Enhanced Production of Cellulosic Ethanol using Thermo and Inhibitor Tolerant Yeasts

Thesis submitted to the Central University of Haryana

for the award of the degree of

DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY

HEMANSI

(Roll No. 9042)

DEPARTMENT OF MICROBIOLOGY

CENTRAL UNIVERSITY OF HARYANA

MAHENDERGARH, HARYANA-123031

INDIA

October, 2021

Enhanced Production of Cellulosic Ethanol using Thermo and Inhibitor Tolerant Yeasts

Thesis submitted to the Central University of Haryana for the award of the degree of

DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY

October, 2021

By

HEMANSI
(Roll No. 9042)

Approved By:

Chairperson, RAC	**:**	**Dr. Jitendra Kumar Saini** _____ **Assistant Professor** **Department of Microbiology**
Members, RAC	**:**	**Dr. Puja Yadav** _____ **Assistant Professor** **Department of Microbiology**
		Dr. Manoj Kumar Gupta _____ **Department of Chemistry**
Head of Department	**:**	**Dr. Gunjan Goel** _____ **Associate Professor** **Department of Microbiology**

Department of Microbiology
Central University of Haryana
Mahendergarh-123031
Haryana, India

Ref._____ **Date:** _____

Certificate/Declaration

This is to certify that the material embodied in the present work, entitled "**Enhanced production of cellulosic ethanol using thermo and inhibitor tolerant yeasts"**, is based on my original research work. It has not been submitted, in part or full, for any other diploma or degree of any University/Institution Deemed to be University and College/Institution of National Importance. References from other works have been duly cited at the relevant places.

Hemansi
(Roll No. 9042)
(Candidate)

Dr. Jitendra Kumar Saini
(Supervisor)

Dr. Gunjan Goel
(Head of the Department)

Certificate/Declaration

This is to certify that the material embodied in the present work, entitled "**Enhanced production of cellulosic ethanol using thermo and inhibitor tolerant yeasts"** is based on my original research work. It has not been submitted, in part or full, for any other diploma or degree of any University/Institution Deemed to be University and College/Institution of National Importance. References from other works have been duly cited at the relevant places.

Hemansi
(Rol No. 9042)
(Candidate)

Dr. Jitendra Kumar Saini
(Supervisor)
Department of Microbiology
Central University of Haryana
Mahendergarh-123029
Haryana, India

Dr. Gunjan Goel
(Head of the Department)
Department of Microbiology
Central University of Haryana
Mahendergarh-123029
Haryana, India

Department of Microbiology
Central University of Haryana
Mahendergarh-123031
Haryana, India

Date:

Certificate of Originality

The research work embodied in this thesis entitled **"Enhanced production of cellulosic ethanol using thermo and inhibitor tolerant yeasts"** submitted for consideration for the award of Doctorate of Philosophy in Microbiology is an original work carried out by me at the **Department of Microbiology**, **Central University of Haryana, Mahendergarh, India**. The manuscript has been subjected to plagiarism check by Turnitin software.

Hemansi

(Roll No. 9042)
(Candidate)

हरियाणा केंद्रीय विश्वविद्यालय

Central University of Haryana

(संसद के अधिनियम सं. 25(2009) के तहत स्थापित)

(Established vide Act no. 25 (2009) of Parliament)

जांट-पाली, महेंद्रगढ़(हरियाणा)-१२३०३१

Jant-Pali, Mahendergarh (Haryana)-123031

No./CUH/2021/LIB./1620 Date: 25/10/2021

To

The Head

Department of Microbiology

Central University of Haryana

Mahendergarh-123031 (Haryana)

Plagiarism Analysis Report

1. Please find enclosed herewith plagiarism analysis report as given below for your reference & further necessary action.

(a) Name of the Researcher : **Hemansi**

Name of the Course : Ph.D

Name of the Department : Microbiology

Class Roll No : 9042

Name of the Supervisor : Dr. Jitendra Saini (Assistant Professor)

Title of the Thesis : **Enhanced Production of Cellulosic Ethanol using Thermo and Inhibitor Tolerant Yeasts**

(b) Reference

S.N.	Software	Submission ID	Date	Word Count	Similarity Index
1.	Turnitin	1683293902	25/10/21	37285	01%

2. A report generated by http://turnitin.com is attached herewith.

25/10/2021

University Librarian

University Librarian
Central Library
Central University of Haryana
Mahendergarh 123031 (Hr.)

Copy to:-

(i) Controller of Examinations, CUH

(ii) Dr. Gunjan Goel, Associate Professor & Head, Department of Microbiology, CUH

(iii) Dr. Jitendra Saini, Assistant Professor, Department of Microbiology, CUH

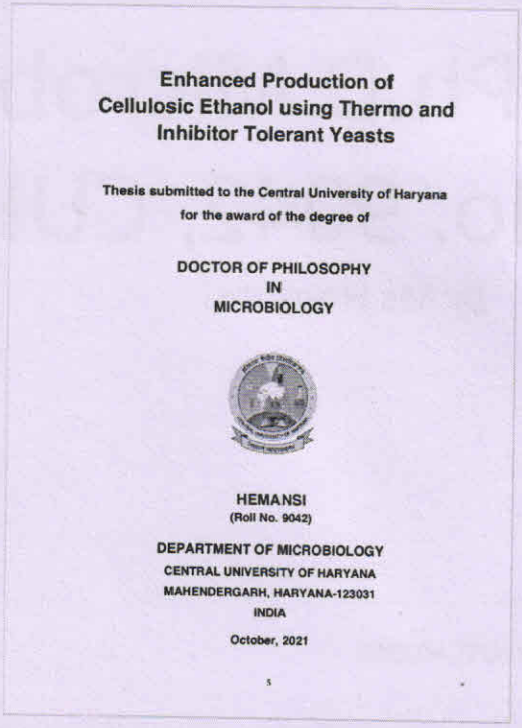
turnitin

Digital Receipt

This receipt acknowledges that Turnitin received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

Submission author:	Ms Hemansi
Assignment title:	Microbiology
Submission title:	Enhanced Production of Cellulosic Ethanol using Thermo an...
File name:	rant_Yeasts_Hemansi,_Ph.D_Microbiology,_Roll_No._9042,_CU...
File size:	25.94M
Page count:	178
Word count:	37,285
Character count:	213,552
Submission date:	25-Oct-2021 10:46AM (UTC+0530)
Submission ID:	1683293902



Enhanced Production of Cellulosic Ethanol using Thermo and Inhibitor Tolerant Yeasts

Thesis submitted to the Central University of Haryana
for the award of the degree of

DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY

HEMANSI
(Roll No. 9042)

DEPARTMENT OF MICROBIOLOGY
CENTRAL UNIVERSITY OF HARYANA
MAHENDERGARH, HARYANA-123031
INDIA

October, 2021

Assistant Librarian
Central Library
Central University of Haryana
Mahendergarh-123029(H.R.)

ACKNOWLEDGEMENT

First and foremost, I would like to express my heartfelt gratitude towards my supervisor Dr. Jitendra Kumar Saini, who has been the guiding light in my journey to achieve this goal. It has been a privilege to have worked under his supervision. I owe an enormous debt of thanks to my supervisor whose gimlet eye turned the first draft of my thesis finally into this far better thesis. His insightful comments significantly improved my thesis and prevented me from making a public spectacle of myself. I thank him for his time and attention when the world was demanding so much from each one of us.

I am extremely grateful to Hon. Vice Chancellor, Central University of Haryana for providing adequate facilities during the course of research work. I would also acknowledge University of Delhi and Bhabha Atomic Research Center (BARC) for providing instrumentation facility for my research.

Special thanks to the support, encouragement and advice extended by Dr. Gunjan Goel, Head of Microbiology Department, Central University of Haryana. I thank Dr. Surender Singh, Dr. Puja, Dr. Manoj Dr. Avijit and Dr. Vinod for providing invaluable research, expertise and feedback.

A special note of thanks goes to Dr. Eiteman, University of Georgia, USA for sharing his invaluable research expertise and new research techniques which helped me extend the domains of my field of study in such short time. Working with him has been a wonderful learning experience in my Ph.D. journey. I am grateful to have found great friends in my colleagues at University of Georgia, USA. Hemshikha, Sarada and Rashmi, thank you for your unending support and making our time together memorable.

I must place on records my gratitude towards Dr. Kavish Kumar Jain, for his wholehearted help and support. His immense scientific knowledge, analytical approach and constant encouragement helped me finish the herculean task of finishing my Ph.D.

I wish to genuinely thank my colleagues, Vikram, Amanjot, Prakriti, Aayush and Himanshu, for their constant support and generous help whenever I needed it.

A special note of thanks goes to my friends Rajkamal Vibhuti and Shalini and Tathagat with whom I shared most of the memories of my stay in Mahendergarh.

Last but not the least I would like to express my deepest gratitude to my parents my sister and my brother (Ram) for their unwavering support, as well as their willingness to let me leave home for so long, cannot go without mention. I am grateful for everything they have done and survived all this with their sense of humour and their love intact.

A sweet thanks to my extended family in Mahendergarh, Dr. Vikas Mohan, Mrs. Harshika, Mrs. Reetu Saini and Advik Saini for cheering me up always in their cute little ways.

Hemansi

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure 4.9: Effect of acetic acid (a), furfural (b), vanillin (c) individually and in combination (d) on the growth of *Pichia kudriavzevii* JKH1 at 42°C for 24 h.
Longer lag phase than the control was considered as inhibition
Figure 4.10 : Effect of different concentrations of various inhibitors on the growth of *P. kudriavzevii* JKH1 on YP agar plates. AA:F:V (1+1+1[Cocktail I]; 2+2+2 [Cocktail II]; 3+3+3[Cocktail III]; 4+4+4 [Cocktail IV]; 1+0.1+0.1 [Cocktail V], 2+0.2+0.2 [Cocktail VI]; 3+0.3+0.3 [Cocktail VII]; 4+0.4+0.4 [Cocktail VIII]; 5+0.5+0.5 [Cocktail IX])

Figure 4.11: Effect of acetic acid (a), furfural (b), vanillin (c) individually and in combination (d) on the growth of *Kluyveromyces marxianus* JKH5 at 42°C. Longer lag phase than the control was considered as inhibition.

Figure 4.12: Effect of different concentrations of various inhibitors on the growth of *K. marxianus* JKH5 in YPD agar plates. AA:F:V (1+1+1[Cocktail I]; 2+2+2 [Cocktail II]; 3+3+3[Cocktail III]; 4+4+4 [Cocktail IV]; 1+0.1+0.1 [Cocktail V], 2+0.2+0.2 [Cocktail VI]; 3+0.3+0.3 [Cocktail VII]; 4+0.4+0.4 [Cocktail VIII]; 5+0.5+0.5 [Cocktail IX])

Figure 4.13: Comparison of growth profile of parent and adapted yeast strain *Pichia kudriavzevii* strain on medium supplemented with inhibitors, AA: acetic acid (6 g/L) (a), F: furfural (3.2 g/L) (b), V: vanillin (2.8 g/L) (c), and cocktail: (acetic acid+furfural+vanillin) (3+1+1 g/L) (d)

Figure 4.14 Comparison of growth profile of parent and adapted yeast strains of *Kluyveromyces marxianus* on medium supplemented with inhibitors, AA: acetic acid (6 g/L) (a), F: furfural (3.2 g/L) (b), V: vanillin (3 g/L) (c), and cocktail: (acetic acid+furfural+vanillin) (3+1+1 g/L) (d)

Figure 4.15: Effect of different glucose concentrations on growth of the adapted yeast *K. marxianus* JKH5 C60

Figure 4.16: Effect of different temperatures on growth of the adapted yeast *K. marxianus* JKH5 C60

Figure 4.17 Effect of ethanol supplementation on growth of *K. marxianus* JKH5 C60

Figure 4.18: Response surface plots of Box-Behnken design for optimization of sequential pretreatment of SCB showing influence of sulphuric acid and temperature (a); temperature and solid loading (b); sulphuric acid and solid loading (c)); sulphuric acid and time (d); temperature and time (e); time and solid loading (f) on the response sugar yield

Figure 4.19 Response surface plots of Box-Behnken design for optimization of sequential pretreatment of SCB showing influence of sulphuric acid and temperature (a); temperature and solid loading (b); sulphuric acid and solid loading (c); sulphuric acid and time (d); temperature and time (e); time and solid loading (f) for the response cellulose content

Figure 4.20: Three dimensional response surface plot showing influence of alkali concentration and solid loading on the response cellulose content for optimization of sequential pretreatment of sugarcane bagasse through D-optimal design

Figure 4.21: FTIR spectra of untreated and pretreated sugarcane bagasse

feeding strategy. Trial D, E and F were conducted at different solid loadings 18, 20 and 30 %, respectively

Figure 4.37: Fed-batch simultaneous saccharification and fermentation of sequentially pretreated sugarcane bagasse by adapted yeast *K. marxianus* JKH5 C60 under shake flask in presence and absence of inhibitory compounds. The solid loading was 20% (dry wt.) and 20 FPU/g enzyme dosage were employed

Figure 4.38: Fed-batch simultaneous saccharification and fermentation of pretreated sugarcane bagasse in the presence of inhibitors by *K. marxianus* JKH5 C60 at bench-top fermenter. The solid loading was 20% (dry wt.) and 20 FPU/g enzyme dosage were employed

Figure 4.39: Fermentation of acid hydrolysate by *Pichia stipitis* NCIM 3499 for ethanol production under shake flask

Figure 4.40: Mass Balance for bioconversion of sequential dilute acid-pretreated sugarcane bagasse to ethanol

ABSTRACT

A major challenge during bioethanol production is the generation of inhibitory compounds during pretreatment of lignocellulosic biomass, which decreases overall ethanol yield and productivity by affecting growth of the fermenting microorganism when using unwashed biomass. Therefore, the major aim of this study was to develop robust bioethanol-producing yeast having superior tolerance to multiple inhibitors. After screening 150 indigenous yeasts, a new robust thermotolerant ethanol fermenting yeast *Kluyveromyces marxianus* JKH5 was selected. It was improved further via adaptive laboratory evolution (ALE) to increase its tolerance to higher concentrations of inhibitory compounds. The developed yeast *K. marxianus* JKH5 C60 had a 3.3-fold higher specific growth rate and 56% reduced lag phase in comparison to the parent strain in presence of inhibitor cocktail containing acetic acid 6 g/L, furfural 3.2 g/L and vanillin 3 g/L. The fermentation efficiency in presence of inhibitors with glucose (100 g/L) was enhanced by 80%, with an ethanol titer of 24.8±1.0 g/L. Further, sequential dilute acid-alkali pretreatment of sugarcane bagasse (SCB) was optimized to reduce its recalcitrance by using Box-Behnken and D-optimal designs. Optimised pretreatment conditions were: H_2SO_4 and NaOH concentrations of 3% (v/v) and 5% (w/v), SCB solid loadings (SLs) of 18 and 15% (w/w), pretreatment time and temperature of 30 min and 121 °C, respectively. The effectiveness of sequential pretreatment was supported by increased cellulose content (83%), drop in hemicellulose (92%), 97.2% removal of lignin, as well as favourable ultrastructural changes in pretreated SCB as confirmed by FT-IR, SEM, EDX, TGA, XRD and SANS analyses. Enzymatic hydrolysis of pretreated bagasse at SL of 15% (w/w) by commercial cellulase (enzyme dose of 20 FPU/gds) resulted in maximum reducing sugar production of 124.8±0.9 g/L. Further, separate hydrolysis and fermentation of sugars by *K. marxianus* JKH5 C60 resulted in ethanol production of 54.9± 1.2 g/L. The detoxified and neutralized pentose-rich (23 g/L) dilute acid hydrolysate was fermented to ethanol (6.8±0.07 g/L) using *Pichia stiptis* NCIM 3499. Batch and fed-batch strategies for simultaneous saccharification and fermentation (SSF) of pretreated bagasse were also optimized at shake flasks and successfully scaled up to 3L lab-scale fermenter. Fed-batch SSF with intermittent feeding of SCB biomass and enzyme emerged as a superior strategy for enhanced bioethanol production at 42 °C, with comparable ethanol production in the presence and absence of inhibitors. Under optimized lab-scale fed-batch SSF at a high solid loading of SCB (dry SL = 20%), the ethanol titer and productivities by the developed yeast (in presence of inhibitors), were 73.4±1.2 g/L and 3.0 g/L, respectively. Mass balance analysis of the whole process of conversion of SCB to bioethanol, using the adapted yeast strain, indicated overall ethanol production of 260.1 kg per tonne of native sugarcane bagasse. Thus, the yeast developed in this study can make bioethanol production process more cost-effective by producing high ethanol titer within a shorter duration, and by decreasing wastewater generation by eliminating the need to wash the pretreated biomass prior to fermentation.

Keywords: Lignocellulose; Sugarcane bagasse; Sequential pretreatment; Small-angle neutron scattering (SANS); Enzymatic saccharification; Bioethanol

सार

बायोएथेनॉल उत्पादन के दौरान एक बड़ी चुनौती लिग्नोसेल्यूलोसिक बायोमास के प्रीट्रीटमेंट के दौरान निरोधात्मक यौगिकों का निर्माण है, जो बिना धुले बायोमास का उपयोग करते समय किण्वित सूक्ष्मजीव के विकास को प्रभावित करके समग्र इथेनॉल उपज और उत्पादकता को कम करता है। इसलिए, इस अध्ययन का प्रमुख उद्देश्य एक मजबूत बायोएथेनॉल-उत्पादक yeast विकसित करना था जिसमें कई अवरोधकों के लिए बेहतर सहनशीलता हो। 150 स्वदेशी यीस्ट की जांच के बाद, एक नया थर्मोटोलरेंट इथेनॉल किण्वन yeast *K. marxianus* JKH5 का चयन किया गया। निरोधात्मक यौगिकों की उच्च सांद्रता के प्रति इसकी सहनशीलता बढ़ाने के लिए अनुकूली प्रयोगशाला विकास (एएलई) के माध्यम से इसे और बेहतर बनाया गया था। विकसित यीस्ट *K. marxianus* JKH5 C60 में एसिटिक एसिड 6 g/L, फुरफुरल 3.2 g/L और वैनिलिन 3 g/L युक्त इनहिबिटर कॉकटेल की उपस्थिति में पैरेंट स्ट्रेन की तुलना में 3.3 गुना अधिक विशिष्ट विकास दर और 56% कम अंतराल चरण था। ग्लूकोज (100 g/L) के साथ अवरोधकों की उपस्थिति में किण्वन दक्षता को 24.8±1.0 g/L के इथेनॉल टिटर के साथ 80% तक बढ़ाया गया था। इसके अलावा, गन्ना खोई (एससीबी) के अनुक्रमिक तनु अम्ल-क्षार प्रीट्रीटमेंट को Box-Behnken and D-optimal डिजाइनों का उपयोग करके इसकी पुनर्गणना को कम करने के लिए अनुकूलित किया गया था। अनुकूलित प्रीट्रीटमेंट स्थितियां थीं: H_2SO_4 और NaOH सांद्रता 3% (v/v) और 5% (w/v), SCB सॉलिड लोडिंग (SL) 18 और 15% (w/w), प्रीट्रीटमेंट समय और 30 मिनट का तापमान और 121°C, क्रमशः। अनुक्रमिक प्रीट्रीटमेंट की प्रभावशीलता बढ़ी हुई सेल्यूलोज सामग्री (83%), हेमिसेल्यूलोज में गिरावट (92%), लिग्निन को 97.2 % हटाने, साथ ही एफटी-आईआर, एसईएम द्वारा पुष्टि के रूप में प्रीट्रीटेड एससीबी में अनुकूल अवसंरचनात्मक परिवर्तनों द्वारा समर्थित थी। EDX, TGA, XRD और SANS विश्लेषण करते हैं। वाणिज्यिक सेल्युलेस (20 FPU/gds की एंजाइम खुराक) द्वारा 15% (w/w) के SL पर प्रीट्रीटेड बैगैस के एंजाइमैटिक हाइड्रोलिसिस के परिणामस्वरूप अधिकतम चीनी उत्पादन 124.8 ± 0.9 g/L कम हो गया। इसके अलावा, *K. marxianus* JKH5 C60 द्वारा अलग हाइड्रोलिसिस और शर्करा के किण्वन के परिणामस्वरूप 54.9± 1.2 g/L का इथेनॉल उत्पादन हुआ। डिटॉक्सिफाइड और न्यूट्रलाइज्ड पेंटोस-रिच (23 g/L) डाइल्यूट एसिड हाइड्रोलाइजेट को *Pichia stipitis* NCIM 3499 का उपयोग करके इथेनॉल (6.8±0.07 g/L) में किण्वित किया गया था। प्रीट्रीटेड खोई के एक साथ सैक्रिफिकेशन और किण्वन (SSF) के लिए बैच और फेड-बैच रणनीतियों को भी शेक फ्लास्क में अनुकूलित किया गया था और सफलतापूर्वक 3L लैब-स्केल किण्वक तक बढ़ाया गया था। एससीबी बायोमास और एंजाइम की आंतरायिक फीडिंग के साथ फेड-बैच एसएसएफ 42 °C पर बायोएथेनॉल उत्पादन को बढ़ाने के लिए एक बेहतर रणनीति के रूप में उभरा, जिसमें अवरोधकों की उपस्थिति और अनुपस्थिति में समान / तुलनीय इथेनॉल उत्पादन होता है। एससीबी (dry SL = 20%) के उच्च ठोस लोडिंग पर अनुकूलित लैब-स्केल फेड-बैच एसएसएफ के तहत, विकसित yeast (अवरोधकों की उपस्थिति में) द्वारा इथेनॉल टिटर और उत्पादकता 73.4±1.2 g/L g/L और 3.0 g/L/h), क्रमशः। अनुकूलित यीस्ट स्ट्रेन का उपयोग करते हुए एससीबी को बायोएथेनॉल में बदलने की पूरी प्रक्रिया का सामूहिक संतुलन विश्लेषण, 260.1 kg per tonne देशी गन्ना खोई के समग्र इथेनॉल उत्पादन को दर्शाता है। इस प्रकार, इस अध्ययन में विकसित yeast बायोएथेनॉल उत्पादन प्रक्रिया को कम अवधि के भीतर उच्च इथेनॉल टिटर का उत्पादन करके और किण्वन से पहले प्रीट्रीटेड बायोमास को धोने की आवश्यकता को समाप्त करके अपशिष्ट जल उत्पादन को कम करके अधिक लागत प्रभावी बना सकता है।

कीवर्ड: *लिग्नोसेल्यूलोज; गन्ने की खोई; अनुक्रमिक दिखावा; स्मॉल-एंगल न्यूट्रॉन स्कैटरिंग (SANS); एंजाइमेटिक सैक्रिफिकेशन; बायोएथेनॉल*

INTRODUCTION

INTRODUCTION

Current scenario of declining fossil fuel reserves and environmental deterioration due to GHG emissions, soaring petroleum prices, concerns over the national energy security and dependence on oil-import have led researchers all over the world to search for alternative transportation fuels termed 'biofuels'. Biofuels, such as bioethanol, can be produced sustainably from biomass resources. Lower cost, and surplus availability of lignocellulosic biomass (LCB), has made it the most appropriate and sustainable feedstock for generating ethanol and other value-added materials via biochemical conversion route (Saini et al., 2015b). Some of the globally abundant LCBs are rice straw, wheat straw and sugarcane bagasse (SCB). LCB, the renewable resource for the production of cellulosic ethanol (Ragauskas et al., 2006), is mainly comprised of cellulose, hemicellulose, and lignin (Kuhad et al., 1997). The total LCB production in India alone exceeds 680 metric ton per annum which accounts for production of approximately 52 billion litres of bioethanol (Jain & Agrawal, 2018). In the year 2017-18, 1500 million litres of ethanol was produced, which could be used for only 5% blending in gasoline (E5). There is still a shortage of 1,100 million litres of ethanol for achieving the mandate of 10% blending (E10). Sugarcane is one of the highly produced crops in India, having annual production of 376 metric ton during 2019-2020 (http://www.fao.org/faostat). Sugar industries generates huge amount of bagasse as a by-product, which can be utilised to overcome the shortage in supply of bioethanol.

The biochemical conversion route involves four major unit operations, including pretreatement, hydrolysis, fermentation and ethanol recovery. The pretreatment process is vital for partial or complete removal of lignin and hemicellulose, reduction in cellulose crystallinity and increasing the porosity of the biomass. Pretreatment makes the cellulose amenable to cellulase enzymes during enzymatic hydrolysis. The resultant hydrolysate

containing monomeric sugars is fermented to ethanol by fermenting microbes. Hydrolysis is a critical step during which cellulolytic enzymes are used to convert complex carbohydrates of biomass into fermentable sugars. Sugars released during hydrolysis can be fermented into ethanol using various fermenting microorganisms via separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF). SHF is employed more commonly by using mesophilic yeast *Saccharomyces cerevisiae* operating at 28-30 °C. However, the major demerit of SHF process is accumulation of monomeric sugars which consequently inhibits cellulases and leads to poor yield of sugar. In contrast, SSF process uses the same reactor for biomass hydrolysis and ethanol production, which improves the process economics by decreasing overall process time. Moreover, obtaining high ethanol concentration is a major challenge at industrial level, as it reduces the energy consumption and associated costs during the recovery of ethanol by distillation. Higher ethanol yields require higher loading of biomass during fermentation. However, high solids cause mixing-problem due to high viscosity and reduce biomass conversion significantly. Therefore, a fed-batch approach for feeding of biomass during SSF is often employed. Fed-batch SSF not only eliminates the technical challenge of mixing and mass transfer, but also reduces end-product inhibition, increases dissolved oxygen and saccharification rate and overall ethanol productivity. The problem of contamination during the fermentation process can be tackled by using a thermotolerant fermenting microorganism capable to ferment at comparatively higher temperature, thereby, eliminating the growth of many mesophilic contaminating microbes.

Another major challenge during bioethanol production is the generation of degradation products during thermochemical pretreatment of biomass (Kang et al., 2014). The LCB-derived inhibitors majorly include furans and its derivatives, phenolics, and weak acids like acetic acid, carboxylic acid, etc. (Wang et al., 2018). Use of pretreatment slurry or the

unwashed biomass during fermentation inhibits fermenting microbes, thereby, reducing ethanol yield and productivity (Wang et al., 2018). Therefore, washing of the pretreated biomass is often needed to remove these inhibitors prior to fermentation, generating large amounts of waste-waters (Lyu et al., 2017). Chemical detoxification is also not a cost-effective method due sugar loss and increased operational time (Lin et al., 2020; López et al., 2004; Shibuya et al., 2017a). As an alternate approach, the fermenting microbes can be potentially improved to tolerate to inhibitors via strain improvement strategies. Genetic engineering based approaches of strain improvement require specific knowledge about underlying principles of tolerance and the target genes or their metabolic functions which makes its application difficult (Wang et al., 2018). Therefore, non-targeted and less complex strain improvement strategy is needed. One such approach for increasing the tolerance of fermenting microorganisms toward multiple inhibitors could be 'adaptive laboratory evolution (ALE)' which relies on accumulation of spontaneous mutations, generation after generation under constant selection pressure (Qin et al., 2016).

In order to address the above mentioned research gaps in cost-effective bioethanol production, the current study hypothesised the following points:

- Can we develop a robust strain of yeast which tolerates multiple stresses?
- Can we enhance production of cellulosic ethanol using the adapted yeast?
- Can we improve the efficiency of the ethanol production by adopting fed-batch fermentation?

Therefore, considering the need to develop a robust yeast cell factory for cost-effective production of cellulosic ethanol, the present study focused on the following objectives:

1. Screening and selection of thermo- and inhibitor tolerant yeast strain(s) for utilizing lignocellulosic wastes for bioethanol production.
2. Enhancing tolerance of potential yeast strain(s) against temperature and inhibitors stress through adaptive laboratory evolution.
3. Optimization of bio-process for maximum bioethanol production from lignocellulosic sugarcane bagasse at shake-flask level using developed yeast strain.
4. Improvisation and scale-up of bio-process for high gravity simultaneous saccharification and fermentation of sugarcane bagasse at bench-scale fermenter.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1. Bioethanol as a renewable fuel

Global concerns over energy security and climate change have necessitated the application of non-conventional resources such as lignocellulosic biomass for generating energy and fuel. The fuel generated from plant or algae biomass is called biofuel, such as bioethanol and biodiesel. Lower cost, surplus availability and renewable nature has made lignocellulosic biomass (LCB) the most appropriate and sustainable feedstock for generating biofuel and other value-added material (Aditiya et al., 2016). Some of the common examples of the globally abundant LCBs are rice straw, wheat straw and sugarcane bagasse (SCB). The total LCB production in India alone exceeds 680 metric ton per annum which accounts for production of approximately 52 billion liters of bioethanol (Jain & Agrawal, 2018). As 40% of the world's energy consumption is in the form of the liquid fuels (Tan et al., 2008), bioethanol has been considered as an alternative to supplement conventional fuels. Bioethanol is a promising renewable and an alternate source of energy produced from lignocellulosic biomass residue and is a sustainable solution to the problems of diminishing petroleum reserves, issues over national security and environmental deterioration due to GHG emissions. Unlike gasoline, bioethanol is an eco-friendly fuel and causes 90% reduction in greenhouse gas emissions compared to petroleum (Sánchez & Cardona, 2008).

Bioethanol is used as a transport fuel, mainly as biofuel additive for gasoline. One overwhelming advantage of bioethanol for the environment is its potential to be carbon neutral on a lifecycle basis – means carbon dioxide (CO_2) emitted during its use is offset by the absorption from the atmosphere during its growth (Sánchez & Cardona, 2008). Ethanol has a higher octane number (113) than petrol (87-93) (https://ethanolrfa.org/) as result pre-ignition does not occur when ethanol is used (Agarwal, 2007; Balat et al., 2008). It improves

the efficiency of engines when compared to petroleum and this enhances its appropriateness for use in advanced vehicles' engines (Balat et al., 2008; Masum et al., 2013).

Ethanol is burnt completely so that hydrocarbon and carbon monoxide emission is drastically lower as compared to petrol. Furthermore, extraction of fossil fuels is hazardous to environment and public health. Ethanol can be mixed with petrol for up to 10-15% (E10 or E15) without alterations to conventional motor engines (Moriarty & Yanowitz, 2015). Countries like United States and Brazil contribute major fraction in world's bioethanol production. The practice of blending ethanol (E5) in India started in 2001. Currently, there is blending of 10% (E10) in petrol. Ministry of petroleum and natural gas, government of India has mandate for increase the blending of ethanol to 20 % (E20) by 2030. Apart from its environmental benefits, the use of bioethanol as a fuel also has economic benefits like creating new jobs, supporting agrarian economy and helps meet the energy needs of developing countries (Quintero et al., 2013).

2.2. Lignocellulosic biomass as feedstock for second generation bioethanol

Lignocellulose is a renewable organic material and is the most promising feedstock considering its great availability, low cost and non-competence with the food demands. It is a natural complex of three biopolymers: cellulose (polymer of α-d-glucose), hemicellulose (heteropolymer of C5 and C6 sugars) and lignin (heteropolymer of phenylpropanoid units) (Juturu & Wu, 2014; Kuhad et al., 1997; Singhania et al., 2009). Numerous lignocellulosic biomasses can be successfully utilized for producing bioethanol. Some of them with their compositions are listed in Table 2.1.In general, the lignocellulosic biomass contains 40-45 % cellulose, 20 to 35% hemicelluloses and 5 to 30% lignin. In addition, small amounts of other materials such as ash, proteins and pectin can be found in lignocellulosic residues, in varied proportion based on the source (Menon & Rao, 2012). 2.9 $\times 10^3$ million tons of lignocellulosic residues are available that are waste products of cereal crops while waste from

pulses and oil crops amounts to be around 3×10^3 million tons. Similarly, plantation also generates waste that generates almost 5.4×10^2 million tons of lignocellulosic waste annually (Saini et al., 2015b; Singhania et al., 2009). The different types of lignocellulosic feedstock commonly used for ethanol production are sugarcane bagasse, wheat straw, rice straw, cotton stalk, rice husks, wheat straw, cotton stalks, corncob, coconut shells and municipal solid waste (MSW), forestry waste counting bark and wood chips. Few prominently used lignocellulosic feedstock are shown in Figure 2.1. The availability of lignocellulosic biomass in Indian context is shown in Figure 2.2.

Sugarcane bagasse (SCB) is one of the largest agricultural remnants in the world and according to the Indian state, it produces 100 million tons per year annually from 600 sugar mills in India (Konde et al., 2021). Since SCB mainly consists of cellulose, hemicellulose, and lignin, it can easily be utilized for generating bioethanol after pretreatment.

Therefore, the efficient use of residual bagasse requires immediate attention from the sugar industry and the scientific community around the world. While newly developed technologies have demonstrated promising prospects for the sustainable transformation of SCB into ethanol and additive chemicals, there is a lack of consensus among the scientific community on technological understanding and commercial use of current SCB transformation technologies (Gao et al., 2018).

Industries and research laboratories in India are working on the development of technology to produce bioethanol from lignocellulosic biomass. Significantly, India is the fourth largest producer of ethanol; therefore, the use of SCB for ethanol production will improve this industry. Therefore, recent efforts led by the Indian government led to the possible growth of SCB technology into a commercial level unit. However, to achieve this goal, Indian industries will need to regard the SCB as a resource for residual waste (Jain and Aggarwal 2018).

Figure 2.1: Different lignocellulosic feedstock used worldwide for bioethanol production. (a-Sugarcane bagasse, b-Wheat Straw, c-Rice Straw, d-Cotton stalk, e-*Prosopis juliflora*, f-*Lantana camara*, g-Willow, h-*Gracillaria verrucosa*)

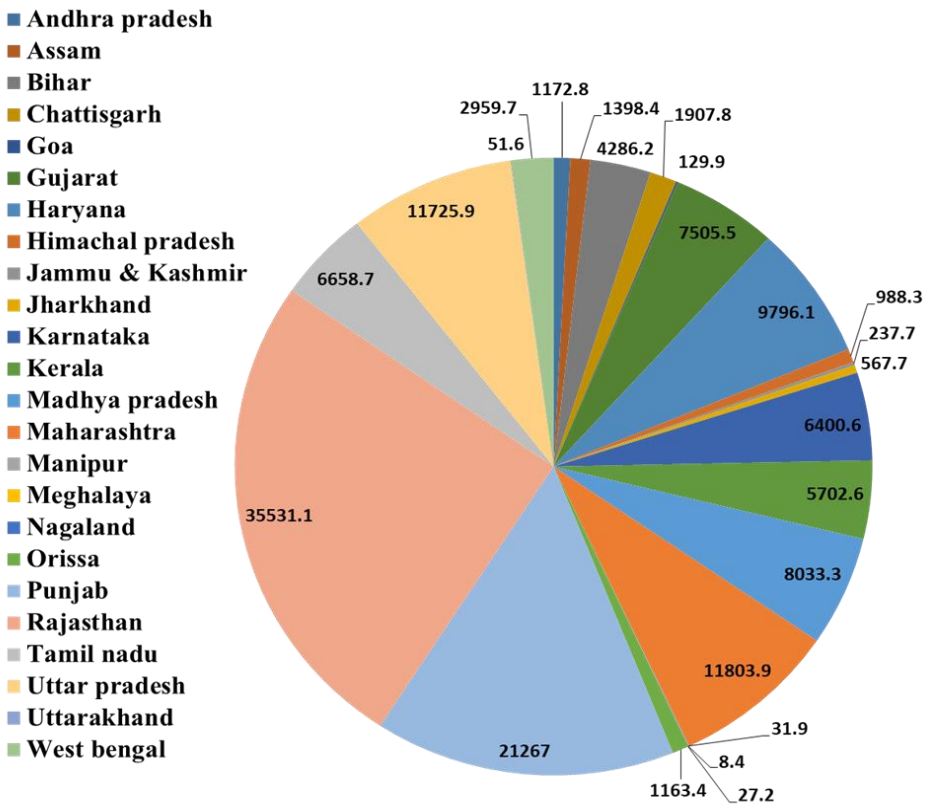


Figure 2.2: Availability of lignocellulosic biomass (kilo ton per year) in India (Hemansi et al., 2019)

2.3. Second generation bioethanol process

Second generation bioethanol is derived from lignocellulosic resources in contrast to first generation bioethanol derived from sugar or starch based resources. The conversion of lignocellulosic biomass to bioethanol is a multi-step process. The structural carbohydrate polymers in lignocellulose, i.e. cellulose and hemicellulose are first depolymerized through pretreatment and saccharification and the obtained monomeric sugars are subsequently fermented to ethanol. Lignocellulose conversion to bioethanol can be carried out in various manners, such as by employing biochemical/microbial/enzymatic route (Kang et al., 2014).

The biochemical conversion route of lignocellulosic biomass to ethanol is milder and environmental friendly and broadly comprises four sequential steps: deconstruction of biomass (pretreatment), saccharification, conversion of sugar to ethanol (fermentation) and purification of the product (Figure 2.3) (Kuhad et al., 1997; Lynd et al., 1999).

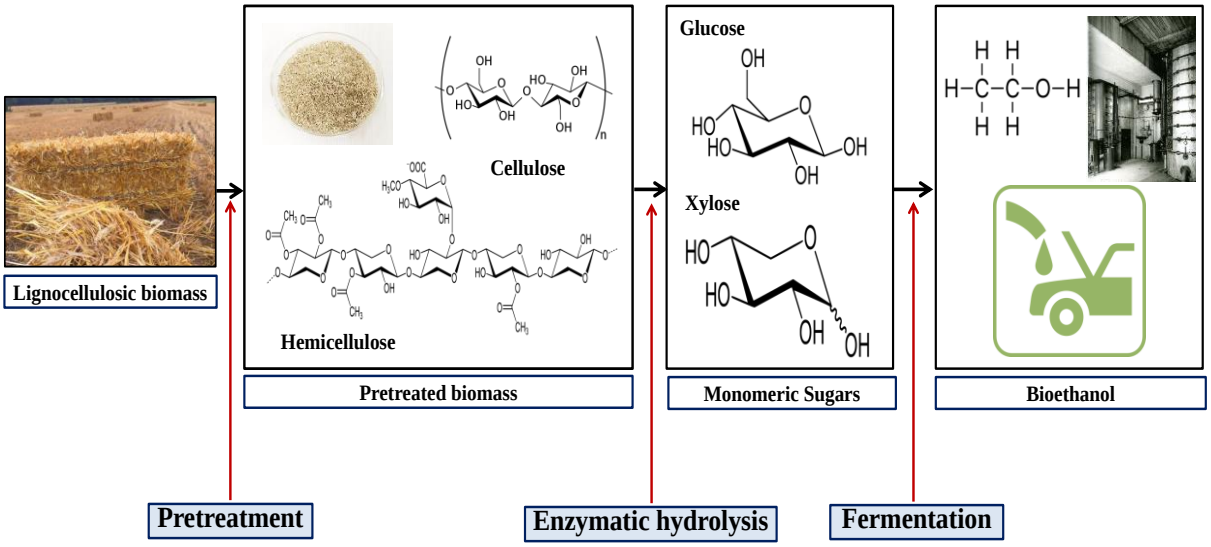


Figure 2.3: Schematic illustration of process of conversion of lignocellulosic biomass to bioethanol

Table 2.1: Composition of various lignocellulosic biomass used for bioethanol production (Hemansi et al., 2018)

Substrate	% composition (dry wt.)			Substrate	% composition (dry wt.)		
	Hexosans	**Pentosans**	**Lignin**		**Hexosans**	**Pentosans**	**Lignin**
Bamboo	49-50	18-20	23	Oat straw	41	16	11
Banana waste	13.2	14.8	14	Olive tree waste	25.2	15.8	19.1
Barley hull	34	36	19.3	Paper	85-99	0-5	0-15
Barley pulp	69.9	18.3	10.9	Pepper stalks	35.7	26.2	18.3
Bean stalks	31.1	26.0	16.7	Pine	41	10	27
Bermuda grass	25	35.7	6.4	Poplar	40	14	20
Birch wood	40	33	21	Reed	49.40	31.50	8.74
Chilli stalks	37.5	28.3	17.3	Rice husk	36	15	19
Coffee pulp	33.7-36.9	44.2-47.5	15.6-19.1	Rice straw	32	24	13
Corn cobs	42	39	14	Rye straw	31	25	7
Corn Stover	38	26	19	Salix	41.5	22-25	25
Cotton seed hair	80-95	5-20	0-5	Saw dust	55	14	21
Cotton stalks	41.7	27.3	18.7	Soft wood stem	45-50	25-35	25-35
Douglas fir	35-48	20-22	15-21	Sorghum straw	33	18	15
Eucalyptus	45-51	11-18	29	Soybean stalks	34	25	20
Flax sheaves	35	24	22	Spruce	45	26	28
Grapevine stems	43.1	19.4	26.6	Sugarcane bagasse	33	30	29
Grasses	25-40	35-50	10-30	Sweet sorghum	23	14	11
Groundnut shells	38	36	16	Switch grass	37	29	19
Hemp	53.86	10.60	8.76	Waste paper	60-70	10-20	5-10
Jute fibers	45-53	18-21	21-26	Water hyacinth	18.4	49.2	-
Miscanthus	43	24	19	Wheat straw	30	24	18
Municipal solids	8-15	NA	24-29	Willow	55.9	14	19

2.3.1. Pretreatment of lignocellulosic biomass

Production of bioethanol from second generation biomass requires efficient depolymerization of structural carbohydrate polymers to be fermented to ethanol. However, lignocellulosic biomass has evolved complex structural and chemical mechanisms, which provide recalcitrance to its structural sugars from the microbial and enzymatic attack. Therefore, a deconstruction of biomass is required to change the biomass size and structure as well as chemical composition so that hydrolysis of the carbohydrate portion to monomeric sugars can be attained rapidly with higher yields. The main aims of pretreatment are as follows:

(1) To improve sugar yields during enzymatic hydrolysis by reduction of crystallinity of cellulose and enhanced porosity of the biomass;

(2) To minimize the emergence of fermentation inhibitors during deconstruction;

(3) To retrieve lignin from hydrolysate for converting it into valuable by-products and

(4) To make the process economic by making the operation easier (Aditiya et al., 2016).

Broadly, pretreatment strategies are categorized into physical, physico-chemical, chemical and biological. With every different feedstock used for bioethanol production, the selection of pretreatment method varies due to distinct chemical composition and physical structure of feedstock. Factors like cellulose crystallinity, lignin content, cell wall porosity, hemicellulose side chain branching and crosslinking are critical in choosing the pretreatment method. Most chemical pretreatment modifies cellulose ultrastructure through certain physico-chemical modification, though it is possible to fractionate cellulose, hemicellulose and lignin by using pretreatment with some catalysts. A list of common pretreatment strategies used and their advantages and disadvantages are shown in Table 2.2.

Table 2.2: Various pretreatment strategies with their specifications (Hemansi et al., 2018)

Pretreatment	**Action**	**Advantages**	**Disadvantages**
Dilute Acid	Hydrolyses hemicelluloses, Alters lignin structure	Hemicellulosic removal	Low removal of lignin, Low enzymatic hydrolysis (30-40%), Inhibitor generation
Dilute Alkali	Eliminates lignin and hemicelluloses, Enhances surface area exposure for enzyme access	High digestibility, high lignin removal	Hemicellulosic sugar loss, low enzymatic hydrolysis (50-60%), Inhibitor generation
Ammonia fibre expansion (AFEX)	surface area for access to enzyme upsurges after treatment, removes out hemicellulose and lignin	Small amount of inhibitors formation	Not proficient for biomass with high level of lignin, high price of ammonia
Ionic liquid	Decreases cellulose crystallinity, removes lignin	High dissolution, green solvents	applications at large-scale are under exploration
Alkaline peroxide	Removes lignin and solubilizse most of the hemicellulose	Cellulose isolation	Loss of hemicellulosic sugars, loss of lignin
Acid-chlorite	Reduces lignin content	Isolation of hemicellulose and cellulose	Loss of lignin, Costly method of pretreatment
Ammonia	Opens up cell wall and exposes celluloses and	Lignin removal (partial)	Hemicellulosic sugar loss, Low enzymatic

	hemicelluloses		hydrolysis (50-60%), Inhibitor generation
Steam explosion	Causes lignin transformation, and causes hemicelluloses solubilisation	Deconstruction of structural polymer, recovery of lignin, lower loss of hemicellulose, less amount of inhibitors generated, higher yield of hemicellulose and, economic process	Generation of inhibitors, generation of inhibitory compounds, partial hemicellulose degradation, disrupted lignin-carbohydrate matrix is lacking
Biological	Degrades lignin and hemicellulose	Partial deconstruction of lignocellulosics, low energy consumption	Longer fermentation time

The biochemical conversion of the cellulose (or hemicellulose) requires action of lignocellulolytic enzymes, thereby releasing mono-, di- and oligo-saccharides which are further fermented by microbes to the desired product. Complete as well as economic conversion of LCBs to its constituent saccharides is still considered a major challenge due to inherent biomass recalcitrance (Agbor et al., 2011). For counteracting the recalcitrance, LCB must be deconstructed using physical-chemical processes prior to its enzymatic hydrolysis by lignocellulolytic enzymes. Pretreatment enhances accessibility of biomass components to lignocellulolytic enzymes, thereby, resulting in maximum product recovery from LCB and improved economics of lignocellulosic biorefineries (Kumar et al., 2009).

More commonly employed pretreatment methods are dilute-acid (DA), alkali (DB) and steam-explosion. Dilute sulphuric acid pretreatment results in breakdown of the rigid structure of LCB by solubilization of its hemicelluloses (Martiniano et al., 2014). DB pretreatment involves hydroxides of sodium, potassium, calcium, and ammonium and

facilitates the delignification, swelling of cellulose and partial decrystallization and solubilisation of cellulose and hemicellulose, respectively (Brodeur et al., 2011; Silverstein et al., 2007). DA pretreatment employing sodium hydroxide is more commonly applied for effective disruption and removal of lignin from LCB (Brodeur et al., 2011; Kumar et al., 2009). The pretreatment processes should enhance the relative proportion of cellulose and reduce both the hemicellulose and lignin contents of LCB for better conversion of cellulose during enzymatic hydrolysis, which is generally not possible when a single step pretreatment is used alone. Therefore, sequential acid-alkali (DAB) pretreatment has also been attempted as a better method for pretreating various lignocellulosic agro-wastes, such as rice straw (Kim et al., 2013), corn stover (Lee et al., 2015), oil palm fruit waste (Kim et al., 2012) and SCB for enhancing the sugar yield during hydrolysis (Giese et al., 2013; Isaac et al., 2018; Philippini et al., 2019). In this method, cellulignin obtained after acid pretreatment is further treated with alkali for lignin removal which in turn maximises the surface area of cellulose available for cellulase during hydrolysis for biorefining operations (Chandel et al., 2014; Keshav et al., 2016). Previous study on sequential DAB pretreatment of SCB reported a final cellulose content of 76.5% along with 20.0% lignin (Chandel et al., 2014). Even this much lignin content could still cause hindrance in economic hydrolysis of the biomass. Therefore, sequential DAB pretreatment of SCB needs further investigations. Furthermore, dilute alkali-acid pretreatment of LCBs such as cotton stalk has also been reported (Rocha et al., 2012), but the studies are only a few in the literature. Pretreatment induces macro to nano-scale structural changes in lignocellulosic plant biomass, including removal and reorganization of constituents, increased pore size and density, altered crystallinity, etc. Such structural variations in LCB are monitored by analytical tools spanning multiple length scales, such as scanning electron microscopy (SEM), Fourier transforming infrared spectroscopic (FT-IR) method, wide or small angle X-ray diffraction (XRD) method, thermo-gravimetry (TGA),

etc. Recently, small angle neutron scattering (SANS) has emerged as a powerful technique for deeper understanding of biomass recalcitrance (Pingali et al., 2017; Yuan et al., 2017), but the reports on application of SANS for lignocellulose monitoring during pretreatment are still very scarce. Further studies on pore size and distribution using SANS are crucial for better understating of the lignocellulose deconstruction, especially for the surplus crop residues like sugarcane bagasse (SCB).

2.3.2. Enzymatic hydrolysis of lignocellulosic biomass

The hydrolysis of pretreated biomass is the most crucial step in the bioethanol production process. Although hydrolysis of biomass can be accomplished by using acid or enzymes, saccharification using enzymes is preferred due to milder processing conditions and environment-friendly nature. Depolymerization of biomass via enzymatic hydrolysis is a multi-enzymatic process with high complexities. In nature, lignocellulosic biomass can be depolymerized by a number of hydrolytic enzymes that are produced by diverse fungi and bacteria. Cellulases are the representative class of enzymes involved in depolymerizing lignocellulosic substrate by synergistic action of all three enzymes present in the complex.

Cellulase complex consists of exoglucanases (cellobiohydrolases, CBH), endoglucanases (EG) and β-glucosidases (cellobiase, BG) (Behera & Ray, 2016). EG acts upon cellulose chains and hence creates two types of reactive ends for CBHs. CBH I acts on reducing ends and CBH II on non-reducing ends of cellulose fragments thereby, catalysing step wise degradation of cellulose to cellobiose. BG utilizes cellobiose and converts it into glucose (Kuhad et al., 2011). CBH gets inhibited by cellobiose, therefore; BG plays a key role in reducing end-product inhibition and depolymerizing the cellulose completely. Modular structure with concluding catalytic and carbohydrate binding molecules (CBM) is a common feature of most of cellulases. The carbohydrate binding molecules facilitate hydrolysis of biomass by fetching the catalytic domain in contiguity to the insoluble cellulose. Thus, the

rate of enzymatic hydrolysis of the biomass is subjective to the substrate properties and catalytic performance both. The scheme of mechanistic action of cellulases over cellulose is shown in Figure 2.4.

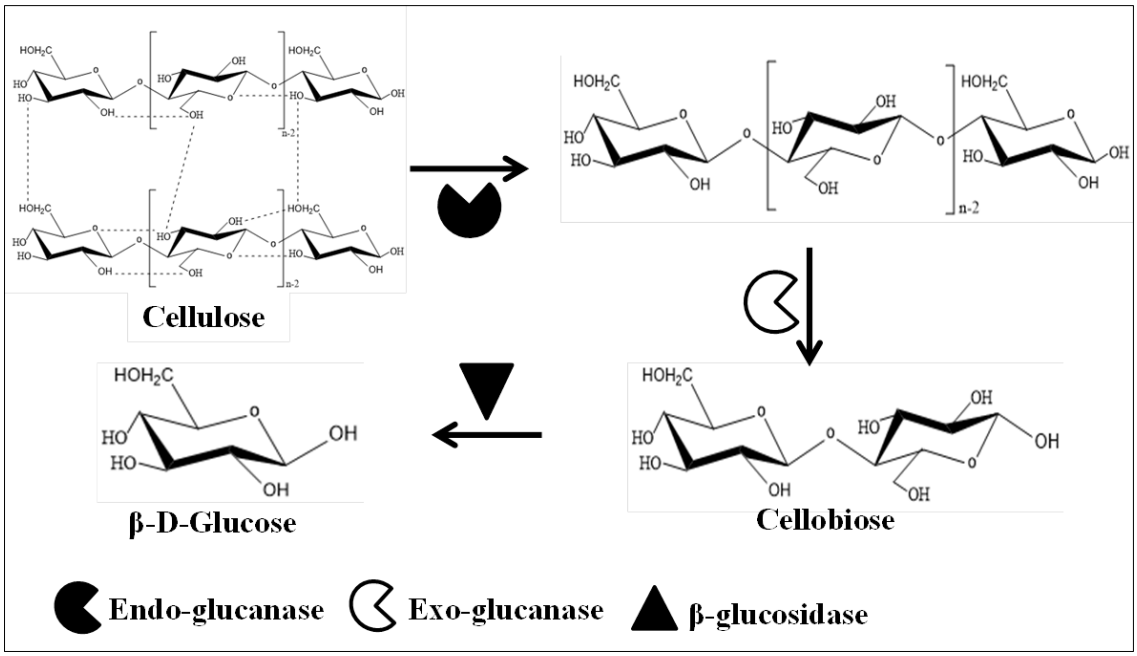


Figure 2.4: Schematic diagram showing mechanism of enzymatic hydrolysis (Hemansi et al., 2019)

Although saccharification using enzymes has more scope for improvement than those using chemicals, the high cost of cellulases is still a technical barrier (Culbertson et al., 2013; Hong et al., 2013). Fall in the cost of cellulase could be obtained by (a) intensive efforts which enquire more than a few aspects of enzymes with improved hydrolytic properties such as binding affinity, thermostability, etc. (b) by improvement of technologies for which are proficient for hydrolysis including of superior cocktails of enzyme and conditions for hydrolysis. In addition to enzyme characteristics, substrate features such as the degree of polymerization, cellulose crystallinity and the existence of lignin and hemicellulose also affect the enzymatic hydrolysis.

Therefore, to improve the overall process, upgrading in cellulase performance and enhancing the substrate-enzyme interaction are prerequisite. Industrially, among all probable strategies, the optimization of the characteristics of cellulases like thermostability and end-product

inhibition is crucial for large scale application. Also, optimizing production medium by altering its components is an approach to enhance the enzymatic hydrolysis. Development of multi-enzyme cocktail secreted by various strains of fungi is also a good choice for improving the performance of cellulase as a complete system. Several studies have reported that synergistic action of cellulase is linked with the ratio of every enzyme in the system (Berlin et al., 2007; Hemansi et al., 2018).

The constraint of cellulases is constantly increasing due to its miscellaneous applications; high demand also produces some challenges. The recalcitrant nature of lignocellulosic biomass is a major complication.

2.3.3. Fermentation

As compared to simpler fermentation process of sugars derived from food-based feedstock, crop-waste based feedstock to ethanol conversion process is very tedious and involves many critical steps. Pentose-rich sugar syrup and hexose rich sugars coming from hydrolysis of hemicellulose and cellulose, respectively, are the major substrates after initial hydrolysis that can be further fermented to produce ethanol. There are many desirable characteristics of an ideal fermenting microorganism, such as high conversion efficiency both with respect to substrate utilized and time, robustness against inhibitory compounds and ability to withstand high ethanol concentrations.

Several laboratories have established the process of utilizing pentose sugars as well as hexose sugars by various yeasts, fungi and bacteria for the production of fermentation products including alcohols. Among these, the most common and efficient glucose fermenting microbes are brewer's yeast *Saccharomyces cerevisiae* and *Zymomonas mobilis* (Hahn-Hägerdal et al., 2006), while for pentose fermentation are *Pichia stipitis* and *Candida shehatae.*

The process of ethanol production not always requires aerobic conditions. It is required only for the production of biomass (Agbogbo & Wenger, 2007). Further to enhance the ethanol production from pentose sugars, different detoxification strategies have been used by various researchers (Chandel et al., 2007). The elimination of inhibitors from fermentation broth considerably improved the yield and productivity of ethanol as compared to un-detoxified hydrolysate. Moreover, utilization of all the sugars including hexoses (C6; glucose, galactose, and mannose) and pentoses (C5 sugars; xylose and arabinose) in a single reactor can be another option to reduce the cost of producing cellulosic bioethanol.

Scientists around the world have employed different fermentation strategies for cost-effective processes for ethanol production from lignocellulosic biomass in a single reactor. These processes include separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), consolidated bioprocessing (CBP), and simultaneous saccharification, filtration and fermentation (SSFF). All the processes have been shown in Figure 2.5.

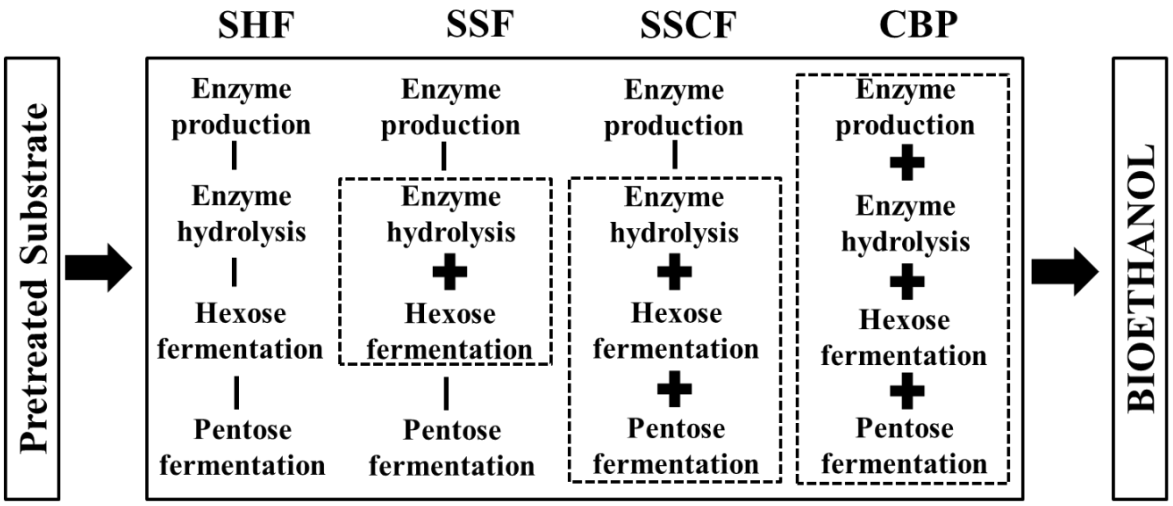


Figure 2.5: Overview of various fermentation strategies (Hemansi et al., 2019)

Above mentioned methods (SSF, SSCF, and CBP) are preferred over separate enzymatic deconstruction and fermentation (SHF) strategy. Despite it, in the current scenario, SHF is the mostly used method for bioethanol production. During the first step of SHF, cocktail of lignocellulolytic enzymes is produced so that lignocellulosic biomass can be converted into syrup of monomeric sugars (hexoses/pentoses). This solution is further used to produce bioethanol with the help of pentose/hexose fermenting microbes in a separate step. For the first step, i.e. hydrolysis, optimum temperature ranges from 45 to 50 °C, whereas for fermentation, the optimal range is near 30 °C, so both steps are performed sequentially. In SSF, the enzymatic hydrolysis of pretreated lignocellulosic biomass to release monomeric sugars for subsequent microbial conversion to ethanol is performed in the same vessel. Hallmark of this type of process is the compromise between optimum temperatures of both hydrolysis and fermentation (Choudhary et al., 2016). SSF is important over SHF as it delimits repression of cellulases (by glucose) via feedback inhibition, so improves the efficiency of saccharification as well as ethanol yield.

This is interesting to note that while performing chemical-based pretreatments, generation of various fermentation inhibitors (furfural, hydroxymethyl furfural, phenolics, acetic acid, etc.) takes place. Therefore, prior to fermentation, removal of these inhibitors seems necessary. Several detoxification strategies such as liming, activated charcoal adsorption, ion-exchange resin treatment and enzymatic detoxification have been used to remove these fermentation inhibitors. An alternative and more sustainable way to tackle the problem of inhibitors is to use inhibitor resistant or tolerant enzymes and microbial strains.

2.4. Challenges associated with bioethanol generation process

Bioethanol is generated by harvesting the plant materials that are otherwise thrown away, then using it to create fuel without adding chemicals or significant amounts of greenhouse gases into the atmosphere. Lignocellulosic biomass is a promising feedstock for bioethanol

production. But there are challenges associated with making biodegradable lignocellulosic biomass, which result in lower ethanol yields than the ideal process. Therefore, technological bottlenecks in commercial production of lignocellulosic ethanol need to be addressed to make ethanol production cost-effective.

2.4.1. Challenges related to feedstock

Major challenges related to feedstock are its collection, storage and transportation. It is also essential to make the feedstock available throughout the year. As the agricultural fields are distributed unevenly within a country, it is very difficult to collect and store the biomass at a single location for round the year use. Since, location of the industries may not be closer to the biomass generation site; the transportation of biomass to distant places can significantly increase the overall bioethanol production process cost (Saini et al., 2015).

2.4.2. Challenges involve with hydrolytic enzyme

Enzyme related challenges during bioethanol production include restricted enzyme mobility, reduced adsorption of cellulase onto cellulose, unproductive binding that hinders the enzymatic action on biomass. High levels of end products like cellobiose and glucose inhibit cellulases by feedback mechanism and lower the rate and yield of saccharification. To achieve desired saccharification efficiency, more enzyme dosages are required, which increases the overall cost. Therefore, the crucial objective for decreasing the enzyme cost is the high level production of in-house enzymes by employing hyper enzyme producing strains, inexpensive raw material and cost-efficient production technologies (Hemansi et al., 2018).

2.4.3. Challenges during biomass pretreatment

One of the significant disadvantages of the pretreatment process is the generation of a variety of chemical compounds due to undesired degradation of its LCB components, formation of by products, use of chemicals/solvents and heat (Figure 2.6). Major degradation products

formed are furans, phenolics, weak acids, etc. (Mankar et al., 2021; Wang et al., 2018). These inhibitory compounds affect the normal functioning of both enzymes and fermenting microorganisms, thereby, inhibiting hydrolysis and fermentation processes, respectively (Bhatia et al., 2021; Koppram et al., 2014). Furans such as, furfural and 5-hydroxymethyl-2-furaldehyde (HMF), inhibit yeast fermentation by blocking their oxidative metabolism and cell-biomass formation (Wang et al., 2018). Acetic acid, the major weak acid generated during pretreatment, causes yeast cell membrane disintegration and increased osmotic pressure, thereby reducing carbohydrate metabolism and fermentation. Similarly, phenolic compounds also reduce fermentation efficiency by affecting yeast growth. The most notable phenolic compounds include vanillin, catechol, coniferyl alcohol and aldehyde, syringaldehyde, and p-coumaric acid. These inhibitors are present in the pretreatment slurry/hydrolysate and also remain adsorbed to the biomass after filtration or centrifugation for solid recovery, thereby, inhibiting the fermenting microbes and decreasing the ethanol yield and productivity (Wang et al., 2018). Therefore, washing of the biomass after pretreatment has become more or less a necessary operational step for removal of these inhibitors prior to hydrolysis and fermentation. But it costs enormous amount of clean water and generates chemical rich waste water needing further treatment (Lyu et al., 2020). Detoxification is also not an economic option, as it causes loss of fermentable sugars, and increases operational time and complexity (Shibuya et al., 2017; Yu et al., 2020). Therefore, there is a need of robust yeast strains which can ferment the glucose in presence of these inhibitors.

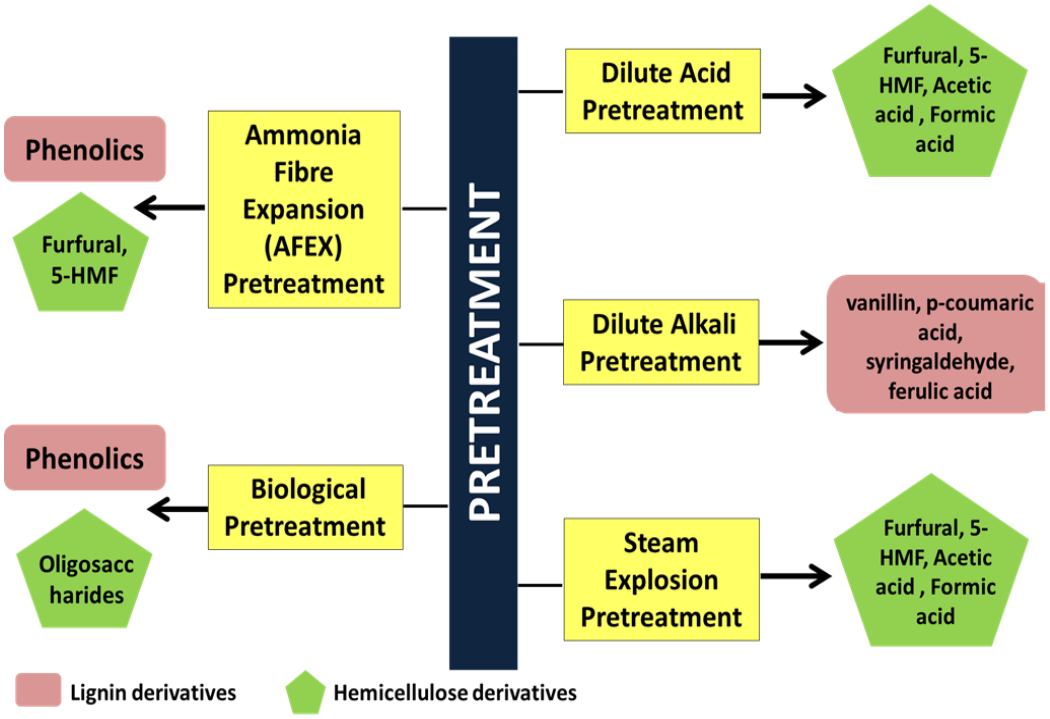


Figure 2.6: Various inhibitors generated during pretreatment of lignocellulosic biomass

2.4.4. Challenges during fermentation

Slower glucose consumption by yeasts creates an osmotic stress for the cells. Few compounds which releases during pretreatment of lignocellulosic biomass, act as inhibitors to the yeast cells while fermentation. These compounds decrease the fermentation efficiency of the cells by increasing their lag phase. Moreover, the presence of a high amount of toxic inhibitors has an impact on the performance of both enzymes and yeast. Due to the high viscosity of substrate solutions, poor mixing and heat transfer difficulties occur. In the SSF process, a compromise between ideal saccharification and fermentation temperatures leads to poor overall conversion of biomass to ethanol.

During cellulosic ethanol fermentation, SSF is the configuration of choice, as it provides many advantages including faster metabolism, high productivity, alleviation of enzyme inhibition by feedback mechanisms, less contamination, lower costs of cooling, and less energy constraints in mixing and recovery of product (Arora et al., 2019). However, use of conventional mesophilic yeasts like *Saccharomyces cerevisiae*, decreases the overall bioconversion efficiency of SSF due to larger mismatch between optimal temperatures of hydrolysis and fermentation processes. Thermotolerant yeasts like *Klyuveromyces marxianus*

provide an upper edge during SSF, as the fermentation can be carried out at a temperature (≥ 42 °C) which is near the optimal range of hydrolysing enzymes (around ≥ 50 °C) (Saini et al., 2015a). Though, thermotolerant yeasts exhibit better ethanol production under temperature stress, low tolerance to multiple fermentation inhibitors at high temperature remains bottleneck to realising their full potential in lignocellulosic biorefineries (Arora et al., 2019; Hemansi et al., 2021).

Microbial strain improvement can be carried out using genetic engineering, metabolic engineering, and synthetic biology based rational approaches, which requires the accurate knowledge about underlying principles of tolerance and the target genes or their metabolic functions (Wang et al., 2018). An alternate strain improvement strategy based upon 'evolutionary engineering' principle is known as 'adaptive laboratory evolution (ALE)' (Mo et al., 2019; Qureshi et al., 2015; Wu et al., 2021). ALE relies upon forced selection of the specific phenotypes after accumulation of spontaneous mutations, generation after generation under constant selection pressure, for developing an improved microbial strain (Yamakawa et al., 2018). It is a powerful strategy to acquire desired phenotype of inhibitor tolerance in the fermenting microorganism by cultivating it under progressively increasing levels of inhibitors followed by screening of the tolerant strains (Sandberg et al., 2019). Ideally, the developed strain would show better growth, and improved ethanol yield, titer and productivity in the presence of inhibitors. Tolerance engineering in fermenting microbes by ALE doesn't require detailed understanding of the inhibitory mechanism and complex interaction of inhibitor with biochemical or genetic networks. Moreover, the metabolic burden exerted due to heterologous expression of genes/proteins and the need for stringent control of genetic expression required in the rational approaches can be avoided by evolving tolerant strains through ALE (Mohamed et al., 2017). Most of the previous studies on ALE applied to improve biofuel fermentation have focused on improving microbial tolerance to a single

stress, such as phenolic compounds, furfural, ionic liquids, ethanol, and acetic acid (Matsusako et al., 2017; Shui et al., 2015; Wang et al., 2018). However, only a few studies on ALE have concentrated on simultaneous improvement of tolerance to multiple stresses and inhibitors, which is practically more advantageous (Wallace-Salinas & Gorwa-Grauslund, 2013).

2.5. Strategies to address current challenges during bioethanol production process

2.5.1 Strain engineering of microbes for improved bioethanol production

One of the major challenges for economic conversion of lignocellulose to bioethanol is to generate robust yeast strains. These strains should be able to cope with inhibitory compounds as well as fermentation conditions while keeping proper catalytic functions for biomass conversion to ethanol. Here, we have tabulated some of the methods such as adaptive evolution, random mutagenesis and metabolic engineering as strategies for acquiring stress tolerant strains suitable for industrial use (Table 2.3).

Control of a strong constitutive promoter showed particularly high ethanol production from xylose and low xylitol yield by fermentation of not only xylose as the sole carbon source, but also a mixture of glucose and xylose (Watanabe et al., 2007). Additionally, an ethanologenic *E. coli* mutant that is, devoid of foreign genes, has also been developed by combining the activities of pyruvate dehydrogenase and the fermentative alcohol dehydrogenase and the mutant was found able to ferments glucose or xylose to ethanol with 82% ethanol yield under anaerobic conditions (Kim et al., 2007).

Table 2.3: Various strategies employed for improving tolerance of fermenting microorganisms

Strategy	Potential drawbacks/Considerations	Approaches	References
Evolutionary engineering	The inhibition problems vary depending on the feedstock and pretreatment	The Adaptive evolution with specific hydrolysate and inhibitors of lignocellulosic	Almario et al. (2013); Koppram et al. (2012); Smith et al. (2014)
Metabolic engineering/genetic engineering	Genetically modified microorganisms-based process	Phenolics, furfural and carboxylic acid resistance engineering	Larsson et al. (2001); Sanda et al. (2011); Wang et al. (2013)
Microorganism selection	Specific productivity and product yields should be selected primarily	Screening of natural or industrial microbial collections	Favaro et al. (2013); Wimalasena et al. (2014)
Conditioning/Det-oxification	More chemicals are needed; some methods require a further step in the process	Chemical additives, such as alkaline therapy, reduction agents, polymers	Alriksson et al. (2011); Alriksson et al. (2006); Cannella et al. (2014)
Feedstock selection and engineering	Wanted to use a wide variety of feedstocks; option for biorefinery, & ,short rotation crops through sugar platform process	Use of less recalcitrant feedstocks and feedstocks that generate less pretreatment inhibitors	Larsen et al. (2012); Studer et al. (2011).
Culturing schemes	Effects on productivity and product output; inoculums adds to industrial process costs	SSF/CBP reduces sugar inhibition of feedstock; uses large sizes of inoculum	den Haan et al. (2013); Hoyer et al. (2010); Olofsson et al. (2010); Olson et al. (2012); Pienkos and Zhang (2009)
Bioabatement	Could take time and affect the sugar content	Microbial treatment	Cao et al. (2013).

Adaptive laboratory evolution (ALE), also known as evolutionary engineering, is a continuous process which is based on the selection of desired strains (Sauer, 2001). ALE requires three key components: (1) select for desired phenotypes; (2) evolve organisms; and (3) generate progeny within a lab that can be screened, according to the selection criteria. It is the approach through which diversity in performance of the microbial strains can be developed which is actually the initial point for metabolic engineering. It is an excellent tool for the production of yeast strains with various biotechnological applications such as production of fuels and chemicals (Querol et al., 2003). Lack of complete understanding of the physiology of microbes and exploiting evolution phenomena naturally to develop strains of interest are driving researchers to prefer this approach. This method can be employed easily on classical or random mutants that could be selected by providing selective pressure for growing on higher inhibitory conditions.

Environmental stress induces several alterations in bacterial or yeast genome and fitter strains that are adapted evolutionarily survived and selected (Galhardo et al., 2007). Connecting growth with production could be achieved with environmental manipulations, and has been seen quick improvements in microbial strains in several cases. The method is being successful in improving industrial yeasts strains which are not considered as GMO and can be commercialised easily. Adaptive evolution can be accomplished by delivering continuous culture conditions via chemostat and controlling the duplication rate of yeast strains, by keeping the environment fixed with the desired metabolites or inhibitors concentrations (Steensels & Verstrepen, 2014).

The technique adaptive evolution is suitable for microbes due to numerous reasons such as less generation time, less generation time, reproducibility, easy maintenance, large population size etc., (Elena & Lenski, 2003). Moreover, it has advantages in process which is simple passaging and do not require specialized equipment. However, the simple manipulations can be easily automated, and thus scaled up to hundreds of simultaneous experiments. It has been

successfully proven as a foundation of molecular & mechanistic cores of evolution. Albeit, variability in selection, making the organism cripple and improved in single feature are few drawbacks of this technique.

Several studies have reported improvement of bioethanol producing microorganisms by adopting ALE as shown in Table 2.4. The major applications of adaptive evolution in yeasts can be categorized into two; one for substrate utilization (glucose/xylose/co-fermentation) and another stress resistance against various inhibitors of the process. In order to enhance the yield of ethanol, it is required that yeast strain should utilize both pentoses and hexoses. Generally, single strain could not be able to utilize both of the sugars simultaneously. Recent studies have been concentrating on producing ethanol using *S. stipitis* (Melo-Ferreira et al., 2014) by xylose at industrial scale. The genes involved in this pathway XYL2 and XYL1 were transferred in *S. cerevisiae* using metabolic engineering and a recombinant strain developed which could utilize both glucose and xylose. Developing xylose utilizing strain through evolutionary engineering was also attempted by many researchers. In a study by Sonderegger and Sauer (2003), mutant with xylose utilization and 19% enhanced ethanol yield was developed using selection of strain after 460 generations under chemostat. Moreover, in spite of general fact that *S. cerevisiae* does not utilize xylose as sole carbon source, applying this evolutionary engineering approach, in a study by Attfield and Kletsas (2000), *S. cerevisiae* strains were growing on xylose

S. cerevisiae has been successfully evolved via adaptive evolution for more efficient lactose and arabinose fermentations (Guimarães et al., 2008). The genetic foundation for the lactose-fermentative adaptive strain's enhanced phenotype was investigated, and mutational events such as deletions in the promoter sequence of LAC genes, plasmid copy number reductions, and transcriptional differentiations were discovered (Guimarães et al., 2008). For multiple-stress resistant yeast mutants, batch selection for freezing–thawing stress resistance found to be the optimum technique. The best evolved strain had a 62-fold increase in ethanol stress

resistance, an 89-fold increase in temperature tolerance, and a considerable increase in freezing–thawing and oxidative stress tolerance (102-fold and 1429-fold, respectively).

In a study by Shui et al. (2015), furfural- and acetic acid-tolerant bacterial strains were developed by ALE. Under a 3 g/L furfural stress environment, the best mutant ZMF3-3 obtained a theoretical ethanol production of 94.84%, significantly higher than the 9.89% yield from ZM4. Given that furfural creates DNA-damaging free radicals in hydrolysates, the underlying mechanism could be that the hydrolysate acts as a mutagen capable of generating genetic variety in the chosen strain.

Furthermore, evolutionary adaptation procedures have been used to improve the fermentation capabilities of recombinant strains. By sub-culturing xylose-fermenting recombinant strains of *Z. mobilis* 39767 in a medium containing 10-50 percent hydrolysate, Lawford and his team improved the strains' tolerance to higher concentrations of acetic acid, and the adapted isolates showed a significant increase in ethanol productivity when compared to un-adapted strains (Lawford & Rousseau, 1999). Similarly, employing a long-term adaptation method of different serial selections for liquid and solid medium, a modified *E. coli* KO11 was created to withstand high ethanol concentrations. The mutants (LY01, LY02, and LY03) showed a survival rate of more than 50% in 10% ethanol (0.5 min exposure) and a reduction in fermentation time (Yomano et al., 1998) . Almost every prior attempt at evolutionary adaptation began with genetic engineering, which was then followed by adaptive selection (Kuyper et al., 2005; Sonderegger & Sauer, 2003; Wisselink et al., 2009).

However, a new technique involving genetic engineering, EMS mutation, and two-step evolutionary adaptation (under consecutive aerobic and oxygen-limited settings) has recently been tried (Liu & Hu, 2010). In comparison to the parental strain, the strain generated thus demonstrated a four-fold increase in its specific growth rate. Surprisingly, the activity of key xylose metabolism enzymes (XR, XDH, and XK) remains intact, implying that chemical mutagenesis and evolutionary adaption may have resulted in a new genetic characteristic that makes mutants capable of xylose metabolism (Liu & Hu, 2010).

Table 2.4: Strain improvement of ethanol producing yeasts for inhibitor tolerance via adaptive laboratory evolution

Inhibitory Conditions	**Fermentation Process**	**Microorganism**	**Improvements achieved**	**References**
High sugar (Osmotic stress)	Batch fermentation	*Saccharomyces cerevisiae*	Utilising 50% Glucose for ethanol production	Zhang et al. (2019)
Alternate substrate (Xylose) (Glucose+Xylose)	Batch and Continuous fermentation	*Saccharomyces cerevisiae*	Increased specific consumption of xylose	Koppram et al. (2012)
Inhibitory compounds (Acetic acid, Furfural, HMF Vanillin, Formic acid)	Batch and Continuous fermentation	*Saccharomyces cerevisiae*	Increased conversion of inhibitors, 50% increased ethanol productivity during fermentation	Koppram et al. (2012)
Ethanol	Batch fermentation	*Klyuveromyces marxianus*	Increased ethanol tolerance from 6% to 10%	Mo et al. (2019)
Ethanol + Temperature	Batch fermentation	*Saccharomyces cerevisiae*	Utilising 50% Glucose for ethanol production	Zhang et al. (2019)
Xylose + Acetic acid	Batch	*Saccharomyces cerevisiae*	Tolerating 20% ethanol, growing at 40°C temperature**, e**nhanced production of ethanol from xylose while tolerating 5 g/L of acetic acid	Ko et al. (2020)

2.5.2. Improvisation of fermentation process

A meaningful way to counteract the challenge of a higher cost of bioethanol production is the application of the SSF process by reducing the number of vessels, minimizing the intermittent operations, lowering the processing steps, reducing the enzyme inhibition, and minimizing the overall production time. An SSF process with high solid loading (above 100 g/L) is expected to significantly decrease bioethanol yield by increasing the viscosity, elevates stress for the microbes and enzymes via increased inhibitor concentrations and low mass and heat

exchange (Wingren et al., 2003). However, it is possible to avoid such inhibitions by combining SSF with intermittent feeding, i.e., carrying out the SSF process in a fed-batch mode.

During the fed-batch SSF process, the biomass feedstock is added intermittently in the fermenter, which does not allow the viscosity of the slurry to increase much, as the biomass is continuously liquified to sugars, which in turn are fermented to ethanol by the yeasts (Rudolf et al., 2005). Thus, there is no build-up of glucose or other sugars, and mixing and heat transfer are improved, thereby increasing the efficiency of enzymes and yeasts. The solid loading can be increased up to 200 g/L or even more by using the fed-batch mode, which is more than 2-fold of the solid loading employed in the batch mode. This intermittent substrate loading maintains the slurry viscosity at manageable levels, besides obtaining much better conversion yields and ethanol titers than the batch-SSF process. Moreover, low levels of free glucose in the slurry during FBSSF allow better conditions for co-fermentation of pentose and hexose sugars into ethanol using the recombinant microorganisms or the mixed microbial cultures. Further, the enzymes and the fermenting microorganisms encounter a significantly lesser concentration of the inhibitors during the FBSSF process, especially during the initial phase (Hoyer et al., 2010; Tomás-Pejó et al., 2009). The yeasts cannot only metabolize low concentrations of some inhibitors (furfural or HMF) but also get better adapted when the inhibitor concentrations are at lower levels (Hodge et al., 2008; Taherzadeh et al., 2000). FBSSF is also more effective in making the bioethanol production economics by allowing optimal dosage of enzyme and yeast inoculum (Gao et al., 2014; Wanderley et al., 2013; Zhang & Zhu, 2017).

Several studies demonstrated advantages of fed-batch over batch SSF **(Table 2.5)**. Lesser enzyme feedback inhibition and increased substrate loading are the main factors that improve the ethanol yield in fed-batch SSF (Gao et al., 2018). In most of the studies, commercial

enzyme formulations, mainly Cellic, CTec2 alone or in combination with glucosidase and other accessory enzymes, have been used. The use of thermotolerant yeast and cold-active cellulases can further improve the ethanol yield under SSF (Choudhary et al., 2017; Hemansi et al., 2021; Saini et al., 2015a).

Another research study compared ethanol titers and yields of batch and fed-batch SSF using sweet sorghum bagasse at high solid loadings in which biomass was fed either with or without proportional quantities of the enzyme and yeast inoculum, and fed-batch SSF proved much better than batch SSF (Darkwah et al., 2016). Fed-batch SSF has also been carried out using paddy straw as feedstock for bioethanol production (Shengdong et al., 2006). The fed-batch SSF was performed using the parameters statistically optimized in a batch SSF, and significantly higher ethanol titers were obtained than the batch processes by mitigating the mixing and mass transfer related problems of the batch process when using high biomass (Shengdong et al., 2006). In a recent study, Gao et al. (2018) produced a higher concentration of bioethanol by using high solid loading of the feedstock, i.e., sugarcane bagasse, by carrying out the SSF process fed-batch mode. The authors also reported better titer (75.57 g/L) and productivity of ethanol (Gao et al., 2018)

Table 2.5: Advantages of using fed-batch simultaneous saccharification and fermentation process for bioethanol production.

S. No.	Advantages	References
1.	As the sugars, generated during hydrolysis, are utilised and converted to ethanol instantaneously, the enzyme inhibition is mitigated.	Gao et al. (2014); Wanderley et al. (2013); Zhang and Zhu (2017)
2.	The process gets economic as enzyme loading can be controlled.	
3.	Mixing of the substrate is increased.	Stanbury and Whitaker (1984); Zhang and Zhu (2017)
4.	reduced viscosity due to pulsed addition of substrate, resulted in minimized content of insoluble solids in the medium	
5.	Reduction of substrate inhibition.	
6.	Ethanol yield can be enhanced by increasing the loading	
7.	overcome mass transfer problems	
8.	Reduction in power due to use of single vessel for hydrolysis and fermentation.	Hodge et al. (2008); Taherzadeh and Karimi (2008)
9.	Also, by using thermotolerant yeast for fermentation, energy can be saved which would have used for cooling down the vessel.	
10.	decreased fermentation time	
11.	higher productivity	Sotaniemi et al. (2016)
12.	higher dissolved oxygen in the medium	
13.	reduced toxic effects of the medium components	
14.	Increased yeast viability	

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1. Chemicals used

All the chemicals and kits used in the experimental work were of AR grade are alphabetically listed in Annexure I.

3.2. Medium composition

3.2.1 Enrichment medium

Yeast extract peptone dextrose (YPD) broth (yeast extract: 10 g/L, peptone: 20 g/L and glucose: 50 g/L) containing chloramphenicol: 50 µg/mL, was used for isolation of thermotolerant yeasts.

3.2.2 Growth medium

Yeast extract peptone dextrose (YPD) broth (yeast extract: 10 g/L, peptone: 20 g/L and glucose: 20 g/L) containing antibiotic (chloramphenicol: 50 µg/mL), was used during growth curve experiments.

3.2.3 Screening medium

Tolerance of the isolated thermotolerant yeasts to inhibitors was tested using YPD agar (yeast extract: 10 g/L, peptone: 20 g/L, glucose: 20 g/L and agar: 20 g/L) supplemented with inhibitors individually g/L: acetic acid (4), furfural (0.5) and vanillin (0.5) and ethanol (7) to the medium.

Yeast extract peptone dextrose (YPD) broth (yeast extract: 10 g/L, peptone: 20 g/L and glucose: 150 g/L) containing antibiotic (chloramphenicol: 50 µg/mL), was used for isolation of thermotolerant yeasts in fermentation medium.

3.2.4 Medium for cellulase production

Different media were utilized for cellulase production from cellulytic fungi strains under solid state (SSF) and submerged fermentation (smf).

Mandel-Weber Medium (Mandels & Weber, 1969)

Components	**g/L**
KH_2PO_4	2
$CaCl2.H_2O$	0.3
$MgSO_4.7H_2O$	0.3
$(NH_4)_2SO_4$	1.4
Urea	0.3
Peptone	0.25
Yeast Extract	0.1
$FeSO_4.7H_2O$	0.0005
$MnSO_4.7H_2O$	0.00016
$ZnSO_4.7H_2O$	0.00014
$CoCl2.6H_2O$	0.0002
Tween 80	1 mL
pH	5.5

Reese's minimal medium **(Tiwari et al., 2016)**

Components	**g/L**
KH_2PO_4	2
$CaCl2.H_2O$	0.3
$MgSO_4.7H_2O$	0.3
$(NH_4)_2SO_4$	1.4
$FeSO_4.7H_2O$	0.0005
$MnSO_4.7H_2O$	0.00016
$ZnSO_4.7H_2O$	0.00014
$CoCl2.6H_2O$	0.0002

3.3. Standard Cultures

Standard thermotolerant ethanologenic yeast cultures *Kluyveromyces marxianus* NCIM 3565 and *Kluyveromyces marxianus* MTCC 4136 were procured from National Culture Collection of Industrial Microorganisms (NCIM), Pune, India and Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India were used for benchmarking. The pentose fermenting mesophillic, yeast *Pichia stipites* NCIM 3499 was also procured from NCIM, Pune, India.

Standard fungal strains for cellulase production *Trichoderma reesei* MTCC 3194 and *Penicillium funicolosum* NCIM 1228 were procured from MTCC and NCIM, respectively. *Aspergillus niger* SH3 was a kind gift from Dr. Surender Singh, at IARI, New Delhi. *Penicillium oxalicum* 114-2 and *Penicillium oxalicum* RE-10 were obtained from Prof. Yunibo Qu, at Shandong University, Shandong, China. *Trichoderma reesei* JKR3, a mutant of *T. reesei* MTCC 3194 was obtained from Department of Microbiology, Central University of Haryana.

3.4. Isolation and screening of thermotolerant yeast

3.4.1. Sample collection

A total of 103 different samples comprising samples of juice, molasses, bagasse, and soil from bagasse dumping sites, molasses were collected from Sonipat sugar mill, Haryana. Solid samples from distillery waste dumping sites and whey samples from dairy were also collected from various regions of Haryana during summer, when the temperature of collection sites was approximately 40 °C (Table 3.1).

Table 3.1: Collection sites and types of samples collected for isolation of thermotolerant yeasts

Type of sample	Site of Collection	No. of Samples
Fruits	Fruit market, Mahendergarh, Haryana	5
Fruits	Fruit market, Noida	2
Whey	Mahendergarh, Haryana	2
Whey	Rewari, Haryana	1
Whey	Delhi	1
Whey	Dadri, Haryana	1
Khameer	Mahendergarh, Haryana	1
Mix fruit Juice	Mahendergarh, Haryana	2
Date palm	Mahendergarh, Haryana	2
Sugarcane bagasse	Mahendergarh, Haryana	3
Sugarcane bagasse	Dadri, Haryana	1
Soil under bagasse	Mahendergarh, Haryana	1
Distillery waste	Haridwar, Uttrakhand	8
Distillery waste	Karnal, Haryana	12
Distillery waste	Muzaffarnagar, Uttar Pradesh	2
Distillery waste	Pathankot, Punjab	3
Sugar mill	Site 1-20 Sonipat, Haryana	56
Total no. of samples		**103**

3.4.2. Isolation of thermotolerant yeasts by enrichment culture

To isolate thermotolerant yeasts, the samples were mixed with enrichment medium and incubated overnight at 42 °C with shaking at 200 rpm. The samples were then serially diluted and 100 µL of the diluted samples were plated on YPD agar, followed by incubation at 42 °C for 24 h. Morphologically distinct yeast colonies appearing on plates were streaked on fresh YPD agar plates to obtain pure cultures. All yeasts were maintained on YPD agar maintenance medium and preserved at 4 °C.

3.4.3. Culture preservation and maintenance medium

YPDAplates and slants containing chloramphenicol: 50 µg/mL, were used for maintaining the isolated and identified thermotolerant yeast cultures. The cultures were preserved at 4 ℃. Fungal cultures were maintained on Potato Dextrose agar (PDA) slants and preserved at 4℃. For long term preservation, glycerol stocks were prepared and stored at -80 ℃ in deep

freezer. The cultures were deposited at the culture bank of Lignocellulose Biorefinery Laboratory, Department of Microbiology, Central University of Haryana, Mahendergarh.

3.4.4. Screening of yeast strains for inhibitor tolerance

Tolerance to inhibitors, acetic acid, furfural and vanillin, was tested using screening medium for inhibitors tolerance. The inhibitors stocks were prepared and filter sterilized before addition to the sterile medium. The cells harvested at OD_{600} 0.7-0.9 were used to inoculate the screening medium for determining inhibitor tolerance.

3.4.5. Screening of thermotolerant yeast for ethanol production

Screening of thermotolerant yeast was done on basis of their growth, sugar utilization, and fermentation characteristics. For primary screening, single colony of the freshly grown yeast was inoculated in 250 mL capped flask containing 100 mL YPD screening medium and incubated overnight at 42 °C. Serially diluted cultures (100 µL) from the flasks were plated on YPD agar, and the obtained pure cultures of distinct yeast colonies, after 24 h, were employed for secondary screening.

For secondary screening, capped 250 mL flasks containing screening medium were inoculated with 2% (v/v) inoculum from the seed culture of OD_{600} 0.7-0.9separately for each isolate. The flasks were incubated at 42 °C and 200 rpm for 24 h. Samples (1 mL) were withdrawn at different time intervals (0, 18, and 24 h) and analyzed for cell growth, sugar, and ethanol.

3.5. Identification and characterization of selected yeast

3.5.1. Colony characteristics

For studying the colony characteristics, yeasts were grown on growth medium plate and incubated at 42 °C for 24 h. Thereafter, the colony characteristics such as colony size, color, texture and shape, elevation and edge were observed and recorded.

3.5.2 Cell morphology

Morphological characteristics of yeast isolates were studied after negative staining. Thin smear of the culture was prepared by taking colony from freshly grown colonies mixing it with Nigrosin black dye on clean microscopic glass slide and drying in air. The smear was observed under 40× and 100× magnification using light microscope (Olympus CH20i, India).

3.5.3. Growth monitoring

Growth of the selected isolate was monitored by measuring cell biomass (dry weight) and increase in absorbance (cell OD_{600}) during growth on YPD growth medium. A colony grown on agar medium was inoculated in broth and incubated at 42 °C and 200 rpm. Aliquots (1 mL) of culture were withdrawn every 30 min until constant cell OD_{600}. A plot of OD_{600} and cell biomass versus time was prepared to assess the growth of isolates in terms of specific growth rate (Eq. 1) and doubling time (Eq. 2)

$$\frac{dx}{dt} = \mu X \qquad (1)$$

$$t_d = \frac{0.693}{\mu} \qquad (2)$$

Where, μ = specific growth rate (h^{-1}), t_d = doubling time, and X = biomass

3.5.4. Biochemical characterization

Biochemical characterization of the selected yeasts was performed using KB006 HiCandida™ Identification Kit (Himedia Lab. Pvt Ltd, India) on the basis of carbohydrate utilization characteristics, following manufacturer's protocol. A 50 µL of 16 h old yeast culture (OD_{600} ~ 0.6) was suspended in saline and was inoculated in each well of identification kit. Thereafter, the lids of the kits were closed and allowed to incubate at 37 °C for 24 h. Colour change in each well was observed after 24 h. Change in colour from

orangish-yellow to pink for detection of urease enzyme and from red to yellow for utilization of sugars was interpreted as positive test.

3.5.5. Molecular identification

The yeasts growing splendidly and exhibiting comparatively higher ethanol production, were finally identified on the basis of amplification and sequencing of their internal transcribed spacers (ITS) regions.

3.5.5.1. Genomic DNA isolation

Selected yeast isolate was grown overnight in YPD broth from which 1 mL aliquot was centrifuged at 10,000 rpm for 10 min to obtain cell pellet. Genomic DNA isolation of selected yeasts was performed using the method described by Sambrook and Russell (2006). Thereafter, 5.0 mL of phenol (pH 8.0): $CHCl_3$: Isoamyl alcohol (25:24:1) solution was added to the cell pellet in 2 mL microcentrifuge tubes and shaken vigorously. The tube was centrifuged at 12,000 rpm and 4 °C for 15 min. The supernatant was mixed with 100 µL RNase (2 mg/mL working concentration) and incubated at 37 °C for 30 min. After RNAse treatment, freshly prepared 5.0 mL solution of $CHCl_3$ and Isoamyl alcohol (24:1) was added to the DNA solution and centrifuged at 10,000 rpm at 4 °C for 15 min. The supernatant was separated in fresh tube and 2.5 mL cold isopropanol was added to this. The solution was stored overnight at -20 °C followed by centrifugation at 10,000 rpm at 4 °C for 15 min. The supernatant was discarded and the pellet was gently washed with chilled 70% ethanol. The pellet was air dried in the laminar air flow to remove traces of ethanol. The pellet was dissolved in minimum amount of sterilized milli-Q water and stored at -20 °C. The DNA was further purified by adding equal volume of phenol (pH 8.0):$CHCl_3$ (1:1) and mixed well by gentle inverting. The solution was centrifuged at 10,000 rpm at 4 °C for 15 min. The upper phase was taken and mixed with equal volume of cold isopropanol. It was incubated at -20 °C for at least 2 h and centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant was

decanted and the pellet was air dried at 37 °C. The pellet was dissolved in a minimum amount of sterilized milli-Q water and stored at room temperature.

3.5.5.2 PCR amplification and sequencing

Amplification of 5.8S-ITS rDNA region of the yeasts were performed by polymerase chain reaction (PCR) using forward primer ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse primer ITS-4 **(**5'-TCCTCCGCTTATTGATATGC-3'). PCR reaction was set up in a final volume of 25 µl containing 50 ng genomic DNA, 25 pmol each of forward primer (pITS-1) and reverse primer (pITS-4), 10 mM each of deoxynucleoside triphosphate (dNTP's) (NEB, England) and 1.0 IU of Taq polymerase (NEB, England). The amplification was performed in G-storm PCR machine (USA) with the following cycling parameters:

Initial denaturation	94 °C for 4 min	
Amplifictaion	94 °C for1 min 58 °C for 40 sec 72 °C for1 min	35 cycles
Final extension	72 °C for 8 min	
Cooled to	4 °C	

PCR amplicon was analysed by agarose gel (0.8%, w/v) electrophoresis and eluted using commercial gel extraction kit (mdi Corporation, India). The eluted PCR amplicons sequencing was done through Sanger sequencing method.

3.5.5.3 Phylogenetic studies of the yeast

The obtained ITS-5.8S-rDNA sequences were analysed by the Basic Local Alignment Search Tool (BLASTn) algorithm and deposited in the NCBI GenBank database for obtaining the accession numbers. The phylogenetic tree was constructed by neighbourhood-joining method using Molecular Evolutionary Genetics Analysis (MEGA) version-7 and boot-strap analysis based on 1000 replicates (Talukdar et al., 2016).

3.6. Strain improvement of selected yeasts by adaptive laboratory evolution (ALE)

ALE is an innovative approach for the generation of evolved microbial strains with desired characteristics, by implementing the rules of natural selection (Dragosits & Mattanovich, 2013). Improvement in the inhibitor tolerance capability of the two finally selected superior yeast strains was carried out by ALE via repetitive batches of sequential growth in YPD medium in 250 mL flask containing 100 mL growth medium supplemented with inhibitors

3.6.1. Effect of fermentation inhibitors on growth of yeast strains

The effect of the predominant inhibitors generated during pretreatment process such as acetic acid (AA), furfural (F) and vanillin (V) was investigated on yeast growth in YPD broth and agar medium. Varying concentrations (g/L) of individual inhibitors used for determination of their effect on growth inhibition were: AA (1, 2, 3, 4, 5, 6), F (1, 1.5, 2, 3, 4) and V (1, 1.5, 2, 3, 4). Additionally, the cocktail of inhibitors containing varying concentrations of inhibitors were also employed for assessing the combined effect of the inhibitors. Different combinations of inhibitors (A+F+V) (g/L) were: cocktail I (1+1+1), cocktail II (2+2+2), cocktail III (3+3+3), cocktail IV (4+4+4), cocktail V (1+0.1+0.1), cocktail VI (2+0.2+0.2), cocktail VII (3+0.3+0.3), cocktail VIII (4+0.4+0.4), and cocktail IX (5+0.5+0.5). Filter sterilised solution of inhibitors and their cocktails were added into the medium in individual flasks after sterilisation. After inoculation of the flasks with yeast inoculum (1%, v/v),the flasks were incubated at 42 °C for 24 h. Absorbance of the yeast culture was measured as OD_{600} after 12 h and a graph was plotted between OD_{600} and inhibitor concentrations to determine the effect of inhibitors on yeast cell growth. The medium without any inhibitor served as control.

3.6.2. Adaptive laboratory evolution of yeasts

The first batch of adaptive laboratory evolution (ALE) experiment was initiated by inoculating loopful of cells from overnight grown colonies to 50 mL growth medium. The

flasks were incubated at 42 °C under shaking until OD_{600} reached 0.7-0.9. The cells were harvested by centrifugation and washed with sterile saline. The obtained cells were inoculated (initial cell OD ~ 0.2) into the fresh medium augmented with filter sterilised inhibitor(s) (A, F, V or cocktail). The flasks were incubated at 42 °C under shaking at 200 rpm until log phase (OD_{600} of 0.6-0.8). The adaption process was executed by gradually increasing the inhibitor concentrations during repetitive batch cultures **(Table 3.2)**. The log phase cells harvested from the previous batch were used as inoculum for the consecutive batch. The serial transfer of cells was continued for adaptation in the given inhibitor(s) concentration. When no further improvement was observed in lag phase and growth rate, the improved strains were transferred in the medium with subsequently higher concentration of inhibitor(s) for further improvement. After 60-70 batches of serial transfer, cells of the developed yeasts were harvested by centrifugation. Pure cultures of the developed yeast strains were then stored as 20% glycerol stocks at -80 °C for subsequent experiments.

Table 3.2: Strategy for adaptation of yeasts towards fermentation inhibitors during adaptive laboratory evolution

Inhibitors	Concentration (g/L)	No. of generations passaged
Acetic acid (A)	3.5	1-12
	4	13-20
	4.5	21-30
	5	31-40
	5.5	41-60
	6	61-70
Furfural (F)	2	1-10
	2.5	11-20
	2.8	21-40
	3	41-50
	3.2	51-60
Vanillin (V)	2	1-12
	2.5	13-20
	2.8	21-40
	3	41-60
Cocktail (A+F+V)	3+0.3+0.3	1-10
	3+0.5+0.5	11-20
	3+0.8+0.8	21-40
	3+1+1	41-60/70

The criteria of the fitness of the cells were reduced lag phase and enhanced biomass yield

3.7. Fermentation by the adapted yeast strains under synthetic medium

The fermentation efficiency of the adapted yeast strain was tested in synthetic medium. A colony from each of freshly grown adapted and parent yeast cells were transferred to 250 mL flask containing 100 mL fermentation medium. The flasks were incubated for 12 h under shaking at 42 °C and 150 rpm. The cells were harvested after 12 h and used as inoculum for fermentation at cell concentration of 2 g/L (dry cell wt.). Fermentation was carried out in 250 mL capped flasks containing 50 mL medium under incubation shaking at 42 °C and 150 rpm.

Samples were collected every 12 h and analysed for glucose and ethanol. Ethanol yields and percent theoretical yields were calculated using the equations (3) and (4), respectively:

$$Y_{p/s}\,(g/g) = \frac{Ethanol\ titer\ (g/L)}{Initial\ sugar\ (\ g/L)} \quad (3)$$

$$Y_T\,(\%) = \frac{Practical\ yield\ (Yp/s)}{Theoretical\ yield}\ X\ 100 \quad (4)$$

3.8. Sequential dilute acid-alkali pretreatment of lignocellulosic biomass

Sugarcane bagasse (SCB) was selected as a biomass based on the availability in nearby regions. It was acquired locally from market, sun-dried, milled (Metrex Scientific Instrumentation, Delhi, India) and sieved. The residues retained on 40 mesh (1-2 mm) sieves were collected, washed for removal of dust and stored in sealed plastic bags after drying at 50 °C.

Pretreatment of sugarcane bagasse was performed sequentially using dilute sulfuric acid followed by dilute sodium hydroxide for removal of hemicellulose and then lignin from the biomass, respectively, aiming to utilize the remaining cellulose for bioethanol production (da Silva et al., 2010).

3.9. Optimization of sequential dilute acid-alkali pretreatment of sugarcane bagasse

The sequential acid-alkali pretreatment of SCB was performed in a stepwise manner using dilute acid (H_2SO_4) and alkali (NaOH). The optimization of dilute acid and subsequent dilute alkali pretreatment of SCB was carried out by Response Surface Methodology (RSM). The independent variables were H_2SO_4 concentration (%, v/v), temperature (°C), residence time (min), and solids loading (%, w/v). The experimental data were fit into the following second-order polynomial equation (Eq. 5) to evaluate the effect of each independent variable on the response(s);

$$Y= \beta_0 + \beta_1x_1+ \beta_2x_2+ \beta_3x_3+ \beta_4x_4+ \beta_{12}x_1x_2+ \beta_{13}x_1x_3+ \beta_{14}x_1x_4+ \beta_{23}x_2x_3+ \beta_{24}x_2x_4+ \beta_{34}x_3x_4+ \beta_{11}x_1^2+ \beta_{22}x_2^2+ \beta_{33}x_3^2+ \beta_{44}x_4^2 \quad (5)$$

Where, Y is the predicted response; β_0 is model constant; x_1, x_2, x_3 and x_4 are independent variables; β_1, β_2, β_3 and β_4 are linear coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} are cross-product coefficients; and, β_{11}, β_{22}, β_{33}, and β_{44} are the quadratic coefficients.

During pretreatment, the solids were analysed for cellulose content, while hydrolysates were analysed for released sugars, furan derivatives and phenolics.

The dilute acid pretreatment step of SCB was optimised by Box-Behnken design (BBD) of experiment with 29 runs. As shown in Table 3.3, the four factors chosen for the study and their values were prescribed into two levels, coded +1 and −1 for high and low values, respectively. After completion of dilute acid pretreatment, the acid hydrolysate was filtered through two layered muslin-cloth. The cellu-lignin was washed until pH 7.0 and oven dried at 60 °C.

Table 3.3: Levels of variables tested in the Box-Behnken design for optimization of dilute acid pretreatment

Coding	**Variable**	**Units**	**Coded level**	
			-1	**+1**
A	Sulphuric Acid	%, v/v	0.5	5
B	Temperature	°C	100	121
C	Time	min	10	30
D	Solid loading	%	5	20

After dilute acid pretreatment step, the recovered cellulignin was further delignified with sodium hydroxide at 121 °C for 30 min according to the D-optimal design using sodium hydroxide concentration (%, w/v) and solid loading (%, dry wt.) as the independent variables (Table 3.4). The temperature and time was kept constant after reviewing the literature (Zhu et al., 2016). The solid biomass after the pretreatment was filtered, washed and at 50 °C and kept in zip-lock bags for further use.

Table 3.4: Levels of variables during dilute alkali pretreatment using D-optimal design for optimization of dilute acid pretreatment

Coding	Variable	Units	Coded level	
			-1	**+1**
A	Sodium hydroxide	% (w/v)	0.5	10
B	Solid loading	% (w/v)	5	20

3.10. Characterization and compositional analysis of lignocellulosic biomass

3.10.1. Compositional analysis

The compositional analysis (α-cellulose, klason lignin, pentosans, moisture and ash) of SCB solids before and after pretreatment was carried out gravimetrically using previously described protocols by TAPPI (1992).

3. 10.1.1. Holocellulose

Dried SCB (5 g) was taken in 500 mL Erlenmeyer flask containing 160 mL distilled water. The flasks covered with inverted 100 mL flasks were heated by keeping on hot plate at 100 °C. Thereafter, sodium chlorite (1.5 g) and glacial acetic acid (0.5 mL) were added to the flasks. The addition of sodium chlorite and glacial acetic acid was repeated till the substrate became white. The flasks were then allowed to cool and release the fumes. The treated material thus obtained was filtered through G2 pre-weighed crucible and washed with distilled water. The crucible was dried to constant weight in an oven at 105±3 °C. The holocellulose content of biomass was determined by the following equation:

$$Holocellulose\ (\%) = \frac{Weight\ of\ sample\ (g) - Weight\ of\ oven\ dried\ sample\ (g)}{Weight\ of\ sample} \times 100$$

3. 10.1.2 Cellulose

Dried SCB (2g) was taken in 500 mL Erlenmeyer flask containing distilled 100 mL water. Sodium chlorite (5 g) was then added to the flasks and autoclaved for 30 min. The treated material thus obtained was filtered through pre-weighed G2 crucible, and washed with

distilled water. The crucible was then dried in an oven at 105±3 °C and cooled in desiccator. The cellulose content of the biomass was determined by the following equation:

$$Cellulose\ (\%) = \frac{Weight\ of\ sample\ (g) - Weight\ of\ oven\ dried\ sample(g)}{Weight\ of\ sample\ (g)} \times 100$$

3. 10.1.2 Hemicellulose

Hemicellulose content was calculated from previously obtained holocellulose and cellulose by using the following equation:

$$Hemicellulose\ (\%) = \frac{Holocellulose\ (g) - Cellulose(g)}{Weight\ of\ sample\ (g)} \times 100$$

3. 10.1.3 Lignin

The oven dried SCB (1.0 g) was taken in a 100 mL beaker and treated with 15 mL of 72% sulphuric acid for 2 h at 25 °C (room temperature) with occasional stirring. After 2 h the content was transferred to 1L flask, acid concentration was brought down to 3% by adding distilled water (560 ml) and refluxed for 4 h at 100 °C. The contents were then filtered through G3 crucible and washed with distilled water until acid free. The crucible was dried to constant weight at 105±3 °C in an oven. The klason-lignin content of biomass was determined by the following equation:

$$Lignin\ (\%) = \frac{Weight\ of\ sample\ (g) - Weight\ of\ oven\ dried\ sample(g)}{Weight\ of\ sample\ (g)} \times 100$$

3. 10.1.4. Moisture and ash

For moisture content analysis of 1.0 g SCB sample was taken in a G3 crucible and kept in an oven at 105 ± 3 °C for drying till constant weight. The dry weight of sample was determined by following equation:

$$Moisture\ content\ (\%) = \frac{Weight\ of\ sample\ (g) - Weight\ of\ oven\ dried\ sample(g)}{Weight\ of\ sample\ (g)} \times 100$$

For estimation of ash content, the dried sample (1.0 g) was taken in a silica crucible and kept in furnace at 500 °C for 4 h. The ash content of biomass was determined by following equation:

$$Ash\ content\ (\%) = \frac{Weight\ of\ sample\ (g) - Oven\ dried\ weight\ of\ ash(g)}{Weight\ of\ sample\ (g)} \times 100$$

3.10.2. Biomass characterization

Structural characterizations of untreated and sequentially pretreated biomass was carried out using various biophysical analytical techniques such as Fourier transforming infra-red spectroscopy (FT-IR), Scanning electron microscopy (SEM), Energy dispersive X-ray spectroscopy (EDX), Thermogravimetric analysis (TGA), and Small angle neutron scattering (SANS). FT-IR, SEM, and EDX, TGA experiments were carried out at University of Delhi, North campus, Delhi whereas, small angle neutron scattering (SANS) experiment was performed at Bhabha Atomic Research Center (BARC), Mumbai, India.

3. 10.2.1 Fourier transforming infra-red (FT-IR) spectroscopy

FTIR spectroscopic investigation was carried out in transmittance mode to monitor the relative changes in the biomass functional groups using Thermo Nicollet iS50 FTIR spectroscope. Samples were prepared by mixing with dried KBr (sample: KBr ¼ 1:200) and pelleted under vacuum. Absorption of IR by untreated or pretreated SCB samples was monitored between 4000 and 500 cm^{-1} (Singh et al., 2005).

3.10.2.2. Scanning electron microscopy (SEM) and Energy dispersive x-ray spectroscopy (EDX)

The SEM analysis of untreated and pretreated SCB was performed to reveal ultra-structural changes. Samples were dried, mounted on aluminium stubs and sputter-coated with a gold layer (JEC 300).The scanning and acquisition of microphotographs of the sputter-coated biomass were carried out using JSM-6610LV (JEOL, Japan) scanning electron microscope (Behera et al., 1996).

The chemical identification of elements and their concentrations in untreated and pretreated substrates were carried out using Energy Dispersive X-Ray Spectroscopy (EDX) coupled with SEM. The EDX system used was RONTEC's Model QuanTax 200 which provides an energy resolution of 127 eV at Mn K alpha. During the analysis, elements in the biomass absorb the X-ray beam, which dislocates electrons from their ground state and thus creates a hole filled by other higher energy state electrons and hence difference in energy resulting in peak formation (Phitsuwan et al., 2017).

3. 10.2.3. Thermogravimetric analysis (TGA)

The thermogravimetric analyses of SCB (50 mg) were carried out in TGA analyser (Linesis TGA Hires 1000). . Mass loss and difference in mass loss were calculated as a function of temperature from 10 to 1000 °C with heating rate of 200 °C/min (Varma & Mondal, 2016).

3. 10.2.4 X-Ray diffraction (XRD)

The crystalline nature of SCB samples were studied by obtaining XRD patterns on XRD diffractometer (X-Calibur-S Single Crystal) with Cu kappa platform as X-ray source. The dried samples were scanned in 2θ value ranged from 5° to 45° using step size of 0.02° and Cu/Kα radiations generated 35kV and 35mA. Calculation of crystallinity index (CrI) was done using equation (Eq. 6) (Segal et al., 1959):

$$CrI\ (\%) = (I_{002}\text{-}I_{am})/\ I_{002} \times 100 \qquad (6)$$

I_{002} corresponds to peak height at 22.2-22.5° (2 θ) and I_{am} corresponds to peak height of the amorphous cellulose at 18° (2θ).

3. 10.2.5 Small angle neutron scattering (SANS)

SANS analyses were carried out by using SANS diffractometer facility at the Guide Tube Laboratory located in Dhruva Reactor at Bhabha Atomic Research Centre (BARC), Mumbai, India (Aswal & Goyal, 2000). SANS analyses reveal knowledge about shape/size of scattering entities within 10 to 1000 Å. The angular distribution of neutrons scattered by the sample was recorded using a 1 m long one-dimensional He^3 position sensitive detector. The SANS diffractometer used for this study covered a *Q*-range of 0.015–0.35 $Å^{-1}$. The temperature in all the measurements was kept fixed at 30ºC. The determination of coherent differential scattering cross-section (dΣ/dΩ) per unit volume using SANS was dependent on wave vector transfer *Q* (= 4π sin (θ/2)/λ, (λ = wavelength of incident neutron and θ= scattering angle). The monochromatic beam of neutrons (λ = 5.2 Å) was used with a spread of $\Delta\lambda/\lambda$ ~ 15%.

3.11. Cellulase production by fungi

Cellulase enzyme production was carried out in solid state and submerged fermentation using various cellulytic fungi as described in section 3.3.

3.11.1. Submerged fermentation for cellulase production

Submerged batch cultivation experiments were carried out in 250 mL Erlenmeyer flasks containing 70 mL Mandel Weber medium having pH 5.5, 2.5% wheat bran and 1% avicel as inducers. Flasks were inoculated with spore suspension (1×10^7 spores/mL) of *T. reesei* and *Penicillium* sp. and incubated at 30 °C, 150 rpm. After 8 d, the enzyme was harvested by

centrifugation for 10 min at 5000 rpm at 4 °C. The supernatant was used as a source of cellulase enzyme and stored at 4 °C for further use (Oberoi et al., 2010).

3.11.2. Solid state fermentation for cellulase production

A. niger was grown under SSF in 500 mL Erlenmeyer flasks containing 60 mL RM medium of pH 4.8 using 6 g wheat bran. Each flask was inoculated with four fungal discs (8 mm diameter each) cut from the periphery of 5 day old fungal colony. The flasks were incubated at 30 °C under static condition. After 7 d, the enzyme was harvested by adding 10 volumes of double distilled water containing 0.15% Triton X-100 and kept under shaking at 25 °C for 1 h. The enzyme was harvested by filtering through double layers of muslin cloth. The enzyme solution which was collected after filtration was centrifuged for 10 min at 5000 rpm and 4 °C. The supernatant collected after centrifugation was stored at 4 °C for further use (Tiwari et al., 2016).

3.12. Enzymatic saccharification of biomass

The cellulase enzyme produced by selected fungal strains was employed for the hydrolysis of pre-treated SCB using crude cellulase of *Penicillium* sp., *A. niger, T. reesei* and commercial enzyme (Cellulase blend, Sigma) at an enzyme loading of 10 FPU/gds. The 12.5 mL hydrolysis reaction mixture contained 0.5 g (solid loading: 4%, dry wt.) sugarcane bagasse with 50 mM sodium citrate buffer (pH 5.0) and 0.5% (v/v) Tween 80. Sodium azide (0.01%) was added to check microbial growth during hydrolysis. The flasks were incubated after enzyme addition at 50 °C under shaking at 150 rpm. The samples were taken at various time intervals (0, 6, 12, 24, 48 and 72 h) for analysis of total reducing sugars.

3.13. Optimization of saccharification

Effect of different parameters was evaluated using one factor at a time (OFAT) approach to obtain the maximum release of total reducing sugar during hydrolysis of sequentially

pretreated SCB. The factors considered were enzyme dose (FPU/gds), incubation temperature (°C) for hydrolysis, initial pH and Tween 80 concentration (%, v/v). Each factor was varied once at a time and its optimal values were employed during subsequent experiments.

3.13.1. Effect of Enzyme dose

The effect of different enzyme dosages ranging from 10-25 FPU/gds, on the enzymatic hydrolysis of sequentially pretreated SCB was studied. The enzymatic hydrolysis of substrate was carried out at 15.0 %, (dry wt.) solid loading in 0.05 M sodium citrate buffer (pH 5.0). Before enzyme loading, the slurry to be hydrolysed was supplemented with 1% (v/v) Tween 80 and then acclimatized by incubating at 50 °C on incubator shaker. Thereafter, varied dosages of enzymes were added to the pre-incubated cellulose slurry and reaction was continued till the sugar release became constant. Samples were withdrawn at regular intervals (0, 6, 12, 24, 48 and 72 h), centrifuged at 10,000 rpm for 10 min and the supernatant was analyzed for total reducing sugars. The flask with enzyme dose 20 FPU/gds was considered as control for the experiment.

3.13.2. Effect of incubation temperature

The effect of incubation temperatures on the enzymatic hydrolysis of sequentially pretreated SCB was studied by performing the hydrolysis at 42, 45 and 50 °C. The enzymatic hydrolysis of the substrate was carried out at 15.0 % solid loading in 0.05 M citrate buffer (pH 5.0). Before enzyme loading, the slurry was acclimatized by incubating at various incubation temperatures (42, 45 and 50 °C) on incubator shaker at 150 rpm. Thereafter, a mixture of 20 U FPU/gds was added to the pre-incubated cellulose slurry and reaction was continued till 72 h. Samples were withdrawn at regular intervals, centrifuged at 10,000 rpm for 10 min and the supernatant was analyzed for total reducing sugars. The flask incubated at temperature 42 °C was considered as control for the experiment.

3.13.3. Effect of initial pH

The enzymatic hydrolysis of sequentially pretreated SCB was carried out at 15.0 % solid loading in 0.05 M citrate buffer of different pH values (4.0-5.5). Before enzyme loading, the slurry was acclimatized by incubating at 50 °C on incubator shaker at 150 rpm. Thereafter, a mixture of 20 FPU/gds was added to the cellulose slurry and reaction was continued till 72 h. Samples were withdrawn at regular intervals, centrifuged at 10,000 rpm for 10 min and the supernatant was analyzed for total reducing sugars. The flask with initial pH 5.0 was considered as control for the experiment.

3.13.4. Effect of Tween 80

The effect of different concentrations of surfactant (Tween 80) on the enzymatic hydrolysis of sequentially pretreated SCB was studied by supplementing Tween 80 ranging from 0.1-2.0 % (v/v). The enzymatic hydrolysis of the substrate was carried out at 15.0 % substrate consistency in 0.05 M sodium citrate buffer. Before enzyme loading, the slurry was supplemented with different concentrations of Tween 80 and then acclimatized by incubating at 50 °C on incubator shaker. Thereafter, a mixture of 20 U FPU/gds was added to the slurry and reaction was continued till 72 h. Samples were withdrawn at regular intervals, centrifuged at 10,000 rpm for 10 min and the supernatant was analyzed for total reducing sugars. The flask with 0.5%, v/v Tween 80 was considered as control for the experiment.

3.14. Separate hydrolysis and fermentation (SHF)

Enzymatic hydrolysis was performed at a solid loading of 15% (dry wt.) (7.5g) of sequentially pretreated SCB and enzyme dose of 10, 15, 20 and 25 FPU/gds. Hydrolysis was carried in 250 mL capped flasks with a working volume of 50 mL. The pH was adjusted to 5.0 during hydrolysis by 50mM sodium citrate buffer and flasks were incubated at 50 °C under shaking at 150 rpm for 72 h. After completion of hydrolysis, the enzymatic hydrolysate

was recovered by centrifugation at 4 °C and 10,000 rpm and 48 mL of it was supplemented with yeast extract (10 g/L) and peptone (20 g/L) prior to fermentation.

Fermentation of the enzymatic hydrolysate slurry after nutrient supplementation (yeast extract and peptone) was carried out by inoculating the flasks with 10% v/v of yeast cells having an OD_{600} of 0.6, containing nutrients supplemented enzymatic hydrolysate slurry. The flasks were incubated at 42 °C under shaking at 150 rpm for 72 h. The samples (1 mL) were withdrawn at 0, 12, 24, 48 and 72 h and analysed for ethanol and residual glucose by high performance liquid chromatography (HPLC).

3.15. Batch simultaneous saccharification and fermentation (SSF) under shake-flask

SSF of sequentially pretreated SCB was performed in 250 mL capped flasks with a reaction volume of 50 mL. The flasks were supplemented with: (g/L) yeast extract (10), peptone (20), acetic acid (3), furfural (1), vanillin (1), 0.5 % (v/v) Tween 80 and pH 5 was maintained. The enzyme dose of 20 FPU/gds and solid loading of 15 % (dry wt.) was employed during SSF. The flasks were inoculated with yeast inoculum (10 %, v/v) ($O.D_{600}$ 0.6) and incubated at 42 °C under shaking at 200 rpm. The set of flasks without inhibitors served as a control for the experiment.

Batch SSF at high gravity was performed at solid loadings of 18, 20 and 30%, dry wt. under the same conditions mentioned above, without inhibitors. The samples were withdrawn intermittently at 0, 6, 12, 18, 24, 36, 48, 72 h centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was used for sugars and ethanol estimation by high performance liquid chromatography analysis.

3.16. Batch simultaneous saccharification and fermentation (SSF) at bench-top fermenter level

Fermentation studies were carried out in 3 L capacity bioreactor (Applikon Biotechnology, Netherlands) with 1 L working volume. The vessel of the fermenter was single walled, non-

jacketed and made of glass and. The fermentation by *K. marxianus* JKH5 C60 strain was carried out using 20% (dry wt.) sequentially pretreated SCB supplemented with (g/L) yeast extract (10), peptone (20), acetic acid (3), furfural (1), and vanillin (1) at 42 °C. The initial pH was adjusted to 5.0 prior to sterilization and not adjusted during the experiment. The enzyme dose of 20 FPU/gds was used for biomass hydrolysis. Yeast inoculum (10%, v/v) of 12 h old culture (1.04×10^8 cells/mL) was employed for starting the fermentation and incubation was carried out for 72 h. The agitation speed was 200 rpm and the aeration was not maintained during the fermentation. The samples were withdrawn at regular intervals of 6 h and centrifuged at 10,000 rpm for 10 min at 4 °C. The fermentation without supplementation of inhibitors was served as a control for the experiment. The supernatant was used for the estimation of sugar and ethanol content by HPLC analysis.

3.17. Fed-batch simultaneous saccharification and fermentation under shake-flask

The fed-batch simultaneous saccharification and fermentation of biomass at high solids loading was performed as per trials corresponding to the feeding strategy and solid loadings mentioned in Table 3.5. The feeding of enzyme was carried out in two different manners. In the first method, total enzyme 20 FPU/gds was added at 0 h (Trial A, B, and C). In the second method, 1/3rd FPU/gds of total enzyme dose of was added at 0, 6 and 12 h (Trial D, E and F). In both the cases, the total enzyme load was same i.e. 20 FPU/gds. The pretreated slurry was supplemented with pre-sterilized solutions of yeast extract, and peptone to a final concentration at 10 g/L and 20 g/L under aseptic conditions. The flasks were inoculated with *K. marxianus* JKH5 C60 cells from overnight culture (10% , v/v) and incubated at 42 °C with agitation at 200 rpm.

Table 3.5: Adopted strategies for feeding of enzyme and biomass during fed-batch simultaneous saccharification and fermentation of SCB

Trial	Solid loading (%, dry wt.)	Feeding Time of Biomass					
		0 h		6 h		12 h	
		Biomass (%, dry wt.)	Enzyme (FPU/gds)	Biomass (%, dry wt.)	Enzyme (FPU/gds)	Biomass (%, dry wt.)	Enzyme (FPU/gds)
A	18	6	20	6	-	6	-
B	20	6.6	20	6.6	-	6.6	-
C	30	10	20	10	-	10	-
Trial	**Solid loading (%, dry wt.)**	**Feeding Time of Biomass + Enzyme**					
		0 h		**6 h**		**12 h**	
		Biomass (%, dry wt.)	**Enzyme (FPU/gds)**	**Biomass (%, dry wt.)**	**Enzyme (FPU/gds)**	**Biomass (%, dry wt.)**	**Enzyme (FPU/gds)**
D	18	6	6.6	6	6.6	6	6.6
E	20	6.6	6.6	6.6	6.6	6.6	6.6
F	30	10	6.6	10	6.6	10	6.6

Fed-batch simultaneous saccharification and fermentation with inhibitors was carried out under same conditions mentioned above by supplementating inhibitor cocktail containing: (g/L) acetic acid (3), furfural (1), vanillin (1). The enzyme dose was 20 FPU/gds and solid loading of 20% (dry wt.) were employed by following trial E (Table 3.5) and pH 5. All the flasks were incubated at 42 °C under shaking at 200 rpm. The set of flasks without inhibitors served as a control for the experiment. The samples were withdrawn intermittently at 0, 6, 12, 48 and 72 h, and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was used for the estimation of sugar and ethanol content by HPLC

3.18. Fed-batch simultaneous saccharification and fermentation at bench-top fermenter level

The fed-batch fermentation of pretreated SCB was scaled up to 3 L bioreactor (Applikon Biotechnology, Netherlands). Fed-batch fermentation was carried out with an initial substrate consistency of 6.6 % (w/v) in 50 mM sodium citrate buffer at 42 °C. The initial pH was adjusted to 5.0 prior to sterilization and not adjusted during the experiment. The agitation speed was 200 rpm and the aeration was not maintained during the fermentation. Before

enzyme loading, the slurry was acclimatized to attain 50 °C and thereafter, an enzyme dosage of 6.6 FPU/ gds, 0.5 % (v/v) Tween 80 and solution of (g/L); yeast extract (10) and peptone (20), acetic acid (3), furfural (1), vanillin (1) was added to bioreactor. The strategy 'Trial E' (Table 3.5) was followed for feeding during fermentation at bench-top fermenter level. The feeding of enzyme and biomass was done via opening a valve under sterile conditions. The fermenter run without inhibitors served as a control for the experiment. The samples were withdrawn at regular intervals, centrifuged at 10,000 rpm for 10 min and the supernatant was used for the estimation of sugar and ethanol content by high performance liquid chromatography analysis.

3.19. Pentose fermentation

To make the bioethanol production process cost-effective, a biorefinery approach comprising efficient utilization of all the components of lignocellulosic biomass is a pre-requisite. Xylose, a component of hemicellulose and released during dilute acid pretretment of SCB, was first detoxified and fermented to ethanol using pentose fermenting yeast.

3.19.1. Detoxification of acid hydrolysate

Detoxification of the acid hydrolysate (100 mL) of SCB was done by increasing the pH to 7 using alkali $Ca(OH)_2$ under constant stirringat room temperature. After neutralisation, the hydrolysate was filtered under vacuum and the filtrate was analyzed for sugars and inhibitors. Activated charcoal (2%, w/v) treatment of the neutralized hydrolysate was performed under stirring conditions at room temperature for 30 min, followed by vacuum filtration of the hydrolysate. The filtrate was analysed for sugars and inhibitors (Zhang et al., 2018).

3.19.2. Fermentation

The fermentation of detoxified acid hydrolysate using was carried out in Erlenmeyer flasks with a working volume of 50 mL after supplementation with YP medium and maintaining pH

5.5 for ethanol production. The flasks were then inoculated with 10.0 % (v/v) of 18 h old culture (2.89×10^8 cells/ mL) of *P. stipitis* NCIM 3499 and were incubated at 30 °C under shaking at 150 rpm. The samples were withdrawn intermittently (0, 48 and 72 h) and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was used for the estimation of sugar and ethanol by HPLC analysis

3.20. Analytical methods

3.20.1. Determination of total reducing sugars

The estimation of total reducing sugars was carried out using the 3,5-Dinitrosalicylic acid (DNSA) reagent, as described by Miller (1959). The reaction mixture containing 1.0 mL appropriately diluted sugar solution and 1.0 mL DNSA reagent was incubated at 100 °C for 5 min in a boiling water-bath. The amount of reducing sugars was determined by taking glucose as standard and measuring absorbance at 540 nm against reaction blank.

3.20.2. High performance liquid chromatography (HPLC)

All samples were centrifuged at 10,000 rpm for 10 min to remove the insoluble particles and the supernatant was filtered through a 0.2 mm filter prior to HPLC analysis. Glucose, xylose and ethanol were analysed using HPLC (Cecil, UK) fitted with Bio-Rad Aminex HPX-87H column operating at 50 °C. The mobile phase was 5 mM H_2SO_4 at flow rate of 0.6 mL/min.

3.20.3. Determination of furans

The estimation of furans was carried out using the method described by Martinez et al. (2000).The amount of furans was estimated by measuring the absorbance of samples at 284 and 320 nm and calculated as follows:

$$A_{284} - A_{320} = 0.127 \times \text{Total furans (mg/L)} + 0.05$$

3.20.4. Determination of phenolics

The estimation of phenolics was carried out using the Folin-Ciocalteu's Reagent, as described by Singleton et al. (1999). The reaction mixture containing 3.0 mL distilled water, 50 µL appropriately diluted sample and 250 µL of Folin- Ciocalteu reagent was incubated at 30 °C for 1 min. After incubation, 750 µL Na_2CO_3 (10 %) was added to the reaction mixture followed by incubation at 30 °C for 60 min in dark. The amount of phenolics was determined by measuring absorbance at 760 nm against reaction blank.

3.20.5. Cell biomass estimation

For determination of cell biomass, 1mL sample was centrifuged in pre-weighed micro-centrifuge tubes at 10000 rpm for 5 min. The pellet was washed twice with double distilled water and dried in a vacuum oven at 80 °C to a constant weight and the cell biomass was denoted in g/L.

3.20.6. Cellulase activity

The enzyme assay for overall cellulase activity was estimated by using the IUPAC method (Ghose, 1987). The cellulase activity was determined using Whatman No. 1 filter paper strip (50 mg or 1 cm × 6 cm) as the substrate. The reaction mixture containing 1.0 mL citrate phosphate buffer (50 mM, pH 5.0), 50 g Whatman No. 1 filter paper and 500 µL of appropriately diluted enzyme solution was incubated for 60 minutes at 50 °C in a water bath. The reaction was terminated by addition of 3.0 mL DNS reagent followed by incubation at 100 °C for 5 minutes in a boiling water bath. The release of sugars was determined by measuring absorbance at 540 nm against reagent blank, substrate control and enzyme control prepared under similar conditions. One international unit of cellulase was defined as the amount of enzyme required to release 1 µmol of glucose per min under the standard assay conditions and was expressed as filter paper unit (FPU)mL.

3.20.7. Endoglucanase (Carboxymethyl cellulase; CMCase) activity

The enzyme assay for endoglucanase was carried out using the IUPAC method (Ghose, 1987). The Endoglucanase activity was determined using 2% carboxymethyl cellulose suspension (prepared in 50 mM citrate phosphate buffer, pH 5.0) as the substrate. The reaction mixture containing 0.5 mL substrate suspension and 500 µL of appropriately diluted enzyme solution was incubated for 30 min at 50 °C in a water bath. The reaction was terminated by addition of 3.0 mL DNS reagent followed by incubation at 100 °C for 5 min in a boiling water bath. The release of sugars was determined by measuring absorbance at 540 nm against reagent blank, substrate control and enzyme control prepared under similar conditions. One international unit of CMCase was defined as the amount of enzyme required to release 1 µmol of glucose per minute under the standard assay conditions.

3.20.8. Cellobiase (β-glucosidase) activity

The enzyme assay for β-glucosidase was carried out using the IUPAC method (Ghose, 1987). The β-glucosidase activity was determined using 0.1% paranitrophenyl-β-D-glucopyranoside (prepared in 50 mM citrate phosphate buffer, pH 5.0 as the substrate. The reaction mixture containing 1.0 mL substrate solution, 1.0 mL citrate phosphate buffer (pH 5.0) and 1.0 mL appropriately diluted enzyme solution was incubated for 10 min at 50 °C in a water bath. The reaction was terminated by addition of 2.0 mL 10% Na_2CO_3. The release of *para*-nitrophenol was determined by measuring absorbance at 400 nm against reagent blank, substrate control and enzyme control prepared under similar conditions. One international unit of β-glucosidase was defined as the amount of enzyme required to release 1 µmol of *para*-nitrophenol per min under the standard assay conditions.

3.20.9. Enzymatic saccharification efficiency

The enzymatic saccharification efficiency during the hydrolysis experiment was carried out using the following equation:

$$Saccharification\ (\%) = \frac{Amount\ of\ reducing\ sugar\ released\ (g)}{Amount\ of\ cellulose\ present\ in\ the\ substrate\ (g)} \times 0.9 \times 100$$

RESULTS

RESULTS

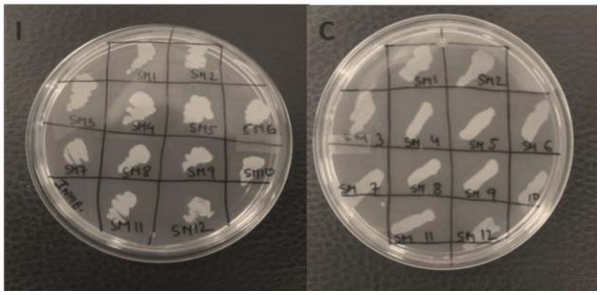
4.1. Isolation, screening and selection of thermotolerant inhibitor tolerant yeasts

In this study a total of one hundred fifty thermotolerant yeasts were isolated. **Table 4.1** shows the yeasts isolated from different samples comprising of citrus fruit and cane juices and fruits (from local market), and bagasse and soil samples (from dumping sites of sugar mill) from northern region of India. The isolates were screened for tolerance towards fermentation inhibitors (acetic acid, furfural, and vanillin). Out of one hundred fifty isolates, thirty six yeast isolates were tolerant to inhibitors and grown well at higher concentrations (200 g/L) of glucose and ethanol (7%, v/v) (**Figure 4.1**). Further, these thirty six yeast isolates were carefully examined and screened for ethanol production at higher temperature (42 °C) **(Table 4.2).**

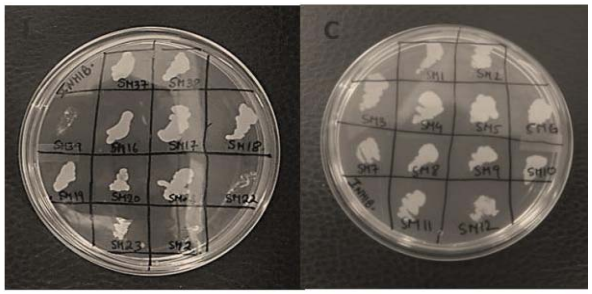
Thirty six screened yeast isolates were studied for ethanol production under shake flask **(Table 4.2**) at higher glucose concentration (150 g/L). Among those, isolates 1A, SM4, SM5 and SM7 produced 55 ± 0.5, 51 ± 1.2, 54 ± 0.7 and 52 ± 0.7 g/L ethanol respectively, after 18 h at 42 °C. The ethanol titers observed were significantly higher than that of others *($p < 0.05$*) and therefore, these four isolates were selected for further fermentation studies. Standard thermotolerant yeast cultures *K. marxianus* NCIM 3565 and *K. marxianus* MTCC 4136 produced 38 ± 0.2 and 32 ± 0.6 g/L ethanol respectively, after 18 h at 42 °C. Isolates 1A, SM 4, SM 5 and SM 7 produced higher ethanol titers than the standard cultures and hence selected for further experiments.

Table 4.1: Isolation of thermotolerant yeasts from various environmental samples

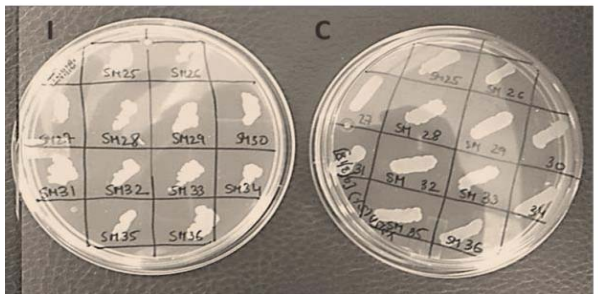
Sample	Site of Collection	No. of Samples	No. of isolates	Name of isolate
Apple	Fruit market, Mahendergarh, Haryana	5	2	5A1, 5A2
Berry	Fruit market, Noida	2	2	B1, B2
Grape	Mahendergarh, Haryana	2	4	G1, G2, G3, G4
Citrus fruit	Rewari, Haryana	1	15	C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14, C15
Sapota	Delhi	1	2	S1 and S2
Blackcurrant	Dadri, Haryana	1	5	BG1, BG2, BG3, BG4, BG5
Whey	Mahendergarh, Haryana	1	11	W1, W2, W3, W4, W5, W6, W7, W8, W9, W10, W11
Citrus Juice	Mahendergarh, Haryana	2	1	K1
Mix fruit Juice	Dadri, Haryana	1	4	MJ1, MJ2, MJ3, MJ4
Date palm	Mahendergarh	2	5	DP1, DP2, DP3, DP4, DP5
Moist bagasse	Mahendergarh, Haryana	3	8	SB11, SB12, SB13, SB14, SB21, SB22, SB23, SB24
Sugarcane bagasse	Mahendergarh, Haryana		4	S1, S2, S3, S4
sugar mill dumping site soil	Haridwar, Uttrakhand	8	12	1A, 4A1, 4A2, 5A1, 5A2, 6B, 7B, 8B1, 8B2, 9A1, 9B2,11A
Distillery waste	Karnal, Haryana	12	12	DW1, DW2, DW3, DW4, DW5, DW6, DW7 DW8, DW9, DW10, DW11, DW12
Distillery waste	Muzaffarnagar, Uttar Pradesh	2	2	DW13, DW14
Distillery waste	Pathankot, Punjab	3	3	DW15, DW16, DW17
Bagasse, soil, cane juice from Sugar mill	Sonipat, Haryana	56	57	SM1, SM2, SM3 SM4, SM5, SM6 SM7, SM8, SM9 SM10, SM11, SM12 SM13, SM14, SM15 SM16, SM17, SM18 SM19, SM20, SM21 SM22, SM23, SM24 SM25, SM26, SM27 SM28, SM29, SM30 SM31, SM32, SM33 SM34, SM35, SM36 SM37, SM38, SM39, SM40, SM41, SM42, SM43, SM44, SM45, SM46, SM47, SM48, SM49, SM50 SM51, SM52, SM53 SM54, SM55, SM56 SM57



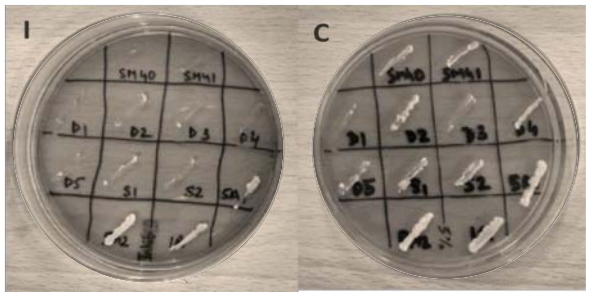
SM1		SM2	
SM3	SM4	SM5	SM6
SM7	SM8	SM9	SM10
SM11		SM12	



SM13		SM14	
SG9	SM16	SM17	SM18
SM19	SM20	SM21	SM22
SM23		SM24	



SM25		SM26	
SM27	SM28	SM29	SM30
SM31	SM32	SM33	SM15
SM35		SM36	



SM40		SM41	
SM42	SM43	SM44	SM45
5A1	SM46	SM47	SM48
1A		5A2	

Figure 4.1: Screening of various thermotolerant yeasts for inhibitor(s) tolerance on agar medium. *I-inhibitor cocktail plates (acetic acid, furfural, vanillin); C-Control plate (without inhibitors)

Table 4.2: Quantitative screening of thermotolerant yeasts for ethanol production

Isolate	Ethanol (g/L)	Isolate	Ethanol (g/L)	Isolate	Ethanol (g/L)
1A (JKH1)	55 ± 0.5	SM 10	18 ± 0.2	SM 23	17 ± 0.4
5A1	26 ± 0.1	SM 11	17 ± 0.3	SM 25	16 ± 0.9
5A2	43 ± 0.1	SM 12	16 ± 0.2	SM 26	19 ± 0.9
SM 1	21 ± 0.1	SM 13	16 ± 0.1	SM 27	34 ± 0.6
SM 2	17 ± 0.1	SM 14	18 ± 2.2	SM 28	16 ± 0.5
SM 3	16 ± 0.1	SM 15	18 ± 0.4	SM 30	16 ± 1.6
SM 4 (JKH 4)	51 ± 1.2	SM 16	18 ± 0.5	SM 31	13 ± 0.4
SM 5 (JKH 5)	54 ± 0.7	SM 17	17 ± 0.5	SM 32	18 ± 0.3
SM 6	49 ± 0.2	SM 18	16 ± 3.8	SM 33	15 ± 0.8
SM 7 (JKH 7)	52 ± 0.7	SM 19	16 ± 0.6	*K. marxianus* NCIM 3565	38 ± 0.2
SM 8	50 ± 0.5	SM 20	19 ± 0.6	*K. marxianus* MTCC 4136	32 ± 0.6
SM 9	21 ± 0.1	SM 21	15 ± 0.4		

4.2 Identification of screened yeast isolates SM, SM4, SM5 and SM7

Yeast isolates SM1, SM4, SM5 and SM7 (renamed as JKH1, JKH4, JKH5 and JKH7) were identified by morphological characteristics and molecular approach based on the ITS-5.8s rDNA sequence phylogenetic characterization.

4.2.1 Colony and cell morphology

Colonies of isolate JKH1 were flat with smooth to lobed margins and had off-white color (Figure 4.2 a) whereas the colonies of remaining isolates were raised, smooth, creamy and round **(**Figure 4.2 d, f & h**)** on YPD agar plates incubated at 42 °C. The cells of isolates were stained with Nigrosin black and observed under microscope for studying morphological features. The cell morphology of negatively stained yeasts under microscope (40X magnification) revealed that isolate JKH1 (Figure 4a) had large ellipsoidal shape and isolates

JKH4, JKH5 and JKH7 (Figure 4 c, e and g) were oval in shape. All the four isolates were unicellular, showed buds or bud scars, indicating budding mode of division/multiplication.

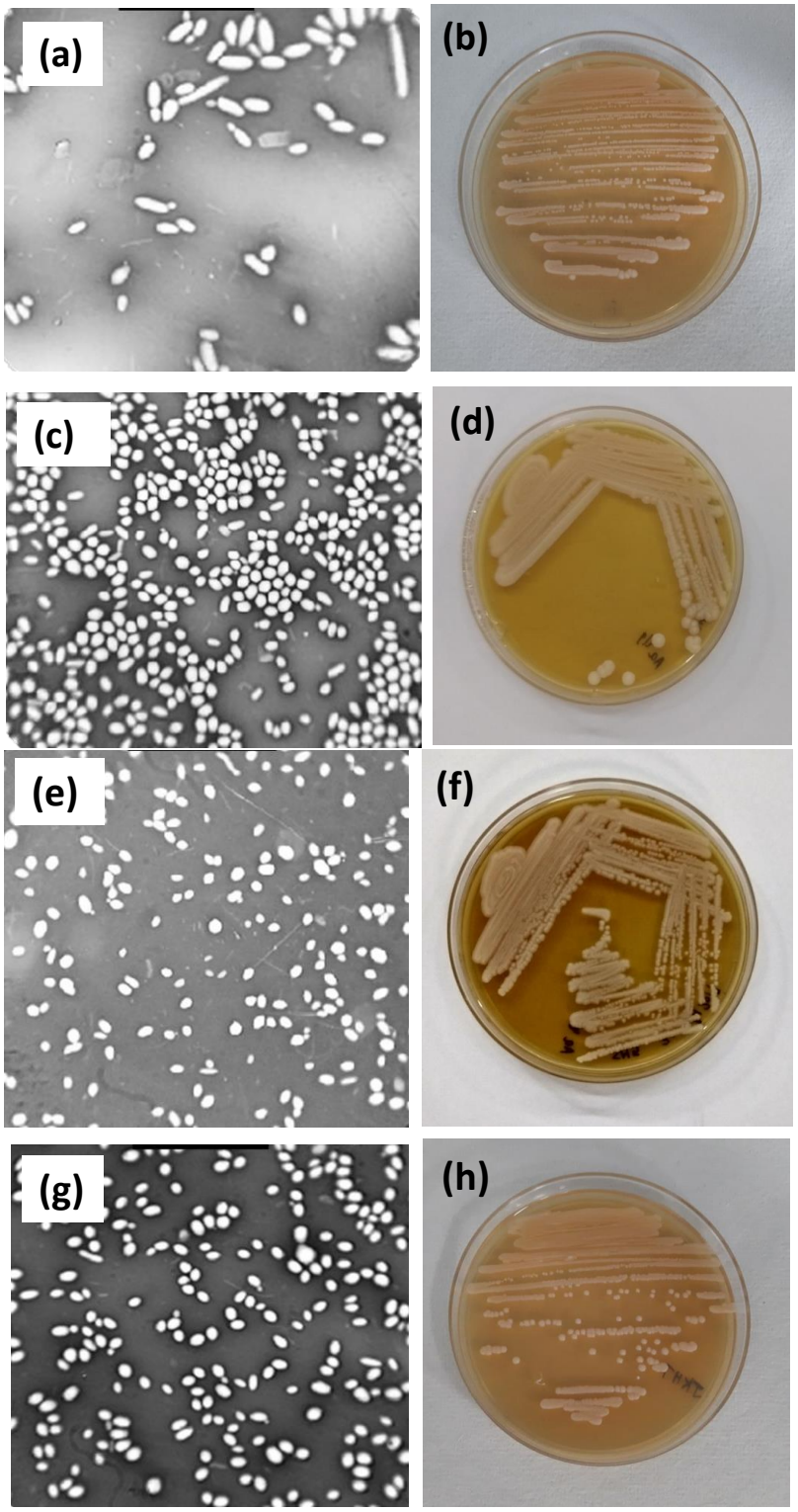


Figure 4.2: Colony characteristics and cell morphology (40× magnification) of yeast isolates JKH1 (a & b), JKH4 (c & d), JKH5 (e & f) and JKH7 (g & h).

4.2.2 Biochemical characterization

Sugar utilization pattern of the isolates JKH1, JKH4, JKH5 and JKH7 are shown in Figure 4.3 and Table 4.3. Results revealed that all four isolates utilized xylose sugar as indicated by change in color from Red color to yellow whereas galactose was utilized by JKH1, JKH5 and JKH7. Dulcitol sugar was only metabolised by isolate JKH5. Moreover, none of the isolates produced urease enzyme.

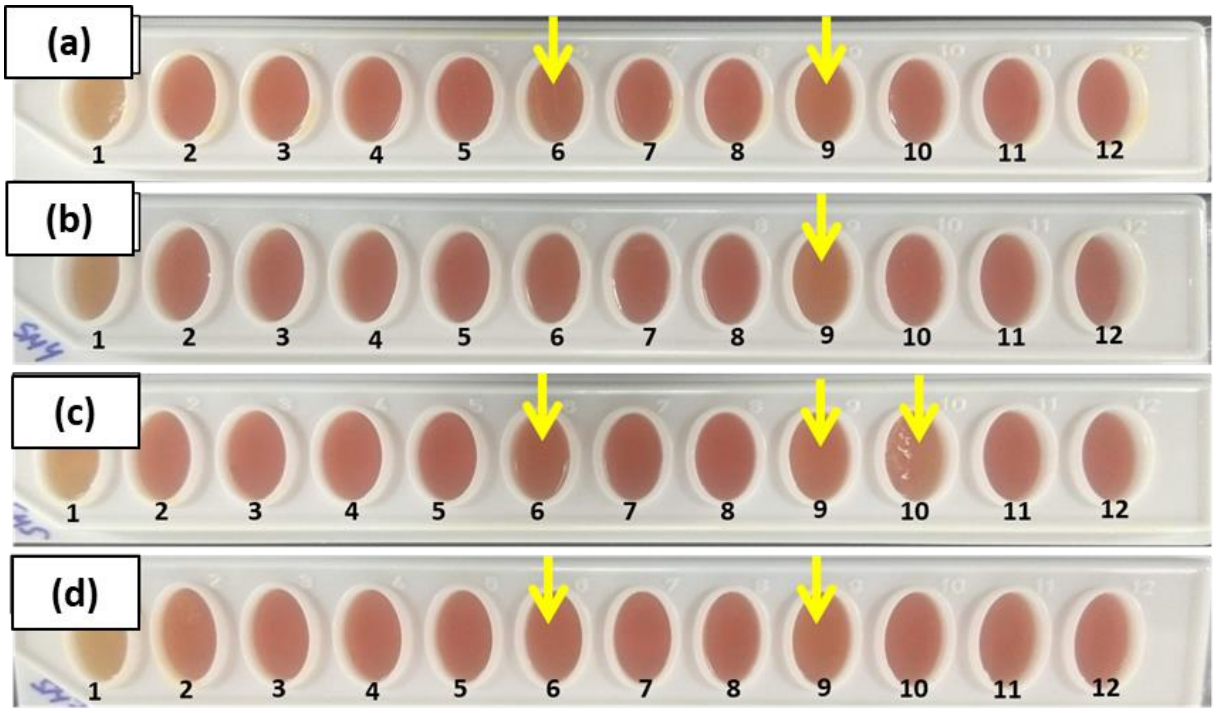


Figure 4.3: Biochemical characterization of selected yeast isolates JKH1 (a), JKH4 (b), JKH5 (c), and JKH7 (d) (1- Urease, 2- Melibiose, 3- Lactose, 4- Maltose, 5- Sucrose, 6- Galactose, 7- Cellobiose, 8- Inositol, 9- Xylose, 10- Dulcitol, 11- Raffinose, 12- Trehalose).

Table 4.3: Sugar utilization and urease production tests by yeasts isolates

S.No.	Test	Results			
		JKH 1	**JKH 4**	**JKH 5**	**JKH 7**
1	Urease	-	-	-	-
2	Melibiose	-	-	-	-
3	Lactose	-	-	-	-
4	Maltose	-	-	-	-
5	Sucrose	-	-	-	-
6	Galactose	+	-	+	+
7	Cellobiose	-	-	-	-
8	Inositol	-	-	-	-
9	Xylose	+	+	+	+
10	Dulcitol	-	-	+	-
11	Raffinose	-	-	-	-
12	Trehalose	-	-	-	-

+ = Color change from red to yellow indicates sugar utilization
- = No color change indicates sugars were not utilized

4.2.3 Molecular identification of yeasts

The yeast isolates were identified based on the variations in the sequences of internal transcriber spacer region (ITS) region. The obtained PCR amplicons of ITS 1, 5.8S rDNA region had molecular wt. of ~ 600-700 bp as shown in Figure 4.4.The obtained sequences (**Appendix I**) were analyzed using nucleotide BLAST algorithm of the DNA Data Bank of NCBI, Genbank. The analyses revealed that isolate JKH1 had 100% similarity with *P. kudriavzevii* KT000038.1 and isolates JKH4, JKH5 and JKH7 showed 100% similarity with *K. marxianus* KJ83098.1, *K. marxianus* P2 KF851351.1, *K. marxianus* MN450878.1, respectively. Hereafter, the isolates were named as *Pichia kudriavzevii* JKH1, *Kluyveromyces marxianus* JKH4, *Kluyveromyces marxianus* JKH5 and *Kluyveromyces marxianus* JKH7. The phylogenetic trees representing the evolutionary relatedness between the yeast species were drawn on the basis of distance matrix of homology sequences of similar

microorganisms by BLAST **(Figure 4.5).** Also, the partial genome sequences of *P. kudriavzevii* JKH1 belonging to *Pichiacea* family and *K. marxianus* JKH4, *K. marxianus* JK5 and *K. marxianus* JKH7, belonging to *Saccharomycetaceae* family and Saccharomycetales order were submitted to NCBI gene bank with GenBank accession numbers MK973094, MK973095, MK973096, and MK973097, respectively.

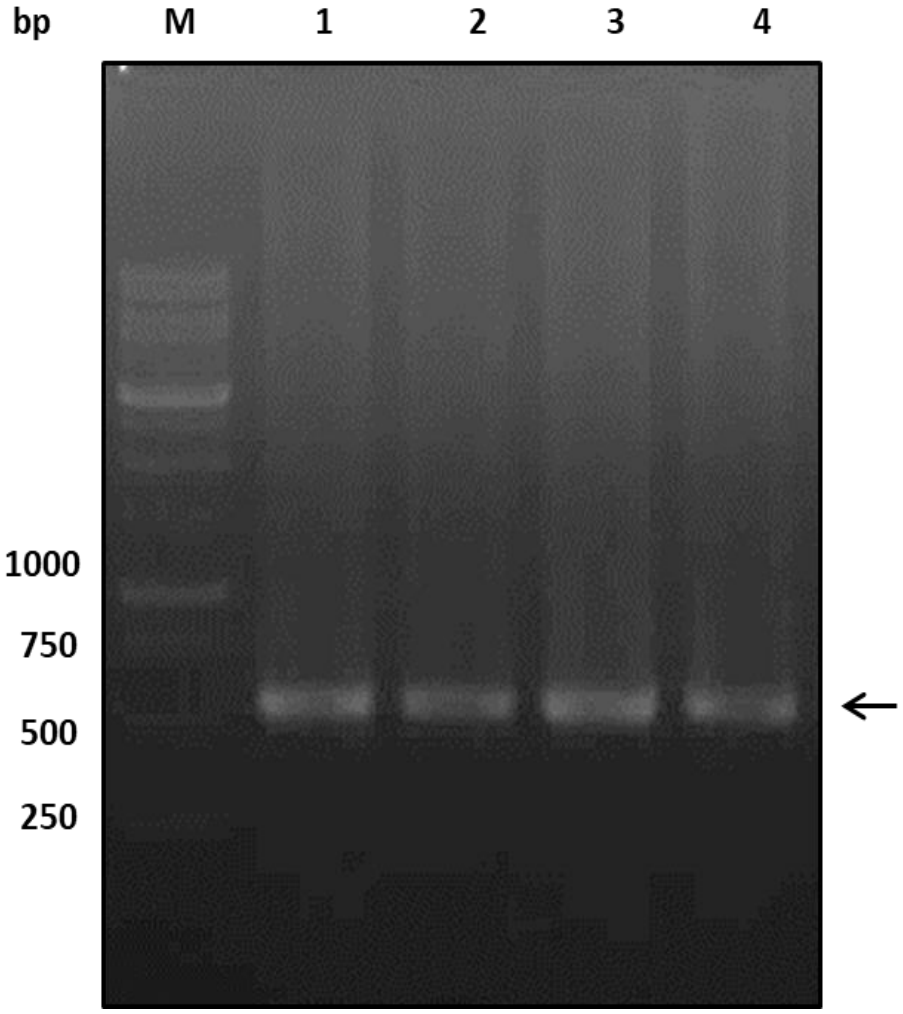


Figure 4.4: Agarose gel electrophoresis representing ITS-5.8S rDNA amplicons of selected yeast isolates. Lane 1: PCR amplicon of JKH1, Lane 2: PCR amplicon of JKH4, Lane 3: PCR amplicon of JKH5, Lane 4: PCR amplicon of JKH7 and Lane M: 1000 b.p. DNA ladder

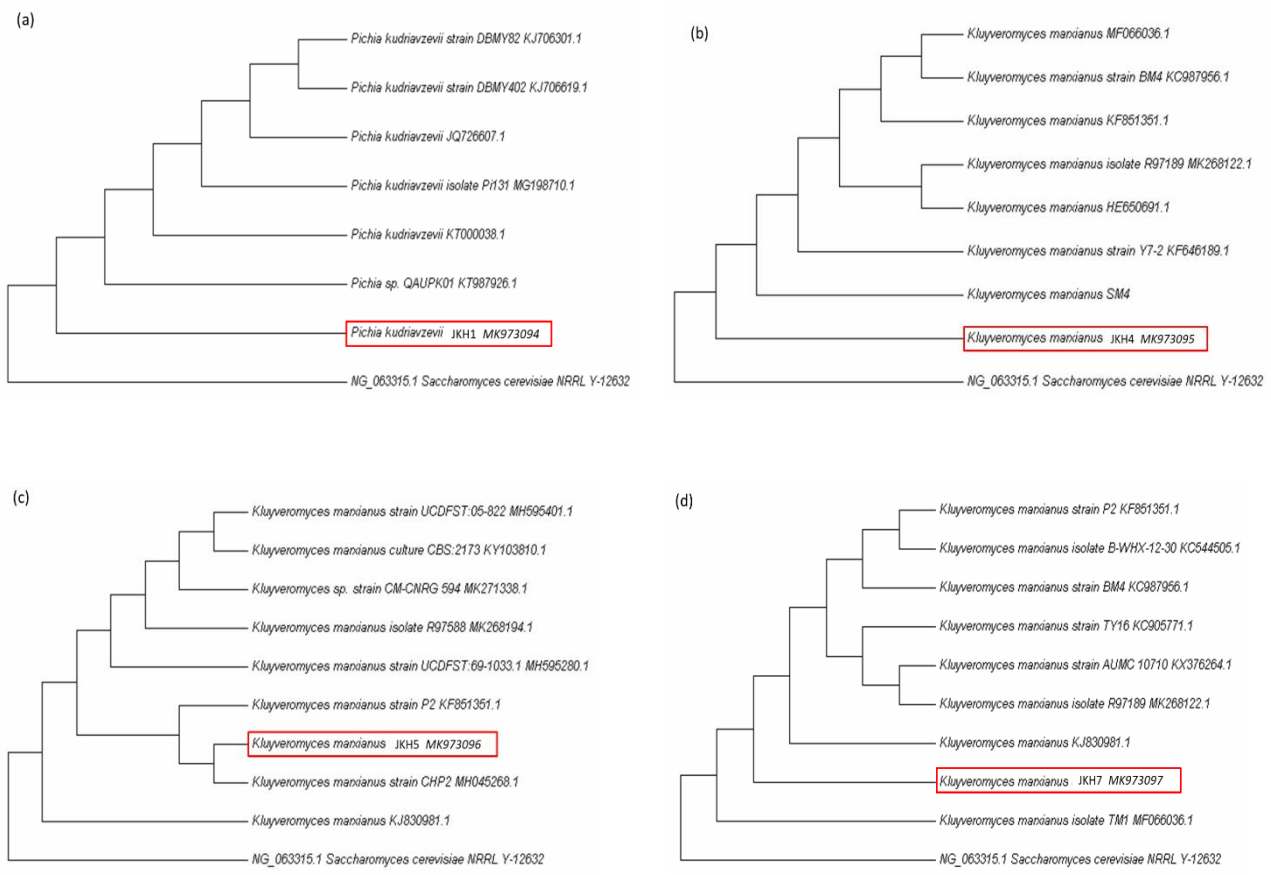


Figure 4.5: The phylogenetic trees showing genetic relatedness of *Pichia kudriavzevii* JKH1 (a), *Kluyveromyces marxianus* JKH4 (b), *Kluyveromyces marxianus* JKH5 (c) and *Kluyveromyces marxianus* JKH7 (d) with other yeasts based on their ITS-5.8S rDNA region

4.3 Fermentation of yeasts under varied initial glucose concentrations at 42 °C

The growth and fermentation of the selected four yeasts were assessed at different glucose concentrations ranging from 50 to 200 g/L. Ethanol production under batch fermentation was performed in YPD media containing various concentrations of glucose as the main carbon source as shown in Figure 4.6 and 4.7**.** *P. kudriavzevii* JKH1 produced 18.2, 38.9, 64.7 and 58.3 g/L at 50, 100, 150 and 200 g/L initial glucose respectively, after 24 h (Figure 4.6a). *K. marxianus* JKH4 produced 12.4, 39.1, 54.7 and 45.3 g/L at 50, 100, 150 and 200 g/L initial glucose respectively, after 24 h (Figure 4.6b). *K. marxianus* JKH5 produced 21.7, 47.8, 57.7 and 70.4 g/L at 50, 100, 150 and 200 g/L initial glucose respectively, after 24 h (Figure 4.6c).

K. marxianus JKH7 produced 25.2, 44.6, 54.7 and 71.7 g/L at 50, 100, 150 and 200 g/L initial glucose respectively, after 24 h (Figure 4.6d). *P. kudriavzevii* JKH1 and *K. marxianus* JKH5 produced higher ethanol titer (64.7 and 57.7 g/L, respectively) among other yeast strains, when initial glucose was 150 g/L.

The glucose consumption by the yeasts was decreased with the increase in initial glucose concentration from 50 to 200 g/L (Figure 4.7). At glucose concentration of 50 and 100 g/L, all four yeasts utilized sugar completely within 20 h. whereas when concentrations were 150 and 200 g/L, only *P. kudriavzevii* JKH1 and *K. marxianus* JKH5 were able to consume complete glucose at 48 h ((Figure 4.7 a and c) and achieved maximum ethanol titer. Other isolates and standard cultures could not consume complete glucose even after 48 h (Figure 4.7 b, d, e and f). Additionally, the average glucose consumption rate by both the strains was 6.2 g/L/h for 24 h of cultivation. Sugar was rapidly assimilated during the first 12 h of fermentation**.** After 24 hours, yeasts (*P. kudriavzevii* JKH1 and *K. marxianus* JKH5) utilized sugars, almost completely, as indicated by residual reducing sugar levels. The rate of utilization of glucose by both the strains was 6.2 g/L/h after 24 h of cultivation.

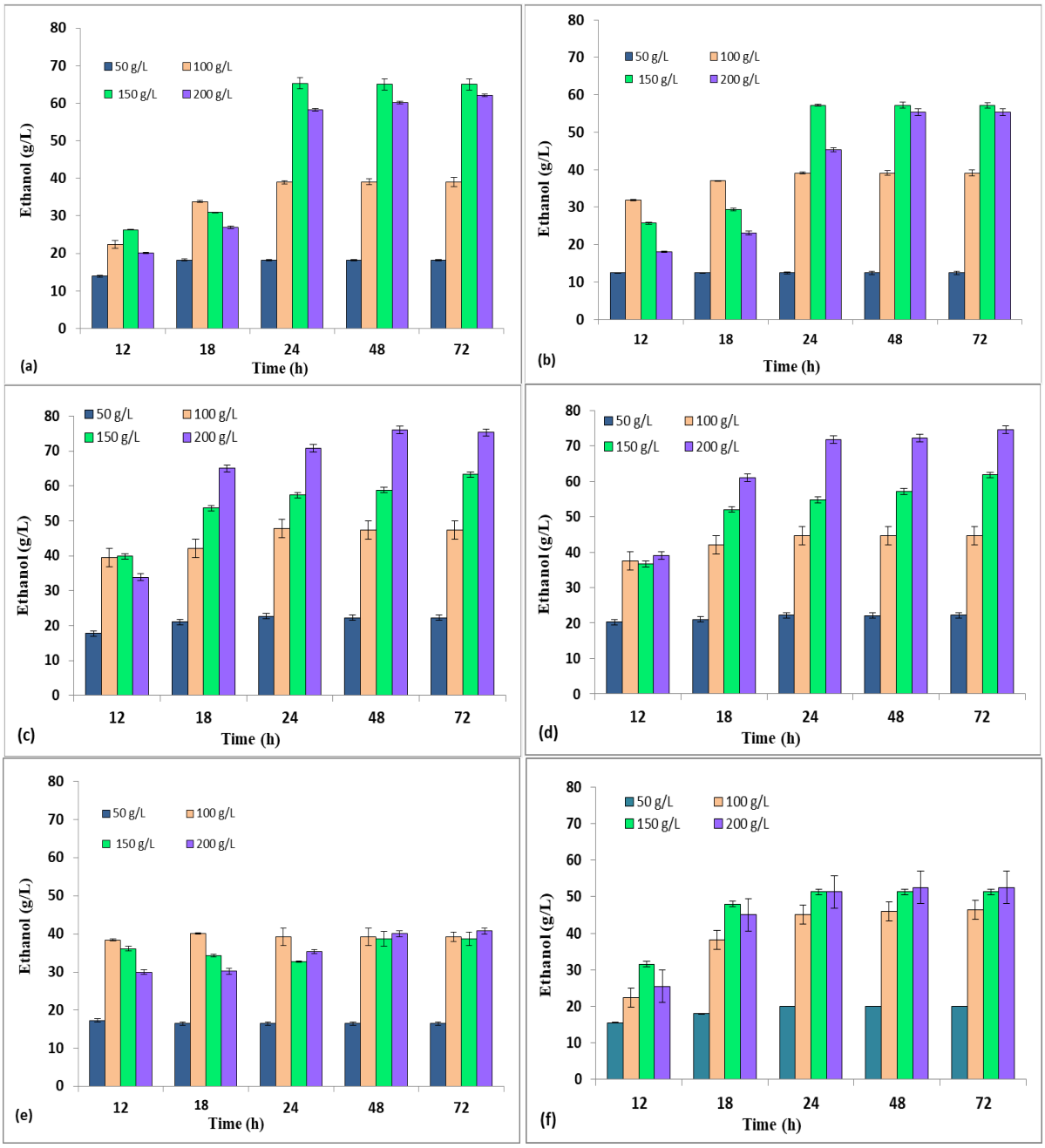


Figure 4.6: Effect of different glucose concentrations on ethanol production by *Pichia kudriavzevii* JKH1 (a), *Kluyveromyces marxianus* JKH4 (b), *Kluyveromyces marxianus* JKH5 (c) and *Kluyveromyces marxianus* JKH7 (d) and standard thermotolerant yeast strains *Kluyveromyces marxianus* NCIM 3565 (e) and *Kluyveromyces marxianus* MTCC 4136 (f).

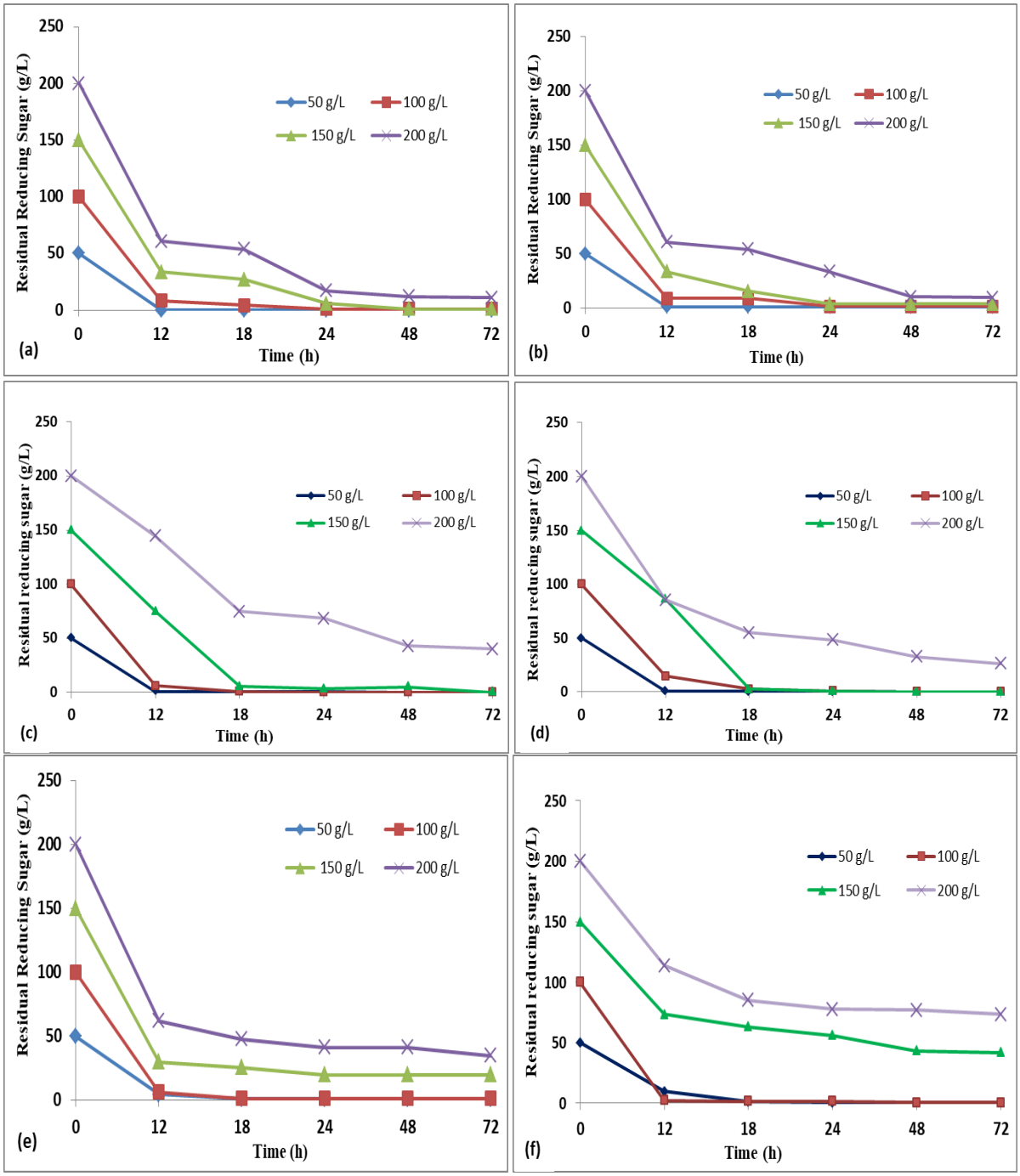


Figure 4.7: Effect of different glucose concentrations on residual reducing sugars during fermentation by *Pichia kudriavzevii* JKH1 (a), *Kluyveromyces marxianus* JKH4 (b), *Kluyveromyces marxianus* JKH5 (c) and *Kluyveromyces marxianus* JKH7 (d) and standard thermotolerant yeast strains *Kluyveromyces marxianus* NCIM 3565 (e) and *Kluyveromyces marxianus* MTCC 4136 (f).

The productivity of *P. kudriavzevii* JKH1 and *K. marxianus* JKH5 were 2.7 and 2.4 g/L/h, respectively **(Table 4.4**). On the basis of fermentation performance, of *Pichia kudriavzevii* JKH1 and *Kluyveromyces marxianus* JKH5 were selected further for improvement through evolutionary engineering. Additionally, both the strains are from two different genera therefore, studying their behaviour under stress was assumed to be more appropriate for the current study.

Table 4.4: Comparison of sugar utilization and ethanol production by selected thermotolerant yeasts

Isolate	Ethanol fermentation			Glucose** utilization	
	Ethanol titer (g/L)	Productivity** (g/L/h)	Yield (g/g)	Concentration (g/L)	Rate of glucose consumption (g/L/h)
Pichia kudriavzevii JKH 1	64.7 ± 2.9	2.7	0.43	149	6.2
Kluyveromyces marxianus JKH 4	54.7 ± 2.1	2.2	0.36	142	3.9
Kluyveromyces marxianus JKH 5	57.7 ± 2.1	2.4	0.38	149	6.2
Kluyveromyces marxianus JKH 7	54.7 ± 2.6	2.2	0.36	136	3.8
Kluyveromyces marxianus MTCC 4136*	45.6 ± 2.6	1.9	0.30	94	2.0
Kluyveromyces marxianus NCIM3565*	34.3 ± 0.2	1.4	0.23	127	1.8

*Standard cultures **Productivity and glucose consumption rate calculated at 24 h with initial glucose concentration of 150 g/L

4.4 Growth kinetics of selected

Growth pattern and cell biomass in terms of cell dry weight of selected yeast strains *P. kudriavzevii* JKH1 and *K. marxianus* JKH5 were studied over a period of 8 h. Growth curve was plotted by measuring cell $O.D_{600}$ and cell biomass against time after a regular interval of 30 min as shown in Figure 4.8 The growth curve of *P. kudriavzevii* JKH1 and *K. marxianus*

JKH5 showed specific growth rate (μ) 0.17 and 0.16 h^{-1}, with doubling time (t_d) 4.10 and 4.37 h, respectively.

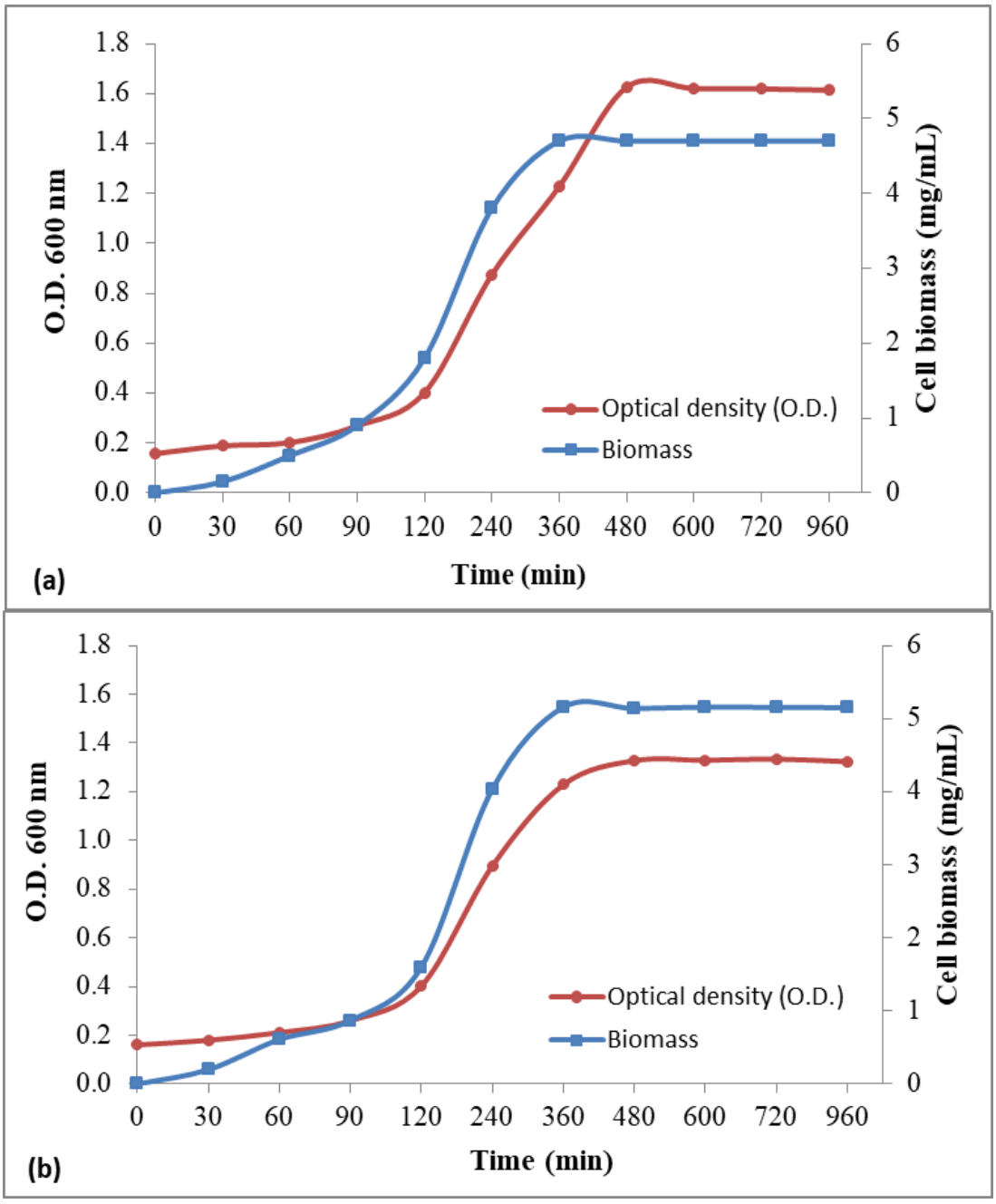


Figure 4.8: Growth curve of *Pichia kudriavzevii* JKH 1 (a) and *Kluyveromyces marxianus* JKH 5 (b)

4.5 Strain improvement of selected yeasts through adaptive laboratory evolution (ALE)

During the pretreatment of lignocellulosic biomass, degradation of components causes release of various chemical compounds such as furfural, vanillin, acetic acid etc. that are

inhibitory to fermenting microbes, by reducing their growth and hence, fermentation efficiency. The detrimental effect of inhibitors is believed to result from its accumulation in the cell, thereby inhibiting enzymes of the central carbon metabolism and disturbing the cells energy balance (Modig et al., 2002; Sárvári Horváth et al., 2003) as well as growth of yeast cells. In the current study, the tolerance of both the strains was studied in the presence of inhibitors (acetic acid, furfural, and vanillin), individually and in combination.

4.5.1 Inhibitor tolerance limit of *P. kudriavzevii* JKH1

In the presence of 4 g/L acetic acid in YPD broth, *P. kudriavzevii* JKH1 grew well, however, at 5 and 6 g/L cell growth was declined sharply (**Figure 4.9).** Likewise, less tolerance to higher concentrations (> 1 g/L) of furfural and vanillin in both YPD broth and agar was observed. However, the growth of the yeast was observed on YPD agar medium even at 5 g/L acetic acid and 3 g/L furfural individually after 36 h **(Figure 4.10**).

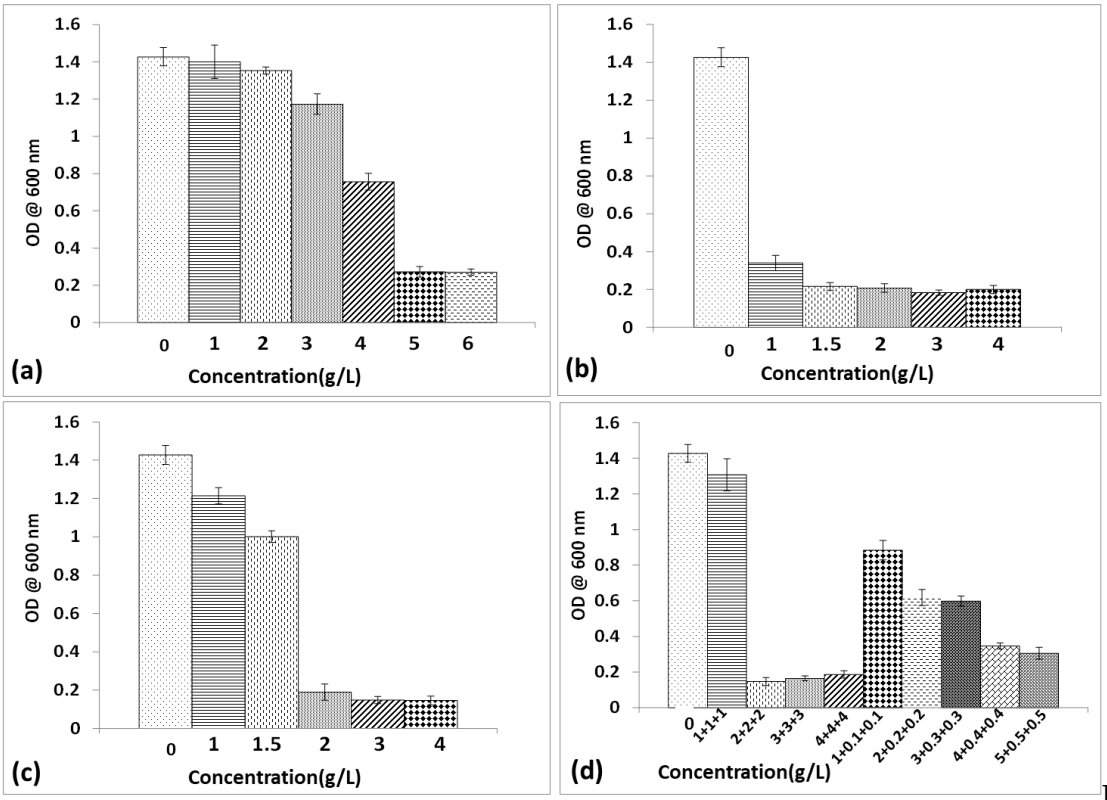
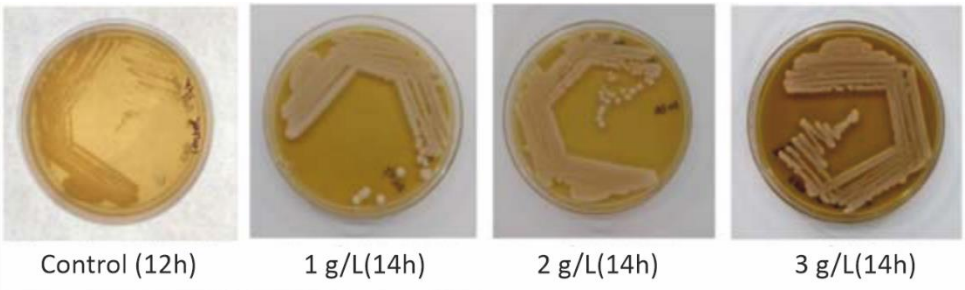
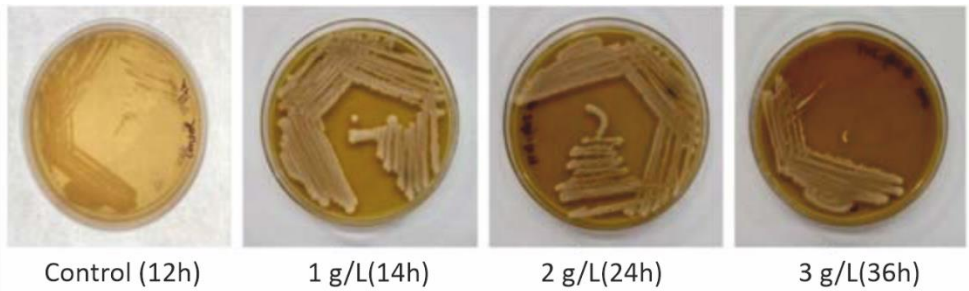


Figure 4.9: Effect of acetic acid (a), furfural (b), vanillin (c) individually and in combination (d) on the growth of *Pichia kudriavzevii* JKH1 at 42°C for 24 h.
Longer lag phase than the control was considered as inhibition

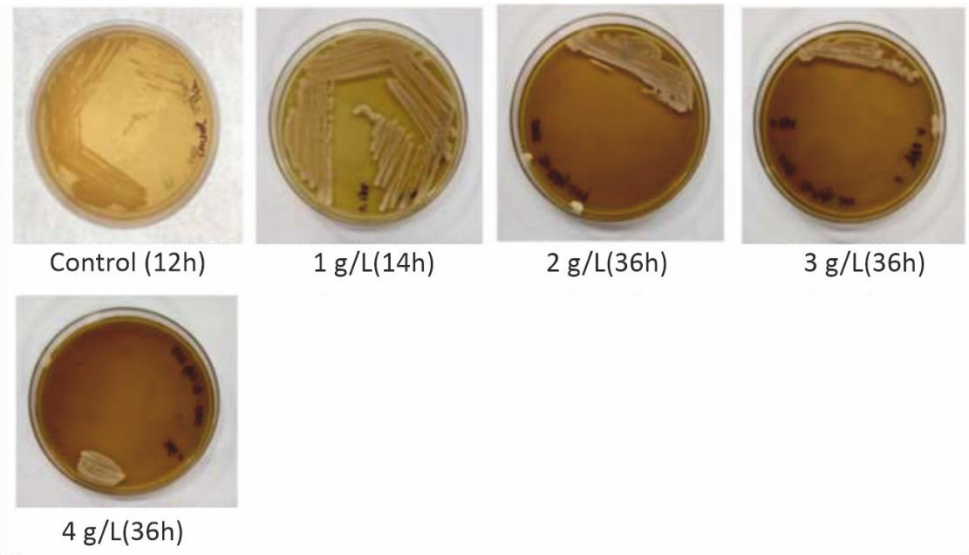
(a) Acetic acid



(b) Furfural



(c) Vanillin



(d) Cocktail (Acetic acid+Furfural+Vanillin)

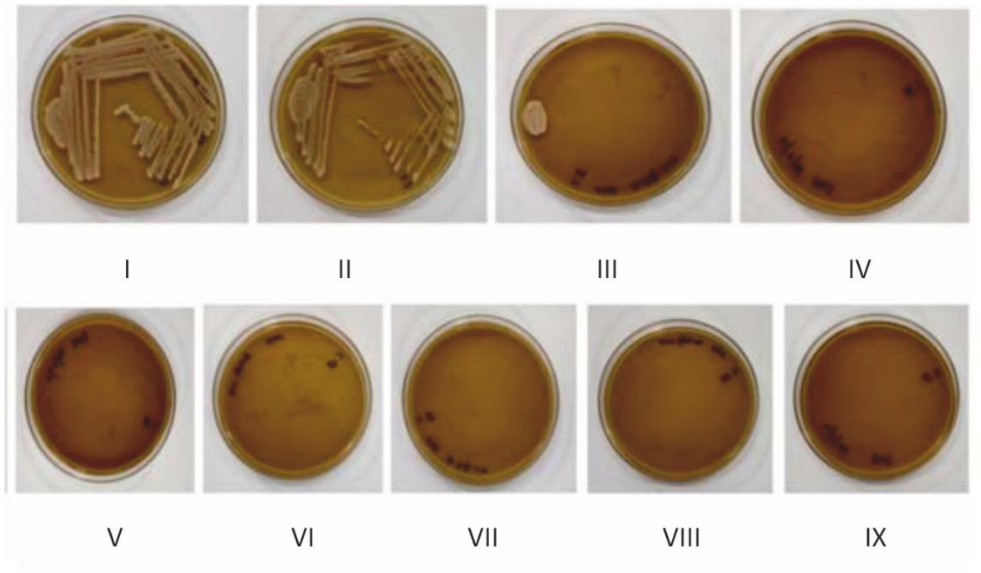


Figure 4.10 : Effect of different concentrations of various inhibitors on the growth of *P. kudriavzevii* JKH1 on YP agar plates. AA:F:V (1+1+1[Cocktail I]; 2+2+2 [Cocktail II]; 3+3+3[Cocktail III]; 4+4+4 [Cocktail IV]; 1+0.1+0.1 [Cocktail V], 2+0.2+0.2 [Cocktail VI]; 3+0.3+0.3 [Cocktail VII]; 4+0.4+0.4 [Cocktail VIII]; 5+0.5+0.5 [Cocktail IX])

4.5.2 Inhibitors tolerance limit of *K. marxianus* JKH5 to inhibitors

As shown in **Figure 4.11a**, the highest concentration of acetic acid which supported maximum growth (OD 0.78) was 4 g/L (v/v), beyond which there was a decline in cell growth. Unlikely, in case of furfural supplemented YPD broth, the cells remained in lag phase and could not grow beyond 1 g/L of furfural. *K. marxianus* JKH5 showed less tolerance to concentrations beyond 1 g/L of vanillin in both YPD broth and agar plate. The toxicity of phenolic compounds lies in the membrane permeation and increasing level of reactive oxygen species. The former attenuates function of selective barriers and substrates exchanging of intercellular membrane of yeasts, and the latter causes cytoskeleton damage, DNA mutagenesis and programmed cell death (Wang et al., 2018). In case of cocktails of inhibitors, no growth inhibition was observed when concentration of A, F and V was (1+1+1) g/L (both in the broth or agar medium) (Figure 4.11d, cocktail I). Conversely, when concentration was (2+2+2) g/L and beyond, the growth of the yeast was adversely affected, which could be attributed to lower tolerance of yeast (Figure 4.11d, cocktail III). However,

the tolerance of the yeast towards cocktail with lower concentration of furfural and vanillin was better (Figure 4.12d, cocktail V).

The tolerance of yeast was also checked in YPD agar plates supplemented with inhibitors. The yeast growth was inhibited till 24 h when the plate was supplemented with 5 g/L acetic acid and full growth on plate took 36 h incubation (F**igure4.12a**). Additionally, few colonies appeared on plates supplemented with furfural, even after 36 h (F**igure 4.12b**).

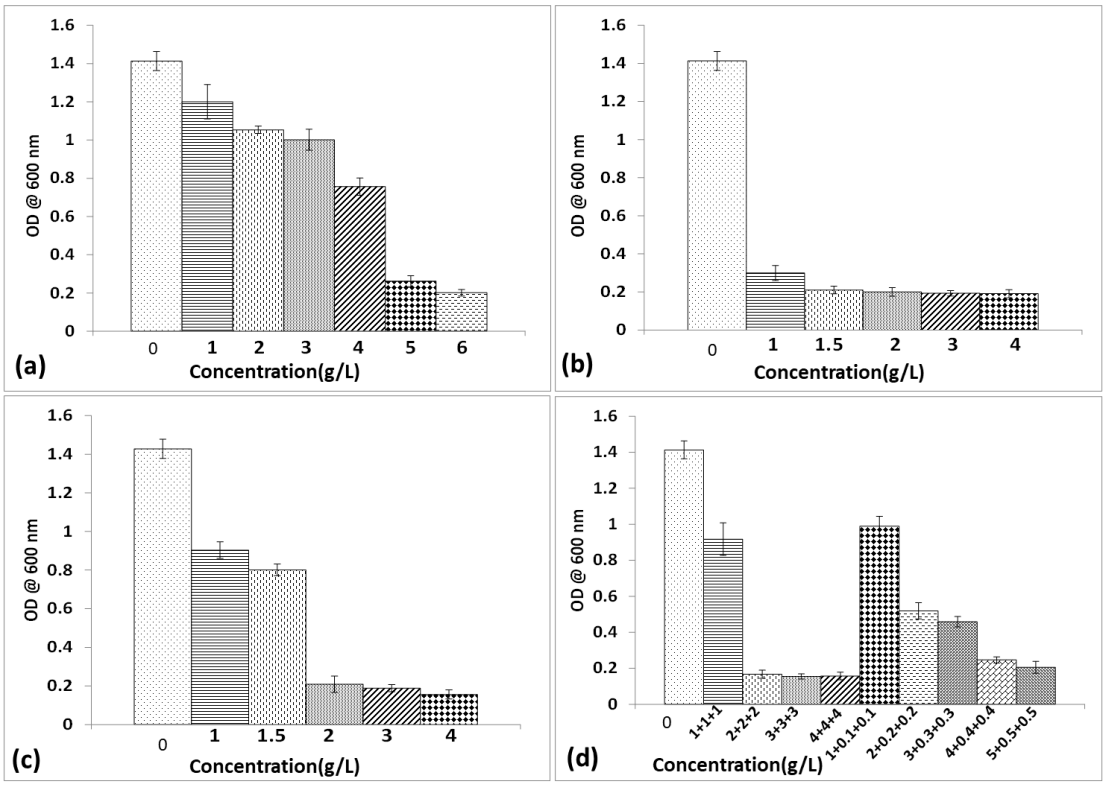
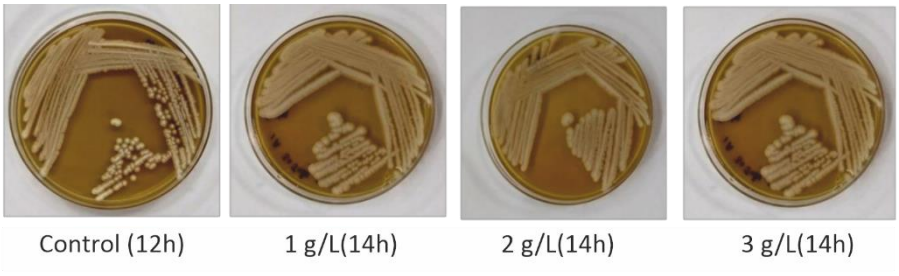
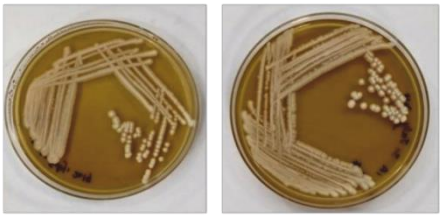


Figure 4.11: Effect of acetic acid (a), furfural (b), vanillin (c) individually and in combination (d) on the growth of *Kluyveromyces marxianus* JKH5 at 42°C. Longer lag phase than the control was considered as inhibition.

(a) Acetic acid

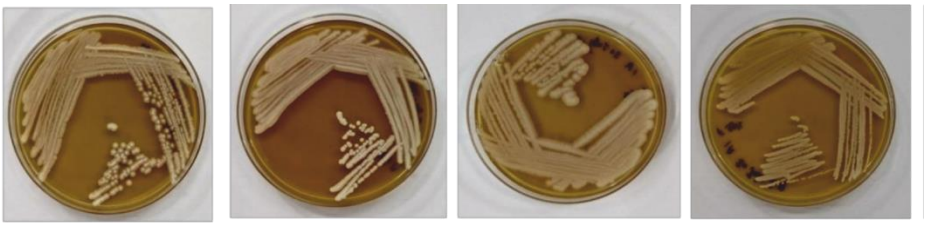


Control (12h) 1 g/L(14h) 2 g/L(14h) 3 g/L(14h)

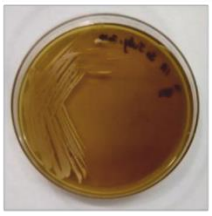


4 g/L(24h) 5 g/L(36h)

(b) Furfural

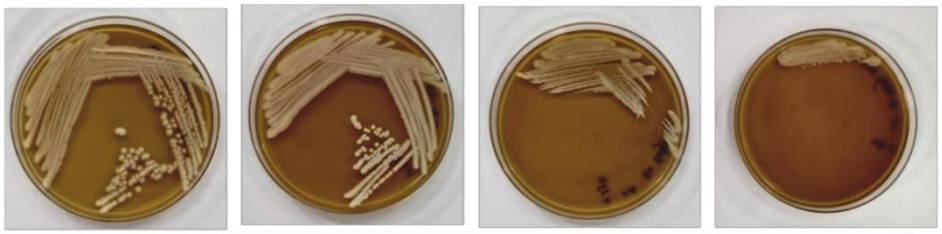


Control (12h) 1 g/L(14h) 2 g/L(24h) 3 g/L(36h)

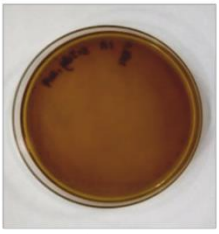


4 g/L(36h)

(c) Vanillin



Control (12h) 1 g/L(14h) 2 g/L(36h) 3 g/L(36h)



4 g/L(36h)

(d) Cocktail (Acetic acid+Furfural+Vanillin)

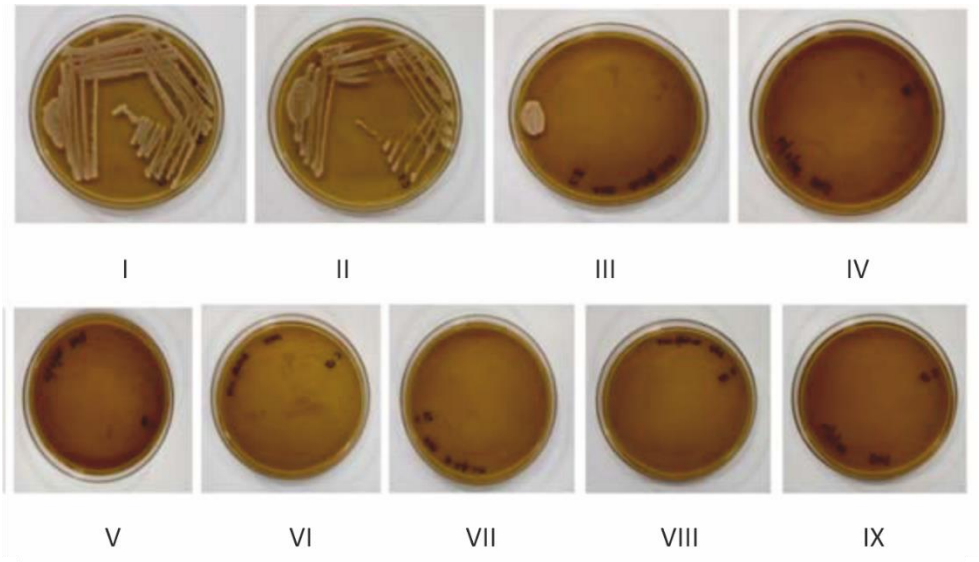


Figure 4.12: Effect of different concentrations of various inhibitors on the growth of *K. marxianus* JKH5 in YPD agar plates. AA:F:V (1+1+1[Cocktail I]; 2+2+2 [Cocktail II]; 3+3+3[Cocktail III]; 4+4+4 [Cocktail IV]; 1+0.1+0.1 [Cocktail V], 2+0.2+0.2 [Cocktail VI]; 3+0.3+0.3 [Cocktail VII]; 4+0.4+0.4 [Cocktail VIII]; 5+0.5+0.5 [Cocktail IX])

4.5.3 Adaptive laboratory evolution of *P. kudriavzevii* JKH1

During the first batch of serial passaging, *P. kudriavzevii* JKH1 showed very less growth due to presence of inhibitor(s) in the medium. In the medium supplemented with acetic acid, JKH1 strain took ten generations (first batch) to get adapted at the initial concentration of 3.5 g/L acetic acid. Thereafter, the cells begin to adapt as indicated by marked reduction in the lag phase. In the second batch of passaging, *P. kudriavzevii* JKH1 took another ten generations to adapt at higher concentration (4 g/L) of acetic acid. *P. kudriavzevii* JKH1 took total of 60 serial transfers to get adapted to the highest concentration of acetic acid (6 g/L) and the adapted strain, *P. kudriavzevii* JKH1 AA60 showed improved specific growth rate (μ) (0.09 h^{-1}) as compared to the parent strain (0.07 h^{1}). Increase in cell density was also witnessed at higher concentrations of acetic acid. **Figure 4.13** shows decrease in the lag phase and doubling time (7 h) of the adapted yeast, *P. kudriavzevii* JKH1 AA60 when compared with parent strain in the presence of acetic acid (6 g/L).

When the parent strain *P. kudriavzevii* JKH1 was cultured for 60 generations in medium with furfural (2-3.2 g/L) at 42 °C, there was a continuous increase in biomass as indicated by higher cell OD_{600}, indicating improved cell survival under stress due to adaptation. Eventually, a furfural tolerant strain *P. kudriavzevii* JKH1 F60 (**Figure 4.13b**) was successfully obtained with improved specific growth (μ) ($0.21h^{-1}$) and doubling time (3.3 h).

In the presence of highest vanillin concentration (2.8 g/L) during adaptation, the developed yeast *P. kudriavzevii* JKH1 V60 showed nearly fivefold increased biomass after 12 h which was evident from its higher specific growth rate ($0.04\ h^{-1}$) than that of the parent strain (**Figure 4.13c**).

During first batch of passaging, in the presence of initial concentrations of inhibitors cocktail (g/L: acetic acid 3; furfural 0.3; vanillin 0.3), the growth of *P. kudriavzevii* JKH1 was repressed by the action of inhibitors. *P. kudriavzevii* JKH1 took twenty generations (first batch) to get adapted to the initial concentrations of inhibitor, after which, the cells began to adapt as indicated by marked reduction in the lag phase. In the next batch of passaging, the inhibitors concentration of the cocktail was increased to (g/L: acetic acid 3; furfural 0.5; vanillin 0.5), and the yeast took thirty generations to get adapted. The final concentration of inhibitors at which *P. kudriavzevii* JKH1 got adapted after 70 serial transfers was (g/L: acetic acid 3; furfural 1; vanillin 1). The tolerance of the adapted yeast *P. kudriavzevii* JKH1 C70 was evident from its nearly fivefold higher biomass and increased specific growth rate ($0.1\ h^{-1}$) than the parent strain (specific growth rate $0.04\ h^{-1}$) (**Figure 4.13d**).

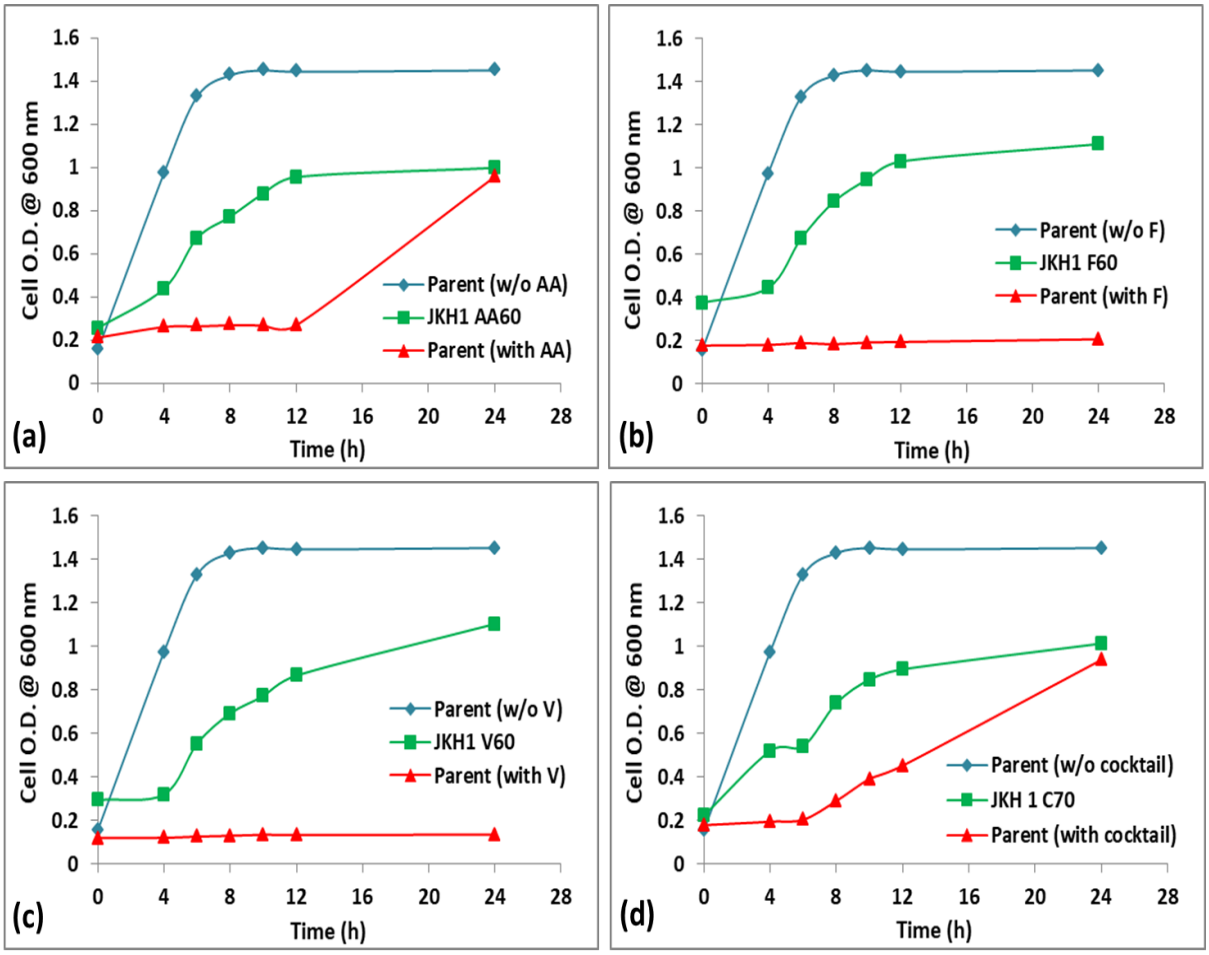


Figure 4.13: Comparison of growth profile of parent and adapted yeast strain *Pichia kudriavzevii* strain on medium supplemented with inhibitors, AA: acetic acid (6 g/L) (a), F: furfural (3.2 g/L) (b), V: vanillin (2.8 g/L) (c), and cocktail: (acetic acid+furfural+vanillin) (3+1+1 g/L) (d)

4.5.4 Adaptive laboratory evolution of *K. marxianus* JKH5 towards fermentation inhibitors

In the medium supplemented with inhibitors, *K. marxianus* JKH5 faced growth inhibition during the initial twelve batches, which was evident from its longer lag phase of 12 h. Once the cells began to adapt during continuous passaging, a marked reduction of the lag phase was observed as shown in **Figure 4.14**. In addition, after 60-70 serial transfers, increase in cell density was also witnessed at the highest concentrations of inhibitors. When acetic acid concentration was maximum (6 g/L) in medium, improved specific growth rate (0.09 h^{-1}) of the adapted yeast, *K. marxianus* JKH5 AA60 was observed as compared to the parent strain

(0.04 h^{-1}). **Figure 4.14a** shows decrease in the lag phase and doubling time of 8 h of the adapted yeast, *K. marxianus* JKH5 AA60 when compared with native strain in the presence of acetic acid.

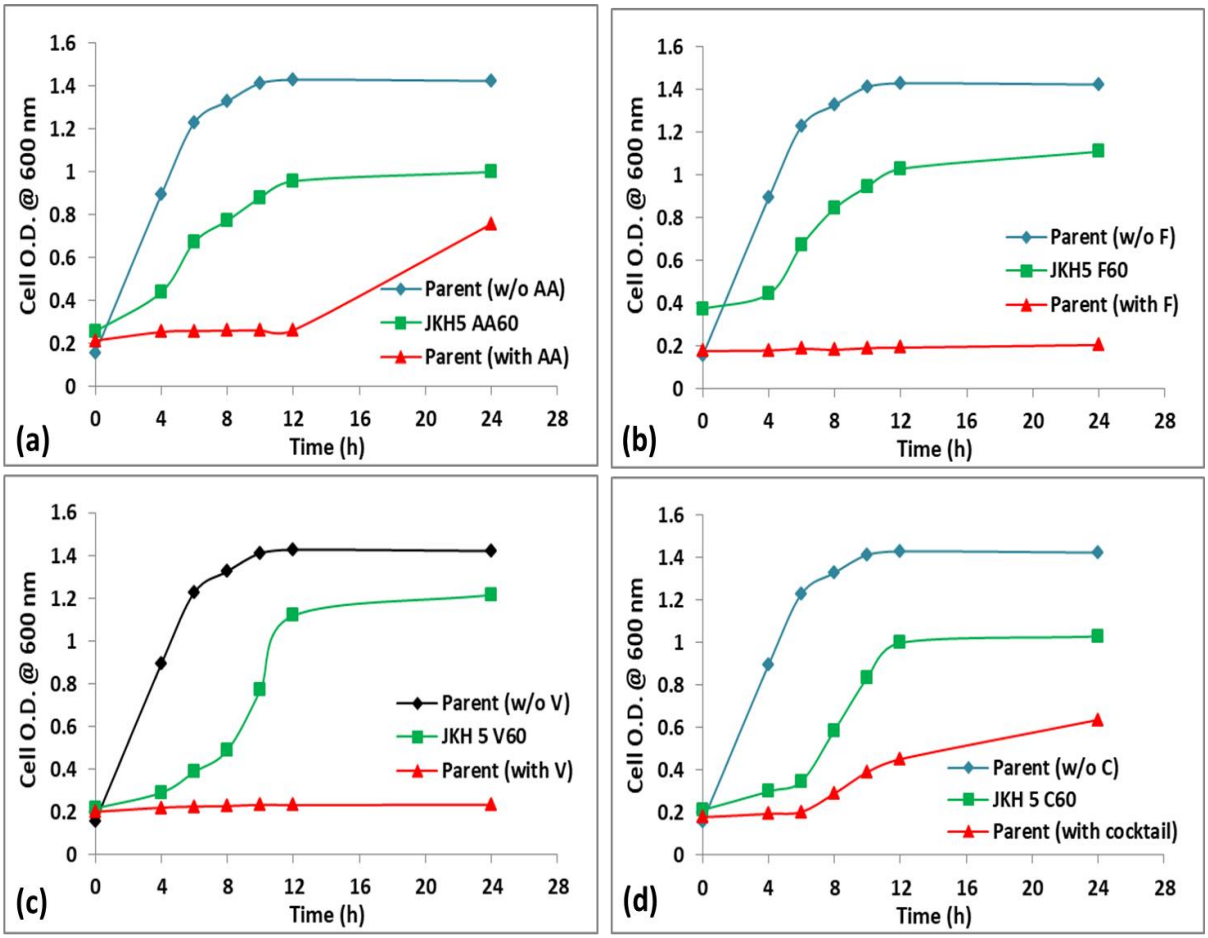


Figure 4.14 Comparison of growth profile of parent and adapted yeast strains of *Kluyveromyces marxianus* on medium supplemented with inhibitors, AA: acetic acid (6 g/L) (a), F: furfural (3.2 g/L) (b), V: vanillin (3 g/L) (c), and cocktail: (acetic acid+furfural+vanillin) (3+1+1 g/L) (d)

The parent strain *K. marxianus* JKH5 was cultured in medium with furfural (2-3.2 g/L) at 42 °C for 60 generations. There was a continuous increase in biomass as indicated by higher cell OD_{600} during the adaptation, which indicated the improved survival adapted cells under inhibitors stress. Eventually, a furfural tolerant strain *K. marxianus* JKH5 F60 (**Figure 4.14b**) was successfully obtained with improved specific growth (μ) (0.15 h^{-1}) and doubling time (4.4 h).In the presence of the highest vanillin concentration (3 g/L), *K. marxianus* JKH5 V60 showed nearly twofold higher biomass and increased specific growth rate (0.04 h^{-1}) after 8 h

than that of *K. marxianus* JKH5 (**Figure 4.14c**). Similarly, in the presence of inhibitors cocktail (g/L: acetic acid 3; furfural 1; vanillin 1), the biomass of the adapted yeast *K. marxianus* JKH5 C60 was nearly fivefold higher and it had higher specific growth rate (0.1 h^{-1}) as compared to the parent strain (0.03 h^{-1}) (**Figure 4.14d**). Moreover, the adapted strains had significantly reduced lag phase.

4.6 Fermentation efficiency of the adapted strains

The strains were adapted to individual inhibitors so that if the yeasts fail to achieve tolerance towards inhibitor cocktail then their adaptation can be achieved through existing adapted strains which are tolerant to individual inhibitors. The adapted strains *P. kudriavzevii* JKH1 C70 and *K. marxianus* JKH5 C60 grew well, with better tolerance to all the inhibitors present together in the cocktail augmented media, and were selected for fermentation experiments.

Both the strains achieved fermentation efficiency of ~80% while producing ethanol at 42 °C with initial glucose concentration of 50 g/L. Moreover, both the strains were able to utilize complete glucose within 18h. The ethanol produced during fermentation by *P. kudriavzevii* JKH1 C70 was 20.8 ± 0.1 g/L whereas the ethanol titer produced by the parent strain *P. kudriavzevii* JKH1 in the presence of inhibitor cocktail was 14.84 ± 3.2 (**Table 4.5**). Moreover, with initial glucose concentration of 100 g/L and with inhibitor cocktail, ethanol produced by *P. kudriavzevii* JKH1 C70 was 25.7 ± 0.4 g/L with productivity of 1.42 g/L/h. Contrastingly, the parent strain *P. kudriavzevii* JKH1 produced 16.45 ± 0.3 g/L ethanol with ~33 % efficiency (**Table 4.6**).

K. marxianus JKH5 C60 produced 20.0 ± 0.3 g/L ethanol at 42 °C with initial glucose concentration of 50 g/L, whereas the parent strain *K. marxianus* JKH5 produced only 8.11 ± 2.8 g/L ethanol (**Table 4.7**). However, with initial glucose concentration of 100 g/L, ethanol produced by *K. marxianus* JKH5 C60 was 24.8 ± 1.8 g/L with 1.4 g/L/h productivity.

Contrastingly, the parent strain *K. marxianus* JKH5 produced 4.90 ± 1.1 g/L ethanol with ~ 9.8 % efficiency (**Table 4.8).**

Out of these two adapted yeasts, *K. marxianus* JKH5 C60 was employed for further experiments due to its comparatively better performance than the parent strain for ethanol production in synthetic medium.

Table 4.5: Comparison of fermentation characteristics of parent and adapted strains of *Pichia kudriavzevii* JKH1 growing on medium supplemented with 50 g/L glucose

Strain	Inhibitor	Initial glucose (50 g/L)				
		Residual sugar (g/L)	Ethanol titre (g/L)	Yield (g/g)	Productivity (g/L/h)	Yield (%)
***P. kudriavzevii* JKH1**	+	2.57 ± 2.5	14.84 ± 3.2	0.30	0.82	58.90
***P. kudriavzevii* JKH1**	-	0.24 ± 0.1	21.53 ± 0.2	0.43	1.20	84.26
***P. kudriavzevii* JKH1 C70**	+	0.27 ± 0.1	20.79 ± 0.1	0.42	1.15	81.33

Inhibitor cocktail (3+1+1) g/L : Acetic acid+Furfural+Vanillin; (+) = inhibitors present; (-)= inhibitors absent

Table 4.6: Comparison of fermentation characteristics of parent and adapted strains of *Pichia kudriavzevii* JKH1 growing on medium supplemented with 100 g/L glucose

Strain	Inhibitor	Initial glucose (100 g/L)				
		Residual sugar (g/L)	Ethanol titre (g/L)	Yield (g/g)	Productivity (g/L/h)	Yield (%)
***P. kudriavzevii* JKH1**	+	46.41 ± 3.9	16.45 ± 0.3	0.16	0.91	31.37
***P. kudriavzevii* JKH1**	-	2.36 ± 1.5	40.95 ± 1.8	0.41	2.26	80.40
***P. kudriavzevii* JKH1 C70**	+	21.31 ± 0.4	25.72 ± 0.4	0.26	1.43	51.0

Inhibitor cocktail (3+1+1) g/L : Acetic acid+Furfural+Vanillin; (+) = inhibitors present; (-)= inhibitors absent

Table 4.7: Comparison of fermentation characteristics of parent and adapted strains of *Kluyveromyces marxianus* JKH5 growing on medium supplemented with 50 g/L glucose

Strain	Inhibitor	Initial Sugar (50 g/L)				
		Residual sugar (g/L)	Ethanol titre (g/L)	Yield (g/g)	Productivity (g/L/h)	Yield (%)
***K. marxianus* JKH5**	+	19.58 ± 0.8	8.11 ± 2.8	0.16	0.45	31.74
***K. marxianus* JKH5**	-	0.94 ± 0.1	21.52 ± 1.5	0.43	1.20	84.22
***K. marxianus* JKH5 C60**	+	4.33 ± 0.1	20.00 ± 0.3	0.40	1.11	78.27

Inhibitor cocktail (3+1+1) g/L : Acetic acid+Furfural+Vanillin; (+) = inhibitors present; (-)= inhibitors absent

Table 4.8: Comparison of fermentation characteristics of parent and adapted strains of *Kluyveromyces marxianus* JKH5 growing on medium supplemented with 100 g/L glucose

Strain	Inhibitor	Initial sugar (100 g/L)				
		Residual sugar (g/L)	Ethanol titre (g/L)	Yield (g/g)	Productivity (g/L/h)	Yield (%)
***K. marxianus* JKH5**	+	80.67 ± 0.1	4.90 ± 1.1	0.05	0.27	9.80
***K. marxianus* JKH5**	-	2.51 ± 1.7	42.28 ± 3.1	0.42	2.35	82.35
***K. marxianus* JKH5 C60**	+	39.95 ± 1.5	24.80 ± 1.8	0.25	1.38	49.02

Inhibitor cocktail (3+1+1) g/L : Acetic acid+Furfural+Vanillin; (+) = inhibitors present; (-)= inhibitors absent

4.7 Characteristics of the adapted strain *K. marxianus* JKH5 C60

Characteristics of the adapted yeast strain *K. marxianus* JKH5 C60 were assessed by analyzing its growth profile on different temperatures and concentrations of glucose, and ethanol. When the adapted strain *K. marxianus* JKH5 C60 was cultured in the medium with different concentrations of glucose (50, 10, 150 and 200 g/L**), t**he maximum cell OD_{600} (7.1) was achieved at 50 g/L glucose **(Figure 4.15).** When glucose was 100 g/L in the medium, the maximum cell OD_{600} was 6.6. Beyond 100 g/L glucose, the growth of strain slowed down and the cell concentrations were significantly lower (p = 0.05) at 150 and 200 g/L glucose.

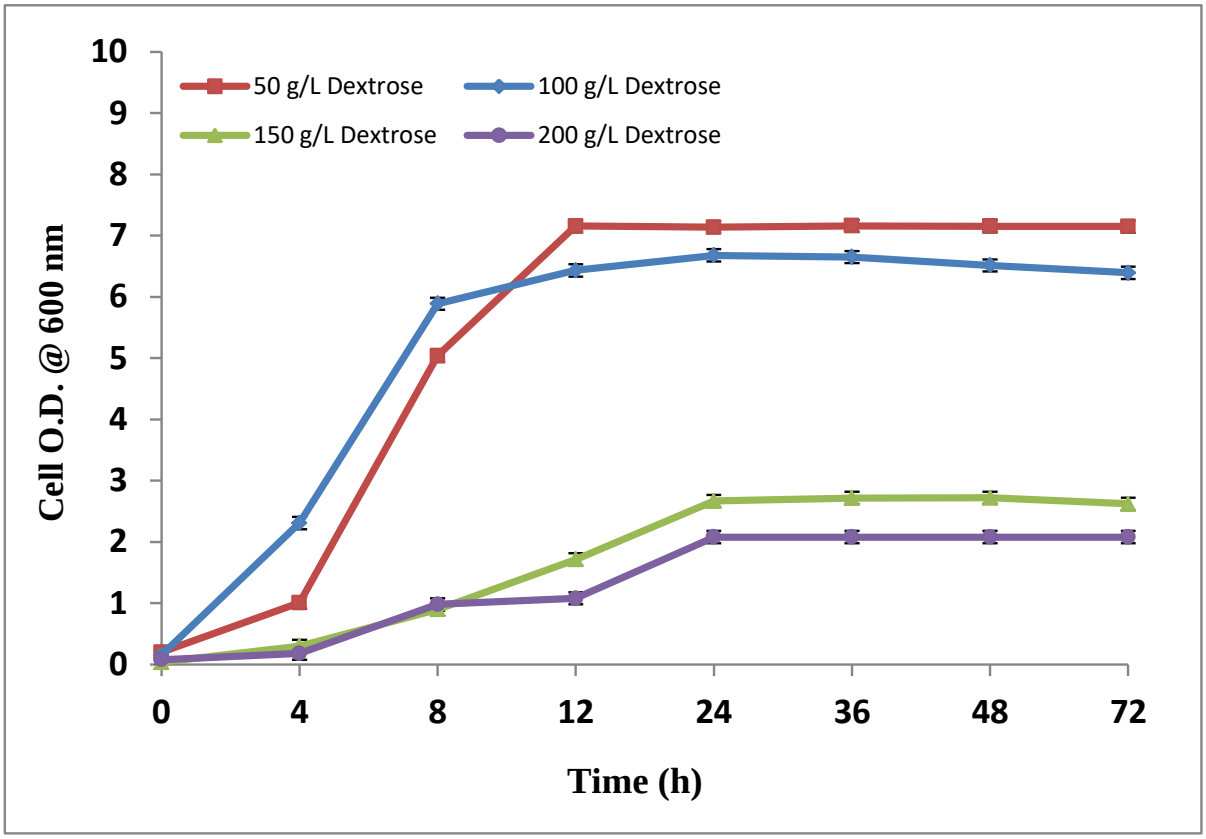


Figure 4.15: Effect of different glucose concentrations on growth of the adapted yeast *K. marxianus* JKH5 C60

Temperature variations influence the ethanol production process by affecting cell metabolism of the yeast. Therefore, to determine the influence of temperature on fermentation, adapted yeast *K. marxianus* JKH5 C60 was cultured in YP medium supplemented with 20 g/L glucose at different temperatures (37, 40, 42 and 45 °C) (**Figure 4.16**). The strain showed maximum growth with a cell OD_{600} of 8.3 at 42 °C. The cell OD_{600} at 37 and 40 °C were 6.6 and 6.0, respectively, which implied that *K. marxianus* JKH5 C60 could grow over a wide temperature range. However, the growth was severely affected at temperature > 42 °C, as indicated by significantly lower (p = 0.05) cell concentration at 45 °C.

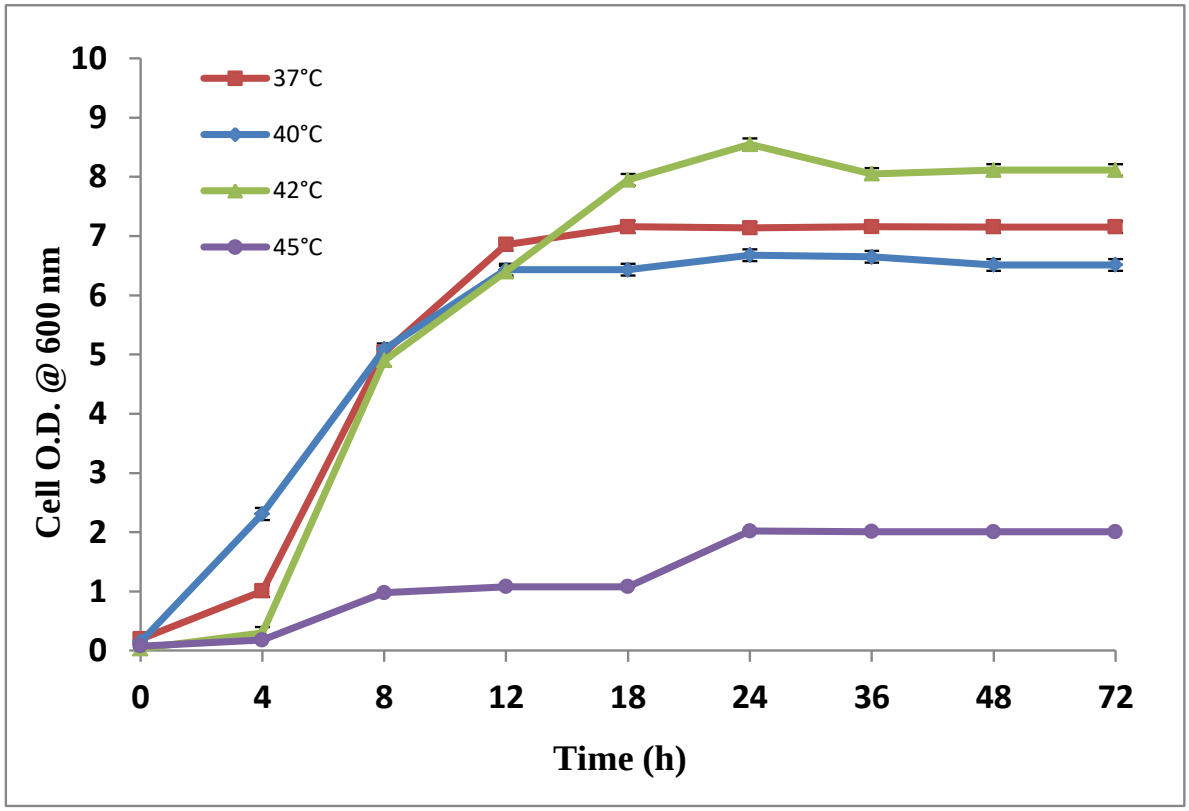


Figure 4.16: Effect of different temperatures on growth of the adapted yeast *K. marxianus* JKH5 C60

The presence of ethanol directly affects significant enzyme functions and structure and also alters the cell membrane, thereby decreasing fermentation efficiency of the yeast. Hence, for assessing the effect of ethanol on growth of *K. marxianus* JKH5 C60, the cells were cultured in medium supplemented with different concentrations of ethanol (5, 7, 8 and 10 %, v/v). Maximum ethanol tolerance was noticed when the adapted strain was incubated with 5%, (v/v) ethanol and the tolerance decreased thereafter **(Figure 4.17).** *K. marxianus* JKH5 C60 could not tolerate ethanol concentrations higher than 7%, v/v.

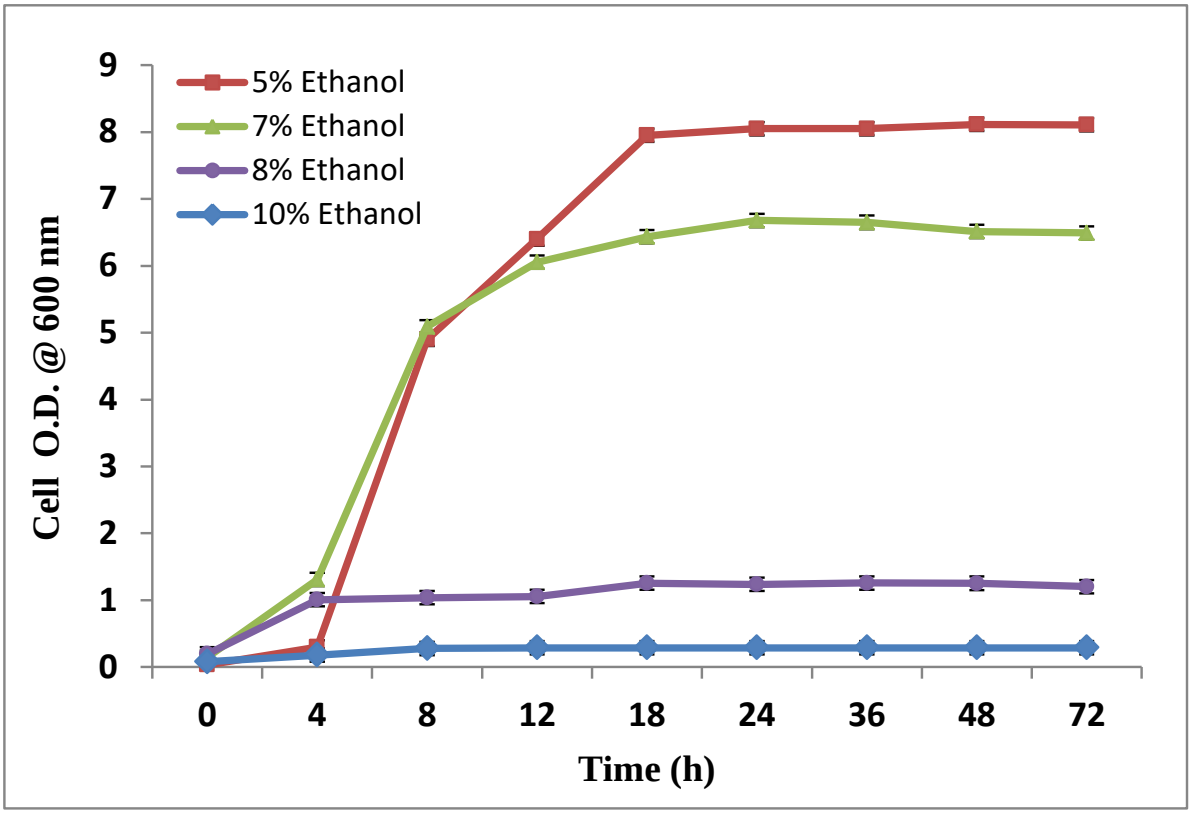


Figure 4.17 Effect of ethanol supplementation on growth of *K. marxianus* JKH5 C60

4.8 Sequential dilute acid-alkali pretreatment of sugarcane bagasse

The pretreatment of SCB was performed sequentially by dilute acid followed by dilute alkali method.

4.8.1 Optimization of dilute acid pretreatment of sugarcane bagasse

Dilute acid pretreatment of lignocellulosic biomass is one of the most effective pretreatment methods which predominantly results in hemicellulose removal with little impact on lignin. The Box-Behnken Design experiments **(Table 4.9)** for dilute acid pretreatment generated second-order polynomial equations (Eq. 3 and 4, respectively) for responses sugar yield (R_1) and cellulose content (R_2), respectively, which were employed for regression analysis:

R_1 =

$143.80+50.15\times A+59.17\times B+17.54\times C-6.64\times D-6.54\times A^2-2.76\times B^2+17.67\times C^2$ 18.00

$\times D^2 16.17\times A\times B-3.18\times A\times C-13.05\times A\times D+1.40\times B\times C-0.68\times B\times D + 8.70\times C\times D$

(3)

$R_2 = 58.00+2.28\times A+3.18\times B+0.42\times C+0.12\times D+0.25\times A^2+0.39\times B^2+1.50\times C^2-0.29\times D^2$

$1.69\times A\times B-0.58\times A\times C -0.63\times A\times D-0.062\times B\times C-0.29\times B\times D+0.72\times C\times D$

(4)

Where, the independent variables A, B, C and D represent concentration of H_2SO_4, temperature, time and solid loading, respectively.

The maximum sugar yield (239.90 ± 0.08 mg/gds) and cellulose content (63.42 ± 0.36) was obtained in run no. 16 in which 12.5% (w/v) biomass (SCB) was treated with 2.75 % (v/v) sulfuric acid at 120 °C for 30 min.

Table 4.9: Box-Behnken design for optimization of dilute acid pretreatment of sugarcane bagasse

Std	Sulfuric Acid (%, v/v)	Temperature (°C)	Time (min)	Solid loading (%, w/w)	Sugar yield (mg/gds)		Cellulose (%, w/w)	
					Actual	Predicted	Actual	Predicted
1	0.5	100	20	12.5	18.80 ± 0.01	13.24	51.32 ± 0.02	51.49
2	5	100	20	12.5	140.10 ± 0.04	145.89	59.25 ± 0.34	59.43
3	0.5	120	20	12.5	164.10 ± 0.03	163.92	60.99 ± 0.01	61.24
4	5	120	20	12.5	220.70 ± 0.05	231.87	62.15 ± 0.01	62.41
5	2.75	110	10	5	138.20 ± 0.03	130.46	59.53 ± 0.01	59.39
6	2.75	110	30	5	162.40 ± 0.03	165.54	58.82 ± 0.02	58.79

7	2.75	110	10	20	110.0 ± 0.12	117.18	57.72 ± 0.01	58.18
8	2.75	110	30	20	169.0 ± 0.42	152.26	59.90 ± 0.02	60.47
9	0.5	110	20	5	45.50 ± 0.02	78.7	55.15 ± 0.03	54.94
10	5	110	20	5	164.20 ± 0.02	179	60.80 ± 0.01	60.76
11	0.5	110	20	20	83.60 ± 0.13	65.42	56.60 ± 0.02	56.43
12	5	110	20	20	150.10 ± 0.06	165.72	59.73 ± 0.06	59.73
13	2.75	100	10	12.5	63.50 ± 0.05	81.17	56.45 ± 0.01	56.22
14	2.75	120	10	12.5	201.40 ± 0.12	199.51	62.43 ± 0.26	62.71
15	2.75	100	30	12.5	96.40 ± 0.01	116.26	57.69 ± 0.11	57.19
16	2.75	120	30	12.5	239.90 ± 0.08	234.59	63.42 ± 0.36	63.44
17	0.5	110	10	12.5	85.10 ± 0.09	90.19	56.56 ± 0.01	56.47
18	5	110	10	12.5	210.80 ± 0.26	190.49	62.46 ± 0.03	62.18
19	0.5	110	30	12.5	119.40 ± 0.11	125.27	58.41 ± 0.07	58.47
20	5	110	30	12.5	232.40 ± 0.01	225.57	62.0 ± 0.03	61.88
21	2.75	100	20	5	99.20 ± 0.02	69.68	54.0 ± 0.02	54.51
22	2.75	120	20	5	201.90 ± 0.06	188.02	61.55 ± 0.36	61.46
23	2.75	100	20	20	59.50 ± 0.12	56.4	55.45 ± 0.12	55.32
24	2.75	120	20	20	159.50 ± 0.02	174.73	61.83 ± 0.02	61.11
25	2.75	110	20	12.5	143.79 ± 0.02	138.73	58.0 ± 0.02	58.0
26	2.75	110	20	12.5	143.80 ± 0.14	138.73	58.01 ± 0.06	58.0

27	2.75	110	20	12.5	143.77 ± 0.09	138.73	58.2 ± 0.03	58.0
28	2.75	110	20	12.5	143.80 ± 0.17	138.73	58.0 ± 0.02	58.0
29	2.75	110	20	12.5	143.81 ± 0.02	138.73	58.0 ± 0.01	58.0

The ANOVA of the models for sugar yield (**Table 4.10)** and for cellulose content (**Table 4.11)** indicated that models were significant *($p < 0.001$*). Moreover, the *$p < 0.001$* values of the model terms indicated the significant contribution of concentration of H_2SO_4 and temperature on pentose sugar yield (increased removal of hemicellulose) and enhancement in cellulose content, respectively, for the two models. The non-significant lack of fit and values of coefficient of determination (R^2) of the models for sugar yield ($R^2 = 0.95$) and cellulose content ($R^2 = 0.99$) indicated good agreement between experimental data and the models, indicating that the models could be used for optimization of dilute acid pretreatment of SCB.

Table 4.10: Analysis of variance (ANOVA) of sugar yield obtained during dilute acid pretreatment of sugarcane bagasse

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	82568.86	7	11795.55	47.83	< 0.0001*
A	30180.27	1	30180.27	122.38	< 0.0001*
B	42008.33	1	42008.33	170.35	< 0.0001*
C	3692.52	1	3692.52	14.97	0.0009
D	529.34	1	529.34	2.15	0.1577
C^2	2532.31	1	2532.31	10.27	0.0043
D^2	1883.72	1	1883.72	7.64	0.0116
AB	1046.52	21	1046.52	4.24	0.0520
Residual	5178.68	17	246.60		
Lack of Fit	5178.68	4	304.63**		
Pure Error	0.000	28	0.000		
Cor Total	87747.54				
R^2	0.956				

***significant; ** non-significant at 95% confidence level. A: sulphuric acid, B: Time, C: Temperature, D: Solid loading. DF = degree of freedom**

Table 4.11: Analysis of variance (ANOVA) of cellulose content obtained during dilute acid pretreatment of SCB

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	220.25	14	15.73	104.24	< 0.0001*
A	62.38	1	62.38	413.32	< 0.0001*
B	121.67	1	121.67	806.13	< 0.0001*
C	2.16	1	2.16	14.30	0.0020
D	0.16	1	0.16	1.05	0.3226
A^2	0.41	1	0.41	2.73	0.1207
B^2	0.99	1	0.99	6.56	0.0226
C^2	14.56	1	14.56	96.48	< 0.0001
D^2	0.55	1	0.55	3.62	0.0777
AB	11.46	1	11.46	75.92	< 0.0001
AC	1.33	1	1.33	8.84	0.0101
AD	1.59	1	1.59	10.52	0.0059
BC	0.016	1	0.016	0.10	0.7524
BD	0.34	1	0.34	2.27	0.1543
CD	2.09	1	2.09	13.83	0.0023
Residual	2.11	14	0.15		
Lack of Fit	2.11	10	0.21**		
Pure Error	0.000	4	0.000		
Cor Total	222.36	28			
R^2	0.991				

***significant; ** non-significant at 95% confidence level, A: sulphuric acid, B: Time, C: Temperature, D: Solid loading. DF = degree of freedom**

The three-dimensional response surface plots between process variables and the obtained response (sugar yield) are shown in Figure 4.18. At higher levels of both H_2SO_4 concentration and temperature, higher sugar yield was obtained (Figure 4.18 a and c). However, lower sugar yield was indicated at solid loading beyond 12.5 %, w/v. Additionally, when solid loading and temperature or time was increased, sugar yield was also enhanced (Figure 4.18 b and f). Increase in sulfuric and temperature, enhanced sugar yield was obtained (Figure 4.18 d and e).

Similarly, higher cellulose content was also indicated at higher level of H_2SO_4 concentration (Figure 4.19 a and c). Additionally, when solid loading was increased with temperature and time, cellulose content was also enhanced (Figure 4.19 b and f). When time of reaction was increased with increasing sulfuric acid or temperature, cellulose content was increased significantly (Figure 4.19 d and e).

After point prediction, the optimized conditions for dilute acid pretreatment were determined as H_2SO_4 concentration, 3% (v/v); solid loading, 18% (dry wt.); pretreatment temperature, 121°C and duration of 30 min. Under optimized conditions the cellulose content in the pretreated biomass was estimated as 63%.

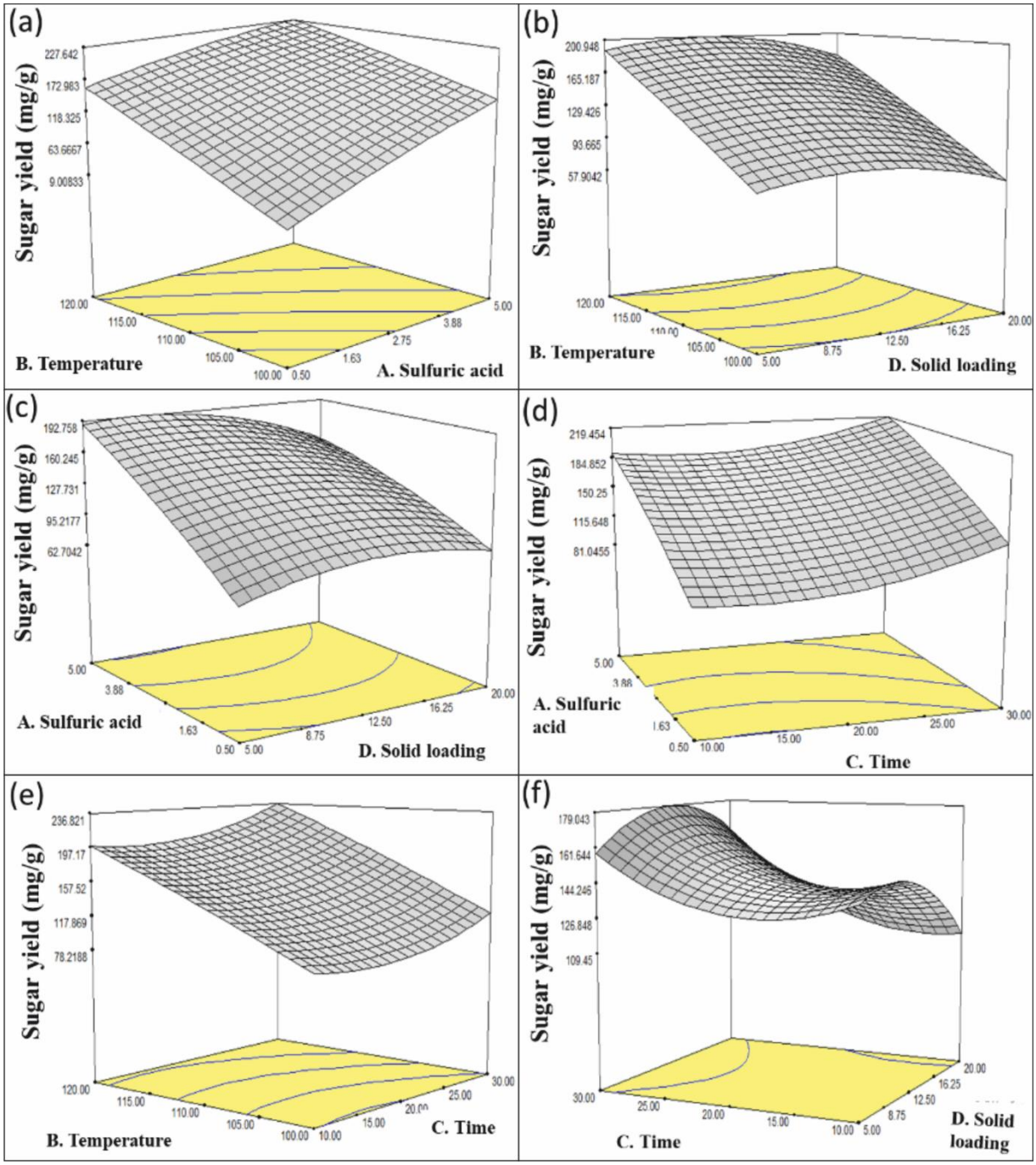


Figure 4.18: Response surface plots of Box-Behnken design for optimization of sequential pretreatment of SCB showing influence of sulphuric acid and temperature (a); temperature and solid loading (b); sulphuric acid and solid loading (c)); sulphuric acid and time (d); temperature and time (e); time and solid loading (f) on the response sugar yield

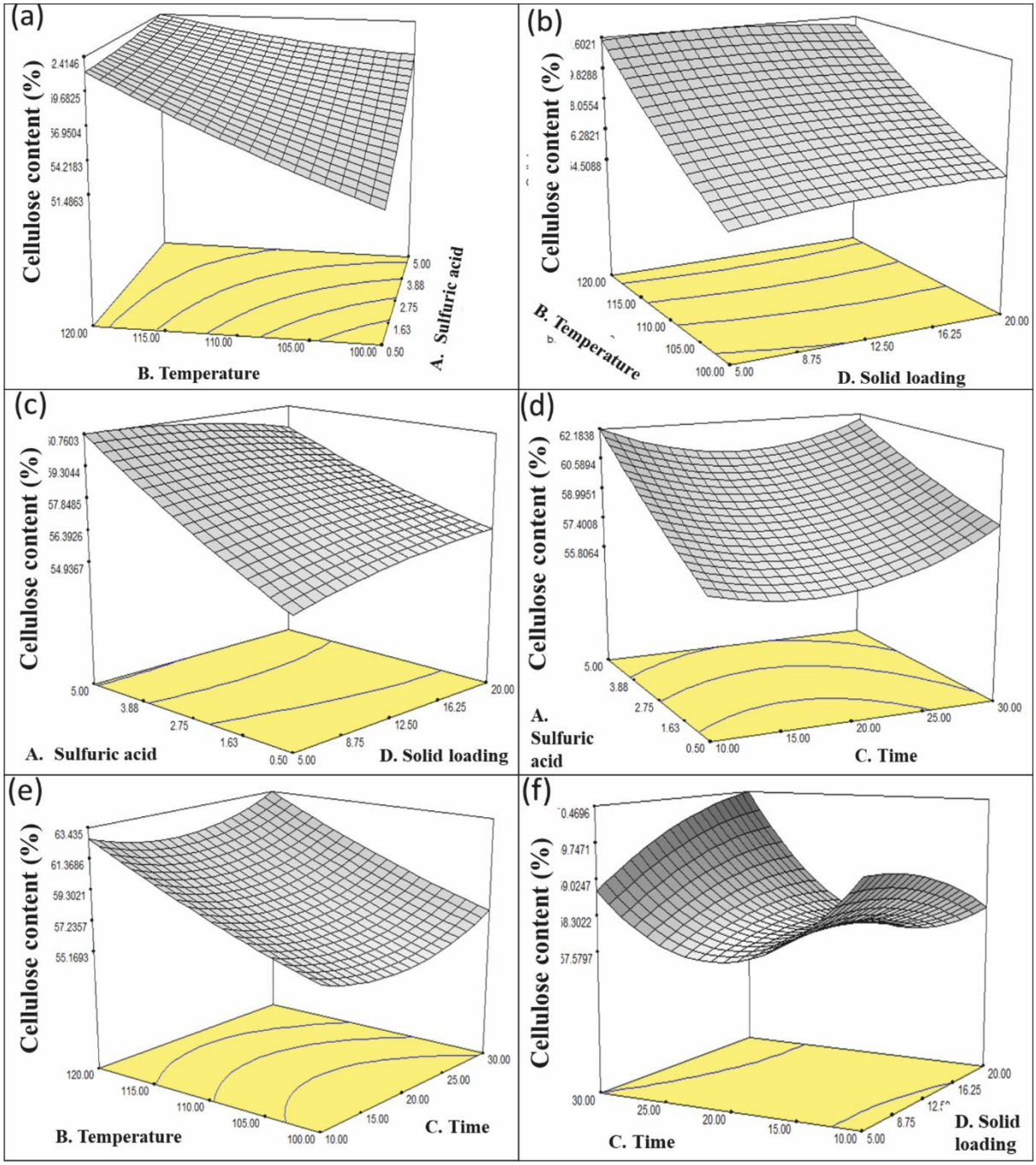


Figure 4.19 Response surface plots of Box-Behnken design for optimization of sequential pretreatment of SCB showing influence of sulphuric acid and temperature (a); temperature and solid loading (b); sulphuric acid and solid loading (c); sulphuric acid and time (d); temperature and time (e); time and solid loading (f) for the response cellulose content

4.8.2 Optimization of dilute alkali pretreatment of sugarcane bagasse

Sequential pretreatment reduced recalcitrance of the biomass due to lignin removal, and hence, enriched the cellulose content in pretreated biomass which ranged from 72.9 to 90.5 %, (w/w) **(Table 4.12)**. The minimum cellulose content was obtained in run no. 1 whereas

the maximum cellulose content was achieved in run no. 6. Overall, a good agreement was observed between the obtained and the predicted cellulose content.

Table 4.12: D-optimal design for sequential dilute acid-alkali pretreatment of sugarcane bagasse

Std	Sodium hydroxide (%, w/v)	Solid loading (%, w/w)	Cellulose content (%, w/w)	
			Actual	Predicted
1	0.5	20	72.90 ± 0.01	72.70
2	10	20	84.61 ± 0.33	84.60
3	5.25	12.5	85.53 ± 0.09	85.40
4	0.5	12.5	76.75 ± 0.12	77.50
5	0.5	5	80.69 ± 0.02	81.0
6	10	5	90.48 ± 0.01	90.30
7	5.25	5	88.79 ± 0.01	88.30
8	10	12.5	88.64 ± 0.07	88.0
9	5.25	20	80.75 ± 0.01	81.40
10	0.5	20	73.22 ± 0.01	72.70
11	0.5	5	81.41 ± 0.02	81.0
12	10	5	89.53 ± 0.03	90.30

The ANOVA of the developed model for dilute alkali pretreatment is presented in **Table 4.13,** which suggested that model was significant ($p< 0.001$) with a non-significant lack of fit and hence, could be utilized for deriving optimized conditions for dilute alkali pretreatment of SCB. Both factors were found to have significant effect on the response i.e. cellulose content. The quadratic model developed for dilute alkali pretreatment was appropriate for predicting cellulose content under different conditions of pretreatment within the range.

Figure 4.20 shows the 3-D response surface plots obtained during D-optimal design for dilute alkali pretreatment. The 3-D plots indicated that higher concentration of sodium hydroxide could be more favorable for attaining higher cellulose content, while lower level of solid loading was correlated with higher cellulose content. Higher alkali concentration significantly reduced available biomass due to high solubilisation of lignin. On the other hand, higher solid loadings of biomass were required to achieve maximum biomass recovery. Therefore, compromise between alkali concentration and biomass loading had to be made. Hence, following conditions were considered as optimum for dilute alkali pretreatment based on the model obtained by D-optimal design through point prediction: solid loading, 15% (dry wt.); sodium hydroxide, 5% (w/v); pretreatment temperature, 121°C and time 30 min. Under optimized conditions of pretreatment, the cellulose content of the biomass (83.31%) was significantly higher than that of untreated SCB ($p < 0.05$).

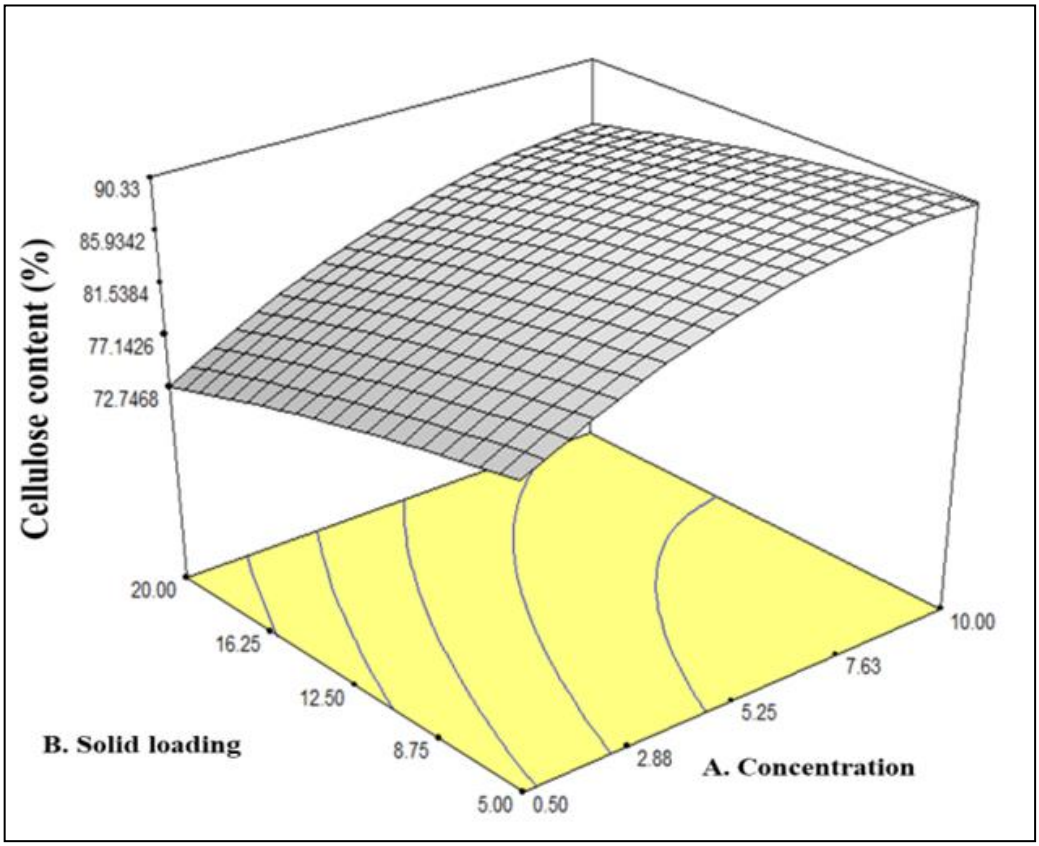


Figure 4.20: Three dimensional response surface plot showing influence of alkali concentration and solid loading on the response cellulose content for optimization of sequential pretreatment of sugarcane bagasse through D-optimal design

Table 4.13: Analysis of variance (ANOVA) of cellulose content during dilute alkali pretreatment of sugarcane bagasse

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	419.37	5	83.87	194.4	< 0.0001*
A	237.5	1	237.5	550.48	< 0.0001*
B	102.59	1	102.59	237.77	< 0.0001*
A^2	15.53	1	15.53	36	0.001*
B^2	0.75	1	0.75	1.74	0.24
AB	2.78	1	2.78	6.44	0.04
Residual	2.59	6	0.43		
Lack of Fit	1.83	3	0.61	2.4	0.25**
Pure Error	0.76	3	0.25		
Cor Total	421.96	11			
R^2	0.99				

***significant, non-significant. A: sodium hydroxide, B: solid loading. DF = Degree of freedom**

4.8.3 Compositional analysis of untreated and pretreated sugarcane bagasse

The compositional analysis revealed that untreated SCB had cellulose, hemicellulose and lignin contents of 51.1, 25.2 and 13.4 %, respectively. The ash content in SCB was 4.3% and moisture content was 6%. Dilute acid pretreatment of SCB enhanced cellulose and lignin contents due to almost complete removal of hemicellulose. While after subsequent dilute alkali pretreatment step, cellulose content was further increased due to significant removal of lignin component of the biomass.

4.9 Characterization of sequentially pretreated sugarcane bagasse

4.9.1 Fourier Transforming Infrared (FT-IR) Spectroscopy

The spectra of untreated and pretreated SCB analysed by Fourier transforming infrared spectroscopy are shown in **Figure 4.21**. The positions of absorption peaks were assigned

according to Singh and co-workers, (2005). The untreated SCB showed adsorption at 680 cm^{-1}, depicting the presence of lignosulfonates. This band was eliminated during alkaline pretreatment due to strong delignification. The dip in the spectra between 840 and1400 cm^{-1} corresponded to an increase in cellulose content. The absence of band at 840 cm^{-1} denoted the formation of amorphous cellulose in sequentially pretreated SCB. The untreated SCB showed adsorption bands at 840, 1386, 1654, 2346, 2900, 3400 cm^{-1} regions. The broad bands at 3400 cm^{-1} and 2900 cm^{-1} were assigned as O-H stretching of H-bonds and C-H bonds, respectively. The decrease in absorption at –OH vibration indicates rupturing in H-bond of cellulose. The peak at 1654 cm^{-1} was ascribed to lignin which was due to C=C stretching and aromatic skeletal vibration of lignin. This peak was however, highly reduced in spectra of sequential dilute acid-alkali pretreated SCB. The characteristic peaks at 1386 cm^{-1} (C-O of syringyl) and 1268 cm^{-1} (C-O of guaicyl ring) were significantly reduced due to the removal of lignin during DAB pretreatment (**Table 4.14**).

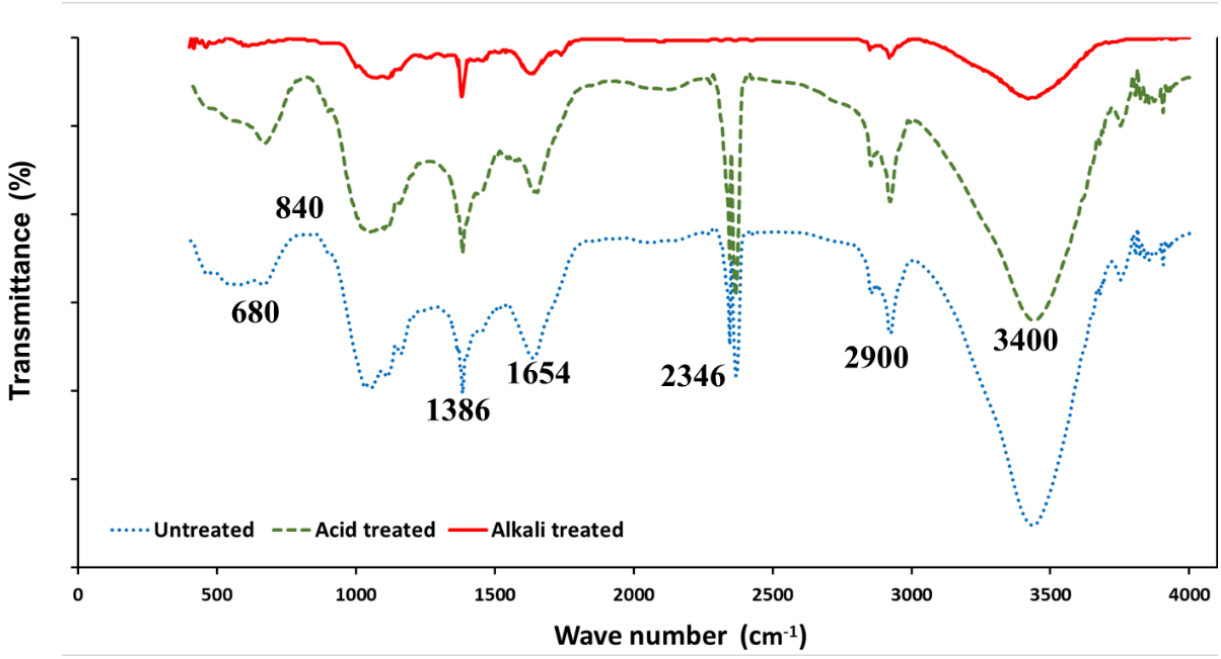


Figure 4.21: FTIR spectra of untreated and pretreated sugarcane bagasse

Table 4.14: Major peaks during FT-IR analysis of pretreated sugarcane bagasse

Wave number (cm^{-1})	Significance of the peak
680	lignosulfonates- lignin removal
840	diminished band in alkali treated indicates amorphous cellulose
1386	present in untreated – reduced in acid and alkali treated- hemicellulose removal
1654	C=O stretch- highly reduced in alkali treated- removal of lignin
2346	CNO stretch vibration- implies removal of hemicellulose

4.9.2 Scanning electron microscopy (SEM)

SEM has been extensively used to study structural changes of lignocellulosic biomass after pretreatment and is one of the methods of choice to investigate the anatomical topographies and deconstruction of cellulose at nano-resolution level. The SEM analysis of pretreated biomass during present study depicted morphological changes in SCB during pretreatment **(Figure 4.22).** Dilute acid pretreatment decreased the hemicellulose content in cell wall of SCB, which resulted in rough surface texture as compared to smooth and intact texture of untreated biomass. Furthermore, sequentially pretreated SCB cell wall appeared ruptured and porous with piths on surface, and had detached fibers as a consequence of delignification. These ruptured cell walls increased the accessible biomass surface, which is highly desirable for enhanced hydrolysis of pretreated LCB by cellulases. SEM analysis clearly indicates better delignification by sodium hydroxide during sequential pretreatment of SCB which is favorable for increasing saccharification yield. Increase in porous appearance, cracks and rupturing of SCB cell walls were due to the better removal of both hemicelluloses and lignin and were correlated with the compositional properties of biomass. Small droplet like structure in the SEM images of sequentially dilute acid-alkali pretreated SCB **(Figure 4.22f)** were probably due to pseudo-lignin formation, contributing to the lignin content of (~8.3%) in the sequentially pretreated biomass.

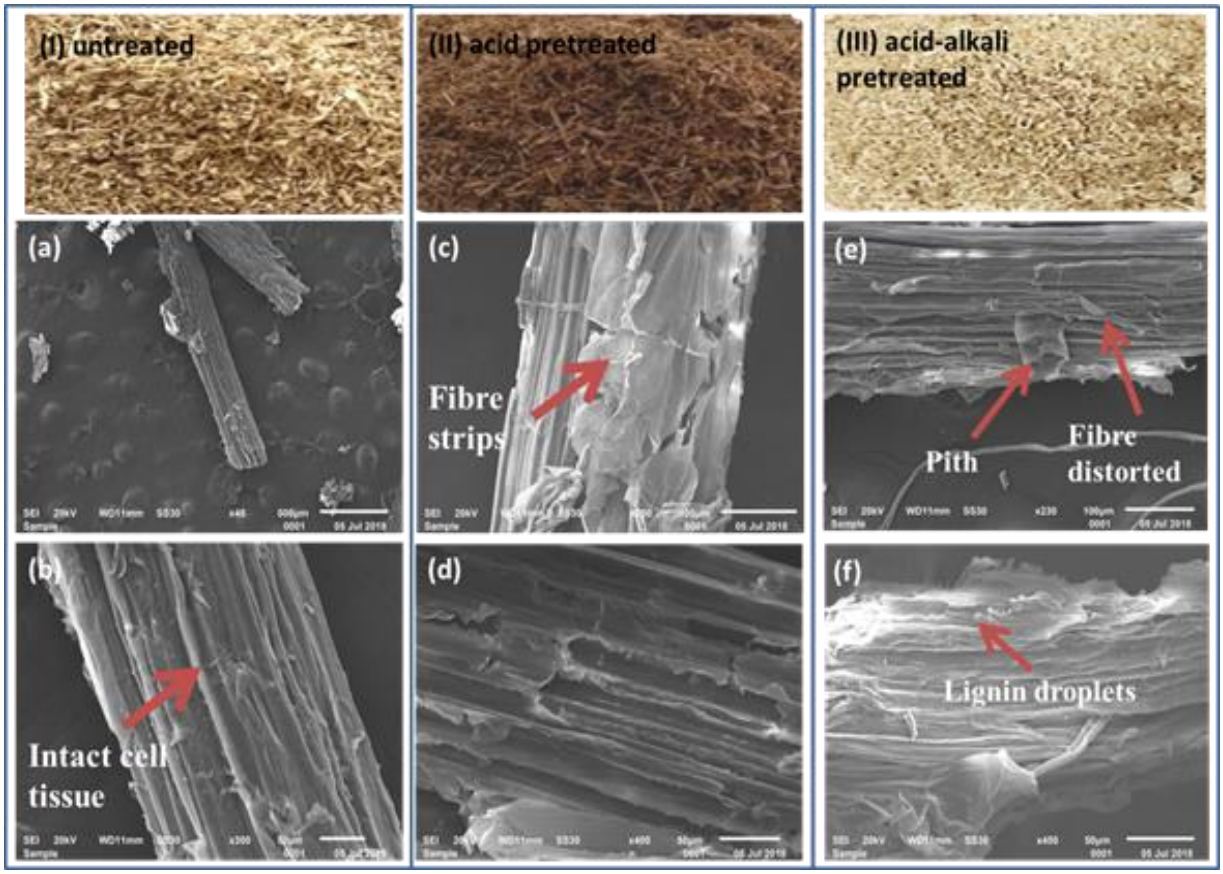


Figure 4.22: Scanning electron micrographs of (i) untreated, (ii) dilute acid pretreated and (iii) sequential dilute acid-alkali pretreated sugarcane bagasse

4.9.3 Energy dispersive X-Ray Spectroscopy (EDX)

The EDX spectrum of SCB was attributed to the presence of mainly carbon and oxygen. It accounted for 100% of the total mass fraction. As shown in the spectrum (**Figure 4.23**) the carbon increased when SCB was pretreated with dilute acid and further with alkali. The increase in cellulose content of the biomass as revealed by EDX analysis suggested the efficacy of the pretreatment method employed during the study.

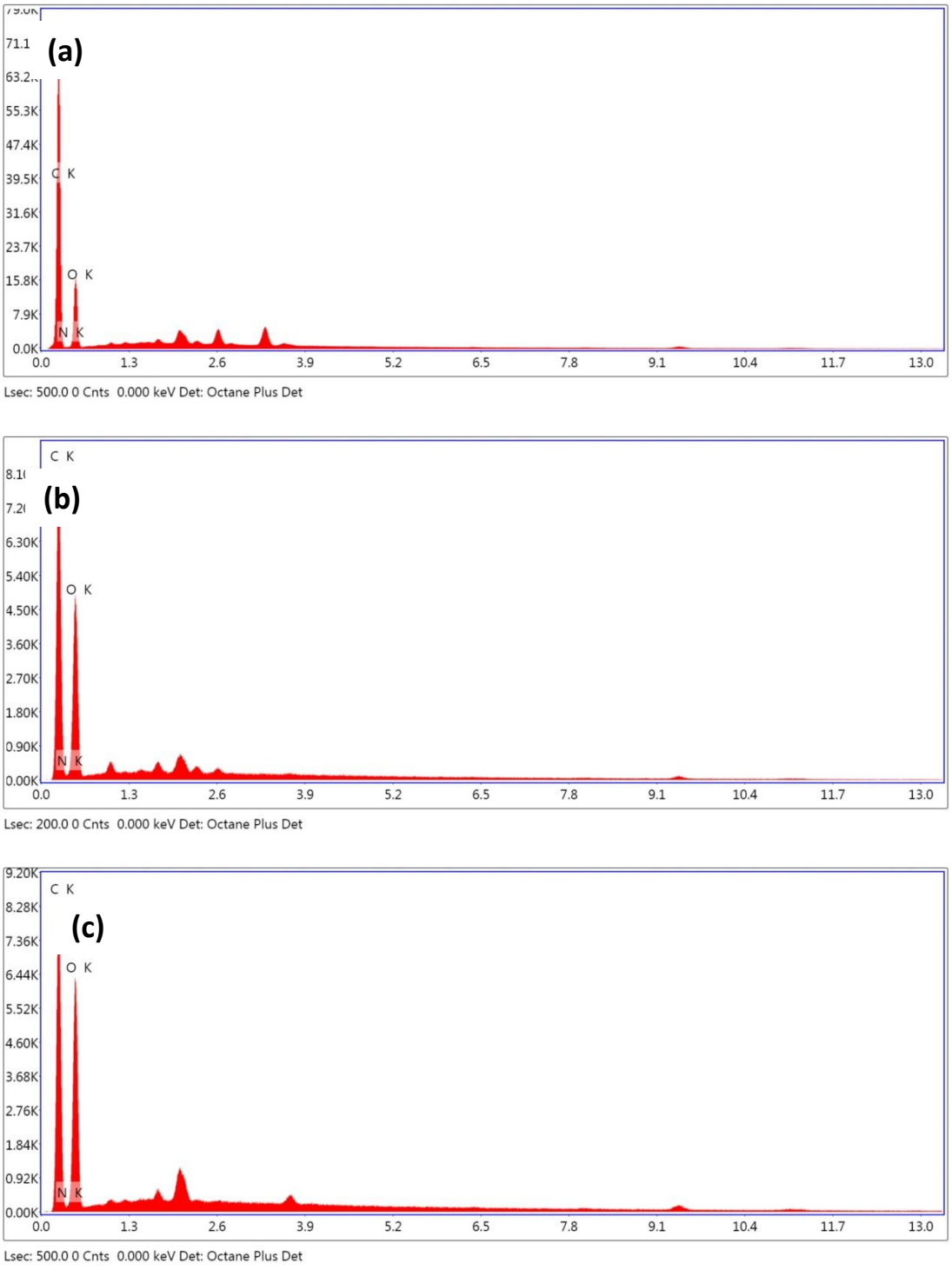


Figure 4.23: EDX spectrum of (a) untreated, (b) dilute acid pretreated and (c) dilute acid- alkali pretreated sugarcane bagasse.

4.9.4 Thermogravimetry (TG) analysis of sugarcane bagasse

TG analysis of lignocellulosic biomass indicates pattern of thermal degradation of the biomass components which help in monitoring the pretreatment induced physiochemical

modifications in LCB and evaluation of the thermal stability of biomass (Varma & Mondal, 2016). During current study, intensive pretreatment resulted in decreased thermal resistance due to degradation of biomass components and de-crystallization. The TG curves of the dilute acid and subsequently dilute alkali pretreated SCB **(Figure 4.24)** exhibited initial decline at 229 to 231°C corresponding to moisture absorption, along with 5% loss in the mass. Thermal degradation of sequentially pretreated SCB occurred at slightly lower temperature than that of dilute acid pretreated SCB. This behavior could be attributed to the increased crystallinity of the biomass resulting in relatively increased of hemicellulose and lignin content which encapsulated the cellulose fibrils. Contrastingly, the sequentially pretreated biomass had its lignin removed by sodium hydroxide, resulting in increased amorphous region. T_{max} represented the maximum temperature at which 90% weight loss was observed. The final decomposition stage for all samples was completed above 400 °C. The values of T_{max} for untreated and dilute acid pretreated SCB were 493 and 495 °C, respectively. Contrastingly, the biomass after sequential pretreatment had significantly lower T_{max} (441°C), which could be attributed to its increased amorphous nature and hence, lowered thermostability.

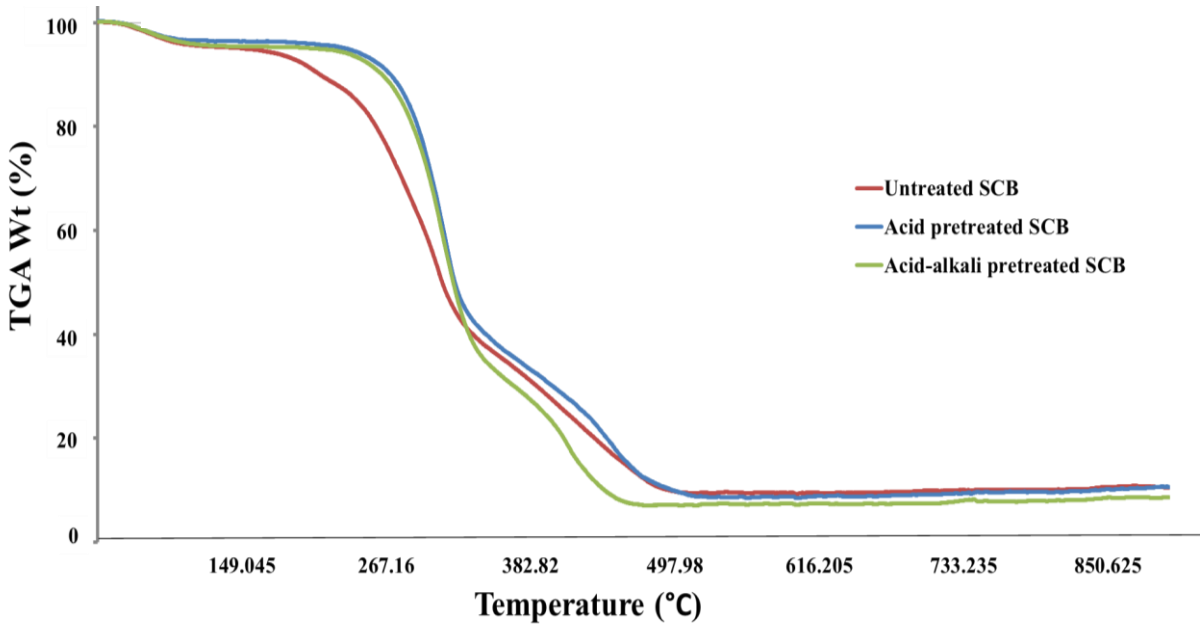


Figure 4.24: Thermal degradation curves of untreated, dilute acid pretreated and sequential dilute acid-alkali pretreated sugarcane bagasse

4.9.5 X-Ray Diffraction (XRD) analysis

Crystallinity of LCB is a crucial factor which directly affects its hydrolysis by lignocellulolytic enzymes. Generally, XRD or the wide angle X-ray scattering (WAXS) is the technique used to reveal crystallinity of LCB after the pretreatment.

The impact of dilute acid and dilute alkali pretreatments on the crystallinity of SCB in the present investigation is shown in **Figure 4.25**. The diffraction pattern of SCB (untreated and pretreated) was similar to cellulose-I lattice as interpreted by three diffraction peaks, the main one at 22.18°, secondary one at 16.26° and smallest one at 34.64°. The widening of peak at 16° and its merger with amorphous scattering peak pointed at distorted cellulose-I and its shift to cellulose-II and was attributed to successful regeneration of amorphous cellulose during sequential pretreatment. On the other hand, dilute acid pretreated biomass had less widened peak at 16°. The observed crystallinity index (CrI %) of untreated SCB was 64.8% which decreased up to 54.7% and 34.7% after sequential dilute acid-alkali pretreatments, respectively, indicating that sequential pretreatment majorly affected the crystal nature of SCB by the reduced crystallinity of cellulose present in SCB.

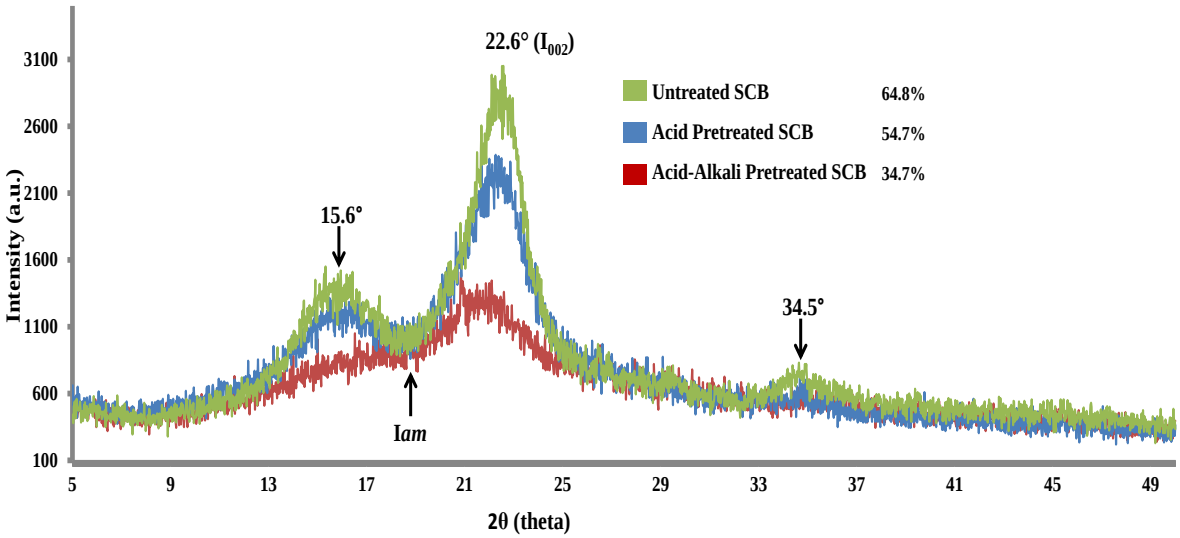


Figure 4.25: X-ray diffraction analysis of untreated and pretreated sugarcane bagasse

4.9.6 Small angle neutron scattering (SANS)

SANS data was fitted to the model for a system of spherical pores. **Figure 4.26** shows the fitted SANS data of untreated, DA and DB pretreated biomass. All the systems were found to consist of pores of two distinct radii. The pore radii for DA pretreated bagasse changed only slightly relative to untreated bagasse. However, sequentially pretreated SCB had increased small pore radii and decreased large pore radii implying increase in number of large pores and hence increased porosity. The relative increase in porosity for SCB samples was obtained by normalizing the scattering invariant of the pretreated samples with that of untreated ones. After DB pretreatment the small pore radii increased from 11.1 Å to 18.5 Å. Increase in porosity was a consequence of the removal or redistribution of lignocellulosic constituents.

The data was further analysed by comparing it with scattering invariant equation which mainly depicted the scattering power of the sample. In the present study, the scattering power was proportional to the density of the pores or the SCB biomass porosity.

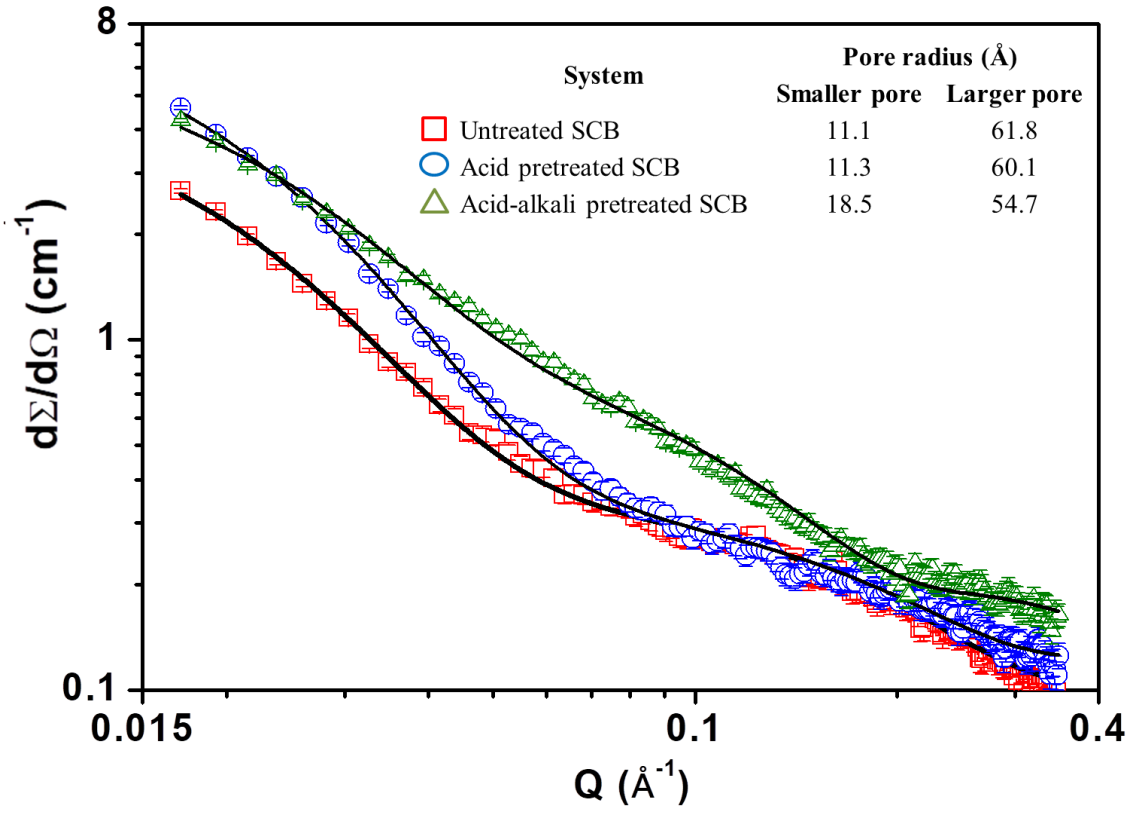


Figure 4.26: SANS analysis of untreated and pretreated sugarcane bagasse

4.10. Enzymatic saccharification of sequentially pretreated SCB

Enzymatic saccharification of pretreated SCB was carried out by using in-house cellulase of cellulolytic fungi as well as the commercial cellulase (cellulase blend, sigma). **Table 4.15** compares the enzyme activities and saccharification potential of various enzymes used in the present study. During saccharification with cellulase of *T. reesei* NCIM 3194, saccharification yield of 23% was obtained, whereas, saccharification yield of 22% was obtained with cellulase of *A. niger* SH3. However, their cellulase cocktail (1:1) significantly increased the saccharification (67 %) of pretreated SCB. However, the saccharification yield was significantly less than that obtained with commercial cellulase (78%). In order to obtain higher ethanol titers during fermentation of pretreated SCB, the commercial cellulase having best saccharification potential among the enzymes used, was preferred for further experiments.

Table 4.15: Enzymatic hydrolysis of sequential dilute acid-alkali pretreated SCB by different cellulases

Enzyme source	Cellulase (IU/mL)	Saccharification (%)
Trichoderma reesei NCIM 3194	1.85 ± 0.05	23
Penicillium chrsogenum MTCC 4392	0.69 ± 0.02	15
Penicillium funiculosum NCIM 1228	0.61 ± 0.08	12
Penicillium oxalicum RE 10	2.1 ± 0.04	25
Penicillium oxalicum 114	2.1 ± 0.06	23
Aspergillus niger SH3	1.13 ± 0.03	22
Trichoderma reesei NCIM 3194 + *Aspergillus niger* SH3	2.2 ± 0.02	67
Cellulase blend (Sigma)	232.4 ± 0.04	78

4.11 Optimization of enzymatic saccharification of sequentially pretreated sugarcane bagasse

Optimization of saccharification is required for achieving the maximum sugar yield and eventually higher conversion into ethanol. Optimization was done using one factor at a time (OFAT) approach. The factors considered for optimization were enzyme dose, temperature for saccharification, initial pH of the enzymatic saccharification reaction and tween 80 concentrations.

4.11.1 Effect of enzyme dosage on the enzymatic saccharification

The effect of different enzyme dosages ranging from10-25 FPU/gds was evaluated for the optimum saccharification of pretreated SCB. The continuous increase in enzyme dose showed increased sugar release however, no significant improvement in the amount of sugar release was observed beyond the enzyme dosage of 20 FPU/gds. The enzymatic saccharification at 20 FPU/gds resulted in optimum saccharification at 72 h with sugar yield of 123.62 ± 2.8 mg/mL (**Figure 4.27**).

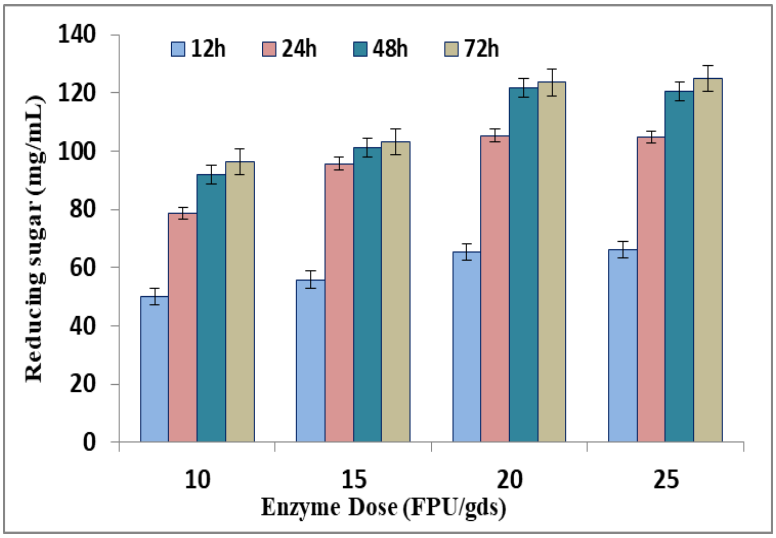


Figure 4.27: Effect of enzyme dosage on enzymatic saccharification of sequentially pretreated sugarcane bagasse

4.11.2 Effect of temperature on the enzymatic saccharification

As the enzyme had to be used under simultaneous saccharification and fermentation at the optimum temperature of yeast growth, therefore, it was required to test the performance of

enzyme at temperatures lower than 50 °C. Therefore, the effect of different temperatures (42, 45 and 50 °C) on saccharification of sequentially pretreated SCB was studied. The results showed that the performance of enzyme was not affected by the temperature variations from at 42, 45 and 50 °C during saccharification (Figure 4.28). The sugar released at 42 °C was 122.9 ± 2.2 mg/mL, which was similar to that obtained at 50 °C (123.6 ± 2.1 mg/mL), indicating that the enzyme could be used without any compromise in sugar yield during SSF carried out at optimal growth temperature of yeast (42 °C).

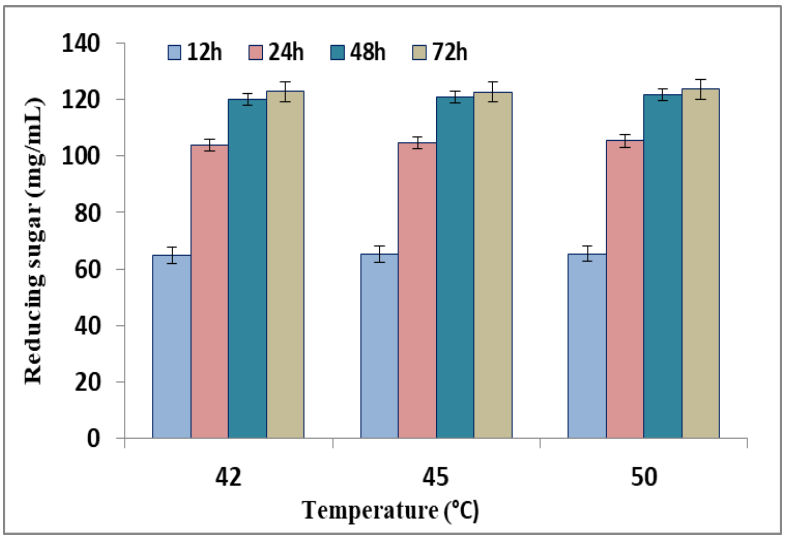


Figure 4.28: Effect of temperature on enzymatic saccharification of sequentially pretreated sugarcane bagasse

4.11.3 Effect of initial pH on the enzymatic saccharification

The effect of initial pH on enzymatic saccharification was examined using citrate buffer (50 mM) of different pH ranging from 4 to 5.5. The maximum sugar (123 ± 3.5 mg/mL) was released when the initial pH was 5.0 during saccharification (Figure 4.29). Further, any increase or decrease in initial pH during saccharification resulted in a decline in the amount of sugar released (Figure 4.29).

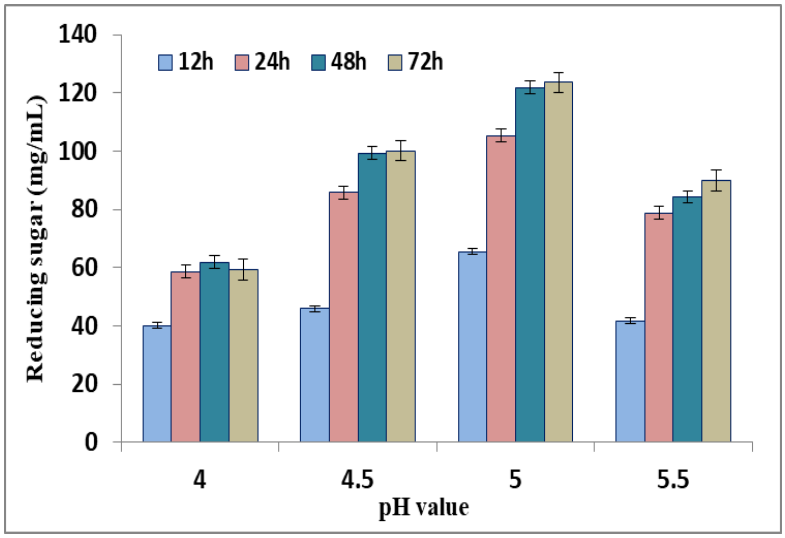


Figure 4.29 Effect of initial pH on enzymatic saccharification of sequentially pretreated sugarcane bagasse

4.11.4. Effect of tween 80 concentration on the enzymatic saccharification

Effect of tween 80 on the enzymatic saccharification of cellulosic biomass was studied by varying its concentrations from 0.1 to 2.0% (v/v). Maximum release of reducing sugar (124.89 ± 3.50 mg/mL) was observed after 72 h when tween 80 was used at 0.5% (v/v). Increasing concentration of Tween 80 beyond 0.5 % (v/v) resulted in gradual decline in release of reducing sugar at all the time points (**Figure 4.30).**

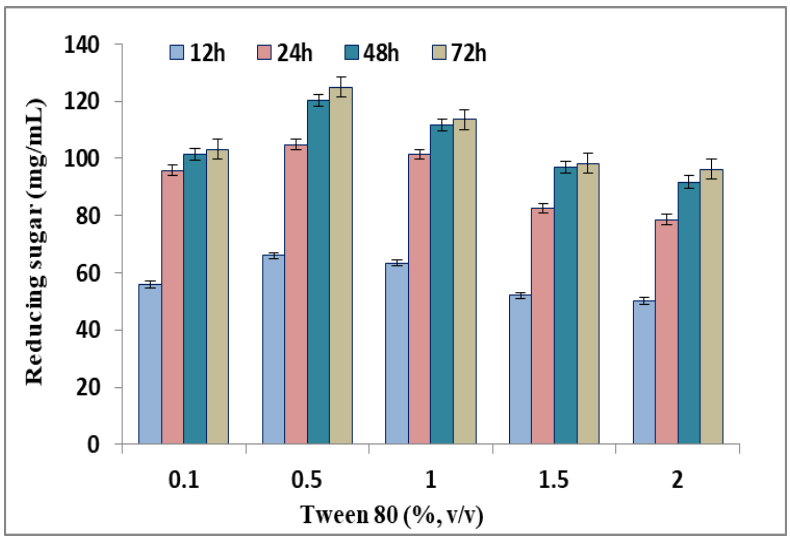


Figure 4.30: Effect of Tween 80 concentration on enzymatic saccharification of sequentially pretreated sugarcane bagasse

4.12 Separate hydrolysis and fermentation of pretreated sugarcane bagasse

The ability of the strain *K. marxianus* JKH5 C60 to produce cellulosic ethanol was examined via SHF under shake flask (Figure 4.31). The SHF of sequential acid-alkali pretreated SCB was performed at 42 °C for 24 h without filtration, centrifugation, autoclaving and vessel change to match the industrial conditions. Enzymatic saccharification of pretreated SCB improved with increasing the enzyme dose from 10 to 20 FPU/gds with no significant improvement further (Figure 4.31a). Sugar release increased with time, additionally, reaching the maximum at 72 h. The highest reducing sugars 123.6 ± 2.10 and 124.8 ± 2.84 g/L were released after 72 h at enzyme doses of 20 and 25 FPU/gds, respectively.

Enzymatic hydrolysate obtained using different enzyme doses (10, 15, 20 and 25 FPU/gds) were further subjected to fermentation by the adapted yeast *K. marxianus* JKH5 C60 resulting in ethanol titers of 45 ± 1.20, 48± 1.50, 54.2± 2.10 and 55.0 ± 2.84 g/L, respectively. The highest fermentation efficiency achieved during SHF was ~ 87%. Since, similar ethanol yield were obtained when SHF was performed with enzyme doses of 20 FPU/gds and 25 FPU/gds, the former enzyme dose was selected for simultaneous saccharification and fermentation (SSF).

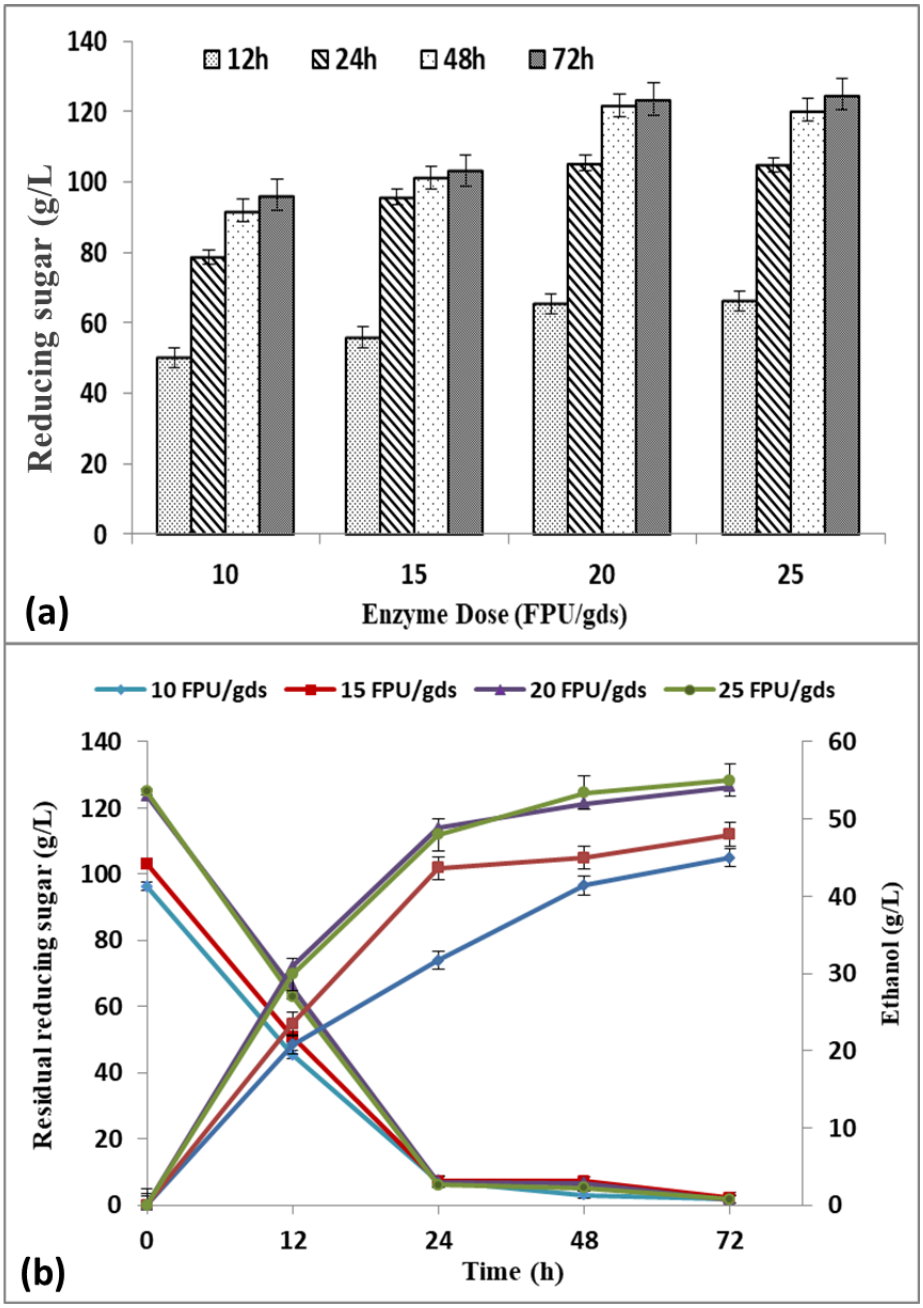


Figure 4.31: Separate hydrolysis and fermentation of sequential pretreated sugarcane bagasse by adapted yeast *K. marxianus* JKH5 C60 (a) enzymatic hydrolysis at different enzyme doses and (b) ethanol fermentation

4.13 Simultaneous saccharification and fermentation of pretreated sugarcane bagasse

4.13.1 Batch SSF at shake flask level

Simultaneous saccharification and fermentation of SCB was performed in the presence and absence of inhibitor cocktail (control) under batch mode, by employing the adapted yeast strain *K. marxianus* JKH5 C60. When fermentation was performed in the absence of inhibitor

cocktail, the ethanol titer of 58.5±0.15 g/L was obtained after 36 h. The corresponding ethanol yield and efficiency were 0.44 g/g and 77.6%, respectively (**Figure 4.32).** SSF in the presence of inhibitor cocktail resulted in production of 54.8±0.9 g/L ethanol after 36 h which was similar to that of the control flask (58.5±0.15 g/L) **(Figure 4.32).** Interestingly, the fermentation performance of *K. marxianus* JKH5 C60 under SSF at 42 °C was similar to that using glucose as added carbon source. No glucose was detected after 6 h, indicating that the yeast utilized the sugar effectively for bioethanol production.

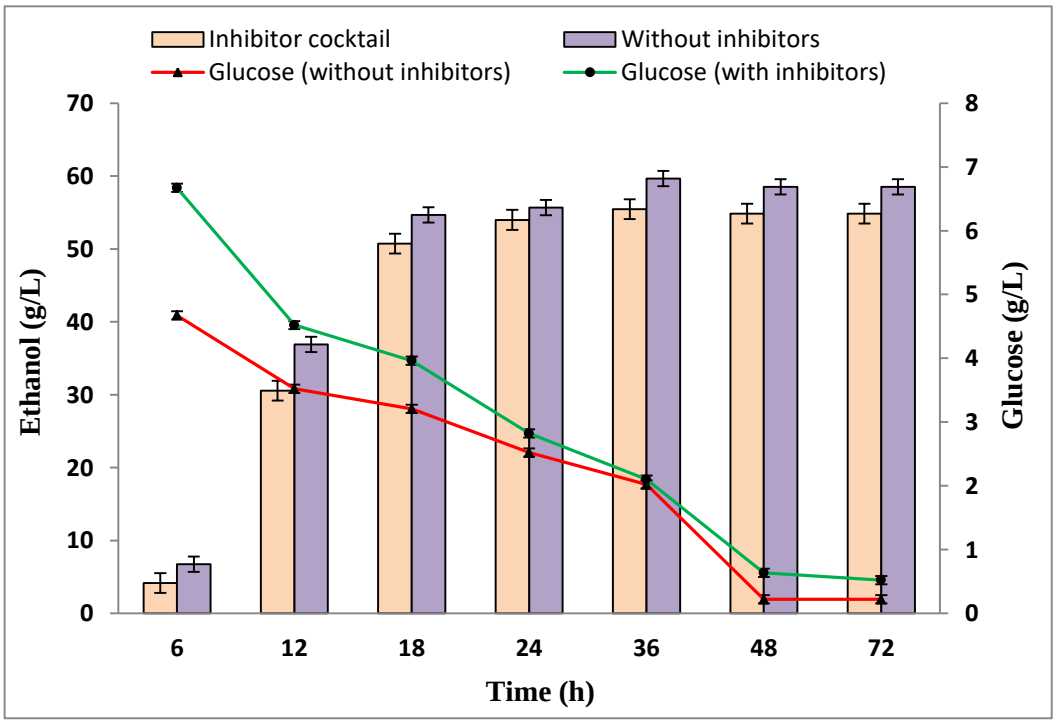


Figure 4.32: Batch simultaneous saccharification and fermentation of sequentially pretreated sugarcane bagasse in the presence of fermentation inhibitors by *K. marxianus* JKH5 C60 under shake flask. The solid loading of 15% (dry wt.), enzyme dosage of 20 FPU/g and inhibitor concentrations: acetic acid+furfural+vanillin (3+1+1) g/L were used

4.13.2 Batch SSF at lab scale fermenter level

Shake flask batch SSF was further scaled up to further 3L bioreactor level, with a working volume of 1L using 15% (w/v) solid loading, in presence and absence of inhibitors (control). In the control experiment, SSF of pretreated SCB resulted in an ethanol production of 67.2 g/L ethanol with an efficiency of 95% **(**Figure 4.33**).** During the fermenter run with inhibitor

cocktail supplementation, the maximum ethanol titer reached to 64.8 ± 1.4 g/L at 36 h. Increase in ethanol production was observed till 24 h, thereafter, it remained almost similar till 72 h **(**Figure 4.33**).** No significant glucose amount was detected during the SSF carried out with or without inhibitors and it never exceeded 5 g/L indicating that the sugars released by the enzymatic hydrolysis were simultaneously utilized by the yeast for ethanol production. The strain had similar fermentation performance in both the conditions (in presence and absence of inhibitors).

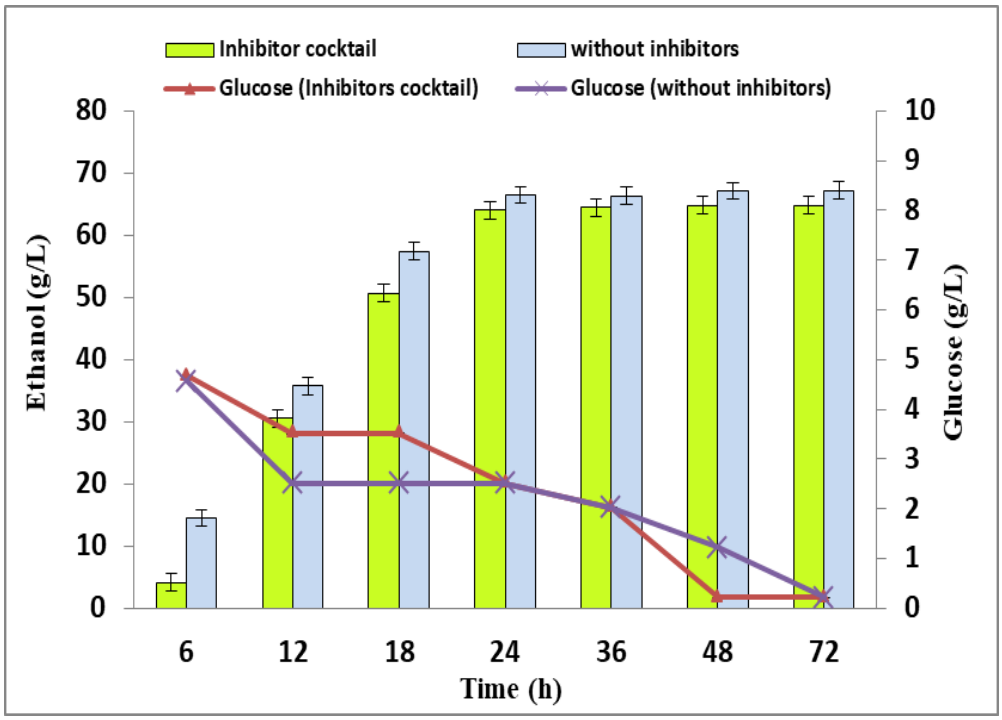


Figure 4.33: Batch simultaneous saccharification and fermentation of sequentially pretreated sugarcane bagasse by the adapted yeast *K. marxianus* JKH5 C60 at lab-scale fermenter in presence and absence of inhibitors. The solid loading was 15% (dry wt.) and 20 FPU/g enzyme dosage

4.14 Simultaneous saccharification and fermentation at high gravity

Effect of different substrate consistencies ranging from 15-30 % (dry wt.) was examined under batch and fed-batch mode of simultaneous saccharification and fermentation, at shake flask level, to select the best run for scale-up.

Batch simultaneous saccharification and fermentation of sequentially pretreated sugarcane bagasse

4.14.1 Batch SSF under shake flask

Simultaneous saccharification and fermentation of sequentially pretreated SCB was performed at higher solid loadings under batch mode. It was observed that with increased solid loading, there was a significant decrease in fermentation efficiency with increase solid loading. Among different substrate consistencies (15-30 %, dry wt.), the maximum ethanol titer (70.1 ± 1.4 g/L) was obtained at solid loading of 20% (dry wt.), however, the fermentation efficiency was compromised (74%). The maximum fermentation efficiency of 80% was achieved when solid loading was 18% (%, dry wt.). Further increase in solid loading reduced the fermentation efficiency (Figure 4.34), which could be attributed to mass transfer limitations due to high viscosity during the batch process.

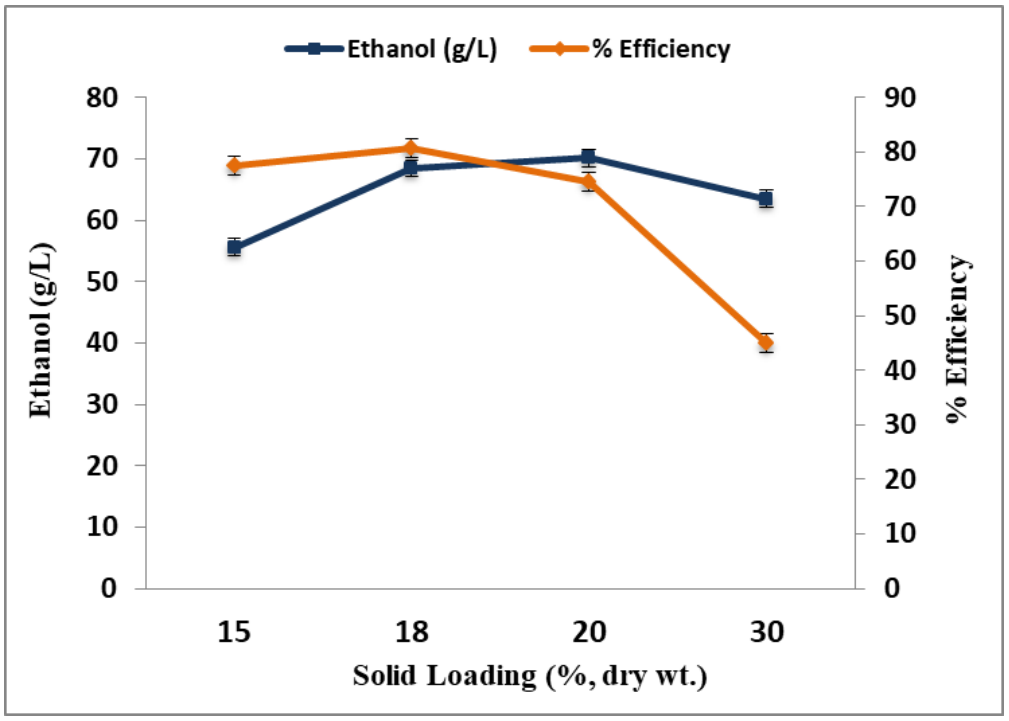


Figure 4.34: Batch simultaneous saccharification and fermentation of sequentially pretreated sugarcane bagasse at high solid loadings by adapted yeast *K. marxianus* JKH5 C60 under shake-flask.

4.14.2 Fed-batch SSF under shake flask

For achieving high ethanol yields and productivity without compromising the efficiency, fed-batch approach for simultaneous saccharification and fermentation was adopted. Fed-batch

SSF at shake flask level was performed in six different sets of experiments (A-F), with different feeding strategies. In trials A, B and C, feeding of biomass was done at regular intervals whereas, the total enzyme was added initially at 0 h (Figure 4.35). In another three trials (D, E and F), feeding of both biomass and enzyme was done at regular intervals proportionately (Figure 4.36**).** The fed-batch SSF of SCB at different solids loadings was performed using commercial cellulase (having an enzyme activity of 230 FPU/mL) at an enzyme loading of 20 FPU/gds. When the solid loading was increased from18% to 30%, concentration of ethanol was also increased. Overall in all the trials, increase in ethanol titer was observed till 24 h, thereafter, it remained almost similar.

Trial A and D with 18% (dry wt.) substrate concentrations were fed with only biomass and biomass plus enzyme at 6 and 12 h, respectively. The ethanol titers observed after 24 h were 75.4± 0.9 g/L and 77.8 ± 0.9 g/L, respectively. When solid loading was 20% (Trial B and E), the ethanol titers observed after 24 h were 81.4± 0.5 and 84.9 ± 1.2 g/L, respectively. When solid loading was 30% (Trial C and F), the ethanol titers observed were 79.7± 0.6 and 72.8.± 0.8 g/L, respectively, after 24 h.

Among different fed-batch trials (A-F), trial E (Figure 4.36) exhibited the best performance, after 24 h. The titer of ethanol obtained was more than 40% of the maximum ethanol titer within 6 h, which indicated better liquefaction. Therefore, the interval of 6 h was chosen for feeding during fed-batch SSF. The maximum ethanol titer and yield achieved during trial E were 84.9 ± 1.2 g/L and 0.50 g/g, respectively. The ethanol production remained constant after 24 h. As Trial E showed maximum ethanol yield and productivity, this strategy was further utilized during scale-up.

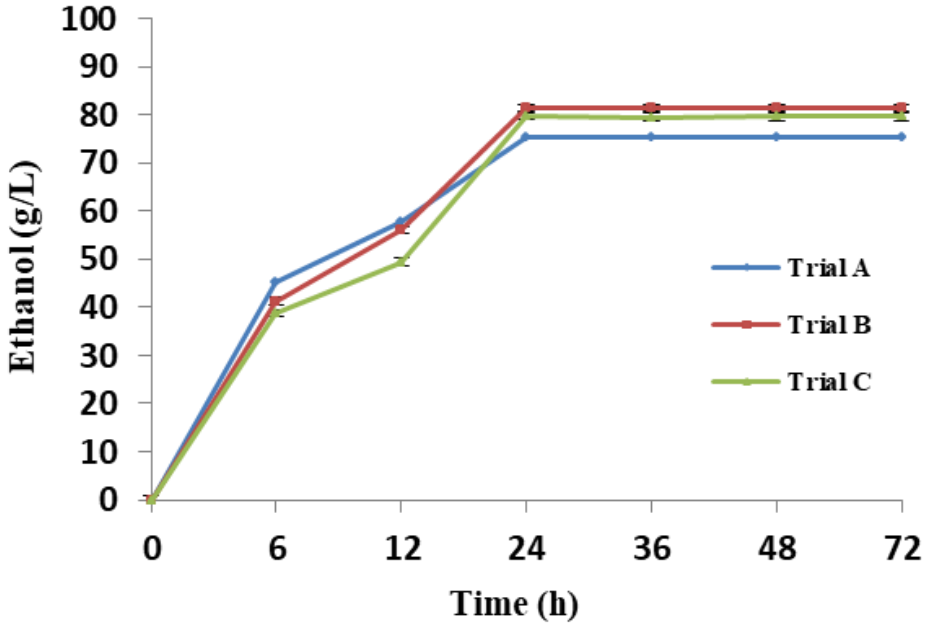


Figure 4.35: Fed-batch simultaneous saccharification and fermentation of sequential pretreated sugarcane bagasse by *K. marxianus* JKH5 C60 under shake flask employing biomass feeding strategy. Trial A, B and C were conducted at different solid loadings 18, 20 and 30 %, respectively

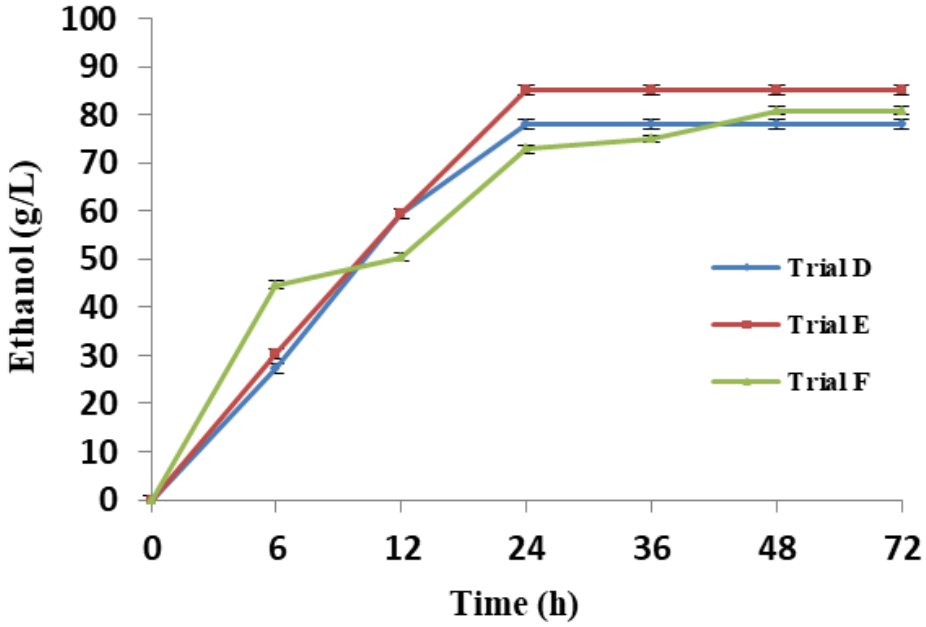


Figure 4.36: Fed-batch simultaneous saccharification and fermentation of sequential pretreated sugarcane bagasse by *K. marxianus* JKH5 C60 under shake flask employing biomass and enzyme feeding strategy. Trial D, E and F were conducted at different solid loadings 18, 20 and 30 %, respectively

4.15 Fed-batch SSF in the presence of inhibitors

Fed-batch simultaneous saccharification and fermentation process of alkali-pretreated sugarcane bagasse was investigated to produce high ethanol titer. The optimal initial solids loading (6.6 %, dry weight), enzyme dose, feeding time and other conditions derived from previous experiments were utilized to check the process efficiency in the presence of inhibitors under shake flask and then at bench-top fermenter.

4.15.1 Fed-batch SSF under shake flask

The flasks were fed at 6 h and 12 h with biomass loading (6.6 %, dry wt.) and enzyme dose (6.6 FPU/gds) in the presence of inhibitors (g/L); acetic acid (3), furfural (1) and vanillin (1) and without inhibitors (control) at initial pH 5. *K. marxianus* JKH5 C60**,** which was adapted against inhibitors cocktail, was employed for fermentation at 10%, v/v. In the control experiment, SSF of pretreated SCB resulted in an ethanol production of 84.9 g/L ethanol with a yield of 0.50 g/g (**Figure 4.37).** During the fermenter run with inhibitor cocktail supplementation, the maximum ethanol titer reached 67.1 ± 1.1 g/L at 48 h. The increase in ethanol production was observed till 24 h, thereafter, it remained almost similar till 72 h in control flask, whereas, in case of inhibitors supplemented flasks, ethanol titer kept increasing till 72 h. **(**Figure 4.37**).** The strain performed similar in both the conditions i.e. in presence and absence of inhibitors.

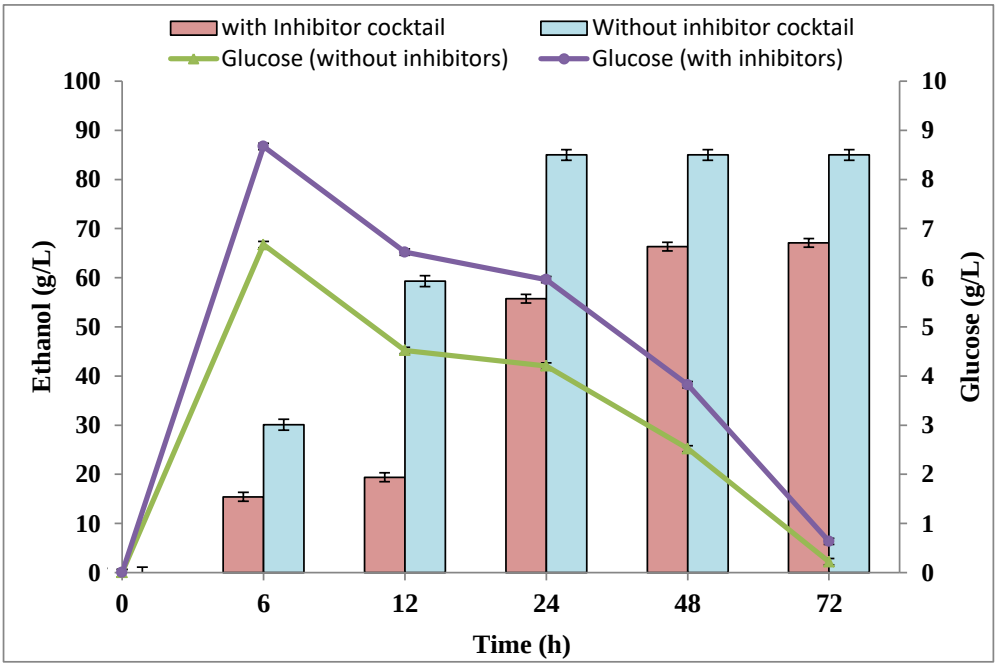


Figure 4.37: Fed-batch simultaneous saccharification and fermentation of sequentially pretreated sugarcane bagasse by adapted yeast *K. marxianus* JKH5 C60 under shake flask in presence and absence of inhibitory compounds. The solid loading was 20% (dry wt.) and 20 FPU/g enzyme dosage were employed

4.15.2 Fed-batch SSF at lab scale fermenter

Shake flask fed-batch SSF was further scaled up to further 3L bioreactor level, with a working volume of 1L. In the control experiment, SSF of pretreated SCB resulted in an ethanol production of 81 g/L ethanol with an efficiency of 86 % **(**Figure 4.38**).** During the inhibitor run with inhibitor cocktail supplementation, the maximum ethanol titer reached 73.4 ± 1.4 g/L at 24 h. The yield of ethanol obtained was 0.44 g/g with 78% conversion efficiency. The ethanol titer in both the cases increased till 72 h **(Figure 4.38).** The glucose amount observed during fed-batch SSF was less than 12 and 8 g/L, in presence an absence of inhibitors, respectively.

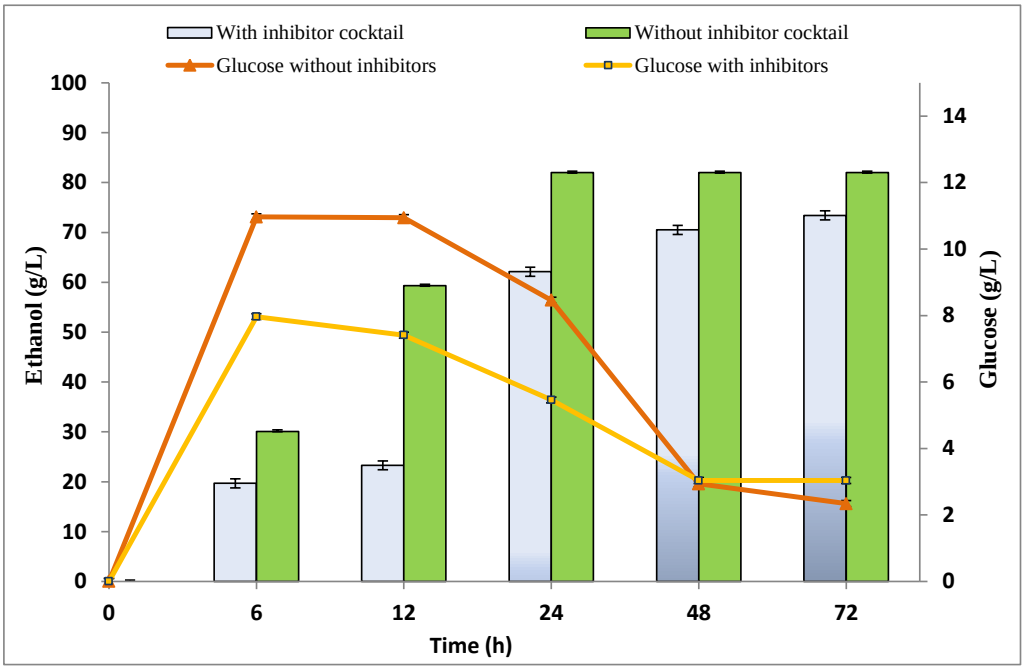


Figure 4.38: Fed-batch simultaneous saccharification and fermentation of pretreated sugarcane bagasse in the presence of inhibitors by *K. marxianus* JKH5 C60 at bench-top fermenter. The solid loading was 20% (dry wt.) and 20 FPU/g enzyme dosage were employed

(a) At 0 h (6.6 % feed) **(b) At 12 h (20% feed; complete**

Fed-batch simultaneous saccharification and fermentation of sequentially pretreated sugarcane bagasse

4.16 Detoxification and fermentation of acid hydrolysate

Acid hydrolysate obtained during dilute acid pretreatment of SCB had xylose concentration of 23 g/L, therefore, it was employed for bioethanol production using pentose fermenting yeast.

4.16.1 Detoxification

Since, acid hydrolysate had low pH and contained inhibitors (furans and phenolics), it was subjected to detoxification and neutralization prior to fermentation. Neutralization of acid hydrolysate by over liming and then detoxification by activated charcoal is an effective method for removal of inhibitors like furans (furfurans and phenolics). The detoxification of the acid hydrolysate resulted in 87-93 % removal of phenolics and 87-96% reduction in furans as shown in Table 4.16.

Table 4.16: Characteristics of dilute acid hydrolysate of sugarcane bagasse before and after detoxification

Components	**Concentration** (g/L)	
	Non-detoxified acid hydrolysate	**Detoxified acid hydrolysate**
Furans	2.17±0.84	0.57±0.002
Phenolics	1.87±0.04	0.1±0.004
Xylose	23±1.2	20±0.076
Glucose	5.8±1.0	4.5±0.05

4.16.2 Pentose fermentation

Fermentation of detoxified acid hydrolysate of SCB was carried out using *Pichia stipitis* NCIM 3499 at shake flask level. Complete xylose was utilized by the pentose fermenting yeast resulting in ethanol titer and yield of 6.8 g/L and 0.33 g/g at 72 h, respectively (Figure 4.39).

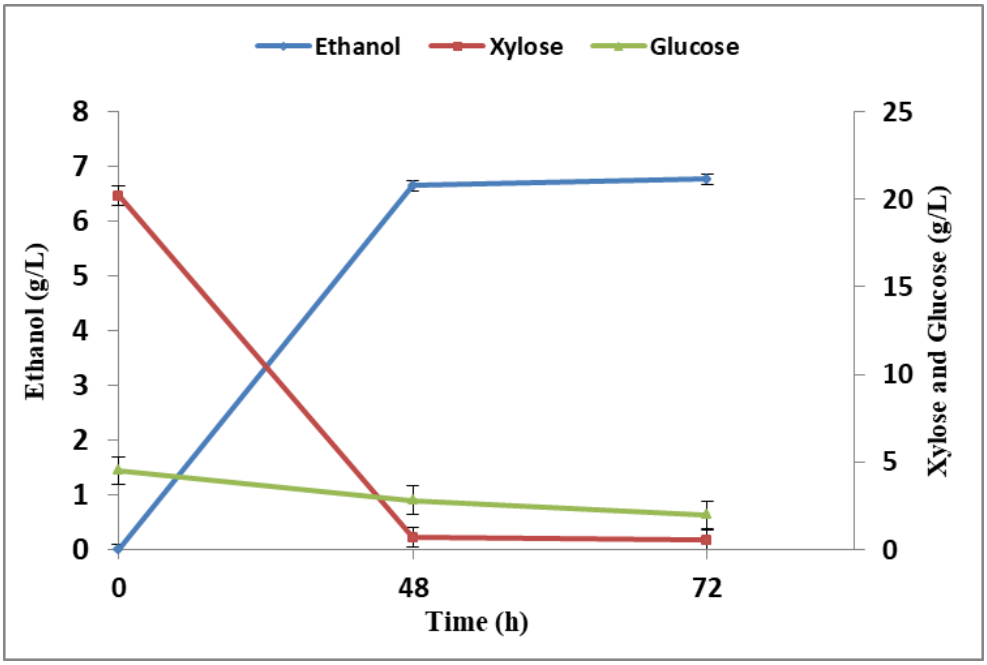


Figure 4.39: Fermentation of acid hydrolysate by *Pichia stipitis* NCIM 3499 for ethanol production under shake flask

4.17 Mass balance analysis

A complete mass balance study was carried out for sugarcane bagasse to evaluate the fate of each component and the results are shown in Figure 4.40. Dilute acid (H_2SO_4) pretreatment of SCB generated a liquid stream called acid hydrolysate and another solid biomass component. The liquid stream had 232 g pentose sugars (majorly xylose) and few inhibitory compounds such as furans (10.68 g) and phenolics (1.65 g), which were derived from breakdown of hemicellulose in the presence of acid at high temperature. The solid component cellulo-lignin was further treated with dilute alkali (NaOH) for removing lignin. The sequential dilute acid-alkali pretreatment released lignin (129.8 g) in the liquid stream leaving behind the cellulose rich biomass, which was hydrolysed into glucose using cellulase. Glucose was further fermented by yeast *K. marxianus* JKH5 C60 to ethanol (181.3 g). The acid hydrolysate was also fermented to ethanol after its detoxification (78.8 g). Thus, using 1000 g initial biomass, a maximum ethanol titer which is possible using the current process was 260.1 g.

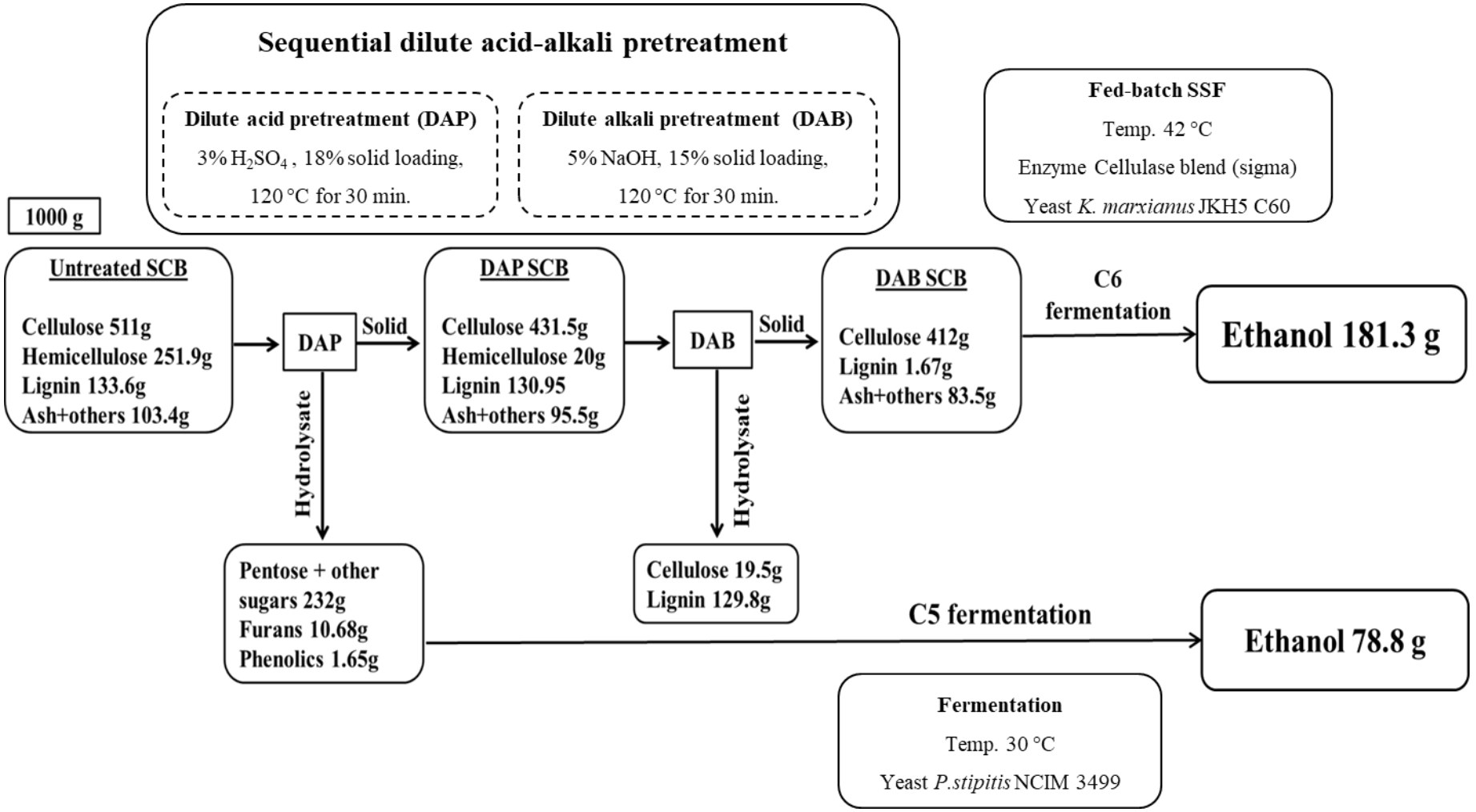


Figure 4.40: Mass Balance for bioconversion of sequential dilute acid-pretreated sugarcane bagasse to ethanol

DISCUSSION

DISCUSSION

The extravagant energy demands of humans have put an enormous pressure on the fossil fuels, current major source of energy. At the current pace of utilization, the non-renewable fossil fuels will be exhausted soon. Moreover, concerns about greenhouse gas (GHG) emissions and increasing carbon foot print in environment have driven the researchers to explore a non-conventional, sustainable fuel (Kuhad et al., 2011a; Saini et al., 2016). Bioethanol is one such biofuel which when blended with petrol provides better thermal efficiency and less CO_2 emissions as compared to unblended petrol.

Bioethanol can be produced by utilizing lignocellulosic agricultural residue through microbial conversion. Though second generation bioethanol has numerous benefits, its production process is complex which involves pretreatment, enzymatic saccharification and fermentation. Each of these steps has its own technical challenges, some of which have been addressed in the present investigation in order to make the whole bioconversion process cost-effective.

Selection of lignocellulosic biomass

Lignocellulosic biomass is considered as the future feedstock for ethanol production because of its low cost and huge availability. The total LCB production in India alone exceeds 680 metric ton per annum which accounts for production of approximately 52 billion litres of bioethanol (Jain & Agrawal, 2018). The LCB biomass chosen in the present investigation was sugarcane bagasse, which is one of the major lignocellulosic biomass generated in large quantities. Sugarcane is one of the highly produced crops in India. Durimg 2019-20, sugarcane crop production was 376 metric ton, resulting in generation of nearly 100 million tons of residual bagasse which could be utilized for bioethanol production (Konde et al., 2021).

Screening and selection of thermotolerant ethanologenic yeasts

The isolation and screening of the efficient thermotolerant ethanol producing yeasts can be helpful in simultaneous saccharification and fermentation of SCB for biofuel production. During the study, 150 thermotolerant yeasts were isolated, among which 36 isolates were found to produce ethanol during glucose fermentation at 42 °C. Isolates *Pichia kudriavzevii* JKH 1 (54 g/L) and *Kluyveromyces marxianus* JKH 5 (55 g/L) were the most potential ethanol producers. Previously, several researchers have reported yeasts isolation from different environmental sources for cost-effective ethanol production. A study by Arora et al. (2015) reported ethanol production by *K. marxianus* at 45 °C. Kaewkrajay et al. (2014) isolated thermotolerant yeast from the soil samples collected from sugarcane, cassava and pineapple plantations. In a recent study by Gao et al. (2018), sugarcane bagasse was utilized for ethanol production by a thermotolerant yeast *K. marxianus*. The main advantage of using thermotolerant yeasts is the faster rate of ethanol production which is an industrial relevant feature.

Adaptive laboratory evolution of yeast for bioethanol

The inhibitors generated during physico-chemical pretreatment of lignocellulosic biomass (furfural, 5-HMF, acetic acid, formic acid, vanillin, etc.) make the process of bioethanol production challenging by reducing the growth of yeast strains and hence, fermentation efficiency while using unwashed pretreated biomass. The effect of three different inhibitors on growth and fermentation of the yeasts clearly showed that the inhibitors were toxic to yeast as evident from reduced specific growth rate and longer lag phase. The lag phase time and specific growth rate were chosen as selection parameters for monitoring the improvement in inhibitor tolerance of the strains during repetitive batch culture in adaptive laboratory evolution experiments (Çakar et al., 2005).

The adapted strains grew well with significantly reduced lag phase in the presence of inhibitors during fermentation. This was most apparent during the lag phase upon inoculation in inhibitors-containing media. *P. kudriavzevii* JKH1 and *K. marxianus* JKH5 both showed longer lag phase in the initial stages of evolutionary experiments. The developed yeast *K. marxianus* JKH5 C60 showed 3.3 folds higher specific growth rate and 56% reduced lag time as compared to parent strains in the presence of inhibitor cocktail. The detrimental effect of inhibitors could be due to inhibition of the enzymes of the central carbon metabolism and disturbance of the cells energy balance, following their accumulation inside the cell (Modig et al., 2002; Sárvári Horváth et al., 2003).

It is expected that the adaptation under medium supplemented with inhibitors and other stresses might introduce new features to the yeast strains, which are favorable for the bioethanol production process. Interestingly, both of the strains *P. kudriavzevii* JKH1 and *K. marxianus* JKH5 displayed almost comparable fermentation yields at 42 °C using glucose as carbon source. As shown in **Table 5.1** ethanol titer and productivity were better than the other previous studies in the presence of inhibitors. Additionally, the strains in the current study are thermotolerant and grown at higher concentrations of glucose and therefore, have multiple stresses to combat. In the present study, higher ethanol yields during fermentation were achieved at elevated temperature (42 °C). Wallace-Salinas and Gorwa-Grauslund (2013) developed *Saccharomyces cerevisiae* (ISO12) for tolerating cocktail of fermentation inhibitors. During the fermentation of spruce hydrolysate at 39 °C, the ethanol produced was 16 g/L. Another study by Narayanan et al. (2016) developed *Saccharomyces cerevisiae* TMB3500 strain tolerance against acetic acid, furfural, 5-HMF and vanillin. Under synthetic medium, the yield of ethanol produced was 0.45 g/g by fermenting glucose.

Table 5.1: Comparison of ethanol production by the adapted yeasts developed in the present study with previously reported yeasts

S. No.	Strain	Fermentation conditions				Ethanol		References
		Concentration Of inhibitors (g/L)	Temperature (°C)	Carbon source	Mode (Source of sugar)	Titer (g/L) and yield (g/g)	Productivity (g/L/h)	
1.	*Saccharomyces cerevisiae* F12	Acetic acid (5.2-6.8) Furfural (1.4-1.6) Vanillin (0.1)	30	Mixture of Xylose and Glucose	Fed-Batch SSF (Prehydrolysate of wheat straw)	27.4/n.d.	0.19	Tomás-Pejó et al. (2010)
2.	*Saccharomyces cerevisiae* (ISO12)	Acetic acid (5.8) Furfural (0.5) HMF (1.5)	39	Majorly Glucose	Batch Simultaneous saccharification and fermentation (Spruce hydrolysate)	16/0.38	0.3	Wallace-Salinas and Gorwa-Grauslund (2013)
3.	*Pichia stipitis* CBS 5776	Acetic acid (2.03) Furfural (0.10) HMF (0.15) Levulinic acid (0.12)	30	Xylose	Separate hydrolysis and fermentation (Prehydrolysate of corn strover)	15.92/ n.d.	0.66	Zhu et al. (2009)
4.	*Saccharomyces cerevisiae* S-adhE	Acetic acid (2)	30	Majorly Xylose	Separate hydrolysis and fermentation (Hydrolysate of corn strover)	41/0.414	0.51	Wei et al. (2013)
5.	*Saccharomyces cerevisiae* S-nc	Acetic acid (2)	30	Majorly Xylose	Separate hydrolysis and fermentation (Hydrolysate of corn strover)	40.0/0.390	0.5	Wei et al. (2013)
6.	*Pichia stipitis* strain NRRL Y-7124	Acetic acid (1.8) Furfural (0.2) HMF (0.3)	25	Mixture of Xylose and Glucose	Separate hydrolysis and fermentation (Hydrolysate of corn	40/ n.d.	0.23	Slininger et al. (2015)

					strover)			
7.	*Saccharomyces cerevisiae* TMB3500	Acetic acid (6) Furfural(1.5) HMF (0.5) Vanillin (1)	30	Glucose	Batch Fermentation (Synthetic medium)	n.d./0.45	n.d.	Narayanan et al. (2016)
8.	*K. marxianus* FIM1	Ethanol (10%, v/v)	30	Glucose	Batch Fermentation (Synthetic medium)	110/n.d	2.2	Mo et al. (2019)
9.	*K. marxianus* FIM1	Ethanol (10%, v/v)	45	Glucose	Batch Fermentation (Synthetic medium)	58/n.d	1.3	Mo et al. (2019)
10.	*K. marxianus* NIRE-K3.1	Xylose (30)	45	Xylose	Batch Fermentation (Synthetic medium)	15.7 (Xylitol) 4.67 (Ethanol)	0.22 and 0.1	Sharma et al. (2017)
11.	*Kluyveromyces marxianus* JKH5 C60	Acetic acid (3) Furfural (1) Vanillin (1)	42	Glucose	Batch Fermentation (Synthetic medium)	20.0/0.40	1.11	Current study
12.	*Pichia kudriavzevii* JKH1 C70	Acetic acid (3) Furfural (1) Vanillin (1)	42	Glucose	Batch Fermentation (Synthetic medium)	20.79/0.40	1.15	Current study

n.d = not determined, HMF – 5-hydroxy – 2 –methyl furfural

Sequential dilute acid-alkali pretreatment of sugarcane bagasse

Present study employed sequential dilute acid-alkali pretreatment of sugarcane bagasse (SCB) for enhancing its bioconversion to ethanol. Box-Behnken and D-optimal designs were used to optimise the process of dilute acid and alkali pretreatments sequentially, resulting in an optimum concentration of 3% (v/v) and 5% (w/v) for H_2SO_4 and NaOH with solid SCB loadings of 18 and 15% (w/w), respectively, for 30 min at 121 °C. The effectiveness of sequential pretreatment was supported by increased cellulose content (83%), drop in hemicellulose, lignin content of the pretreated bioimass. The obtained cellulose content after dilute acid pretreatment in this study was better than that reported in (R.G. et al., 2012), while it was slightly less as compared to obtained in the study by Aguiar et al. (2010) (Table 5.2). Therefore, to further enhance the cellulose content, delignifcation of dilute acid pretreated SCB was attempted using dilute alkali pretreatment method (Kaur et al., 2012). Thus overall sequential pretreatment lead to efficient removal of hemicellulose and lignin due to which higher cellulose content was obtained in comparison to the previous reports (Ahmadi et al., 2016; Binod et al., 2012; Talha et al., 2016) (Table 5.2).The characterization of SCB was done using techniques like FT-IR, XRD, TGA, SEM, SANS which revealed favorable structural changes in crystallinity, porosity, thermostability etc. after pretreatment. . The results of FT-IR analyses showed highly reduced peaks at 1386 cm^{-1} (C-O of syringyl) and 1268 cm^{-1} (C-O of guaicyl ring) in spectra of sequential dilute acid-alkali pretreated SCB were in agreement with previous reports (Pasma et al., 2013; Phitsuwan et al., 2017; Singh et al., 2005).

During SEM analysis, cell wall of sequentially pretreated SCB cell wall appeared ruptured and porous with piths on surface, and had detached fibers as a consequence of delignification. Simiar results have been been reported in previous study by (Zhu et al., 2016). TG analysis of SCB indicated difference in the pattern of thermal degradation of untreated and pretreated

biomass. The final decomposition stage for all samples was completed above 400 °C, which was in correlation with the results by Ávila-Lara et al. (2015). The values of T_{max} for untreated and dilute acid pretreated SCB were 493 and 495 °C, respectively. Contrastingly, the biomass after sequential pretreatment had significantly lower T_{max} (441°C), which could be attributed to its increased amorphous nature and hence, lowered thermostability. The results TG analysis of pretreated SCB were in agreement with Brugnago et al. (2011). Crystallinity is a crucial property of LCB which negatively affects its hydrolysis by lignocellulolytic enzymes. Generally, XRD or the wide angle X-ray scattering (WAXS) is used to reveal crystallinity of LCB after the pretreatment (Zhang et al., 2015). The diffraction pattern of SCB (untreated and pretreated) in the current study was similar to cellulose-I lattice as interpreted by three diffraction peaks, the main one at 22.18°, secondary one at 16.26° and smallest one at 34.64°. This indicated that the crystallnity was significantly decreased after pretreatment as reported in earlier reports (Cheng et al., 2015; Yuan et al., 2017).

SANS has recently emerged as a robust technique for characterization of porous materials and can measure total porosity in a range of 1 to 100 nm. SANS analysis of the pretreated biomass in the present investigation, indicated that the scattering power was proportional to the density of the pores and the porosity of the biomass was increased after pretreatment. The increased pore density favours better accessibility of cellulose to enzymes and hence, enhanced sugar yield (Pingali et al., 2017). Previously, SANS was utilized for assessing the relative porosity of eucalyptus, white poplar and pine samples after pretreatment by ionic liquids (Yuan et al., 2017). There are very limited reports on SANS analysis of pretreated biomass for its characterization and none of the previous studies has focused on SANS analysis of SCB. This is the first report on SANS analyses of SCB carried out in India.

Table 5.2: Compositional analysis of untreated and pretreated sugarcane bagasse

S.No.	Untreated SCB			Pretreated SCB			Method of pretreatment	References
	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)		
1	51.10±1.8	25.19±2.5	13.36±1.4	63.31	2.53	25.53	Dilute Acid	**Current study**
2	51.10±1.8	25.19±2.5	13.36±1.4	83.30	1.26	8.30	Sequential acid alkali	**Current study**
3	46.20	31.20	9.74	50.30	21.80	6.65	Dilute Alkali	Ahmadi et al. (2016)
4	43.20	25.20	22.90	57.50	6.60	32.50	Steam explosion	Rocha et al. (2012)
5	43.20	25.20	22.90	86.80	4.0	6.10	Sequential Steam explosion and Dilute Alkali	Rocha et al. (2012)
6	55.34	25.87	11.21	70.08	5.87	12.22	Dilute Acid	R.G. et al. (2012)
7	55.34	25.87	11.21	53.40	11.98	9.75	Dilute Alkali	Aguiar et al. (2010)
8	55.34	25.87	11.21	59.50	8.46	13.37	Hydrogen peroxide (alkaline)	Aguiar et al. (2010)
9	34.0	27.0	18.0	66.60	26.50	4.90	Microwave assisted alkali	Binod et al. (2012)
10	38.59	27.89	17.79	64.89	9.61	7.85	Acid and alkaline	Guilherme et al. (2017)
11	36.0	28.70	18.0	35.70	18.10	14.0	Alkaline	Carvalho et al. (2016)
12	35.60	32.20	22.50	40.10	8.70	4.70	Alkaline	Talha et al. (2016)

Enzymatic hydrolysis of pretreated sugarcane bagasse

The bioconversion of lignocellulosic biomass to fermentatble sugar is carried by enzymatic hydrolysis using cellulase. Cellulases are complexes of enzymes that work synergistically to bring about the breakdown of cellulose (Lynd Lee et al., 2002). Cellulase producing microbes include various fungi such as *Trichoderma reesei, T. koningii, T. lignorum, Penicillium funiculosum, P. chrysoporeum, P. oxalicum, Aspergillus wenti, A. niger, Fusarium solani,* and bacteria like *Clostridium sp, Psuedomonas sp., Bacillus sp., Serratia marscens* (Deswal et al., 2011; Kuhad et al., 2011c). Most of the cellulase producing microbes do not have complete cellulase system and therefore, could not efficiently hydrolyze lignocellulosic biomass (Ahmad et al., 2013; Hemansi et al., 2018). This shortcoming has been overcome by using commercial cellulase preparations, which have higher hydrolytic efficiency. Few of the commercial cellulases available in market include Spirizyme from Novozymes A/s, Cellic 2 from Novozymes A/s, SacchariSabC6 from Advance Enzymes, Ctec series from Novozymes, Cellulase from Zytex, Accelarase from Dupont, Cellulase blend and Cellulclast from Sigma-Aldrich etc. Though the commercial enzyme formulations are expensive, these are highly efficient in hydrolysis of LCB (Hung et al., 2018; Thite & Nerurkar, 2019).

In the current study, hydrolysis of pretreated SCB was investigated with both in-house cellulase and commercial cellulases. It was found that commercial enzyme (Cellulase blend from Sigma-Aldrich) was more efficient during hydrolysis of pretreated SCB (78% saccharification) than in-house cocktail cellulase (67% saccharification). The lower efficiency of the in-house cellulases of *Trichoderma* sp. *Penicillium* sp. and *Aspergillus* sp. might be due to lower Bgl/FPU ratio and low efficiency of cellobiohydrolase enzyme as has been reported earlier (Kuhad et al., 2011c). However, the commercial enzyme preparation (Sigma) are formulated to have better Bgl/FPU ratio resulting in better hydrolysis (Singhania et al., 2009). Hence, commercial cellulase enzyme was used for further hydrolysis and

fermentation experiments to obtain higher sugar yields and subsequently higher ethanol production.

Bioethanol production from sugarcane bagasse

Separate hydrolysis and fermentation (SHF) of pretreated SCB was performed at optimum conditions of the enzyme and yeast respectively, for hydrolysis and fermentation steps (Singhania et al., 2014) The optimal conditions for release of maximum reducing sugar and ethanol production were further utilized under simultaneous saccharification and fermentation process. In the present study ethanol titer obtained was 54.9 g/L during separate hydrolysis and fermentation (SHF) of SCB which was better than the titer of 33 g/L and 40 g/L reported by Méndez et al. (2019) and Slininger et al. (2015), respectively.

Simultaneous saccharification and fermentation of sugarcane bagasse

SSF process is considered better than SHF for bioethanol production due to use of a single vessel, low inhibition of enzyme by feedback mechanisms and overall better conversion efficiency. However, the hydrolysis temperature has to be compromised during SSF when using a mesophilic fermenting microorganism. Therefore, in order to achieve better ethanol production by alleviating the mismatch of the optimal temperature of the enzymes (near 45-55 °C) and that of the fermenting microorganisms (28-35 °C), a thermotolerant yeast *Kluyveromyces marxianus,* capable of fermentation above 40 °C was employed during current study. Previously, several thermotolerant yeasts belonging mainly to genera *Kluyveromyces* have been successfully used to produce higher ethanol ranging between 40 to 80 g/L under batch SSF (Choudhary et al., 2017; Hacking et al., 1984; Hughes et al., 1984). In the present study, the adapted yeast *Kluyveromyces marxianus* JKH5 C60 was employed for batch SSF of pretreated sugarcane bagasse (20 %, dry wt. SL) at 42 °C, resulting in maximum ethanol titer of 70.1 g/L. The titer reported here was higher than the titer of 63.15 g/L and 12.6 g/L obtained previously under similar conditions of fermentation by Gao et al.

(2018) and Ballesteros et al. (2002), respectively. The ethanol titers of 18 and 14.2 g/L reported by Hoyer and co-workers, (2010) during fermentation of spruce hydrolysate at 10 and 14% solid loading, respectively, were also lower than the current report.

Fed-batch simultaneous saccharification and fermentation of sugarcane bagasse for enhanced bioethanol production

SSF under fed-batch mode has the potential to improve ethanol production at high gravity of LCB. Various strategies have been followed in the past for feeding biomass, enzyme or/and inoculum at different time intervals in order to enhance the conversion efficiency and yield of ethanol (Liu et al., 2020; Zhang & Zhu, 2017). In the current study, strategy of feeding biomass and enzyme at 6 and 12 h produced maximum ethanol during SSF at 42 °C employing *K. marxianus* JKH5 C60. Under batch SSF ethanol production was 70.1 g/L, in comparison to 84.9 g/L under fed-batch SSF at 20 % (dry wt.) solid loading. The fermentation efficiency (~80%) was also increased at high solid loading during the fed-batch process. The enhancement in ethanol production during FBSSF of SCB was comparable with the previous study (Mukasekuru et al., 2018) employing feeding of enzyme and biomass (SCB). Another research by Darkwah et al. (2016) employed fed-batch SSF of sweet sorghum bagasse at variable solid loadings and reported higher ethanol titers and yields than that obtained in the batch process. In a recent study, Gao et al. (2018) also reported a higher concentration (75.57 g/L) of bioethanol during fed-batch SSF of SCB at high solid loading than the titer (62.65 g/L) obtained under batch SSF. The authors also reported that the use of alkali pretreated bagasse was an important factor in improving cost and efficiency of bioethanol production by improving the specific surface area and cellulose accessibility to enzymes, low water consumption, and energy usage. Similarly, in the current study, the sequential dilute acid-alkali pretreatment improved the digestibility of SCB, thereby, enhancing ethanol titer and yield during fermentation.

Under fed-batch SSF in the presence of inhibitors, the ethanol titer (73.4 g/L) was 88% of the maximum titer obtained when no inhibitor was present during fermentation. The reported ethanol production in presence of inhibitors was much higher than the similar studies reported in Table 5.3. For example, Kassim and co-workers (2016) reported an ethanol titer of 10.1 and 9.21 g/L, under batch and fed-batch SSF, respectively. Similarly, (Chang et al., 2012) also suggested higher ethanol yields during FBSSF (32 g/L) than batch SSF (23 g/L) at high solid loading. Comparison of the ethanol production under batch and fed-batch SSF indicates that fed-batch SSF is obviously a better option for large scale bioethanol production at higher solid loadings (>15%) and biomass containing inhibitor.

Thus, the yeast strain *K. marxianus* JKH5 C60 developed in this study can efficiently carry out the fermentation of unwashed biomass after pretreatment and can help decrease the overall cost, time, and wastewater generation during high titer bioethanol production.

Table 5.3: Comparison of Fed-batch and batch SSF processes for cellulosic ethanol production

Substrate	Microorganisms	Enzyme*	Ethanol Titer (g/L)		References
			Fed-Batch	Batch	
Recycled paper	*K. marxianus*	Celluclast	17.7	12.6	Ballesteros et al. (2002)
Spruce	*S. cerevisiae*	Celluclast	~19	~14.2	Hoyer et al. (2010)
Spruce	*S. cerevisiae*	Celluclast	~17	~18	Hoyer et al. (2010)
Newspaper waste	*S. cerevisiae*	Cellulase+Xylanase+ Novozyme 188	14.77	5.64	Kuhad et al. (2010)
Corn-cob	*S. cerevisiae*	Cellulase+ Novozyme 188	32.3	23.0	Chang et al. (2012)
Wheat straw	*S. cerevisiae*	Cellic CTec2 and Cellic HTec2+Laccase	32	19	Moreno et al. (2013)
Sugarcane bagasse	*S. cerevisiae*	Cellic CTec2	75.57	63.15	Gao et al. (2018)
Chlorella sp.	*S. cerevisiae*	Cellulase (Sigma)	10.1	9.21	Kassim et al. (2019)
Sago hampas	*S. cerevisiae*	Spirizyme® fuel HS	111.88	62.65	Muradi et al. (2020)
Sugarcane bagasse (shake-	*K. marxianus* JKH5	Cellulase blend	84.9 ± 2.5	70.1 ± 1.7	Current study
Sugarcane bagasse (fermenter)	*K. marxianus* JKH5	Cellulase blend	81.9 ± 3.4	72.4 ± 3.7	Current study

*The enzymes used in the study are commercially available in market.

Fermentation of pentose sugar present in acid hydrolysate for ethanol

Under biorefinery approach, biomass feedstock is converted into more than one useful product such as fuel and chemicals and there is near zero waste emission. Therefore, biorefinery approach is considered more sustainable for economic and efficient production of bio-based products. In the current study, biorefinery was employed to maximise ethanol production from both hexose (derived from cellulose) and pentose (derived from hemicellulose). The capability to efficiently ferment pentose sugars is not prevalent among microbes and only few yeasts have been reported to be promising pentose fermenters, such as *Candida* sp., *Pichia* sp., and *Pachysolen tannophilus* (Abbi et al., 1996; Gírio et al., 2010; Hahn-Hägerdal et al., 2007; Palmqvist & Hahn-Hägerdal, 2000).

In the current study, *Pichia stipitis* NCIM 3499, previously reported as a potent xylose fermenting yeast (Gupta et al., 2012), was utilised for producing ethanol from dilute acid hydrolysate of SCB. The ethanol titer obtained (6.8 g/L) in the present study, was less than 9.4 g/L from acid hydrolysate of rice straw reported by Kaur and Kuhad (2019) and 11.8 g/L from acid hydrolysate of corn cob reported by Gupta et al., (2012). However, the ethanol yield of 0.34 g/g in the present study was comparable to the yield of 0.37 g/g reported by da Silva et al. (2010). Our results were better than the study of Codato et al. (2018) and Martins et al. (2018) who reported ethanol titers of 5.9 g/L and 6 g/L while fermenting xylose.

Mass Balance

The primary objective of this study is to analyse the mass balance in each unit operations for sorghum biomass to ethanol conversion. Mass balance analysis is necessary for assessing the commercial feasibility of the process since loss of the biomass components occurs during each of the operational step and it is necessary to account for this loss (Akanksha et al. 2016). In the present study, during dilute acid pretreatment, hemicelluloses of SCB were hydrolysed. The acid hydrolysate consisted pentose sugars, majorly xylose, along with inhibitory compounds like acetic acid, furans (furfural and HMF) and phenolics. The cellulignin biomass remaining after acid pretreatment was subsequently pretreated with dilute alkali, which majorly removed the lignin fraction, thereby, enhancing amorphous cellulose content. The overall loss from the native biomass reported here is comparable to the study by Rocha et al. (2012). During the first step of pretreatment, the biomass recovery was 64% and the loss was majorly due to 92 % removal of hemicellulose. After the second step of pretreatment, the biomass recovery was 88.9% and the loss was majorly due to solubilisation of lignin. Thus, during sequential pretreatment, total solid biomass recovery was 54%. Considering all the components (including cellulose, hemicellulose, lignin and degradation products) in solid and liquid fractions, overall material which could be accounted for was 94.1% of the initial biomass, the remaining being lost during sugar degradation and washing while physico-chemical treatments. In a previous study, a total of only 76.4% of the material was accounted for during the mass balance (Rocha et al., 2012), indicating that overall pretreatment was better in our study in terms of less biomass loss. The pretreated biomass having 83% cellulose content was acted upon by cellulases during hydrolysis to release glucose. The glucose and xylose (derived from detoxified acid hydrolysate) were further fermented to ethanol separately, resulting in an overall ethanol production of 91.7 g/L after fermentation.

Pentose fermentation enhanced the overall ethanol yield during the process (Akanksha et al., 2016). Using the developed process during current study a total of 260.1 kg of bioethanol could be produced per tonne.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Isolation and characterization of thermo and inhibitor tolerant yeasts

A total of 150 thermotolerant yeasts were isolated from various fruits, fruit juices, bagasse, and soil samples and waste samples collected from sugar mill and distilleries. Two most potential ethanol fermenting thermotolerant yeast were identified as *Kluyveromyces marxianus* JKH5 and *Pichia kudriavzevii* JKH1 based on their ITS-5.8s rDNA sequence analysis. These two potential yeasts were superior to rest of the isolated yeasts and standard yeast cultures due to their capability to produce ethanol titer of 55 and 54 g/L, respectively, within 18 h incubation at 42°C.

Enhancing tolerance of potential yeast strain(s) against temperature and inhibitors stress through adaptive laboratory evolution

The selected strains *K. marxianus* JKH5 and *P. kudriavzevii* JKH1 had low tolerance to inhibitors and tolerated only 3.5 g/L acetic acid, 2 g/L furfural, 2 g/L vanillin individually, and 2+0.2+0.2 g/L, respectively, of the same inhibitors mixed together in the inhibitor cocktail. Therefore, the strains were further improved to tolerate higher inhibitor concentrations through adaptive laboratory evolution (ALE) by growing them continuously in the presence of gradually increasing levels of inhibitors. After improvement, the strains were able to tolerate 6 g/L acetic acid, 3.2 g/L furfural, 3 g/L vanillin, individually, and 3+1+1 g/L, respectively, of the inhibitors present in the cocktail together. The adapted yeast *K. marxianus* JKH5 C60 had 60% and 80% improved ethanol productivity while fermenting glucose with initial concentration of 50 and 100 g/L, respectively, compared to the performance of its parent strain. In presence of inhibitors, adapted strain had shorter doubling time with reduced lag period and better specific growth rate.

Optimization of bio-process for cellulosic ethanol production by adapted yeast at shake-flask level

The method for sequential dilute acid-alkali pretreatment of sugarcane bagasse was optimized, using dilute sulfuric acid (3%, v/v) and dilute sodium hydroxide (5%, w/v) to overcome the biomass recalcitrance and improve the enzymatic hydrolysis of the lignocellulosic biomass. Upon optimized sequential dilute acid alkali pretreatment of sugarcane bagasse, the cellulose content increased from 51% to 83% and the hemicellulose content decreased from 23 to 1.4% together with a delignification of 97.2%, compared to native sugarcane bagasse. The pretreated biomass was characterized by various biophysical techniques, such as X-Ray diffraction (XRD), Scanning electron microscopy (SEM), Fourier transforming infra-red (FT-IR) spectroscopy, Thermo-gravimetric analysis (TGA) and Small angle neutron scattering (SANS) analysis. The increased cell wall porosity of the pretreated biomass, a favorable factor for improved enzymatic hydrolysis, was confirmed by increase in number of small pores and decreasing in number of large pores as analyzed by small angle neutron scattering. This is the first report on the successful application of SANS for unravelling and monitoring ultra-structural changes during deconstruction of sugarcane bagasse.

The hydrolysis of pretreated sugarcane bagasse resulted in enhanced titre of fermentable sugars at 125 g/L (78% saccharification) due to increased accessibility of biomass to cellulase. Separate hydrolysis and fermentation (SHF) of sequentially pretreated biomass for bioethanol production was performed by employing the adapted strain *K. marxianus* JKH5 C60, resulting in production of ethanol titer of 54 g/L. The same yeast was also employed for simultaneous saccharification and fermentation (SSF) of sequentially pretreated bagasse (at a solid loading of 15%, dry wt.) for enhanced production of bioethanol in presence of inhibitor cocktail. SSF by the adapted yeast resulted in an ethanol titer of 58.5 and 54.8 g/L ethanol,

when fermentation was performed in the absence (control) and presence of inhibitor cocktail, respectively.

Improvisation and scale-up of bio-process for high gravity simultaneous saccharification and fermentation of sugarcane bagasse at bench-scale fermenter

Batch-SSF of pretreated biomass (at a solid loading of 15%, dry wt.) for ethanol production using adapted yeast was successfully scaled-up to 3L lab-scale fermenter of SCB, resulting in enhanced production of ethanol with titers of 67.2 and 64.8 g/L, respectively, in absence (control) and presence of inhibitor cocktail. High solid loadings (18, 20 and 30%, dry wt.) of the pretreated biomass were also evaluated for enhancing the ethanol titer, yield and productivity. But the yield and efficiency of the ethanol production declined drastically from 80% (15 % solid loading during control experiment) to ~40% (30% solid loading) with rise in solid loading. Therefore, fed-batch strategy for enhanced ethanol production bagasse at higher solid loadings was adopted with different feeding strategies, employing feeding of biomass alone or feeding of biomass along with enzyme. The latter approach proved better for enhanced ethanol production at high gravity of biomass under shake-flask and was successfully employed for further scale-up at fermenter level. High gravity Fed-bath SSF of sequentially pretreated sugarcane bagasse (with intermittent feeding of biomass and enzyme and having a final solid loading of 20 %, dry wt.) produced 84.9 g/L ethanol with a productivity of 3.5 g/L/h under shake-flask. Under similar conditions, but in presence of inhibitor cocktail, fed-batch SSF of sequentially pretreated bagasse resulted in an ethanol titer of at 73.4 g/L under lab-scale fermenter.

Pentose sugars, majorly xylose, resulting from the dilute-acid pretreatment of sugarcane bagasse were also employed for ethanol production using pentose fermenting yeast. Before fermentation, the acid-hydrolysate was over-limed and detoxified to remove more than ~80%

inhibitors. Fermentation of the detoxified acid hydrolysate by *Pichia stipitis* NCIM 3499 resulted in production of ethanol with a titer of 6.8 g/L.

Mass balance analysis of the whole bioprocess for conversion of sugarcane bagasse to bioethanol using the adapted yeast strain was also performed to assess overall bioconversion. It indicated that overall, 260.1 kg of bioethanol could be produced per tonne of native sugarcane bagasse.

CONCLUSIONS

This study successfully developed a new robust thermo- and inhibitor tolerant yeast *Kluyveromyces marxianus* JKH5 C60 via adaptive laboratory evolution to tolerate higher concentrations of inhibitory compounds which are generated during pretreatment of biomass. Furthermore, the process of sequential dilute acid alkali pretreatment of sugarcane bagasse was developed to improve its enzymatic digestibility. Sequential pretreatment method could be employed for pretreatment of other lignocellulosic biomass also for enhancing the biomass conversion for biorefinery applications. Batch and fed-batch process of simultaneous saccharification and fermentation of pretreated bagasse were also optimized at shake flask and successfully scaled-up to 3L lab-scale fermenter using the adapted yeast. Comparison of the batch and fed-batch SSF of biomass revealed that fed-batch SSF was a better strategy for producing higher titers of cellulosic ethanol under high-gravity conditions. Pentose sugars retrieved from the dilute acid pretreatment step were also fermented successfully under biorefinery approach, to enhance overall yield of ethanol. Overall results this study indicate the developed yeast can help decrease the overall cost, time, and wastewater generation during high titer bioethanol production by eliminating the need to wash the pretreated biomass prior to fermentation.

Major limitation of the present study was that the fermentation of the unwashed pretreated biomass in the presence of actual acid hydrolysate having inhibitory compounds could not be optimized. Therefore, future studies should consider employing the developed strains for cellulosic ethanol production from unwashed biomass or pretreated biomass slurry (without liquid separation) obtained after dilute acid pretreatment of sugarcane bagasse. Furthermore, the adapted yeast strains can be evolved to ferment xylose present in the acid hydrolysate. The optimized process of enhanced bioethanol production from sequentially pretreated

sugarcane bagasse via fed-batch SSF can be further scaled-up to pilot scale fermenter in future.

REFERENCES

REFERENCES

Abbi, M., Kuhad, R.C., Singh, A. 1996. Bioconversion of pentose sugars to ethanol by free and immobilized cells of *Candida shehatae* (NCL-3501): Fermentation behaviour. *Process Biochemistry*, **31**(6), 555-560.

Aditiya, H.B., Mahlia, T.M.I., Chong, W.T., Nur, H., Sebayang, A.H. 2016. Second generation bioethanol production: A critical review. *Renewable and Sustainable Energy Reviews*, **66**, 631-653.

Agarwal, A.K. 2007. Biofuels (alcohols and biodiesel) applications as fuels for internal combustion engines. *Progress in Energy and Combustion Science*, **33**(3), 233-271.

Agbogbo, F.K., Wenger, K.S. 2007. Production of ethanol from corn stover hemicellulose hydrolyzate using *Pichia stipitis*. *Journal of Industrial Microbiology and Biotechnology*, **34**(11), 723-727.

Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B. 2011. Biomass pretreatment: Fundamentals toward application. *Biotechnology Advances*, **29**(6), 675-685.

Aguiar, M.M., Ferreira, L.F.R., Monteiro, R.T.R. 2010. Use of vinasse and sugarcane bagasse for the production of enzymes by lignocellulolytic fungi. *Brazilian Archives of Biology and Technology*, **53**, 1245-1254.

Ahmad, F., Atiyeh, M.N., Pereira, B., Stephanopoulos, G.N. 2013. A review of cellulosic microbial fuel cells: Performance and challenges. *Biomass and Bioenergy*, **56**, 179-188.

Ahmadi, F., Zamiri, M.J., Khorvash, M., Ziaee, E., Polikarpov, I. 2016. Pre-treatment of sugarcane bagasse with a combination of sodium hydroxide and lime for improving the ruminal degradability: optimization of process parameters using response surface methodology. *Journal of Applied Animal Research*, **44**(1), 287-296.

Akanksha, K., Sukumaran, R.K., Pandey, A., Rao, S.S., Binod, P. 2016. Material balance studies for the conversion of sorghum stover to bioethanol. *Biomass and Bioenergy*, **85**, 48-52.

Almario, M.P., Reyes, L.H., Kao, K.C. 2013. Evolutionary engineering of Saccharomyces cerevisiae for enhanced tolerance to hydrolysates of lignocellulosic biomass. *Biotechnology and Bioengineering*, **110**(10), 2616-2623.

Alriksson, B., Cavka, A., Jönsson, L.J. 2011. Improving the fermentability of enzymatic hydrolysates of lignocellulose through chemical in-situ detoxification with reducing agents. *Bioresource Technology*, **102**(2), 1254-1263.

Alriksson, B., Sjöde, A., Nilvebrant, N.O., Jönsson, L.J. 2006. Optimal conditions for alkaline detoxification of dilute-acid lignocellulose hydrolysates. *Appl Biochem Biotechnol*, **129-132**, 599-611.

Arora, R., Behera, S., Sharma, N.K., Kumar, S. 2019. Evaluating the Pathway for Co-fermentation of Glucose and Xylose for Enhanced Bioethanol Production Using Flux Balance Analysis. *Biotechnology and Bioprocess Engineering*, **24**(6), 924-933.

Arora, R., Behera, S., Sharma, N.K., Kumar, S. 2015. A new search for thermotolerant yeasts, its characterization and optimization using response surface methodology for ethanol production. *Frontiers in microbiology*, **6**, 889-889.

Aswal, V.K., Goyal, P.S. 2000. Small-angle neutron scattering diffractometer at Dhruva reactor. *Current Science*, **79**(7), 947-953.

Attfield, P.V., Kletsas, S. 2000. Hyperosmotic stress response by strains of bakers' yeasts in high sugar concentration medium. *Letters in Applied Microbiology*, **31**(4), 323-327.

Ávila-Lara, A.I., Camberos-Flores, J.N., Mendoza-Pérez, J.A., Messina-Fernández, S.R., Saldaña-Duran, C.E., Jimenez-Ruiz, E.I., Sánchez-Herrera, L.M., Pérez-Pimienta, J.A. 2015. Optimization of Alkaline and Dilute Acid Pretreatment of Agave Bagasse by Response Surface Methodology. *Frontiers in Bioengineering and Biotechnology*, **3**(146).

Balat, M., Balat, H., Öz, C. 2008. Progress in bioethanol processing. *Progress in Energy and Combustion Science*, **34**(5), 551-573.

Ballesteros, M., Oliva, J.M., Manzanares, P., Negro, M.J., Ballesteros, I. 2002. Ethanol production from paper material using a simultaneous saccharification and fermentation system in a fed-batch basis. *World Journal of Microbiology and Biotechnology*, **18**(6), 559-561.

Behera, B.K., Arora, M., Sharma, D.K. 1996. Scanning electron microscopic (SEM) studies on structural architecture of lignocellulosic materials of *Calotropis procera* during its processing for saccharification. *Bioresource Technology*, **58**(3), 241-245.

Behera, S.S., Ray, R.C. 2016. Solid state fermentation for production of microbial cellulases: Recent advances and improvement strategies. *International Journal of Biological Macromolecules*, **86**, 656-669.

Berlin, A., Maximenko, V., Gilkes, N., Saddler, J. 2007. Optimization of enzyme complexes for lignocellulose hydrolysis. *Biotechnology and Bioengineering*, **97**(2), 287-296.

Bhatia, S.K., Jagtap, S.S., Bedekar, A.A., Bhatia, R.K., Rajendran, K., Pugazhendhi, A., Rao, C.V., Atabani, A.E., Kumar, G., Yang, Y.-H. 2021. Renewable biohydrogen production

from lignocellulosic biomass using fermentation and integration of systems with other energy generation technologies. *Science of The Total Environment*, **765**, 144429.

Binod, P., Satyanagalakshmi, K., Sindhu, R., Janu, K.U., Sukumaran, R.K., Pandey, A. 2012. Short duration microwave assisted pretreatment enhances the enzymatic saccharification and fermentable sugar yield from sugarcane bagasse. *Renewable Energy*, **37**(1), 109-116.

Brodeur, G., Yau, E., Badal, K., Collier, J., Ramachandran, K.B., Ramakrishnan, S. 2011. Chemical and physicochemical pretreatment of lignocellulosic biomass: a review. *Enzyme Res*, **2011**, 787532.

Brugnago, R.J., Satyanarayana, K.G., Wypych, F., Ramos, L.P. 2011. The effect of steam explosion on the production of sugarcane bagasse/polyester composites. *Composites Part A: Applied Science and Manufacturing*, **42**(4), 364-370.

Çakar, Z.P., Seker, U.O.S., Tamerler, C., Sonderegger, M., Sauer, U. 2005. Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae*. *FEMS Yeast Research*, **5**(6-7), 569-578.

Cannella, D., Sveding, P.V., Jørgensen, H. 2014. PEI detoxification of pretreated spruce for high solids ethanol fermentation. *Applied Energy*, **132**, 394-403.

Cao, G., Ximenes, E., Nichols, N.N., Zhang, L., Ladisch, M. 2013. Biological abatement of cellulase inhibitors. *Bioresource Technology*, **146**, 604-610.

Carvalho, D.M.d., Queiroz, J.H.d., Colodette, J.L. 2016. Assessment of alkaline pretreatment for the production of bioethanol from eucalyptus, sugarcane bagasse and sugarcane straw. *Industrial Crops and Products*, **94**, 932-941.

Chandel, A.K., Antunes, F.A.F., Anjos, V., Bell, M.J.V., Rodrigues, L.N., Polikarpov, I., de Azevedo, E.R., Bernardinelli, O.D., Rosa, C.A., Pagnocca, F.C., da Silva, S.S. 2014. Multi-scale structural and chemical analysis of sugarcane bagasse in the process of sequential acid–base pretreatment and ethanol production by S*cheffersomyces shehata*e and S*accharomyces cerevisiae*. *Biotechnology for Biofuels*, **7**(1), 63.

Chandel, A.K., Kapoor, R.K., Singh, A., Kuhad, R.C. 2007. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. *Bioresource Technology*, **98**(10), 1947-1950.

Chang, Y.-H., Chang, K.-S., Huang, C.-W., Hsu, C.-L., Jang, H.-D. 2012. Comparison of batch and fed-batch fermentations using corncob hydrolysate for bioethanol production. *Fuel*, **97**, 166-173.

Cheng, G., Zhang, X., Simmons, B., Singh, S. 2015. Theory, practice and prospects of X-ray and neutron scattering for lignocellulosic biomass characterization: towards understanding biomass pretreatment. *Energy & Environmental Science*, **8**(2), 436-455.

Choudhary, J., Singh, S., Nain, L. 2017. Bioprospecting thermotolerant ethanologenic yeasts for simultaneous saccharification and fermentation from diverse environments. *Journal of Bioscience and Bioengineering*, **123**(3), 342-346.

Choudhary, J., Singh, S., Nain, L. 2016. Thermotolerant fermenting yeasts for simultaneous saccharification fermentation of lignocellulosic biomass. *Electronic Journal of Biotechnology*, **21**, 82-92.

Culbertson, A., Jin, M., da Costa Sousa, L., Dale, B.E., Balan, V. 2013. In-house cellulase production from AFEX™ pretreated corn stover using *Trichoderma reesei* RUT C-30. *RSC Advances*, **3**(48), 25960-25969.

da Silva, A.S.A., Inoue, H., Endo, T., Yano, S., Bon, E.P.S. 2010. Milling pretreatment of sugarcane bagasse and straw for enzymatic hydrolysis and ethanol fermentation. *Bioresource Technology*, **101**(19), 7402-7409.

Darkwah, K., Wang, L., Shahbazi, A. 2016. Simultaneous saccharification and fermentation of sweet sorghum after acid pretreatment. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, **38**(10), 1485-1492.

den Haan, R., Kroukamp, H., Mert, M., Bloom, M., Görgens, J.F., van Zyl, W.H. 2013. Engineering *Saccharomyces cerevisiae* for next generation ethanol production. *Journal of Chemical Technology & Biotechnology*, **88**(6), 983-991.

Deswal, D., Khasa, Y.P., Kuhad, R.C. 2011. Optimization of cellulase production by a brown rot fungus *Fomitopsis* sp. RCK2010 under solid state fermentation. *Bioresource Technology*, **102**(10), 6065-6072.

Dragosits, M., Mattanovich, D. 2013. Adaptive laboratory evolution—Principles and applications for biotechnology. *Microbial cell factories*, **12**, 64.

Elena, S.F., Lenski, R.E. 2003. Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nat Rev Genet*, **4**(6), 457-69.

Favaro, L., Jooste, T., Basaglia, M., Rose, S.H., Saayman, M., Görgens, J.F., Casella, S., van Zyl, W.H. 2013. Designing industrial yeasts for the consolidated bioprocessing of starchy biomass to ethanol. *Bioengineered*, **4**(2), 97-102.

Galhardo, R.S., Hastings, P.J., Rosenberg, S.M. 2007. Mutation as a Stress Response and the Regulation of Evolvability. *Critical Reviews in Biochemistry and Molecular Biology*, **42**(5), 399-435.

Gao, Y., Xu, J., Yuan, Z., Jiang, J., Zhang, Z., Li, C. 2018. Ethanol production from sugarcane bagasse by fed-batch simultaneous saccharification and fermentation at high solids loading. *Energy Science & Engineering*, **6**(6), 810-818.

Gao, Y., Xu, J., Yuan, Z., Zhang, Y., Liang, C., Liu, Y. 2014. Ethanol Production from High Solids Loading of Alkali-Pretreated Sugarcane Bagasse with an SSF Process. *BioResources; Vol 9, No 2 (2014)*.

Ghose, T.K. 1987. Measurement of cellulase activities. *Pure and Applied Chemistry*, **59**(2), 257-268.

Giese, E.C., Pierozzi, M., Dussán, K.J., Chandel, A.K., da Silva, S.S. 2013. Enzymatic saccharification of acid–alkali pretreated sugarcane bagasse using commercial enzyme preparations. *Journal of Chemical Technology & Biotechnology*, **88**(7), 1266-1272.

Gírio, F.M., Fonseca, C., Carvalheiro, F., Duarte, L.C., Marques, S., Bogel-Łukasik, R. 2010. Hemicelluloses for fuel ethanol: A review. *Bioresource Technology*, **101**(13), 4775-4800.

Guilherme, A.d.A., Dantas, P., Soares, J., Dos Santos, E., Fernandes, F., De Macedo, G. 2017. Pretreatments and enzymatic hydrolysis of sugarcane bagasse aiming at the enhancement of the yield of glucose and xylose. *Brazilian Journal of Chemical Engineering*, **34**, 937-947.

Guimarães, P.M., François, J., Parrou, J.L., Teixeira, J.A., Domingues, L. 2008. Adaptive evolution of a lactose-consuming *Saccharomyces cerevisiae* recombinant. *Appl Environ Microbiol*, **74**(6), 1748-56.

Gupta, R., Mehta, G., Chander Kuhad, R. 2012. Fermentation of pentose and hexose sugars from corncob, a low cost feedstock into ethanol. *Biomass and Bioenergy*, **47**, 334-341.

Hacking, A.J., Taylor, I.W.F., Hanas, C.M. 1984. Selection of yeast able to produce ethanol from glucose at 40° C. *Applied Microbiology and Biotechnology*, **19**(5), 361-363.

Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M.F., Lidén, G., Zacchi, G. 2006. Bio-ethanol – the fuel of tomorrow from the residues of today. *Trends in Biotechnology*, **24**(12), 549-556.

Hahn-Hägerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I., Gorwa-Grauslund, M.F. 2007. Towards industrial pentose-fermenting yeast strains. *Applied Microbiology and Biotechnology*, **74**(5), 937-953.

Hemansi, Gupta, R., Kuhad, R.C., Saini, J.K. 2018. Cost effective production of complete cellulase system by newly isolated *Aspergillus niger* RCKH-3 for efficient enzymatic

saccharification: Medium engineering by overall evaluation criteria approach (OEC). *Biochemical Engineering Journal*, **132**, 182-190.

Hemansi, Gupta, R., Yadav, G., Kumar, G., Yadav, A., Saini, J.K., Kuhad, R.C. 2019. Second Generation Bioethanol Production: The State of Art. in: *Sustainable Approaches for Biofuels Production Technologies: From Current Status to Practical Implementation*, (Eds.) N. Srivastava, M. Srivastava, P.K. Mishra, S.N. Upadhyay, P.W. Ramteke, V.K. Gupta, Springer International Publishing. Cham, pp. 121-146.

Hemansi, Kaushik, A., Yadav, G., Saini, J.K. 2021. Simultaneous saccharification and fermentation of sequential dilute acid-alkali pretreated cotton (*Gossypium hirsutum* L.) stalk for cellulosic ethanol production. *Journal of Chemical Technology & Biotechnology*, **n/a**(n/a).

Hodge, D.B., Karim, M.N., Schell, D.J., McMillan, J.D. 2008. Soluble and insoluble solids contributions to high-solids enzymatic hydrolysis of lignocellulose. *Bioresource Technology*, **99**(18), 8940-8948.

Hong, Y., Nizami, A.-S., Pour Bafrani, M., Saville, B.A., MacLean, H.L. 2013. Impact of cellulase production on environmental and financial metrics for lignocellulosic ethanol. *Biofuels, Bioproducts and Biorefining*, **7**(3), 303-313.

Hoyer, K., Galbe, M., Zacchi, G. 2010. Effects of enzyme feeding strategy on ethanol yield in fed-batch simultaneous saccharification and fermentation of spruce at high dry matter. *Biotechnol Biofuels*, **3**, 14.

Hughes, D.B., Tudroszen, N.J., Moye, C.J. 1984. The effect of temperature on the kinetics of ethanol production by a thermotolerant strain of *Kluveromyces marxianus*. *Biotechnology Letters*, **6**(1), 1-6.

Hung, H.C., Adeni, D.S.A., Johnny, Q., Vincent, M. 2018. Production of bioethanol from sago hampas via Simultaneous Saccharification and Fermentation (SSF). *Nusantara Bioscience*, **10**(4), 240-245.

Isaac, A., Antunes, F.A.F., Conti, R., Montoro, L.A., Malachias, A., Massara, P., Kitten, G., Markötter, H., Manke, I., Silva, S.S.d. 2018. Unveiling 3D physicochemical changes of sugarcane bagasse during sequential acid/alkali pretreatments by synchrotron phase-contrast imaging. *Industrial Crops and Products*, **114**, 19-27.

Jain, L., Agrawal, D. 2018. Performance evaluation of fungal cellulases with dilute acid pretreated sugarcane bagasse: A robust bioprospecting strategy for biofuel enzymes. *Renewable Energy*, **115**, 978-988.

Juturu, V., Wu, J.C. 2014. Microbial cellulases: Engineering, production and applications. *Renewable and Sustainable Energy Reviews*, **33**, 188-203.

Kaewkrajay, C., Dethoup, T., Limtong, S. 2014. Ethanol production from cassava using a newly isolated thermotolerant yeast strain. *ScienceAsia*, **40**(4), 268-277.

Kang, Q., Appels, L., Tan, T., Dewil, R. 2014. Bioethanol from Lignocellulosic Biomass: Current Findings Determine Research Priorities. *The Scientific World Journal*, **2014**, 298153.

Kassim, M.A., Tan, K.M., Serri, N.A. 2019. Enzymatic hydrolysis of dilute alkaline pretreated *Chlorella* sp. biomass for biosugar production and fed-batch bioethanol fermentation. *Asia-Pacific Journal of Science and Technology*, **24**(1).

Kaur, A., Kuhad, R.C. 2019. Valorization of Rice Straw for Ethanol Production and Lignin Recovery Using Combined Acid-Alkali Pre-treatment. *BioEnergy Research*, **12**(3), 570-582.

Kaur, U., Oberoi, H.S., Bhargav, V.K., Sharma-Shivappa, R., Dhaliwal, S.S. 2012. Ethanol production from alkali- and ozone-treated cotton stalks using thermotolerant *Pichia kudriavzevii* HOP-1. *Industrial Crops and Products*, **37**(1), 219-226.

Keshav, P.K., Shaik, N., Koti, S., Linga, V.R. 2016. Bioconversion of alkali delignified cotton stalk using two-stage dilute acid hydrolysis and fermentation of detoxified hydrolysate into ethanol. *Industrial Crops and Products*, **91**, 323-331.

Kim, N., Abdulovic, A.L., Gealy, R., Lippert, M.J., Jinks-Robertson, S. 2007. Transcription-associated mutagenesis in yeast is directly proportional to the level of gene expression and influenced by the direction of DNA replication. *DNA Repair*, **6**(9), 1285-1296.

Kim, S., Park, J.M., Seo, J.-W., Kim, C.H. 2012. Sequential acid-/alkali-pretreatment of empty palm fruit bunch fiber. *Bioresource Technology*, **109**, 229-233.

Kim, S.B., Lee, S.J., Lee, J.H., Jung, Y.R., Thapa, L.P., Kim, J.S., Um, Y., Park, C., Kim, S.W. 2013. Pretreatment of rice straw with combined process using dilute sulfuric acid and aqueous ammonia. *Biotechnology for Biofuels*, **6**(1), 109.

Ko, J.K., Enkh-Amgalan, T., Gong, G., Um, Y., Lee, S.-M. 2020. Improved bioconversion of lignocellulosic biomass by S*accharomyces cerevisiae* engineered for tolerance to acetic acid. *GCB Bioenergy*, **12**(1), 90-100.

Konde, K.S., Nagarajan, S., Kumar, V., Patil, S.V., Ranade, V.V. 2021. Sugarcane bagasse based biorefineries in India: potential and challenges. *Sustainable Energy & Fuels*, **5**(1), 52-78.

Koppram, R., Albers, E., Olsson, L. 2012. Evolutionary engineering strategies to enhance tolerance of xylose utilizing recombinant yeast to inhibitors derived from spruce biomass. *Biotechnology for Biofuels*, **5**(1), 32.

Koppram, R., Tomás-Pejó, E., Xiros, C., Olsson, L. 2014. Lignocellulosic ethanol production at high-gravity: challenges and perspectives. *Trends in Biotechnology*, **32**(1), 46-53.

Kuhad, R., Gupta, R., Khasa, Y. 2011a. Bioethanol production from lignocellulosics: an overview. *Wealth from waste*, 53-106.

Kuhad, R.C., Gupta, R., Khasa, Y.P., Singh, A., Zhang, Y.H.P. 2011b. Bioethanol production from pentose sugars: Current status and future prospects. *Renewable and Sustainable Energy Reviews*, **15**(9), 4950-4962.

Kuhad, R.C., Gupta, R., Singh, A. 2011c. Microbial cellulases and their industrial applications. *Enzyme research*, **2011**, 280696.

Kuhad, R.C., Mehta, G., Gupta, R., Sharma, K. 2010. Fed batch enzymatic saccharification of newspaper cellulosics improves the sugar content in the hydrolysates and eventually the ethanol fermentation by *Saccharomyces cerevisiae*. *Biomass & Bioenergy - BIOMASS BIOENERG*, **34**, 1189-1194.

Kuhad, R.C., Singh, A., Eriksson, K.-E.L. 1997. Microorganisms and enzymes involved in the degradation of plant fiber cell walls. in: *Biotechnology in the Pulp and Paper Industry*, (Eds.) K.E.L. Eriksson, W. Babel, H.W. Blanch, C.L. Cooney, S.O. Enfors, K.E.L. Eriksson, A. Fiechter, A.M. Klibanov, B. Mattiasson, S.B. Primrose, H.J. Rehm, P.L. Rogers, H. Sahm, K. Schügerl, G.T. Tsao, K. Venkat, J. Villadsen, U. von Stockar, C. Wandrey, Springer Berlin Heidelberg. Berlin, Heidelberg, pp. 45-125.

Kumar, R., Mago, G., Balan, V., Wyman, C.E. 2009. Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. *Bioresource Technology*, **100**(17), 3948-3962.

Kuyper, M., Toirkens, M.J., Diderich, J.A., Winkler, A.A., van Dijken, J.P., Pronk, J.T. 2005. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting Saccharomyces cerevisiae strain. *FEMS Yeast Research*, **5**(10), 925-934.

Larsen, J., Haven, M.Ø., Thirup, L. 2012. Inbicon makes lignocellulosic ethanol a commercial reality. *Biomass & Bioenergy*, **46**, 36-45.

Larsson, S., Cassland, P., Jönsson Leif, J. 2001. Development of a *Saccharomyces cerevisiae* Strain with Enhanced Resistance to Phenolic Fermentation Inhibitors in Lignocellulose Hydrolysates by Heterologous Expression of Laccase. *Applied and Environmental Microbiology*, **67**(3), 1163-1170.

Lawford, H.G., Rousseau, J.D. 1999. The Effect of Glucose on High-Level Xylose Fermentations by Recombinant *Zymomonas* in Batch and Fed-Batch Fermentations. in: *Twentieth Symposium on Biotechnology for Fuels and Chemicals: Presented as Volumes 77–79 of Applied Biochemistry and Biotechnology Proceedings of the Twentieth Symposium on Biotechnology for Fuels and Chemicals Held May 3–7, 1998, Gatlinburg, Tennessee*, (Eds.) B.H. Davison, M. Finkelstein, Humana Press. Totowa, NJ, pp. 235-249.

Lee, J.W., Kim, J.Y., Jang, H.M., Lee, M.W., Park, J.M. 2015. Sequential dilute acid and alkali pretreatment of corn stover: Sugar recovery efficiency and structural characterization. *Bioresource Technology*, **182**, 296-301.

Lin, S.-P., Kuo, T.-C., Wang, H.-T., Ting, Y., Hsieh, C.-W., Chen, Y.-K., Hsu, H.-Y., Cheng, K.-C. 2020. Enhanced bioethanol production using atmospheric cold plasma-assisted detoxification of sugarcane bagasse hydrolysate. *Bioresource Technology*, **313**, 123704.

Liu, E., Hu, Y. 2010. Construction of a xylose-fermenting Saccharomyces cerevisiae strain by combined approaches of genetic engineering, chemical mutagenesis and evolutionary adaptation. *Biochemical Engineering Journal*, **48**(2), 204-210.

Liu, Y., Yu, Q., Xu, J., Yuan, Z. 2020. Evaluation of structural factors affecting high solids enzymatic saccharification of alkali-pretreated sugarcane bagasse. *Cellulose*, **27**(3), 1441-1450.

López, M.J., Nichols, N.N., Dien, B.S., Moreno, J., Bothast, R.J. 2004. Isolation of microorganisms for biological detoxification of lignocellulosic hydrolysates. *Applied Microbiology and Biotechnology*, **64**(1), 125-131.

Lynd Lee, R., Weimer Paul, J., van Zyl Willem, H., Pretorius Isak, S. 2002. Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiology and Molecular Biology Reviews*, **66**(3), 506-577.

Lynd, L.R., Wyman, C.E., Gerngross, T.U. 1999. Biocommodity Engineering. *Biotechnology Progress*, **15**(5), 777-793.

Lyu, H., Lv, C., Zhang, M., Liu, J., Meng, F., Geng, Z.F. 2017. Kinetic studies of the strengthening effect on liquid hot water pretreatments by organic acids. *Bioresour Technol*, **235**, 193-201.

Lyu, H., Zhang, J., Zhai, Z., Feng, Y., Geng, Z. 2020. Life cycle assessment for bioethanol production from whole plant cassava by integrated process. *Journal of Cleaner Production*, **269**, 121902.

Mandels, M., Weber, J. 1969. The Production of Cellulases. in: *Cellulases and Their Applications*, Vol. 95, AMERICAN CHEMICAL SOCIETY, pp. 391-414.

Mankar, A.R., Pandey, A., Modak, A., Pant, K.K. 2021. Pretreatment of lignocellulosic biomass: A review on recent advances. *Bioresource Technology*, **334**, 125235.

Martinez, A., Rodriguez, M.E., York, S.W., Preston, J.F., Ingram, L.O. 2000. Effects of Ca(OH)2 treatments ("overliming") on the composition and toxicity of bagasse hemicellulose hydrolysates. *Biotechnology and Bioengineering*, **69**(5), 526-536.

Martiniano, S.E., Philippini, R.R., Chandel, A.K., Rosa, C.A., Pagnocca, F.C., da Silva, S.S. 2014. Evaluation of Rice Bran Extract as a Nitrogen Source for Improved Hemicellulosic Ethanol Production from Sugarcane Bagasse by New Xylose-Fermenting Yeast Strains Isolated from Brazilian Forests. *Sugar Tech*, **16**(1), 1-8.

Martins, G.M., Bocchini-Martins, D.A., Bezzerra-Bussoli, C., Pagnocca, F.C., Boscolo, M., Monteiro, D.A., Silva, R.d., Gomes, E. 2018. The isolation of pentose-assimilating yeasts and their xylose fermentation potential. *Brazilian Journal of Microbiology*, **49**(1), 162-168.

Masum, B.M., Masjuki, H.H., Kalam, M.A., Rizwanul Fattah, I.M., Palash, S.M., Abedin, M.J. 2013. Effect of ethanol–gasoline blend on NOx emission in SI engine. *Renewable and Sustainable Energy Reviews*, **24**, 209-222.

Matsusako, T., Toya, Y., Yoshikawa, K., Shimizu, H. 2017. Identification of alcohol stress tolerance genes of *Synechocystis* sp. PCC 6803 using adaptive laboratory evolution. *Biotechnology for biofuels*, **10**, 307-307.

Melo-Ferreira, J., Vilela, J., Fonseca, M.M., da Fonseca, R.R., Boursot, P., Alves, P.C. 2014. The elusive nature of adaptive mitochondrial DNA evolution of an arctic lineage prone to frequent introgression. *Genome biology and evolution*, **6**(4), 886-896.

Méndez, J., Passos, D.d.F., Wischral, D., Modesto, L.F., Pereira, N. 2019. Second-generation ethanol production by separate hydrolysis and fermentation from sugarcane bagasse with cellulose hydrolysis using a customized enzyme cocktail. *Biofuels*, 1-7.

Menon, V., Rao, M. 2012. Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. *Progress in Energy and Combustion Science*, **38**(4), 522-550.

Miller, G.L. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry*, **31**(3), 426-428.

Mo, W., Wang, M., Zhan, R., Yu, Y., He, Y., Lu, H. 2019. Kluyveromyces marxianus developing ethanol tolerance during adaptive evolution with significant improvements of multiple pathways. *Biotechnology for Biofuels*, **12**(1), 63.

Modig, T., LidÉN, G., Taherzadeh, M.J. 2002. Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. *Biochemical Journal*, **363**(3), 769-776.

Mohamed, E.T., Wang, S., Lennen, R.M., Herrgård, M.J., Simmons, B.A., Singer, S.W., Feist, A.M. 2017. Generation of a platform strain for ionic liquid tolerance using adaptive laboratory evolution. *Microbial Cell Factories*, **16**(1), 204.

Moreno, A.D., Tomás-Pejó, E., Ibarra, D., Ballesteros, M., Olsson, L. 2013. Fed-batch SSCF using steam-exploded wheat straw at high dry matter consistencies and a xylose-fermenting *Saccharomyces cerevisiae* strain: effect of laccase supplementation. *Biotechnology for Biofuels*, **6**(1), 160.

Moriarty, K., Yanowitz, J. 2015. E15 and Infrastructure.

Mukasekuru, M.R., Hu, J., Zhao, X., Sun, F.F., Pascal, K., Ren, H., Zhang, J. 2018. Enhanced High-Solids Fed-Batch Enzymatic Hydrolysis of Sugar Cane Bagasse with Accessory Enzymes and Additives at Low Cellulase Loading. *ACS Sustainable Chemistry & Engineering*, **6**(10), 12787-12796.

Muradi, N.A., Adeni, D.S.A., Suhaili, N. 2020. Enhancement of very high gravity bioethanol production via fed-batch fermentation using sago hampas as a substrate. *AsPac J. Mol. Biol. Biotechnol*.

Narayanan, V., Sànchez I Nogué, V., van Niel, E.W.J., Gorwa-Grauslund, M.F. 2016. Adaptation to low pH and lignocellulosic inhibitors resulting in ethanolic fermentation and growth of *Saccharomyces cerevisiae*. *AMB Express*, **6**(1), 59-59.

Oberoi, H.S., Chavan, Y., Bansal, S., Dhillon, G.S. 2010. Production of Cellulases through Solid State Fermentation Using Kinnow Pulp as a Major Substrate. *Food and Bioprocess Technology*, **3**(4), 528-536.

Olofsson, K., Palmqvist, B., Lidén, G. 2010. Improving simultaneous saccharification and co-fermentation of pretreated wheat straw using both enzyme and substrate feeding. *Biotechnology for Biofuels*, **3**(1), 17.

Olson, D.G., McBride, J.E., Joe Shaw, A., Lynd, L.R. 2012. Recent progress in consolidated bioprocessing. *Current Opinion in Biotechnology*, **23**(3), 396-405.

Palmqvist, E., Hahn-Hägerdal, B. 2000. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. *Bioresource Technology*, **74**(1), 17-24.

Pasma, S.A., Daik, R., Maskat, M.Y., Hassan, O. 2013. Application of Box-Behnken Design in Optimization of Glucose Production from Oil Palm Empty Fruit Bunch Cellulose. *International Journal of Polymer Science*, **2013**, 104502.

Philippini, R.R., Martiniano, S.E., Chandel, A.K., de Carvalho, W., da Silva, S.S. 2019. Pretreatment of Sugarcane Bagasse from Cane Hybrids: Effects on Chemical Composition and 2G Sugars Recovery. *Waste and Biomass Valorization*, **10**(6), 1561-1570.

Phitsuwan, P., Permsriburasuk, C., Baramee, S., Teeravivattanakit, T., Ratanakhanokchai, K. 2017. Structural Analysis of Alkaline Pretreated Rice Straw for Ethanol Production. *International Journal of Polymer Science*, **2017**, 4876969.

Pienkos, P.T., Zhang, M. 2009. Role of pretreatment and conditioning processes on toxicity of lignocellulosic biomass hydrolysates. *Cellulose*, **16**(4), 743-762.

Pingali, S.V., Urban, V.S., Heller, W.T., McGaughey, J., O'Neill, H., Foston, M.B., Li, H., Wyman, C.E., Myles, D.A., Langan, P., Ragauskas, A., Davison, B., Evans, B.R. 2017. Understanding Multiscale Structural Changes During Dilute Acid Pretreatment of Switchgrass and Poplar. *ACS Sustainable Chemistry & Engineering*, **5**(1), 426-435.

Qin, D., Hu, Y., Cheng, J., Wang, N., Li, S., Wang, D. 2016. An auto-inducible Escherichia coli strain obtained by adaptive laboratory evolution for fatty acid synthesis from ionic liquid-treated bamboo hydrolysate. *Bioresource Technology*, **221**, 375-384.

Querol, A., Belloch, C., Fernández-Espinar, M.T., Barrio, E. 2003. Molecular evolution in yeast of biotechnological interest. *Int Microbiol*, **6**(3), 201-5.

Quintero, J.A., Moncada, J., Cardona, C.A. 2013. Techno-economic analysis of bioethanol production from lignocellulosic residues in Colombia: A process simulation approach. *Bioresource Technology*, **139**, 300-307.

Qureshi, A.S., Zhang, J., Bao, J. 2015. High ethanol fermentation performance of the dry dilute acid pretreated corn stover by an evolutionarily adapted Saccharomyces cerevisiae strain. *Bioresource Technology*, **189**, 399-404.

R.G., C., Godoy, Gonçalves. 2012. Study of Sugarcane Bagasse Pretreatment with Sulfuric Acid as a Step of Cellulose Obtaining. *World Academy of Science, Engineering and Technology International Journal of Nutrition and Food Engineering*.

Ragauskas, A.J., Nagy, M., Kim, D.H., Eckert, C.A., Hallett, J.P., Liotta, C.L. 2006. From wood to fuels: Integrating biofuels and pulp production. *Industrial Biotechnology*, **2**(1), 55-65.

Rocha, G.J.M., Gonçalves, A.R., Oliveira, B.R., Olivares, E.G., Rossell, C.E.V. 2012. Steam explosion pretreatment reproduction and alkaline delignification reactions performed on a pilot scale with sugarcane bagasse for bioethanol production. *Industrial Crops and Products*, **35**(1), 274-279.

Rudolf, A., Alkasrawi, M., Zacchi, G., Lidén, G. 2005. A comparison between batch and fed-batch simultaneous saccharification and fermentation of steam pretreated spruce. *Enzyme and Microbial Technology*, **37**(2), 195-204.

Saini, J.K., Agrawal, R., Satlewal, A., Saini, R., Gupta, R., Mathur, A., Tuli, D. 2015a. Second generation bioethanol production at high gravity of pilot-scale pretreated wheat straw employing newly isolated thermotolerant yeast *Kluyveromyces marxianus* DBTIOC-35. *RSC Advances*, **5**(47), 37485-37494.

Saini, J.K., Patel, A.K., Adsul, M., Singhania, R.R. 2016. Cellulase adsorption on lignin: A roadblock for economic hydrolysis of biomass. *Renewable Energy*, **98**, 29-42.

Saini, J.K., Saini, R., Tewari, L. 2015b. Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. *3 Biotech*, **5**(4), 337-353.

Sambrook, J., Russell, D.W. 2006. Purification of nucleic acids by extraction with phenol: chloroform. *Cold Spring Harbor Protocols*, **2006**(1), pdb. prot4455.

Sánchez, Ó.J., Cardona, C.A. 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresource Technology*, **99**(13), 5270-5295.

Sanda, T., Hasunuma, T., Matsuda, F., Kondo, A. 2011. Repeated-batch fermentation of lignocellulosic hydrolysate to ethanol using a hybrid S*accharomyces cerevisiae* strain metabolically engineered for tolerance to acetic and formic acids. *Bioresource Technology*, **102**(17), 7917-7924.

Sandberg, T.E., Salazar, M.J., Weng, L.L., Palsson, B.O., Feist, A.M. 2019. The emergence of adaptive laboratory evolution as an efficient tool for biological discovery and industrial biotechnology. *Metabolic Engineering*, **56**, 1-16.

Sárvári Horváth, I., Franzén Carl, J., Taherzadeh Mohammad, J., Niklasson, C., Lidén, G. 2003. Effects of Furfural on the Respiratory Metabolism of Saccharomyces cerevisiae in Glucose-Limited Chemostats. *Applied and Environmental Microbiology*, **69**(7), 4076-4086.

Sauer, U. 2001. Evolutionary Engineering of Industrially Important Microbial Phenotypes. in: *Metabolic Engineering*, (Eds.) J. Nielsen, L. Eggeling, J. Dynesen, M. Gárdonyi, R.T. Gill, A.A. de Graaf, B. Hahn-Hägerdal, L.J. Jönsson, C. Khosla, R. Licari, R. McDaniel,

M. McIntyre, C. Miiller, J. Nielsen, R.R. Cordero Otero, H. Sahm, U. Sauer, D.E. Stafford, G. Stephanopoulos, C.E. Wahlbom, K.S. Yanagimachi, W.H. van Zyl, Springer Berlin Heidelberg. Berlin, Heidelberg, pp. 129-169.

Segal, L., Creely, J.J., Martin, A.E., Conrad, C.M. 1959. An Empirical Method for Estimating the Degree of Crystallinity of Native Cellulose Using the X-Ray Diffractometer. *Textile Research Journal*, **29**(10), 786-794.

Sharma, N.K., Behera, S., Arora, R., Kumar, S. 2017. Evolutionary Adaptation of *Kluyveromyces marxianus* NIRE-K3 for Enhanced Xylose Utilization. **5**(32).

Shengdong, Z., Yuanxin, W., Yufeng, Z., Shaoyong, T., Yongping, X., Ziniu, Y., Xuan, Z. 2006. Fed-batch simultaneous saccharification and fermentation of microwave/acid/alkali/H2O2 pretreated rice straw for production of ethanol. *Chemical Engineering Communications*, **193**(5), 639-648.

Shibuya, M., Sasaki, K., Tanaka, Y., Yasukawa, M., Takahashi, T., Kondo, A., Matsuyama, H. 2017a. Development of combined nanofiltration and forward osmosis process for production of ethanol from pretreated rice straw. *Bioresource Technology*, **235**, 405-410.

Shibuya, M., Yasukawa, M., Sasaki, K., Tanaka, Y., Takahashi, T., Kondo, A., Matsuyama, H. 2017b. Up-concentration of sugars in pretreated-rice straw by an osmotic pressure-driven method. *Biochemical Engineering Journal*, **121**, 13-16.

Shui, Z.-X., Qin, H., Wu, B., Ruan, Z.-y., Wang, L.-s., Tan, F.-R., Wang, J.-L., Tang, X.-Y., Dai, L.-C., Hu, G.-Q., He, M.-X. 2015. Adaptive laboratory evolution of ethanologenic Zymomonas mobilis strain tolerant to furfural and acetic acid inhibitors. *Applied Microbiology and Biotechnology*, **99**(13), 5739-5748.

Silverstein, R.A., Chen, Y., Sharma-Shivappa, R.R., Boyette, M.D., Osborne, J. 2007. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. *Bioresource Technology*, **98**(16), 3000-3011.

Singh, R., Singh, S., Trimukhe, K.D., Pandare, K.V., Bastawade, K.B., Gokhale, D.V., Varma, A.J. 2005. Lignin–carbohydrate complexes from sugarcane bagasse: Preparation, purification, and characterization. *Carbohydrate Polymers*, **62**(1), 57-66.

Singhania, R.R., Patel, A.K., Soccol, C.R., Pandey, A. 2009. Recent advances in solid-state fermentation. *Biochemical Engineering Journal*, **44**(1), 13-18.

Singhania, R.R., Saini, J.K., Saini, R., Adsul, M., Mathur, A., Gupta, R., Tuli, D.K. 2014. Bioethanol production from wheat straw via enzymatic route employing Penicillium janthinellum cellulases. *Bioresource Technology*, **169**, 490-495.

Singleton, V.L., Orthofer, R., Lamuela-Raventós, R.M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. in: *Methods in Enzymology*, Vol. 299, Academic Press, pp. 152-178.

Slininger, P.J., Shea-Andersh, M.A., Thompson, S.R., Dien, B.S., Kurtzman, C.P., Balan, V., da Costa Sousa, L., Uppugundla, N., Dale, B.E., Cotta, M.A. 2015. Evolved strains of *Scheffersomyces stipitis* achieving high ethanol productivity on acid- and base-pretreated biomass hydrolyzate at high solids loading. *Biotechnology for Biofuels*, **8**(1), 60.

Smith, J., van Rensburg, E., Görgens, J.F. 2014. Simultaneously improving xylose fermentation and tolerance to lignocellulosic inhibitors through evolutionary engineering of recombinant S*accharomyces cerevisiae*harbouring xylose isomerase. *BMC Biotechnology*, **14**(1), 41.

Sonderegger, M., Sauer, U. 2003. Evolutionary Engineering of *Saccharomyces cerevisiae* for Anaerobic Growth on Xylose. *Applied and Environmental Microbiology*, **69**(4), 1990-1998.

Sotaniemi, V.-H., Taskila, S., Ojamo, H., Tanskanen, J. 2016. Controlled feeding of lignocellulosic substrate enhances the performance of fed-batch enzymatic hydrolysis in a stirred tank reactor. *Biomass and Bioenergy*, **91**, 271-277.

Stanbury, P., Whitaker, A. 1984. Principles of Fermentation Technology, edition. ed, Pergamon, Oxford Oxfordshire, New York.

Steensels, J., Verstrepen, K.J. 2014. Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations. *Annu Rev Microbiol*, **68**, 61-80.

Studer, M.H., DeMartini, J.D., Davis, M.F., Sykes, R.W., Davison, B., Keller, M., Tuskan, G.A., Wyman, C.E. 2011. Lignin content in natural Populus variants affects sugar release. *Proceedings of the National Academy of Sciences*, **108**(15), 6300.

Taherzadeh, M.J., Gustafsson, L., Niklasson, C., Lidén, G. 2000. Inhibition effects of furfural on aerobic batch cultivation of *Saccharomyces cerevisiae* growing on ethanol and/or acetic acid. *Journal of Bioscience and Bioengineering*, **90**(4), 374-380.

Taherzadeh, M.J., Karimi, K. 2008. Pretreatment of Lignocellulosic Wastes to Improve Ethanol and Biogas Production: A Review. *International Journal of Molecular Sciences*, **9**(9).

Talha, Z., Ding, W., Mehryar, E., Hassan, M., Bi, J. 2016. Alkaline Pretreatment of Sugarcane Bagasse and Filter Mud Codigested to Improve Biomethane Production. *BioMed Research International*, **2016**, 8650597.

Talukdar, S., Talukdar, M., Buragohain, M., Yadav, A., Yadav, R.N.S., Bora, T.C. 2016. Enhanced candicidal compound production by a new soil isolate P*enicillium verruculosum* MKH7 under submerged fermentation. *BMC Microbiology*, **16**(1), 288.

Tan, K.T., Lee, K.T., Mohamed, A.R. 2008. Role of energy policy in renewable energy accomplishment: The case of second-generation bioethanol. *Energy Policy*, **36**(9), 3360-3365.

TAPPI. 1992. Test Methods Program. *Process & Product Quality Conference Proceedings*.

Thite, V.S., Nerurkar, A.S. 2019. Valorization of sugarcane bagasse by chemical pretreatment and enzyme mediated deconstruction. *Scientific Reports*, **9**(1), 15904.

Tiwari, R., Pranaw, K., Singh, S., Nain, P.K.S., Shukla, P., Nain, L. 2016. Two-step statistical optimization for cold active β-glucosidase production from *Pseudomonas lutea* BG8 and its application for improving saccharification of paddy straw. *Biotechnology and Applied Biochemistry*, **63**(5), 659-668.

Tomás-Pejó, E., Ballesteros, M., Oliva, J.M., Olsson, L. 2010. Adaptation of the xylose fermenting yeast *Saccharomyces cerevisiae* F12 for improving ethanol production in different fed-batch SSF processes. *Journal of Industrial Microbiology and Biotechnology*, **37**(11), 1211-1220.

Tomás-Pejó, E., Oliva, J.M., González, A., Ballesteros, I., Ballesteros, M. 2009. Bioethanol production from wheat straw by the thermotolerant yeast *Kluyveromyces marxianus* CECT 10875 in a simultaneous saccharification and fermentation fed-batch process. *Fuel*, **88**(11), 2142-2147.

Varma, A.K., Mondal, P. 2016. Physicochemical Characterization and Pyrolysis Kinetic Study of Sugarcane Bagasse Using Thermogravimetric Analysis. *Journal of Energy Resources Technology*, **138**(5).

Wallace-Salinas, V., Gorwa-Grauslund, M.F. 2013. Adaptive evolution of an industrial strain of *Saccharomyces cerevisiae* for combined tolerance to inhibitors and temperature. *Biotechnol Biofuels*, **6**(1), 151.

Wanderley, M.C.d.A., Martín, C., Rocha, G.J.d.M., Gouveia, E.R. 2013. Increase in ethanol production from sugarcane bagasse based on combined pretreatments and fed-batch enzymatic hydrolysis. *Bioresource Technology*, **128**, 448-453.

Wang, L., Littlewood, J., Murphy, R.J. 2013. Environmental sustainability of bioethanol production from wheat straw in the UK. *Renewable and Sustainable Energy Reviews*, **28**, 715-725.

Wang, S., Sun, X., Yuan, Q. 2018. Strategies for enhancing microbial tolerance to inhibitors for biofuel production: A review. *Bioresour Technol*, **258**, 302-309.

Watanabe, S., Saleh, A.A., Pack, S.P., Annaluru, N., Kodaki, T., Makino, K. 2007. Ethanol production from xylose by recombinant S*accharomyces cerevisia*e expressing protein engineered NADP+-dependent xylitol dehydrogenase. *Journal of Biotechnology*, **130**(3), 316-319.

Wei, N., Quarterman, J., Kim, S.R., Cate, J.H.D., Jin, Y.-S. 2013. Enhanced biofuel production through coupled acetic acid and xylose consumption by engineered yeast. *Nature Communications*, **4**(1), 2580.

Wimalasena, T.T., Greetham, D., Marvin, M.E., Liti, G., Chandelia, Y., Hart, A., Louis, E.J., Phister, T.G., Tucker, G.A., Smart, K.A. 2014. Phenotypic characterisation of *Saccharomyces* spp. yeast for tolerance to stresses encountered during fermentation of lignocellulosic residues to produce bioethanol. *Microbial Cell Factories*, **13**(1), 47.

Wingren, A., Galbe, M., Zacchi, G. 2003. Techno-economic evaluation of producing ethanol from softwood: comparison of SSF and SHF and identification of bottlenecks. *Biotechnol Prog*, **19**(4), 1109-17.

Wisselink, H.W., Toirkens Maurice, J., Wu, Q., Pronk Jack, T., van Maris Antonius, J.A. 2009. Novel Evolutionary Engineering Approach for Accelerated Utilization of Glucose, Xylose, and Arabinose Mixtures by Engineered *Saccharomyces cerevisiae* Strains. *Applied and Environmental Microbiology*, **75**(4), 907-914.

Wu, Y., Jameel, A., Xing, X.-H., Zhang, C. 2021. Advanced strategies and tools to facilitate and streamline microbial adaptive laboratory evolution. *Trends in Biotechnology*.

Yamakawa, C.K., Qin, F., Mussatto, S.I. 2018. Advances and opportunities in biomass conversion technologies and biorefineries for the development of a bio-based economy. *Biomass and Bioenergy*, **119**, 54-60.

Yomano, L.P., York, S.W., Ingram, L.O. 1998. Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production. *Journal of Industrial Microbiology and Biotechnology*, **20**(2), 132-138.

Yu, K.L., Chen, W.-H., Sheen, H.-K., Chang, J.-S., Lin, C.-S., Ong, H.C., Show, P.L., Ling, T.C. 2020. Bioethanol production from acid pretreated microalgal hydrolysate using microwave-assisted heating wet torrefaction. *Fuel*, **279**, 118435.

Yuan, X., Duan, Y., He, L., Singh, S., Simmons, B., Cheng, G. 2017. Characterization of white poplar and eucalyptus after ionic liquid pretreatment as a function of biomass loading using X-ray diffraction and small angle neutron scattering. *Bioresource Technology*, **232**, 113-118.

Zhang, J., Zhang, X., Li, C., Zhang, W., Zhang, J., Zhang, R., Yuan, Q., Liu, G., Cheng, G. 2015. A comparative study of enzymatic hydrolysis and thermal degradation of corn stover: understanding biomass pretreatment. *RSC Advances*, **5**(46), 36999-37005.

Zhang, M.-M., Xiong, L., Tang, Y.-J., Mehmood, M.A., Zhao, Z.K., Bai, F.-W., Zhao, X.-Q. 2019. Enhanced acetic acid stress tolerance and ethanol production in *Saccharomyces cerevisiae* by modulating expression of the de novo purine biosynthesis genes. *Biotechnology for Biofuels*, **12**(1), 116.

Zhang, T., Zhu, M.-J. 2017. Enhanced bioethanol production by fed-batch simultaneous saccharification and co-fermentation at high solid loading of Fenton reaction and sodium hydroxide sequentially pretreated sugarcane bagasse. *Bioresource Technology*, **229**, 204-210.

Zhang, Y., Xia, C., Lu, M., Tu, M. 2018. Effect of overliming and activated carbon detoxification on inhibitors removal and butanol fermentation of poplar prehydrolysates. *Biotechnology for Biofuels*, **11**(1), 178.

Zhu, J.-J., Yong, Q., Xu, Y., Chen, S.-X., Yu, S.-Y. 2009. Adaptation fermentation of *Pichia stipitis* and combination detoxification on steam exploded lignocellulosic prehydrolyzate. *Natural Science*, **Vol.01No.01**, 8.

Zhu, Z., Rezende, C.A., Simister, R., McQueen-Mason, S.J., Macquarrie, D.J., Polikarpov, I., Gomez, L.D. 2016. Efficient sugar production from sugarcane bagasse by microwave assisted acid and alkali pretreatment. *Biomass and Bioenergy*, **93**, 269-278.

ANNEXURES

Annexure I

S. No.	Chemical	Source
1.	Dinitrosalicylic acid, Glucose, Xylose, Carboxymethyl cellulose, Furfural, Hydroxymethyl furfural,Vanillin, Cellulase enzyme blend, pnitrophenyl p-nitrophenol, p-nitrophenyl-β-D-glucopyranoside, Congo Red, Tween 80, Triton X-100, Xylose, Avicel, Carboxy Methyl cellulose, ethanol, Acetic acid, Acetonitrile, phosphoric acid, , sulphuric acid, Acetone	Sigma-Merck,USA
2.	Yeast Extract, Peptone, Beef Extract, Dextrose, Ammonium sulphate, Ammonium bicarbonate, Ammonium chloride, Acetone, Magnesium sulphate, Potassium dihydrogen sulphate, di-potassium hydrogen sulaphte, di-sodium hydrogen orthophosphate, Citric acid, Sodium nitrate, Sodium nitrite, Sodium bicarbonate, Sodium hydroxide, Urea, Copper sulphate, Ferrous sulphate, Manganese sulphate, Zinc sulphate, Sodium azide, Sodium sulphite, sodium potassium tartarate	Hi-Media, India
3.	Tween 80, Triton X 100, Sodium carbonate, Follin-Ciocalteu reagent	Sisco research laboratories, Central Drug House Pvt. Ltd. Ansari road, New Delhi, India
4.	Chloramphenicol	SRL
5.	Phenol	Qualigen fine chemicals, Mumabi, India
6.	Wheat bran and sugarcane bagasse	Locally
7.	Genomic DNA isolation kit, gel extraction kit	MDI Pvt. Ltd., India and Promega, USA
8.	*Taq* DNA polymerase, DNAase, RNAse	NEB, UK

Annexure II

Pichia kudriavzevii JKH 1 similarity sequences

Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident (%)	Acc. Len	Accession
Pichia kudriavzevii	865	865	94%	0	100	521	MT599316.1
Pichia kudriavzevii	865	865	94%	0	100	481	MN371845.1
Pichia kudriavzevii	865	865	94%	0	100	496	MK329990.1
Pichia kudriavzevii	865	865	94%	0	100	536	LC389030.1
Pichia kudriavzevii	865	865	94%	0	100	537	LC389007.1
Pichia sp.	865	865	94%	0	100	514	MF662390.1
Pichia kudriavzevii	865	865	94%	0	100	504	MG015964.1
Pichia kudriavzevii	865	865	94%	0	100	512	KT175182.1
Suhomyces xylopsoci	865	865	94%	0	100	521	MZ269243.1
Trichophyton erinacei	865	865	94%	0	100	521	MZ266318.1
Pichia kudriavzevii	865	865	94%	0	100	510	MZ020643.1
Saccharomycetes sp.	865	865	94%	0	100	484	MZ089524.1
Pichia kudriavzevii	865	865	94%	0	100	513	JX174414.1
saccharomycete sp. KCH	865	865	94%	0	100	517	EU315760.1
[Candida] inconspicua	865	865	94%	0	100	515	EU315757.1
Pichia kudriavzevii	865	865	94%	0	100	516	EU315751.1
Saccharomycetes sp.	863	955	94%	0	100	580	MG748838.1
Pichia kudriavzevii	857	857	93%	0	100	520	MT875240.1
Pichia kudriavzevii	857	857	93%	0	100	490	MT772077.1
Pichia kudriavzevii	857	857	93%	0	100	490	MT772076.1
Pichia kudriavzevii	857	857	93%	0	100	490	MT772075.1
Pichia kudriavzevii	857	857	93%	0	100	490	MT772074.1
Pichia kudriavzevii	857	857	93%	0	100	490	MT772073.1
Pichia kudriavzevii	857	857	93%	0	100	490	MT772072.1

Kluyveromyces marxianus JKH 4 similarity sequences

Scientific Name	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Accession
Kluyveromyces marxianus	1441	1441	62%	0	100	780	MN450867.1
Kluyveromyces marxianus	1225	1225	53%	0	99.7	711	KY611850.1
Kluyveromyces marxianus	1225	1225	53%	0	99.7	717	LC269188.1
Kluyveromyces marxianus	1225	1225	53%	0	99.7	854	KY103837.1
Kluyveromyces marxianus	1225	1225	53%	0	99.7	723	KY103833.1
Kluyveromyces marxianus	1225	1225	53%	0	99.7	686	KY103831.1
Kluyveromyces marxianus	1225	1225	53%	0	99.7	857	KY103795.1
Kluyveromyces marxianus	1225	1225	53%	0	99.7	686	HG532087.1
Kluyveromyces marxianus	1225	1225	53%	0	99.7	679	HG532083.1
Kluyveromyces marxianus	1225	1225	53%	0	99.7	698	KX833106.1
Kluyveromyces sp.	1225	1225	53%	0	99.7	702	MK713462.1
Kluyveromyces marxianus	1225	1225	53%	0	99.7	700	JQ425345.1
Kluyveromyces marxianus	1225	1225	53%	0	99.7	715	JQ083435.1
Kluyveromyces marxianus	1223	1223	53%	0	99.7	700	MG009532.1
Kluyveromyces marxianus	1225	1225	53%	0	99.55	756	KY103808.1
Kluyveromyces marxianus	1225	1225	53%	0	99.55	703	KY103803.1
Kluyveromyces marxianus	1225	1225	53%	0	99.55	726	KX376261.1
Kluyveromyces marxianus	1225	1225	53%	0	99.55	713	KP132326.1
Kluyveromyces marxianus	1234	1234	54%	0	99.41	744	MK268122.1
Kluyveromyces marxianus	1223	1223	53%	0	99.41	712	HQ014729.2
Kluyveromyces marxianus	1223	1223	53%	0	99.7	670	KF851354.1
Kluyveromyces sp.	1223	1223	53%	0	99.7	694	MK713485.1
Kluyveromyces marxianus	1221	1221	53%	0	99.7	748	KY103790.1
Kluyveromyces sp.	1219	1219	53%	0	99.7	730	MK271338.1
Kluyveromyces marxianus	1221	1221	54%	0	99.26	731	KY103816.1
Kluyveromyces marxianus	1221	1221	53%	0	99.41	714	KP132325.1
Kluyveromyces marxianus	1221	1221	54%	0	99.26	727	HQ014731.1
Kluyveromyces	1232	1232	54%	0	99.13	687	KF646189.

marxianus							1
Kluyveromyces marxianus	1232	1232	54%	0	99.13	1005	KC905771.1

Kluyveromyces marxianus JKH 5 similarity sequences

Scientific Name	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Accession
Kluyveromyces marxianus	1142	1142	97%	0	99.68	938	MN450878.1
Kluyveromyces marxianus	1138	1138	97%	0	99.68	656	MH045268.1
Kluyveromyces marxianus	1134	1134	97%	0	99.36	708	KJ830981.1
Kluyveromyces marxianus	1129	1129	97%	0	99.36	697	MN985331.1
Kluyveromyces marxianus	1127	1127	95%	0	99.84	780	MN450867.1
Kluyveromyces marxianus	1127	1127	96%	0	99.52	693	MN371852.1
Kluyveromyces marxianus	1127	1686	96%	0	99.52	1277	KF851351.1
Kluyveromyces marxianus	1127	1127	96%	0	99.52	721	MW828328.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	652	MT448651.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	664	MT136538.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	663	MT136537.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	634	MT136535.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	664	MT136534.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	664	MT136533.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	697	MT321271.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	639	MT187615.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	733	MT187614.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	726	LR738883.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	726	LR738882.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	726	LR738881.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	726	LR738880.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	726	LR738879.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	726	LR738878.1
Kluyveromyces	1125	1125	96%	0	99.52	726	LR738877.

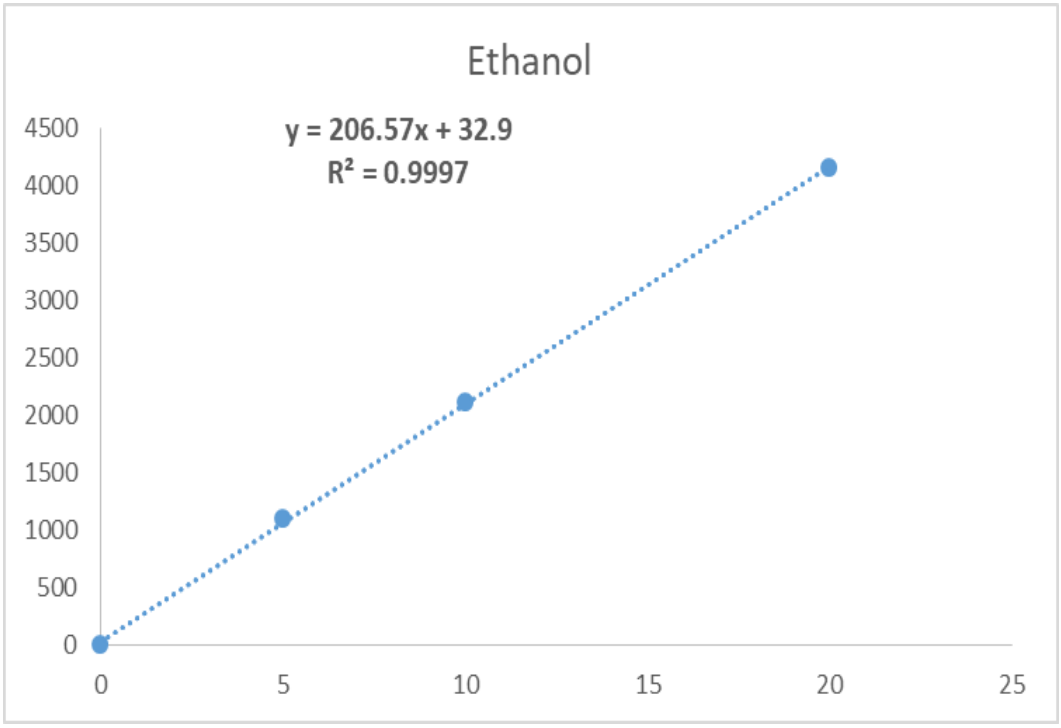
marxianus							1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	726	LR738876.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	726	LR738875.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	726	LR738874.1
Kluyveromyces marxianus	1125	1125	96%	0	99.52	726	LR738873.1

Kluyveromyces marxianus JKH 7 similarity sequences

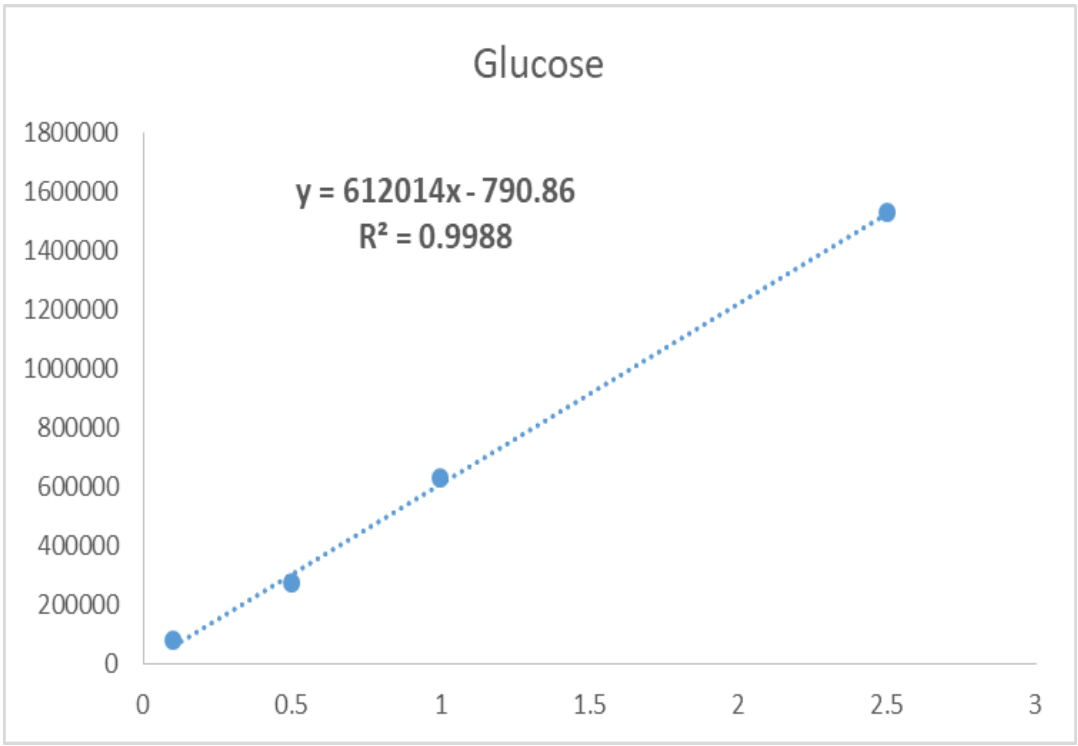
Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident (%)	Acc. Len	Accession
Kluyveromyces marxianus	1733	1733	98%	0	100	938	MN450878.1
Kluyveromyces marxianus	1271	1271	74%	0	99.02	721	MW828328.1
Kluyveromyces marxianus	1229	1229	72%	0	99.12	693	MN371852.1
Kluyveromyces marxianus	1229	1229	71%	0	99.12	716	KF646169.1
Kluyveromyces marxianus	1229	1229	72%	0	98.98	694	KC544505.1
Kluyveromyces marxianus	1225	1225	72%	0	98.84	697	MN985331.1
Kluyveromyces marxianus	1225	1225	71%	0	99.12	725	KX376264.1
Kluyveromyces marxianus	1225	1225	71%	0	99.41	695	MW618876.1
Kluyveromyces marxianus	1223	1223	70%	0	99.55	744	MK268122.1
Kluyveromyces marxianus	1223	1223	71%	0	99.26	708	KJ830981.1
Kluyveromyces marxianus	1219	1219	70%	0	99.41	756	KY103808.1
Kluyveromyces marxianus	1219	1219	70%	0	99.41	703	KY103803.1
Kluyveromyces marxianus	1219	1219	70%	0	99.41	713	KP132326.1
Kluyveromyces marxianus	1218	1218	70%	0	99.55	717	LC269188.1
Kluyveromyces marxianus	1218	1218	70%	0	99.55	854	KY103837.1
Kluyveromyces marxianus	1218	1218	70%	0	99.55	723	KY103833.1
Kluyveromyces marxianus	1218	1218	70%	0	99.55	686	KY103831.1
Kluyveromyces marxianus	1218	1218	70%	0	99.55	857	KY103795.1
Kluyveromyces marxianus	1218	1218	70%	0	99.55	698	KX833106.1
Kluyveromyces marxianus	1218	1218	70%	0	99.55	686	HG532087.1

Kluyveromyces marxianus	1218	1218	70%	0	99.55	679	HG532083.1
Kluyveromyces sp.	1218	1218	70%	0	99.55	702	MK713462.1
Kluyveromyces marxianus	1218	1218	70%	0	99.55	715	JQ083435.1
Kluyveromyces marxianus	1216	1216	70%	0	99.55	700	MG009532.1
Kluyveromyces marxianus	1216	1216	70%	0	99.55	670	KF851354.1
Kluyveromyces sp.	1216	1216	70%	0	99.55	694	MK713485.1
Kluyveromyces marxianus	1214	1214	70%	0	99.55	748	KY103790.1
Kluyveromyces marxianus	1214	1214	70%	0	99.26	714	KP132325.1
Kluyveromyces marxianus	1214	1214	70%	0	99.4	687	KF646189.1
Kluyveromyces marxianus	1214	1214	70%	0	99.26	727	HQ014731.1

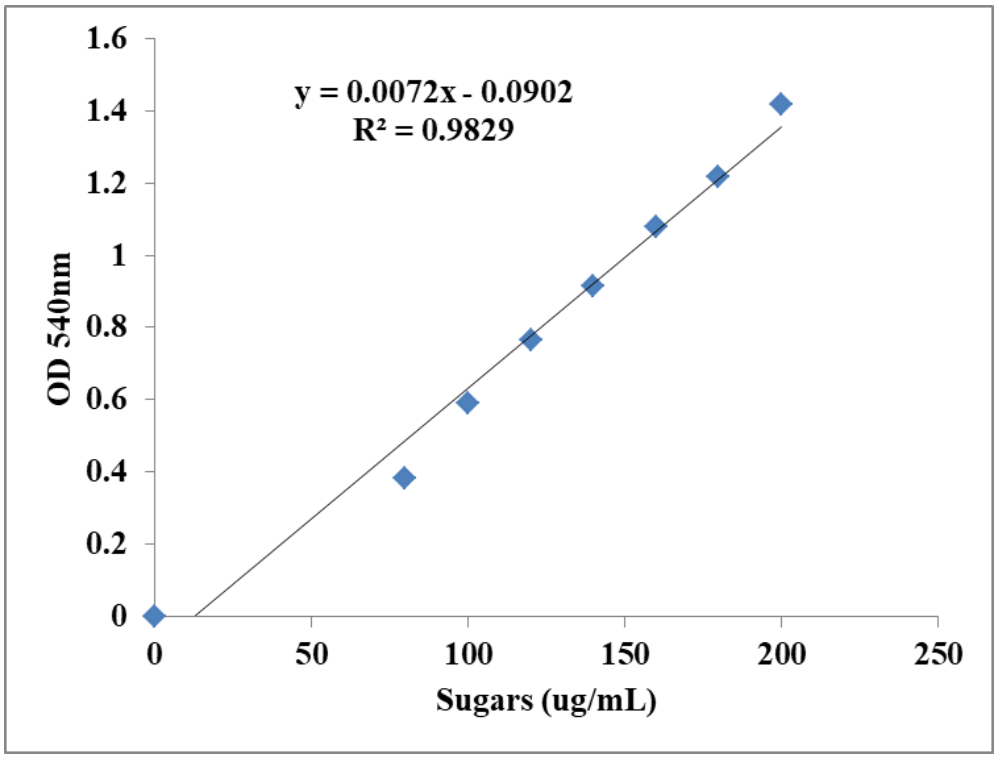
Annexure III



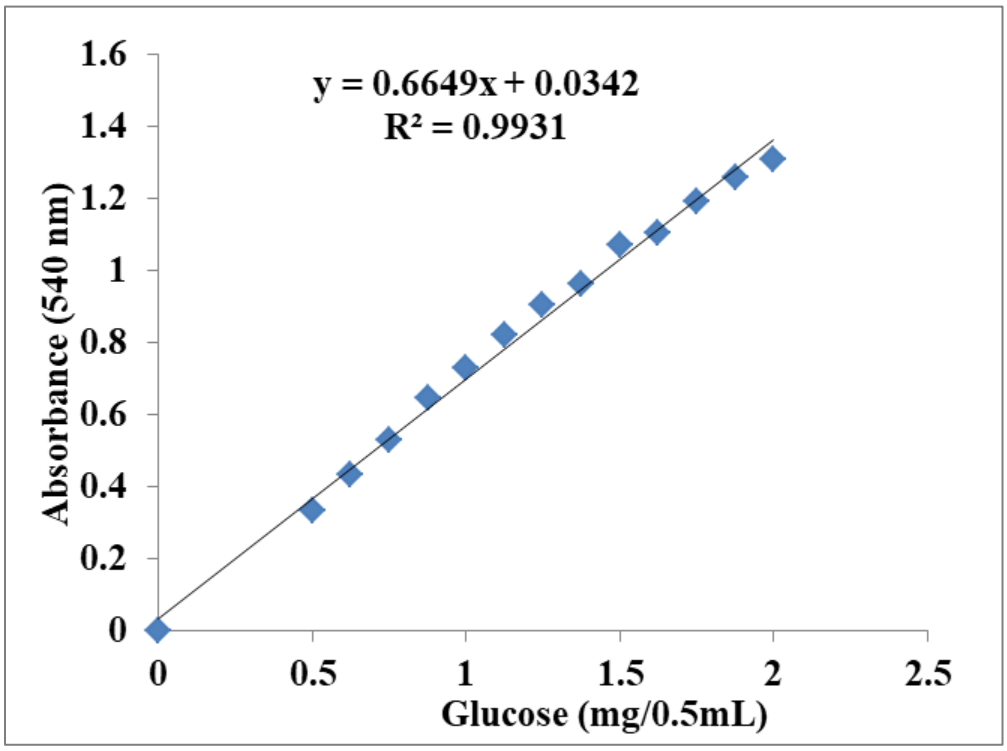
Standard curve of ethanol for estimation of ethanol by HPLC



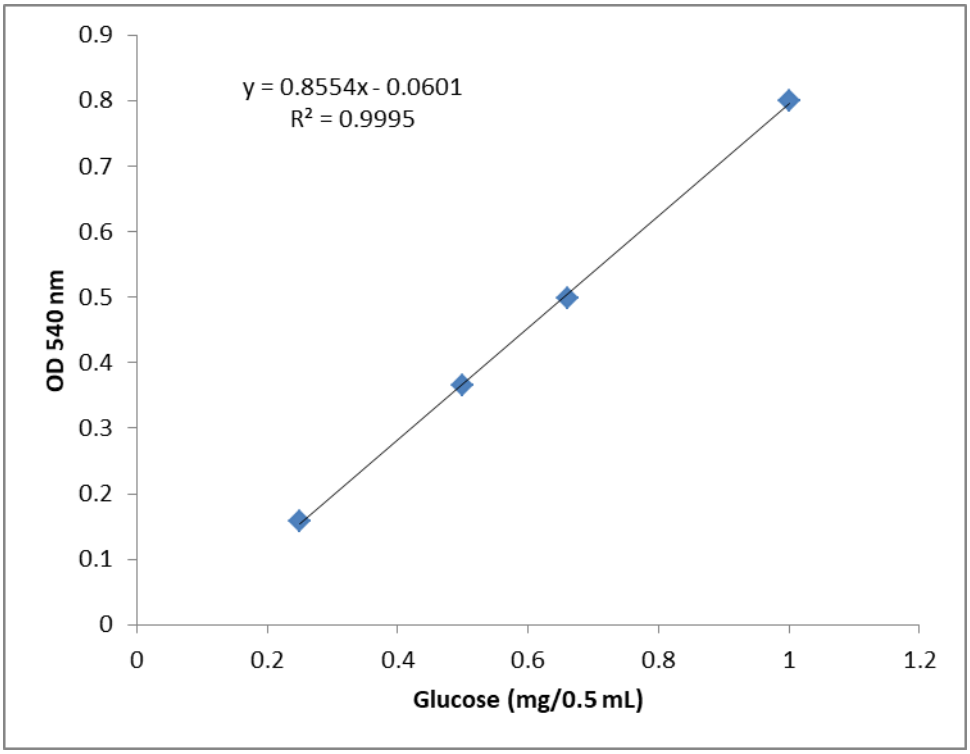
Standard curve of glucose for estimation of glucose by HPLC



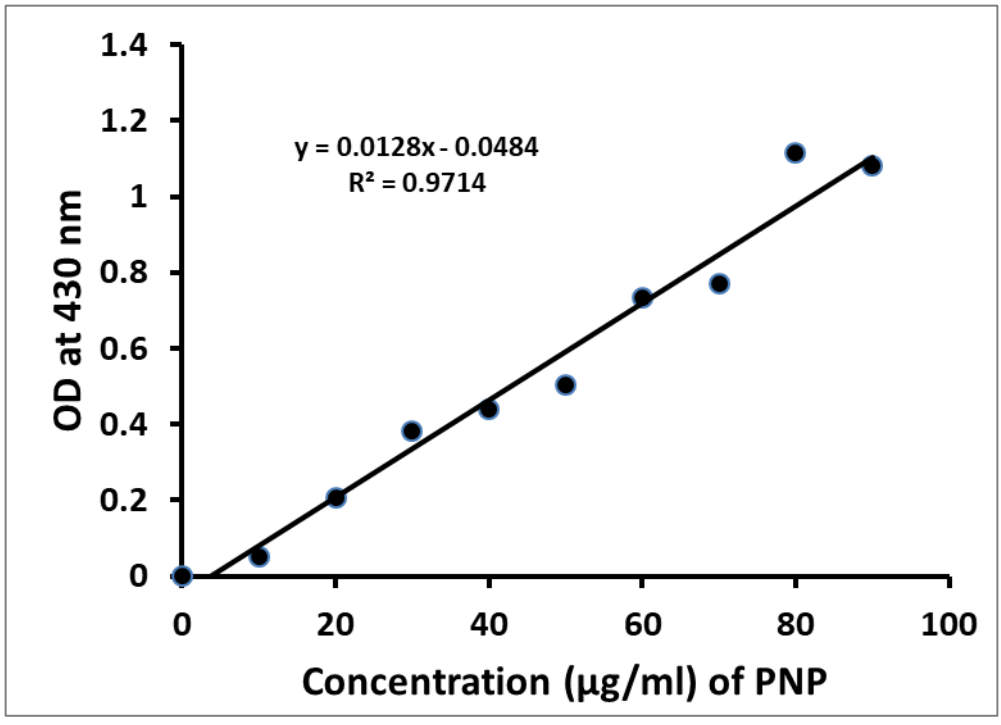
Standard curve of glucose for estimation of reducing sugars



Standard curve of glucose for estimation of Filter Paper Cellulase (exoglucanase)



Standard curve of glucose for estimation of Carboxy-methyl Cellulase (endoglucanase)



Standard curve of p-nitro phenol for estimation of β-glucosidase