and ethanol production using native rice straw (0.5 mm) as feedstock, keeping all the parameters same.

3.3.5. Statistical analysis

All the experiments were performed in triplicate and the data were analyzed by analysis of variance (ANOVA) using GraphPad prism (5.0) software followed by the Tukey's honestly significant difference test. Results were expressed as mean $\pm$ S.D (n=3).

CHAPTER IV. RESULTS AND DISCUSSION

4.1. Screening and characterization of cellulose degrading microorganism

Cellulose degrading bacteria were isolated and screened from different habitat such as compost, paper pulp and sugarcane bagasse. Out of 32 bacterial isolate, eight cellulolytic bacteria were selected for morphological, physiological and biochemical characterization. Biolog's GEN III MicroPlate identification and molecular characterization of these eight isolates were done using 16S rDNA gene sequence analysis and phylogenetic positions were determined.

4.1.1. Isolation of cellulose degrading bacteria

The bacterial isolates from varying sources (Table 4.1.1) such as compost, paper pulp and sugarcane bagasse were tested for their ability to grown in nutrient broth supplemented with 0.5% (w/v) CMC. Physico-chemical characterization of these samples showed pH in the range of 6.32 to 7.62 and Electrical conductivity (EC) in the range of 1.24 to 2.56 mS m⁻¹ (Table 4.1.1). EC was highest (2.56 mS m⁻¹) in compost followed by sugarcane bagasse and paper pulp which was 2.37 and 1.24 mS m⁻¹, respectively. Total nitrogen was also highest (1.06%) in compost. C:N ratio was highest in paper pulp (73) followed by sugarcane bagasse (60) and compost (28).

Table 4.1.1. Physico-chemical characteristics of different samples.

Site	Sample source	pH	EC (mS m ⁻¹)	Organic C (%)	Total N (%)	C/N
1	Compost	7.15 ± 0.21	2.56 ± 0.11	29.72 ± 2.42	1.06 ± 0.03	28
2	Paper pulp	7.62 ± 0.32	1.24 ± 0.09	41.23 ± 3.15	0.56 ± 0.02	73
3	Sugarcane bagasse	6.32 ± 0.29	2.37 ± 0.12	37.58 ± 1.82	0.63 ± 0.04	60

Values are mean ± SD (n=3)

Native bacterial population (Table 4.1.2) was highest in compost followed by sugarcane bagasse and paper pulp samples. Most of the bacterial colonies appeared at 10⁻³ dilution, however no growth was observed at 10⁻⁷ and 10⁻⁸ dilution. The results indicated that an active population of these bacteria persisted in all the samples collected.

Table 4.1.2. Population of bacteria in different sources per gram of sample.

Site	Sample source	Location	Bacteria ($\times 10^3$)
1	Compost	Thapar University, Patiala, Punjab, India.	3.6
2	Paper pulp	Vishal Paper Industries Pvt. Ltd. Patiala, Punjab, India.	0.07
3	Sugarcane bagasse	K M Sugar Mills, Kanpur, Uttar Pradesh, India.	2.1

Though bacterial population and diversity varies with different physico-chemical characteristics and sources, all of them are subjected to grow at neutral pH (7.0). Ubiquitous nature of bacteria makes them promising candidates for isolating them from diverse habitat.

4.1.2. Screening of cellulose degrading bacteria

Bacteria grown in nutrient broth enriched with (0.5%, w/v) CMC were further screened for production of cellulase on nutrient agar plate supplementary with CMC (0.5% w/v). After 24 h of incubation at 37°C, out of 32, eight cellulose degrading bacteria (designated as NA8, NA9, NA11, NA14, NA15, DGA, DGB and DGC) were screened on the basis of Congo red staining and selected for further studies on the basis of clear zone of hydrolysis around colonies (**Figure 4.1.1**). The maximum zone of hydrolysis (14 mm) was shown by isolate NA15 followed by isolate NA14, NA11, NA9, NA8, DGB, DGC and DGA which was observed to be 11, 10, 9, 8, 8, 5 and 4 mm, respectively. Negative control without any inoculum (Figure 4.1.1.a) and *Paenibacillus polymyxa* MTCC 3088, an efficient cellulase (β -glucosidase) producer (Chakraborti et al., 2000) was used as positive control (Figure 4.1.1.b) for reference.

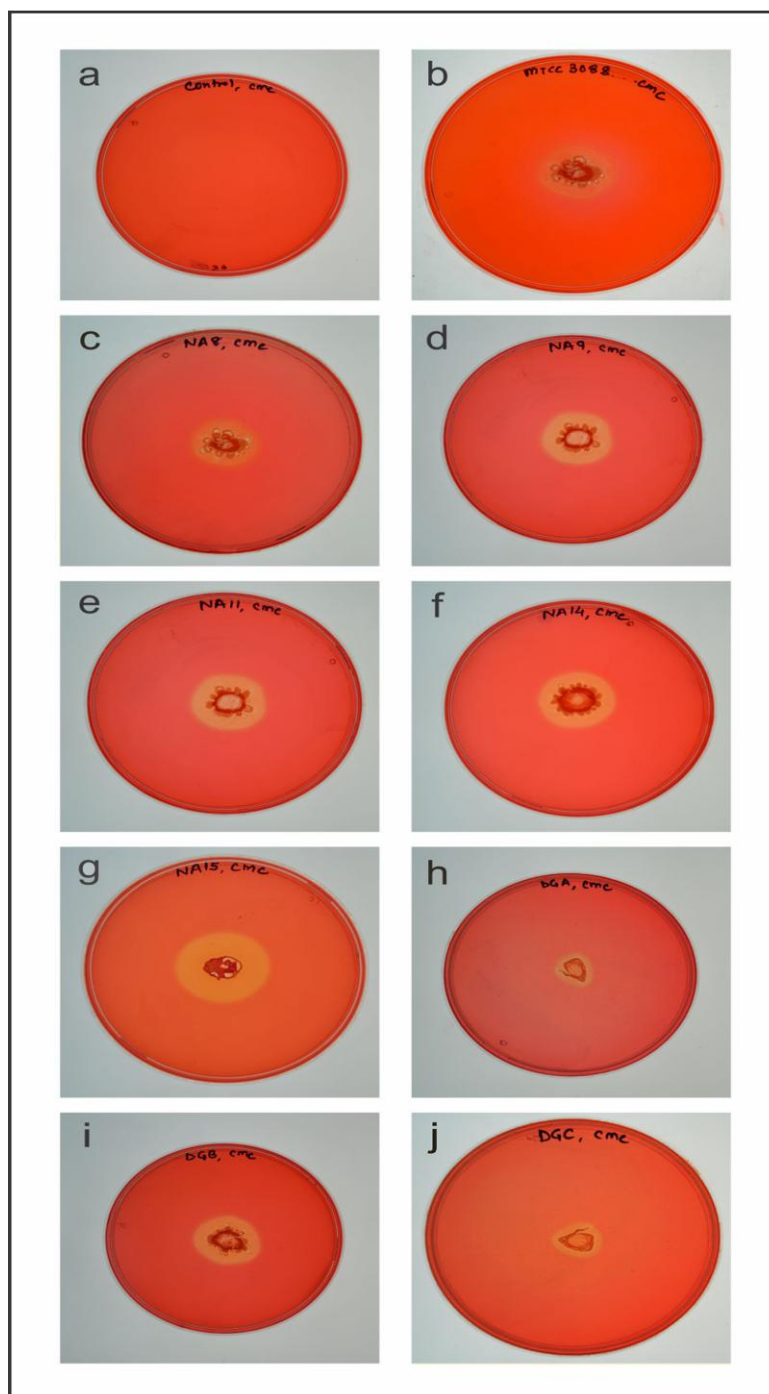


Figure 4.1.1. Screening of cellulose degrading bacteria on the basis of hydrolysis zone using Congo red staining technique.

a: Control; b: *P. polymyxa* MTCC3088; c: NA8, d: NA9;
e: NA11; f: NA14; g: NA15; h: DGA; i: DGB; j: DGC.

Table 4.1.3. Comparison of cellulase activity in cell free supernatant of different bacterial isolates.

Bacterial isolate	Sample source	CMCase (U/mL)	FPase (U/mL)	β -glucosidase (U/mL)
<i>P. polymyxa</i>	IMTECH	0.03 $\pm$ 0.002d	0.01 $\pm$ 0.002d	0.06 $\pm$ 0.001a
NA8	Paper pulp	0.03 $\pm$ 0.001d	0.01 $\pm$ 0.001d	0.008 $\pm$ 0.001g
NA9	Paper pulp	0.04 $\pm$ 0.003c	0.02 $\pm$ 0.003c	0.01 $\pm$ 0.003de
NA11	Compost	0.05 $\pm$ 0.002b	0.03 $\pm$ 0.002b	0.02 $\pm$ 0.002cd
NA14	Compost	0.05 $\pm$ 0.001b	0.03 $\pm$ 0.001b	0.02 $\pm$ 0.001bc
NA15	Compost	0.06 $\pm$ 0.002a	0.04 $\pm$ 0.002a	0.01 $\pm$ 0.002b
DGA	Sugarcane bagasse	0.02 $\pm$ 0.001e	0.008 $\pm$ 0.001d	0.005 $\pm$ 0.001g
DGB	Sugarcane bagasse	0.04 $\pm$ 0.001c	0.02 $\pm$ 0.001e	0.01 $\pm$ 0.002ef
DGC	Sugarcane bagasse	0.03 $\pm$ 0.001d	0.009 $\pm$ 0.001d	0.01 $\pm$ 0.001fg

Values are mean $\pm$ SD (n =3), bearing different letters in the same column are significant at P<0.05.

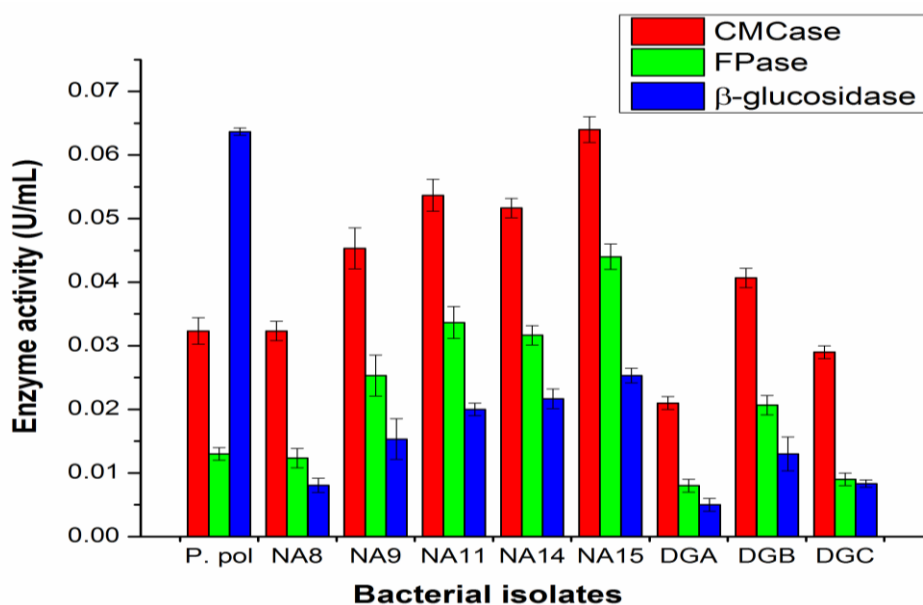


Figure 4.1.2. CMCase, FPase and β -glucosidase activity in cell free supernatant of different bacterial isolates. The *vertical bars* designate the standard deviation for the mean of three replicative test.

After primary screening, all the eight bacterial isolates were checked for quantitative estimation of cellulase. Maximum CMCase and FPase activity in cell free supernatant of overnight grown culture (0.6 OD at 600 nm), incubated at 37°C (120 rpm) of isolate NA15 was 0.06, 0.04 U/mL, respectively followed by NA11 and NA14 which was 0.05 and 0.03 U/mL for CMCase and FPase (Table 4.1.3, Figure 4.1.2). Eight efficient bacterial isolates were characterized on the basis of biochemical properties and molecular phylogeny based on 16S rRNA gene sequence analysis. Based on these characteristics, the most efficient cellulose degrading isolate (NA15) was selected for optimization and biochemical characterization of cellulase and further to application in ethanol production via SSF.

4.1.3. Morphological, biochemical and physiological characterization of bacteria

4.1.3.1. Growth kinetics of bacterial isolates

The growth kinetics of all eight bacterial isolates was found similar. All bacterial isolates spend 10-14 h in lag phase, attained log phase at about 20-24 h of incubation and then reached stationary phase after completion of 20-24 h (Figure 4.1.3.) in nutrient broth supplemented with 0.5% (w/v) CMC at an incubation temperature of 37°C under shaking condition (120 rpm).

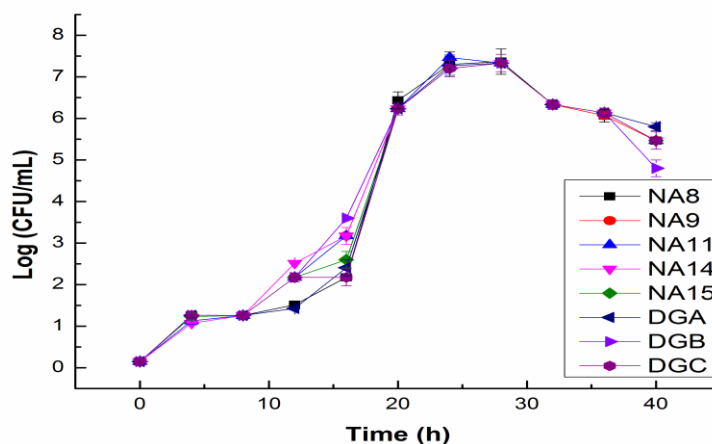


Figure 4.1.3. Growth profiles of bacterial isolates over a period of 40 h incubated at 37°C under shaking condition (120 rpm). The *vertical bars* designate the standard deviation for the mean of three replicative tests.

To investigate the maximum cellulase production, most efficient cellulase producer isolate NA15 were grown in nutrient broth supplemented with 0.5% (w/v) at an incubation temperature of 37 °C over a time period of 40 h under shaking condition (120 rpm). CMCase activity and protein concentration were monitored in cell free supernatant of isolate NA15 in terms of growth (OD at 600 nm). Maximum CMCase activity (0.06 U/mL) with 0.11 mg/mL protein (Figure 4.1.4) concentration was obtained after 24 h of incubation. Similar results were reported by cellulase producing *B. pumilus* EB3 (Ariffin et al., 2006), *Bacillus* sp. AS3 (Deka et al., 2011) and *B. amyloliquefacines* SS35 (Singh et al., 2013) confirming the fact that cellulase is a growth-associated product.

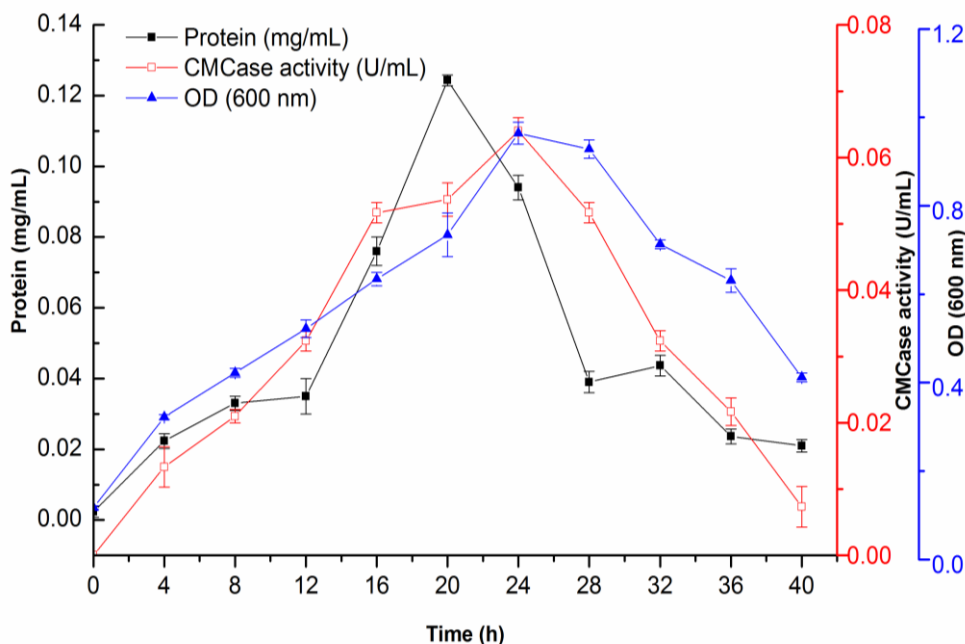


Figure 4.1.4. CMCase activity and protein concentration in cell free supernatant of isolate NA15 during the growth for a period of 40 h incubated at 37°C under shaking condition (120 rpm).

4.1.3.2. Morphological and biochemical characterization

All the eight bacterial isolates were identified to be Gram positive, rod shaped bacteria lacking capsule (Table 4.1.4). Also, all were capable of producing catalase and oxidase. All the eight isolates except DGA were nitrate reductase producers. Isolate NA15 was found to be urease, DNase and Bile-Esculin positive.

Table 4.1.4. Biochemical characterization of cellulose degrading bacterial isolates.

Bacterial Isolates	Gram Staining	Capsule	Catalase	Oxidase	Nitrate Reduction	DNase	Citrate Utilization	Bile-Esculin	V-P reaction	Urease
NA8	+	-	+	+	+	-	+	-	+	-
NA9	+	-	+	+	+	-	+	-	+	-
NA11	+	-	+	+	+	-	±	-	+	-
NA14	+	-	+	+	+	-	±	+	+	-
NA15	+	-	+	+	+	+	-	+	-	+
DGA	+	-	+	+	-	+	+	-	-	+
DGB	+	-	+	+	+	±	-	-	+	-
DGC	+	-	+	+	+	-	-	-	+	-

+, Positive; -: negative and ±: weak positive

Except NA15, DGB and DGC, all the isolates were able to utilize citrate as carbon source. Other than NA15 and DGA, all the isolates were found to be positive for V-P reaction.

4.1.3.3. Antibiotic sensitivity test

Bacterial isolates were tested for their sensitivity towards different antibiotics (**Table 4.1.5**). NA8, NA9, NA14 and NA15 were sensitive for all the twenty antibiotics used. DGB and DGC showed resistance to many antibiotics, while NA11 was quite resistant to only two antibiotics Cefaperazone (75 µg) and Penicillin (10 units). Cefuroxime (30 µg), Cefoperazone (75 µg), Ampicillin/Cloxacillin (10/10 µg) and Penicillin (10 units) were inefficient to inhibit the growth of DGA. There were noticeable variations in sensitivity patterns to antibiotics among all the bacterial isolates. Few *Bacillus* sp. have been reported to be resistant to penicillin G antibiotics, whereas few have been reported to show sensitivity to Cephalosporin C, Polymyxin B, Ampicillin, Bacitracin, Rifampicin, Tetracycline, Kanamycin, Streptomycin, Chloramphenicol, and Erythromycin (Claus and Berkeley, 1986). The antibiotic profile of all the isolates matched well with sensitivity profile of *Bacillus* species.

Table 4.1.5. Antibiotic profiling of bacterial isolates of the present study.

Bacterial isolate	Antibiotic resistance*
NA8	--
NA9	--
NA11	Cefaperazone (75 µg), Penicillin (10 units)
NA14	--
NA15	--
DGA	Cefuroxime (30 µg), Cefoperazone (75 µg), Ampicillin/Cloxacillin (10/10 µg), Penicillin (10 units)
DGB	Cefuroxime (30 µg), Ciprofloxacin (5 µg), Roxithromycin (30 µg), Ampicillin/Cloxacillin (10/10 µg), Penicillin (10 units)
DGC	Cefuroxime (30 µg), Ceftazidime (30 µg), Claritromycin (15 µg), Azithromycin (15 µg), Ampicillin/Cloxacillin (10/10 µg), Penicillin (10 units)

*Total 20 antibiotics were used with isolated bacteria using ICOSA, Universal -1 Kit (HiMedia, Mumbai, India).

4.1.3.4. Salinity, alkalinity and temperature tolerance test

Upon subjecting all the bacterial isolates to salinity and temperature tolerance (Table 4.1.6), NA11, NA14, DGB and DGC were able to survive in the NaCl concentration range of 0-7%. Minimum tolerance for NaCl concentration was observed for DGA (0-2%) followed by NA15

(0-5%), whereas NA8 and NA9 were the most osmotolerant isolates among all and survived up to 12 and 10% NaCl concentration, respectively.

The bacterial isolates were investigated for growth in solid and liquid media buffered with different pH. All the isolates were able to grow between pH 5 - 8 except DGA, DGB and DGC which showed a narrow range of pH (6-7) (Table 4.1.6). Two isolates NA8 and NA9 showed wide pH tolerance and grew up to a pH range of 4-8. When subjected to different temperatures (25, 30, 37, 45, 50, 55 and 60°C), NA8, NA11 and NA14 were able to grow in range of 25 - 60°C, NA9 in 25 - 55°C, NA15 and DGA in 25 - 50°C while DGB and DGC in the range of 30 - 55°C (Table 4.1.6).

Table 4.1.6. Effect of sodium chloride concentration, temperature and pH on the growth of bacterial isolates.

Bacterial isolate	NaCl (%)	Temperature (°C)	Growth in different media pH					
			4	5	6	7	8	9
NA8	0-12	25-60	+	+++	+++	+++	+	-
NA9	0-10	25-55	+	++	++	+++	+	-
NA11	0-7	25-60	-	++	++	+++	+	-
NA14	0-7	25-60	-	++	++	+++	+	-
NA15	0-5	25-50	-	+++	+++	+++	+	-
DGA	0-2	25-50	-	-	++	+++	-	-
DGB	0-7	30-55	-	-	++	+++	-	-
DGC	0-7	30-55	-	-	++	+++	-	-

-: no growth, +: poor growth, ++: good growth, +++: luxurious growth

Salt tolerance, growth temperature range as well as growth pH range are important taxonomic criteria which are used to differentiate species in the genus *Bacillus* (Claus and Berkeley, 1986). All the bacterial isolates of the present study thrived well in slightly acidic environments.

4.1.3.5. Scanning electron microscopy (SEM) of bacterial isolate

Scanning electron micrograph of most efficient cellulase producer (isolate NA15) confirmed the rod shaped structure and revealed an approximate size of $2.2 \times 0.6 \mu\text{m}$ at magnification 5,000X (Figure 4.1.5).

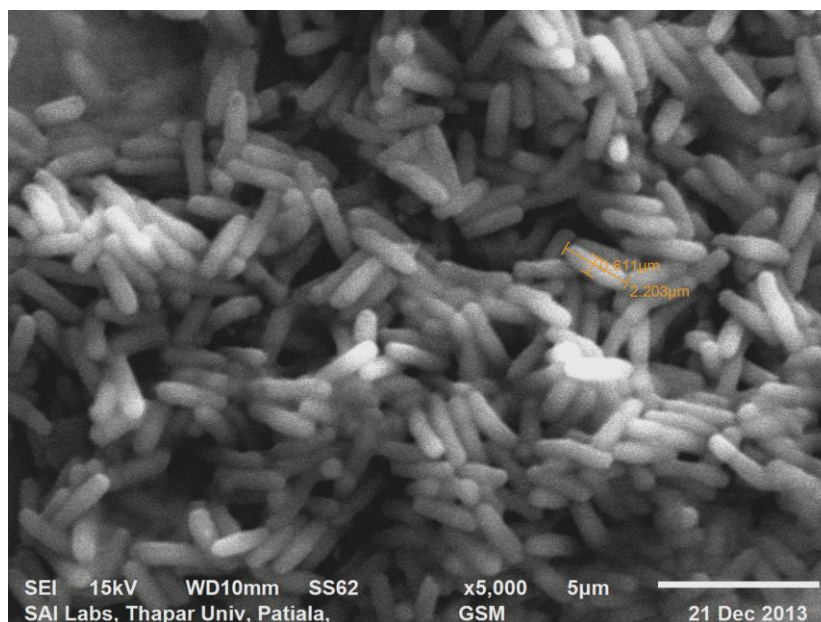


Figure 4.1.5. Scanning electron micrograph of *B. subtilis* NA15 (Magnification: 5,000X)

4.1.3.6. *Biolog GEN III MicroPlate™* identification of bacterial isolates

Biolog's identification method is an easiest, rapid, cost effective and extensively used method to characterize microbial community by determining the phenotypic fingerprint. On the basis of phenotypic pattern (Table 4.1.8) in the GEN III MicroPlate™ analyzed by Phenotype MicroArray (PM) Software, strain NA8, NA9, NA11, NA14, DGB and DGC were identified as a member of *B. licheniformis*. NA15 and DGA revealed their identity as *Bacillus subtilis* and *Lysinibacillus fusiformis* (Table 4.1.7).

Table 4.1.7. Microbial identification using GEN III MicroPlate™

Bacterial isolate	Source	Biolog GEN III MicroPlate™ identification
NA8	Paper pulp	<i>Bacillus licheniformis</i>
NA9	Paper pulp	<i>Bacillus licheniformis</i>
NA11	Compost	<i>Bacillus licheniformis</i>
NA14	Compost	<i>Bacillus licheniformis</i>
NA15	Compost	<i>Bacillus subtilis</i>
DGA	Sugarcane bagasse	<i>Lysinibacillus fusiformis</i>
DGB	Sugarcane bagasse	<i>Bacillus licheniformis</i>
DGC	Sugarcane bagasse	<i>Bacillus licheniformis</i>

Table 4.1.8. Substrate utilization of cellulolytic bacteria using Biolog GEN III MicroPlate™.

Properties	Bacterial isolates							
	NA8	NA9	NA11	NA14	NA15	DGA	DGB	DGC
Negative Control	-	-	-	-	-	-	-	-
Dextrin	+	+	+	+	+	+	+	+
D-maltose	+	+	+	+	+	-	+	+
D-trehalose	+	+	+	+	+	-	+	+
D-cellobiose	+	+	+	+	+	+	+	+
Gentiobiose	+	+	+	+	+	-	+	+
Sucrose	+	+	+	+	+	-	+	+
D-turanose	+	+	+	+	+	-	+	+
Stachyose	+	+	+	+	+	-	-	+
Positive Control	+	+	+	+	+	+	+	+
pH 6	+	+	+	+	+	+	+	+
pH 5	+	+	+	+	+	-	+	+
D-Raffinose	+	+	+	+	+	-	+	+
α -D-Lactose	+	+	-	+	+	-	-	-
D-Melibiose	+	+	-	+	+	-	-	+
β -Methyl-D Glucoside	+	+	+	+	+	-	+	+
D-Salicin	+	+	+	+	+	-	+	+
N-Acetyl-D-Glucosamine	+	+	+	+	+	+	+	+
N-Acetyl- β -D Mannosamine	+	+	-	+	+	-	-	-
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-	-
N-Acetyl Neuraminic Acid	-	-	-	-	-	-	-	-
1% NaCl	+	+	+	+	+	+	+	+
4% NaCl	+	+	+	+	+	+	+	+
8% NaCl	+	+	+	+	+	+	+	+
α -D-Glucose	+	+	+	+	+	-	+	+
D-Mannose	+	+	+	+	+	-	+	+
D-Fructose	+	+	+	+	+	+	+	+
D-Galactose	+	+	-	+	+	-	-	-
3-Methyl Glucose	-	-	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	+	-	-
L-Fucose	-	-	-	-	-	+	-	-
L-Rhamnose	+	+	+	+	+	-	-	+
Inosine	+	+	-	+	+	+	-	-
1% Sodium Lactate	+	+	+	+	+	+	+	+
Fusidic Acid	-	-	-	-	-	-	-	-
D-Serine	-	-	-	-	-	+	-	-
D-Sorbitol	+	+	+	+	+	-	+	+

D-Mannitol	+	+	+	+	+	-	+	+
D-Arabitol	-	-	-	-	-	+	-	-
myo-Inositol	+	+	+	+	+	+	-	+
Glycerol	+	+	+	+	+	+	+	+
D-Glucose- 6-PO ₄	+	-	-	+	+	+	-	-
D-Fructose- 6-PO ₄	+	-	-	+	+	+	+	+
D-Aspartic Acid	+	+	+	+	+	+	-	-
D-Serine	-	-	-	-	-	+	-	-
Troleandomycin	-	-	-	-	-	-	-	-
Rifamycin SV	-	-	+	-	+	-	-	-
Minocycline	-	-	-	-	-	-	-	-
Gelatin	+	+	+	+	-	+	+	+
Glycyl-L-Proline	+	+	+	+	+	+	-	+
L-Alanine	+	+	+	+	+	+	-	+
L-Arginine	+	+	+	+	+	+	-	+
L-Aspartic Acid	+	+	+	+	+	+	-	+
L-Glutamic Acid	+	+	+	+	+	+	-	+
L-Histidine	+	-	-	+	+	-	-	-
L-Pyroglutamic Acid	+	-	-	+	+	-	-	-
L-Serine	+	+	-	+	-	+	-	+
Lincomycin	-	-	+	-	-	+	+	+
Guanidine HCl	+	+	+	+	-	+	+	+
Niaproof 4	-	-	-	-	-	-	-	-
Pectin	+	+	+	+	+	-	+	+
D-Galacturonic Acid	+	+	+	+	+	+	+	+
L-Galactonic Acid	-	-	-	-	-	+	-	-
Lactone								
D-Gluconic Acid	+	+	+	+	+	+	+	+
D-Glucuronic Acid	+	+	+	+	+	+	+	+
Glucuronamide	-	+	-	+	-	+	+	+
Mucic Acid	+	+	+	+	+	+	-	+
Quinic Acid	-	-	-	-	-	+	-	-
D-Saccharic Acid	+	+	+	+	+	+	-	+
Vancomycin	-	-	-	-	-	-	-	-
Tetrazolium Violet	-	-	-	-	-	+	+	+
Tetrazolium Blue	-	-	-	-	-	-	-	-
p-Hydroxy- Phenylacetic Acid	-	-	-	-	-	-	-	-
Methyl Pyruvate	+	+	+	+	+	+	-	-
D-Lactic Acid Methyl Ester	+	-	-	+	+	+	-	-
L-Lactic Acid	+	+	+	+	+	+	+	+
Citric Acid	+	+	+	+	+	+	+	+
α -Keto-Glutaric Acid	+	-	-	+	+	+	-	-
D-Malic Acid	+	-	-	+	-	+	-	-

L-Malic Acid	+	+	+	+	+	+	+	+
Bromo-Succinic Acid	+	+	+	+	+	+	+	+
Nalidixic Acid	-	-	-	-	-	+	-	+
Lithium Chloride	+	+	+	+	+	+	+	+
Potassium Tellurite	+	+	+	+	+	+	+	+
Tween 40	+	+	-	+	+	+	-	+
γ -Amino-Butyric Acid	+	+	-	+	+	+	-	+
α -Hydroxy- Butyric Acid	+	+	+	+	-	+	-	-
β -Hydroxy- D,LButyric Acid	-	-	-	-	+	+	-	-
α -Keto-Butyric Acid	+	-	-	+	-	+	-	-
Acetoacetic Acid	+	+	+	+	+	+	+	+
Propionic Acid	+	+	+	+	-	+	-	+
Acetic Acid	+	+	+	+	+	+	-	+
Formic Acid	+	-	-	+	+	+	-	+
Aztreonam	+	+	+	+	+	+	+	+
Sodium Butyrate	+	+	+	+	+	+	+	+
Sodium Bromate	-	-	-	-	-	+	-	-

+: Positive reaction; -: Negative reaction.

The identification of herbicide degrading *Serratia marcescens* SE1 (Abel et al., 2012) and cellulose degrading *Bacillus* sp. C1 (Sadhu et al., 2013) were found to be reliable using GEN III MicroPlate™ and Biolog GP2 plate, respectively.

4.1.4. Molecular characterization of bacterial isolates

After the biochemical characterization and identification based on phenotypic pattern using Biolog GEN III MicroPlate™ of bacterial isolates, molecular characterization of these bacteria was carried out by PCR based amplification of 16S rDNA using universal primers. 16S rRNA gene is widely used as phylogenetic marker due to extent of divergence in the sequence of this gene provides a reliable estimate of phylogenetic distance between different species (Wakabayashi et al., 1999). Amplification of 16S rDNA region showed amplicons of about 1.5 kb in all isolates (Fig. 4.1.6).

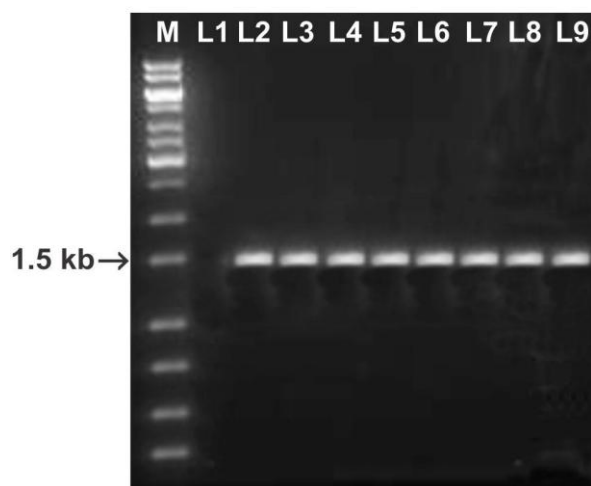


Figure 4.1.6. 16S rDNA amplification of bacterial isolates. Lane M: 1 kb DNA ladder;
L1: Control; L2: NA8; L3: NA9; L4: NA11; L5: NA14; L6: NA15; L7: DGA;
L8: DGB; L9: DGC

4.1.4.1. Identification of the bacterial isolates

16S rDNA PCR products were extracted, purified and then sequenced using automated DNA sequencer AB3500 xL (Applied Biosystem, USA). The sequences were analyzed by multiple sequence alignment to check the similarities among the isolates. The homologies among the sequences varied from 85 to 99% between isolates. Minimum of 85% similarity was found in NA8 with DGA, NA11 with DGA, NA14 with DGA, DGA with DGC, DGA with DGB, whereas maximum (99%) similarity was found between DGB and DGC (Table 4.1.9).

The sequences were further compared for the similarity in the GenBank DNA database using BlastN (NCBI) (Altschul et al., 1997). The related sequences showing similarity in BLAST were retrieved from GenBank and RDPII and aligned using Clustal W (Thompson et al., 1994). The resulting multiple alignments were optimized visually and evolutionary distances were calculated using Kimura 2 parameter. Phylogenetic tree was constructed using neighbour joining method of MEGA 5 package (Tamura et al., 2007). Gaps were treated as missing data. It has been reported that sequence identities of over 98% represent same species while the sequences that share an identity below 98% are usually considered to be part of the same genus (Sadowsky et al., 1996). Blast results revealed that 16S rDNA of the bacterial isolates in present study had high similarity with the sequences of NCBI database (Table 4.1.10).

Table 4.1.9. Percentage similarity of 16S rDNA sequences of bacterial isolates using multiple sequence alignment (ClustalW)

Seq A	Name	Length (bp)	Seq B	Name	Length (bp)	Score
1	NA8	1451	2	NA9	1455	96
1	NA8	1451	3	NA11	1464	95
1	NA8	1451	4	NA14	1485	96
1	NA8	1451	5	NA15	1486	95
1	NA8	1451	6	DGA	1464	85
1	NA8	1451	7	DGB	1471	98
1	NA8	1451	8	DGC	1487	97
2	NA9	1455	3	NA11	1464	97
2	NA9	1455	4	NA14	1485	97
2	NA9	1455	5	NA15	1486	96
2	NA9	1455	6	DGA	1464	86
2	NA9	1455	7	DGB	1471	98
2	NA9	1455	8	DGC	1487	98
3	NA11	1464	4	NA14	1485	95
3	NA11	1464	5	NA15	1486	94
3	NA11	1464	6	DGA	1464	85
3	NA11	1464	7	DGB	1471	96
3	NA11	1464	8	DGC	1487	96
4	NA14	1451	5	NA15	1486	93
4	NA14	1451	6	DGA	1464	85
4	NA14	1451	7	DGB	1471	96
4	NA14	1451	8	DGC	1487	95
5	NA15	1486	6	DGA	1464	86
5	NA15	1486	7	DGB	1471	97
5	NA15	1486	8	DGC	1487	97
6	DGA	1464	7	DGB	1471	85
6	DGA	1464	8	DGC	1487	85
7	DGB	1471	8	DGC	1487	99

NA8, NA9, DGB and DGC had shown 99% similarity with *Bacillus licheniformis* LZBL8, *Bacillus licheniformis* X2, *Bacillus licheniformis* BAB1836 and *Bacillus licheniformis* MS514. NA11 showed maximum similarity with *Bacillus* sp. CK1 (96%), whereas NA14 showed 99% identity with *Bacillus licheniformis* BPRIST030. Isolate NA15 had maximum (99%) similarity with *Bacillus subtilis* HB1 while 99% similarity with *Lysinibacillus fusiformis* N139 was observed with DGA (Table 4.1.10).

Table 4.1.10. Bacterial isolates and their closest relative species inferred from 16S rRNA gene sequences of NCBI database.

Bacterial isolate	Nearest match	Phylum	Query coverage (%)	E value	% Identity to closest related bacterial sp.
NA8	<i>Bacillus licheniformis</i> LZBL8 (JX847114)	Firmicutes	100	0.0	99
NA9	<i>Bacillus licheniformis</i> X2 (HQ917117)	Firmicutes	100	0.0	99
NA11	<i>Bacillus</i> sp. CK1 (HM589859)	Firmicutes	100	0.0	96
NA14	<i>Bacillus licheniformis</i> BPRIST030 (JF700489)	Firmicutes	99	0.0	99
NA15	<i>Bacillus subtilis</i> HB1 (JQ518358)	Firmicutes	100	0.0	99
DGA	<i>Lysinibacillus fusiformis</i> N139 (JQ900546)	Firmicutes	99	0.0	99
DGB	<i>Bacillus licheniformis</i> BAB1836 (KF535152)	Firmicutes	100	0.0	99
DGC	<i>Bacillus licheniformis</i> MS514 (EU718490)	Firmicutes	100	0.0	99

Phylogenetic analysis showed similar result as Biolog GEN III MicroPlate™ identification method, revealed that all the eight bacterial isolates belong to Phylum Firmicutes. Seven isolates were associated with the genera *Bacillus* while one was associated with *Lysinibacillus* sp. (Figure 4.1.7). The majority of bacterial isolates of the present study were found to be *Bacillus* genera. Numerous reports are already available that cite high amounts of cellulase production such as *B. subtilis* YJ1 (Yin et al., 2010) *B. amyloliquefaciens* DL-3 (Lee et al., 2008), *B. subtilis* A-53 (Kim et al., 2009), *Bacillus* sp. M-9 (Bajaj et al., 2009), *B. licheniformis* (Bischoff et al., 2006), *Bacillus* sp. PDV (Sharma et al., 1990), *Bacillus* sp. CH43 and *Bacillus* sp. HR68 (Mawazda et al., 2006), *B. subtilis* GN156 (Apiraksakorn, 2008), *Bacillus* sp. C1 (Sadhu et al., 2013), *Bacillus* sp. (Vijayaraghvan et al., 2012), *Bacillus* sp. DUSELR13 (Rastogi et al., 2010), *B. flexus* NT (Trivedi et al., 2011). Besides *Bacillus* spp., *Paenibacillus barcinonensis* MG7 (Asha et al., 2012), *Caldibacillus cellulovorans* MG7 (Huang and Monk, 2004) and *Rhizobium fredii* J1 (Chen et al., 2004) are also known to produce cellulase. No efficient cellulase producers have been reported from genus *Lysinibacillus* to the best of our knowledge. Isolate NA15 belonged to *Bacillus subtilis*, was selected for further studies due to its higher cellulase activity.

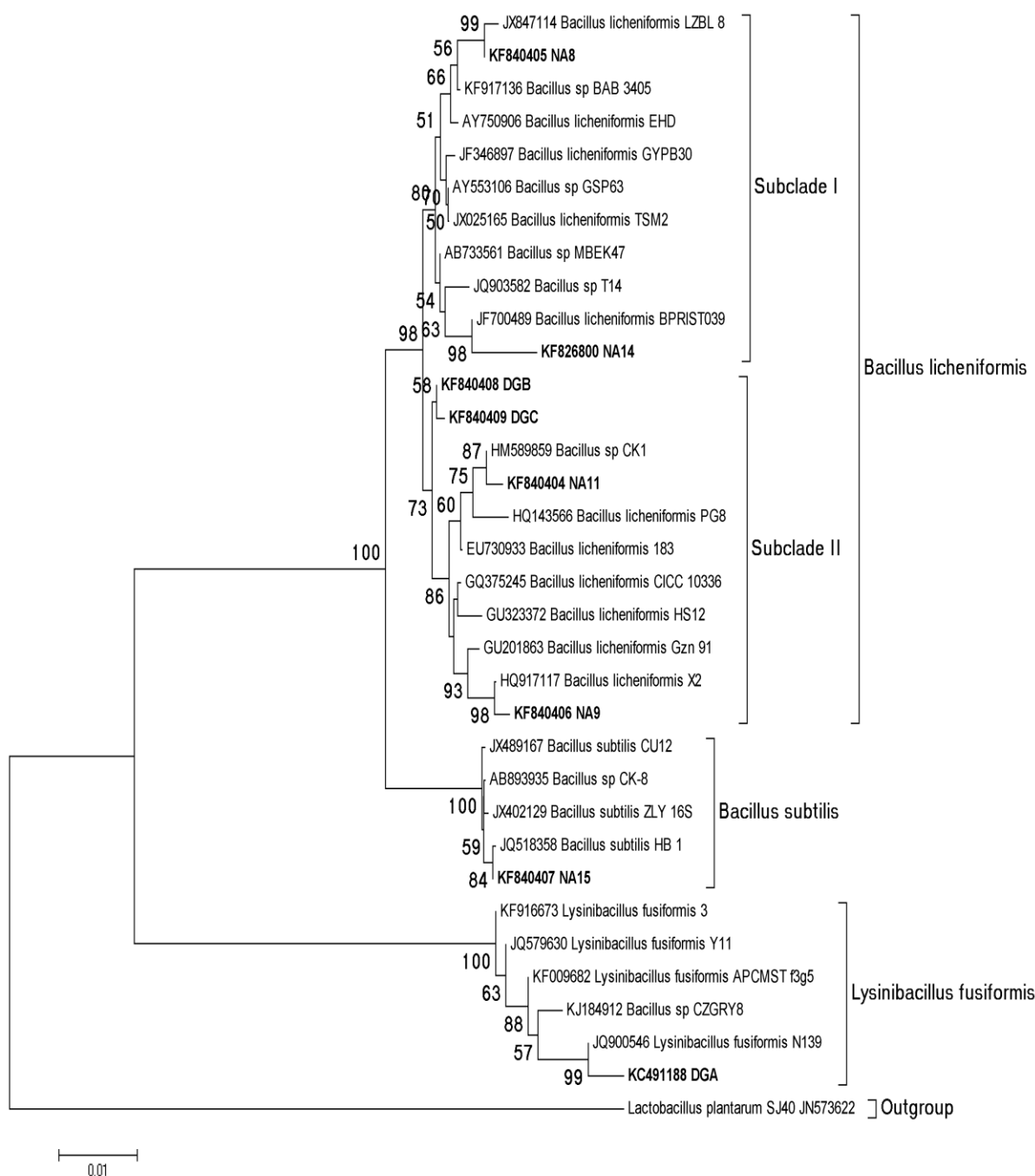


Figure 4.1.7. Neighbor-joining tree based on bacterial 16S rRNA sequence data from different bacterial isolates of current study along with sequences available in GenBank database. Numerical values indicate bootstrap percentile from 1,000 replicates. *Lactobacillus plantarum* was used outgroup taxa.

The 16S rDNA gene sequences of all 8 cellulose degrading isolates determined in this study were deposited in the GenBank of NCBI data library under the accession numbers KF840405, KF840406, KF840404, KF826800, KF840407, KC491188, KF840408 and KF840409 for NA8, NA9, NA11, NA14, NA15, DGA, DGB and DGC, respectively (Appendix III).

Conclusion

Efficient cellulose degrading bacteria from different habitat such as compost, paper pulp and sugarcane bagasse were isolated. The bacterial load of samples from different sites varied from 0.07×10^3 to 3.6×10^3 per gm of samples. Though the presence of cellulase producing bacteria in carbon rich biomass waste materials is not surprising, but their high number proved their adaption to the particular niche. Out of 32, eight cellulose degrading bacteria screened and were identified to be Gram positive, rod shaped lacking capsule. Also, all were gram positive and rod shaped, showed positive result for catalase and oxidase test. Based on phylogenetic analysis, all the eight isolates were identified as the member of phylum Firmicutes. NA8, NA9, NA11, NA14 DGB and DGC were found to show maximum similarity with *Bacillus licheniformis*. Isolate NA15 had maximum similarity with *Bacillus subtilis*, while DGA belong to a different genera *Lysinibacillus*.

4.2. Biochemical characterization of cellulose degrading enzymes

Prior to biochemical characterization of cellulose degrading enzymes produced by most efficient bacteria *Bacillus subtilis* NA15, media ingredients were optimized for enhance production of CMCase using statistical method. *B. subtilis* NA15 was grown in optimized media and then cell free supernatant was subjected to partial purification using ammonium sulfate and further to DEAE-Sepharose column chromatography. Purified CMCase was analyzed for molecular weight determination using SDS-PAGE which was confirmed by Zymogram. Purified protein was then characterized for optimum pH, temperature and their stability, effects of various additives, substrate specificity and enzyme kinetics.

4.2.1. Statistical optimization of media ingredients for enhanced production of CMCase

4.2.1.2. Diagnostic experimental design of CMCase production by *B. subtilis* NA15

The screening of 11 nutrients was done using Plackett-Burman design to explicate the parameters that significantly influence the CMCase production. As a result, the CMCase activity in cell free supernatant of *B. subtilis* NA15 after 24 h of incubation showed variation from 0.006 to 0.38 U/mL (Table 4.2.1). This variation reflected the significance of factors on the response (CMCase activity). The analysis of regression coefficients and *t*-value of five ingredients were shown in Table 4.2.2. In general, a large *t*-value associated with a low *P*-value of a variable indicates a high significance of the corresponding model terms. ANOVA through Fisher's test was used to evaluate the effect of independent variables on the response and significant result were identified by a *P*-value of <0.05. Out of eleven, four variables (CMC, peptone, yeast extract and MnCl₂) above 95% confidence level were selected for further interactive study (Table 4.2.3).

Table 4.2.1. Response in terms of CMCase activity of screening of media constituents using Plackett-Burman design.

Runs		CMCase activity (U/mL)
Std	Run	Experimental values
8	1	0.283
6	2	0.069
3	3	0.258
1	4	0.246
11	5	0.266
4	6	0.106
5	7	0.07
7	8	0.223
2	9	0.153
9	10	0.384
10	11	0.156
12	12	0.006

Neglecting the variables which had less or no contribution, the model equation for CMCase activity can be written as:

$$\text{CMCase activity (Y)} = 0.185118 + 0.091784 * A + 0.036299 * B + 0.029499 * C + 0.013868 * G + 0.011216 * J$$

Where, Y = CMCase activity, A = CMC, B = peptone, C = yeast extract, D = $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$

Different nitrogen sources, such as yeast extract and peptone were chosen, as these have been reported to significantly affect the cellulase production (Lee et al., 2008). Enhancement of cellulase production using various organic and inorganic nitrogen sources (yeast extract, peptone, ammonium chloride, ammonium sulfate, ammonium nitrate, and potassium nitrate) was also reported by Asha et al. (2012). The biosynthesis of cellulases by *T. reesei* was very high in medium supplemented with CMC as carbon source as reported by Ahamed and Vermette (2008). In addition to CMC, yeast extract and peptone showed significant effect on cellulase production (Li and Gao, 1997).

Table 4.2.2. Model coefficient estimated by multiple linear regressions.

Source	Sum of squares	Mean square	F value	Computed t-values
A:CMC	0.101092	0.101092	288.205	75.86
B:Peptone	0.015812	0.015812	45.0774	11.86
C:Yeast Extract	0.010442	0.010442	29.7705	7.84
G: $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.002308	0.002308	6.57904	1.73
J:KCl	0.00151	0.001510	4.30357	1.13

Table 4.2.3. Statistical analysis of Plackett-Burman design.

Source	Sum of Squares	Degree of freedom	Mean Square	F- Value	p-value Prob> F	Confidence level
Model	0.131163	5	0.026233	74.78709	< 0.0001	99.99
A-CMC	0.101092	1	0.101092	288.2049	< 0.0001	99.99
B-Peptone	0.015812	1	0.015812	45.07743	0.0005	99.95
C-Yeast- Extract	0.010442	1	0.010442	29.77045	0.0016	
G- $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.002308	1	0.002308	6.579035	0.0426	95.74
J-KCl	0.00151	1	0.00151	4.303571	0.0834	91.66
Residual	0.002105	6	0.000351			
Cor Total	0.133268	11				

Manganese is a micronutrient required as a cofactor in many enzyme systems (Graffin, 1994). Enhanced CMCase activity was also reported by Yin et al. (2010) due to presence of Mn^{2+} and Co^{+} , confirming the presence of SH group on the active site, however metal ions such as K^{+} , Na^{+} , NH_4^{+} did not affect the cellulase activity. The present study is in good correspondence as $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ showed positive effect on CMCase production. Enhanced cellulase activity of *Cellulomonas* sp. ASN2 (Irfan et al., 2012) and *B. subtilis* YJ1 (Li et al., 2010) was also reported in presence of Mn^{2+} and Co^{2+} . Previous study revealed that K^{+} , Na^{+} and NH_4^{+} showed null effect on the cellulase produced by *Rhizopus oryzae* (Murashima et

al., 2002), whereas, Co^{2+} and Mn^{2+} enhanced cellulase activity of *Mucor circinelloides* (Saha, 2004) and *Chalara paradoxa* (Lucas et al., 2001), respectively.

4.2.1.2. Optimizing of media constituents using Central composite design

Response surface methodology is a stepwise approach, used to evaluate the interaction effects of the screened parameters for optimization of CMCase production by *B. subtilis* NA15.

Table 4.2.4. Coded experimental design and results for the response surface of maximum CMCase activity of *B. subtilis* NA15 as a function of CMC, Peptone, Yeast Extract, and $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$.

Runs		CMCase activity (U/mL)
Number	Order	Experimental
29	1	0.274
13	2	0.065
15	3	0.107
8	4	0.434
26	5	0.269
5	6	0.087
14	7	0.451
23	8	0.293
30	9	0.276
10	10	0.333
25	11	0.236
28	12	0.269
18	13	0.465
6	14	0.398
24	15	0.284
4	16	0.327
7	17	0.115
19	18	0.214
16	19	0.414
12	20	0.397
2	21	0.228
17	22	0.010
3	23	0.181
9	24	0.087
11	25	0.225
27	26	0.256
20	27	0.267
1	28	0.047
21	29	0.165
22	30	0.216

Analysis of the screened variables from a five-level response surface design was complex but led to identification of the key variables affecting CMCase production. The results obtained were fed into the Design-Expert software (Version 8.0.7.1, Stat-Ease, Minneapolis, Minn. USA) and analyzed using ANOVA as appropriate to the experimental design used. The actual values ranges of the screened variables were presented in Table 4.2.4.

Table 4.2.5. R-Squared, Adj R-Squared, Pred R-Squared and Adeq Precision Value of the model.

Std. Dev.	0.02	R-Squared	0.983
Mean	0.24	Adj R-Squared	0.967
C.V. %	9.04	Pred R-Squared	0.915
PRESS	0.038	Adeq Precision	31.48

Table 4.2.6. Analysis of variance (ANOVA) for response surface quadratic model.

Source	Sum of squares	df	Mean square	F value	Probability > F	
Model	0.441	14	0.031	63.46	< 0.0001	Significant
A:CMC	0.369	1	0.369	743.47	< 0.0001	
B:Peptone	0.015	1	0.015	30.89	< 0.0001	
C:Yeast Extract	0.005	1	0.005	10.15	0.006	
D:MnCl ₂ .4H ₂ O	0.002	1	0.002	4.91	0.04	
AB	0.002	1	0.002	4.11	0.06	
AC	0.021	1	0.021	42.29	< 0.0001	
AD	0.001	1	0.001	2.98	0.10	
BC	0.008	1	0.008	16.85	0.0009	
BD	0.000	1	0.0004	0.97	0.33	
CD	0.004	1	0.004	8.18	0.01	
A ²	0.0005	1	0.0008	1.80	0.19	
B ²	0.0006	1	0.0006	1.36	0.26	
C ²	0.008	1	0.008	16.83	0.0009	
D ²	0.001	1	0.001	2.72	0.11	
Residual	0.007	15	0.0004			Not Significant
Lack of fit	0.006	10	0.0006	2.77	0.13	

To test the fit of the model equation, the regression-based determination coefficient R^2 was evaluated. The predicted R^2 (0.91) was in close proximity with adjusted R^2 (0.97), advocated for greater significance of model (Table 4.2.5). The statistical analysis allowed us to determine the contribution of experimental factors (signals) in comparison to noise, where the signal should be fairly large in comparison to noise. Thus, the estimated adequate precision of 31.48 for CMCase production, represented the signal to noise ratio, is an adequate signal. The model F value of 63.46 as well as values of $P > F$ (<0.0001) indicated that the model terms

(A, B, C, D AB, BC, AC, CD, C²) were significant (Table 4.2.6). The application of RSM yielded the following regression equation (Equation 1), which established an empirical relationship between CMCase Activity (Y) and the test variables in coded units:

$$\text{CMCase Activity (Y)} = 0.2633 + 0.124 * A + 0.025 * B + 0.0145 * C + 0.0100 * D - 0.011 * A * B + 0.036 * A * C + 0.009 * A * D - 0.022 * B * C - 0.005 * B * D - 0.015 * C * D - 0.005 * A^2 - 0.004 * B^2 - 0.017 * C^2 - 0.007 * D^2$$

(Equation 1)

The contour plots were generated to investigate the interaction among the four significant parameters and to evaluate their combined effects on the response of CMCase activity. Based on the interactions between the variables, an increase in CMCase activity on increasing the concentration of each variable reached to optimum level. From Figure 4.2.1, it was observed that at a concentration of 13.8 g/L CMC (A) and 6.75 g/L of yeast extract (C), the CMCase activity was maximum (0.41 U/mL). Similarly in an interactive study of peptone (B) and yeast extract (C), maximum CMCase activity was observed at their respective concentrations of 6.05 g/L and 4.6 g/L, respectively (Figure 4.2.2).

A number of numerical solutions were suggested by the Design-Expert software within the experimental range of parameters for maximum CMCase activity. In order to validate the predicted model, experiments suggested were conducted in duplicates. The suggested solutions revealed that the variables were optimized at points located in the rightmost region of the plot (Figure 4.2.3). The maximum experimental value for CMCase activity was 0.47 U/mL, while the predicted response based on RSM was estimated to be 0.45 U/mL at CMC (18 g/L), peptone (5 g/L), yeast extract (5 g/L) and MnCl₂ (0.5 g/L). Simplified medium with only four ingredients showed seven-folds increase in CMCase activity as compared to unoptimized medium with 11 ingredients. The excellent correlation between predicted and experimental values of experiment conducted justified the validity of the regression model obtained. The optimised cellulase activity of *B. subtilis* NA15 (0.47 U/mL) obtained in this study was higher than that of *Geobacillus* sp. (Abdel-Fattah et al., 2007), *Brevibacillus* sp., *Bacillus* sp. (Rastogi et al., 2010), *B. subtilis* AS3 (Deka et al., 2013) with maximum CMCase activity of 0.12, 0.02, 0.01, 0.43 U/mL, respectively under optimised conditions using RSM. Additions of yeast extract and ammonium sulphate showed two-fold increase in cellulase produced by *Geobacillus* sp. under optimised conditions (Abdel-Fattah et al., 2007).

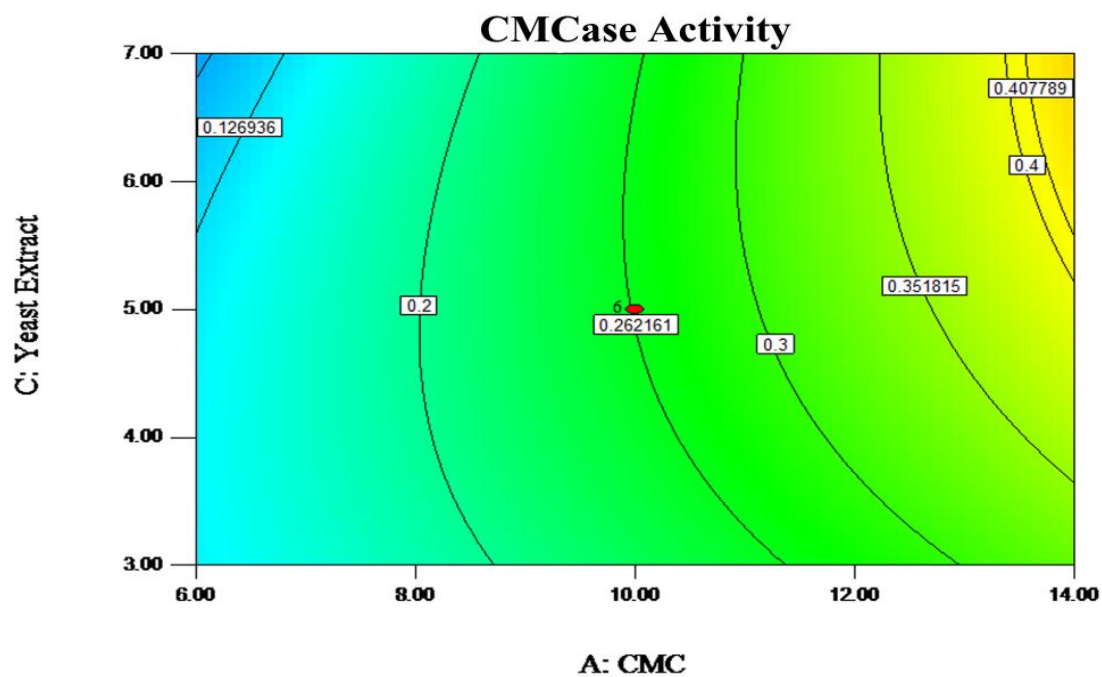


Figure 4.2.1. 2D contour-plot showing relative effect of concentration of A: CMC and C: Yeast Extract on CMCase activity.

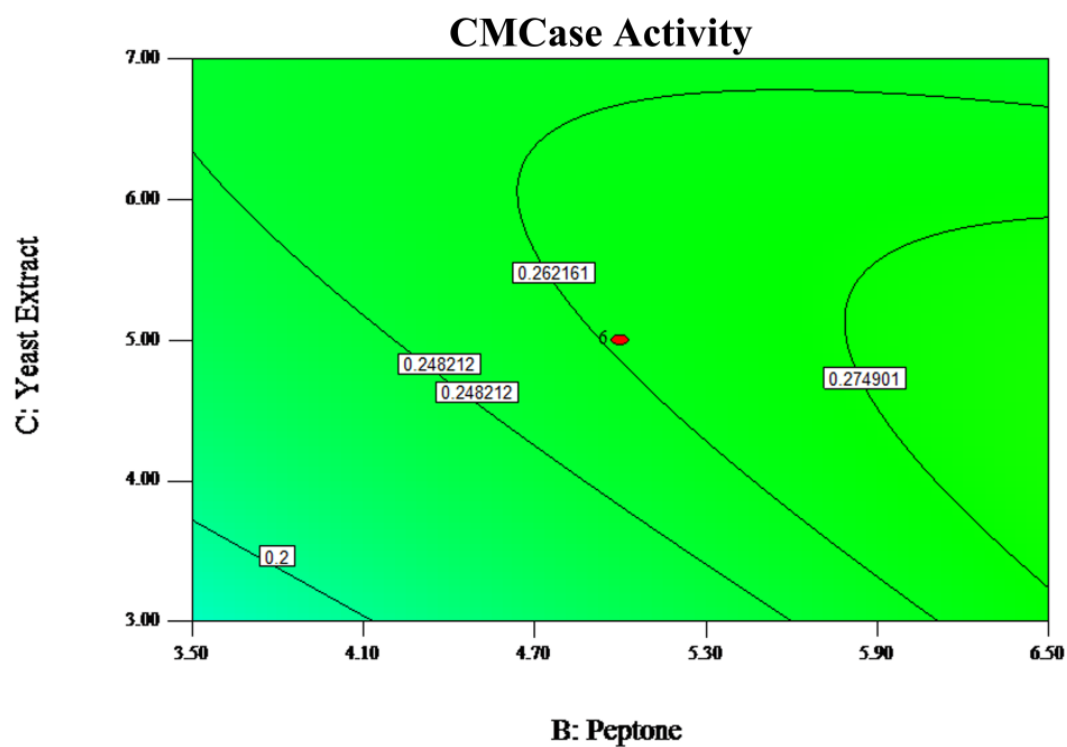


Figure 4.2.2. 2D contour-plot showing relative effect of concentration of B: Peptone and C: Yeast Extract on CMCase activity

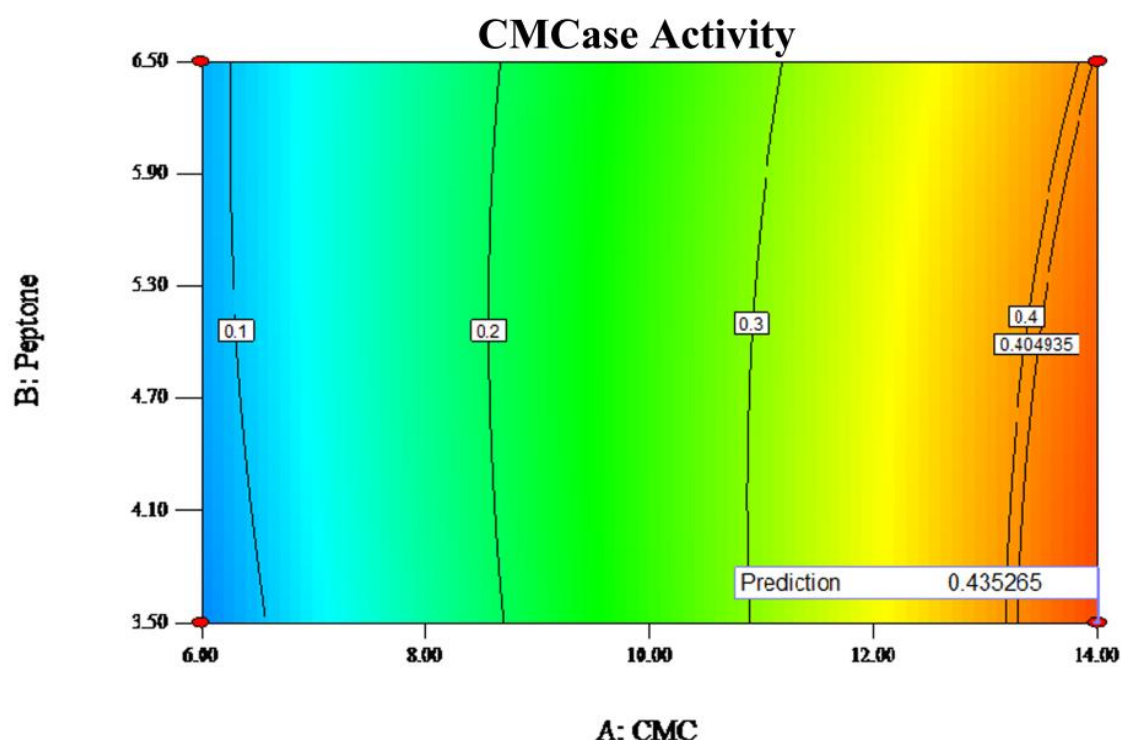


Figure 4.2.3. 2D desirability contour-plot showing the predicted optimum CMCase activity relative to B: Peptone and A: CMC.

4.2.2. Purification of CMCase

4.2.2.1. Ammonium sulfate precipitation and DEAE-Sepharose ion exchange chromatography

Crude extract from the culture medium was taken through a two step purification, partially by ammonium sulfate precipitation and finally by ion exchange chromatography with DEAE-Sephadex fast flow column. The purification fold of the cellulase was 11.48 times with recovery yield of 43.75% (Table 4.2.7). Active fraction showed maximum CMCase activity (3.24 U/mL) in presence of 1% (w/v) CMC dissolved in potassium phosphate buffer (50 mM, pH 7.0). A brief summary of the purification steps was presented in Table 4.2.7. Cellulase was purified from *Bacillus* sp. C1 using DEAE Sepharose column chromatography (Sadhu et al., 2013), *Bacillus* sp. M-9 using DEAE-cellulose chromatography (Bajaj et al. 2009) and from *Cellulomonas* sp. YJ5 using Sephacryl S-100 chromatography (Yin et al. 2010) with 8.5, 9.06 and 17.5 folds increments, respectively.

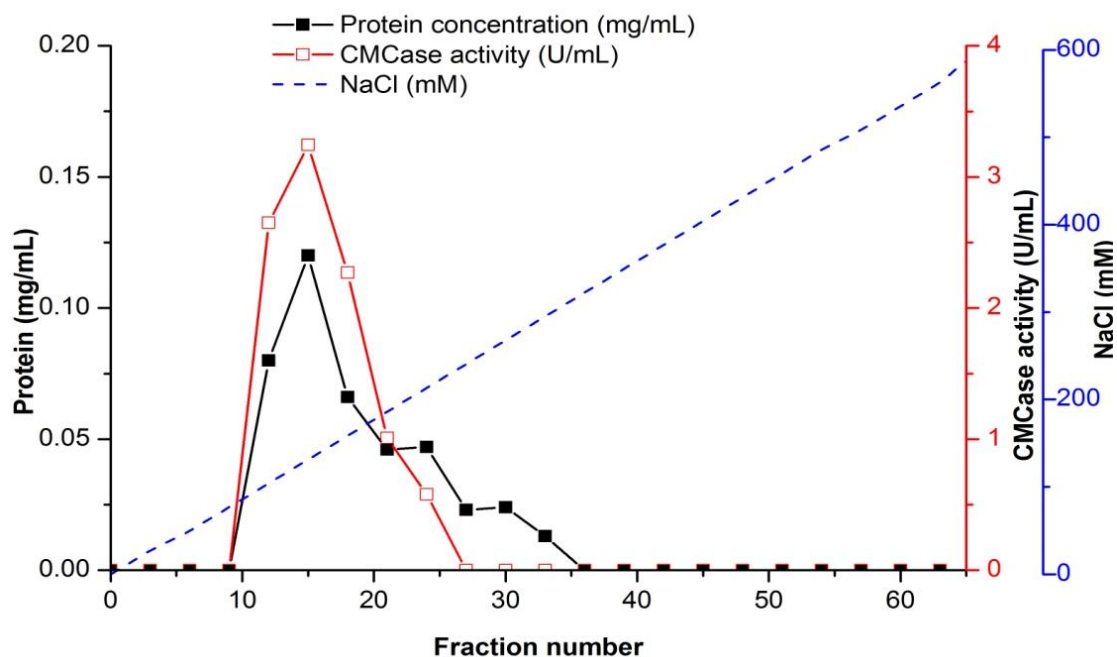


Figure 4.2.4. Chromatogram of cellulase from *B. subtilis* NA15 using DEAE-Sepharose chromatography (1.5 x 10 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.0) and eluted with linear gradient of 0 - 600 mM NaCl.

Table 4.2.7. Purification of CMCase produced by *B. subtilis* NA15.

Sample	Volume (ml)	Total protein (mg)	Protein concentration (mg/ml)	Total activity (Units)	Specific activity (U/mg)	Purification (folds)	Yield (%)
Crude culture broth	90	16.8	0.19	44.5	2.65	1	100
Ammonium sulfate precipitate (after dialysis)	15	1.25	0.08	25.14	20.12	7.59	56.49
After DEAE Sepharose chromatography	6	0.64	0.11	19.47	30.42	11.48	43.75

4.2.2.2. SDS-PAGE and Zymography

The molecular mass of the purified cellulase was estimated to be about 45 kDa (Figure 4.2.5). This molecular mass was in close proximity with 37 - 43 kDa of the cellulases from *B. licheniformis* (Bischoff et al., 2006), but smaller than CMCase of 97 kDa of *Bacillus* sp. C1 (Sadhu et al., 2013), 61-80 kDa of the cellulases from *Bacillus* sp. AC-1 (Li et al., 2006), *Bacillus* sp. HSH-810 where it is 80 kDa (Kim et al., 2005). In *Pseudomonas fluorescens* it was of 36 kDa (Bakare et al., 2005) while in *B. amyloliquefaciens* DL-3 and *Bacillus* sp. M-9 it was of 54 kDa (Lee et al., 2008; Bajaj et al., 2009). Protein profiling of *Thermomonospora* sp. (George et al., 2001) and *Cellulomonas* sp. YJ5 (Yin et al., 2010) showed molecular mass

of 38 and 43.7 kDa, respectively. The activity band of CMCase showed clear zone of hydrolysis against a dark blue background.

Zymogram analysis of protein from *Streptomyces* sp. B-PNG 23 also showed five different CMCase with molecular mass of 97 kDa, 66 kDa, 62.5 kDa, 53 kDa and 50 kDa (Azzeddine et al., 2013), while it was of 24 kDa and 40 kDa from digestive fluid of *Scolytus* sp. and *Spharagemon bolli*, respectively (Oppert et al., 2010). An endoglucanase activity band of 200 kDa was also associated with multienzyme complex from bacterium *Tepidemicrobium xylanilyticum* BT14 (Phitsuwan et al., 2012).

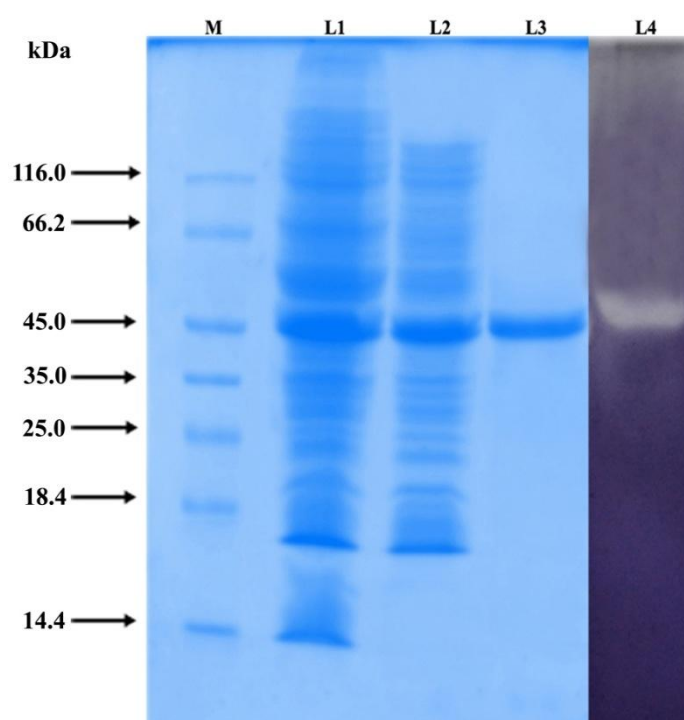


Figure 4.2.5. SDS-PAGE and Zymogram of protein produced by *B. subtilis* NA15.

M: Molecular weight marker (kDa); L1: Cell free supernatant;

L2: Partially purified protein after dialysis; L3: Active fraction of purified protein after DEAE-Sepharose ion exchange chromatography;

L4: Zymogram of purified CMCase.

4.2.3. Biochemical characterization of purified cellulase

4.2.3.1. Effect of pH on CMCase activity and stability

CMCase activity of purified protein from *B. subtilis* NA15 exhibited highest activity (3.24 U/mL) when assayed at pH 7.0 and was stable over a pH range of 5-7 (Figure 4.2.6). Enzyme was active at and retained its 82% of activity at pH 5 after an incubation of 12 h; its

activity gradually decreased beyond pH 7.0 and was totally inactive at pH 9.0 after 16 h of incubation (Figure 4.2.7).

Optimally active cellulase at pH range of 7.0 - 7.5 was also reported from *B. subtilis* and *B. circulans* (Ray et al. 2007), *Bacillus* sp C1 (Sadhu et al., 2013) and *B. amyloliquefaciens* (Lee et al., 2008).

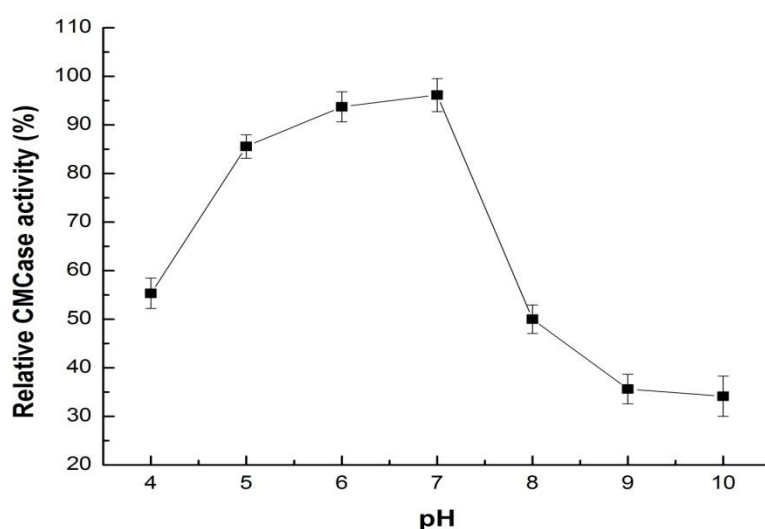


Figure 4.2.6. CMCase activity of *B. subtilis* NA15 at different pH. The *vertical bars* designate the standard deviation for the mean of three replicative tests.

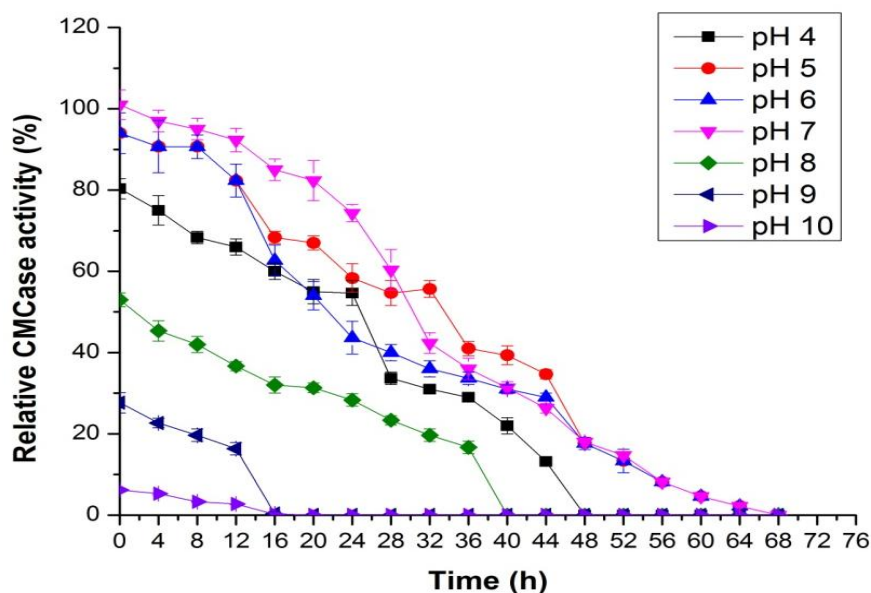


Figure 4.2.7. CMCase activity of *B. subtilis* NA15 when incubated at different pH over a period of 72 h. The *vertical bars* designate the standard deviation for the mean of three replicative tests.

However, cellulase produced from *B. subtilis* YJ1 (Yin et al. (2010) showed optimum value at pH 6-6.5. In contrast, *Bacillus* sp. AS3 (Deka et al., 2011), *B. flexus* NT (Trivedi et al., 2011) and *Bacillus* sp. HSH-810 (Kim et al., 2005) showed maximum activity at alkaline pH.

4.2.3.2. Effect of temperature on CMCase activity and thermal stability

The effect of temperature on CMCase of purified cellulase of *B. subtilis* NA15 was observed over a temperature range of 30 to 80°C (Figure 4.2.8). The maximum CMCase activity (3.24 U/mL) was found at when assay was performed at 50°C (pH 7.0), and beyond that it gradually decreased and remained 25% of its activity at assay condition of 80°C (pH 7.0). The optimum temperature of cellulase at 50°C was also found in *Bacillus* sp. C1, (Sadhu et al., 2013), *B. subtilis* A-53 (Kim et al., 2009), *B. amyloliquefaciens* DL3 (Lee et al., 2008) and *Thermomonospora* sp. (George et al. 2001). The optimum temperature was higher for cellulase obtained from *Bacillus* strains as it was 60°C in M-9 (Bajaj et al. 2009), 65°C in CH43, 70°C in HR68 (Mawadza et al. 2000). However, CMCase of *B. licheniformis* C108 was highly stable up to 100°C (Ashabdil et al. 2011).

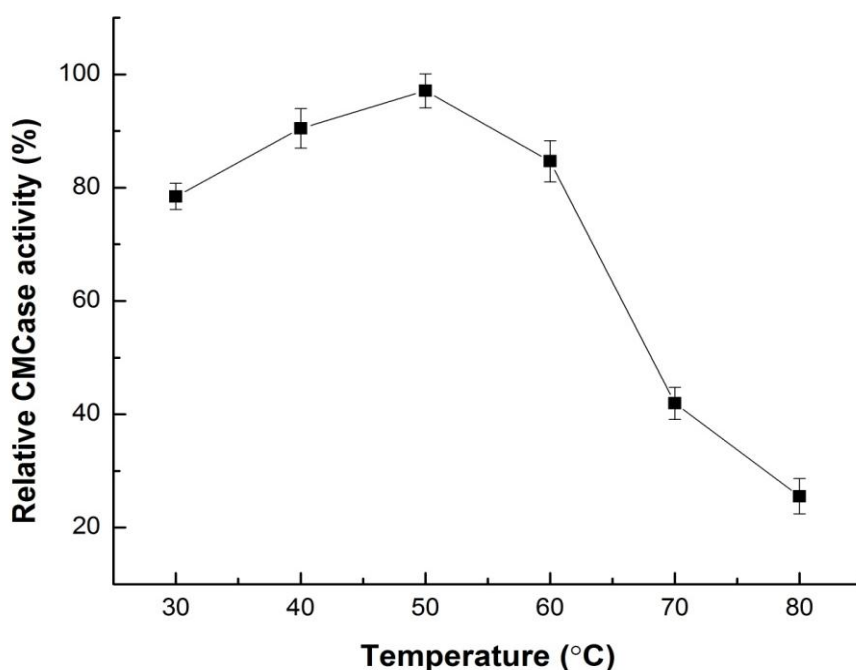


Figure 4.2.8. Effect of temperature on CMCase activity of *B. subtilis* NA15. The vertical bars designate the standard deviation for the mean of three replicative tests.

Purified CMCase from *B. subtilis* NA15 was thermally stable at an incubation temperature of 30, 40 and 50°C for 16 h, retaining its activity to 85, 75 and 60%, respectively and beyond that decreased drastically due to denaturation of the enzyme (Figure 4.2.9). Beyond 60°C of incubation temperature, CMCase activity decreased with increase in time of incubation.

CMCase activity totally disappeared after 20 h of incubation at 80°C. Thermal stability of the enzyme was also reported by Tai et al. (2004), according to which the activity of CMCase of *Geobacillus* sp. dropped rapidly after a slight increase of temperature beyond 70°C. The CMCase from *B. amyloliquefaciens* DL-3 was stable in the temperature range of 40-80°C (Lee, 2008) and in between 30 to 70°C in cellulase from *B. pumilis* EB3 (Ariffin et al., 2006) and *Bacillus* sp. C1 (Sadhu et al., 2013).

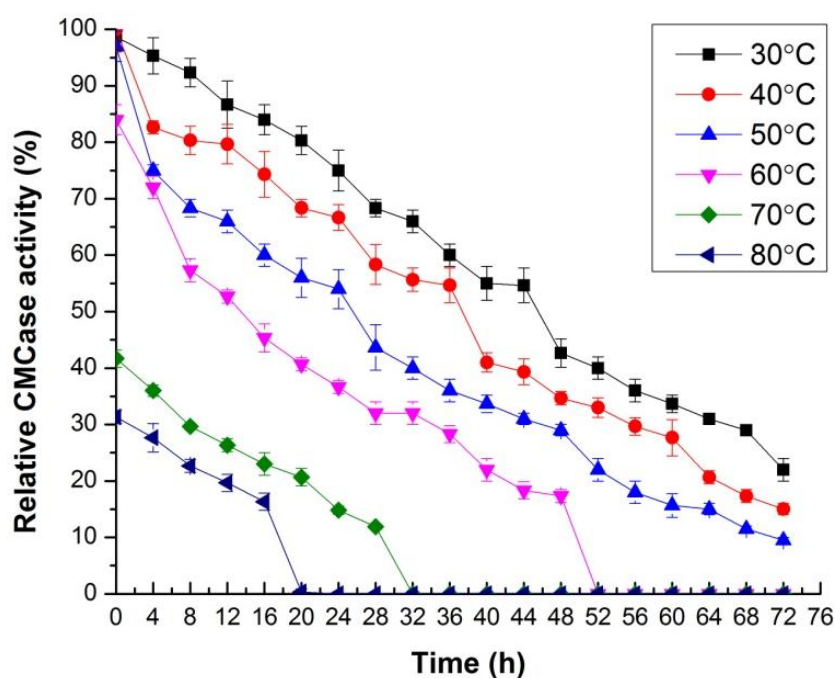


Figure 4.2.9. Thermal stability of CMCase of *B. subtilis* NA15 when incubated at different temperature over a period of 72 h. The vertical bars designate the standard errors for the mean of three replicative tests.

4.2.3.2. Effect of various chemicals on purified CMCase

The relative change in CMCase activity in presence of various metal ions and additives was studied and it was found that CMCase activity was enhanced in the presence of Mn^{2+} , Co^{2+} , Mg^{2+} , Na^+ , K^+ and Tween-80 and the relative activities obtained were 132, 116, 109, 105 103 and 102%, respectively (Table 4.2.8) at 1 mM concentration. Cu^{2+} , Hg^{2+} , Pb^{2+} , SDS, Ag^+ , Zn^+ , EDTA, Ca^{+2} and Fe^{+2} affected the CMCase activity inversely and the relative activities obtained in presence of these ions were 42, 43, 51, 66, 72, 78, 80, 88 and 90%, respectively at 1 mM concentration. Suppressive effect of Co^{2+} higher concentration (10 mM) and Tween-80 at 5 mM and 10 mM concentration was observed. However, no CMCase activity was observed in presence of Hg^{+2} and Pb^{+2} at 10 mM concentration.

Table 4.2.8. Effect of various modulators on CMCase activity.

Metal ions /additives	Relative CMCase activity (%)		
	1 mM	5 mM	10 mM
Ag ⁺	72	84.9	57.6
Ca ²⁺	88	93.3	104.4
Hg ²⁺	42.6	10.47	0
Pb ²⁺	51.5	14.07	0
Cu ²⁺	42	31.6	8.1
K ⁺	103	115.5	134.9
Na ⁺	104.7	112	117.3
Mg ²⁺	109	124.4	127.3
Mn ²⁺	132.5	148.6	169.2
Fe ²⁺	90	68.7	22.8
Co ²⁺	116	121.8	99.2
Zn ⁺	77.6	89.65	65.5
EDTA	79.5	73	14.4
SDS	66	37.1	21.2
Tween-80	102.4	82.4	56.7

[#]Control experiment was performed without any additive

These results matched fairly well with some already studied characteristics of CMCase from *Bacillus* spp. such as *Bacillus subtilis* YJ-1 (Yin et al., 210), *Bacillus* sp. VG1 (Singh et al., 2004), *Bacillus* sp. C1 (Sadhu et al., 2013), *B. amyloliquefaciens* DL-3 (Lee, 2008) and *B. flexus* NT (Trivedi et al., 2011). Study on endoglucanases from *B. subtilis* GN156 showed the inhibition effect on enzyme activity by Co²⁺ and Mn²⁺ ions (Apiraksakorn et al., 2008). On contrary, positive effect of Mn²⁺ on cellulase activity was shown by *Paenibacillus barcinonensis* (Asha et al., 2012). Hg²⁺ at 1 mM concentration completely inhibited CMCase activity of recombinant *Escherichia coli* in which endoglucanase gene of thermophilic *Bacillus* sp. PDV was cloned (Sharma et al., 1997).

4.2.3.4. Substrate specificity of partially purified enzyme

The activities of the purified enzyme with various substrates, viz. CMC (3.23 U/mL), pNPG (0.0 U/mL), barley β-D-Glucan (4.38 U/mL), lichenan (2.20 U/mL), hydroxymethyl cellulose (1.6 U/mL), avicel (0.0 U/mL), starch (2.50 U/mL) and xylans (for birchwood, oat spelt, and beechwood, the activities were 0.16, 0.18 and 0.06 U/mL, respectively) are listed in Table 4.2.9. In comparison to CMC the relative activities of purified enzyme with barley β-D-Glucan (135.6%), starch (77.4%) and lichenan (68.1%) were significantly higher than the activities towards hydroxyethyl cellulose (49.5%) and xylans (for birchwood, oat spelt, and beechwood were 4.9%, 5.6% and 1.9%, respectively).

Table 4.2.9. Specificity of the enzyme with different substrate.

Substrate	Enzyme activity [#] (U/mL)	Relative activity (%)
CMC	3.23 ± 0.12	100
pNPG	0.0 ± 0.0	0
β-D-Glucan (barley)	4.38 ± 0.21	135.6
Lichenan	2.20 ± 0.05	0
Hydroxyethylcellulose	1.6 ± 0.07	49.54
Avicel	0.0 ± 0.0	0
Starch	2.50 ± 0.05	77.4
Xylan (birchwood)	0.16 ± 0.03	4.95
Xylan (oat spelt)	0.18 ± 0.02	5.57
Xylan (beechwood)	0.06 ± 0.01	1.86

[#] Values are mean ± SD (n=3)

No detectable activities were observed with avicel (crystalline cellulose) and pNPG, indicated that the enzyme lacks exoglucanase and β-glucosidase activity. All the substrates have β-1,4-linked sugar monomers, whereas barley β-D-Glucan and lichenan has β-1,3-1,4-linkages. These results indicated that the enzyme was a β-1,3-1,4-glucanase or endoglucanase (EC 3.2.1.4). These results showed similarity with the other cellulases produced by *Bacillus* sp. such as CMCase from *B. subtilis* AS3 where barley β-D-Glucan hydrolysis resulted to 212% relative activity (Deka et al., 2013). In another study by Kim et al. (2009), CMCase produced by *B. subtilis subsp. subtilis* A-53. CMCase from *B. amyloliquefaciens* DL-3 hydrolysed CMC, avicel, barley β-D-Glucan and xylan but could not hydrolyse pNPG showing the absence of cellobiase activity (Lee et al., 2008).

4.2.4. Enzyme kinetics of purified CMCase

After investigating the CMCase activity of DEAE-Sepharose purified protein, the next step was to investigate the kinetics of purified CMCase of *B. subtilis* NA15. The Lineweaver - Burk plot was linear and indicated that hydrolysis of CMC by the enzyme CMCase followed Michaelis-Menten kinetics in purified enzyme. The graph gave two kinetic parameters, *K_m* and *V_{max}*, which indicate the substrate affinity of enzyme. The *K_m* and *V_{max}* of the purified CMCase was found to be 0.124 mg/mL and 2.272 μmoles/mg/min, respectively (Figure 4.2.9, Table 4.2.10). A lower *K_m* and higher *V_{max}* value is desirable for an enzyme to show higher affinity towards a specific substrate.

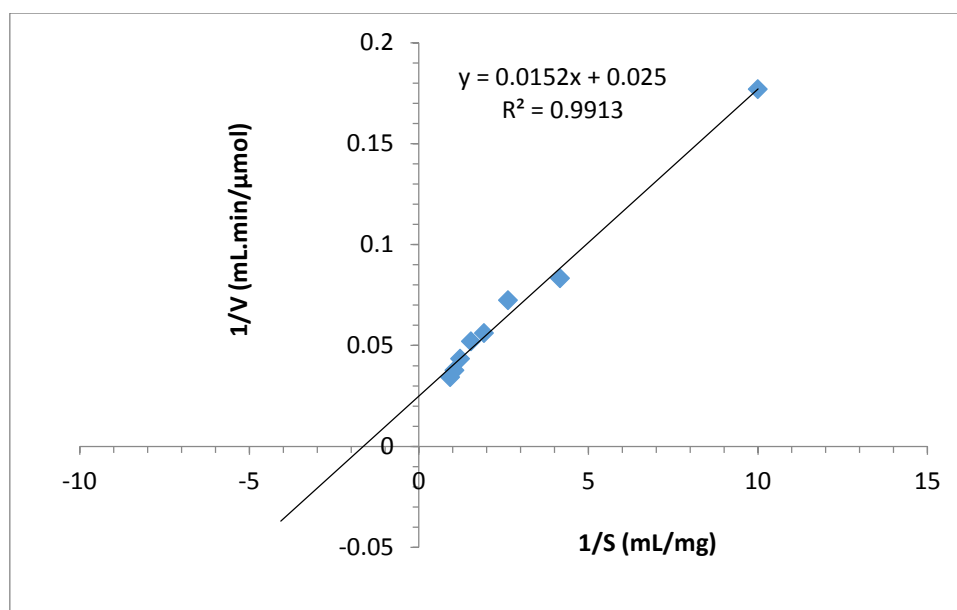


Figure 4.2.9. Lineweaver-Burk plot for determination of kinetics of purified CMCase.

Table 4.2.10. Comparison of various characteristics of cellulases from different *Bacillus* spp.

Bacteria	Molecular mass (kDa)	K _m (mg/ml)	V _{max} (μmoles/mg/min)	Reference
<i>B. subtilis</i> NA15	45	0.12	2.27	This study
<i>B. subtilis</i> YJ1	32.5	ND	ND	Yin et al., 2010
<i>B. amyloliquefaciens</i> DL-3	54	ND	ND	Lee et al., 2008
<i>B. subtilis</i> A-53	56	ND	ND	Kim et al., 2009
<i>Bacillus</i> sp. M-9	54	ND	ND	Bajaj et al., 2009
<i>B. licheniformis</i>	37	ND	ND	Bischoff et al., 2006
<i>Bacillus</i> sp. PDV	33	0.588	ND	Sharma et al., 1990
<i>Bacillus</i> sp. CH43	40	1.5	0.00093	Mawadza et al., 2000
<i>Bacillus</i> sp. HR68	40	1.7	0.0017	Mawadza et al., 2000
<i>B. subtilis</i> AS3	30	0.13	3.38	Deka et al., 2013
<i>B. subtilis</i> GN156	25	1.53	0.0085*	Apiraksakorn et al., 2008
<i>Bacillus</i> sp. C1	97	0.25	20 [#]	Sadhu et al., 2013
<i>Bacillus</i> sp.	58	ND	ND	Vijayaraghavan et al., 2012
<i>Bacillus</i> sp. DUSELR13	ND	3.11	0.56*	Rastogi, et al., 2010
<i>B. flexus</i> NT	97	6.18	370.17	Trivedi et al., 2011

*Value in U/mL, [#]Value in μmoles/mL/min

The K_m value of 0.124 mg/mL was in close proximity with CMCase produced by *Bacillus* sp. AS3 (0.13 mg/mL) (Deka et al., 2013), while it was lower than that of *Bacillus* sp. C1 (0.25 mg/mL) (Sadhu et al., 2013), *Bacillus* sp. PDV (0.588 mg/mL) (Sharma et al., 1990), *Bacillus* sp. CH43 (1.5 mg/mL) (Mawadza et al., 2000), *B. subtilis* GN156 (1.53 mg/mL) (Apirasakorn et al., 2008), *Bacillus* sp. HR68 (1.7 mg/mL) (32) *Bacillus* sp. DUSELR13 (3.11 mg/mL) (Rastogi, et al., 2010) and *B. flexus* NT (6.18 mg/mL) (Trivedi et al., 2011) (Table 4.2.10).

The value of V_{max} (2.27 μ moles/mg/min) obtained in this study was higher than that showed by CMCase produced from, *Bacillus* sp. CH3 (0.00093 μ moles/mg/min) (Mawadza et al., 2006), *Bacillus* sp. HR68 (0.0017 μ moles/mg/min) (Mawadza et al., 2006) and lower than CMCase from *B. subtilis* AS3 (3.38 μ moles/mg/min) (Deka et al., 2013), *B. flexus* NT (370.17 μ moles/mg/min) (Trivedi et al., 2011) (Table 4.2.10).

Conclusion

The aim of the present work was to optimize media ingredients for enhanced CMCase production and further characterization of CMCase from *B. Subtilis* NA15. The screening of 11 nutrients was done using Plackett-Burman design to explicate the parameters that significantly influence the carboxy methyl cellulase (CMCase) production. Maximum CMCase production of 0.47 U/mL was obtained using Response Surface Methodology (RSM) with carboxy methyl cellulose (CMC): 18 g/L, peptone: 5 g/L, yeast extract: 5 g/L and $MnCl_2$: 0.5 g/L. The model predicted the maximum CMCase activity (0.45 U/mL) which was in good agreement with the experimental value of 0.47 U/mL showing 7-fold increase as compared to unoptimized medium. Presence of $MnCl_2$ in the medium significantly enhanced the CMCase production. The enzyme was purified by ion exchange chromatography and 11.5 fold increase in CMCase activity with protein yield of 43.75% was obtained. The molecular weight of the CMCase was approximately 45 kDa as determined by SDS-PAGE and zymogram analysis. The purified CMCase hydrolyzed carboxymethylcellulose (CMC), barley- β -D-Glucan, lichenan, hydroxyethylcellulose, starch and xylan but not avicel and p-nitrophenyl- β -D-glucopyranoside (pNPG). The optimal temperature and pH for CMCase activity was 50°C and 7.0. Purified CMCase was highly stable up to 12 h at pH 5.0 and 30°C by retaining its activity to 82 and 87%, respectively. The robust nature of this enzyme made them efficient in hydrolysis while use during SSF process.

4.3. Optimization of ethanol production by simultaneous saccharification and fermentation (SSF)

To optimize the ethanol production via SSF, agricultural and leaf litter biomass were collected, washed to remove adhering debris, dried, grounded and then sieved to a mesh size of 0.5 mm. Biomass samples from different sources such as rice and wheat straw and leaf litters of Mango, Poplar, Ashoka and Eucalyptus trees were characterized for physico-chemical properties to gather a clue for selecting promising substrate for production of ethanol. Different combinatorial pre-treatment strategies were applied to enhance sugar yield from these biomasses to obtain fermentable sugar for ethanol production. Further, different parameters were optimized using Taguchi design to obtain greater yield of ethanol via SSF using pre-treated biomass as feedstock.

4.3.1. Physico-chemical characterization of agricultural waste and leaf litter biomass

4.3.1.1. Moisture and ash content

Moisture content plays a considerable role regarding selection of energy conversion process technology (Cuiping et al., 2004). Among agricultural waste biomass, wheat and rice straw showed moisture content of 6.5 and 5.6%, respectively, whereas leaf litters from Mango, Poplar, Ashoka, and Eucalyptus had moisture content of 7.6, 8.3, 7.3 and 4.4%, respectively (Table 4.3.1). Poplar leaf litters showed highest moisture content of 8.3% followed by Mango leaf litter (7.6%). Comparative low moisture content (<15%) feedstock is preferred for thermo-chemical conversion process, while bio-conversion can utilize biomass with high or optimum moisture content (Nanda et al., 2013).

Ash content in wheat and rice straw, Mango, Poplar, Ashoka and Eucalyptus leaf litter biomass was found to be 2.4, 8, 9.5, 3.7, 8.7 and 3%, respectively (Table 4.3.1). Ash content of wheat straw was observed to be minimum (2.4%), among all six biomasses under study. Ash, the incombustible solid mineral matter of the biomass, mainly contains oxides of silica (SiO), Alumina (AlO), Iron (FeO), calcium (CaO) and Magnesium (MgO). The chemical compositions of the ash are closely related to operational problems such as slagging, fouling, sintering and corrosion, due to high amount of alkali. A significant fraction (up to one-fifth) of herbaceous biomass consists of ash, which cannot be converted to energy. Greater ash content is believed to hinder enzymatic saccharification of lignocellulosic biomass (Bin and Hongzang, 2010). Utilization of biomasses as biofuel feedstock with higher ash content requires washing to remove

water soluble and loosely adhered inorganic materials and appropriate pre-treatment approach to minimize ash content.

Moisture and ash content of different lignocellulose biomass falls in the range of 5.0-7.9 and 1.1-9.8%, respectively (Naik et al., 2010). *Areca catechu*, *Ziziphus rugosa* and *Albizia lucida* biomass showed moisture content of 6.6, 5.2 and 5.8% and ash content of 2.9, 1.3 and 1.5%, respectively (Sasmal et al., 2012).

4.3.1.2. Volatile matter and Fixed carbon

Volatile matter in wheat straw, rice straw, Mango, Poplar, Ashoka and Eucalyptus leaf litter biomass was found to be 78.5, 68.4, 75, 76, 75.3 and 80.8%, respectively (Table 4.3.1). Highest volatile matter (80.8%) was observed for Eucalyptus leaf litter biomass followed by wheat straw, which was 78.5%. Wheat and rice straw, Mango, Poplar, Ashoka and Eucalyptus leaf litter biomass showed 12.5, 17.9, 7.7, 11.2, 7.7 and 12.1% fixed carbon, respectively. Highest fixed carbon (17.9%) was observed for rice straw followed by wheat straw, which had 12.5% fixed carbon.

Volatile matter and fixed carbon of Canadian biomass samples were reported in the range of 77-82% and 4.8-14.4%, respectively (Naik et al., 2010). Biomass from *Areca catechu*, *Ziziphus rugosa* and *Albizia lucida* also showed volatile matter of 82.4, 78.3 and 75%, respectively and fixed carbon content of 8, 15 and 17%, respectively (Sasmal et al., 2012). In biomass fuel, chemical energy is stored mainly in the form of fixed carbon and volatile matter. The portion driven-off as a gas (including moisture) by heating to 950°C for 7 min is volatile matter whereas the non volatile organic mass which may also contain oxygen with hydrogen, excluding the ash and moisture contents is fixed carbon (Parikh et al., 2007). The fixed carbon is used to relate the heating value of the product and co-products. The significance of the volatile matter and fixed carbon contents is that they provide a measure of the ease with which the biomass can be ignited and subsequently gasified, or oxidised, depending on how the biomass is to be utilized as an energy source (McKendry, 2002). Both volatile matter and fixed carbon have positive effect on the calorific value (Majumder et al., 2008).

Table 4.3.1. Proximate analysis of different biomass.

Biomass	*Proximate analysis (wt.%)			
	Moisture	Ash	Volatile matter	Fixed carbon
Wheat straw	6.50 ± 0.35bc	2.45 ± 0.18c	78.50 ± 5.57a	12.51 ± 0.88b
Rice straw	5.65 ± 0.43cd	8.04 ± 0.63b	68.43 ± 3.79a	17.88 ± 1.21a
Mango leaf litter	7.57 ± 0.47ab	9.46 ± 0.60a	75.01 ± 4.77a	7.71 ± 0.4c
Poplar leaf litter	8.32 ± 0.70a	3.70 ± 0.28c	76.05 ± 6.25a	11.23 ± 0.78b
Ashoka leaf litter	7.34 ± 0.69ab	8.67 ± 0.65ab	75.34 ± 4.18a	7.66 ± 0.71c
Eucalyptus leaf litter	4.45 ± 0.37d	3.06 ± 0.13c	80.84 ± 6.04a	12.17 ± 0.56b

*Values are mean ± SD (n =3), bearing different letters in the same column are significant at P<0.05.

Table 4.3.2. Ultimate analysis and calorific value of biomass.

Biomass	*Ultimate analysis (wt. %)					Calorific value (MJ/kg)
	C	H	N	S	O	
Wheat straw	39.28 ± 2.43a	4.53 ± 0.25b	0.64 ± 0.02d	0.06 ± 0.004c	55.46 ± 3.0ab	15.55
Rice straw	32.45 ± 1.30b	5.17 ± 0.23ab	0.95 ± 0.04c	0.20 ± 0.010a	61.23 ± 3.67a	13.17
Mango leaf litter	39.32 ± 2.40a	5.25 ± 0.20ab	0.28 ± 0.01e	0.10 ± 0.008b	55.05 ± 3.95ab	18.37
Poplar leaf litter	40.68 ± 3.08a	4.81 ± 0.49ab	1.40 ± 0.03b	0.02 ± 0.002e	53.09 ± 2.55ab	18.50
Ashoka leaf litter	44.78 ± 2.39a	5.55 ± 0.15a	1.48 ± 0.09b	0.09 ± 0.004b	48.10 ± 2.5b	19.32
Eucalyptus leaf litter	45.41 ± 2.10a	5.15 ± 0.35ab	2.49 ± 0.12a	0.04 ± 0.002d	46.91 ± 2.91b	19.91

*Values are mean ± SD (n =3), bearing different letters in the same column are significant at P<0.05.

4.3.1.3. CHNSO analysis

Ultimate analysis illustrates the biomass composition in correspondence to the top five element carbon (C), hydrogen (H), nitrogen (N), sulphur (S) and oxygen (O) and total mineral matter (Shukla,1997). Ultimate composition together with calorific value of different biomass is presented in Table 4.3.2. The percentage of carbon in wheat straw, rice straw, Mango, Poplar, Ashoka and Eucalyptus leaf litter biomass was 39.3, 32.4, 39.3, 40.7, 44.8 and 45.4%, respectively. The oxygen, hydrogen, nitrogen and sulphur content showed variation in the range of 46-61, 4.5-5.5, 0.2-2.5 and 0.02-0.2%, respectively.

Carbon, nitrogen and oxygen are the main constituent of solid fuels, carbon is the principal element among them, accrued from atmospheric CO₂ during the process of photosynthesis and majorly contributes to total heating value of biomass. Amount of carbon relates to total carbohydrate content of lignocelluloses, whereas N and S correspond to gaseous emissions from feedstock. Ultimate analysis is particularly important in evaluating fuel feedstock in terms of its pollution potential, higher heating value and also theoretical air-fuel ratio (Mansaray, 1997). In contrast to conventional fuels, biofuels demonstrate higher proportion of O and H, compared with C, deteriorating fuel energy value due to lower energy contained in C=O and C-H bonds, in comparison to C-C bonds (McKendry 2002). High nitrogen and ash content reduce hydrocarbon yield (Sanderson, 1996) as composition of biomass affect the conversion process (Naik et al., 2010). In response, it can be advocated that lower nitrogen content (<0.3%) of native lignocellulosic biomass suits enzymatic saccharification for ethanol production. Similar studies were carried out to determine the CHNSO analysis of Canadian biomasses (Naik et al., 2010) and biomasses of North-East Indian region (Sasmal et al., 2012) and biomass and biochars of North America (Nanda et al., 2013) for assessing potential of lignocellulosic feedstocks.

4.3.1.4. Calorific value

Wheat straw, rice straw, Mango, Poplar, Ashoka and Eucalyptus leaf litter exhibited calorific value of 15.5, 13.2, 18.4, 18.5, 19.3 and 19.9 MJ/kg, respectively (Table 4.3.2). Eucalyptus leaf litter biomass showed highest (19.9 MJ/kg) whereas rice straw showed minimum calorific value of 13.1 MJ/kg.

The calorific value or gross calorific value (GCV) of lignocellulosic feedstocks is foremost aspect which signifies energy or amount of heat released when ignited in presence of excess air. Demirbas (2001) reported HHV of 18.60 kJg⁻¹ for holocellulose (cellulose and hemicelluloses),

whereas it was in the range of 23.26 to 26.58 kJg⁻¹ for lignin due to higher state of oxidation. Gasoline and diesel has heating value of about 47 and 45 MJ/kg, respectively, used as fuel for running internal combustion engines, whereas, heating values of 15-25 MJ/kg was observed with different biomasses (Capareda, 2009). High calorific value of wheat straw (20.3 MJ/kg), pinewood (19.6 MJ/kg) (Naik et al., 2010), biomass of *Ziziphus rugosa* (21.24 MJ/kg) and *Albizia lucida* (20.3 MJ/kg) (Sasmal et al., 2012) makes the biomass more efficient for the production of bio-energy.

4.3.1.5. Extractive in different biomass

The yield of hexane, alcohol and water extracts from different biomass samples are presented in Table 4.3.3. Higher water extractives were observed in Mango leaf litter (13.39%) whereas more hexane (15.2%) and ethanol (12.3%) extractive were found in Eucalyptus leaf litter biomass. The elevated amount of total extractive (37.9%) was observed in Eucalyptus leaf litter biomass followed by Mango and Ashoka leaf litter biomass which was 31.7 and 29.7%, respectively. The total extractives in the Canadian biomass and biomass of North-East India was reported in the range of 14.1-19% (Naik et al., 2010) and 14.07-18.84% (Sasmal et al., 2012), respectively. Extractives present in biomass comprised of non-structural aromatic compounds, which possess one or more phenolic hydroxyl groups (Senelwa and Sims, 1999). The knowledge of the amount of extractives helps to estimate exact amount of fermentable sugars present in biomass (Sasmal et al., 2012). Extractives from lignocellulose biomass include polar and non-polar compounds such as resins, terpene, waxes etc. which are responsible for the protection of plants from microorganisms and termites. Other than biofuel production, these compounds have other value as green chemical. Extraction of non polar compounds, hexane and ethanol extraction was carried out followed by extraction of polar compounds from raffinate biomass with water (Sasmal et al., 2012).

Table 4.3.3. Extractive in g from 100 gm of biomass.

Biomass	Hexane extract	Ethanol extract	Water extract	Cellulose	Hemicellulose	Lignin
Wheat straw	1.92 ± 0.27e	2.47 ± 0.18e	11.38 ± 0.64b	36.06 ± 2.22b	28.81 ± 1.63ab	18.45 ± 1.05a
Rice straw	6.34 ± 0.16d	6.85 ± 0.11d	9.33 ± 0.79bc	43.38 ± 3.35a	24.39 ± 2.08bc	9.45 ± 0.69c
Mango leaf litter	9.65 ± 0.55bc	8.68 ± 0.55c	13.39 ± 0.92a	31.23 ± 1.71bc	28.02 ± 3.02ab	10.56 ± 0.79bc
Poplar leaf litter	8.60 ± 0.54c	9.79 ± 0.52bc	9.95 ± 0.73c	37.75 ± 4.21ab	21.85 ± 1.55cd	11.95 ± 0.80b
Ashoka leaf litter	10.59 ± 0.81b	10.26 ± 0.66b	8.84 ± 0.53c	26.65 ± 1.64c	32.04 ± 2.19a	10.63 ± 1.18bc
Eucalyptus leaf litter	15.19 ± 0.95a	12.34 ± 0.54a	10.40 ± 0.63bc	31.46 ± 2.51bc	17.65 ± 2.20d	12.77 ± 0.38b

*Values are mean ± SD (n =3), bearing different letters in the same column are significant at P<0.05.

4.3.1.6. Cellulose, hemicellulose and lignin content

Cellulose content of rice straw and Poplar leaf litter biomass was found to be 43.4 and 37.7%, respectively, followed by wheat straw (36.1%), Eucalyptus (31.5%), Mango (31.2%) and Ashoka leaf litter biomass (26.6%) (Table 4.3.3). Compared to other biomasses under study more hemicellulose content (32%) was depicted in Ashoka leaf litter biomass, whereas minimum lignin content (9.4%) was observed in rice straw. The hemicellulose content was found less in all four leaf litter biomass studied than the reported value by Prasad et al. (2007).

Naik et al. (2010) showed cellulose content of 34.6, 39.0 and 32.5% for wheat straw, pine wood and barley straw, respectively. North-East Indian biomasses from *Albizia lucida*, *Ziziphus rugosa* and *Areca catechu* also showed cellulose content of 53.46, 51.56 and 44.46%, which may be potential feedstock for renewable fuel. Cellulose and hemicellulose represent potentially fermentable carbohydrate fraction in form of hexoses and pentoses and for bio fuel generation but lignin retards conversion process owing to its recalcitrance and interlinking properties with hemicelluloses.

4.3.1.7. Scanning electron microscopy (SEM)

Physical appearance of all six native biomass samples (0.5 mm) was observed using SEM at different magnifications (Figure 4.3.1). More compact surface structure was seen in rice straw (Figure 4.3.1 b) and Poplar leaf litter (Figure 4.3.1 d). Micrographs of wheat straw (Figure 4.3.1 a), Mango leaf litter (Figure 4.3.1 c), Ashoka leaf litter (Figure 4.3.1 e) and Eucalyptus leaf litter (Figure 4.3.1 f) showed less compact surface structure which may be more prone towards hydrolytic enzymes. Although slight variation in surface structure was found during observation at different magnifications, probably due to difference in the sample texture of various component of the same leaf.

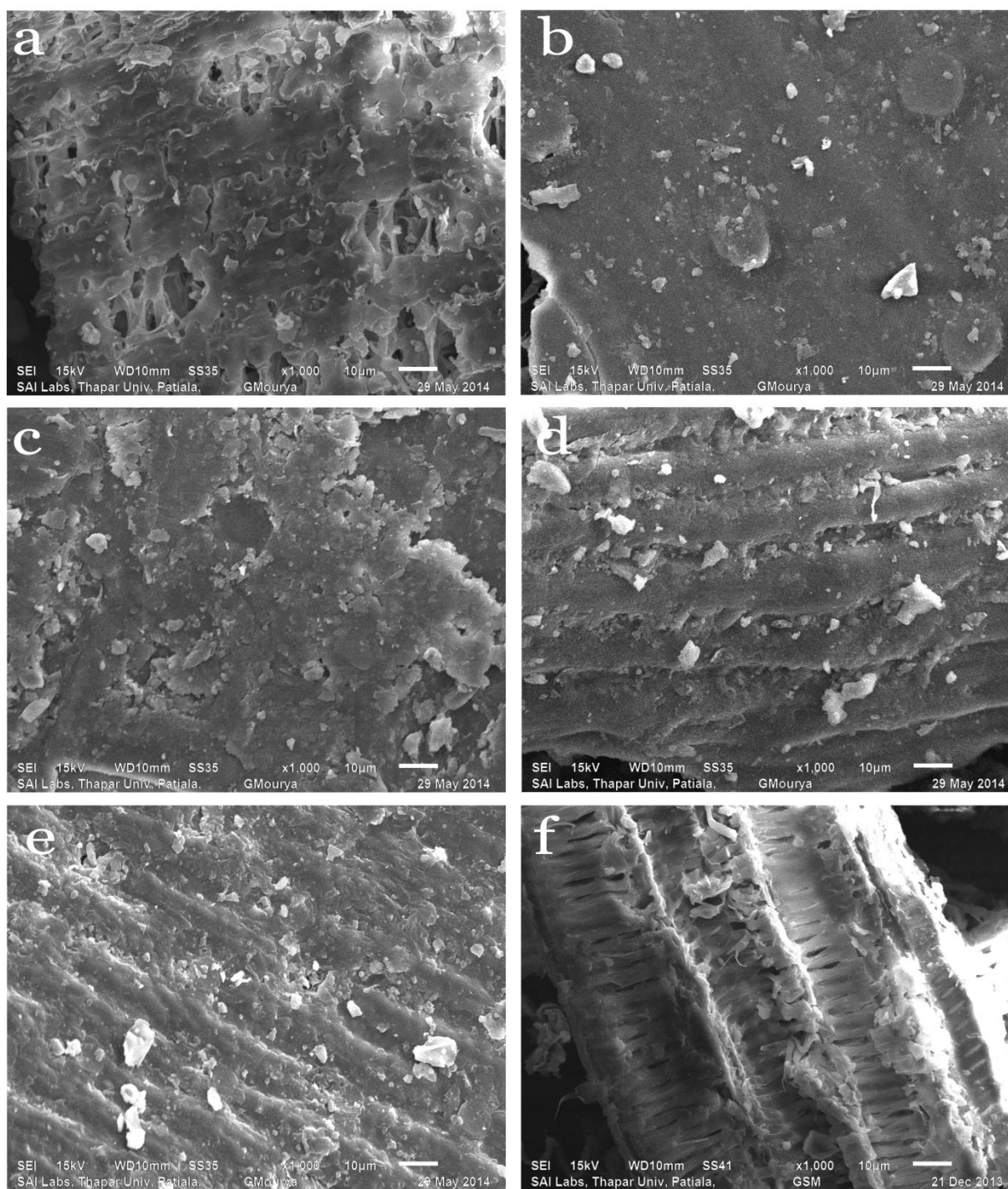


Figure 4.3.1. Scanning electron micrographs of different native biomass samples (0.5 mm).

a: Wheat straw; b: Rice straw; c: Mango leaf litter; d: Poplar leaf litter;
e: Ashoka leaf litter; f: Eucalyptus leaf litter. (Magnification: 1,000X)

4.3.1.8. X-ray diffraction analysis (XRD)

XRD analysis of various biomass samples are presented in Figure 4.3.2. Crystallinity index (CrI) of these samples along with crystalline and amorphous peak is presented in Table 4.3.4. Maximum CrI was observed in wheat straw (46.63%), followed by rice straw (39.55%), Eucalyptus (32.23%), Mango (23.13 %), Ashoka (20.94 %) and Poplar leaf litter biomass (20.93%). The lower value of CrI advocated that Ashoka leaf litter biomass has properties of easy degradability.

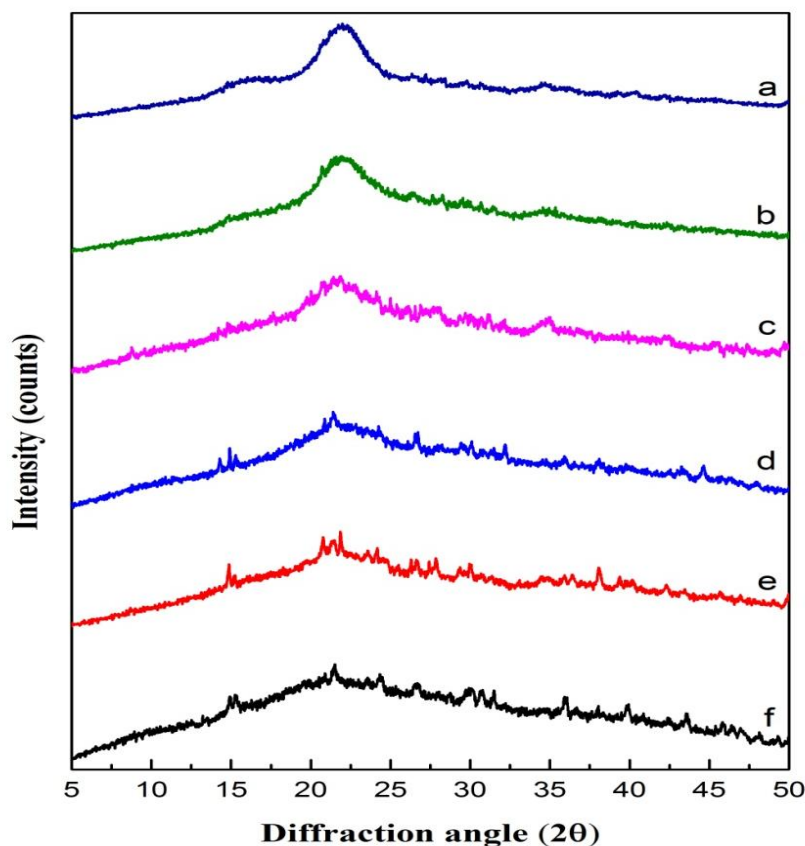


Figure 4.3.2. XRD analysis of different native biomass samples.

a: Wheat straw; b: Rice straw; c: Mango leaf litter;
d: Poplar leaf litter; e: Ashoka leaf litter; f: Eucalyptus leaf litter.

Table 4.3.4. Peak at crystalline and amorphous region and *CrI* of different native biomass.

Biomass	Crystalline portion peak (I_{002})	Amorphous portion peak (I_{am})	<i>CrI</i> (%)
Wheat straw	22.34	18.52	46.63
Rice straw	22.25	18.05	39.55
Mango leaf litter	22.89	18.45	23.13
Poplar leaf litter	22.82	18.62	20.93
Ashoka leaf litter	22.30	18.49	20.94
Eucalyptus leaf litter	21.51	18.44	32.23

All six biomass under study showed peak of crystalline region at 2θ angle range of 21.51- 22.89, whereas amorphous portion peak in the range of 18.05-18.62. Overall biomass crystallinity depends on cellulose, hemicellulose and lignin content (Meshitsuka and Isogai et al., 1996). Highly crystalline of biomass corresponded to higher *CrI*, hinders digestibility of cellulose. However, due to the heterogeneous nature of celluloses, presence of hemicelluloses and lignin, it is not the only factor plays an effective enzymatic in hydrolysis of biomass (Taherzadeh and Karimi, 2008). *CrI* of 48% was observed for sugarcane bagasse (Corrales et al., 2012), 33% for corn stover (Zeng et al., 2011), 46.43% for *Albizia lucida* and 42.46% for *Ziziphus rugosa* and 63.84% for *Areca catechu* husk (Sasmal et al., 2012). Amorphous cellulose is readily accessed by cellulase as compared to crystalline microfibrils of cellulose (Taherzadah and Karimi, 2008) and it is widely accepted that decrease in crystallinity increases the digestibility of lignocelluloses (Fan et al., 1980; Chang and Holtzapple, 2000; Nanda et al., 2013). On contrary, more crystalline lignocelluloses have been reported to show more digestibility (Grethelin 1985; Wyman, 1996; Xiao et al., 2011; Kuila et al., 2011).

4.3.1.9. Thermal gravimetric analysis (TGA)

The TGA of agricultural and leaf litter biomasses showed onset temperature of devolatilization in the range of 200-250°C for all the six biomass studied, which corresponds to about 10% weight loss with respect to the final weight loss (Figure 4.3.3). The main weight loss ends at 350 -390°C followed by a slow and continuous loss. The former step was due to the primary devolatilization, where as the later was attributed to the degradation of heavier chemical structures in the solid matrix (Fisher et al., 2002). The hemicellulose and cellulose part of

biomass usually start to decompose at around 206°C and 315°C, respectively, while the decomposition of lignin start above 330°C (Hornof et al., 1977; Ledakowicz and Stolerak 2002). The thermal decomposition study was conducted on rapeseed (*Brassica napus* L), sponge ground fibers, sugarcane bagasse, banana fibers by several researchers and their study revealed that the thermal decomposition of hemicelluloses occurred at temperatures ranging from 150 to 350°C, cellulose decomposed at temperature range of 275-350°C, and lignin decomposed gradually between the temperatures 250°C and 500°C (Guimaraes et al., 2009; Chen et al., 2010).

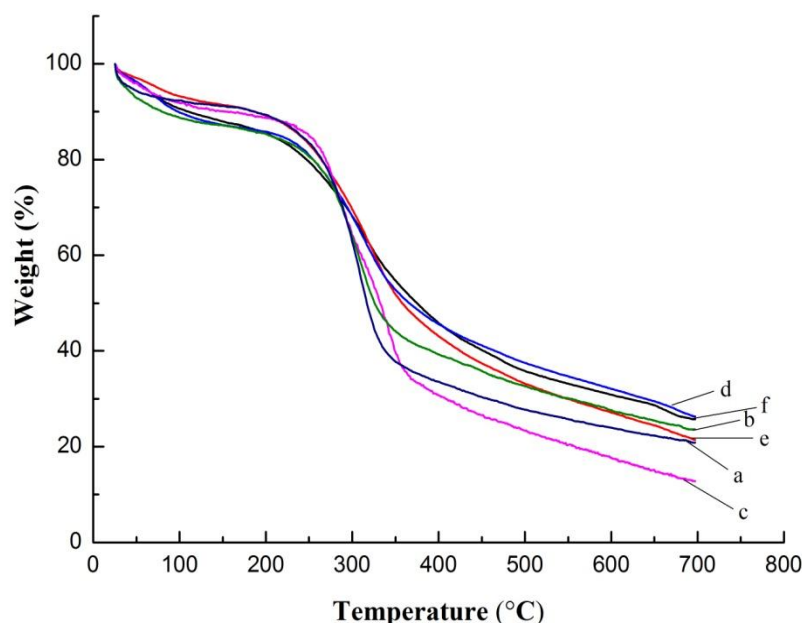


Figure 4.3.3. TGA of different native biomass samples.

a: Wheat straw; b: Rice straw; c: Mango leaf litter;
d: Poplar leaf litter; e: Ashoka leaf litter; f: Eucalyptus leaf litter.

4.3.1.10. Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared (FTIR) spectroscopy analysis was carried out to detect changes in functional groups of different biomass. The obtained spectrum is represented in Figure 4.3.4. The main characteristics were attributed to different chemical group present in cellulose, hemicellulose and lignin components of biomass. The peaks in fingerprint are based on literature values (Adel et al., 2010; Xiao et al., 2011; Meng et al., 2012; Zhang et al., 2013) which is summarised in Table 4.3.5. Most prominent bands in all six biomass sample were 3402, 2910, 2843, 1635, 1375, 1232 and 1043 cm^{-1} (Figure 4.3.4).

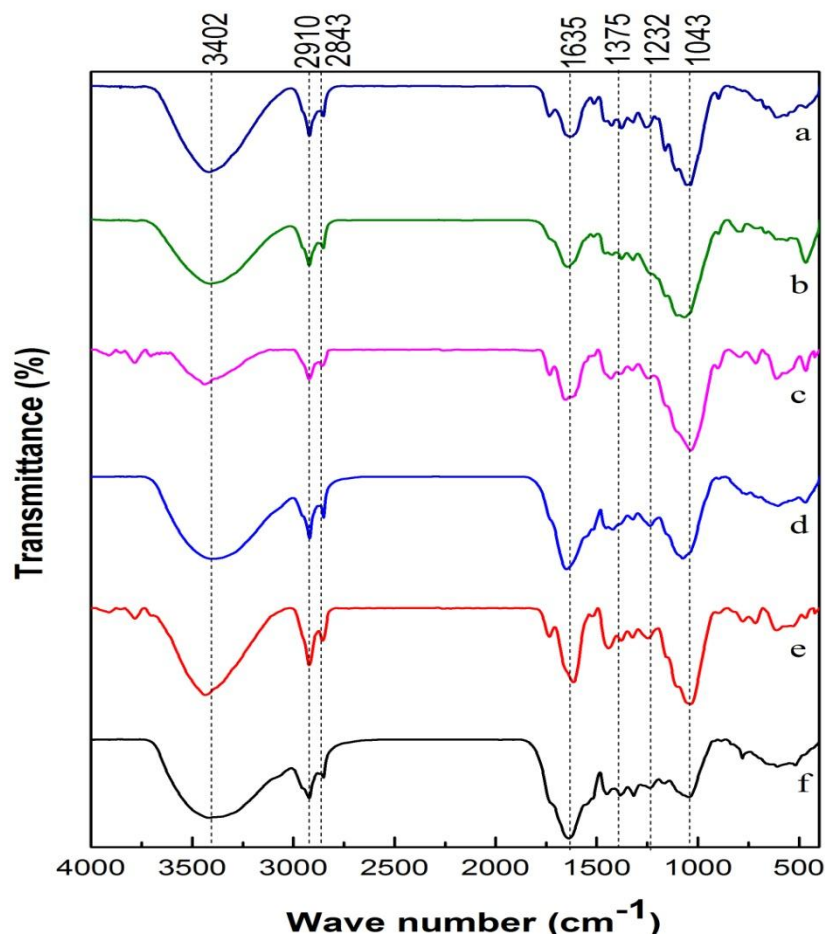


Figure 4.3.4. FTIR spectra of native biomass samples.

a: Wheat straw; b: Rice straw; c: Mango leaf litter;
d: Poplar leaf litter; e: Ashoka leaf litter; f: Eucalyptus leaf litter.

Table 4.3.5. Assignment of FTIR bands of functional groups in lignocellulosic biomass samples

Name of characteristic group	Wavenumber (cm ⁻¹)
O-H stretching vibration (hydrogen bonded) groups and C-H stretching from cellulose, lignin and hemicelluloses	3402
	2910
Methoxyl (-OCH ₃), C-H stretching	2843
Bending mode of the absorbed water and the stretching of C=O in lignin	1635
Aliphatic C-H deformation vibration in cellulose and hemicelluloses	1375
Syringyl ring breathing and C-O stretching out of lignin and xylan	1232
C-O stretching vibration in cellulose and hemicelluloses	1043

The band in the range of 1500-1700 cm^{-1} represents the aromatic ring stretch of lignin, 3600-3200 cm^{-1} is related to O-H stretching vibrations of alcohol and phenol, 1400 to 900 cm^{-1} is designated to vibrations of variety of bonds such as C-H, C-O, C-N and P-O, O-H and C-H symmetric and asymmetric stretching bands appears in the region of 2900 cm^{-1} . Pronounced peak at 1043 and 1375 cm^{-1} which are assigned to vibration for cellulose and hemicellulose chemical groups, conveys more carbohydrate content of lignocellulose biomass whereas peak at 1635 and 1232 cm^{-1} attribute higher lignin content. It is evident (Figure 4.3.4) that Poplar leaf litter biomass and wheat straw are enriched with more carbohydrate in comparison to other biomass while Ashoka leaf litter showed greater lignin content. FTIR of wheat straw was reported by Zhang et al. (2008) with intense peaks at 1045, 1130, 1249 and 1270 cm^{-1} and 3320, 2916, 2859, 1733, 1648, and 1600 cm^{-1} for rice straw (Li et al., 2012).

4.3.1.11. Solid state ^{13}C CP/MAS NMR spectroscopy

Solid state ^{13}C CP/MAS NMR spectra provide characteristic chemical shift value ascribed to cellulose, hemicellulose and lignin which is represented in Figure 4.3.5. Signal assignment for various biomasses has been assigned based on the literatures (Sun et al., 2004; Rezende et al., 2011; Shi et al., 2011; Kohn et al., 2011) and summarised in Table 4.3.6. Intense peaks were observed at 172, 104, 72, 63 and 32 ppm. Peaks assigned in the region of 50 to 120 ppm are attributed mostly to cellulose carbons, but also with contributions from hemicellulose and lignin signals. Signals from lignin are concentrated mainly between 110 to 160 ppm. However, an aliphatic region for lignin lies from 0-95 ppm whereas, an aromatic region falls in between 100 and 160 ppm (Himmelsbach et al., 1983).

Table 4.3.6. Signal assignments for chemical shifts obtained from solid state ^{13}C CP/MAS NMR spectroscopy of different biomass.

Chemical shift (ppm)	Assignments
173	Carboxyl group of hemicelluloses
104	Anomeric carbon (C1) of cellulose
83	C4 of amorphous cellulose and hemicelluloses
72	C2, C3, C5 of cellulose, $\text{OC}_\alpha\text{H}_2$ carbon of lignin
63	C6 of crystalline cellulose
32	Methoxyl ($-\text{OCH}_3$) and methylene ($-\text{CH}_2$) groups in lignin
23	CH_3 in acetyl group of hemicelluloses

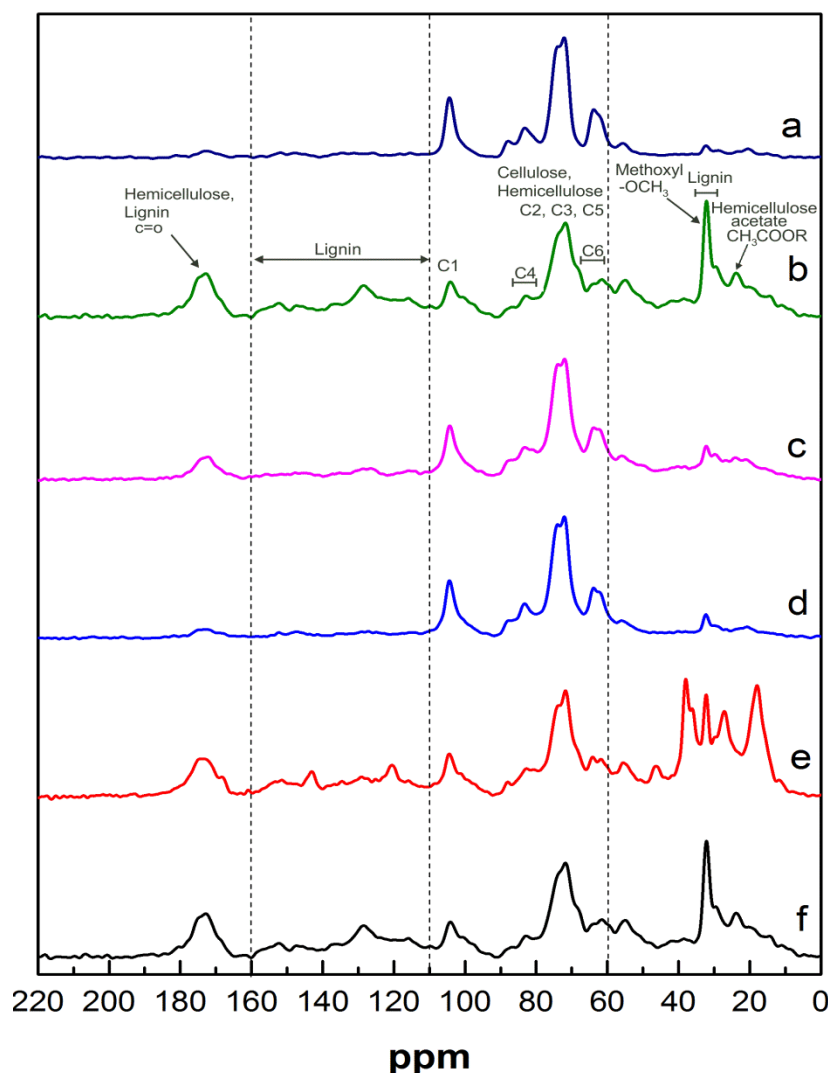


Figure 4.3.5. Solid state ^{13}C CP/MAS NMR spectra of different native biomass samples.

a: Wheat straw; b: Rice straw; c: Mango leaf litter;

d: Poplar leaf litter; e: Ashoka leaf litter; f: Eucalyptus leaf litter.

Intense peak at 32 ppm due to acetyl and methyl group of lignin was observed for rice straw, Ashoka and Eucalyptus leaf litter biomass. Peak at 23 ppm is assigned for acetyl group of hemicelluloses and prominent in rice straw and Ashoka leaf litter biomass. Prominent peak at 172 ppm in rice straw and Mango, Eucalyptus and Ashoka leaf litter biomass and 83 ppm in wheat straw, rice straw and Poplar leaf litter biomass, renders a clue for more cellulose and hemicellulose content. Chemical shift obtained at 104, 63 and 83 ppm represent greater carbohydrate content for different biomass samples. Biomass with high cellulose content showed intense peak at 72 ppm, assigned for C2, C3 and C5 of cellulose, respectively. Peak at 172 and

104 ppm correspond to acetyl groups of hemicellulose and C1 of cellulose. Sharp signals at 105, 89 and 65 ppm correspond to ordered cellulose which has been observed in *Tamarix ramosissima* (Xiao et al., 2011). Similar signal were reported for native and pre-treated biomass of lime wood (Popescu et al., 2011).

Based on various physico-chemical properties of biomasses, rice and wheat straw and Poplar leaf litter appear to be a good candidate for ethanol production with cellulose content of 43.4, 36 and 37.8%, respectively. Correlating the effects of multiple characteristics may lead to conflicting or even erroneous conclusions. Final selection of promising substrate can only be made after employing suitable pre-treatment strategy and further characterization of treated biomass with enhanced reducing sugar yield. Solid state ^{13}C CP/MAS NMR spectroscopy was most reliable technique to determine biochemical changes before and after pre-treatment of biomass.

4.3.2. Pre-treatment of agricultural waste and leaf litter biomass

Conversion of lignocellulosic biomass to biofuel and biobased products remains bottleneck at industrial scale. Although some chemical and thermo-chemical pre-treatment technique have been employed in bioconversion process, all with limited success in development of an economically feasible technology. In this context, intelligent combination of pre-treatment techniques has been applied to enhance the yield of fermentable sugar.

Present study is focused on four different pre-treatment strategies viz. fungal pretreatment followed by alkaline fractionation, fungal pre-treatment followed by solvolysis, dilute H_2SO_4 -NaOH-pre-treatment and microwave-alkali-acid pre-treatment to enhance reducing sugar yield and enzyme accessibility of feedstock from different agricultural wastes and leaf litter biomasses. Cellulose, hemicellulose, lignin and reducing sugar content was determined in the recovered biomass after pre-treatment.

4.3.2.1. Effect of pre-treatment on wheat straw

Pulverized wheat straw (0.5 mm) was subjected to four different pre-treatment. Maximum increase in cellulose content (48.6%) was found in microwave-acid-alkali pre-treatment, followed by dilute acid-alkali, fungal solvolysis and fungal alkaline fractionation which was found 29, 20.1 and 8.8%, respectively as compared to native biomass (Table 4.3.7). Decrease in the hemicellulose and lignin content was observed after all pre-treated biomass. Reduction in case of hemicellulose was observed to be 10.5, 18.9, 23.5 and 48.4% with fungal alkaline

fractionation, fungal solvolysis dilute acid-alkali and microwave-acid-alkali pre-treatment, respectively. Whereas, decrease in the lignin also followed the same pattern as that of hemicellulose with maximum decrease (68.4%) in microwave-acid-alkali pre-treated wheat straw, whereas reducing sugar was enhanced from 73 to 201.3 mg/g.

Table 4.3.7. Cellulose, hemicellulose, lignin and reducing sugar content of native and pre-treated wheat straw.

Pre-treatment	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reducing sugar (mg/g)
Native biomass	34.79 ± 2.16d	28.5 ± 1.0a	18.4 ± 0.8a	73.0 ± 3.2e
Fungal-alkaline fractionation	38.17 ± 1.02cd	25.5 ± 0.4b	13.5 ± 0.4b	97.7 ± 3.1d
Fungal-solvolysis	41.8 ± 1.3bc	23.1 ± 0.4c	11.7 ± 0.6c	112.1 ± 5.0c
Dilute acid-alkali	44.9 ± 1.1b	21.8 ± 0.6c	9.02 ± 0.3d	161.2 ± 7.0b
Microwave-acid-alkali	51.7 ± 2.01a	14.7 ± 0.5d	5.8 ± 0.2e	201.3 ± 7.2a

Values are mean ± SD (n =3), bearing different letters in the same column are significant at P<0.05.

4.3.2.2. Effect of pre-treatment on rice straw

Maximum increase in cellulose content (41.2%) was found in microwave-acid-alkali pre-treatment, followed by dilute acid-alkali, fungal solvolysis, fungal alkaline fractionation which was found 23.4, 13.4 and 4.7%, respectively as compared to native biomass (Table 4.3.8). Decrease in hemicellulose and lignin content was observed after all pre-treatments. Reduction in case of hemicellulose was observed to be 7.8, 20.1, 29.5 and 39.2% with fungal alkaline fractionation, fungal solvolysis dilute acid-alkali, microwave-acid-alkali pre-treatment, respectively. Decrease in lignin content followed the same pattern as that of hemicellulose with maximum decrease (50.9%) in microwave-acid-alkali pre-treated wheat straw, whereas reducing sugar was enhanced from 81 to 246 mg/g of biomass.

Table 4.3.8. Cellulose, hemicellulose, lignin and reducing sugar content of native and pre-treated rice straw.

Pre-treatment	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reducing sugar (mg/g)
Native biomass	42.5 ± 2.0d	24.5 ± 0.68a	9.16 ± 0.43a	81.13 ± 4.8d
Fungal-alkaline fractionation	44.5 ± 1.7cd	22.6 ± 0.77a	8.0 ± 0.40ab	116.18 ± 9.5c
Fungal-solvolysis	48.2 ± 1.3bc	19.56 ± 0.78b	6.8 ± 0.37b	136.6 ± 9.8c
Dilute acid-alkali	52.43 ± 1.4ab	17.27 ± 0.53c	5.4 ± 0.47c	186.3 ± 7.1b
Microwave-acid-alkali	60 ± 1.1a	14.9 ± 0.73c	4.5 ± 0.40c	246.3 ± 9.5a

Values are mean ± SD (n =3), bearing different letters in the same column are significant at P<0.05.

4.3.2.3. Effect of pre-treatment on Mango leaf litter biomass

Among four different pre-treatment employed to pulverize Mango leaf litter biomass (0.5 mm), maximum increase (43.1%) in cellulose content was found in microwave-acid-alkali pre-treatment, followed by dilute acid-alkali, fungal solvolysis, fungal alkaline fractionation which was found 27.5, 21 and 16%, respectively as compared to native biomass (Table 4.3.9). Decrease in the hemicellulose and lignin content was observed after all pre-treatments. Reduction in case of hemicellulose was observed to be 12.6, 20, 40 and 46.7% with fungal alkaline fractionation, fungal solvolysis dilute acid-alkali, microwave-acid-alkali pre-treatment, respectively. Decrease in lignin content followed the same pattern as that of hemicellulose with maximum decrease (61.5%) in microwave-acid-alkali pre-treated wheat straw, whereas reducing sugar was enhanced from 45 to 126 mg/g of biomass.

Table 4.3.9. Cellulose, hemicellulose, lignin and reducing sugar content of native and pre-treated Mango leaf litter.

Pre-treatment	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reducing sugar (mg/g)
Native biomass	29.97 ± 1.23c	27.80 ± 1.11a	13.75 ± 0.97a	45.3 ± 2.87d
Fungal-alkaline fractionation	34.76 ± 1.04b	24.3 ± 1.52ab	10.53 ± 0.62b	55 ± 3.89d
Fungal-solvolysis	36.26 ± 1.45b	22.2 ± 1.61b	10.3 ± 0.70b	67.66 ± 3.38c
Dilute acid-alkali	38.2 ± 1.55ab	16.7 ± 1.16c	7.10 ± 0.45c	102.76 ± 4.05b
Microwave-acid-alkali	42.9 ± 1.62a	14.8 ± 0.92c	5.30 ± 0.36c	126.26 ± 7.71a

Values are mean ± SD (n =3), bearing different letters in the same column are significant at P<0.05.

4.3.2.4. Effect of pre-treatment on Poplar leaf litter biomass

Pulverized Poplar leaf litter biomass (0.5 mm) was subjected to four different pre-treatment. Maximum increase (38.8%) was found in microwave-acid-alkali pre-treatment, followed by dilute acid-alkali and fungal solvolysis pre-treatment in which it was 17% as compared to native biomass (Table 4.3.10). Decrease in the hemicellulose and lignin content was observed after all pre-treatments. Reduction in case of hemicellulose was observed to be 8.4, 12.1, 28.2 and 41% with fungal alkaline fractionation, fungal solvolysis dilute acid-alkali, microwave-acid-alkali pre-treatment, respectively. Decrease in lignin content followed the same pattern as that of hemicellulose with maximum decrease (50.2%) in microwave-acid-alkali pre-treated wheat straw, whereas reducing sugar was enhanced from 51 to 138 mg/g of biomass.

Table 4.3.10. Cellulose, hemicellulose, lignin and reducing sugar content of native and pre-treated Poplar leaf litter.

Pre-treatment	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reducing sugar (mg/g)
Native biomass	35.38 ± 1.96c	20.55 ± 1.10a	11.12 ± 1.22a	51.13 ± 4.44d
Fungal-alkaline fractionation	41.33 ± 2.25bc	18.83 ± 0.80a	9.0 ± 0.46b	62.3 ± 3.02cd
Fungal-solvolysis	41.33 ± 2.73bc	18.06 ± 0.59a	8.17 ± 0.19b	68 ± 4.97c
Dilute acid-alkali	44.36 ± 1.8ab	14.76 ± 0.98b	6.10 ± 0.22c	125.6 ± 4.34b
Microwave-acid-alkali	49.1 ± 1.4a	12.13 ± 1.32b	5.54 ± 0.23c	138.16 ± 6.72a

Values are mean ± SD (n =3), bearing different letters in the same column are significant at P<0.05.

4.3.2.5. Effect of pre-treatment on Ashoka leaf litter biomass

Pulverized Ashoka leaf litter biomass (0.5 mm) was subjected to four different pre-treatment. Maximum increase (54%) was found in microwave-acid-alkali pre-treatment, followed by dilute acid-alkali, fungal alkaline fractionation, fungal solvolysis which was 35, 24.6 and 12%, respectively, as compared to native biomass (Table 4.3.11). Decrease in the hemicellulose and lignin content was observed after all pre-treatments. Reduction in case of hemicellulose was observed to be 7.3, 14.3, 25 and 33.5 in hemicellulose was found with fungal alkaline fractionation, fungal solvolysis dilute acid-alkali and microwave-acid-alkali pre-treatment, respectively. Decrease in lignin content followed the same pattern as that of hemicellulose with maximum decrease (48%) in microwave-acid-alkali pre-treated wheat straw, whereas reducing sugar was enhanced from 38 to 127 mg/g of biomass.

Table 4.3.11. Cellulose, hemicellulose, lignin and reducing sugar content of native and pre-treated Ashoka leaf litter.

Pre-treatment	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reducing sugar (mg/g)
Native biomass	25.25 ± 1.08d	30.61 ± 1.19a	10.0 ± 0.72a	38.10 ± 1.03d
Fungal-alkaline fractionation	28.26 ± 0.78cd	28.36 ± 0.90ab	8.06 ± 0.37b	43.90 ± 1.20d
Fungal-solvolysis	31.46 ± 1.07bc	26.24 ± 0.88b	7.30 ± 0.32bc	61.40 ± 3.02c
Dilute acid-alkali	34.06 ± 1.29b	22.96 ± 0.80c	6.33 ± 0.36cd	74.93 ± 3.58b
Microwave-acid-alkali	38.86 ± 2.51a	20.36 ± 0.89d	5.20 ± 0.23d	86.56 ± 3.50a

Values are mean ± SD (n =3), bearing different letters in the same column are significant at P<0.05.

4.3.2.6. Effect of pre-treatment on *Eucalyptus* leaf litter biomass

A maximum increase in cellulose content (50.6%) was found in microwave-acid-alkali pre-treatment, followed by dilute acid-alkali, fungal solvolysis and fungal alkaline fractionation, which was 38, 19.1 and 12.4%, respectively, as compared to native biomass (Table 4.3.12). Increased percent reduction of 14.3, 20.7, 34.7 and 48.8 in hemicellulose content was found with fungal alkaline fractionation, fungal solvolysis dilute acid-alkali and microwave-acid-alkali pre-treatment, respectively. Decrease in lignin content was maximum (50.7%) in microwave-acid-alkali pre-treated wheat straw, whereas reducing sugar was enhanced from 54 to 103 mg/g.

Table 4.3.12. Cellulose, hemicellulose, lignin and reducing sugar content of native and pre-treated *Eucalyptus* leaf litter.

Pre-treatment	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reducing sugar (mg/g)
Untreated BLL	30.15 ± 1.29c	18.55 ± 0.74a	12.79 ± 0.62a	54.43 ± 4.10c
Fungus-alkaline fractionation	33.90 ± 1.26bc	15.90 ± 0.54b	9.20 ± 0.44b	55.20 ± 3.10c
Fungus-solvolysis	35.90 ± 1.20b	14.70 ± 1.0b	10.17 ± 0.44b	63.70 ± 3.07c
Dilute acid-alkali	41.60 ± 1.41a	12.13 ± 0.68c	6.6 ± 0.44c	87.86 ± 3.30b
Microwave-acid-alkali	45.40 ± 1.79a	9.50 ± 0.69d	6.30 ± 0.43c	102.90 ± 5.69a

Values are mean ± SD (n =3), bearing different letters in the same column are significant at P<0.05.

Pre-treatment of biomass samples were performed with fungus and solvolysis to break rigid structure of lignocellulose. The process consists of biological treatments with selective lignin-degrading fungi *P. chrysosporium* MTCC 787 following subsequent solvolysis. *P. chrysosporium*, a lignolytic white rot fungi, known to produces lignolytic enzymes including manganese peroxidase (MnP) and lignin peroxidase (LiP) (Bak et al., 2009). Increase in cellulose content of 55% and decrease in hemicellulose and lignin content of 61 and 36%, respectively, were also found with alkali pre-treated rice straw (Zhang and Cai, 2008). Ethanolysis reactions are effective in lignin solubilization to enhance enzymatic saccharification of cellulosic feedstock (Teramoto et al., 2008), advocated lignin reduction and increment in cellulose content in the current study. Pre-treatment of cotton stalks with *P. chrysosporium* led to 35.5% reduction in the initial lignin; however, the enzymatic digestibility of the pretreated cotton stalks was lower than that of untreated stalks (Shi et al., 2008). It was also reported that the enzymatic digestibility of corn stover pretreated with *Cyathus stercoreus* and *P. chrysosporium* were 3.75 and 1.26 times greater, respectively, than that of untreated corn stover (Keller et al., 2003).

Goyal et al. (1992) optimized the ethanolysis treatment for pulping of hardwood, and concluded that treatment with aqueous 60% (v/v) ethanol at 195°C gave the highest pulp yield with minimum loss of viscosity. Baba et al. (2011) reported pre-treatment of Japanese cedar wood by white rot fungi and ethanolysis for ethanol production with increase in sugar production by 7 times. Pre-treatment of beech wood by ethanolysis and white rot fungi (*Ceriporiopsis subvermispora*) for ethanol production by SSF was also observed by Itoh et al. (2003). *P. chrysosporium* has been reported to be a promising white rot fungus for biological pre-treatment of lignocellulosic waste. In this method, biological pre-treatment followed by ethanol-alkaline extraction was adopted to deconstruct complex structure of biomass samples. Combination of biological pre-treatment with physical or chemical processes improves efficiency of pre-treatment (Rabelo et al., 2008; Zhao et al., 2008b). Enhanced delignification and xylan removal from lignocellulose is attributed to synergistic effect of biological pre-treatment and alkaline fractionation (Yu et al., 2010a). Mineralization of lignin in all biomass samples was probably due to lignin depolymerisation and modification of lignin macromolecules. Results obtained were in fair agreement with study carried by Yang et al. (2013) for improved bioconversion of Poplar wood by white rot fungus and alkaline fractionation. Yang et al. (2013) presented total lignin reduction from 24.3 to 10.6% after 16 weeks of pre-treatment. Yu et al. (2009b) reported the total sugar yield of rice hull after the combination pre-treatment of hydrogen peroxide and fungal treatment was greater than that after the sole pretreatment. Biological pre-treatment for 15 days of cornstalk with mild alkaline pre-treatment was observed to enhance delignification and xylan loss to reduce recalcitrance, improving enzymatic digestibility (Yu et al., 2010).

High ethanol concentration can be achieved by increasing the concentration of fermentable sugar by employing pre-treatment which improves cellulose availability by removing hemicellulose and lignin. Alkali treatment breaks the ester bonds between lignin, hemicellulose and cellulose, and avoiding fragmentation of the hemicellulose polymers (Gaspar et al., 2007) whereas acid disrupt lignin and increase cellulose accessibility to enzyme hydrolysis (Wyman, 1996; Yang and Wyman, 2004). However acid treatment might result in formation of fermentation inhibitors such as furfural and hydroxymethyl furfural (Taherzadeh and Karimi, 2008). Results presented by Zhang et al. (2010) for dilute-acid-alkali pre-treatment of corn cob were observed in reasonable degree of agreement with current investigation, showing 81% reduction in lignin content and 53.8% increase in cellulose. Consecutive acid and alkali pre-treatment of sugarcane

bagasse was carried by Giese et al. (2013) and reported increase in the activity of cellulase enzymes towards cellulose yielding significant amount of reducing sugars.

Among all four different pre-treatments investigated, microwave-acid-alkali was the best and most promising pre-treatment method, as it can reduce the pre-treatment time at higher temperature. Microwave based pre-treatment approach utilizes both thermal and non-thermal effects due to intermolecular collision as a result of realignment of polar molecules with microwave oscillations and thus accelerates physical, chemical and biological processes (Maa et al., 2009). Binod et al. (2012) have reported reducing sugar yield of 0.66 g/g dry biomass with microwave treatment of sugarcane bagasse with 1% NaOH at 600 W for 4 min followed by enzymatic hydrolysis, whereas combining microwave-alkali-acid treatments with 1% NaOH followed by 1% sulphuric acid resulted in an increase in reducing sugar yield to 0.83 g/g dry biomass.

The positive effects of the presence of alkali and microwave irradiation had also been studied by Keshwani and Cheng (2010) on hydrolysis of switch grass. The application of microwave irradiation for 10 min at 250 W to switch grass immersed in 3% NaOH (w/v) produced the highest yields of reducing sugars. Optimization of microwave pre-treatment to study the effect of factors including the ratio of biomass to NaOH solution, pre-treatment time, microwave power, and the concentration of NaOH solution with three different levels on the chemical composition, cellulose/hemicellulose recoveries, and ethanol concentration from wheat straw was investigated by Xu et al. (2011). Balcu et al. (2011) investigated the use of acid in combination with microwave. They reported maximum reducing sugar yield at 140°C with 0.82% aqueous solution of sulphuric acid.

4.3.3. Characterization of native and microwave-acid-alkali pre-treated rice straw

After pre-treatment of all six biomass samples with different combined pre-treatment technique, microwave-acid-alkali pre-treatment is found effective and promising combinatorial pre-treatment approach for all six biomass taken under study. Maximum reducing sugar yield was 246 mg/g of biomass and 51 and 39% reduction in lignin and hemicellulase was found with microwave-acid-alkali pre-treated rice straw. Therefore, microwave-acid-alkali pre-treated rice straw was selected as substrate for ethanol production via SSF. In order to investigate the effect of physical and chemical changes which took place during the pre-treatment process, SEM,

XRD, TGA, FTIR and solid state ^{13}C CP/MAS NMR spectroscopy analysis was carried out for microwave-acid-alkali pre-treated rice straw and compared with native rice straw.

4.3.3.1. Scanning electron microscopy (SEM)

Physical changes in native and microwave-alkali-acid pre-treated rice straw were observed by SEM. SEM observation of native (Figure 4.3.6 a) and pre-treated biomass (Figure 4.3.6 b) showed pre-treatment induced structural change in the biomass. SEM revealed that native rice straw exhibited ordered and compact surface structure with intact morphology which was destroyed in pretreated rice straw. Similar structural changes were observed for sugarcane bagasse pretreated with microwave alkali pretreatment (Binod et al., 2012), sugarcane top pre-treated with ultrasound assisted with surfactant (Sindhu et al., 2012), hot compressed water treatment of *Tamarix ramosissima* biomass (Xiao et al., 2011).

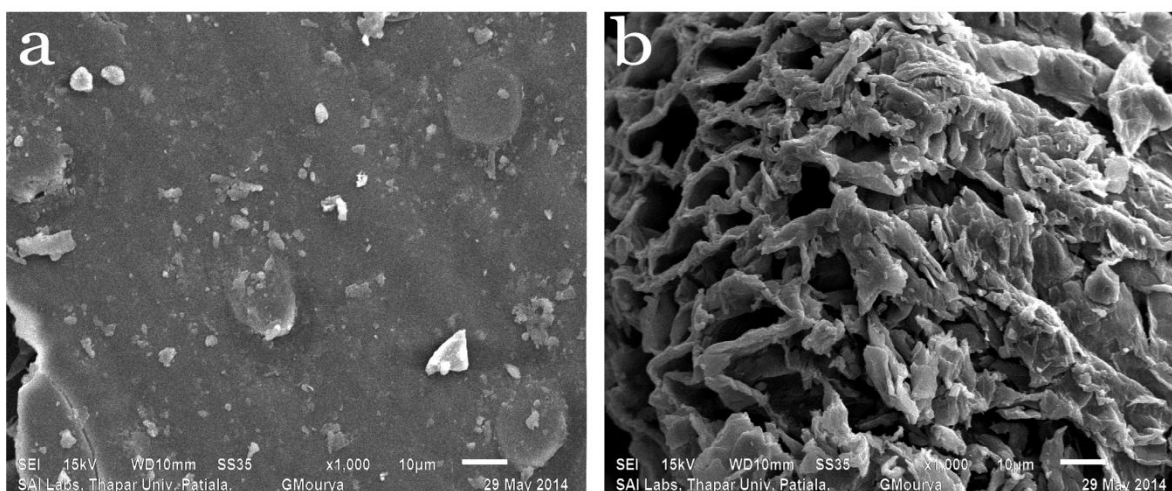


Figure 4.3.6. Scanning electron micrograph of (a) native and (b) Microwave-acid-alkali pre-treated rice straw (magnification: 1,000X).

4.3.3.2. X-ray diffraction analysis (XRD)

The XRD profile of native and microwave-alkali-acid pre-treated rice straw is presented in Figure 4.3.7. *CrI* in native and microwave-acid-alkali pre-treated biomass were 39.5 and 48.52%, respectively. A significant increase (11.4%) in *CrI* was recorded in pre-treated biomass as compared to native biomass. Overall biomass crystallinity depends on cellulose, hemicellulose and lignin content (Meshitsuka and Isogai et al., 1996). Highly crystalline biomass hinders enzymatic hydrolysis whereas biomass with low crystallinity exhibits improved lignocellulose digestibility (Fan et al., 1980).

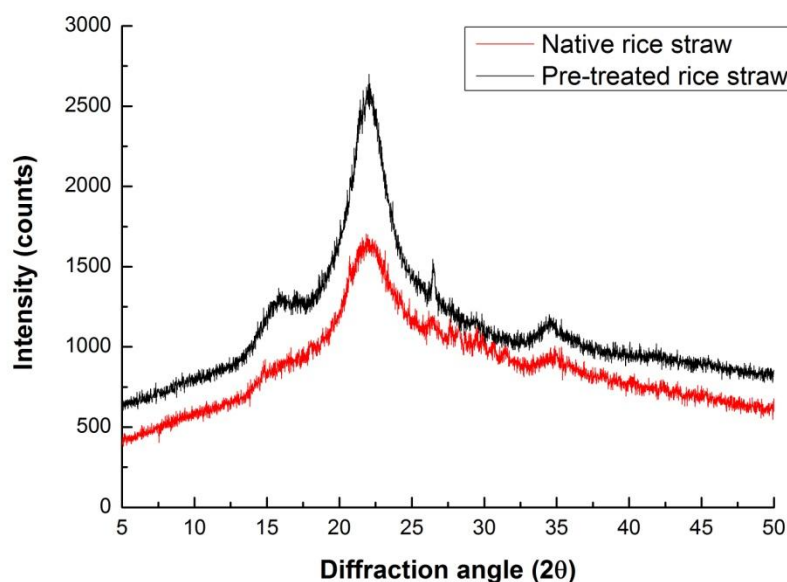


Figure 4.3.7. XRD profile of native and microwave-alkali-acid pre-treated rice straw.

The result was in close proximity with the earlier report of surfactant-assisted ultrasound pretreatment of sugarcane tops (Sindhu et al., 2012) with 48.85 and 65% *CrI* of pre-treated biomass. Treatment of microwave-acid, microwave-alkali and microwave-alkali followed by acid pretreatment of sugarcane bagasse also showed 5.35, 11.85 and 12.11% increase in *CrI* (Binod et al., 2012). Hot compressed water pre-treated *Tamarix ramosissima* demonstrated significant increase in *CrI* as compared to untreated biomass (Xiao et al., 2011). Increase in *CrI* by 6% was also reported in enzyme pretreated *Lantana camara* biomass (Kuila et al., 2011). The crystallinity is mainly related to the molecular mass of hydrocarbons and fatty components of biomass (Zhang et al., 2008). Pre-treatment is believed to eliminate hemicellulose from hydrolysis surface and changes cellulose crystallinity (Xiao et al., 2011). Probably, crystallinity has been damaged owing to the synergistic effect of alkali, microwave and acid treatment, leading to the enhanced saccharification of rice straw. Hemicellulose and lignin solubilization together with less ordered cellulose might be the main reason for increase in cellulose crystallinity.

4.3.3.3. Thermal gravimetric analysis (TGA)

To understand the changes in thermal properties of rice straw before and after microwave-alkali-acid pre-treatment, TGA of native and pre-treated biomass was carried out. Comparative rapid devolatilization in the temperature range of 300 - 700°C in pre-treated rice straw advocated less

lignin content as compared to native (Figure 4.3.8). The main weight loss ends at 350-390°C followed by a slow and continuous weight loss. The former step was due to the primary devolatilization, whereas the later was attributed to the degradation of heavier chemical structures in the solid matrix (Fisher et al., 2002). Similar devolatilization properties were also found with hot water pretreated biomass of *Tamarix ramosissima* (Xiao et al., 2011) and heat pretreated sugarcane bagasse (Chen et al., 2010). Flattening of tail beyond 500°C was observed mainly due to removal of lignin in pre-treated biomass, as long tail was observed due to high percentage of mannose or lignin content (Naik et al., 2010).

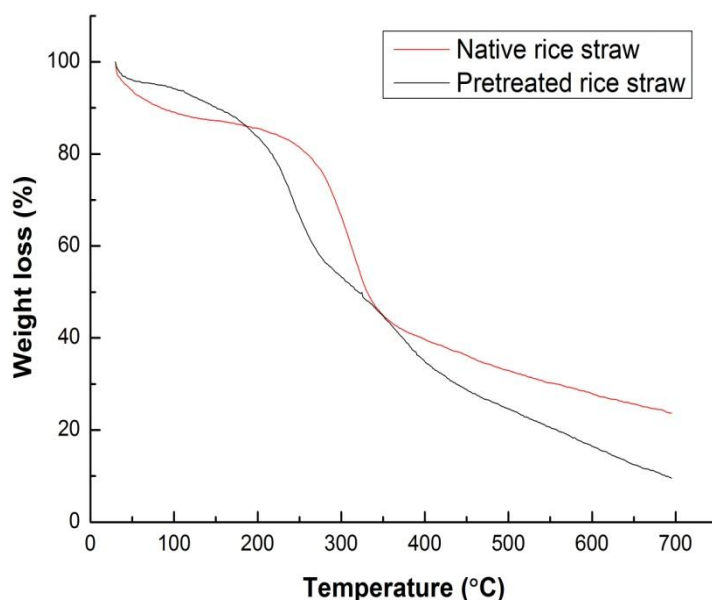


Figure 4.3.8. TGA of native and microwave-alkali-acid pre-treated rice straw.

The behaviour of biomass materials during devolatilization was related to the presence of chemical constituents such as cellulose, hemicellulose and lignin (Raveendran et al., 1996). The hemicellulose and cellulose part of biomass usually start to decompose at around 206°C and 315°C while the decomposition of lignin start above 330°C respectively (Ledakowicz and Stolerak 2002; Hornof et al., 1977). The thermal decomposition study was conducted on rapeseed (*Brassica napus* L), sponge ground fibers, sugarcane bagasse, banana fibers by several researchers and their study revealed that the thermal decomposition of hemicelluloses occurred at temperatures ranging from 150°C to 350°C, cellulose was decomposed at temperature range of 275-350°C, and lignin was gradually decomposed between the temperatures 250°C and 500°C (Chen et al., 2010; Guimaraes et al., 2009).

4.3.3.4. Fourier transform infrared (FTIR) spectroscopy analysis

Fourier transform infrared (FTIR) spectroscopy analysis was carried out to detect changes in functional groups of biomass and spectra as shown in Figure 4.3.9. The main characteristics were attributed to different chemical groups of cellulose, hemicellulose and lignin. Most prominent bands in biomass sample were 3402, 2910, 1741, 1631, 1045 and 856 cm^{-1} . The peaks in fingerprint were based on literature (Zhang et al., 2008; Adel et al., 2010; Xiao et al., 2011; Meng et al., 2012; Kim et al., 2012) as summarised in Table 4.3.13. The band in the range of 1500-1700 cm^{-1} represents the aromatic ring stretch of lignin, 3200-3600 cm^{-1} is related to O-H stretching vibrations of alcohol and phenol, 1400 to 900 cm^{-1} is designated to vibrations of variety of bonds such as C-H, C-O, C-N and P-O and O-H and C-H symmetric and asymmetric stretching bands appears in the region of 2900 cm^{-1} .

Deep band at 3402 cm^{-1} is related to O-H stretching vibrations caused by presence of alcoholic and phenolic hydroxyl group involved in hydrogen bond. The O-H stretching band was changed to higher wave number and somewhat broadened as a result of pre-treatment which is an indication of weaker intra and intermolecular hydrogen bonding and thereby lower crystallinity (Jeihanipour et al., 2009). The band position at 2910 cm^{-1} is attributed to C-H stretching vibration showed enhancement in intensity of peak, indicating that the methyl and methylene portions of cellulose were ruptured (Xiao et al., 2011). Reduction in peak intensity was observed at 1631 cm^{-1} , attributed to hemicellulose acetyl and uronic ester groups or linkages in lignin and/or from ester linkage of carboxylic group of the ferulic and *p*-coumaric acids of lignin and/or hemicellulose (Sindhu et al., 2012).

Table 4.3.13. Assignment of the FTIR bands of functional groups in biomass samples

Name of characteristic group	Wavenumber (cm^{-1})
O-H stretching vibration (hydrogen bonded)	3402
Stretching of $-\text{CH}_2$ and $-\text{CH}_3$	2910
C=O stretching of unconjugated ketone, carbonyls, and ester groups; C=O in xylan acetates (hemicelluloses)	1741
Bending mode of the absorbed water and the stretching of C=O in lignin	1631
C–O stretching vibration in cellulose and hemicellulose	1045
Glycosidic linkage of hemicelluloses	856

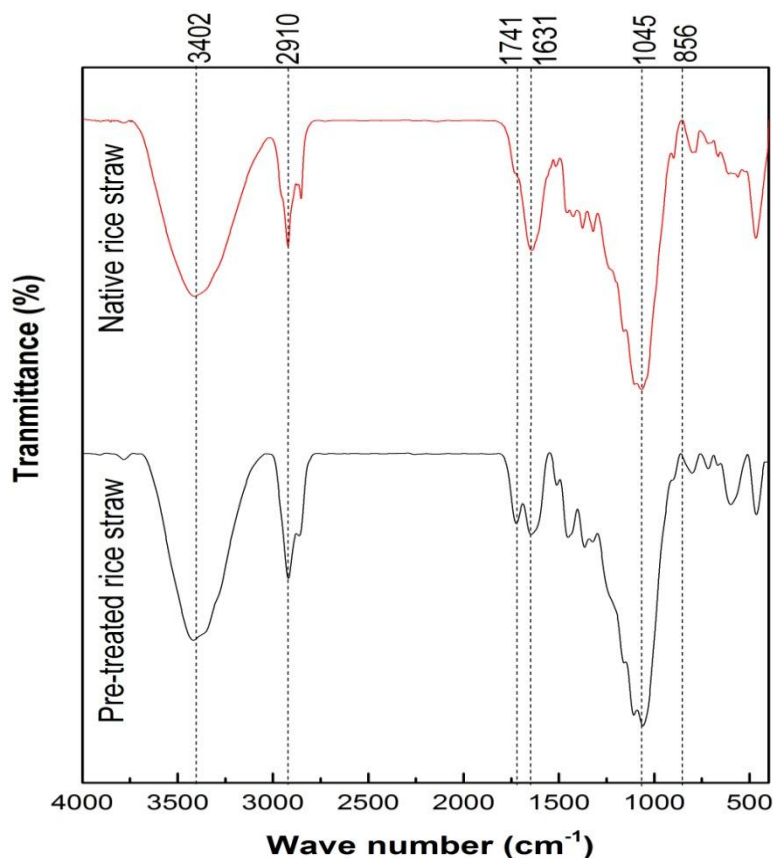


Figure 4.3.9. FTIR spectra of native and microwave-alkali-acid pre-treated biomass.

4.3.3.5. Solid state ^{13}C CP/MAS NMR spectroscopy

Solid state ^{13}C CP/MAS NMR spectroscopy is an important tool to characterize chemical shift values ascribed to cellulose, hemicellulose and lignin component of lignocellulosic biomass. A solid state ^{13}C CP/MAS NMR spectrum of the native and microwave-acid-alkali biomass is presented in Figure 4.3.10. Signal assignment for various components of biomasses has been assigned based on the literatures (Sun et al., 2004; Rezende et al., 2011; Shi et al., 2011; Kohn et al., 2011) as summarised in Table 4.3.14.

The region between 60 and 110 ppm was dominated by prominent signals, which were assigned mostly to cellulosic carbons. The sharp signal at 104 ppm corresponds to ordered cellulose (C1, C4, C6) while signal at 83 and 63 ppm were due to disordered cellulose carbons. Difference in relative signal intensities between native and microwave-acid-alkali pre-treated rice straw biomass reflected the changes owing to pre-treatment. The higher cellulose content of the pre-treated biomass was seen as distinct peak (72 ppm) assigned for C2, C3 and C5 of cellulose. Less

prominent peak at 172.2 ppm in pre-treated biomass corresponds to acetyl groups of hemicellulose, ascribed to hemicellulose removal.

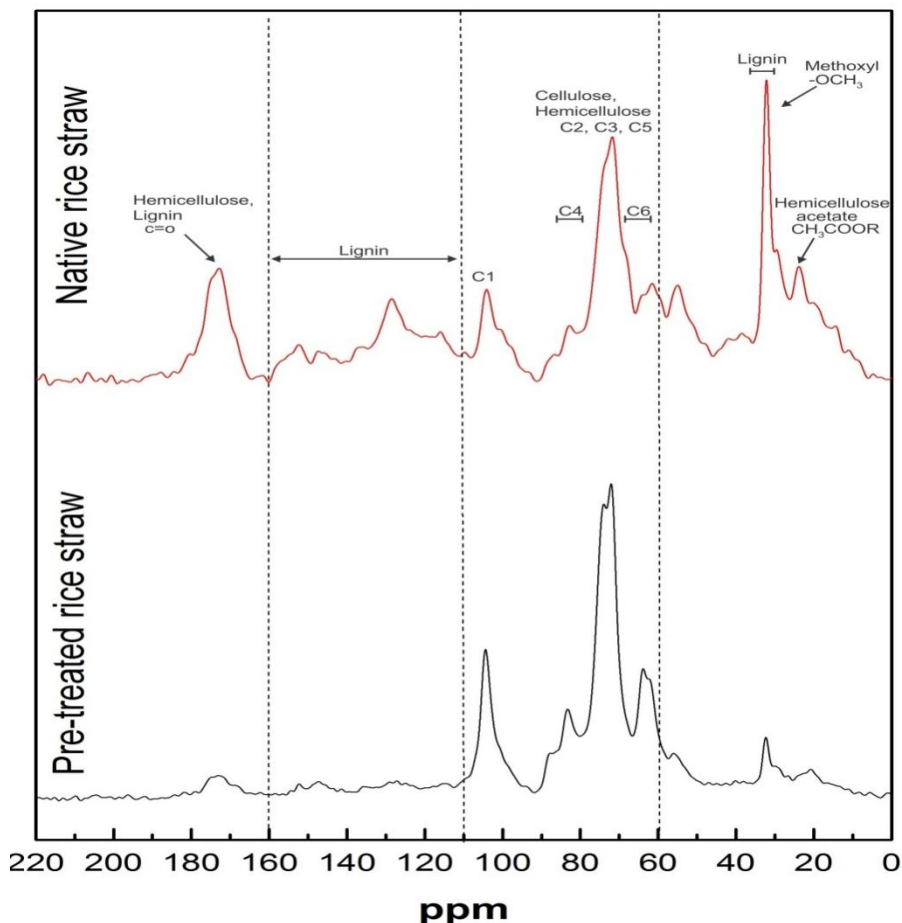


Figure 4.3.10. Solid state ^{13}C CP/MAS NMR spectra of native and microwave-acid-alkali pre-treated rice straw.

Table 4.3.14. Signal assignments for chemical shifts obtained from solid state ^{13}C CP/MAS NMR spectroscopy of biomass

Chemical shift (ppm)	Assignments
173	Carboxyl group of hemicelluloses
104	Anomeric carbon (C1) of cellulose
83	C4 of amorphous cellulose and hemicelluloses, $\text{OC}_\beta\text{H}_2$ carbon of lignin
72	C2, C3, C5 of cellulose
63	C6 of crystalline cellulose
32	Methoxyl (OCH_3) and methylene (CH_2) groups in lignin
23	CH_3 in acetyl group in lignin

Very less intense peak at 23 ppm in pre-treated biomass, ascribed to acetyl group in lignin indicates considerable reduction in lignin level of microwave-alkali-acid pre-treated biomass. The results were in good agreement with hot water pretreated biomass of *Tamarix ramosissima* (Xiao et al., 2011) and biological pretreated lime wood (Delmotte et al., 2008). It can be concluded that main changes occurring in structure of microwave-alkali-acid pretreated rice straw biomass were due to degradation of hemicelluloses and lignin structure. Obtained results were in good concord with ^{13}C CP/MAS NMR study of limewood (Popescu et al., 2011).

4.3.4. Ethanol production via SSF using orthogonal design

After successful accomplishment of pre-treatment study of different substrate used in this study viz. wheat straw, rice straw, Mango, Poplar, Ashoka and Eucalyptus leaf litter, the best pre-treatment was found to be microwave-acid-alkali in terms of release of reducing sugar. To monitor the ethanol production, all six microwave-acid-alkali pre-treated samples were subjected to SSF for selection of suitable substrate.

4.3.4.1. Screening of substrate for ethanol production via SSF

Among all the microwave-acid-alkali treated biomass samples, maximum ethanol production (12 g/L) was found with rice straw followed by wheat straw which was 10.4 g/L, while among leaf litters, Poplar and Mango, Ashoka, Eucalyptus leaf litter biomass showed 8.5, 7.2, 6.2 and 3.1 g/L ethanol, respectively after 24 h and remains almost constant till 48 h of incubation (Figure 4.3.11). Microwave-acid-alkali pre-treated Poplar leaf litter appear to be the best among all leaf litter biomass studied, with 8.5 g/L of ethanol after 24 h of incubation, may also be used as feedstock for ethanol production.

4.3.4.2. Optimization of process parameters of SSF using Taguchi design

To optimize process parameters of SSF, microwave-acid-alkali pre-treated rice straw was selected on the basis of maximum ethanol production. Taguchi orthogonal array design was used for its optimization as it is a cost effective, time saving and efficient option to determine optimal combination of factor levels to facilitate analysis of design. The fermentation process parameters, namely, temperature, pH, enzyme volume, fermentative microbe inoculum size play an important role in ethanol production (Latifian et al., 2007) using lignocellulosic feedstock. In the present study, the effect of 6 factors on SSF process of pre-treated rice straw was investigated using Taguchi experimental design in 25 runs (Table 4.3.15). Response was obtained in terms of

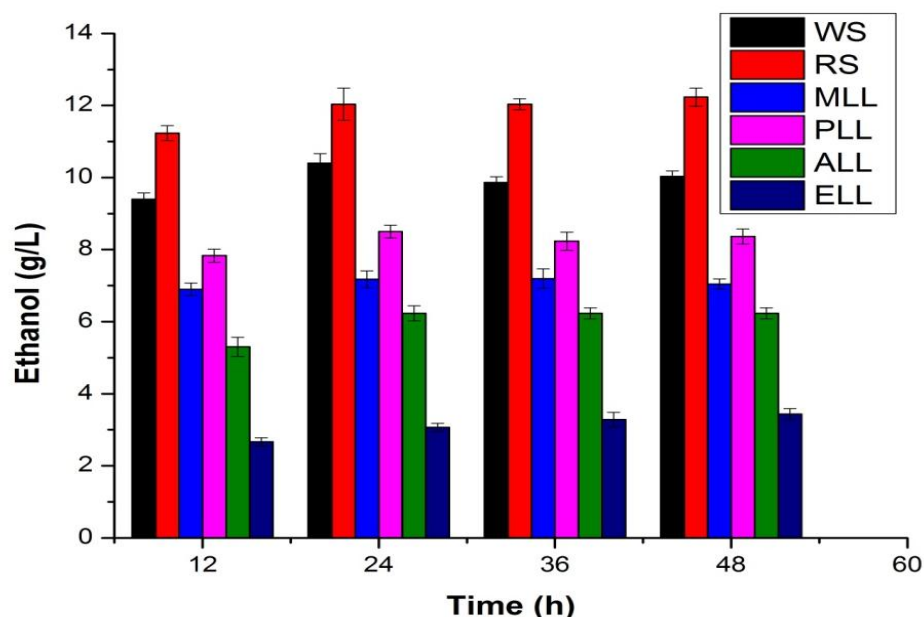


Figure 4.3.11. Ethanol production using microwave-acid-alkali pre-treated substrate via SSF.

WS: Wheat straw; RS: Rice straw; MLL: Mango leaf litter; PLL: Poplar leaf litter; ALL: Ashoka leaf litter; ELL: Eucalyptus leaf litter. The *vertical bars* designate the standard deviation for the mean of three replicative tests.

ethanol concentration (g/L), as function of six factors, viz, substrate loading (v/v), temperature (°C), pH, time (h), enzyme loading (v/v) and yeast inoculum size (v/v). Ethanol concentration falls in range of 3.2 to 16.2 g/L as combined effect of six factors in their specified range. In run 6 (std. 24), with a combination of 11% (w/v) substrate loading, 1.0% (v/v) enzyme loading, 10% (v/v) yeast inoculum at pH 5.0, maximum ethanol concentration (16.2 g/L) was observed after 12 h of incubation at 35°C.

Analysis of the screened variables from a five-level orthogonal array design led to identification of the key factors affecting ethanol production. The results obtained were fed into the Design-Expert software (Version 8.0.7.1, Stat-Ease, Minneapolis, MN) and analyzed using ANOVA. The actual value ranges of the screened variables were presented in Table 4.3.16. To test the fit of the model equation, the regression-based determination coefficient R^2 was evaluated. The predicted R^2 (0.94) was in close proximity with adjusted R^2 (0.99), advocated for greater significance of model. The statistical analysis allowed us to determine the contribution of experimental factors (signals) in comparison to noise, where the signal should be fairly large in comparison to noise. Thus, the estimated adequate precision of 42.26 for ethanol production,

represented the signal to noise ratio, is an adequate signal. The model F value of 155.16 as well as values of $P > F$ (<0.0001) indicated that the model terms (A : substrate loading, B : pH, C : Temperature, E : Time, F : Inoculum size) were significant (Table 4.3.16).

Table 4.3.15. Response values of L_{25} Taguchi orthogonal array design for SSF optimization

Runs		Response	
Std	Run	Ethanol concentration (g/L)	Ethanol yield (%)
2	1	6.4	15.1
23	2	15.2	35.8
6	3	7.5	17.6
22	4	8.8	20.7
1	5	3.2	7.5
24	6	16.2	38
8	7	8.4	19.8
7	8	4.4	10.3
17	9	12.7	29.9
20	10	4.2	9.9
16	11	4.7	11
19	12	5.2	12.2
4	13	4.5	10.6
10	14	5.9	13.9
9	15	6	14.1
25	16	5.9	13.9
15	17	11.3	26.6
5	18	4.3	10.1
11	19	8.8	20.7
3	20	4.6	10.9
18	21	9.6	22.6
13	22	4.1	9.7
21	23	6.1	14.3
12	24	7.8	18.4
14	25	5.3	12.5

Results showed that run 2 [substrate loading (11%, w/v), pH 4.5, temperature (30°C), enzyme loading (0.5%, v/v), time 72 h, inoculums size (8% v/v)] yielded 15.2 g/L of ethanol whereas maximum ethanol yield of 16.2 g/L was observed with run 6 [substrate loading (11%, w/v), pH 5.0, temperature (35°C), enzyme loading (1.0% v/v), time 24 h, inoculums size (10% v/v)] (Table 4.3.15). Results presented by Zhu et al. (2007) to optimize ethanol production from pre-treated rice straw at 10% (w/v) loading using SSF with 54% ethanol yield was also found to be in

reasonable agreement. An overall ethanol yield of 70% was reported by Ohgren et al. (2006) from corn stover at 10% water insoluble solid.

Table 4.3.16. Analysis of variance (ANOVA) for selected factorial mode of Taguchi design.

Source	Sum of square	df	Mean of square	F-Value	p-value Prob > F
Model	289.848	220	14.4924	155.1649	0.002708
A: Substrate loading (w/v)	89.7336	4	22.4334	43.27431	0.001507
B: pH	20.3096	4	5.0774	9.794367	0.024157
C:Temperature (°C)	26.4856	4	6.6214	12.77276	0.01505
E:Time (h)	66.8816	4	16.7204	32.25386	0.002659
F:Inoculum size (v/v)	83.7376	4	20.9344	40.38272	0.001724

Increase in pH and temperature beyond 5 and 35°C, respectively exerts a negative impact on overall ethanol production. pH optimization for ethanol production from wild grass using Taguchi design shows an optimum pH of 4.3 as reported by Das et al. (2014). Similar result of ethanol production (17.4 g/L) at pH 4.5 from bagasse pith hydrolysate was also reported (Dasgupta et al., 2013). Findings were supported by the studies carried out by Wan et al. (2012) for softwood conversion to ethanol at 30°C and 5 pH resulting in ethanol yield of 85.1% and 51% yield from Tapioca stem at 30°C and 5.5 pH (Magesh et al., 2011). Ethanol titre of 32.6 g/L was obtained from sugarcane bagasse at optimized process conditions of 35°C, 5.5 pH and 72 h fermentation time (Sasikumar and Viruthagiri, 2008) and of 59.2 g/L for Miscanthus biomass at 32°C with 7% inoculums and 24% substrate loading after 48 h of incubation.

A number of numerical solutions were suggested by the Design-Expert software (Version 8.0.7.1, Stat-Ease, Minneapolis, MN) within the experimental range of parameters for maximum ethanol production. The model predict, response based on RSM and estimates ethanol concentration of 18.744 g/L at substrate loading (11%, w/v), pH (4.5), temperature (30°C), enzyme loading (0.5% v/v), inoculum size (8%, v/v) and time (24 h).

4.3.4.3. Validation of experimental model for ethanol production

In order to validate, the predicted model experiment was conducted in triplicates following these condition with microwave-acid-alkali pre-treated rice straw. After 24 h of incubation at 30°C, samples were withdrawn to estimate ethanol concentration. The maximum experimental value for ethanol production at model predicted experiment condition was 18.774 g/L. The excellent correlation between predicted (18.744 g/L) and experimental values (18.9 g/L) of experiment

conducted at combination of parameters predicted by model, justified the validity of the regression model obtained (Figure 4.3.12).

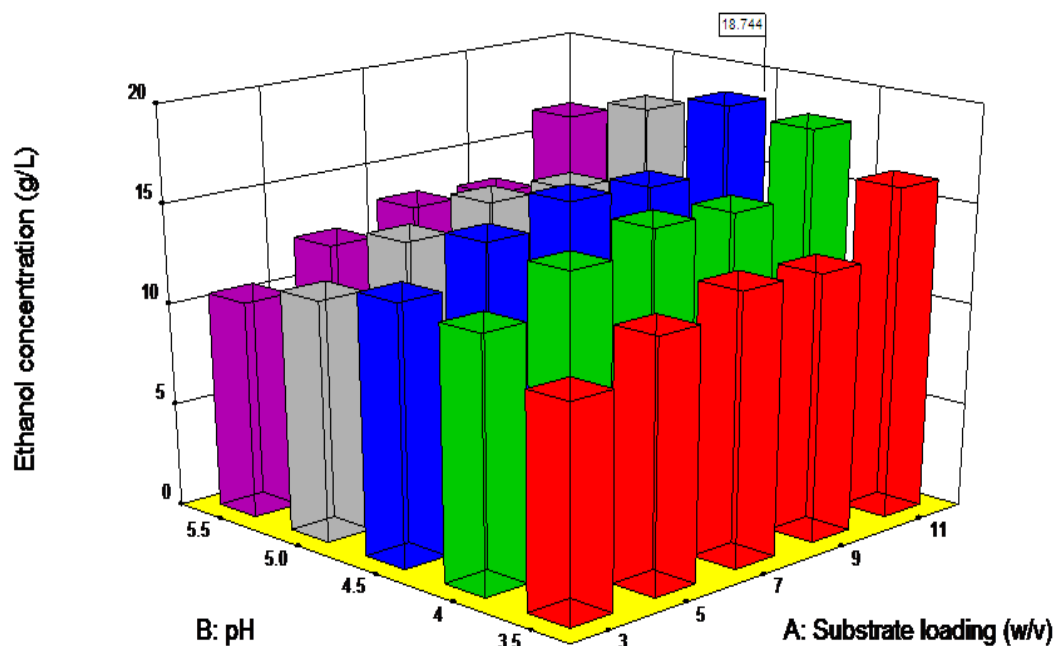


Figure 4.3.12. 3D Contour plot showing interaction between pH and substrate loading for ethanol concentration.

Figure 4.3.12 shows time course for reducing sugar and ethanol concentration during SSF where reducing sugar was converted to ethanol in time dependent manner. During SSF process, reducing sugar released from lignocellulose was enhanced owing to enzymatic action which was further converted to ethanol.

4.3.4.4. Ethanol production in 3-L bioreactor via SSF

Ethanol production using microwave-acid-alkali pre-treated rice straw was subjected to SSF following the optimized condition [substrate loading (11 % w/v), pH (4.5), temperature (30°C), enzyme loading (0.5%, v/v), inoculum size (8%, v/v)] in a total volume of 2 L in a bioreactor for a period of 72 h. After regular interval of 12 h, sample was withdrawn and investigated for reducing sugar and ethanol content. After 12 h of SSF process, reducing sugar was 43.1 g/L, gradually decreases to 23.4 g/L after the completion of 24 h and then remains constant (Figure 4.3.13). Ethanol concentration at the end of 24 h reached to 25.2 g/L which corresponded to ethanol yield of 59%. Enhance ethanol yield of 21% was observed with controlled pH condition in a bioreactor as compared to bench scale in which optimum yield was 38%. Beyond 24 h of

fermentation, no significant increase in ethanol production was observed. Ethanol production was enhanced by 18 g/L using microwave-acid-alkali pre-treated rice straw as feed stock as compared to untreated biomass in which ethanol production was 7.2 g/L at the end of 24 h.

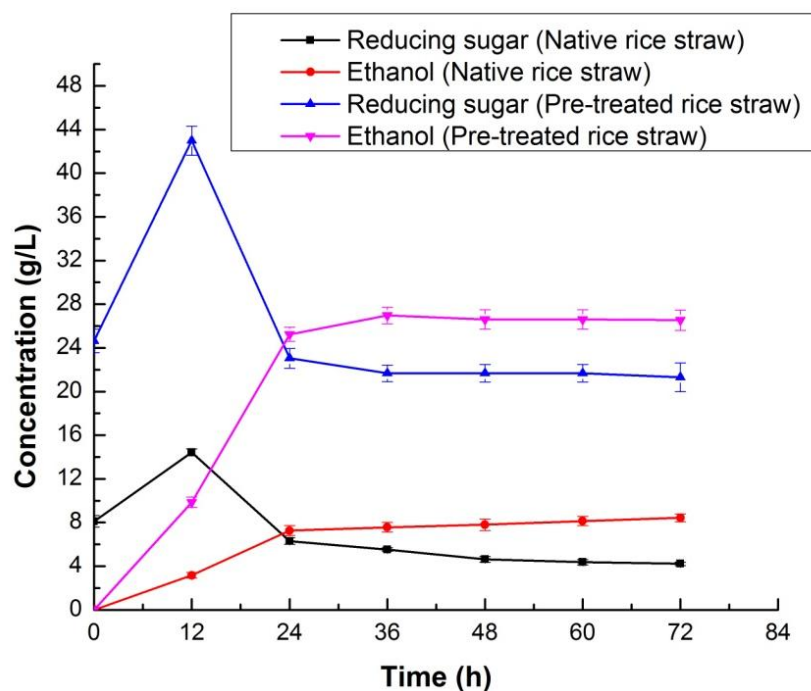


Figure 4.3.13. Reducing sugar and ethanol concentration over a period of 72 h.

In the first few hours, a higher reducing sugar concentration appeared in reaction mixture due to action of cellulase. Reducing sugar concentration reached its peak at 12 h and decreased gradually because the fermentation rate reached the rate of enzyme hydrolysis. Hydrolytic efficiency of cellulase is negatively affected by catabolite repression and increase in lignin concentration (Berlin et al., 2006; Mussatto et al., 2008). After 36 h, enzyme accessibility to cellulose and hemicellulose was reduced due to lignin and ash (Oberoi et al., 2012). Increase in reducing sugar production till 12 h (40 g/L) and maximum ethanol concentration of 24.25 g/L at end of 24 h was reported by Oberoi et al. (2012). The result was in good agreement with previously reported ethanol concentration of 12.7 g/L using rice straw (Ko et al., 2009), 11.2 g/L using wheat straw (Qi et al., 2010), 12.97 g/L using orange peels (Oberoi et al., 2010), 17.40 g/L using bagasse pith hydrolysate (Dasgupta et al., 2013), 27.4 g/L using softwood (Wan et al., 2012), 59.2 g/L using Miscanthus biomass (Han et al., 2011).

Using partially purified bacterial cellulase from *B. subtilis* NA15 in SSF for hydrolysis of biomass, made the process more economical as compared to commercial cellulase, the same could be also used for hydrolysis of various lignocellulosic wastes to achieve enhanced ethanol yield at pilot scale fermentation.

Conclusions

Characterization strategy may identify the specific pretreatment strategy to be adopted for generation of effective renewable fuel. SEM analysis revealed the compact surface morphology of rice straw and Poplar leaf litter biomass. Rice straw and Poplar leaf litter biomass showed cellulose content of 43 and 38%, respectively. XRD analysis showed low *CrI* (21%) of Poplar and Ashoka leaf litter biomass advocated that these biomasses may be prone towards enzymatic hydrolysis. Solid state ^{13}C CP/MAS NMR spectroscopy analysis showed pronounced peak at 1043 and 1375 cm^{-1} which are assigned to vibration for cellulose and hemicellulose chemical groups, conveys more carbohydrate content of lignocellulose biomass whereas peak at 1635 and 1232 cm^{-1} attribute higher lignin content. NMR study concluded that Poplar leaf litter biomass and wheat straw are enriched with carbohydrate in comparison to other biomass while Ashoka leaf litter showed greater lignin content. To correlate the effect of multiple physico-chemical characteristics may lead to conflicting or even erroneous conclusions towards combined and integrated effect of an array of compositional analysis, crystallinity, recalcitrance and degree of polymerization. Pre-treatment of all six biomass samples with different combined pre-treatment technique showed that microwave-acid-alkali pre-treatment was found to be effective and promising combinatorial pre-treatment approach for biomass taken under study with maximum reducing sugar (246 mg/g of biomass) from rice straw. Physical and chemical changes in the microwave-acid-alkali pre-treated rice straw was characterized using SEM, XRD, TGA, FTIR and Solid state ^{13}C CP/MAS NMR spectroscopy to reveal the efficiency of pre-treatment. Ethanol production from microwave-acid-alkali treated rice straw via SSF was optimized using Taguchi design and the maximum ethanol (18.9 g/L) was obtained with optimized condition [substrate loading (11%, w/v), pH (4.5), temperature (30°C), enzyme loading (0.5%, v/v), inoculum size (8%, v/v), time 24 h]. An effort was made to scale up the ethanol production in 3-L bioreactor using microwave-acid-alkali pre-treated rice straw under the same optimized conditions which resulted into 25.2 g/L ethanol corresponding to 59%, after 24 h of fermentation process.

1. Out of 32, eight bacterial isolates (NA8, NA9, NA11, NA14, NA15, DGA, DGB and DGC) from different sources such as compost, paper pulp and sugarcane bagasse were identified as cellulose degraders.
2. Phylogenetic analysis revealed that NA8, NA9, NA11, NA14, DGB and DGC showed maximum similarity with *Bacillus licheniformis*, isolate NA15 had maximum similarity with *Bacillus subtilis* whereas, DGA shared maximum similarity with *Lysinibacillus fusiformis*. These conclusions were drawn using two different techniques, Biolog GEN III MicroPlate™ identification and PCR based amplification of 16S rRNA gene analysis.
3. Isolate NA15 (*Bacillus subtilis* NA15) was found to be the best cellulose degrader among all the eight screened isolates which showed maximum CMCase activity (0.06 U/mL) after 24 h of incubation at 37°C.
4. Screening of 11 media ingredients using Plackett-Burman design was done to explicate the parameters that significantly influence the carboxy methyl cellulase (CMCase) production was done and maximum CMCase activity of 0.47 U/mL was obtained using Response surface methodology (RSM) with carboxy methyl cellulose (CMC): 18 g/L, peptone: 5 g/L, yeast extract: 5 g/L and MnCl₂: 0.5 g/L. The model predicted the maximum CMCase activity (0.45U/mL) which was in good agreement with the experimental value of 0.47 U/mL showing 7-fold increase as compared to unoptimized medium.
5. CMCase from *Bacillus subtilis* NA15 was purified using DEAE-Sepharose ion exchange chromatography with increase in CMCase activity from 2.65 to 30.42 U/mg of protein (11.48-fold) with a yield of 43.7%. The approximate molecular mass of the purified cellulase was 45 kDa. Purified CMCase exhibited highest activity of 3.24 U/mL at pH 7.0, which was stable at pH 5-7 and thermally stable up to 60°C.
6. Solid state ¹³C CP/MAS NMR spectroscopy was most reliable technique to determine biochemical changes before and after pre-treatment of biomass.
7. Microwave-acid-alkali pre-treatment was found to be the most effective for all six biomass samples such as wheat and rice straw and Mango, Poplar, Ashoka, Eucalyptus leaf litter biomass, taken under investigation. Microwave-acid-alkali pre-treated rice straw was

selected as feedstock for ethanol production with maximum reducing sugar yield of 246 mg/g of biomass.

8. The second most effective strategy for pre-treatment of biomasses involved use of dilute-acid-alkali, in which 186 mg/g of biomass reducing sugar was obtained from rice straw, which can be an effective feedstock for ethanol production and to make the process economically feasible.
9. Microwave-acid-alkali pre-treated Poplar leaf litter appear to be the best among all leaf litter biomass studied, resulting into 8.5 g/L of ethanol production after 24 h of fermentation process.
10. Ethanol production using microwave-acid-alkali pre-treated rice straw as a feedstock was done under optimized conditions [substrate loading (11%, w/v), pH (4.5), temperature (30°C), enzyme loading (0.5%, v/v), inoculum size (8%, v/v)] of SSF and maximum ethanol concentration (25.2 g/L) corresponding to 59% yield was obtained after 24 h of fermentation process under controlled conditions in a 3-L bioreactor.

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APPENDIX I

A. NUTRIENT BROTH

Ingredients	Quantity (g/L)
Peptic digest of animal tissue	5
Sodium chloride	5
Beef extract	1.5
Yeast extract	1.5

B. NUTRIENT AGAR

Ingredients	Quantity (g/L)
Peptic digest of animal tissue	5
Sodium chloride	5
Beef extract	1.5
Yeast extract	1.5
Agar	15

C. SIMMONS CITRATE AGAR

Ingredients	Quantity (g/L)
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1
Dipotassium phosphate	1
Sodium citrate	2
Sodium chloride	5
Bromothymol blue	0.08
Agar	15
Final pH (at 25°C)	6.8 ± 0.2

D. UREA AGAR BASE

Ingredients	Quantity (g/L)
Peptic digest of animal tissue	1
Dextrose	1
Sodium chloride	5
Monopotassium phosphate	2
Phenol red	0.012
Agar	15
Final pH (at 25°C)	6.8 ± 0.2

E. MGYP MEDIUM

Ingredients	Quantity (g/L)
Malt extract	3
Glucose	10
Yeast extract	3
Peptone	5

F. MR-VP MEDIUM

Ingredients	Quantity (g/L)
Buffered peptone	7
Dextrose	5
Dipotassium phosphate	5
Final pH (at 25°C)	6.9 ± 0.2

G. BILE-ESCULIN AGAR BASE

Ingredients	Quantity (g/L)
Buffered peptone	7
Dextrose	5
Dipotassium phosphate	5
Final pH (at 25°C)	6.9 ± 0.2

H. NITRATE AGAR

Ingredients	Quantity (g/L)
Agar	12
Beef extract	3
Peptic digest of animal tissue	5
Potassium nitrate	1

I. GYE MEDIUM

Ingredients	Quantity (g/L)
Glucose	10
Yeast extract	1
(NH ₄) ₂ SO ₄	5
KH ₂ PO ₄	1
MgSO ₄ .7H ₂ O	0.5

J. POTATO DEXTROSE AGAR

Ingredients	Quantity (g/L)
Potato extract	4
Dextrose	20
Agar	15

K. DNase TEST AGAR

Ingredients	Quantity (g/L)
Enzymatic Digest of Casein	15
Enzymatic Digest of Animal Tissue	5
Deoxyribonucleic Acid	2
Sodium Chloride	5
Agar	15
Final pH(at 25°C)	7.3 ± 0.2

All chemicals and reagents used for microbiological and chemical determinations were of the highest analytical grade and purchased from Sigma unless otherwise specified. Standard media components were purchased from Fisher Scientific (USA) or Sigma Aldrich (USA) and Hi-Media (Mumbai, India). Enzymes, Restriction enzymes and other chemicals of molecular biology grade were procured from New England Biolabs, UK unless otherwise specified. Sterilization of the media and chemicals were done in an autoclave at 121°C (15 lbs) for 15 min unless otherwise specified.

APPENDIX II

(A) BUFFERS

1. 50 mM Sodium Citrate buffer (pH 4.0, 5.0 and 6.0)

Stock solutions: A: 0.1 M citric acid; B: 0.1 M sodium citrate.

Use x mL A+ y mL B and dilute to 100 mL with 50 mL distilled water.

A (mL)	B (mL)	pH
37	13	3.5
33.0	17.0	4
25.5	24.5	4.5
20.5	29.5	5
13.7	36.3	5.5
9.5	41.5	6

2. 50 mM Potassium phosphate buffer (pH 7.0 and 6.0)

Stock solutions: A: 1M K₂HPO₄; B: 1 M KH₂PO₄

Use x mL A+ y mL B and dilute to 1000 mL with 995 mL distilled water.

A (mL)	B (mL)	pH
3.075	1.925	7
0.66	4.34	6

3. Glycine-NaOH buffer (pH 8.0, 9.0 and 10.0)

Stock solutions: 0.2 M glycine; 0.2 M NaOH.

Combine 25 mL glycine stock with x mL 0.2 M NaOH and dilute with distilled water to make 100 mL solution.

0.2 M NaOH	pH
2	8
6	9
19.3	10

4. DNS Reagent

Distilled Water	1415 mL
3, 5-Dinitrosalicylic acid	10.5 g
NaOH	19.8
Rochelle salts (Na-K tartarate)	306 g
Dissolve the above and then add:	
Rochelle salts (Na-K tartarate)	306 g
Phenol (melt at 50°)	7.6 mL
Sodium metabisulphite	8.3 g

(B) TBE buffer (10X)

Tris-HCl	0.09 M (pH 8.0)
Boric acid	0.9 M
EDTA	0.02 M (pH 8.0)

(C) Agarose gel loading dye (6X)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30.0%

(D) SDS-PAGE

Analysis of proteins were done through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the method as developed by Laemmli (1970).

All solutions for SDS-PAGE was made as follows:

1. 10 X SDS-PAGE running buffer

30.3 g Tris (Hydroxymethyl) Aminomethane
144.0 g Glycine
10.0 g Sodium dodecyl sulphate (SDS)

These compounds were dissolved in 1 L distilled water and stored at room temperature until required. When required for electrophoresis, the running buffer was diluted 10 times with distilled water to obtain the required volume.

2. 10% SDS stock solution

10 g SDS was dissolved in 100 mL distilled water.

3. 10% Ammonium persulphate (APS) solution

0.1 g APS was dissolved in 1 mL distilled water

This solution was prepared freshly prior to preparing a gel.

4. 30% Acrylamide stock solution

29.8 g acrylamide

0.2 g bis-acrylamide

These compounds were dissolved in distilled water and then made up to 100 mL volume. The bottle was wrapped in aluminium foil to protect it against light and stored at 4°C.

5. Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)

6 g Tris (Hydroxymethyl) Aminomethane

This was dissolved in distilled water and the pH adjusted with hydrochloric acid (HCl) to a pH of 6.8 before the solution was made up to 100 mL. This buffer was stored at 4°C.

6. Resolving gel buffer (1.5 M Tris-HCl, pH 8.8)

18.15 g Tris (Hydroxymethyl) Aminomethane

This was dissolved in distilled water and the pH adjusted with hydrochloric acid (HCl) to a pH of 8.8 before the solution was made up to 100 mL. This buffer was stored at 4°C.

7. SDS sample buffer (5x)

2.5 mL distilled water

1 mL 0.5 M Tris-HCl buffer (pH 6.8)

3 mL glycerol

2 mL 10% SDS stock solution (w/v)

1 mL 1% bromophenol blue (w/v)

The sample buffer was stored at room temperature. Prior to preparing samples for electrophoresis, 5 µL β-mercaptoethanol was added to 95 µL of SDS sample buffer. SDS sample buffer (3 µL) was added to 15 µL of protein samples before boiling and electrophoresis.

8. Coomassie Brilliant Blue protein staining solution

The staining solution was prepared by dissolving 0.075% (w/v) Coomassie Brilliant Blue R250 in a solution of 30% methanol and 10% glacial acetic acid. Staining of polyacrylamide gels was done for a minimum of 1 h or overnight on a platform shaker.

9. Coomassie destain solution

Destain solution was prepared by mixing 30% methanol, 60% distilled water and 10% glacial acetic acid. Gels were placed in destain solution on a platform shaker until protein bands appeared against a clear background. Gels were then placed in distilled water and then photographs were taken.

10. Preparation of SDS-PAGE gels

Resolving gel

The 12.5% SDS-PAGE resolving gels were prepared in a small beaker by adding the following solutions in sequence:

- 3.13 mL distilled water
- 2.5 mL 1.5 M Tris-HCl buffer (pH 8.8)
- 4.17 mL 30% acrylamide stock solution
- 0.1 mL 10% SDS stock solution
- 0.1 mL 10% APS solution
- 0.05 mL TEMED

The solution was gently mixed by swirling the beaker before pouring the gel. Gels were then covered with about 50-100 μ L of isopropanol and allowed to set. For zymogram, 0.5 mL of a 2% (w/v) stock solution of CMC was added to the resolving gel prior to addition of APS and the distilled water reduced by the same volume.

The 4% stacking gels were prepared in a small beaker by adding the following solutions in the following sequence:

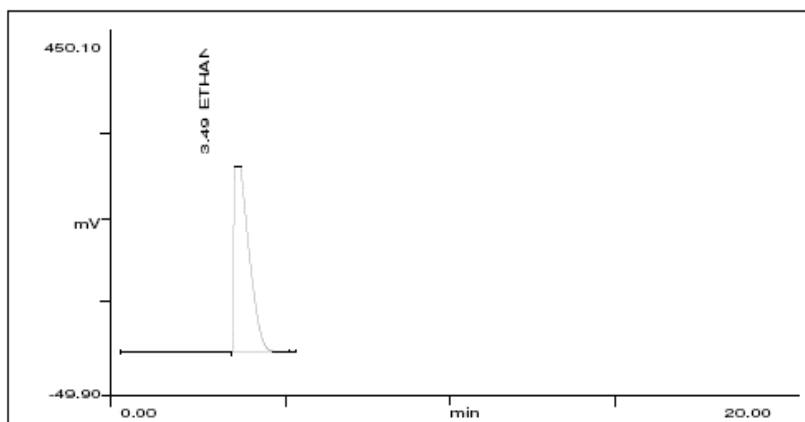
- 3.6 mL distilled water
- 0.63 mL 0.5 M Tris-HCl buffer (pH 6.8)
- 0.66 mL 30% acrylamide stock solution
- 0.05 mL 10% SDS stock solution
- 0.01 mL 10% APS solution
- 0.005 mL TEMED

The isopropanol was removed from the resolving gel with filter paper. The stacking gel solution was poured on top of the resolving gel before plastic combs were inserted to form the wells.

REPORT

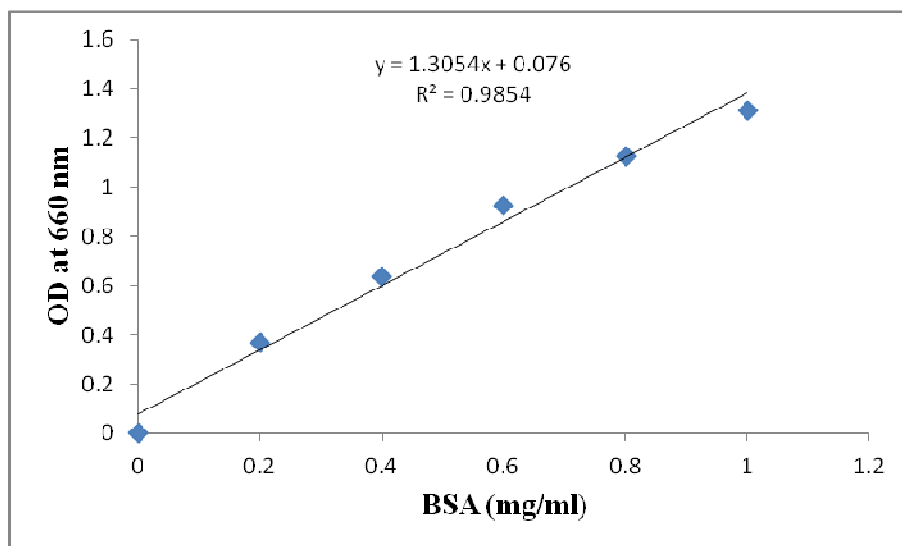
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 Method File: .MET
 Detector: FID. System: GC
 Date: 26 Jun 2013 Time: 15:6:14
 Run: ch1: 0
 Type of Analysis : External On Area
 Report printed on : 26/6/2013 at : 16:01:09

Pk.Width	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
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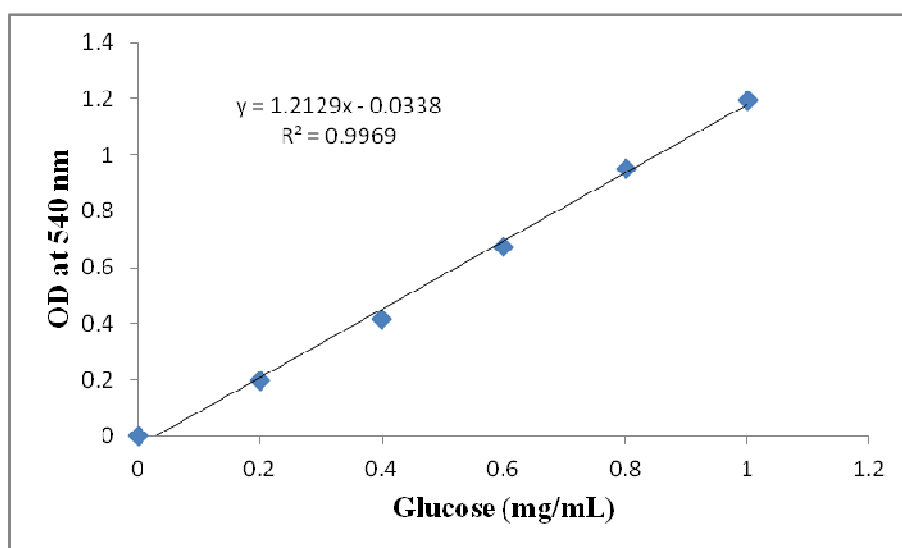


No.	R.T.	Area	Resp Fact	Con Amt	Pk Ty	Comp Name
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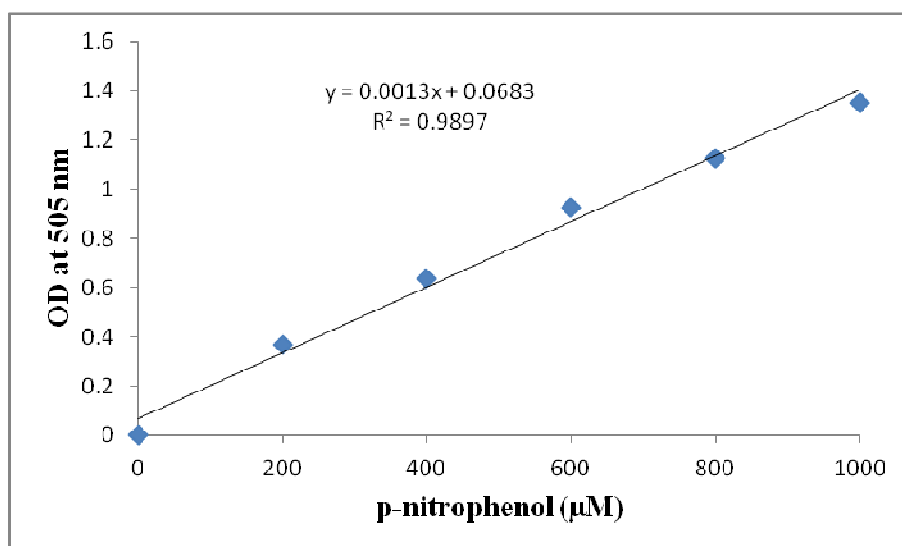
Peak of ethanol (100%) and corresponding area obtained using Gas chromatography.



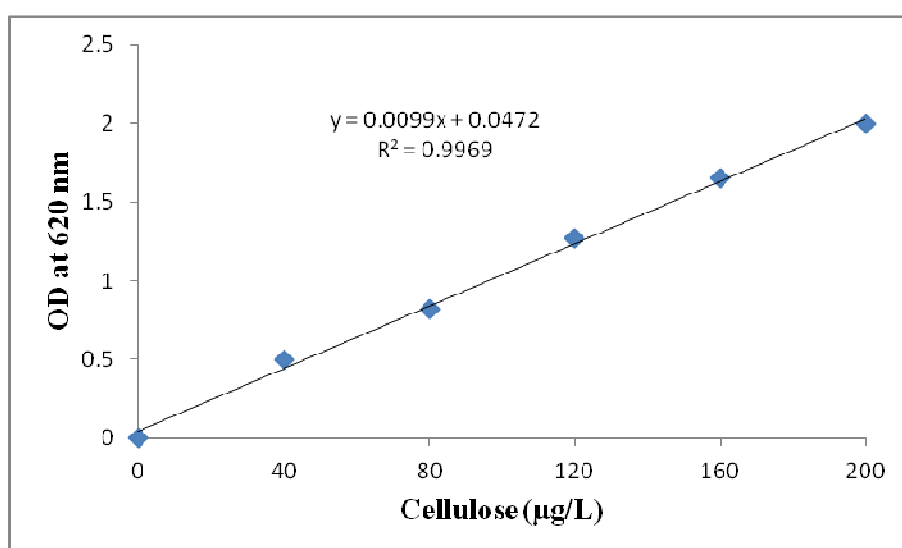
Standard curve of protein



Standard curve of Glucose



Standard curve of p-nitro phenol



Standard curve of cellulose

Appendix-III

NCBI GenBank details of bacterial isolates

Bacillus licheniformis strain NA8 16S ribosomal RNA gene, partial sequence

GenBank: KF840405.1

[FASTA Graphics](#)

[Go to:](#)

LOCUS KF840405 1451 bp DNA linear BCT 09-FEB-2014

DEFINITION Bacillus licheniformis strain NA8 16S ribosomal RNA gene, partial sequence.

ACCESSION KF840405

VERSION KF840405.1 GI:580961272

KEYWORDS .

SOURCE Bacillus licheniformis

ORGANISM [Bacillus licheniformis](#)
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.

REFERENCE 1 (bases 1 to 1451)

AUTHORS Akhtar,N. and Goyal,D.

TITLE Cellulose degrading bacteria from paper pulp

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1451)

AUTHORS Akhtar,N. and Goyal,D.

TITLE Direct Submission

JOURNAL Submitted (11-NOV-2013) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India

COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

FEATURES Location/Qualifiers

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/collected_by="Nadeem Akhtar"

[rRNA](#) <1..>1451
/product="16S ribosomal RNA"

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Bacillus licheniformis strain NA9 16S ribosomal RNA gene, partial sequence

GenBank: KF840406.1

[FASTA Graphics](#)

[Go to:](#)

LOCUS KF840406 1455 bp DNA linear BCT 09-FEB-2014

DEFINITION Bacillus licheniformis strain NA9 16S ribosomal RNA gene, partial sequence.

ACCESSION KF840406

VERSION KF840406.1 GI:580961273

KEYWORDS .

SOURCE Bacillus licheniformis

ORGANISM [Bacillus licheniformis](#)
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.

REFERENCE 1 (bases 1 to 1455)
AUTHORS Akhtar,N. and Goyal,D.
TITLE Cellulose degrading bacteria from paper pulp
JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1455)
AUTHORS Akhtar,N. and Goyal,D.
TITLE Direct Submission
JOURNAL Submitted (11-NOV-2013) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India

COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

FEATURES Location/Qualifiers

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/collected_by="Nadeem Akhtar"

[rRNA](#) <1..>1455
/product="16S ribosomal RNA"

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Bacillus licheniformis strain NA11 16S ribosomal RNA gene, partial sequence

GenBank: KF840404.1

[FASTA Graphics](#)

[Go to:](#)

LOCUS KF840404 1464 bp DNA linear BCT 09-FEB-2014

DEFINITION Bacillus licheniformis strain NA11 16S ribosomal RNA gene, partial sequence.

ACCESSION KF840404

VERSION KF840404.1 GI:580961271

KEYWORDS .

SOURCE Bacillus licheniformis

ORGANISM [Bacillus licheniformis](#)
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.

REFERENCE 1 (bases 1 to 1464)

AUTHORS Akhtar,N. and Goyal,D.

TITLE Cellulose degrading bacteria from agricultural waste biomass

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1464)

AUTHORS Akhtar,N. and Goyal,D.

TITLE Direct Submission

JOURNAL Submitted (11-NOV-2013) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India

COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

FEATURES Location/Qualifiers

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[rRNA](#) <1..>1464
/product="16S ribosomal RNA"

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Bacillus licheniformis strain NA14 16S ribosomal RNA gene, partial sequence

GenBank: KF826800.1

[FASTA Graphics](#)

[Go to:](#)

LOCUS KF826800 1485 bp DNA linear BCT 09-FEB-2014

DEFINITION Bacillus licheniformis strain NA14 16S ribosomal RNA gene, partial sequence.

ACCESSION KF826800

VERSION KF826800.1 GI:580961277

KEYWORDS .

SOURCE Bacillus licheniformis

ORGANISM [Bacillus licheniformis](#)
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.

REFERENCE 1 (bases 1 to 1485)

AUTHORS Akhtar,N. and Goyal,D.

TITLE Cellulose degrading bacteria from agricultural waste biomass

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1485)

AUTHORS Akhtar,N. and Goyal,D.

TITLE Direct Submission

JOURNAL Submitted (07-NOV-2013) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India

COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

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[rRNA](#) <1..>1485

/product="16S ribosomal RNA"

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Bacillus subtilis strain NA15 16S ribosomal RNA gene, partial sequence

GenBank: KF840407.1

[FASTA Graphics](#)

[Go to:](#)

LOCUS KF840407 1486 bp DNA linear BCT 09-FEB-2014

DEFINITION Bacillus subtilis strain NA15 16S ribosomal RNA gene, partial sequence.

ACCESSION KF840407

VERSION KF840407.1 GI:580961274

KEYWORDS .

SOURCE Bacillus subtilis

ORGANISM [Bacillus subtilis](#)
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.

REFERENCE 1 (bases 1 to 1486)

AUTHORS Akhtar,N. and Goyal,D.

TITLE Cellulose degrading bacteria from agricultural waste biomass

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1486)

AUTHORS Akhtar,N. and Goyal,D.

TITLE Direct Submission

JOURNAL Submitted (11-NOV-2013) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India

COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

FEATURES Location/Qualifiers

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/product="16S ribosomal RNA"

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Lysinibacillus fusiformis strain DGA 16S ribosomal RNA gene, partial sequence

GenBank: KC491188.1

[FASTA Graphics](#)

[Go to:](#)

LOCUS KC491188 1464 bp DNA linear BCT 10-MAR-2013

DEFINITION Lysinibacillus fusiformis strain DGA 16S ribosomal RNA gene, partial sequence.

ACCESSION KC491188

VERSION KC491188.1 GI:459219254

KEYWORDS .

SOURCE Lysinibacillus fusiformis

ORGANISM [Lysinibacillus fusiformis](#)
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Lysinibacillus.

REFERENCE 1 (bases 1 to 1464)

AUTHORS Akhtar,N. and Goyal,D.

TITLE Cellulose degrading bacterial isolates from sugar industry of Kanpur

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1464)

AUTHORS Akhtar,N. and Goyal,D.

TITLE Direct Submission

JOURNAL Submitted (15-JAN-2013) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India

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Assembly Method :: multalign v. 1.1
Sequencing Technology :: Sanger dideoxy sequencing
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Bacillus licheniformis strain DGB 16S ribosomal RNA gene, partial sequence

GenBank: KF840408.1

[FASTA Graphics](#)

[Go to:](#)

LOCUS KF840408 1471 bp DNA linear BCT 09-FEB-2014

DEFINITION Bacillus licheniformis strain DGB 16S ribosomal RNA gene, partial sequence.

ACCESSION KF840408

VERSION KF840408.1 GI:580961275

KEYWORDS .

SOURCE Bacillus licheniformis

ORGANISM [Bacillus licheniformis](#)
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.

REFERENCE 1 (bases 1 to 1471)

AUTHORS Akhtar,N. and Goyal,D.

TITLE Cellulose degrading bacteria from sugarcane bagasse

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1471)

AUTHORS Akhtar,N. and Goyal,D.

TITLE Direct Submission

JOURNAL Submitted (11-NOV-2013) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India

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Sequencing Technology :: Sanger dideoxy sequencing
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Bacillus licheniformis strain DGC 16S ribosomal RNA gene, partial sequence

GenBank: KF840409.1

[FASTA Graphics](#)

[Go to:](#)

LOCUS KF840409 1487 bp DNA linear BCT 09-FEB-2014

DEFINITION Bacillus licheniformis strain DGC 16S ribosomal RNA gene, partial sequence.

ACCESSION KF840409

VERSION KF840409.1 GI:580961276

KEYWORDS .

SOURCE Bacillus licheniformis

ORGANISM [Bacillus licheniformis](#)
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.

REFERENCE 1 (bases 1 to 1487)

AUTHORS Akhtar,N. and Goyal,D.

TITLE Cellulose degrading bacteria from sugarcane bagasse

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1487)

AUTHORS Akhtar,N. and Goyal,D.

TITLE Direct Submission

JOURNAL Submitted (11-NOV-2013) Department of Biotechnology and Environmental Sciences, Thapar University, Bhadson Road, Patiala, Punjab 147004, India

COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

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Simplification and Optimization of Media Ingredients for Enhanced Production of CMCase by Newly Isolated *Bacillus subtilis* NA15

Nadeem Akhtar,^a Aanchal,^a Dinesh Goyal,^a and Arun Goyal^b

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Published online 00 Month 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/ep.12004

Cellulose degrading bacterium was isolated from composting biomass and identified as Bacillus subtilis on the basis of phenotypic fingerprint using GEN III MicroPlate™ and 16S ribosomal RNA (rRNA) gene sequence analysis. 16S rRNA gene sequence similarity analysis showed 99% similarity with Bacillus subtilis. The screening of 11 nutrients was done using Plackett-Burman design to explicate the parameters that significantly influence the carboxy methyl cellulase (CMCase) production. Maximum CMCase production of 0.47 U/mL was obtained using response surface methodology with carboxy methyl cellulose (CMC): 18 g/L, peptone: 5 g/L, yeast extract: 5 g/L and MnCl₂: 0.5 g/L. The model predicted the maximum CMCase activity (0.45 U/mL), which was in good agreement with the experimental value of 0.47 U/mL showing sevenfold increase as compared to unoptimized medium. Presence of MnCl₂ in the medium significantly enhanced the CMCase production. © 2014 American Institute of Chemical Engineers Environ Prog, 00: 000–000, 2014

Keywords: *Bacillus subtilis* NA15, carboxy methyl cellulase, response surface methodology, 16S rRNA

INTRODUCTION

Cellulose, the most common natural renewable biopolymer, is a linear condensation polymer consisting of D-anhydroglucopyranose joined by β-1, 4-glycosidic bonds with a degree of polymerization from 100 to 20,000 [1]. Cellulases, historically, been divided into three major groups: endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) [2] and the synergistic actions of these enzyme is widely accepted mechanism for cellulose hydrolysis [3]. These enzymes can either be free in aerobic microorganisms, or grouped in a multicomponent enzyme complex, cellulosome, in anaerobic cellulolytic bacteria [4]. Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization [5] and it contribute to 8% of the worldwide industrial enzyme demands and the demand is expected to increase by 100% within 2014 [6]. The cellulase enzyme market is expected to expand dramatically with its application in hydrolysis of pretreated cellulosic materials to sugars and further to bioethanol and bio-based products [7].

Over the years, characterization of cellulases and hemicellulases producing bacteria and fungi has been investigated extensively [8], capable of utilizing various cellulosic substrate such as carboxymethyl cellulose, avicel, lichenan, xylan, sugarcane bagasse, rice straw, wheat straw, and leaf litter biomass. *Bacillus* spp., resistant to wide range of physiological and environmental conditions [9], constitutes the dominant bacterial workhorses in the production and secretion of large quantity of extracellular enzymes in microbial fermentations [10]. Cellulase producing bacteria have been isolated from a wide variety of sources such as composting heaps, decaying plant material, feces of ruminants, and hot springs [11]. Bacteria, which have the high growth rate as compared to fungi, have the good potential to be used in cellulase production [12]. Many aerobic microorganisms, both bacterial and fungal, use the cell free cellulase to digest cellulose [13]. Cellulose degradation is largely an aerobic process, selected primary aerobic cellulolytic bacterial isolates are *Bacillus* strain C1 [14], *Bacillus* sp. AS3 [15], *B. subtilis* GN156 [16], and *B. amyloliquefaciens* DL-3 [17]. Therefore, for optimum production and effective utilization of extracellular bacterial cellulase, the medium ingredients have to be optimized, as its production in microorganisms is significantly influenced by medium constituents [18,19], temperature, pH, aeration, and other physical parameters [20]. Also, the relationship between these parameters has marked significant affect on the production of the cellulase and the same has been reported in bacteria [21] and fungi [22]. However, optimization of medium ingredients by classical method involves changing single independent variable at a time while keeping others at a constant level. This is time-consuming and expensive for large number of variables [23] and may result in unacceptable conclusions [24]. Plackett-Burman design plays a key role in screening of many variables and allows obtaining unbiased estimate of linear effects of all factors with maximum accuracy for a given number of observations. Response surface methodology (RSM) has been widely applied in many areas of biotechnology such as ethanol production using *Kluyveromyces* sp. [25], bacteriocin production by *Lactobacillus casei* [26] and cellulase production by *B. subtilis* AS3 [18] and *B. amyloliquefaciens* DL-3 [19].

The objective of the present work was to design an optimal medium for enhanced extracellular carboxy methyl cellulase (CMCase) production by a newly isolated *B. subtilis* NA15. The designing of medium constituents was done

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preliminary through screening of significant parameters using Plackett-Burman design followed by optimization of significant parameters by central composite design (CCD).

MATERIALS AND METHODS

Sample Collection, Screening, and Isolation of Cellulolytic Bacteria

Samples of compost from degraded mixed agricultural waste biomass were collected from Thapar University, Patiala, Punjab (India) using alcohol-sterilized implements. Bacterial strains were isolated and purified by enrichment culture technique using Nutrient Agar (NA), supplemented with 1% (w/v) carboxymethyl cellulose (CMC) using incubation temperature of 37°C. Clear zone of hydrolysis formed around the bacterial colonies after 24–48 h of growth were screened by staining it with aqueous solution of Congo red (0.1%, w/v) for 15 min followed by destaining with 1 M NaCl [27]. Out of eight bacteria screened, only one efficient cellulase producing strain (designated as NA15) was selected for further studies.

Biochemical and Molecular Characterization of Bacterial Isolate NA15

Pure culture obtained as above was subjected to Gram stain, capsule stain, growth on MacConkey agar, KOH, oxidase, catalase, nitrate reduction, citrate utilization, Bile Esculin, Voges-Proskauer (V-P) reaction, Indole, DNase and urease tests as per standard methods [28] with *E. coli* DH5 α as control. To reveal the bacterial size and morphology, sample was prepared as per protocol discussed by Akhtar et al. [15] and visualized under scanning electron microscope (SEM) (Model: JEOL JSM-6510 LV) under different magnifications.

Biolog GEN III MicroPlate™ Identification

Microbial identification was done using Biolog GEN III MicroPlate™ (Biolog, Hayward, California) as per manufacture's instruction. The bacterial isolate NA15 to be identified was grown on Biolog recommended agar medium (agar with 5% sheep blood) and then further suspended in a proprietary inoculating fluid at the recommended cell density (90–98% transmittance). The cell suspension was inoculated (100 μ L per well) into the GEN III MicroPlate™ and then incubated at 37°C under aerobic condition. After 22 h of incubation, the phenotypic fingerprint of GEN III MicroPlate™ was analyzed using GEN III OmniLog® Combo Plus System (Biolog, Hayward, California).

16S rRNA Gene Sequencing and Phylogenetic Analysis

The chromosomal DNA was extracted from the overnight grown culture of strain NA15 using saline-EDTA by the method described by Akhtar et al. [15] and used as the template for 16S rRNA gene amplification. The 16S rRNA gene fragment of the DNA was amplified by polymerase chain reaction (PCR) using 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1510R (5'-GGTTACCTTGTTACGACTT-3') primers [29] with GeneAmp® 2700 PCR (Applied Biosystems). Reaction mixture was prepared for PCR to a final volume of 100 μ L containing 1X PCR buffer (Invitrogen), each deoxynucleotide triphosphate at a concentration of 200 μ M, 1.5 mM MgCl₂, each primer at a concentration of 0.1 μ M, 2.5U of *Taq* DNA polymerase (Invitrogen) and 2 μ L (~50 ng) of template DNA. The PCR was performed with an initial denaturation at 92°C for 2 min followed by 36 temperature cycles as follows: 92°C for 1 min, 48°C for 30 s, 72°C for 2 min, and a final extension at 72°C for 6 min. Amplification was verified by electrophoresis of 5 μ L aliquot of PCR product on a 0.7% agarose gel and the amplicon was purified using QIA gel extraction kit (Qiagen) as per manufacture's instruction. The

purified PCR product was sequenced using automated DNA sequencer AB3500 xL (Applied Biosystem). The gene sequence was compared with Ribosomal Database Project-II (RDP-II) [30] and with those from GenBank (<http://www.ncbi.nlm.nih.gov/>) using the BLASTN program [31]. The sequences were aligned using the CLUSTALW program [32], evolutionary distance was calculated by Kimura 2 parameter and a phylogenetic tree was constructed by the neighbor-joining method. Bootstrap analysis was based on 1000 resamplings and a phylogenetic tree was constructed using the MEGA 5.0 software [33].

Production and Assay of Extracellular CMCase

The bacterial isolate NA15 was grown in an unoptimized medium containing 11 different ingredients: peptone (5 g/L), yeast extract (5 g/L), K₂HPO₄ (0.01 g/L), MgSO₄·5H₂O (0.025 g/L), FeSO₄·7H₂O (0.025 g/L), MnCl₂·4H₂O, NaCl (0.025 g/L), KCl (0.025 g/L), NaH₂PO₄ (0.025 g/L), Na₂HPO₄ (0.025 g/L) and supplemented with 1.0% CMC. Culture medium (100 mL) in 250-mL flask was inoculated with 1% (v/v) overnight grown culture (O.D. 0.6 at 600 nm) and

Table 1. Biochemical characteristics of the bacterial isolate NA15.

Characteristics/biochemical test	Observation*
Gram reaction	+
Capsule stain	–
Morphology	Rods
Growth on MacConkey's Agar	+
Indole test	–
DNase	+
KOH	–
Oxidase	+
Catalase	+
NO ₃ reduction into NO ₂	+
Citrate utilization	–
Bile Esculin	+
Voges-Proskauer (V-P) reaction	–
Urease	+

*+, positive reaction; –, negative reaction

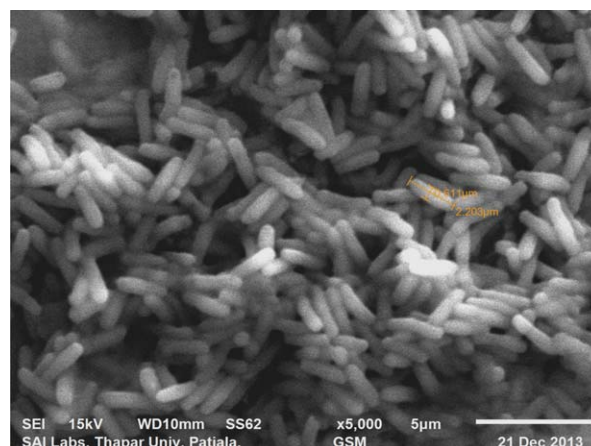


Figure 1. Scanning electron micrograph of *B. subtilis* NA15 (magnification: 5000 $\times$). [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.wileyonlinelibrary.com).]

Table 2. Substrate utilization of *B. subtilis* NA15 using Biolog GEN III MicroPlate™.

Properties	Result*	Properties	Result*	Properties	Result*
Negative control	–	Inosine	+	D-Glucuronic acid	+
Dextrin	+	1% Sodium lactate	+	Glucuronamide	–
D-maltose	+	Fusidic acid	–	Mucic acid	+
D-trehalose	+	D-Serine	–	Quinic acid	–
D-cellobioase	+	D-Sorbitol	+	D-Saccharic acid	+
Gentiobiose	+	D-Mannitol	+	Vancomycin	–
Sucrose	+	D-Arabitol	–	Tetrazolium violet	–
D-turanose	+	myo-Inositol	+	Tetrazolium blue	–
Stachyose	+	Glycerol	+	p-Hydroxy-phenylacetic acid	–
Positive control	+	D-Glucose-6-PO ₄	+	Methyl pyruvate	+
pH 6	+	D-Fructose-6-PO ₄	+	D-Lactic acid methyl ester	+
pH 5	+	D-Aspartic acid	+	L-Lactic acid	+
D-Raffinose	+	D-Serine	–	Citric acid	+
α-D-Lactose	+	Troleandomycin	–	α-Keto-glutaric acid	+
D-Melibiose	+	Rifamycin SV	+	D-Malic acid	–
β-Methyl-D glucoside	+	Minocycline	–	L-Malic acid	+
D-Salicin	+	Gelatin	–	Bromo-succinic acid	+
N-Acetyl-D-glucosamine	+	Glycyl-L-proline	+	Nalidixic acid	–
N-Acetyl-β-D-mannosamine	+	L-Alanine	+	Lithium Chloride	+
N-Acetyl-D-galactosamine	–	L-Arginine	+	Potassium tellurite	+
N-Acetyl neuraminic Acid	–	L-Aspartic acid	+	Tween 40	+
1% NaCl	+	L-Glutamic acid	+	γ-Amino-butyric acid	+
4% NaCl	+	L-Histidine	+	α-Hydroxy-butyric acid	–
8% NaCl	+	L-Pyroglutamic acid	+	β-hydroxy-D,L-butyric acid	+
α-D-Glucose	+	L-Serine	–	α-Keto-butyric acid	–
D-Mannose	+	Lincomycin	–	Acetoacetic acid	+
D-Fructose	+	Guanidine HCl	–	Propionic acid	–
D-Galactose	+	Niaproof 4	–	Acetic acid	+
3-Methyl glucose	–	Pectin	+	Formic acid	+
D-Fucose	–	D-Galacturonic Acid	+	Aztreonam	+
L-Fucose	–	L-Galactonic acid lactone	–	Sodium butyrate	+
L-Rhamnose	+	D-Gluconic acid	+	Sodium bromate	–

*+: Positive reaction, –: Negative reaction

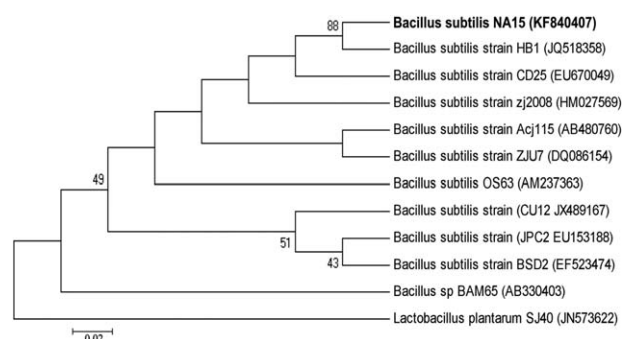


Figure 2. 16S rRNA gene sequence-based dendrogram constructed by the neighbor joining method showing the phylogenetic position of *B. subtilis* NA15 along with sequences available in GenBank database. Numerical values indicate bootstrap percentile from 1000 replicates. *Lactobacillus plantarum* was used as outgroup taxa. Bar: 0.02 substitutions per nucleotide.

incubated at 37°C under shaking condition (120 rpm) for 24 h. After 24 h of incubation, cell free supernatant collected after centrifugation (7000g, 20 min) was investigated for CMCase activity. The assay conditions for extracellular CMCase produced by strain N15 was optimized (described in

detail in the Supporting Information) and the activity was found maximum (0.06 U/mL) at 50°C in presence of 1% (w/v) CMC dissolved in phosphate buffer (50 mM, pH 7.0). The reaction mixture (2 mL) contained 0.2 mL of cell free supernatant in 1.8 mL of 1% (w/v) CMC dissolved in phosphate buffer (50 mM, pH 7.0). The amount of reducing sugar liberated was quantified by 3, 5-dinitrosalicylic acid (DNS) method [34] using glucose as standard. One unit (U) of CMCase is defined as the amount of enzyme that liberates 1 μM of reducing sugar per minute.

Experimental Design of RSM

The present study for optimization of CMCase production was accomplished in two consecutive steps: first, a Plackett-Burman design was executed to determine the significant parameters affecting CMCase production. Whereas, in the second stage, a five-level RSM design (CCD) with eight axial points and six replicates at the center point was performed to estimate linear and quadratic effects and the corresponding response surface.

Plackett-Burman Design

A preselection of the medium constituents that might affect CMCase activity was performed using a two level screening Plackett-Burman design for 11 different medium constituents such as CMC, peptone, yeast extract, K₂HPO₄, MgSO₄·5H₂O, FeSO₄·7H₂O, MnCl₂·4H₂O, NaCl, KCl, NaH₂PO₄, and Na₂HPO₄ using Design-Expert (Version

8.0.7.1, Stat-Ease, Minneapolis, MN). The effect of each factor was assessed in terms of the difference between the average of the measurements made at the high level of factor and the average of the measurements made at the low level of that factor, which was determined using the following equation:

$$E_{(Xi)} = 2 \left(\sum P_{i+} - \sum P_{i-} \right) / N$$

where $E_{(Xi)}$ is the effect of the tested variable, P_{i+} and P_{i-} are the activities from the trials whereas the variable (X_i) measured were present at high and low level, respectively, and N is the number of trials (experiments) presented in Table 4. Standard error (SE) of the concentration effect was the square root of the variance of an effect, and the significance level (P -value) of the effect of each concentration was determined using student's t -test as given by the equation:

$$t_{(Xi)} = E_{(Xi)} / SE$$

Designing of Optimal Medium using CCD

To derive the optimum level of screened variables, a CCD with five coded levels (Table 3) and four variables was used to study the combined influence of CMC (A), peptone (B), yeast extract (C), and $MnCl_2 \cdot 4H_2O$ (D). For the four factors, the design was made up of a full 2^2 factorial design with its four points augmented with six replications of the center points (all factors at level 0) and the four star points, that is, points having for one factor an axial distance to the center of $\pm a$, whereas the other two factors are at level 0. The axial distance was chosen to be 2.0 to make this design orthogonal. Thirty experiments were used in the present study. Using least squares, the regression model was fit to evaluate

the linear interaction and the quadratic effects of screened variables. The second order polynomial model was described as follows:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j$$

The model behavior was explained by above quadratic equation. Where Y is predicted response (CMase activity), β_0 is the model constant, β_i is the linear coefficient, β_{ij} is the coefficient for interaction effect, and X_i is the dimensionless coded value if X_i (independent variable).

The results were analyzed by the experimental design model and two-dimensional (2D) contour, plots using the statistical software package Design-Expert (Version 8.0.7.1, Stat-Ease, Minneapolis, MN). The fit quality of the model was evaluated by R^2 and analysis of variance (ANOVA). The robustness of the model and optimal values of parameters was assessed by the determination coefficient, correlation coefficient (R), or F -test [35].

RESULTS AND DISCUSSION

Screening and Characterization of Bacterial Strain NA15

Bacterial strain NA15 was isolated from compost and screened by hydrolysis zones in Congo red staining. Strain NA15 was Gram positive and rod-shaped, showed positive tests for oxidase, catalase, DNase, capsule stain, urease, nitrate reduction and Bile Esculin, and negative for KOH, citrate utilization, indole and Voges-Proskauer (V-P) reaction (Table 1). SEM confirmed the rod-shaped structure and revealed an approximate size of $2.2 \times 0.6 \mu m$ at magnification 5000 $\times$ (Figure 1).

Biolog GEN III MicroPlate™ Identification

On the basis of phenotypic pattern in the GEN III MicroPlate™ analyzed by Phenotype MicroArray (PM) Software (Table 2), strain NA15 was identified as a member of *B. subtilis*. Biolog's identification method is an easiest, rapid, cost effective, and extensively used method to characterize microbial community by determining the phenotypic fingerprint. The identification of herbicide degrading *Serratia marcescens* SE1 [36] and cellulose degrading *Bacillus* sp. C1 [14] have found to be reliable using GEN III MicroPlate™ and Biolog GP2 plate, respectively. Out of eight bacteria screened, only one efficient cellulase producing strain (designated as NA15) was selected for further studies.

Table 3. Range of values for response surface method.

	Independent variables (g/L)	Coded levels				
		$-\alpha$	-1	0	1	$+\alpha$
A	CMC	2	6	10	14	18
B	Peptone	2	3.5	5	6.5	8
C	Yeast extract	1	3	5	7	9
D	$MnCl_2 \cdot 4H_2O$	0.01	0.2575	0.505	0.75	1

Table 4. Coded experimental design layout of Plackett-Burman design for the screening of media constituents.

Runs		Coded values (g/L)											CMCase activity (U/mL)
Std	Run	A: CMC	B: peptone	C: yeast extract	D: K_2HPO_4	E: $MgSO_4$	F: $FeSO_4$	G: $MnCl_2 \cdot 4H_2O$	H: NaCl	J: KCl	K: $NaHSO_4$	L: Na_2HPO_4	Experimental values
8	1	18	8	1	0.5	0.05	0.45	0.01	0.5	1	0.01	0.2	0.283
6	2	2	2	1	2	0.05	0.45	1	0.02	1	0.1	0.2	0.069
3	3	18	2	9	2	0.05	0.45	1	0.5	0.01	0.01	0.05	0.258
1	4	18	8	1	2	0.45	0.45	0.01	0.02	0.01	0.1	0.05	0.246
11	5	18	2	9	2	0.45	0.05	0.01	0.02	1	0.01	0.2	0.266
4	6	2	8	1	2	0.45	0.05	1	0.5	1	0.01	0.05	0.106
5	7	2	2	9	0.5	0.45	0.45	0.01	0.5	1	0.1	0.05	0.07
7	8	18	2	1	0.5	0.45	0.05	1	0.5	0.01	0.1	0.2	0.223
2	9	2	8	9	0.5	0.45	0.45	1	0.02	0.01	0.01	0.2	0.153
9	10	18	8	9	0.5	0.05	0.05	1	0.02	1	0.1	0.05	0.384
10	11	2	8	9	2	0.05	0.05	0.01	0.5	0.01	0.1	0.2	0.156
12	12	2	2	1	0.5	0.05	0.05	0.01	0.02	0.01	0.01	0.05	0.006

Phylogenetic Analysis of the 16S rRNA Gene Sequence

PCR amplified 16S rRNA partial gene (~1.5 Kb) sequence of strain NA15 was submitted to GenBank (Accession number KF840407). The morphological, biochemical, and molecular characterization of 16S rDNA similarity analysis also showed 99% similarity with *B. subtilis*. Phylogenetic analysis revealed that these sequences were clustered into one group and the sequences of NA 15 clustered with *B. subtilis* (Figure 2). 16S rRNA gene is the universal target for bacterial identification, which provides reliable, reproducible, and accurate method for identification of bacteria [37]. Sometimes, using a single Biolog GEN III MicroPlate™ identification method may mislead [36], therefore, to confirm the identification, 16S rRNA gene sequencing and phylogenetic analysis have been investigated and the result was in good agreement with Biolog's GEN III MicroPlate™ identification.

Table 5. Model coefficient estimated by multiple linear regressions.

Source	Sum of squares	Mean square	F value	Computed t-values
A:CMC	0.101092	0.101092	288.2049	75.86
B:Peptone	0.015812	0.015812	45.07743	11.86
C:Yeast extract	0.010442	0.010442	29.77045	7.84
G:MnCl ₂ .4H ₂ O	0.002308	0.002308	6.579035	1.73
J:KCl	0.00151	0.00151	4.303571	1.13

Diagnostic Experimental Design of CMCase Production by *B. subtilis* NA15

The CMCase activity of the cell free supernatant of *B. subtilis* NA15 after 24 h of incubation showed variation from 0.006 to 0.38 U/mL (Table 4). This variation reflected the significance of factors on the response (CMCase activity). The analysis of regression coefficients and *t*-value of five ingredients were shown in Table 5. In general, a large *t*-value associated with a low *P*-value of a variable indicates a high significance of the corresponding model terms [38]. ANOVA through Fisher's test was used to evaluate the effect of 11 independent variables on the response and significant results were identified by a *P*-value of <0.05. CMC, peptone, yeast extract, and MnCl₂ showed confidence level above 95%, were selected for further interactive study (statistical analysis of Plackett-Burman design is provided in Supplementary Information).

Neglecting the variables which had less or no contribution, the model equation for CMCase activity can be written as:

Table 7. *R*-Squared, Adj *R*-Squared, Pred *R*-Squared, and Adeq Precision value of the model

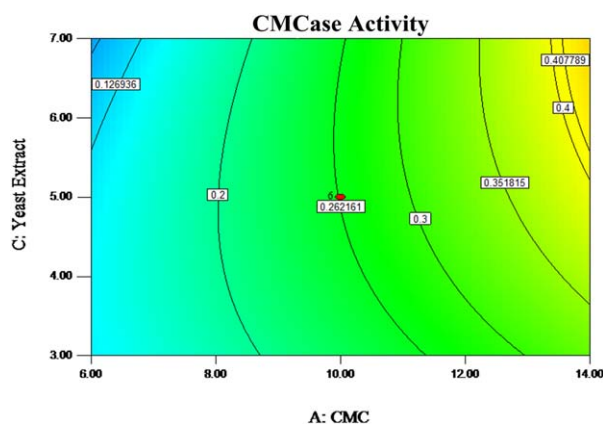
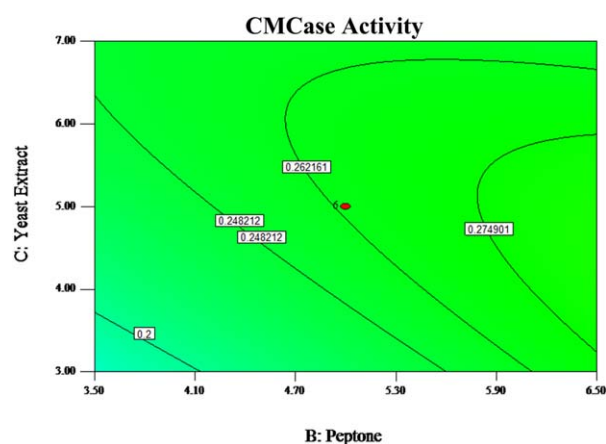
Std. Dev.	0.02	<i>R</i> -Squared	0.983
Mean	0.24	Adj <i>R</i> -Squared	0.967
C.V. %	9.04	Pred <i>R</i> -Squared	0.915
PRESS	0.038	Adeq Precision	31.48

Table 6. Coded experimental design and results for the response surface of maximum CMCase activity of *B. subtilis* NA15 as a function of CMC, Peptone, Yeast Extract, and MnCl₂.4H₂O.

Runs		Coded values (g/L)				CMCase activity (U/mL)
Number	Order	A:CMC	B:Peptone	C:Yeast extract	D:MnCl ₂ .4H ₂ O	Experimental predicted
29	1	10	5.0	5	0.505	0.274
13	2	6	3.5	7	0.752	0.065
15	3	6	6.5	7	0.752	0.107
8	4	14	6.5	7	0.257	0.434
26	5	10	5.0	5	0.505	0.269
5	6	6	3.5	7	0.257	0.087
14	7	14	3.5	7	0.752	0.451
23	8	10	5.0	5	0.010	0.293
30	9	10	5.0	5	0.505	0.276
10	10	14	3.5	3	0.752	0.333
25	11	10	5.0	5	0.505	0.236
28	12	10	5.0	5	0.505	0.269
18	13	18	5.0	5	0.505	0.465
6	14	14	3.5	7	0.257	0.398
24	15	10	5.0	5	1.00	0.284
4	16	14	6.5	3	0.257	0.327
7	17	6.0	6.5	7	0.257	0.115
19	18	10	2.0	5	0.505	0.214
16	19	14	6.5	7	0.752	0.414
12	20	14	6.5	3	0.752	0.397
2	21	14	3.5	3	0.257	0.228
17	22	2	5.0	5	0.505	0.01
3	23	6	6.5	3	0.257	0.181
9	24	6	3.5	3	0.752	0.087
11	25	6	6.5	3	0.752	0.225
27	26	10	5.0	5	0.505	0.256
20	27	10	8.0	5	0.505	0.267
1	28	6	3.5	3	0.257	0.047
21	29	10	5.0	1	0.505	0.165
22	30	10	5.0	9	0.505	0.216

Table 8. Analysis of variance (ANOVA) for response surface quadratic model.

Source	Sum of squares	df	Mean square	F value	Probability>F	
Model	0.441	14	0.031	63.46	<0.0001	Significant
A:CMC	0.369	1	0.369	743.47	<0.0001	
B:Peptone	0.015	1	0.015	30.89	<0.0001	
C:Yeast Extract	0.005	1	0.005	10.15	0.006	
D:MnCl ₂ ·4H ₂ O	0.002	1	0.002	4.91	0.04	
AB	0.002	1	0.002	4.11	0.06	
AC	0.021	1	0.021	42.29	<0.0001	
AD	0.001	1	0.001	2.98	0.10	
BC	0.008	1	0.008	16.85	0.0009	
BD	0.000	1	0.0004	0.97	0.33	
CD	0.004	1	0.004	8.18	0.01	
A ²	0.0005	1	0.0008	1.80	0.19	
B ²	0.0006	1	0.0006	1.36	0.26	
C ²	0.008	1	0.008	16.83	0.0009	
D ²	0.001	1	0.001	2.72	0.11	
Residual	0.007	15	0.0004			
Lack of fit	0.006	10	0.0006	2.77	0.13	Not Significant

**Figure 3.** 2D contour-plot showing relative effect of concentration of A: CMC and C: yeast extract of CMCase activity. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]**Figure 4.** 2D contour-plot showing relative effect of concentration of B: peptone and C: yeast extract on CMCase activity. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

$$\begin{aligned} \text{CMCase activity (Y)} = & 0.185118 + 0.091784 * A \\ & + 0.036299 * B + 0.029499 * C \\ & + 0.013868 * G + 0.011216 * J \end{aligned}$$

Where, Y = CMCase activity, A = CMC, B = peptone, C = yeast extract, D = MnCl₂·4H₂O.

Among different organic (yeast extract and peptone) and inorganic (ammonium chloride, ammonium sulfate, ammonium nitrate, and potassium nitrate) sources of nitrogen [39], yeast extract, and peptone were chosen, as these have been reported to significantly affect the cellulase production [17,40]. Cellulase production by *Trichoderma reesei* was enhanced in medium supplemented with CMC as carbon source [41]. In addition to CMC, yeast extract and peptone also showed significant effect on cellulase production from *Cytophaga* sp. [40]. Manganese required as a cofactor in many enzyme systems [42] led to increase in CMCase activity of *B. subtilis* NA15. Presence of Mn²⁺ and Co²⁺ were reported to enhance CMCase activity of *Cellulomonas* sp. ASN2 [43], *B. subtilis* YJ1 [44], and *Rhizopus oryzae* [45]; however presence of K⁺, Na⁺, NH₄⁺ had no effect [8]. Co²⁺ and Mn²⁺ also enhanced cellulase activity of *Mucor circinelloides* [46] and *Chalara paradoxa* [47].

Optimizing of Media Constituents using CCD

RSM is a stepwise approach, used to evaluate the interaction effects of the screened parameters for optimization of CMCase production by *B. subtilis* NA15. Analysis of the screened variables from a five-level response surface design was complex but led to identification of the key variables affecting CMCase production. The results obtained were fed into the Design-Expert software (Version 8.0.7.1, Stat-Ease, Minneapolis, MN) and analyzed using ANOVA as appropriate to the experimental design used. The actual values ranges of the screened variables were presented in Table 6. To test the fit of the model equation, the regression-based determination coefficient R^2 was evaluated. The predicted R^2 (0.98) was in close proximity with adjusted R^2 (0.97), advocated for greater significance of model (Table 7). The statistical analysis allowed us to determine the contribution of experimental factors (signals) in comparison to noise, where the signal should be fairly large in comparison to noise. Thus, the estimated adequate precision of 31.48 for CMCase production, represented the signal to noise ratio, is an adequate signal. The model F value of 63.46 as well as values of $P > F$ (<0.0001) indicated that the model terms (A, B, C, D AB,

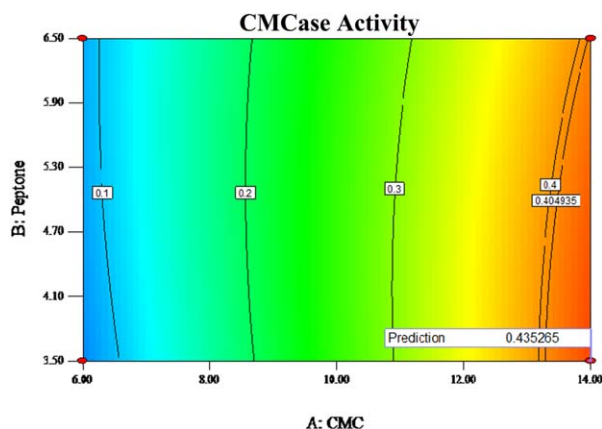


Figure 5. 2D desirability contour-plot showing the predicted optimum CMCase activity relative to *B*: peptone and *A*: CMC. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

BC, *AC*, *CD*, C^2) were significant (Table 8). The application of RSM yielded the following regression equation (Eq. 1), which established an empirical relationship between CMCase Activity (*Y*) and the test variables in coded units:

$$\text{CMCase Activity (Y)} = 0.2633 + 0.124 * A + 0.025 * B + 0.0145 * C + 0.0100 * D - 0.011 * A * B + 0.036 * A * C + 0.009 * A * D - 0.022 * B * C - 0.005 * B * D - 0.015 * C * D - 0.005 * A^2 - 0.004 * B^2 - 0.017 * C^2 - 0.007 * D^2(1)$$

The contour plots were generated to investigate the interaction among the four significant parameters and to evaluate their combined effects on the response of CMCase activity. Based on the interactions between the variables, an increase in CMCase activity on increasing the concentration of each variable reached to optimum level. From Figure 3, it was observed that at a concentration of 13.8 g/L CMC (*A*) and 6.75 g/L of yeast extract (*C*), the CMCase activity was maximum (0.41 U/mL). Similarly in an interactive study of peptone (*B*) and yeast extract (*C*), maximum CMCase activity was observed at their respective concentrations of 6.05 and 4.6 g/L, respectively (Figure 4).

A number of numerical solutions were suggested by the Design-Expert software within the experimental range of parameters for maximum CMCase activity. In order to validate the predicted model experiments suggested was conducted in duplicates. The suggested solutions revealed that the variables were optimized at points located in the right-most region of the plot (Figure 5). The maximum experimental value for CMCase activity was 0.47 U/mL, while the predicted response based on RSM was estimated to be 0.45 U/mL at CMC (18 g/L), peptone (5 g/L), yeast extract (5 g/L), and MnCl_2 (0.5 g/L). Simplified medium with only four ingredients showed sevenfolds increase in CMCase activity as compared to unoptimized medium with 11 ingredients. The excellent correlation between predicted and experimental values of experiment conducted justified the validity of the regression model obtained. Using RSM, optimized CMCase activity (0.47 U/mL) of *B. subtilis* NA15 obtained in this study was higher than that of *Geobacillus* sp. [10], *Brevibacillus* sp. [48], and *Bacillus* sp. [18] which was 0.12, 0.02, 0.01, 0.43 U/mL, respectively. Additions of yeast extract and ammonium sulfate showed twofold increase in cellulase produced by *Geobacillus* sp. under optimized conditions [10].

CONCLUSIONS

Aerobic, Gram positive, cellulase producing bacterium was isolated from composting biomass and identified on the basis of phenotypic fingerprinting using GEN III MicroPla-

teTM and phylogenetic analysis as *B. subtilis* NA15. The optimum media ingredients for production of CMCase with higher activity in cell free supernatant of *B. subtilis* NA15 were different from those of unoptimized medium. Most probably *B. subtilis* NA15 is highly specific toward its media ingredients qualitatively as well as quantitatively toward enhanced extracellular CMCase activity. A 2^2 full factorial CCD was applied to study the overall effect of media ingredients. A sevenfold increase in CMCase activity was observed with the optimum medium for which the maximum predicted CMCase activity of 0.45 U/mL, which was in perfect agreement with experimental value of 0.47 U/mL.

ACKNOWLEDGMENTS

The authors are thankful to Directors, Thapar University and IIT Guwahati for providing infrastructural support. The research work was supported by joint project grant from Department of Biotechnology, Ministry of Science and Technology, Government of India to A. Goyal and D. Goyal.

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Characterization of Cellulase Producing *Bacillus* sp. for Effective Degradation of Leaf Litter Biomass

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Published online 16 November 2012 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/ep.11726

Cellulose degrading Bacillus spp. (AS1, AS2, and AS3) were isolated from cow dung and identified phylogenetically by 16S ribosomal RNA (16S rRNA) sequence analysis. Bacillus sp. AS1 showed 99% homology with Bacillus megaterium whereas, Bacillus sp. AS2 and AS3 were similar to B. subtilis. The activity of extracellular carboxymethyl cellulase in presence of 1% carboxymethyl cellulose at 50°C was 0.04 U/mL for Bacillus sp. AS1 and 0.06 U/mL for Bacillus sp. AS2 at pH 7.0, whereas higher activity of 0.08 U/mL was observed for Bacillus sp. AS3 at pH 9.0. Crude cellulase from all the three Bacillus sp. were stable over a wide range of pH (5.0–9.0) and thermally stable up to 60°C. Carboxymethyl cellulase, filter paperase, and β -glucosidase activity of Bacillus sp. AS3 was 0.07, 0.02, and 0.04 U/ml, respectively. Degradation of leaf litter biomass treated by Bacillus sp. AS3 showed 47% drop in carbon:nitrogen (C:N) ratio and x-ray diffractograph revealed decline in crystalline structure. © 2012 American Institute of Chemical Engineers Environ Prog, 32: 1195–1201, 2013

Keywords: cellulase, *Bacillus* sp., carboxymethyl cellulose, 16S rRNA gene

INTRODUCTION

Cellulose is a linear condensation polymer consisting of D-anhydroglucopyranose joined together by β -1,4-glycosidic bonds with a degree of polymerization from 100 to 20,000 [1]. Cellulose biodegradation by cellulases and cellulosomes, produced by numerous microorganisms, represents a major carbon flow from fixed carbon sinks to atmospheric CO₂ [2]. The widely accepted mechanism for enzymatic cellulose hydrolysis involves synergistic actions by endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and β -glucosidase (EC 3.2.1.21). These enzymes can either be free, particularly in aerobic microorganisms, or grouped in a multicomponent enzyme complex, cellulosome, such as in anaerobic cellulolytic bacteria [3]. The market for cellulase enzyme is increasing owing to its large-scale application in hydrolysis of cellulosic biomass to sugars, which can be further fermented to bioethanol and biobased products [4].

Over the years, culturable, cellulase-producing bacteria have been isolated from a wide variety of sources such as composting heaps, decaying plant material, feces of ruminants, and hot springs [5]. Bacteria having high growth rate

as compared to fungi has the good potential for use in cellulase production [6]. Many aerobic microorganisms, both bacterial and fungal, use the cell free cellulase to digest cellulose [7]. Cellulose degradation is largely an aerobic process and the primary cellulolytic bacterial isolates are *Pseudomonas* sp. [8], *Bacillus subtilis* [9], *Bacillus licheniformis* [10], and *Bacillus brevis* [11]. *Fibrobacter succinogenes* from the rumen is also an important cellulose degrader [12].

Microorganisms using a free cellulase mechanism secrete a set of individual cellulases, most of which contain a carbohydrate-binding module joined by a flexible linker to the catalytic domain, although additional domains are present in some enzymes [13]. Most of the anaerobic microorganisms digest cellulose by cellulosome, which is a large cellulase complex present on the outer surface of the cell wall [14]. Cellulosome from different organisms have different architecture wherein *Clostridium thermocellum* cellulosome consists of several copies of high molecular weight scaffold in protein that contains carbohydrate-binding module, multiple cohesin domains, a dockerin domain, and/or a cell wall-binding domain [15].

In composting cattle manure, filamentous bacteria belong to genera *Micromonospora*, *Cytophaga*, and *Sporocytophaga* were the most numerically abundant as compared to fungi [16], since ruminants mainly feed on cellulose, and their digestive tract harbors different type of microorganisms that are capable of digesting cellulose. *B. subtilis* GN156 isolated from silage was able to produce various hydrolytic enzymes including carboxymethyl cellulase (CMCase) [17].

Bioconversion of cellulose to sugars is a prerequisite for the subsequent production of bioenergy and can be readily fermented to fuel ethanol [18]. Use of lignocellulosic agricultural residues such as grasses and forestry wastes for ethanol production [19] is more economical. The aim of the present study was to characterize cellulases from cellulose-degrading aerobic bacteria from cow dung and its application in recycling of leaf litter biomass.

MATERIALS AND METHODS

Sample Collection, Screening, and Isolation

Cow dung from farming community was collected from the nearby village of Patiala, Punjab, India, using alcohol-sterilized implements. Samples (10% wt/vol) were suspended in 100 mL of sterile distilled water, and serial dilutions were

made. Bacteria were isolated by plating each dilution on nutrient agar (NA) plate. Colonies that developed on the plates were subcultured repeatedly to obtain pure cultures. The bacterial colonies grown on NA supplemented with 1% (wt/vol) carboxymethyl cellulose (CMC) kept at 37°C under observation for 3–4 days were screened for the hydrolysis efficiency by staining with aqueous solution of Congo red (0.1%) for 15 min followed by destaining with 1M NaCl [20]. Cellulose hydrolysis was checked as the clear zone around the colonies. Out of 32 bacteria screened, only three prominent cellulase producers were further studied.

Characterization of Bacterial Isolates

Pure cultures obtained as above were subjected to microscopic examination for shape and size. Each isolate was characterized for its cellulose hydrolyzing capacity by analyzing cellulase activity as described later. Gram staining, growth on MacConkey agar and KOH, oxidase, catalase, nitrate reduction, citrate utilization, Bile Esculin, Voges–Proskauer (V–P) reaction, and urease tests were performed as per standard methods [21] with *Escherichia coli* DH5 α as reference.

Scanning Electron Microscopy

Overnight grown cultures of bacterial isolates were centrifuged at 7000g for 10 min. Cell pellets were washed with 0.85% saline and resuspended in 2.5% glutaraldehyde in 50 mM sodium phosphate buffer for fixation and incubated at 4°C for 3 h [22]. Bacterial cells were then dehydrated with 75% ethanol followed by absolute alcohol and air dried for 5 h and then completely dried in a desiccator for 12 h under vacuum. The cells were then coated with gold sputter and examined under the scanning electron microscope (model: LEO1430VP; Carl Zeiss, India).

16S rRNA Gene Sequencing and Phylogenetic Analysis

For sequence analysis, genomic DNA was extracted from overnight grown bacterial cells in nutrient broth (NB) (2 mL). Bacterial pellet was suspended in 800 μ L of saline EDTA (0.15M NaCl and 0.1M EDTA of pH 8.0) and lysed by crystalline lysozyme (~100 μ g) followed by incubation at 37°C for 1 h. To this suspension, 2.0% sodium dodecyl sulfate was added and incubated at 65°C for 15 min. DNA was recovered by phenol:chloroform:isoamyl alcohol (25:24:1) extraction followed by precipitation with isopropanol. DNA pellet was resuspended in 50 μ L of TE buffer (10 mM Tris of pH 8; 1 mM EDTA of pH 8.0) and analyzed by agarose (0.7%) gel electrophoresis.

Polymerase chain reaction (PCR) was performed in GenAmp[®] PCR system (Applied Biosystem) using genomic DNA as template. The following primer pair: 27f (5'-AGAGTTT-GATCCTGGCTCAG-3') and 1492r (5'-ACGGGCGGTGTGTC-3') [23] were used to amplify the 16S rRNA gene sequence. PCR reaction mixture (100 μ L) containing 1 $\times$ PCR buffer (Invitrogen) with all four deoxynucleotide triphosphate (200 μ M), MgCl₂ (1.5 mM), both 27f and 1492r primer (0.1 μ M each) and 2.5 U of *Taq* DNA polymerase (Invitrogen) was prepared. Reaction mixture containing template DNA (~10 ng) underwent initial denaturation (92°C for 120 s) followed by 36-cycle episode of denaturation (92°C for 60 s), annealing (48°C for 30 s), and elongation (72°C for 120 s). Final extension was allowed at 72°C for 370 s. The amplified PCR product was cloned into the pGEM-T Easy subunit vector as per manufacturer's instruction (Promega). The ligation mixture was used to transform competent *E. coli* DH5 α cells by heat shock method. After screening of the recombinant clone, the sequence was generated by chain termination method using Applied Biosystem automated sequencer. The 16S rRNA gene sequence obtained was compared with Ribosomal Database Project-II [24] and with those from GenBank

using the BLASTN program [25]. Phylogenetic analysis was done by alignment of the sequences using CLUSTALW program [26]. The evolutionary distance was calculated by Kimura two parameter and a phylogenetic tree was constructed by the neighbor-joining method. Bootstrap analysis was based on 1,000 resamplings. The MEGA 4.0 package [27] was used to generate a neighbor-joining tree.

Production and Assay of Crude Cellulase

The bacterial isolates were grown in medium containing 0.5% tryptone, 0.5% yeast extract, 0.1% K₂HPO₄, 0.25% MgSO₄·5H₂O, and 0.25% FeSO₄·7H₂O supplemented with 0.5% CMC. Culture medium (100 mL) in 250 mL flask was inoculated with 1% overnight grown culture (OD₆₀₀ = 0.6) and incubated at 37°C under shaking condition at 120 rpm for 24 h. Cellulase activity in cell-free supernatant collected after centrifugation at 7000g for 20 min was analyzed. CMCase activity was carried out by incubating cell-free supernatant with substrate CMC for 30 min at 50°C. The reaction mixture (2 mL) contained 0.2 mL of cell-free supernatant in 1.8 mL of 1% (wt/vol) CMC dissolved in 50 mM phosphate buffer (pH 7.0). The amount of reducing sugar liberated was quantified by 3,5-dinitrosalicylic acid (DNS) method [28] using glucose as standard. Whatman no. 1 filter paper strip (1 $\times$ 3 cm) soaked in 1.8 mL of 50 mM phosphate buffer (pH 7.0) was used as substrate for filter paperase (FPase) activity. Cell-free supernatant (0.2 mL) was added in presoaked filter paper and then incubated at 50°C for 60 min. One unit (U) of enzyme activity was defined as the amount of enzyme that released one micromolecule of glucose per minute. β -glucosidase activity was determined by adding 0.2 mL of cell-free supernatant 1.8 mL of 0.15 g/L of *p*-nitrophenyl- β -D-glucopyranoside as substrate, prepared in 50 mM phosphate buffer (pH 7.0) and incubated at 50°C for 30 min. One unit of β -glucosidase activity was determined as 1 μ mole of *p*-nitrophenol liberated per milliliter enzyme per minute. The protein concentration was measured as per Lowry *et al.* [29] using bovine serum albumin as standard.

Biochemical Characterization of CMCase

CMCase activity in cell-free supernatant of bacterial strains (AS1, AS2, and AS3) was checked at different pH and temperature range. The optimum pH for CMCase was determined by incubating the cell-free supernatant (0.2 mL) in 1.8 mL of CMC in the presence of appropriate buffers for different pH such as 50 mM sodium acetate (pH 4.0 and 5.0), 50 mM potassium phosphate (pH 6.0 and 7.0), and 50 mM glycine–NaOH buffer (pH 8.0, 9.0, and 10.0). The reaction mixture of various pH buffers were incubated for 30 min at 50°C. The enzyme assays were carried out by determining the amount of reducing sugars released as described above. The optimum temperature for the activity of crude cellulase was determined by assaying the enzyme at temperatures ranging from 30°C to 80°C in 2 mL reaction mixture containing 1% CMC dissolved in 50 mM phosphate buffer (pH 7.0) with incubation time of 30 min.

Thermal stability of CMCase was determined after pre-treatment of cell-free supernatant at temperatures ranging from 30°C to 80°C for 30 min, thereafter enzyme activity was assayed at 50°C using CMC (1%) as substrate dissolved in 50 mM phosphate buffer (pH 7.0) by determining release of reducing sugars as described.

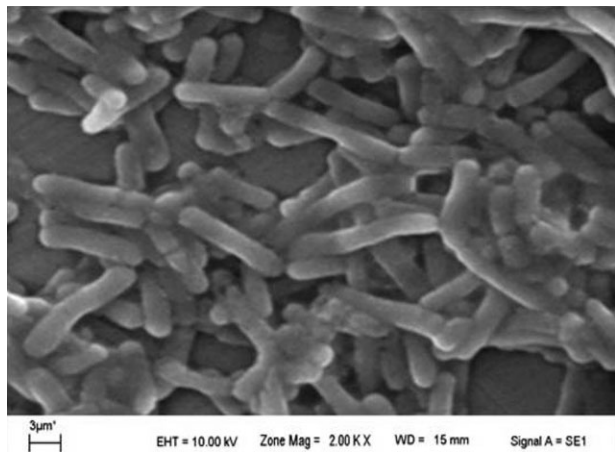
Degradation of Leaf Litter Biomass

Leaf litter biomass from different tree species such as *Hevea brasiliensis*, *Eucalyptus marginata*, *Polyalthia longifolia*, *Tectona grandis*, and *Mangifera indica* were collected from different places of Thapar University campus, Patiala, Punjab (India), located at 30°19'48"N, 76°24'0"E and 310 m

Table 1. Biochemical characteristics of the bacterial isolates.

Characteristics/ biochemical test	Observation		
	Strains		
	AS1	AS2	AS3
Gram reaction	+	+	+
Morphology	Rods	Rods	Rods
Growth on McConkey's agar	+	+	+
KOH	—	—	—
Oxidase	+	+	+
Catalase	+	+	+
NO ₃ reduction into NO ₂	+	+	+
Citrate utilization	—	—	—
Bile Esculin	+	+	+
Voges–Proskauer (V–P) reaction	—	—	—
Urease	+	—	—

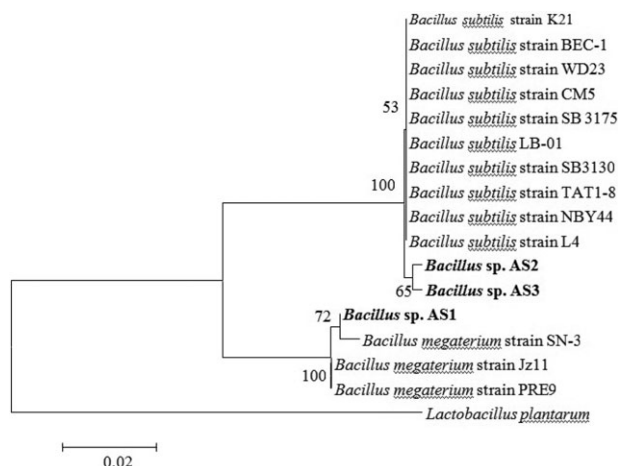
+, Positive reaction; —, negative reaction.

**Figure 1.** Scanning electron micrograph of *Bacillus* sp. AS3 (10,000×).

above the sea level. Equal amount of leaf litters of different tree species by weight were taken, washed to remove adhering debris, and were air dried and milled by mechanical blender to a size of 2–3 cm for further degradation process. One kilogram of milled leaf litter biomass was taken in round plastic tubs of diameter 14.5 in. and depth of 6.5 in., in triplicate and kept under shade at an ambient temperature. *Bacillus* sp. AS3 from NA plate was inoculated in 5 mL of NB and was grown at 37°C with rotary agitation at 160 rpm for 24 h; 1% (vol/vol) of the inoculum was further transferred into 100 mL of NB and incubated at 37°C for 24 h. Thereafter, the whole bacterial inoculum (7×10^9 cfu/mL) was inoculated one time to 1 kg of biomass and uninoculated biomass was kept as control. Moisture was maintained at 40–50% in each tub by gravimetric method and mixing was done at an interval of 3–4 days for 90 days. pH was neutral (pH 7.1) to slightly alkaline (pH 7.8) throughout the degradation process.

Cellulose Degradation and C:N Ratio

Biomass was treated for 90 days and degraded sample was milled and sieved to 2 mm size and analyzed for cellulose by Anthrone method [30], reducing sugar by DNS

**Figure 2.** 16S rRNA gene sequence-based dendrogram constructed by the neighbor-joining method showing the phylogenetic position of the bacterial isolates (AS1, AS2, and AS3) along with sequences available in GenBank database. Numerical values indicate bootstrap percentile from 1000 replicates. *Lactobacillus plantarum* was used as outgroup taxa. Bar, 0.02 substitutions per nucleotide.

method [28], organic carbon by Walkley and Black [31] and total nitrogen by Kjeldahl method [32].

X-Ray Diffractographic analysis

Effect of treatment by *Bacillus* sp. AS3 on the crystalline structure of leaf litter biomass was analyzed by x-ray diffraction [33]. Treated and untreated biomass was vacuum dried to remove the water content from the cellulosic substrate. Crystallinity was determined by x-ray diffraction at room temperature in the range of $5^\circ \leq 2\theta \leq 40^\circ$ at the scan speed of $5^\circ/\text{min}$ using a PANalytical X'Pert PRO system with Ni-filter. Diffractometer operated at 45 KV/40 mA with λ (Cu K α) = 1.5406 Å [34].

RESULTS AND DISCUSSION

Morphology and Biochemical Characterization

Cellulolytic bacteria have been isolated from diverse habitats [35]. In the present study, 32 bacterial strains were isolated from cow dung, among which three (designated as AS1, AS2, and AS3) were efficient producer of extracellular cellulases as observed by clear hydrolysis zones after Congo red staining. Similarly, lignocellulolytic bacteria were reported from goat's feces [36]. Aerobic bacterial strains (AS1, AS2, and AS3) were Gram positive and rod shaped and showed positive tests for oxidase, catalase, nitrate reduction, and bile esculin and negative for KOH, citrate utilization, and V–P reaction, whereas strain AS1 gave the positive test for urease in contrast to AS2 and AS3 (Table 1). Scanning electron micrograph revealed rod-shaped morphology with average cell dimension of $5.5 \times 1.2 \mu\text{m}$ for strain AS3 (Figure 1).

Phylogenetic Analysis of the 16S rRNA Gene Sequence

The 1.5-Kb 16S rRNA gene of the three isolates was amplified from the genomic DNA and sequences were submitted to GenBank with the accession numbers as EU754023, EU754024, and EU754025 for AS1, AS2, and AS3, respectively. AS1 had the maximum similarity to *Bacillus megaterium* strain PRE9 whereas AS2 and AS3 exhibited maximum similarity to *B. subtilis* strain L4 (Figure 2). All the three strains

Table 2. Cellulase activity of different isolates in cell free supernatant at 50°C in the presence of 50 mM potassium phosphate buffer (pH 7.0).

Bacterial isolate	Cellulase activity* (U/mL)			
	CMCase	FPase	β -glucosidase	Protein (mg/mL)
<i>Bacillus</i> sp. AS1	0.04 $\pm$ 0.009	0.01 $\pm$ 0.003	0.02 $\pm$ 0.004	0.04 $\pm$ 0.003
<i>Bacillus</i> sp. AS2	0.06 $\pm$ 0.001	0.03 $\pm$ 0.004	0.05 $\pm$ 0.006	0.06 $\pm$ 0.001
<i>Bacillus</i> sp. AS3	0.07 $\pm$ 0.004	0.02 $\pm$ 0.002	0.04 $\pm$ 0.003	0.07 $\pm$ 0.007

*Values are mean of triplicate with standard error.

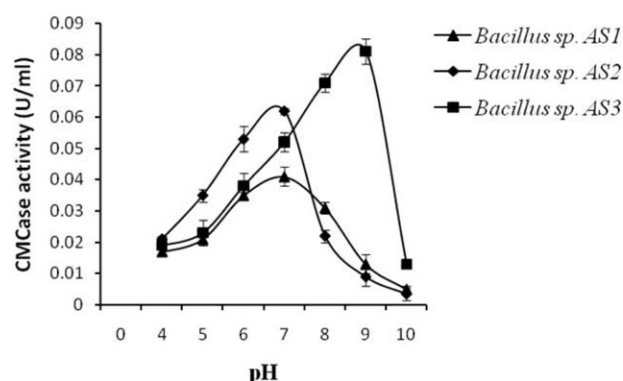


Figure 3. CMCase activity of *Bacillus* sp. AS1, *Bacillus* sp. AS2, and *Bacillus* sp. AS3 at 50°C at different pH. The vertical bars designate the standard errors for the mean of three replicative tests.

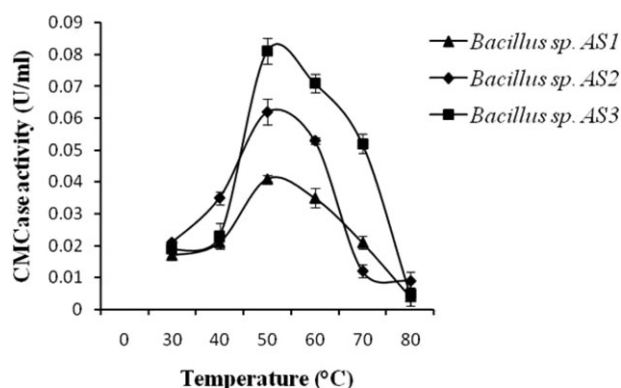


Figure 4. Effect of temperature on the CMCase activity of *Bacillus* sp. AS1, *Bacillus* sp. AS2, and *Bacillus* sp. AS3. The vertical bars designate the standard errors for the mean of three replicative tests.

are closely related and support their phylogenetic position in the genus *Bacillus*. Morphological, biochemical, and molecular characterization using 16S rRNA gene sequence analysis of all isolates revealed that they belong to *Bacillus*.

Characterization of Cellulases From Bacterial Isolates

CMCase, FPase, and β -glucosidase activity and protein concentration in cell-free supernatant of 24-h-old grown cultures was investigated (Table 2). The FPase, CMCase, and β -glucosidase activities were found to be similar to *Bacillus pumilus* EB3 after 24 h of fermentation at pH 7.0 [37]. The cell-free supernatant of *Bacillus* sp. exhibited endoglucanase, exoglucanase, as well as β -glucosidase activity and their synergy results in hydrolysis of cellulose to glucose [3].

Effect of pH

CMCase activity in cell-free supernatant of strains (AS1, AS2, and AS3) at varying pH was 0.04 U/mL for AS1 and 0.06 U/mL for AS2 at pH 7.0 in 50 mM potassium phosphate buffer, whereas for strain AS3, it was highest 0.08 U/mL at pH 9.0 in 50 mM glycine-NaOH buffer (Figure 3).

The optimum pH for the strains AS1 and AS3 was found to be 7.0, which is in accordance with the previous study by Ray *et al.* [38], where pH 7.0–7.5 was more suitable for cellulase production by *B. subtilis* and *Bacillus circulans*. In contrast to that, strain AS3 showed maximum activity (0.08 U/mL) at pH 9.0, suggesting its alkaline nature [39]. Yin *et al.* [40] also reported cellulase from *B. subtilis* YJ1, which was active at an optimum pH of 6.0 and stable in the pH range of 6.5–7.5. Cellulases from *B. pumilus* EB3 had an optimum pH of 7.0 and were stable at pH range 6.0–9.0. This is in contrast with most of the fungal cellulase having an optimum

pH of 4.0–6.0 [37]. Cellulase from AS1, AS2, and AS3 has an advantage in terms of stability over broad pH range.

Effect of Temperature on CMCase Activity and Its Thermal Stability

Bacillus sp. (AS1, AS2, and AS3) were found to have the temperature tolerance up to 60°C. The effect of temperature on cellulase activity by AS1, AS2, and AS3 was observed over a temperature range of 30°C to 80°C (Figure 4). The maximum CMCase activity was found in AS3 (0.08 U/mL) at 50°C and pH 9.0 followed by AS2 (0.07 U/mL) and AS1 (0.06 U/mL) at 50°C and pH 7.0. The enzymes were thermally stable at temperature from 30°C to 60°C, beyond which a sharp decline in activity was found due to denaturation of the enzyme with no activity at 80°C (Figure 5).

Enzyme activity at 40°C was optimum for *B. subtilis* and *B. circulans* [38] and maximum endoglucanase activity was observed in *Cellulomonas*, *Bacillus*, and *Micrococcus* sp. at 40°C and neutral pH [41]. Thermal stability of the cellulases produced by *Bacillus* sp. AS3 in the degradation of leaf litter biomass is required to improve the bioconversion [42], and periodic release of enzyme would favor sugar formation [43].

Thermal stability of CMCase produced by *Geobacillus* sp. dropped rapidly beyond 70°C [44]. Enzyme activity was optimum at 50°C in case of *Bacillus amyloliquefaciens* DL-3 and found stable at 40–80°C [45]. The medium optimized for *Bacillus* sp. AS3 by response surface methodology showed six-fold increase in the cellulase activity and also the strain was found to be efficient lichenase producer [39].

Degradation of Leaf Litter Biomass by *Bacillus* sp. AS3

Degradation of leaf litter biomass over a period of 90 days was studied with and without treatment with *Bacillus* sp.

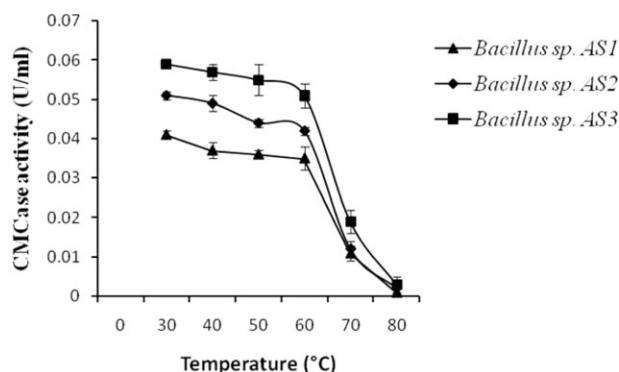


Figure 5. Thermal stability of CMCase activity of *Bacillus* sp. AS1, *Bacillus* sp. AS2, and *Bacillus* sp. AS3 determined after pre treatment of crude cellulase at different temperature (30–80°C) for 30 min. The vertical bars designate the standard errors for the mean of three replicative tests.

Table 3. Decomposition of leaf litter biomass by *Bacillus* sp. AS3 after 90 days.

Analytical parameter	Control	Biomass treated by <i>Bacillus</i> sp. AS3
Residual cellulose (mg g ⁻¹)	168.37 ± 3.09	157.13 ± 2.92
Reducing sugar (mg g ⁻¹)	3.16 ± 0.12	3.45 ± 0.09
Carbon (%)	20.73 ± 0.91	15.64 ± 0.06
Nitrogen (%)	0.46 ± 0.02	0.49 ± 0.03
C:N ratio	45:1 ± 2.71	24:1 ± 1.87

Values are means of triplicate with standard error. At 0 day, residual cellulose: 174.46 mg g⁻¹; reducing sugar: 2.93 mg g⁻¹; carbon: 21.22%; nitrogen: 0.43%.

AS3. Eight percent increase in reducing sugar content and 7% decrease in residual cellulose was observed with respect to control in treated biomass after 90 days of degradation (Table 3). Cellulases from *Bacillus* sp. AS3 hydrolyzed the leaf litter biomass, resulting into release of reducing sugar. Release of reducing sugar by using commercial Celluclast from spruce biomass [46] and pretreated olive pulp [47] has been reported earlier. Organic carbon was 20.73% in the untreated and 12.64% in the treated biomass after 90 days, whereas nitrogen content was found to be 0.46% in the untreated and 0.52% in the treated biomass. The C:N ratio of leaf litter biomass was reduced from 45:1 to 24:1 by *Bacillus* sp. AS3. The increase in nitrogen may be due to concentration effect caused by degradation of labile organic carbon compounds [48]. In addition, when loss of organic matter is greater than loss of NH₃, nitrogen concentration usually increases [49]. The drop in the C:N ratio by *Bacillus* sp. AS3-treated biomass was 47%, which was also reported while degradation of sugarcane trash by *Trichoderma reesei* [50].

After 90 days of biodegradation, x-ray diffractograph of untreated leaf litter biomass showed intense peak at 2θ angle of 21° (Figure 6). The calculated area of untreated and treated biomass was 9,68,256 and 2,83,024, respectively, in comparative square unit. A significant decrease in the area of this peak was observed in the treatment of biomass comprising of *Bacillus* sp. AS3. X-ray diffraction analysis showed decrease in cellulosic crystalline structure of leaf litter biomass after treatment with *Bacillus* sp. AS3. Crystallinity of

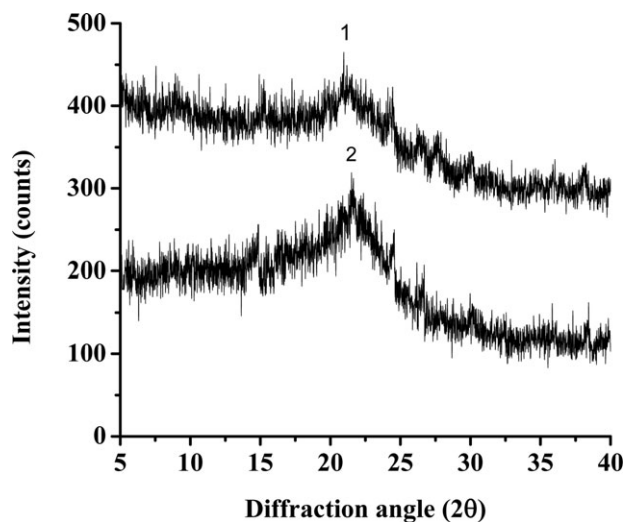


Figure 6. X-ray diffractographic analysis of leaf litter biomass. Peak 1 represents the crystalline complexity in untreated biomass and peak 2 represents the reduced crystalline complexity in treated biomass after 90 days of treatment.

biomass depends on complex nature of bonding among cellulose, hemicelluloses, and lignin [51], and this crystalline structure of the leaf litter biomass is damaged because of degradation by microbial treatment leading to enhanced digestibility of the cellulosic biomass [46,52].

CONCLUSION

The present work reveals isolation, identification, and application of natural isolates for recycling of cellulosic agricultural waste biomass. *Bacillus* spp. (AS1, AS2, and AS3) isolated from cow dung were potential cellulase producers. Morphological, biochemical, and molecular characterization using 16S rRNA gene sequence analysis of the isolates revealed that all the three isolates belong to *Bacillus*. Extracellular cellulase from AS1 and AS2 was optimally active at 50°C and pH 7.0, whereas in AS3, it was at pH 9.0. Cellulase activity was retained over a wide range of temperature (30–60°C) and pH (5.0–9.0), which allows the manipulation of process parameters. Crude cellulases from *Bacillus* sp. were thermally stable, which makes it applicable for large-scale recycling of cellulosic biomass. Treatment of leaf litter biomass by *Bacillus* sp. AS3 led to decrease in cellulose content by 7% with simultaneous increase in reducing sugar content by 8%. There was also 47% drop in the C:N ratio with decline in cellulosic crystalline structure.

ACKNOWLEDGMENTS

The authors are thankful to Directors, Thapar University, and IIT Guwahati and Science and Technology Entrepreneur's Park (STEP), Thapar University, for providing infrastructural support. The research work was supported by joint project grant from Department of Biotechnology, Ministry of Science and Technology, Government of India to D. Goyal and A. Goyal.

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Abstract: Leaf litter biomass from Eucalyptus globulus was pretreated using a novel surfactant assisted microwave-acid pretreatment (SAMAP) technique for enhanced enzymatic release of sugars. Screening of parameters for SAMAP technique was carried out using Taguchi experimental design followed by enzymatic saccharification and the critical parameters identified were time, acid concentration and surfactant concentration. To optimize the saccharification of SAMAP biomass combined effect of significant parameters were further investigated using response surface methodology (RSM). As compared to reducing sugar yield of 0.05g/g in native biomass, enzymatic hydrolysis of recovered solid fraction of SAMAP biomass (0.5 mm) showed 93% increase in reducing sugar (0.69 g/g), when treated with 1.25% H₂SO₄ and 0.2% Tween-80 followed by 80 FPU/g of enzymatic hydrolysis for 42 h. Composition of native biomass was 31% cellulose, 18% hemicellulose and 12% total lignin. Delignified SAMAP biomass after recovery showed 33% increase in cellulose content whereas, hemicellulose and lignin content was reduced by 39 and 66%, respectively. Native and pretreated biomass were characterized by scanning electron microscopy (SEM), x-ray diffraction (XRD), thermal gravimetric analysis (TGA), fourier transform infrared analysis (FTIR) and solid state ¹³C cross polarizing magic angle spinning (CP/MAS) NMR spectroscopy. ;

**SURFACTANT ASSISTED MICROWAVE-ACID PRETREATMENT OF LEAF LITTER
BIOMASS FOR ENHANCED ENZYMATIC RELEASE OF SUGARS**

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ABSTRACT

Leaf litter biomass from *Eucalyptus globulus* was pretreated using a novel surfactant assisted microwave-acid pretreatment (SAMAP) technique for enhanced enzymatic release of sugars. Screening of parameters for SAMAP technique was carried out using Taguchi experimental design followed by enzymatic saccharification and the critical parameters identified were time, acid concentration and surfactant concentration. To optimize the saccharification of SAMAP biomass combined effect of significant parameters were further investigated using response surface methodology (RSM). As compared to reducing sugar yield of 0.05g/g in native biomass, enzymatic hydrolysis of recovered solid fraction of SAMAP biomass (0.5 mm) showed 93% increase in reducing sugar (0.69 g/g), when treated with 1.25% H₂SO₄ and 0.2% Tween-80 followed by 80 FPU/g of enzymatic hydrolysis for 42 h. Composition of native biomass was 31% cellulose, 18% hemicellulose and 12% total lignin. Delignified SAMAP biomass after recovery showed 33% increase in cellulose content whereas, hemicellulose and lignin content was reduced by 39 and 66%, respectively. Native and pretreated biomass were characterized by scanning electron microscopy (SEM), x-ray diffraction (XRD), thermal gravimetric analysis (TGA), fourier transform infrared analysis (FTIR) and solid state ¹³C cross polarizing magic angle spinning (CP/MAS) NMR spectroscopy.

Key words: Hydrolysis, leaf litter biomass, microwave, pretreatment, surfactant

1 INTRODUCTION

2 Lignocellulose biomass is most abundant, incredible, renewable and low cost promising substrate for
3 bioethanol production. Agricultural residues serve as a low cost feed stock for the production of
4 biofuels.¹ Utilization of these feed stocks for biofuel production requires development of pretreatment
5 techniques to break up lignin structures and to enhance enzymatic saccharification of cellulose.² The
6 deep encapsulation of cellulose by lignin and hemicelluloses and highly crystalline structure of native
7 lignocelluloses resists enzymatic hydrolysis for bioconversion to sugars and further to ethanol.
8 Pretreatment is an essential step towards the development and industrialization of efficient second
9 generation lignocellulosic ethanol processes.³ The major challenge involved in the pretreatment of
10 lignocelluloses is to develop novel strategies to improve enzymatic release of sugars for bioethanol
11 production. Pretreatment processes separate lignin from polysaccharides and reduce cellulose
12 crystallinity making the glycoside bonds more accessible to the hydrolytic enzymes.⁴ Pretreatment of
13 lignocellulosic biomass using acid, alkali, steam explosion, hot water and ammonia fibre explosion
14 (AFEX) techniques has been tested for different types of biomass, however cannot be used for all
15 feedstocks, due to large variation in the composition of biomass from different sources.⁵
16 Several reports are available on pretreatment of lignocellulosic biomass using acid¹, alkali⁶, ionic
17 liquids,⁷ organosolvent,⁸ organic acids,⁹ microwave,¹⁰ ultrasound,¹¹ biological¹² and combined
18 pretreatment.¹³
19 *Eucalyptus* species managed as short-rotation crops for bioenergy, are of increasing interest in many
20 parts of the world.¹⁴ Out of 300 species of *Eucalyptus* all over world, *Eucalyptus globulus*, an
21 evergreen tree, frequently found to be grown in India in a cool, moist, equitable climate and deep
22 soil.¹⁵ The tree bears a lot of leaves which are simple, entire, gland-dotted, and pendulous, plays an
23 important role in cellulose production¹⁶ which may be used as potential feedstock for sugars.
24 The use of microwave radiation is a promising pretreatment process that utilizes thermal and
25 nonthermal effects generated by microwaves in aqueous environments,¹⁷ as it break down lignin-
26 hemicellulose complex and expose more accessible surface area of cellulose to cellulase.¹⁸ Surfactant
27 assisted ultrasound pretreatment of sugarcane tops¹⁹, microwave-assisted alkali pretreatment of corn

cob²⁰ and microwave-assisted alkali pretreatment of switch grass¹⁷, have also been reported for improved enzymatic release of sugars. However, surfactant assisted microwave-acid pretreatment (SAMAP) of lignocellulosic feedstock have not been exploited to enhance the enzymatic hydrolysis to the best of our knowledge.

The objective of the present study was development of a novel process for enhancement of reducing sugar yield from *Eucalyptus* leaf litter biomass by adopting a SAMAP technique. The optimization of process parameters for pretreatment and hydrolysis was done using response surface methodology (RSM) and features of native and pretreated samples were investigated by scanning electron microscopy (SEM), x-ray diffraction (XRD), thermal gravimetric analysis (TGA), fourier transform infrared (FTIR) spectroscopy analysis and solid state cross polarizing magic angle spinning (CP/MAS) ¹³C NMR spectroscopy.

EXPERIMENTAL

Leaf litter biomass

Eucalyptus globulus leaf litter was collected from Thapar University campus, Patiala, Punjab (India), located at 30°19'48"N, 76°24'0"E and 310 m above the sea level. Leaf litter was washed to remove adhered debris, dried, grounded and sieved to particle size of 0.5 mm. Sieved sample was stored in an air tight container at room temperature for further use. The compositional analysis of native and pretreated leaf litter was carried out by two-step acid hydrolysis as per standard protocol from National Renewable Energy Laboratory.²¹

Pretreatment of leaf litter biomass

Primary screening

Pretreatment was performed in 150 ml stoppered conical flasks with biomass loading of 10% (w/v). The samples were soaked with different surfactant (1% w/v) like Tween-80, Tween-40, Tween-20, Triton X-100, PEG 6000, SDS and CTAB for 60 min. Soaked biomass samples (10% w/v) were placed in proprietary express vessel and subjected to microwave pretreatment using Microwave Accelerated Reaction System (MARS6®, CEM, Buckingham, United Kingdom) with an operating frequency of 2450 MHz and power range of 100 - 1800 W. It was initially carried out for 60 s at 200

W and 150 °C and further autoclaved (121 °C, 15 lb) for 60 min. Samples were neutralized using tap water devoid of sugar with two consecutive wash and then dried at room temperature (32 ± 2 °C). Reducing sugar was analysed in native and recovered solid fraction of biomass using 2, 5-dinitrosalicylic acid method.²²

Optimization of process parameters for pretreatment by Taguchi experimental design

Taguchi statistical design was used to find out the best pretreatment condition for SAMAP of *Eucalyptus* leaf litter biomass. According to the orthogonal array, five variables in 16 experiments were used to evaluate the pretreatment efficiency. The design and execution of experiments were carried out to generate experimental data considering all possible interactions between different process variables (Table 1). The statistical software Design-Expert (Version 8.0.7.1, Stat-Ease, Minneapolis, Minn. USA) was used for experimental design and validation. The pretreated samples were washed with tap water, dried at room temperature (32 ± 2 °C) and reducing sugar was estimated in recovered biomass as per protocol discussed earlier.²²

Characterization of native and pretreated leaf litter biomass

Scanning electron microscopy (SEM)

Physical changes in native and SAMAP biomass were observed by SEM. Images of the native and treated biomass taken using SEM (Model: JEOL JSM-6510 LV, USA) at magnification 1,000X. The samples were coated with gold using gold sputter at a voltage of 10-15 kV.

X-ray diffraction (XRD) analysis

X-ray diffraction (XRD) analysis reveals the crystallinity of native and SAMAP biomass, were analyzed at room temperature in the scanning angle of 5-50° at the scan speed of 5° min⁻¹ using a PANalytical X'Pert PRO diffractometer (Netherlands) with Ni-filter, operated at 45 KV and 40 mA with λ (Cu K α) = 1.5406 Å. The crystallinity index (*CrI*) of the leaf litter biomass was calculated and determined as described by Segal et al.²³ as follows:

$$CrI = [(I_{002} - I_{am}) / I_{002}] \times 100$$

Where I_{002} is the intensity for the crystalline portion of biomass (i.e., cellulose) at about 2θ of 22.5° , and I_{am} is the peak for the amorphous portion (i.e., cellulose, hemicellulose, and lignin) at about 2θ of 18° in most literatures.²⁴⁻²⁵

Thermal gravimetric analysis (TGA)

To understand the devolatilization characteristics, TGA of native and SAMAP biomass were performed using Mettler Toledo instrument (Model: TGA/SDTA 851e). The devolatilization of biomasses were studied from 20 to 700 $^\circ\text{C}$ with a heating rate of 10 $^\circ\text{C min}^{-1}$ with purge gas (Nitrogen) flow rate of 60 ml min^{-1} .

Fourier transform infrared (FTIR) spectroscopy analysis

Fourier transform infrared (FTIR) spectroscopy analysis was carried out to detect changes in functional groups of native and SAMAP biomass, were obtained using Nicolet instrument (Model: MAGNA 550, USA). The biomass (10 mg) was well mixed with 200 mg of KBr and the mixture was compressed for preparation of pellets. Each spectrum was the average of 64 scans with a total scan time of 15 s in the IR range of 400 - 4000 cm^{-1} at 1 cm^{-1} .

Solid state CP/MAS ^{13}C NMR spectroscopy

Solid state ^{13}C CP/MAS NMR spectroscopy is an important tool to analyze the structural features of lignocellulosic biomass. Solid state ^{13}C CP/MAS NMR spectra of native and SAMAP biomass were obtained at 75.475 MHz using a Bruker AV-300 FT NMR spectrometer (Germany). Dry samples were spun throughout at a rate of 10 kHz using 4 mm zirconia (ZrO_2) rotor with CP pulse programme. Typically 18,000 transients were accumulated in the time domain with contact time of 1 ms and a recycle delay of 3 s. Tetramethylsilane (TMS) was used as an external standard for the calibration of the ^{13}C chemical shift.

Enzymatic hydrolysis

Enzymatic saccharification of SAMAP leaf litter biomass was carried out in 150 ml stoppered conical flasks by incubating 10% (w/v) of biomass with commercial cellulase (Zytext India Private Limited, Mumbai, India). The enzyme loading was 50 filter paper unit (FPU)/g of pretreated biomass, 20 μl of 1x antibiotic solution (Penicillin-Streptomycin cock tail), 0.2% w/v surfactant (Tween-80) and 0.5%

v/v acid (H₂SO₄) concentration. Total reaction volume was made up to 20 ml with citrate buffer (100 mM, pH 4.8). Samples were incubated at 50 °C for 42 h under shaking condition (120 rpm). Samples were centrifuged (10,000 rpm, 10 min) after incubation to remove unhydrolyzed biomass and reducing sugar was analyzed as discussed earlier.²² The reducing sugar yield was expressed in g/g pretreated biomass taken into consideration of material loss during pretreatment stage.

Optimization of enzymatic saccharification by Box-Behnken design

Box-Behnken (BB) design was executed to study the effect of independent variables on the response and interaction of factors with different combination of variables. The critical parameters that affect enzymatic hydrolysis were enzyme loading, acid concentration, surfactant concentration and incubation time.

In BB experimental design, the total number of experimental combinations is $N=K^2+K+C_p$, where K is the number of independent variables and C_p is rotatable central point number. Table 2 represents 29 experimental runs design for optimization of enzymatic saccharification, in which each variable was tested in three different levels coded as -1, 0 and +1 which corresponds to lower, middle and higher values, respectively.

In the developing regression equation factors were coded according to the equation:

$$X_i = (x_i - x_{oi}) / x_i$$

Where X_i is coded value of i^{th} independent variable, x_i is the natural value of i^{th} independent variable; x_{oi} is the natural value of the i^{th} independent variable at the centre point. Reducing sugar yield was determined for each of trial and a second order polynomial was fitted to the response data obtained from the design. The general polynomial equation is as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2$$

Where Y is the predicted response (reducing sugar in g/g of biomass), β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the coefficient for the interaction effect, and X_i is a dimensionless coded value of x_i (independent variable).

RESULTS AND DISCUSSION

The composition analysis of native and SAMAP was carried out determining cellulose, hemicellulose

and total lignin content. Native biomass contained 31.25% cellulose, 17.58% hemicellulose, 16.34% total lignin and reducing sugar of 0.05 g/g which were 46.56, 10.76, 4.18% and 0.50 g/g for cellulose, hemicellulose, total lignin and reducing sugar, respectively for SAMAP biomass (Table 3). The recovery of the SAMAP biomass was 64% after pretreatment which was mainly due to removal of hemicellulose and lignin. SAMAP biomass showed increase in cellulose and reducing sugar content by 33 and 90%, respectively, whereas decrease in hemicellulose and lignin content was found to be 39 and 74%, respectively. Identical observations were earlier reported with surfactant assisted ultrasound pretreatment of sugarcane tops¹⁹ and alkali assisted microwave pretreatment of cotton plant residue.¹³

Primary screening

Among different surfactant screened, Tween-80 was found to be more effective agent in terms of reducing sugar yield (0.426 g/g). Other surfactants such as Tween-40 and Tween-20 also found to be potent but reducing sugar yields were 0.378 g/g and 0.393 g/g respectively, which was low as compared to Tween-80 pretreatment. PEG 6000, Triton X-100 and CTAB gave almost same reducing sugar yield (0.349, 0.346 and 0.353 g /g). Positive effect of Tween on pretreatment of corn stover and sugarcane tops was also reported by Qing et al.²⁶ and Sindhu et al.,¹⁹ respectively. Non-ionic surfactant improves cellulose conversion by increasing available cellulose surface area and preventing nonspecific binding of cellulase enzyme.²⁶

Effect of different process parameters for pretreatment by Taguchi experimental design

Taguchi design was adopted for optimization of selected parameters such as time, temperature, power, acid concentration (v/v) and surfactant concentration (w/v). Run order 2 and 3 gave nearly identical reducing sugar yield (0.254 and 0.261 g/g). Maximum reducing sugar was observed to be 0.502 g/g with run order 11 conditions (Table 1), were selected for further enzymatic hydrolysis studies.

Among all variables studied, analysis of variance (ANOVA) result predicted three statistically significant factors such as time, acid concentration and surfactant concentration. The F-value computed for Taguchi model was 30.47 which reflect strength of model (Table 4). Analysis of sugar yield from SAMAP biomass using Taguchi design revealed that time, acid concentration and surfactant concentration contributed the maximum impact with P (probability) > F less than 0.05

among all selected factors. Predicted R^2 (0.76) and adjusted R^2 (0.95) were in reasonable agreement with each other. The model has an adequate precision of 19.874, this suggest that the model can be used to navigate the design space. The adequate precision value is an index of the signal to noise and a value >4 is an essential prerequisite for a model to be a good fit. Effect of interaction between surfactant concentration and acid concentration on reducing sugar yield of SAMAP biomass is depicted in figure 1.

Non-ionic surfactants modify the biomass surface and interact hydrophobically with lignin to remove it and increases accessible area for cellulose.²⁶ Identical observation was earlier reported with surfactant assisted ultrasound pretreatment of sugarcane tops.¹⁹ Dilute acid pretreatment break down lignin shield surrounding lignocellulose and cause hemicellulose solubilization to open structure of biomass which improves enzyme accessibility to cellulosic fraction.²⁷ Decrease in sugar yield was observed with increase in acid concentration beyond 1% as it imposed negative effect which may be due to formation of inhibitors such as furfural, acetic acid and hydroxyl-methyl furfural.²⁸

Effect of non-ionic surfactant assisted dilute acid pretreatment of wheat straw on enzymatic hydrolysis and simultaneous saccharification and fermentation for ethanol production was reported by Qi et al.²⁹ Comparison of three types of microwave pretreatment using sugarcane bagasse as the lignocellulosic biomass has been evaluated for enzymatic saccharification efficiency and lignin removal.¹⁰ Study on comparison of alkali assisted microwave pretreatment of cotton plant residue with high pressure reactor pretreatment and modelling of alkali assisted microwave pretreatment followed by evaluation of enzymatic saccharification was also carried out by Vani et al.¹³

Characterization of native and SAMAP biomass

SEM and XRD analysis

SEM observation of untreated (Fig. 2a) and SAMAP biomass (Fig. 2b) showed pretreatment induced structural change in the biomass. SEM revealed ordered and compact structure which was destroyed in pretreated biomass. Similar structural changes were observed for sugarcane bagasse pretreated with microwave alkali pretreatment¹⁰ and sugarcane top pretreated with ultrasound assisted with surfactant.¹⁹

1 The XRD profile of native and SAMAP biomass is presented in figure 3. *CrI* in native and SAMAP
2 biomass were 32.23 and 45.5%, respectively (Table 3). A significant increase (13.27%) in *CrI* was
3 recorded in SAMAP biomass as compared to native biomass. The result was in close proximity with
4 the earlier report of surfactant-assisted ultrasound pretreatment of sugarcane tops.¹⁹ Treatment of
5 microwave-acid, microwave-alkali and microwave-alkali followed by acid pretreatment of sugarcane
6 bagasse also showed 5.35, 11.85 and 12.11% increase in *CrI*.¹⁰ Increase in *CrI* by 6% was also
7 reported in enzyme pretreated *Lantana camara* biomass.²⁵ The crystallinity is mainly related to the
8 molecular mass of hydrocarbons and fatty components of biomass.³⁰ Probably, crystallinity has been
9 damaged owing to the synergistic effect of surfactant, microwave and acid treatment, leading to the
10 enhanced saccharification of leaf litter biomass.

11 **Thermal gravimetric analysis (TGA)**

12 The TGA of leaf litter biomass showed onset temperature of devolatilization in the range of 200-250
13 °C, which corresponded to 10% weight loss with respect to the final weight loss (Fig. 4). The
14 behaviour of biomass materials during devolatilization was related to the presence of chemical
15 constituents such as cellulose, hemicellulose and lignin.³¹ The main weight loss ends at 350 - 390 °C
16 followed by a slow and continuous weight loss. The former step was due to primary devolatilization,
17 whereas the later was attributed to the degradation of heavier chemical structures in the solid matrix.³²
18 Similar devolatilization properties were also found with hot water pretreated biomass of *Tamarix*
19 *ramosissima*³³ and heat pretreated sugarcane bagasse.³⁴ Flattening of tail beyond 500 °C was observed
20 mainly due to removal of lignin in SAMAP biomass, as long tail was observed due to high percentage
21 of mannose or lignin content.³⁵

22 **Fourier transform infrared (FTIR) spectroscopy analysis**

23 The changes in functional groups of native and SAMAP biomass were presented by FTIR spectra
24 (Fig. 5) and peak assignments have been described based on literatures.³⁶⁻³⁹ Most prominent bands in
25 native sample were 3416, 2911, 1714, 1458, 731cm⁻¹. The broad band at 3416 cm⁻¹ is related to O-H
26 stretching vibrations caused by presence of alcoholic and phenolic hydroxyl group involved in
27 hydrogen bond. The O-H stretching band was changed to higher wave number and somewhat

1 broadened as a result of pretreatment which is an indication of weaker intra and intermolecular
2 hydrogen bonding and thereby lower crystallinity.⁴⁰ The band position at 2911 cm⁻¹ is attributed to C-
3 H stretching vibration showed slight enhancement in intensity of peak, indicating that the methyl and
4 methylene portions of cellulose were ruptured. Highest reduction was observed in the 1714 cm⁻¹ band,
5 attributed to hemicellulose acetyl and uronic ester groups or linkages in lignin and/or from ester
6 linkage of carboxylic group of the ferulic and p-coumaric acids of lignin and/or hemicellulose.
7 Almost absence of this signal in SAMAP indicated that pretreatment cleaved ester bond from the
8 hemicellulose and/or lignin. Absorption peak at 1458 cm⁻¹ represents absorption due to C-H
9 deformation within the methoxyl group of lignin and hemicellulose. Since the adsorption of the band
10 greatly reduced in SAMAP, suggested release of acid-soluble lignin. Bands in the range of 1000 -
11 1200 cm⁻¹ were related to structural features of cellulose and hemicelluloses. The enhancement of
12 absorption peaks at 1076 cm⁻¹ after pretreatment indicated the increase in cellulose content in the solid
13 residue.¹⁰

14 **Solid state CP/MAS ¹³C NMR spectroscopy**

15 Solid state CP/MAS ¹³C NMR spectra of the native and SAMAP biomass are presented in figure 6.
16 Peaks have been assigned based on the literatures.^{33, 41-43} The peak at 22.4 ppm can be attributed to the
17 -CH₃ in acetyl group of hemicellulose and lignin, was more prominent in native biomass. The peak at
18 31.6 and 55.9 assigned to aryl methoxyl carbons of lignin, have been greatly reduced after
19 pretreatment. The prominent peaks in the SAMAP biomass as compared to native in the region
20 between 60 and 110 ppm are predominantly due to the different carbons of cellulose. Signals from the
21 lignin phenolic groups arising from syringyl (S) and guaiacyl (G) type lignin are present from 110-
22 160 ppm. In this region, the peaks from 110 to 120 ppm can be assigned to carbons (C) of G lignin
23 and peaks between 120-140 ppm correspond to quaternary carbon in S and G lignin. The higher
24 cellulose contents of the SAMAP biomass was seen as distinct peak (72 ppm) assigned for C2, C3 and
25 C5 of cellulose. Less prominent peak at 172.2 ppm in SAMAP biomass corresponds to acetyl groups
26 of hemicellulose, ascribed to hemicellulose degradation. The results were in good agreement with hot
27 water pretreated biomass of *Tamarix ramosissima*³³ and biological pretreated lime wood.⁴²

Optimization of enzymatic saccharification by Box-Behnken design

Box-Behnken design was used for optimization of enzymatic hydrolysis of SAMAP biomass. The polynomial equation was derived to represent the reducing sugar yield as a function of the independent variables tested.

$$\begin{aligned}\text{Reducing sugar (g/g)} = & 0.5652 + 0.010917*A + 0.031083 *B + 0.136083 *C + 0.056417 *D \\ & -0.03475 *A *B - 0.006*A*C + 0.0115*A*D + 0.0205 *B*C \\ & + 0.018 *B*D + 0.0232 *C*D - 0.01264*A^2 + 0.010608 *B^2 \\ & - 0.09914 *C^2 - 0.14689 *D^2.\end{aligned}$$

Where A, B, C, D are coded values for acid concentration, surfactant concentration, enzyme loading, incubation time, respectively. Testing of the model was performed by the Fisher's statistical test for ANOVA using Design Expert software (Table 5).

ANOVA of the quadratic regression model suggests that the model is significant with a computed *F* value of 31.56. The values of *P* > *F* (<0.0001) implies that the model terms (enzyme loading and incubation time) are significant. Equation obtained from ANOVA indicates *R*² value of 0.96 which is in reasonable agreement with predicted *R*² (0.83) and adjusted *R*² (0.93). A lower value for the coefficient of variation (CV) suggests higher reliability of the experiment and obtained CV value of 7.11% demonstrates a greater reliability of the trials.

The effect of interaction between acid and surfactant concentration is shown in contour plot (Fig. 7).

It was observed that reducing sugar yield decreases after 42 h of incubation with enzyme loading of 20-80 FPU/g. As compared to reducing sugar yield of 0.05g/g in native biomass, enzymatic hydrolysis of recovered solid fraction of SAMAP biomass (0.5 mm) showed 93% increase in reducing sugar (0.69 g/g), when treated with 1.25% H₂SO₄ and 0.2% Tween-80 followed by 80 FPU/g of enzymatic hydrolysis for 42 h (Table 2). The initial increase in the reducing sugars with increase in enzyme dose from 20 to 50 FPU/g and slow increase in reducing sugars afterwards was observed which might be due to the less adsorption efficiency for higher enzyme concentrations than for diluted ones,⁴⁴ probably due to saturation of substrate surface with enzyme. Low reducing sugar yield with increased hydrolysis time after 42 h may be due to inhibition of the enzyme action by the accumulated

1 hydrolysis products.⁴⁵ The addition of non-ionic surfactant (Tween-80) improved enzymatic
2 hydrolysis effectively as it reduces non-productive adsorption of enzyme to lignin component of
3 lignocellulose biomass by removing lignin. Enhanced yield of sugar was also reported from
4 hydrolysis of sugarcane tops using Tween-40 by Sindhu et al.¹⁹

5 **CONCLUSION**

6 Surfactant (Tween-80) assisted microwave-acid pretreatment of *Eucalyptus globulus* leaf litter
7 biomass was effective for deriving sugar after enzymatic saccharification. Maximum yield of reducing
8 sugar (0.69 g/g) was obtained with optimum surfactant concentration of 0.2 % (w/v), 80 FPU/g of
9 enzyme loading after 42 h of incubation. SEM, XRD, TGA, FTIR, solid state CP/MAS ¹³C NMR
10 spectroscopy and composition analysis revealed differences between native and pretreated biomass.
11 To the best of our knowledge, this is the first report on surfactant assisted microwave acid
12 pretreatment of leaf litter biomass. The technique was found to be highly effective in removing
13 hemicellulose and lignin to provide accessible area for enzymatic hydrolysis. Further studies need to
14 be carried out to determine the overall process efficiencies and feasibility of the adopted strategy
15 using different kind of lignocellulosic biomass.

16 **ACKNOWLEDGEMENTS**

17 The authors are thankful to Directors, Thapar University and IIT Guwahati for providing
18 infrastructural support. The research work was supported by joint project grant from Department of
19 Biotechnology, Ministry of Science and Technology, Government of India to A. Goyal and D. Goyal.

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Table1.
Taguchi design for optimization of SAMAP leaf litter

Runs		Factors					Reducing sugar (g/g)	
Number	Order	A:Time (min.)	B: Temperature (°C)	C: Power (watt.)	D: Acid (% v/v)	E: Surfactant (% w/v)	Experimental	Predicted
7	1	10	50	200	0.5	0.05	0.211	0.208
12	2	10	100	400	1	0.1	0.254	0.269
5	3	10	150	600	1.5	0.15	0.261	0.266
14	4	10	200	800	2	0.2	0.301	0.283
6	5	20	50	400	1.5	0.2	0.398	0.413
4	6	20	100	200	2	0.15	0.443	0.44
2	7	20	150	800	0.5	0.1	0.465	0.447
9	8	20	200	600	1	0.05	0.487	0.492
16	9	30	50	600	2	0.1	0.311	0.316
13	10	30	100	800	1.5	0.05	0.421	0.403
15	11	30	150	200	1	0.2	0.502	0.521
11	12	30	200	400	0.5	0.15	0.478	0.484
8	13	40	50	800	1	0.15	0.377	0.359
3	14	40	100	600	0.5	0.2	0.412	0.417
1	15	40	150	400	2	0.05	0.198	0.214
10	16	40	200	200	1.5	0.1	0.168	0.165

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Table 2.
Box-Behnken design for enzymatic hydrolysis.

Runs		Factors				Reducing sugar (g/g)	
Number	Order	A: Acid (% v/v)	B: Surfactant (% w/v)	C: Enzyme (FPU/g)	D: Time (h)	Experimental	Predicted
5	1	1.25	0.125	20	24	0.164	0.149
7	2	1.25	0.125	20	60	0.256	0.216
14	3	1.25	0.2	20	42	0.321	0.351
27	4	1.25	0.125	50	42	0.562	0.565
3	5	0.5	0.2	50	42	0.612	0.618
26	6	1.25	0.125	50	42	0.576	0.565
20	7	2	0.125	80	42	0.583	0.594
21	8	1.25	0.05	50	24	0.317	0.359
28	9	1.25	0.125	50	42	0.581	0.565
16	10	1.25	0.2	80	42	0.689	0.664
6	11	1.25	0.125	80	24	0.354	0.375
12	12	2	0.125	50	60	0.446	0.484
4	13	2	0.2	50	42	0.563	0.570
24	14	1.25	0.2	50	60	0.563	0.534
13	15	1.25	0.05	20	42	0.301	0.330
22	16	1.25	0.2	50	24	0.376	0.385
15	17	1.25	0.05	80	42	0.587	0.561
9	18	0.5	0.125	50	24	0.384	0.349
11	19	0.5	0.125	50	60	0.41	0.439
8	20	1.25	0.125	80	60	0.539	0.534
29	21	1.25	0.125	50	42	0.566	0.565
1	22	0.5	0.05	50	42	0.512	0.486
2	23	2	0.05	50	42	0.602	0.577
17	24	0.5	0.125	20	42	0.298	0.300
10	25	2	0.125	50	24	0.374	0.348
19	26	0.5	0.125	80	42	0.563	0.584
23	27	1.25	0.05	50	60	0.432	0.436
18	28	2	0.125	20	42	0.342	0.334

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Table 3.
Cellulose, hemicellulose, total lignin, **reducing sugar** and *CrI* of native and SAMAP biomass.

Biomass	Cellulose (%)	Hemicellulose (%)	Total lignin (%)	Reducing sugar (g/g)	<i>CrI</i> (%)
Native	31.25	17.58	12.34	0.05	32.23
SAMAP	46.56	10.76	4.18	0.50	45.5

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Table 4.
Analysis of variance (ANOVA) for selected factorial mode of Taguchi design.

Source	Sum of squares	df	Mean square	F value	Probability > F	
Model	0.299882	12	0.02499	30.47267	0.0084	Significant
A:Time	0.167349	3	0.055783	68.02103	0.0029	
B:Temperature	0.0135	3	0.0045	5.487349	0.0979	
D:Acid	0.060295	3	0.020098	24.50777	0.013	
E:Surfactant	0.058737	3	0.019579	23.8754	0.0135	
Residual	0.00246	13	0.00082			
Cor. Total	0.302342	15				

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Table 5.
Analysis of variance (ANOVA) for selected factorial of Box-Behnken mode.

Source	Sum of squares	df	Mean square	F value	Probability > F	
Model	0.478098	14	0.03415	31.56848	< 0.0001	Significant
A:Acid	0.00143	1	0.00143	1.321983	0.2695	
B:Surfactant	0.011594	1	0.011594	10.71768	0.0055	
C:Enzyme	0.222224	1	0.222224	205.4261	< 0.0001	
D:Incubation time	0.038194	1	0.038194	35.30698	< 0.0001	
AB	0.00483	1	0.00483	4.46513	0.053	
AC	0.000144	1	0.000144	0.133115	0.7207	
AD	0.000529	1	0.000529	0.489013	0.4958	
BC	0.001681	1	0.001681	1.553933	0.233	
BD	0.001296	1	0.001296	1.198035	0.2922	
CD	0.002162	1	0.002162	1.998805	0.1793	
A ²	0.001037	1	0.001037	0.958259	0.3442	
B ²	0.00073	1	0.00073	0.67479	0.4252	
C ²	0.063756	1	0.063756	58.93679	< 0.0001	
D ²	0.13996	1	0.13996	129.3804	< 0.0001	
Residual	0.015145	14	0.001082			
Lack of fit	0.014182	10	0.001418	5.891982	0.0511	Not-significant
Pure error	0.000963	4	0.000241			
Cor total	0.493243	28				

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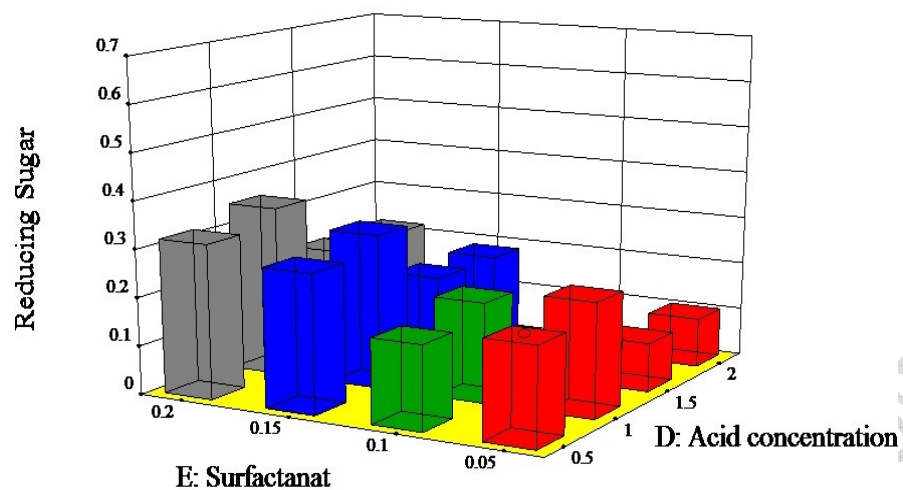
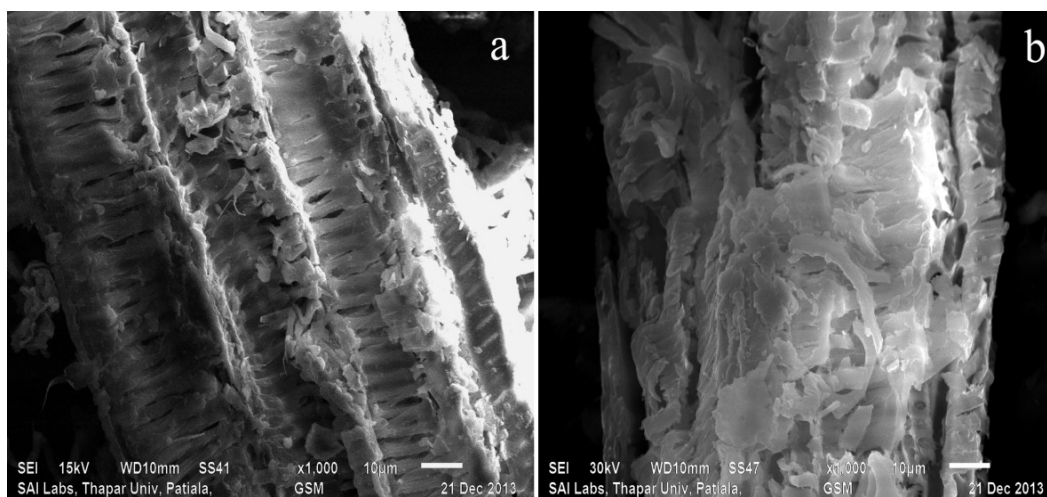


Figure 1: 3D contour plot showing interaction between acid and surfactant concentration.

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Figure 2: Scanning electron micrograph of (a) native and (b) SAMAP biomass
(magnification: 1,000X).

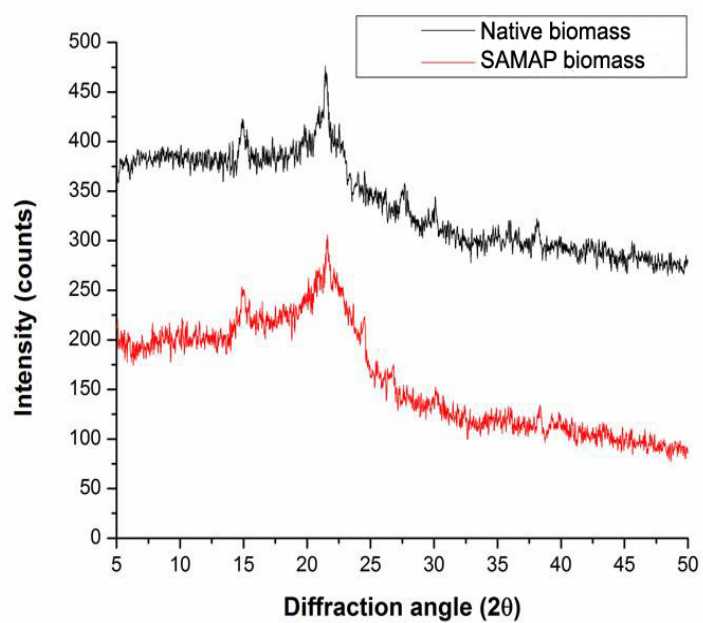


Figure 3: XRD profile of native and SAMAP biomass.

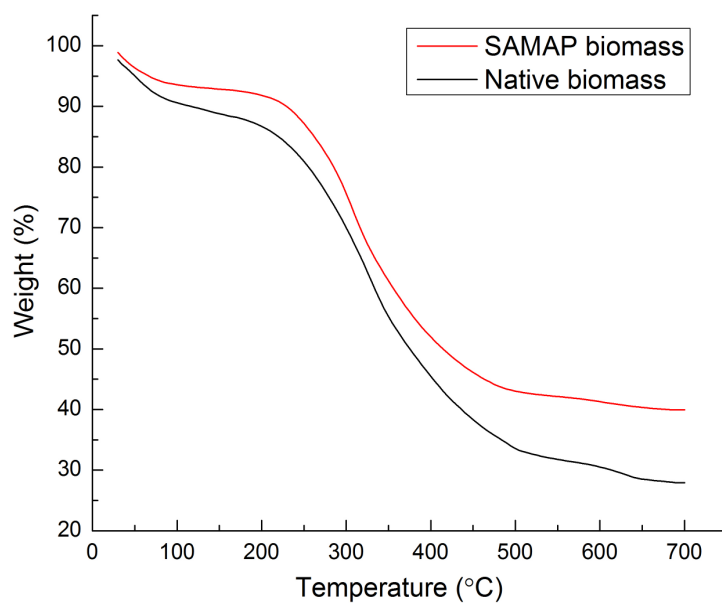


Figure 4: TGA of native and SAMAP biomass.

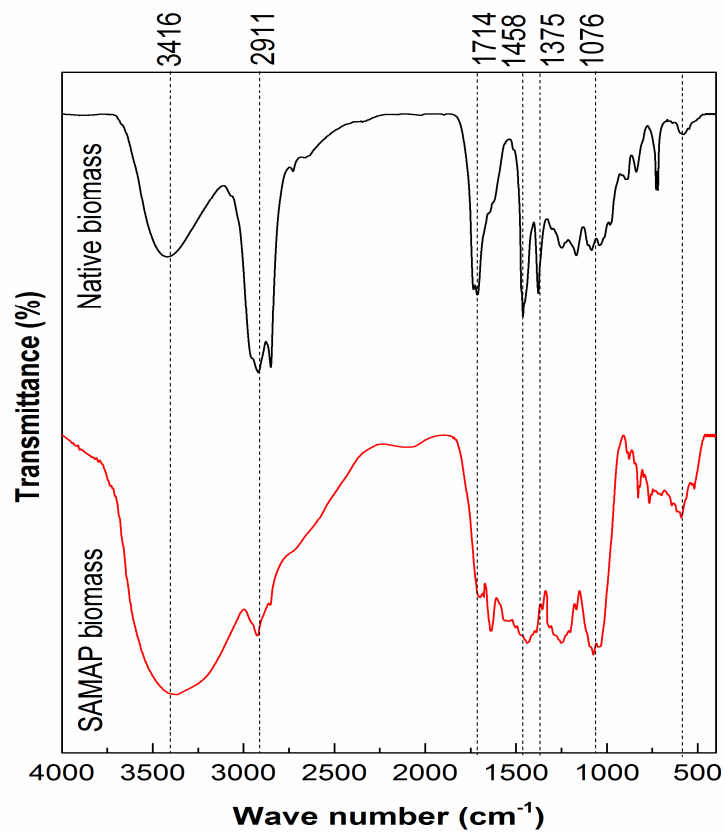


Figure 5: FTIR spectra of native and SAMAP biomass.

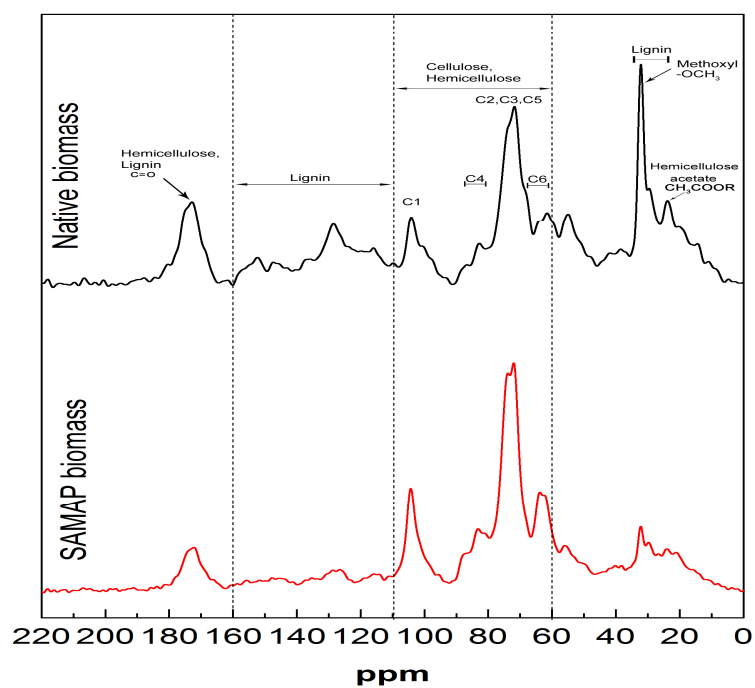


Figure 6: Solid state CP/MAS ^{13}C NMR spectra of native and SAMAP biomass.

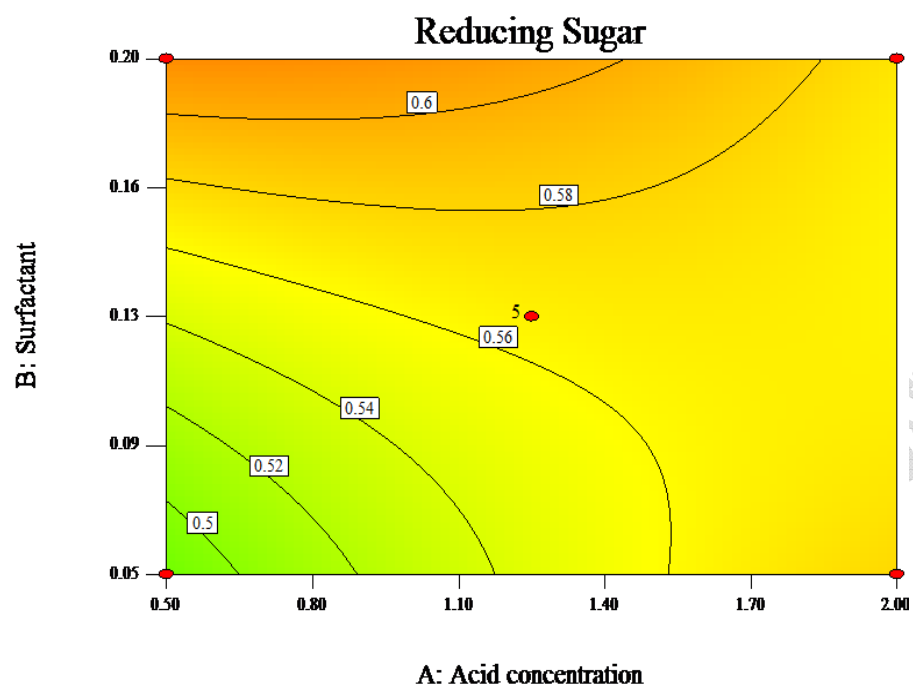


Figure 7: 3D contour plot showing interaction between acid and surfactant concentration



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**Biodegradation of leaf litter biomass by combination of *Bacillus* sp. and
Trichoderma reesei MTCC164**

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Acknowledgements

The authors are thankful to Directors, Thapar University and IIT Guwahati for providing infrastructural support. The research work was supported by joint project grant from Department of Biotechnology, Ministry of Science and Technology, Government of India to A. Goyal and D. Goyal.

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Abstract

Aim. Rapid biodegradation of leaf litter biomass from different tree species using different treatments including combination of *Bacillus* sp. AS3 and *Trichoderma reesei* MTCC164 over a period of 90 days.

Methods. Treated and untreated biomass was analyzed for pH, cellulose, reducing sugar, total organic carbon and total nitrogen over a period of 90 days. Scanning Electron Microscopy (SEM) and X-ray diffraction (XRD) analysis was performed to judge the microbial degradation of leaf litter biomass before and after treatment.

Results. Initially leaf litter biomass had cellulose content of 306.9 mg g^{-1} biomass and 47.6% organic carbon, which were reduced to 127.4 mg g^{-1} and 23%, respectively. Reducing sugar was increased from 5.47 to 10.94 mg g^{-1} of biomass and total nitrogen from 0.49 to 1.85%. Carbon:Nitrogen (C:N) ratio was narrowed from 97:1 to a range of 12:1 to 24:1 in different treatments and was maximally reduced to 86% by *Bacillus* sp. AS3 and *T. reesei* MTCC164 in 90 days. XRD analysis of leaf litter biomass showed 8.2% increase in CrI and SEM revealed the degraded structure after the action of microbial cellulases.

Discussion.

Co-inoculation of *Bacillus* sp. and *Trichoderma* sp. was effective for large scale rapid recycling of agricultural waste residues by reducing the degradation period from 90 days to 30 days. The co-inoculation strategy shall render easy accessibility of its cellulosic components for bioconversion to fermentable sugars.

Key words. Cellulose degradation, leaf litter, C:N ratio, X-ray diffraction

Introduction

Cellulose content in the plant biomass is typically in the range of a 35 to 50% of plant dry weight and is the only foreseeable sustainable source of fuels and materials available to humanity¹. Cellulose is a homopolymer of β -1,4 linked glucose units having chain length in the range of 100-14,000 residues, is the most abundant renewable natural biological resource in the biosphere². Many microorganisms are capable of degrading and using cellulose and hemicellulose as carbon and energy sources³.

Cellulose biodegradation by cellulases plays a key role in several agricultural and waste treatment processes⁴. Effective conversions of recalcitrant lignocelluloses to fermentable sugars require three sequential steps: (a) size reduction, (b) pretreatment/fractionation and (c) enzymatic hydrolysis⁵. Microorganisms to hydrolyze and metabolize insoluble cellulose, extracellular cellulases must be produced that are either free or cell associated⁶. Culturable, cellulase producing bacteria have been isolated from a wide variety of sources such as composting heaps, decaying plant material, feces of ruminants and hot springs⁷. *Bacillus cereus* MRLB1 isolated from soil⁸ and *Bacillus* sp AS3⁹ isolated from cow dung are known to produce various cellulase. Both bacteria and fungi, utilize cellulose through the production of substantial amounts of extracellular cellulase¹⁰, although these enzymes are occasionally present in complexes at the cell surface¹¹.

The complete cellulolytic enzyme systems for breakdown of crystalline cellulose to glucose have been observed by white-rot fungi *Phanerochaete chrysosporium* and soft-rot fungi *T. reesei* and *Penicillium pinophilum*¹². Fungi have also been shown to produce ligninolytic enzymes during the biodegradation of lignocellulosic materials¹³. *A. niger* and *Trichoderma viride* are known to produce extracellular enzymes¹⁴ with homologous

and heterologous proteins¹⁵. The activities of lignin peroxidases, manganese peroxidases, and laccase during solid-state fermentation of lignocellulosic materials were also reported by Dinis et al¹⁶.

Consortium of fungi such as *A. niger* MSK7 and *T. viridae* MSK10¹⁷ and bacteria (*Clostridium straminisolvens* CSK1, *Clostridium* sp. strain FG4, *Pseudoxanthomonas* sp. strain M1-3, *Brevibacillus* sp. strain M1-5 and *Bordetella* sp. strain M1-6) have also been studied for degradation of cellulose by Kato et al¹⁸.

The proper management and disposal of leaf litter biomass is a serious concern, although attention has been focused for safe disposal in a short period of time. Microbial biodegradation is an effective and ecofriendly way to promote sustainable agriculture and environmental protection, improving the soil's physical, chemical, and biological properties²⁰, which ultimately results in better plant growth and yield. Bioconversion of cellulose into fermentable sugars²¹ using lignocellulosic agricultural residues such as grasses, forestry wastes for ethanol production²² are more economical.

Since this type of biomass generated in large quantities and for their efficient recycling, present work has been focused on degradation of litter biomass obtained from different tree species was studied by *Bacillus* sp. AS3 and *T. reesei* MTCC164 and by co-inoculation in terms of reduction in crystalline complex and increase in reducing sugar content.

Materials and Methods

Leaf litter

Leaf litter biomass from different tree species such as *Eucalyptus marginata*, *Polyalthia longifolia*, *Saraca asoca*, *Populus deltoides* and *Mangifera indica* were collected from

different places of Thapar University, Patiala, Punjab (India), located at 30°19'48"N , 76°24'0"E and 310 m above the sea level. Equal amounts of leaf litter of different tree species by weight were taken, washed to remove adhering debris and were air dried and milled by mechanical blender to the size of 2-3 cm for further degradation process.

Microorganisms and media

Bacillus sp. AS3 (GenBank accession No. EU754025), isolated from cow dung [9Akhtar] was maintained on Nutrient Agar (NA) plate and subcultured every four weeks. A loopful of culture from NA plate was added to 5 ml of Nutrient Broth (NB) and was grown under aerobic condition with rotary agitation at 120 rpm for 24 h at 37°C. A 1 % (v/v) of the inoculum was transferred into 100 ml of Nutrient broth (NB) and incubated at 37 °C for 24 h at 120 rpm. After 24 h of incubation, 100 ml of culture (7×10^8 CFU/ml) was inoculated one time to one kg biomass for degradation. *T. reesei* MTCC164, used in the present study was procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. The strain was maintained on Potato Dextrose Agar (PDA) plate. For suspending spores, sterilized water (5 ml) was applied to the culture plate. After gentle shaking, the suspension was taken in a separate sterile tube and spore suspensions were set to 5×10^7 spores/ml using hemocytometer. A 1 % (v/v) of it was added to 100 ml of PDB (Potato Dextrose Broth) and incubated at 28°C for 48 h with rotatory agitation (120 rpm). After 48 h of incubation, the culture was inoculated one time to one kg of biomass for degradation process.

Biodegradation of leaf litter biomass was studied in eight different treatments as given in table 1. One kilogram of milled leaf litter biomass was taken in round plastic tubs of diameter 14 inches and depth of 6 inches, in triplicates and kept under shade at an

ambient temperature in the range of 26-32°C. Moisture was maintained at 40-50% in each tub by gravimetric method²³.

Biomass was treated over a period of 90 days and samples were withdrawn at 15 days of interval from each tub and were dried, milled and sieved to 0.5 mm size and analyzed for pH²⁴, cellulose by Anthrone method²⁵, reducing sugar by 3, 5-dinitrosalicylic acid (DNS) method²⁶, total organic carbon and total nitrogen²⁴.

Scanning Electron Microscopy (SEM)

SEM of dried untreated and pretreated leaf litter biomass was carried out using Scanning Electron Microscope (Model: EV- 40, Carl Zeiss, India) at 20 kV. Prior to viewing, the samples were sputter coated with thin layer gold. Micrographs were taken at different magnifications from 1,000 $\times$ to 10,000 $\times$ depending upon the feature to be traced.

X-ray Diffraction (XRD) analysis of leaf litter biomass

The effect of treatment by co-inoculation of *Bacillus* sp. AS3 and *T. reesei* MTCC164 on the crystalline structure of leaf litter biomass was determined by X-ray diffraction at room temperature in the scanning angle of 5-50° at the scan speed of 0.5 min⁻¹ using a PANalytical X'Pert PRO system with Ni-filter and diffractometer operating at 45 kV and 130 mA with λ (CuK α) = 1.5406 Å°. The crystallinity index (*CrI*) of the leaf litter biomass was calculated and determined as described by Segal et al.²⁷, as follows:

$$CrI = [(I_{002} - I_{am}) / I_{002}] \times 100$$

Where I_{002} is the intensity for the crystalline portion of biomass (i.e. cellulose) at about 2 θ of 21-23°, and I_{am} is the peak for the amorphous portion (i.e. cellulose, hemicellulose, and lignin) at about 2 θ of 15-18° in most literatures²⁷⁻³¹.

Statistical analysis

One way analysis of variance (ANOVA) with Duncan's Multiple Range Test (DMRT) was used to compare the means³².

Results

During microbial mediated degradation under shade, the moisture content in different treatments over a period of 90 days was maintained in the range of 40-50%. pH of leaf litter biomass gradually increased from initial pH of 6.86 ± 0.4 in all the treatments and varied from 7.68 ± 0.5 to 8.12 ± 0.5 .

Cellulose and reducing sugar

Cellulose content of leaf litter biomass at the start of experiment was 360.9 mg g^{-1} of biomass which decreased gradually in all the treatment (Table 2) and residual cellulose content varied from 127.4 to 198.1 mg g^{-1} of biomass after 90 days of treatment. Cellulose content was maximally decreased by 65% when biomass was treated with *Bacillus* sp. AS3 in combination with *T. reesei* MTCC164 followed by treatment of *T. reesei* MTCC164 with activator DAP, which was 62%.

Estimation of reducing sugars showed gradual increase over a period of 90 days in all treatments (Table 3). However, after 15 days of treatment there was a slight decrease in reducing sugar content and then onwards there gradual increase in reducing sugar was observed due to hydrolysis of cellulose. After 90 days, reducing sugar content was found in the range of 5.31 to 10.94 mg g^{-1} of biomass in different treatments as compared to 5.31 mg g^{-1} in control. The maximum rise in reducing sugar was by 59% in the treatment comprising of *Bacillus* sp. AS3 in combination with *T. reesei* MTCC164 followed by treatment of *T. reesei* MTCC164 with activator DAP which was 55%.

Total organic carbon

Total organic carbon content was found in the range of 23.1 to 29.21% in all the treatment which was 31.20% in control after 90 days of treatment (Table 4). The decrease in organic carbon content was highest by 51% in the treatment comprising of *Bacillus* sp. AS3 in combination with *T. reesei* MTCC164 followed by treatment of *T. reesei* MTCC164 with activator DAP (49%), after 90 days of treatment.

Nitrogen content

There was significant change in the total nitrogen content and it varied from 1.85% to 1.09% (Table 5). The maximum increase (74%) in the total nitrogen content was observed in treatment comprising of *Bacillus* sp. AS3 in combination with *T. reesei* MTCC164 followed by treatment of *T. reesei* MTCC164 along with DAP which was 71%.

C:N ratio

Over a period of 90 days there was a significant change in the C:N ratio of treated leaf litter biomass and it varied in the range of approximately 12:1 to 29:1 (Figure 1) due to different bacterial and fungal treatment as compared to initial C:N ratio (97:1). The maximum drop in C:N ratio was observed due to action of *Bacillus* sp. AS3 in combination with *T. reesei* MTCC164 (88%), followed by *T. reesei* MTCC164 along with activator DAP (84%), as compared to C:N ratio at 0 day of treatment. Co-inoculation led to an overall decrease in degradation time by attaining C:N ratio of 25:1 at the end of 30 days of the experiment which was in close proximity after 90 days in case of control, reduced the degradation period from 90 days to 30 days.

SEM and XRD analysis

SEM images of untreated (Figure 2a) leaf litter biomass revealed ordered and compact structure whereas after treatment with *Bacillus* sp. AS3 and *T. reesei* MTCC 164 the ordered structures were destroyed (Figure 2b) and area accessible for cellulase was increased due to removal of lignin and hemicellulose³³.

X-ray diffraction analysis showed intense crystalline peak at 2θ angle of 21.5° (Figure 3). After 90 days of biodegradation, a significant increase (8.2%) in the *CrI* was recorded in biomass treated with *Bacillus* sp. AS3 and *T. reesei* MTCC164 under natural environmental conditions.

Discussion

Paddy straw and soyabean trash also showed alkaline pH of 7.5 after 90 days of biodegradation²¹. Substantial cellulose hydrolysis occurs by ruminal bacteria at pH below 6.0, once the bacteria have adhered to cellulose, synthesizes a glycocalyx and initiates its growth at higher pH³⁴ which may enhance degradation process. DAP was used in very less amount (0.01 g/g of biomass) as source of nitrogen and phosphorus, when mixed with lignocellulosic substrate favors degradation process³⁵, however, their utilization efficiency remains low due to loss by volatilization, denitrification and conversion into unavailable forms³⁶.

Cellulose and reducing sugar

Reduction of 29% cellulose was also observed with the degradation of rice straw by *A. niger* and *T. viride* after 45 days of composting³⁷. Degradation of sugarcane trash using *T. reesei* also showed similar result with 31% increase in the reducing sugar after 45 days of treatment³⁸. Because of low β -glucosidase level in the enzyme complex produced by *T.*

reesei MTCC164 alone leads to retardation of cellulose hydrolysis due to catabolite repression³⁹. Therefore, cellulases produced by *Bacillus* sp. AS3, a rich source of β -glucosidase⁹ may overcome the inhibition in co-inoculation. Presence of DAP elicited cellulose degradation by 14% when used with *T. reesei* MTCC164 in comparison to *T. reesei* MTCC164 alone, whereas inhibition in cellulose degradation by 6% was observed in treatment comprised of *Bacillus* sp. AS3 and DAP as compared to *Bacillus* sp. AS3 alone, after 90 days of biodegradation. Probably, DAP caused inhibition of bacterial growth which in turns hindered biodegradation of leaf litter biomass. Presence of urea and DAP also showed toxic effect on growth of *Pseudomonas aeruginosa* GRC₂³⁶.

Total organic carbon

The reduction in organic carbon in biodegradation process was mainly due to conversion of organic carbon to energy and CO₂ as metabolic end products³⁷. Thus, the organic carbon content of substrate decreased as degradation proceeds which were in accordance with the composting of sawdust⁴⁰. Fungal consortium also showed 36% and 25% decrease in organic carbon in biodegradation of soybean supplemented paddy straw and urea amended paddy straw, respectively after 45 days of treatment²¹.

Nitrogen content

The increase in nitrogen content in early stages of experiment could be due to loss of nitrogen in the form of ammonia or microbial immobilization⁴¹. It might be due to the formation of new cell structure, enzymes, hormones and nitrification by the microorganisms⁴². Increased total nitrogen content during the biodegradation process was in agreement with the composting of lignocellulosic materials amended with chicken

manure⁴³. Composting of paddy straw using *A. niger* and *T. viridae* also showed of 46% increment in total nitrogen content after 45 days of treatment³⁷.

C:N ratio

The increased C:N ratio of treated biomass was in good agreement with composting of paddy straw by *A. niger* and *T. viridae* which showed 54% decrease in C:N ratio after 45 days of treatment³⁷. Fungi have wider C:N ratio of 30:1 as compared to 10:1 of the bacteria, hence fungi are more capable of degrading any lignocellulosic material as their dependency on nitrogen is comparatively less⁴⁴. Singh et al.³⁸ observed that the drop in C:N ratio of sugarcane trash by the action of *T. reesei* was 47 %, by *A. terreus* it was 61%, and by *Cellulomonas uda* it was 52%. It is believed that C:N ratio carries importance for degradation of lignocellulosic material since to degrade each molecule of carbon, a definite proportion of nitrogen is required by the microorganisms³⁸.

SEM and XRD analysis

SEM reveled the degraded structure and XRD analysis showed an increase in *CrI* of treated leaf litter biomass. Comparative more increase in *CrI* was observed in treated biomass of *Lantara camara* using *Pleurotus* sp. and *T. reesei* Rut C30³¹, treated biomass of *Tamarix ramosissima* using hot compressed water at 140°C³⁰ and treated leaf litter biomass using *Bacillus* sp. AS3 alone⁹. The crystallinity of the surface is mainly related to the high molecular mass hydrocarbons and fatty components in the biomass¹³ which may be damaged because of degradation owing to the microbial treatment leading to the enhanced digestibility of the cellulosic biomass. The second highest peak (Figure 3) after 21.5° was at 15° which correspond to the amorphous region^{45, 46}. Crsytallinity was also

observed at 2 θ angle of 20° in Canadian biomass²⁹, 2 θ angle of 25° in *L. camara* biomass³¹ and *T. ramosissima* biomass³⁰.

Conclusion

Overall, among different treatments, treatment with *Bacillus* sp. AS3 along with *T. reesei* MTCC 164 was found to be the most effective in terms of cellulose hydrolysis, high reducing sugar production and narrowing C:N ratio in leaf litter biomass, followed by *T. reesei* MTCC164 in presence of DAP. Co-inoculation strategy using *Bacillus* sp. AS3 along with *T. reesei* MTCC164 shall render easy accessibility of its cellulosic components for bioconversion to fermentable sugars. XRD analysis of biomass showed decrease in crystalline structure and SEM revealed increased accessible area for cellulase after microbial treatment of leaf litter biomass.

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(a) treated and (b) untreated ($5,000\times$).

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Table 1. Bacterial and fungal inoculum per kg of leaf litter biomass

S.No.	Treatment
1.	Control (Leaf litter biomass)
2.	DAP
3.	<i>Bacillus</i> sp. AS3
4.	<i>T. reesei</i> MTCC164
5.	<i>Bacillus</i> sp. AS3 + <i>T. reesei</i> MTCC164
6.	<i>Bacillus</i> sp. AS3 + DAP
7.	<i>T. reesei</i> MTCC164 + DAP
8.	<i>Bacillus</i> sp. AS3 + <i>T. reesei</i> MTCC164 + DAP

Bacillus sp. AS3 @ 100 ml/kg of biomass

T. reesei MTCC164 @ 100 ml/kg of biomass

DAP: Diammonium phosphate @ 0.1g/kg of biomass

Table 2. Residual cellulose (mg g⁻¹) in leaf litter biomass at different intervals of decomposition.

Treatments	No. of days					
	15	30	45	60	75	90
C	342.81 ^a ± 11.40	332.56 ^a ± 12.10	282.21 ^a ± 13.36	251.19 ^a ± 13.40	198.07 ^a ± 12.38	196.21 ^a ± 11.24
DAP	336.14 ^{ab} ± 12.31	322.56 ^b ± 10.11	271.87 ^{ab} ± 11.13	241.18 ^{ab} ± 11.44	188.06 ^{ab} ± 10.18	186.48 ^{ab} ± 14.13
B	316.14 ^c ± 10.04	302.54 ^d ± 11.35	251.88 ^{cd} ± 10.29	221.21 ^{cd} ± 14.49	167.74 ^{cd} ± 9.43	166.28 ^{cd} ± 13.57
T	305.0 ^d ± 9.54	292.55 ^e ± 10.24	241.98 ^{de} ± 9.48	211.19 ^{de} ± 10.19	157.39 ^{de} ± 10.02	156.51 ^{de} ± 10.21
B + T	284.68 ^e ± 8.43	265.35 ^g ± 9.27	217.71 ^g ± 14.53	185.53 ^f ± 9.19	133.25 ^g ± 9.40	127.41 ^g ± 12.36
B + DAP	326.14 ^{bc} ± 12.27	312.56 ^c ± 13.29	261.87 ^{bc} ± 12.25	231.19 ^{bc} ± 13.28	178.07 ^{bc} ± 14.16	176.89 ^{bc} ± 13.18
T + DAP	288.02 ^e ± 13.28	227.02 ^g ± 11.26	222.05 ^{fg} ± 10.26	194.30 ^{ef} ± 10.61	138.25 ^{fg} ± 12.26	137.41 ^{fg} ± 9.32
B + T + DAP	294.0 ^{de} ± 10.33	282.30 ^f ± 12.46	231.98 ^{ef} ± 14.17	201.19 ^{ef} ± 9.55	147.38 ^{ef} ± 11.22	146.25 ^{ef} ± 10.25

C: Control; B: *Bacillus* sp. AS3; T: *T. reesei* MTCC164; DAP: Diammonium phosphate

Cellulose content on 0 day: 360.89 ± 13.28 mg g⁻¹

Values are mean ± S.E., Mean values followed by the same superscript within each column do not differ significantly at $P \leq 0.05$ by Duncan's Multiple Range Test (DMRT).

Table 3. Reducing sugar content (mg g⁻¹) in leaf litter biomass at different intervals of decomposition.

Treatments	No. of days					
	15	30	45	60	75	90
C	4.41 ^a ± 0.24	4.76 ^{bc} ± 0.31	4.79 ^d ± 0.41	5.18 ^{ef} ± 0.46	5.21 ^g ± 0.32	5.31 ^g ± 0.47
DAP	4.33 ^a ± 0.31	4.50 ^{cd} ± 0.21	4.92 ^d ± 0.33	6.18 ^{cd} ± 0.37	6.23 ^{ef} ± 0.41	6.29 ^f ± 0.49
B	4.16 ^a ± 0.27	2.17 ^g ± 0.19	3.53 ^e ± 0.25	4.41 ^f ± 0.25	6.09 ^f ± 0.51	7.96 ^{de} ± 0.62
T	3.43 ^b ± 0.34	3.17 ^f ± 0.17	4.52 ^d ± 0.35	5.42 ^{de} ± 0.26	7.09 ^d ± 0.66	8.56 ^{cd} ± 0.56
B + T	2.10 ^d ± 0.19	6.17 ^a ± 0.54	7.54 ^a ± 0.47	8.34 ^a ± 0.64	10.1 ^a ± 0.85	10.94 ^a ± 1.10
B + DAP	4.33 ^a ± 0.23	3.81 ^e ± 0.25	3.92 ^e ± 0.36	5.18 ^{ef} ± 0.43	6.73 ^{de} ± 0.53	7.57 ^c ± 0.41
T + DAP	2.71 ^c ± 0.18	5.77 ^b ± 0.37	6.59 ^b ± 0.42	7.41 ^b ± 0.54	9.10 ^b ± 0.73	9.94 ^b ± 0.83
B + T + DAP	3.21 ^{bc} ± 0.31	4.17 ^{de} ± 0.35	5.53 ^c ± 0.45	6.42 ^c ± 0.47	8.10 ^c ± 0.54	8.93 ^c ± 0.64

C: Control; B: *Bacillus* sp. AS3; T: *T. reesei* MTCC164; DAP: Diammonium phosphate

Reducing sugar at 0 day: 5.47 ± 0.43 mg g⁻¹

Values are mean ± S.E., Mean values followed by the same superscript within each column do not differ significantly at $P \leq 0.05$ by Duncan's Multiple Range Test (DMRT).

Table 4. Organic carbon content (%) in leaf litter biomass at different intervals of decomposition.

Treatment	No. of days					
	15	30	45	60	75	90
C	46.32 ^a ± 2.18	42.85 ^a ± 2.80	38.53 ^a ± 3.07	33.71 ^a ± 1.88	31.61 ^a ± 2.10	31.20 ^a ± 2.16
DAP	46.27 ^a ± 2.12	41.82 ^b ± 2.13	36.53 ^b ± 2.34	31.94 ^b ± 2.04	30.28 ^b ± 1.94	29.21 ^b ± 2.03
B	44.27 ^{bc} ± 2.26	39.82 ^d ± 2.29	34.53 ^d ± 2.08	30.27 ^{cd} ± 1.83	28.28 ^d ± 1.60	26.21 ^e ± 2.07
T	43.27 ^{cd} ± 1.96	38.82 ^e ± 1.84	33.40 ^e ± 1.90	29.07 ^{de} ± 1.66	27.32 ^e ± 1.87	26.29 ^e ± 1.56
B + T	40.02 ^f ± 1.89	36.02 ^g ± 1.14	30.03 ^h ± 1.19	26.05 ^g ± 1.69	24.23 ^h ± 1.21	23.13 ^h ± 1.95
B + DAP	45.27 ^{ab} ± 2.10	40.82 ^c ± 3.02	35.53 ^c ± 2.22	31.04 ^{bc} ± 2.24	29.28 ^c ± 1.91	27.20 ^d ± 1.95
T + DAP	41.28 ^e ± 2.65	37.07 ^f ± 2.14	31.34 ^g ± 1.96	27.05 ^{fg} ± 2.10	25.23 ^g ± 1.38	24.13 ^g ± 2.08
B + T + DAP	42.27 ^{de} ± 2.40	38.05 ^e ± 2.64	32.40 ^f ± 1.87	28.05 ^{ef} ± 1.87	24.24 ^f ± 0.20	25.15 ^f ± 2.10

C: Control; B: *Bacillus* sp. AS3; T: *T. reesei* MTCC164; DAP: Diammonium phosphate

Carbon content at 0 day: 47.58 ± 2.12%

Values are mean ± S.E., Mean values followed by the same superscript within each column do not differ significantly at $P \leq 0.05$ by Duncan's Multiple Range Test (DMRT).

Table 5. Nitrogen content (%) in leaf litter biomass at different intervals of decomposition.

Treatments	No. of days					
	15	30	45	60	75	90
C	0.54 ^g ± 0.02	0.62 ^f ± 0.03	1.62 ^h ± 0.07	0.86 ^e ± 0.04	0.95 ^g ± 0.06	1.09 ^h ± 0.06
DAP	0.65 ^f ± 0.02	0.76 ^e ± 0.04	0.83 ^g ± 0.04	0.97 ^e ± 0.06	1.04 ^f ± 0.07	1.22 ^g ± 0.05
B	0.73 ^d ± 0.03	0.85 ^e ± 0.03	1.05 ^e ± 0.05	1.52 ^{ab} ± 0.08	1.24 ^d ± 0.05	1.56 ^d ± 0.05
T	0.74 ^{cd} ± 0.03	0.95 ^d ± 0.04	1.16 ^d ± 0.07	1.25 ^{cd} ± 0.07	1.37 ^c ± 0.06	1.52 ^d ± 0.04
B + T	0.92 ^a ± 0.05	1.44 ^a ± 0.08	1.62 ^a ± 0.09	1.74 ^a ± 0.08	1.81 ^a ± 0.09	1.85 ^a ± 0.08
B + DAP	0.67 ^{ef} ± 0.03	0.81 ^e ± 0.05	0.95 ^f ± 0.06	1.06 ^{de} ± 0.04	1.14 ^e ± 0.06	1.42 ^e ± 0.06
T + DAP	0.84 ^b ± 0.03	1.23 ^b ± 0.07	1.34 ^b ± 0.08	1.45 ^{bc} ± 0.07	1.56 ^b ± 0.07	1.67 ^b ± 0.05
B + T + DAP	0.79 ^{bc} ± 0.04	1.08 ^c ± 0.04	1.26 ^c ± 0.05	1.36 ^{bc} ± 0.06	1.44 ^c ± 0.05	1.60 ^c ± 0.06

C : Control; B: *Bacillus* sp. AS3; T: *T. reesei* MTCC164; DAP: Diammonium phosphate

Nitrogen content at 0 day: 0.49 ± 0.02 %

Values are mean ± S.E., Mean values followed by the same superscript within each column do not differ significantly at $P \leq 0.05$ by Duncan's Multiple Range Test (DMRT).

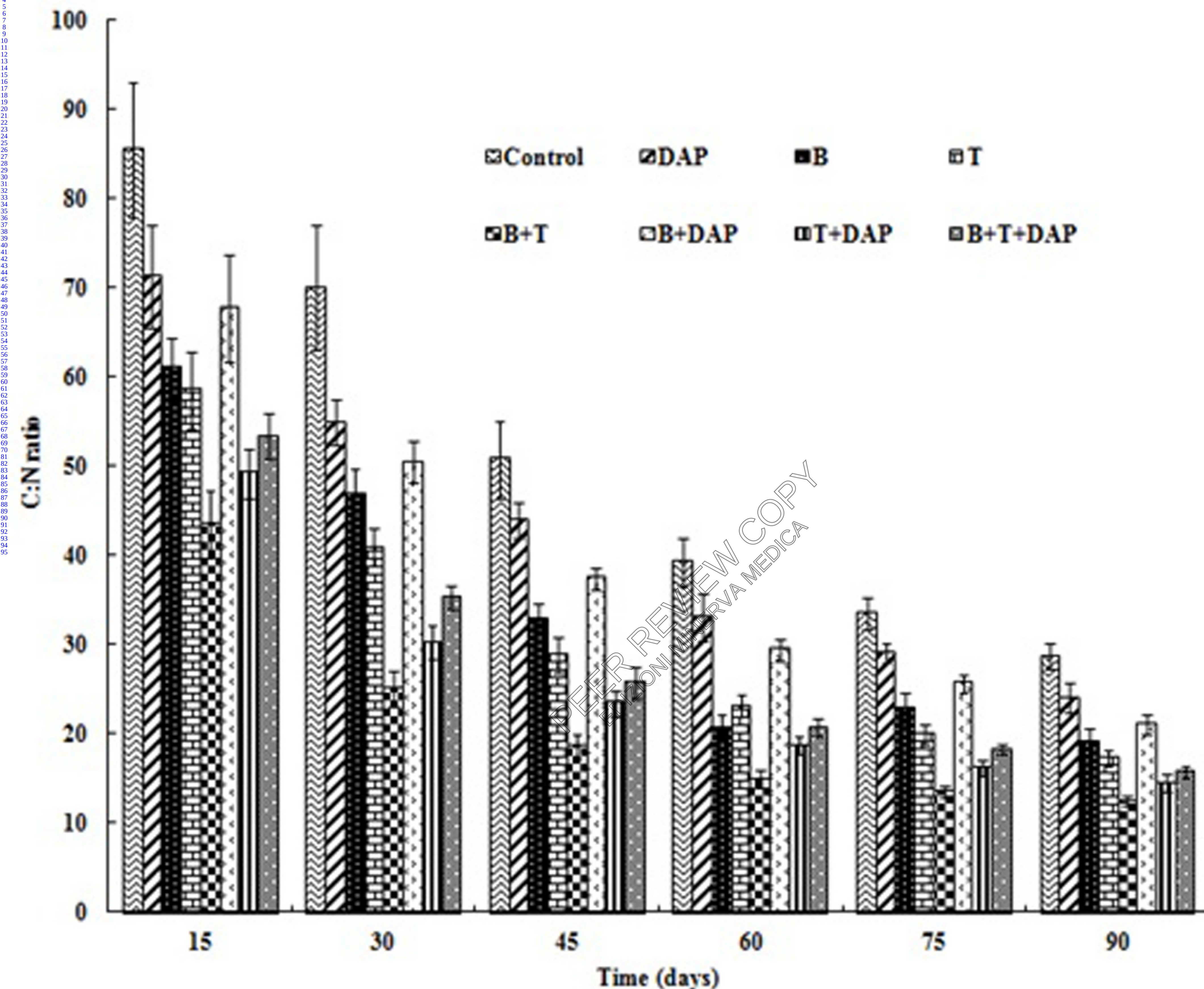


Figure 1. C:N ratio of leaf litter biomass at different interval of decomposition.

C:N ratio at 0 day: 97:1

B: *Bacillus* sp. AS3; T: *T. reesei* MTCC164; DAP: Diammonium phosphate

The vertical bars designate the standard errors for the mean of three replicative tests

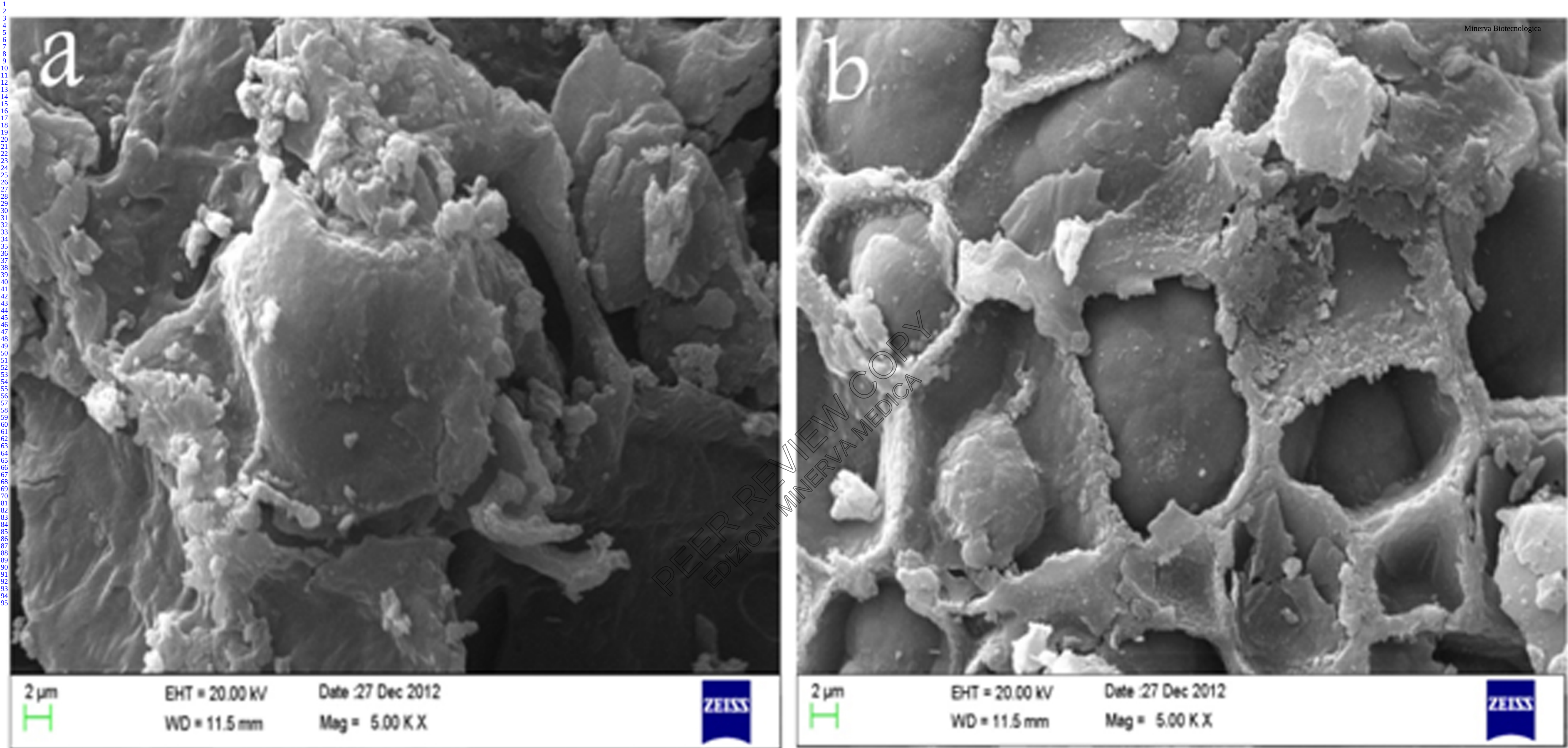


Figure 2. SEM micrographs of leaf litter biomass after 90 days.

(a) treated and (b) untreated (5, 000 x).

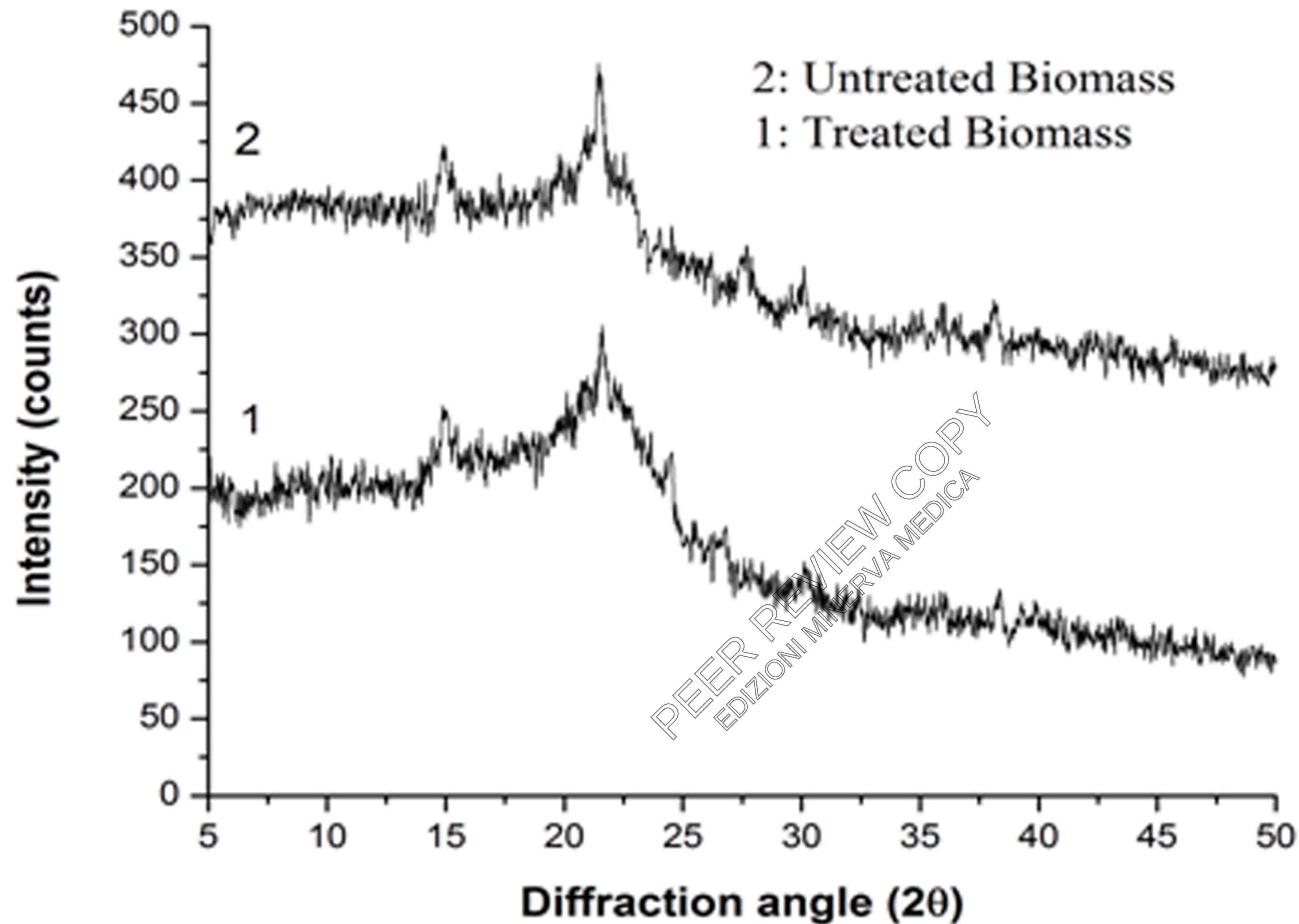


Figure 3. XRD analysis of treated and untreated leaf litter biomass after 90 days of treatment.