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

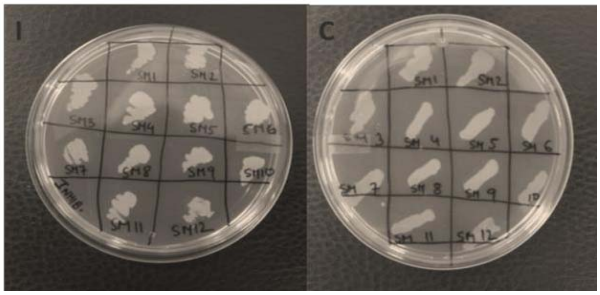
4.1. Isolation, screening and selection of thermotolerant inhibitor tolerant yeasts

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).**

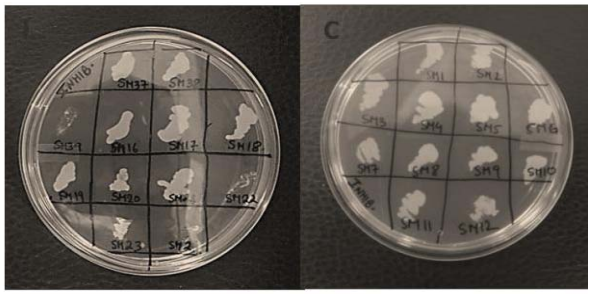
Thirty six screened yeast isolates were studied for ethanol production under shake flask **(Table 4.2**) at higher glucose concentration (150 g/L). Among those, isolates 1A, SM4, SM5 and SM7 produced 55 ± 0.5, 51 ± 1.2, 54 ± 0.7 and 52 ± 0.7 g/L ethanol respectively, after 18 h at 42 °C. The ethanol 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

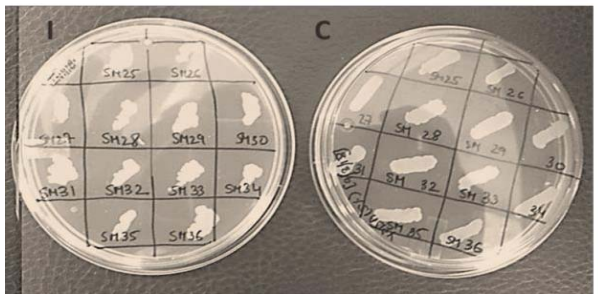
Sample	Site of Collection	No. of Samples	No. of isolates	Name of isolate
Apple	Fruit market, Mahendergarh, Haryana	5	2	5A1, 5A2
Berry	Fruit market, Noida	2	2	B1, B2
Grape	Mahendergarh, Haryana	2	4	G1, G2, G3, G4
Citrus fruit	Rewari, Haryana	1	15	C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, 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



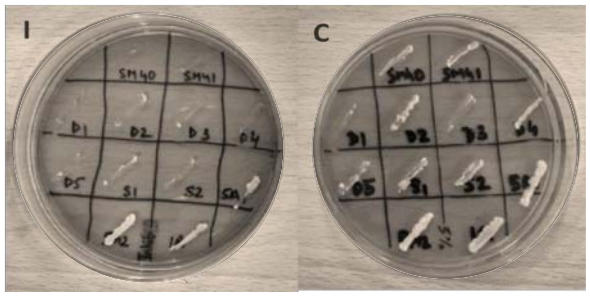
SM1		SM2	
SM3	SM4	SM5	SM6
SM7	SM8	SM9	SM10
SM11		SM12	



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.

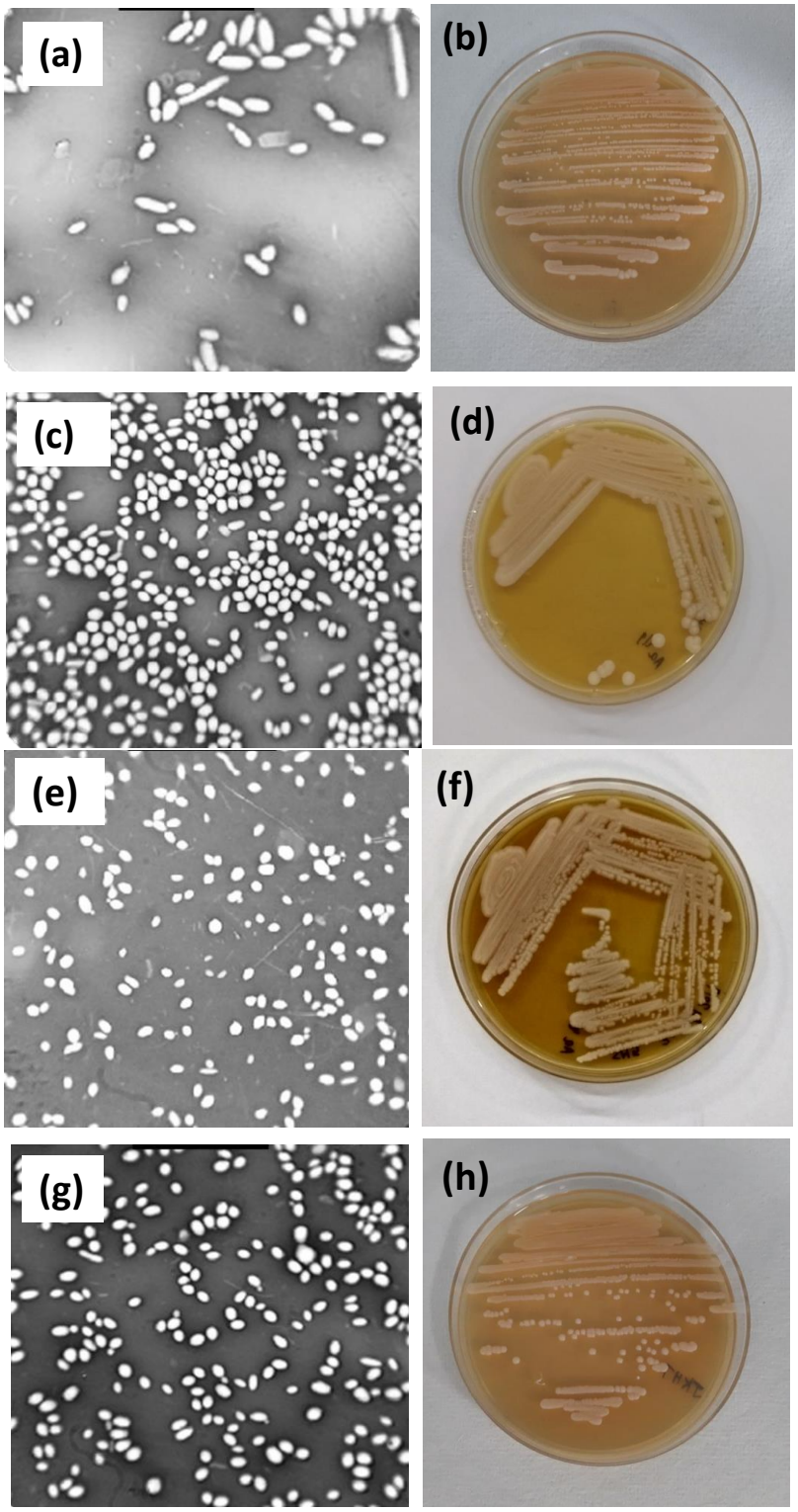


Figure 4.2: Colony characteristics and cell morphology (40× magnification) of yeast isolates JKH1 (a & b), JKH4 (c & d), JKH5 (e & f) and JKH7 (g & h).

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.

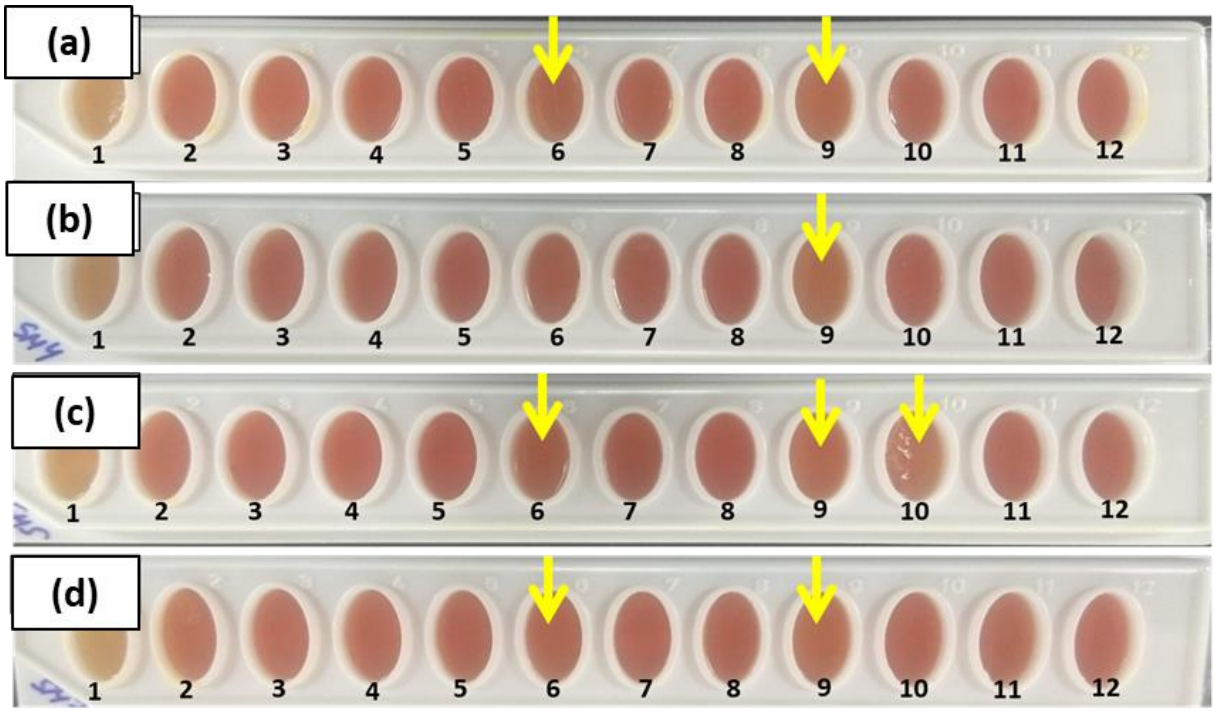


Figure 4.3: Biochemical characterization of selected yeast isolates JKH1 (a), JKH4 (b), JKH5 (c), and JKH7 (d) (1- Urease, 2- Melibiose, 3- Lactose, 4- Maltose, 5- Sucrose, 6- Galactose, 7- Cellobiose, 8- Inositol, 9- Xylose, 10- Dulcitol, 11- Raffinose, 12- Trehalose).

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.

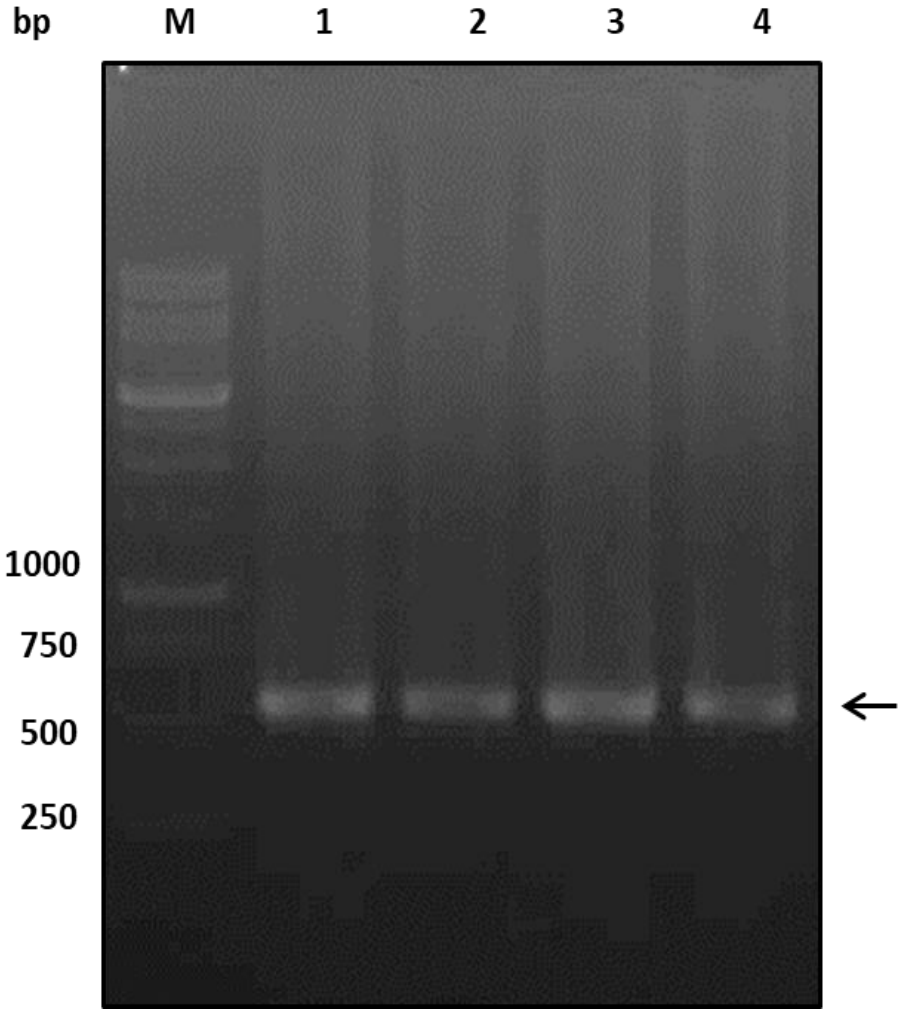


Figure 4.4: Agarose gel electrophoresis representing ITS-5.8S rDNA amplicons of selected yeast isolates. Lane 1: PCR amplicon of JKH1, Lane 2: PCR amplicon of JKH4, Lane 3: PCR amplicon of JKH5, Lane 4: PCR amplicon of JKH7 and Lane M: 1000 b.p. DNA ladder

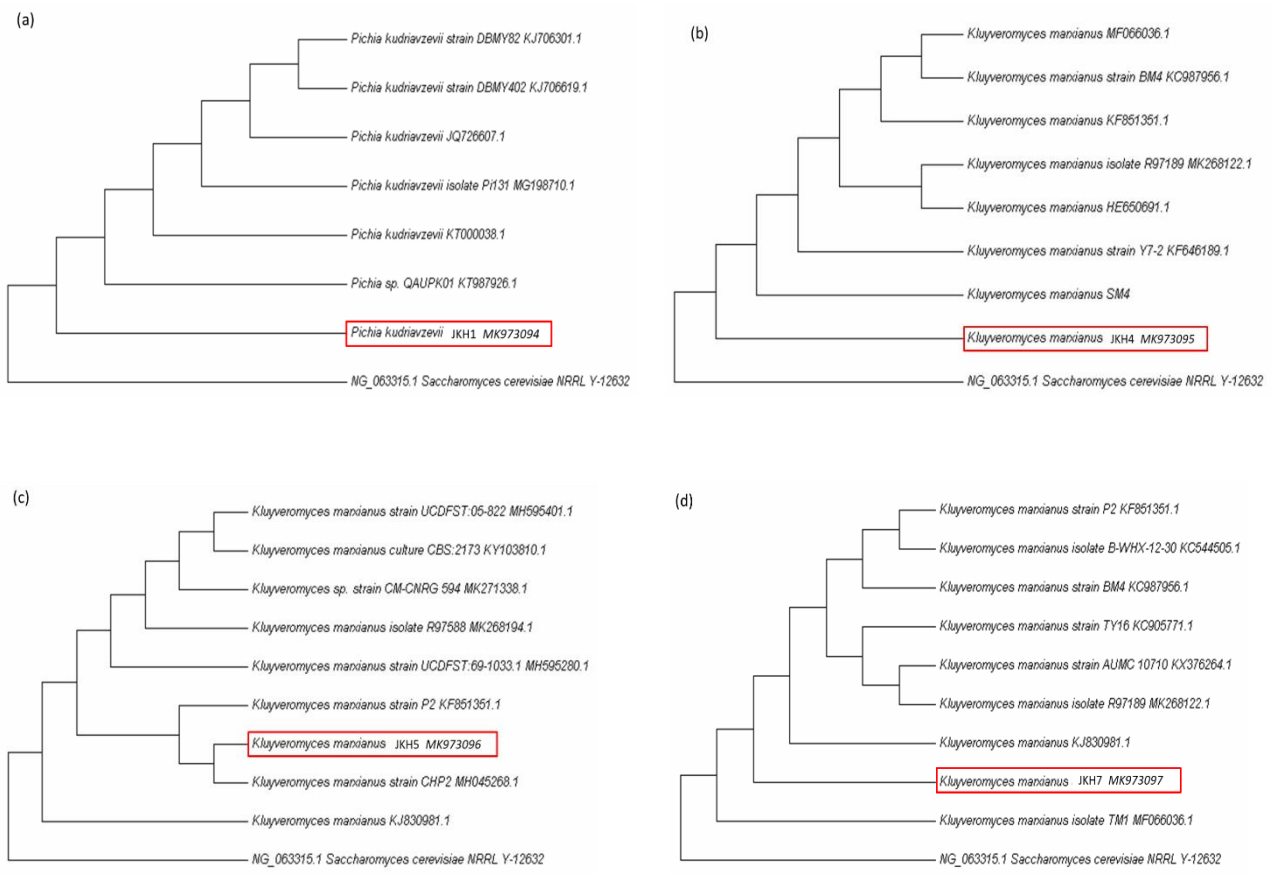


Figure 4.5: The phylogenetic trees showing genetic relatedness of *Pichia kudriavzevii* JKH1 (a), *Kluyveromyces marxianus* JKH4 (b), *Kluyveromyces marxianus* JKH5 (c) and *Kluyveromyces marxianus* JKH7 (d) with other yeasts based on their ITS-5.8S rDNA region

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.

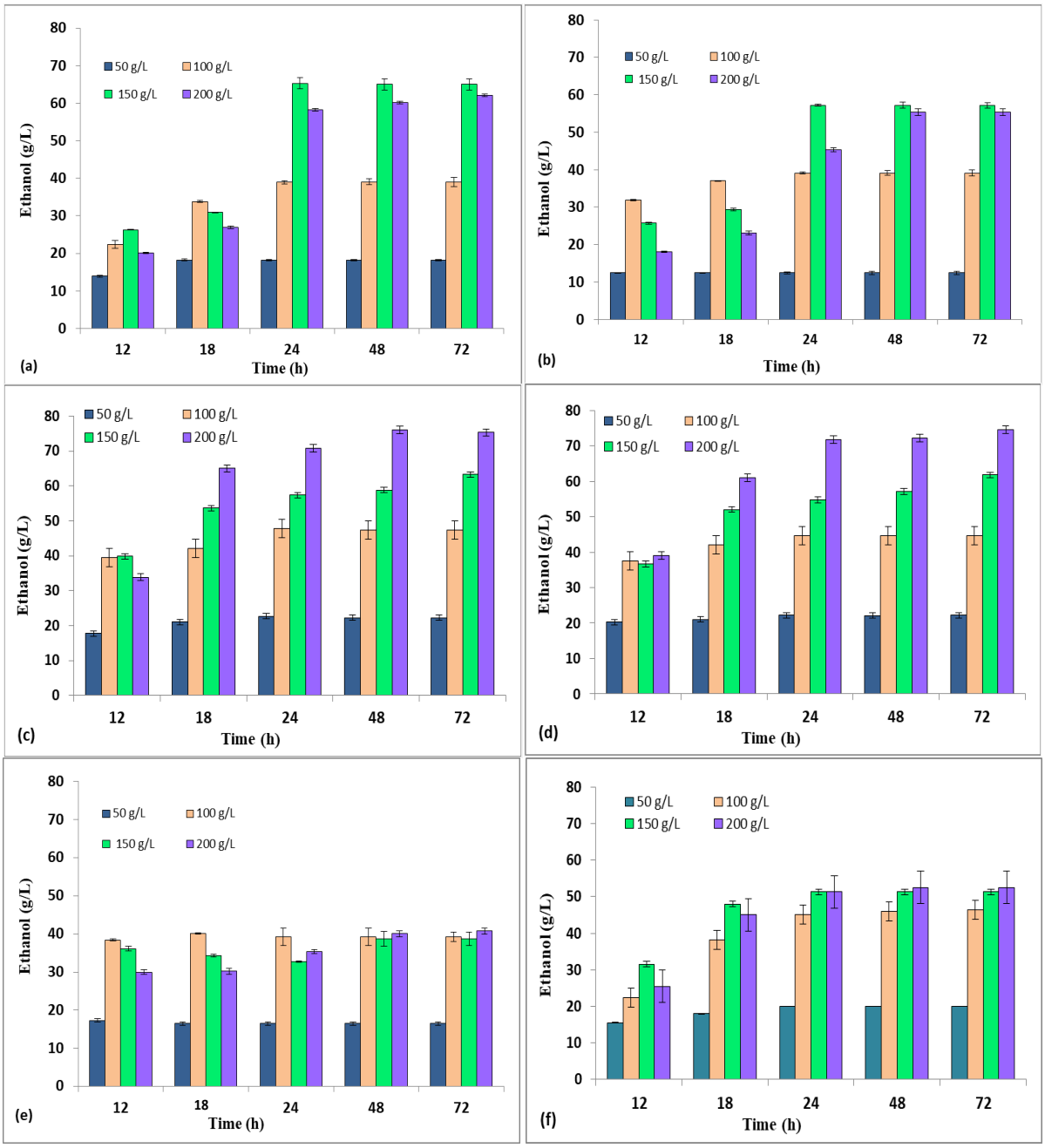


Figure 4.6: Effect of different glucose concentrations on ethanol production by *Pichia kudriavzevii* JKH1 (a), *Kluyveromyces marxianus* JKH4 (b), *Kluyveromyces marxianus* JKH5 (c) and *Kluyveromyces marxianus* JKH7 (d) and standard thermotolerant yeast strains *Kluyveromyces marxianus* NCIM 3565 (e)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

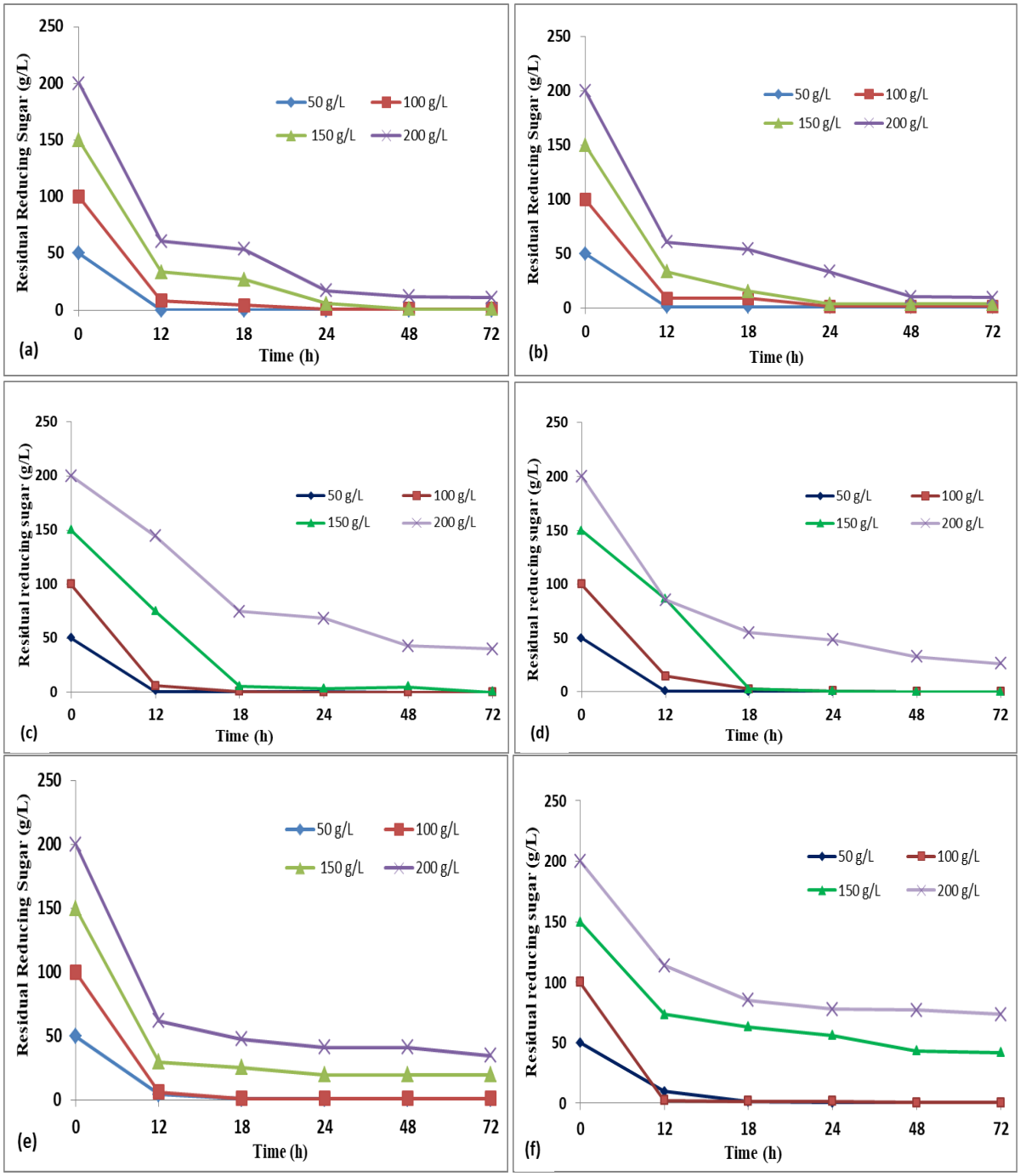


Figure 4.7: Effect of different glucose concentrations on residual reducing sugars during fermentation by *Pichia kudriavzevii* JKH1 (a), *Kluyveromyces marxianus* JKH4 (b), *Kluyveromyces marxianus* JKH5 (c) and *Kluyveromyces marxianus* JKH7 (d) and standard thermotolerant yeast strains *Kluyveromyces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

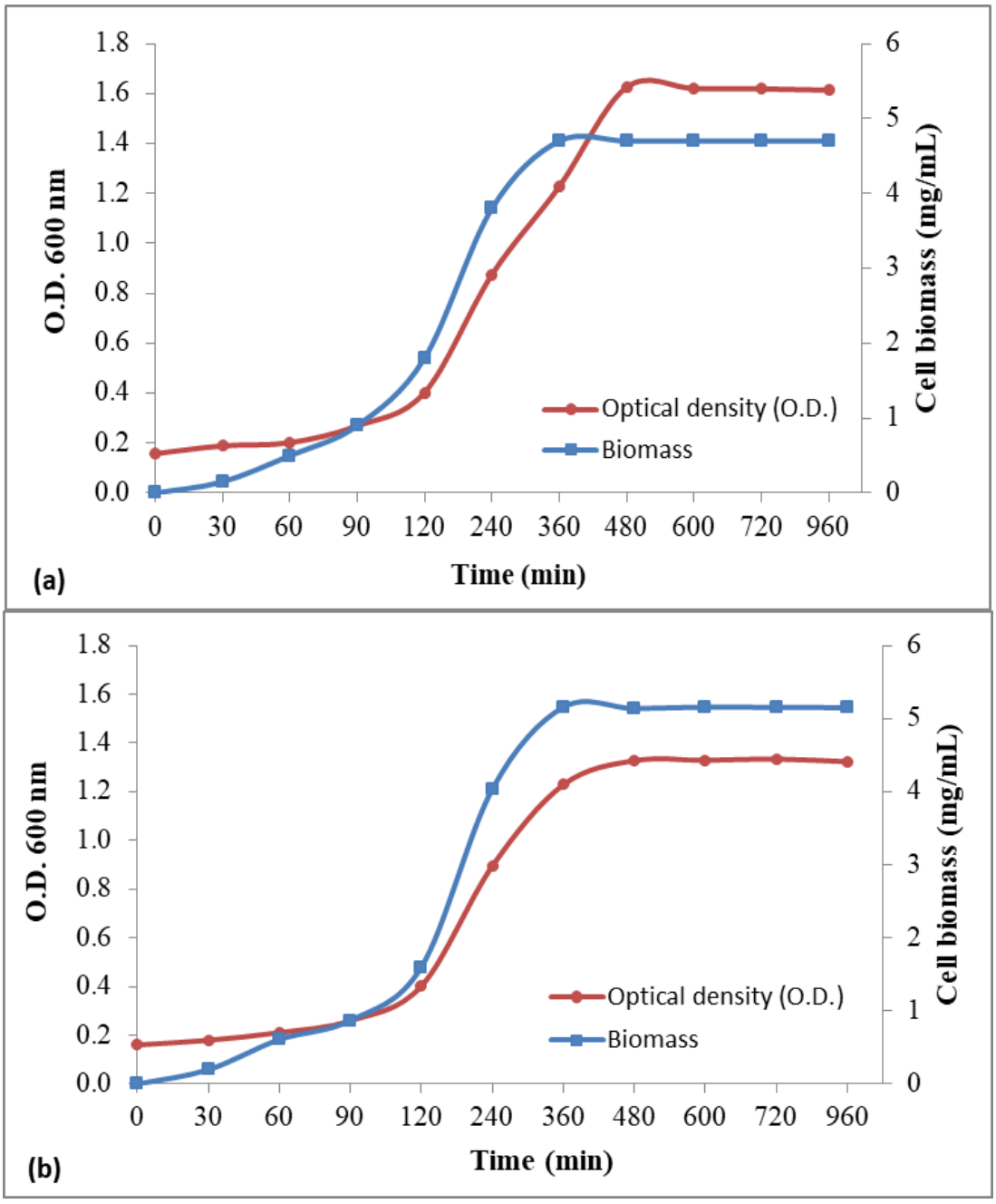


Figure 4.8: Growth curve of *Pichia kudriavzevii* JKH 1 (a) and *Kluyveromyces marxianus* JKH 5 (b)

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**).

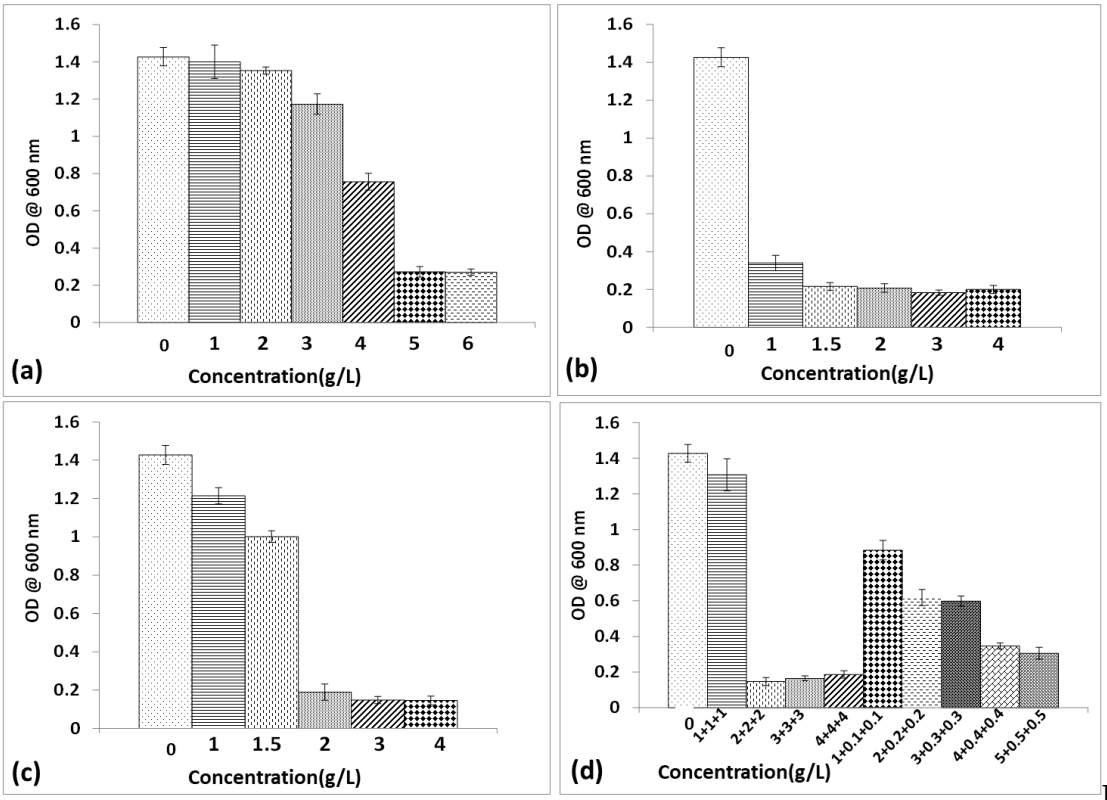
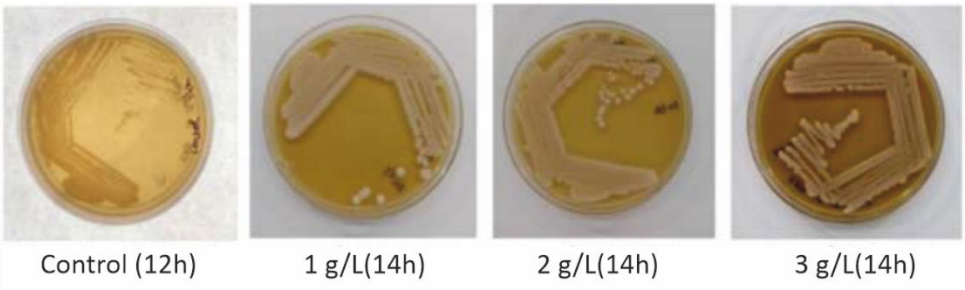
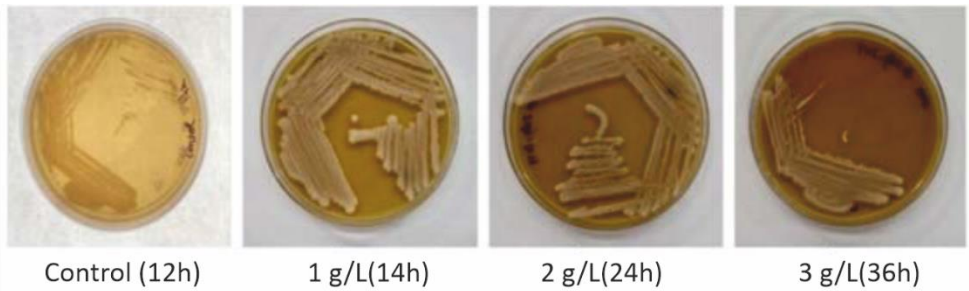


Figure 4.9: Effect of acetic acid (a), furfural (b), vanillin (c) individually and in combination (d) on the growth of *Pichia kudriavzevii* JKH1 at 42°C for 24 h.
Longer lag phase than the control was considered as inhibition

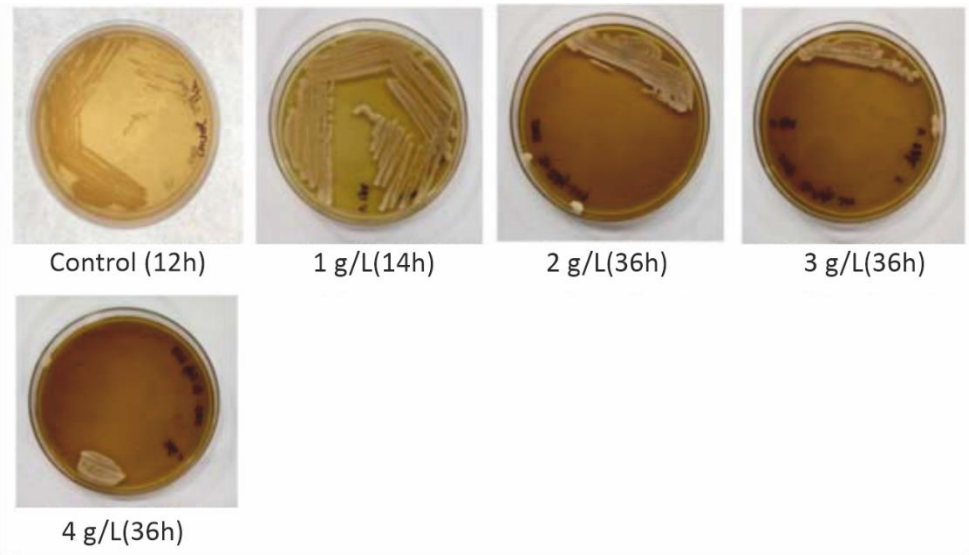
(a) Acetic acid



(b) Furfural



(c) Vanillin



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

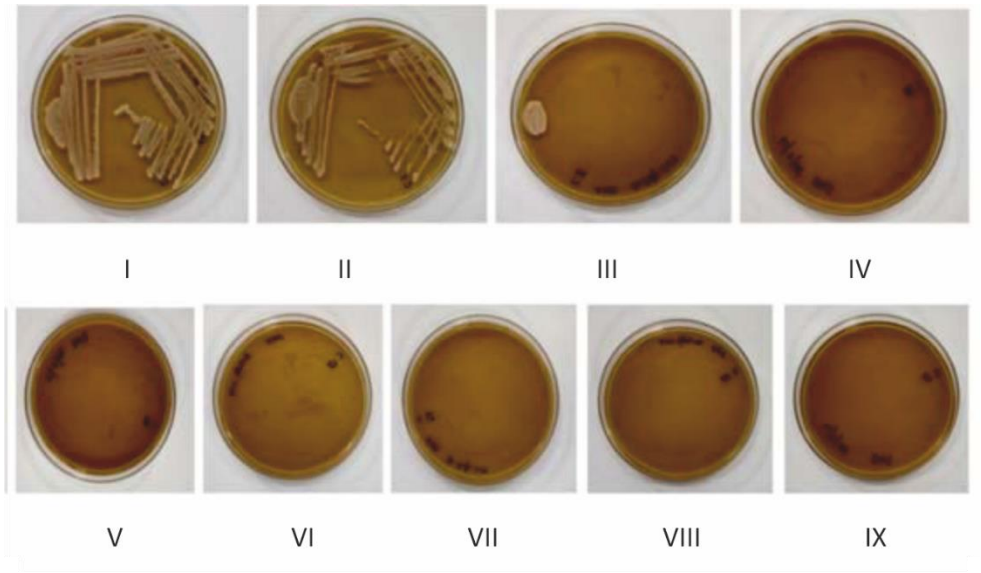


Figure 4.10 : Effect of different concentrations of various inhibitors on the growth of *P. kudriavzevii* JKH1 on YP agar plates. AA:F:V (1+1+1[Cocktail I]; 2+2+2 [Cocktail II]; 3+3+3[Cocktail III]; 4+4+4 [Cocktail IV]; 1+0.1+0.1 [Cocktail V], 2+0.2+0.2 [Cocktail VI]; 3+0.3+0.3 [Cocktail VII]; 4+0.4+0.4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

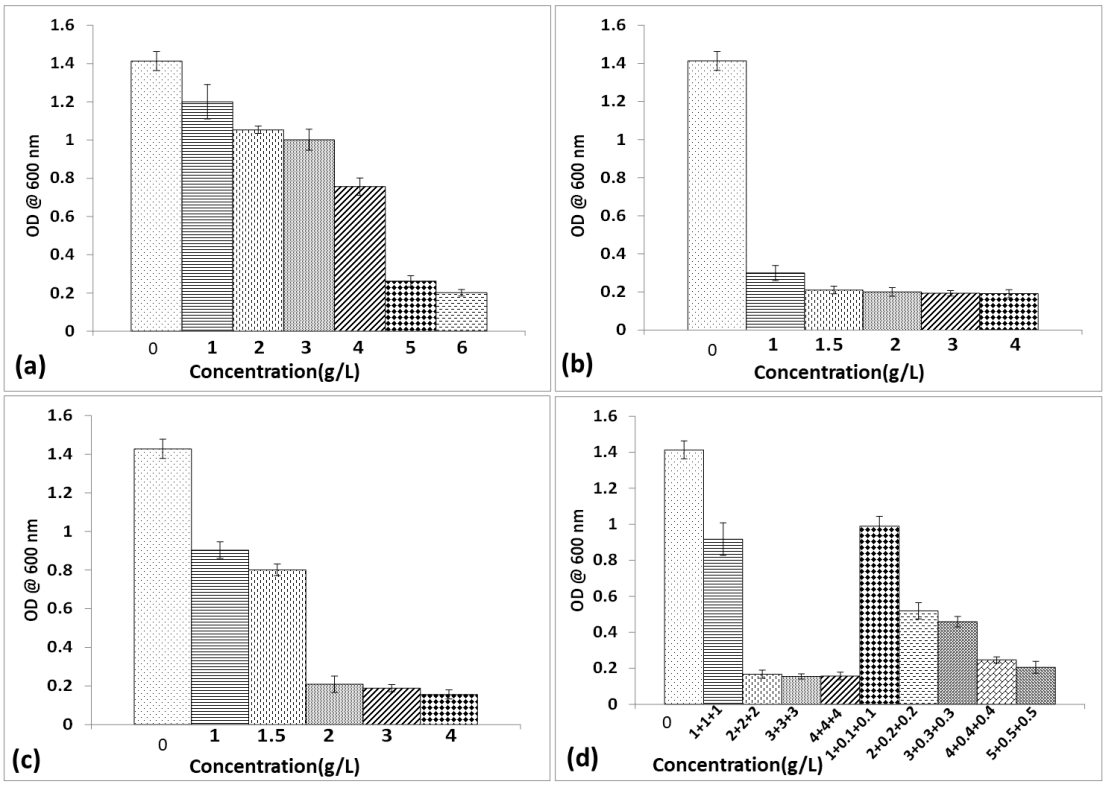
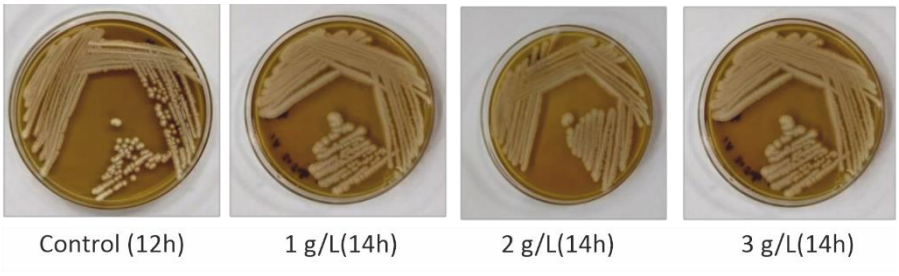
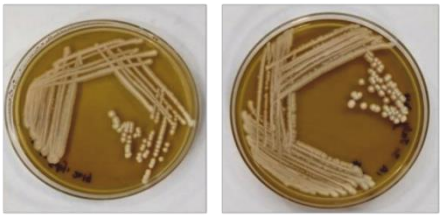


Figure 4.11: Effect of acetic acid (a), furfural (b), vanillin (c) individually and in combination (d) on the growth of *Kluyveromyces marxianus* JKH5 at 42°C. Longer lag phase than the control was considered as inhibition.

(a) Acetic acid

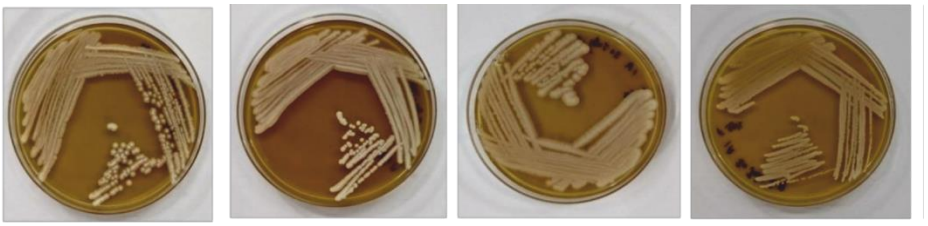


Control (12h) 1 g/L(14h) 2 g/L(14h) 3 g/L(14h)

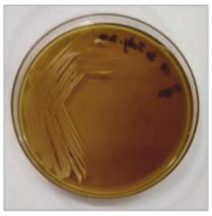


4 g/L(24h) 5 g/L(36h)

(b) Furfural

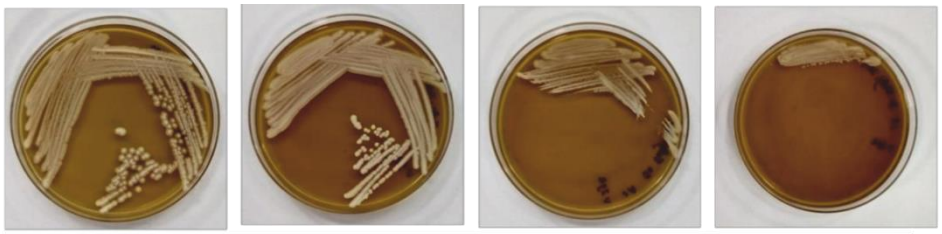


Control (12h) 1 g/L(14h) 2 g/L(24h) 3 g/L(36h)

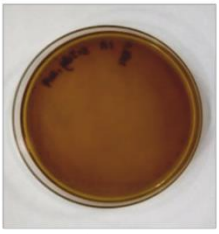


4 g/L(36h)

(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)

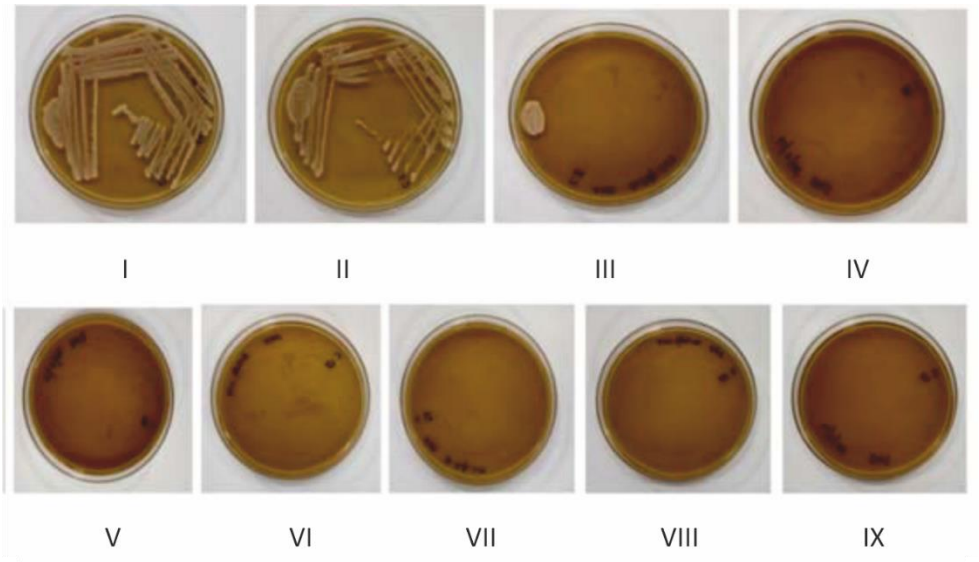


Figure 4.12: Effect of different concentrations of various inhibitors on the growth of *K. marxianus* JKH5 in YPD agar plates. AA:F:V (1+1+1[Cocktail I]; 2+2+2 [Cocktail II]; 3+3+3[Cocktail III]; 4+4+4 [Cocktail IV]; 1+0.1+0.1 [Cocktail V], 2+0.2+0.2 [Cocktail VI]; 3+0.3+0.3 [Cocktail VII]; 4+0.4+0.4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

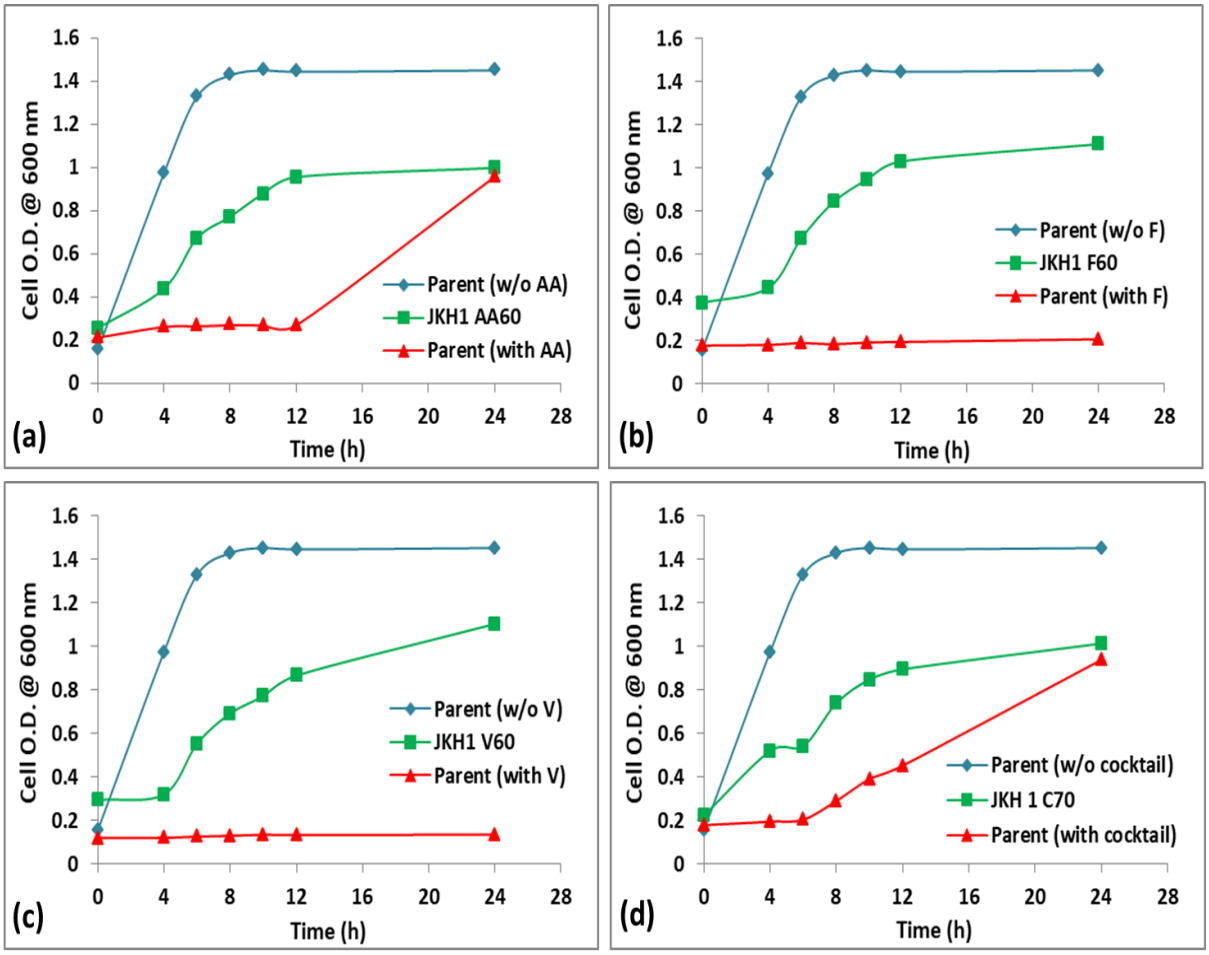


Figure 4.13: Comparison of growth profile of parent and adapted yeast strain *Pichia kudriavzevii* strain on medium supplemented with inhibitors, AA: acetic acid (6 g/L) (a), F: furfural (3.2 g/L) (b), V: vanillin (2.8 g/L) (c), and cocktail: (acetic acid+furfural+vanillin) (3+1+1 g/L) (d)

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.

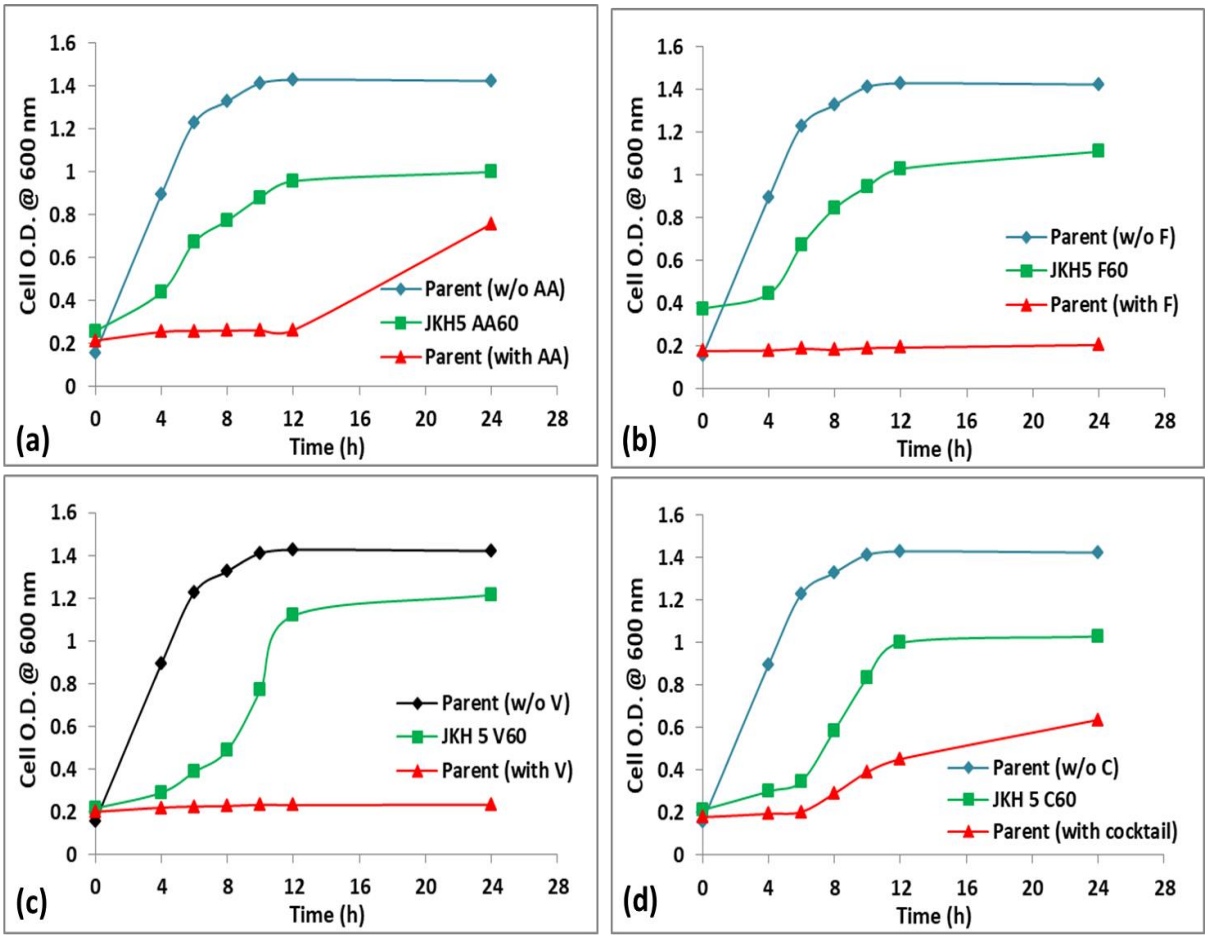


Figure 4.14 Comparison of growth profile of parent and adapted yeast strains of *Kluyveromyces marxianus* on medium supplemented with inhibitors, AA: acetic acid (6 g/L) (a), F: furfural (3.2 g/L) (b), V: vanillin (3 g/L) (c), and cocktail: (acetic acid+furfural+vanillin) (3+1+1 g/L) (d)

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.

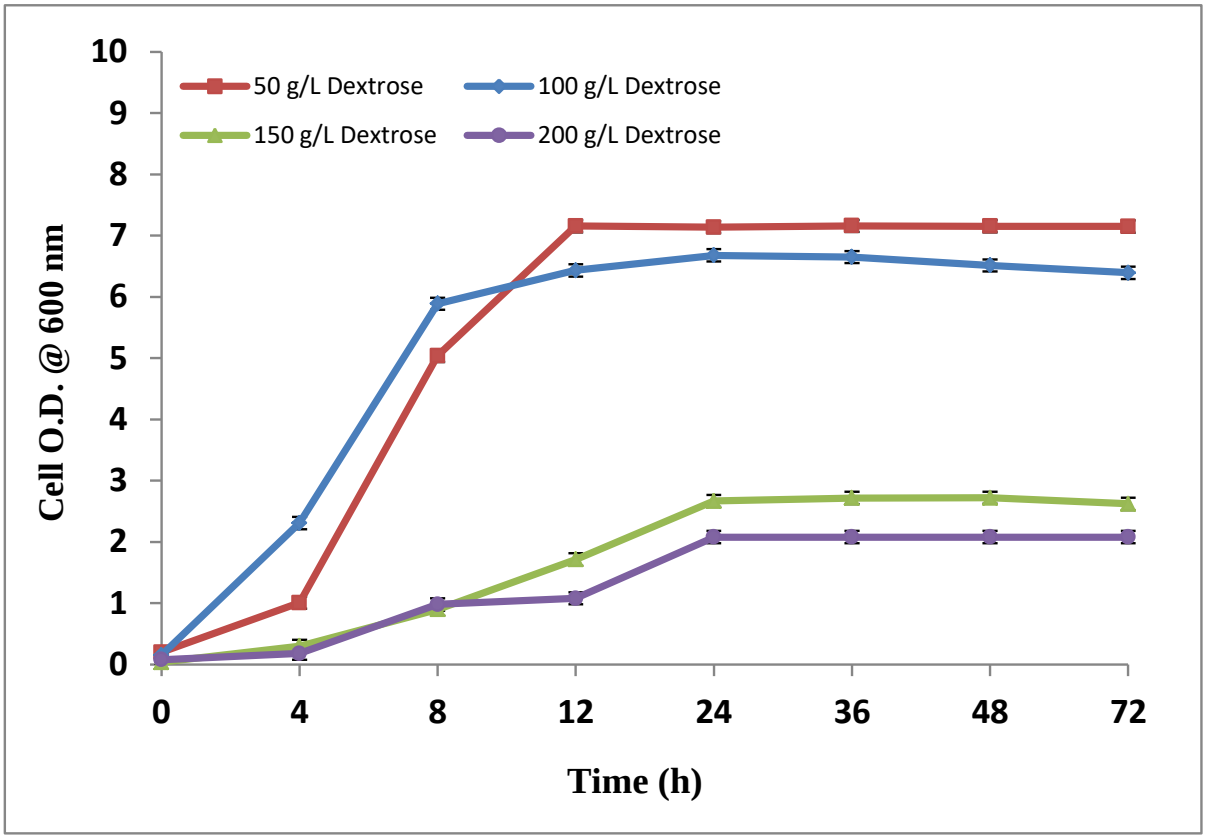


Figure 4.15: Effect of different glucose concentrations on growth of the adapted yeast *K. marxianus* JKH5 C60

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.

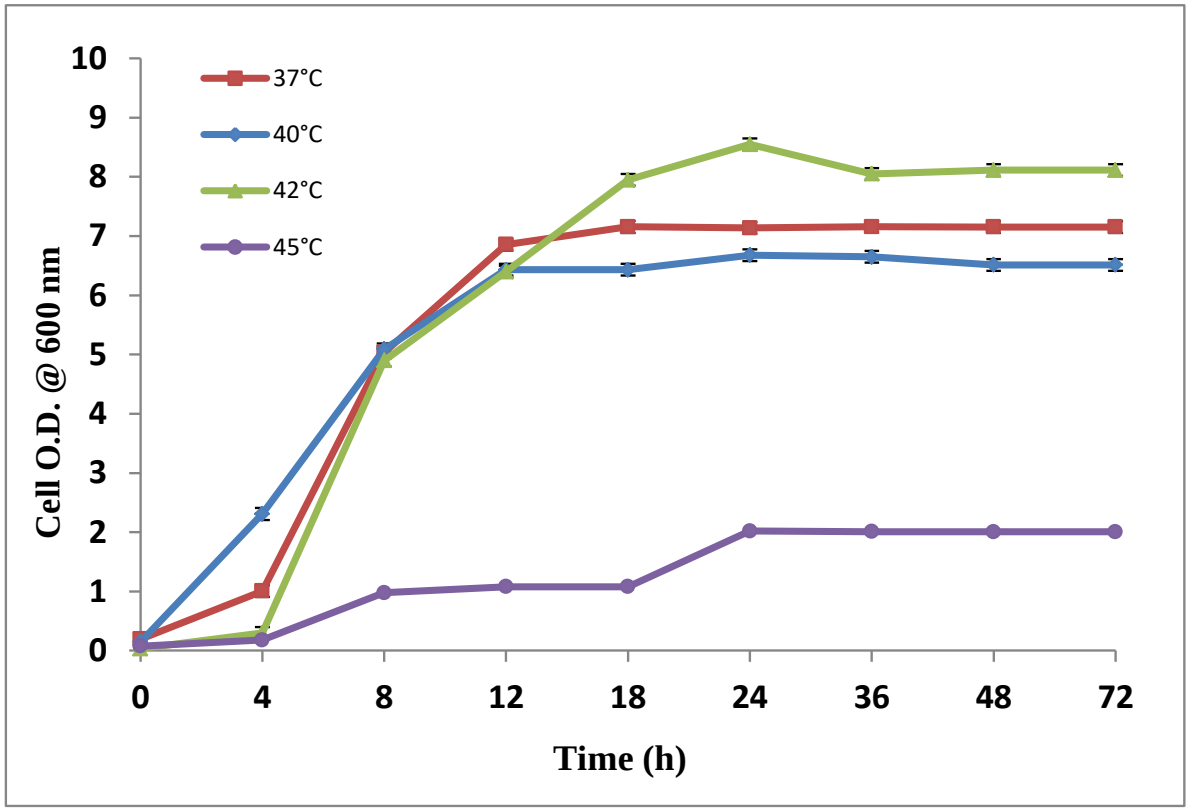


Figure 4.16: Effect of different temperatures on growth of the adapted yeast *K. marxianus* JKH5 C60

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.

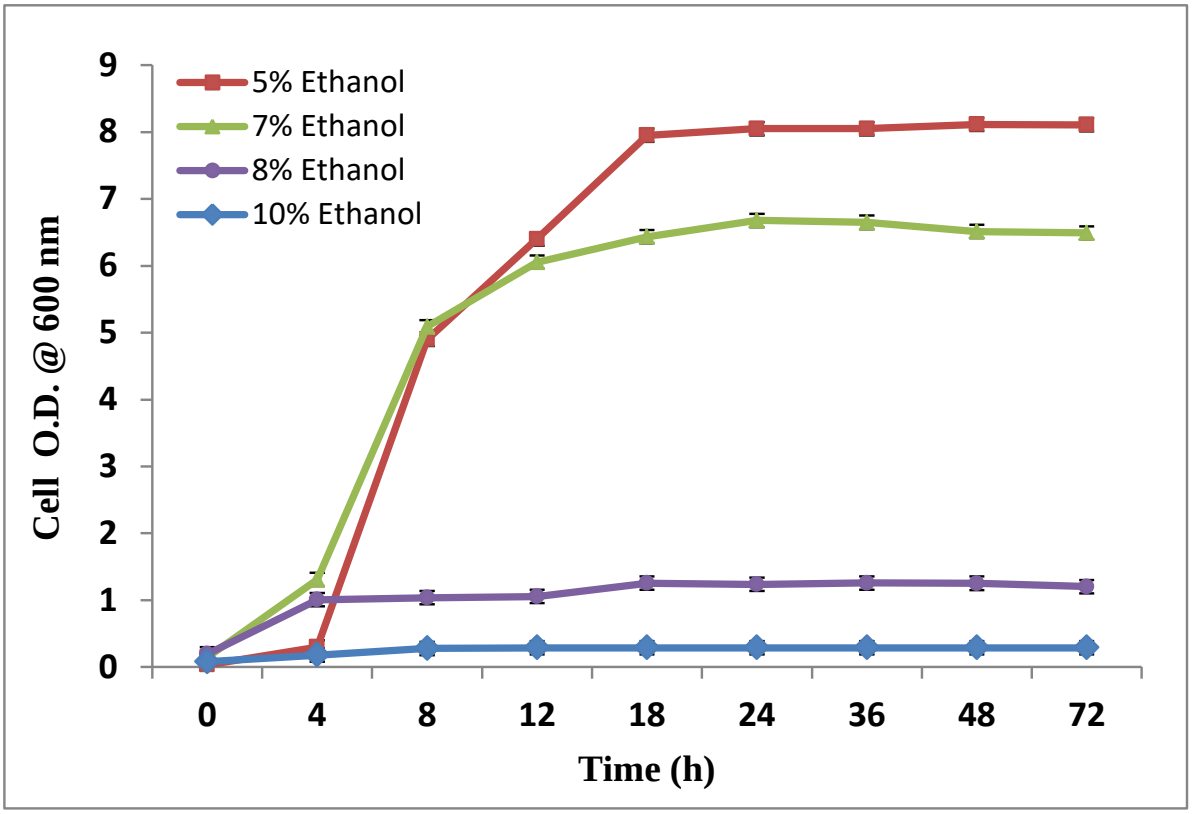


Figure 4.17 Effect of ethanol supplementation on growth of *K. marxianus* JKH5 C60

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%.

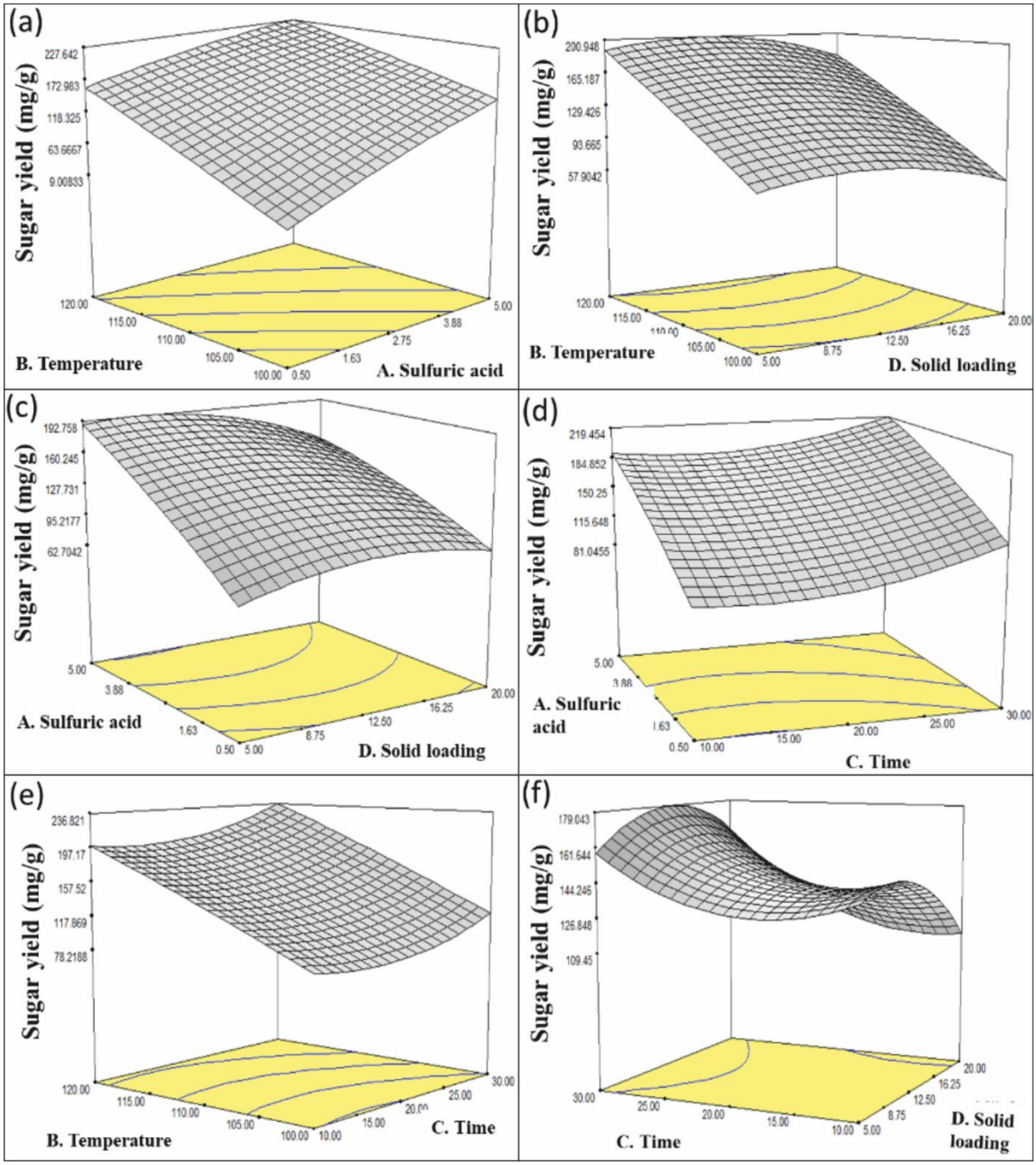


Figure 4.18: Response surface plots of Box-Behnken design for optimization of sequential pretreatment of SCB showing influence of sulphuric acid and temperature (a); temperature and solid loading (b); sulphuric acid and solid loading (c)); sulphuric acid and time (d); temperature and time (e); time and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

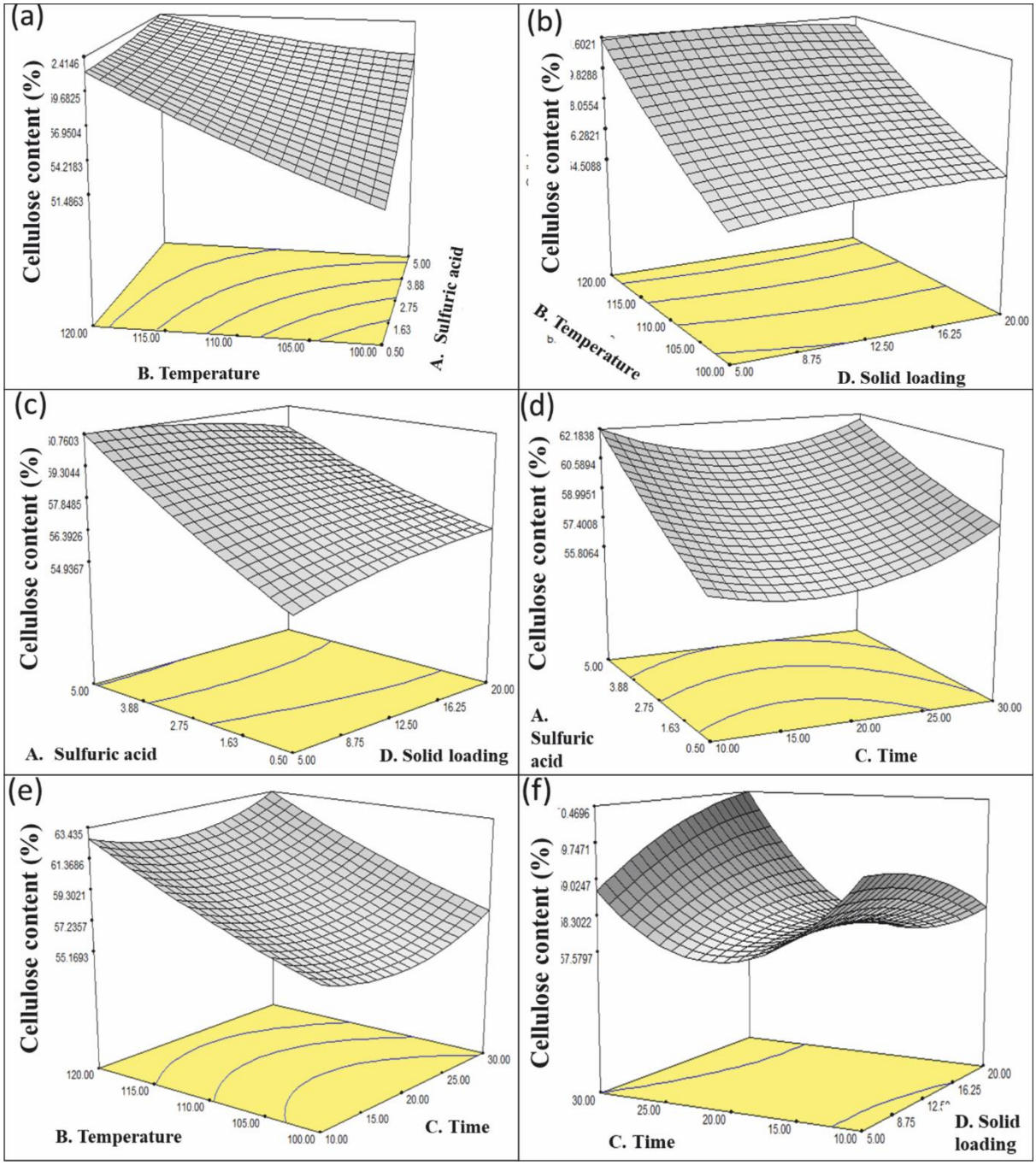


Figure 4.19 Response surface plots of Box-Behnken design for optimization of sequential pretreatment of SCB showing influence of sulphuric acid and temperature (a); temperature and solid loading (b); sulphuric acid and solid loading (c); sulphuric acid and time (d); temperature and time (e); time and 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 ($p < 0.05$).

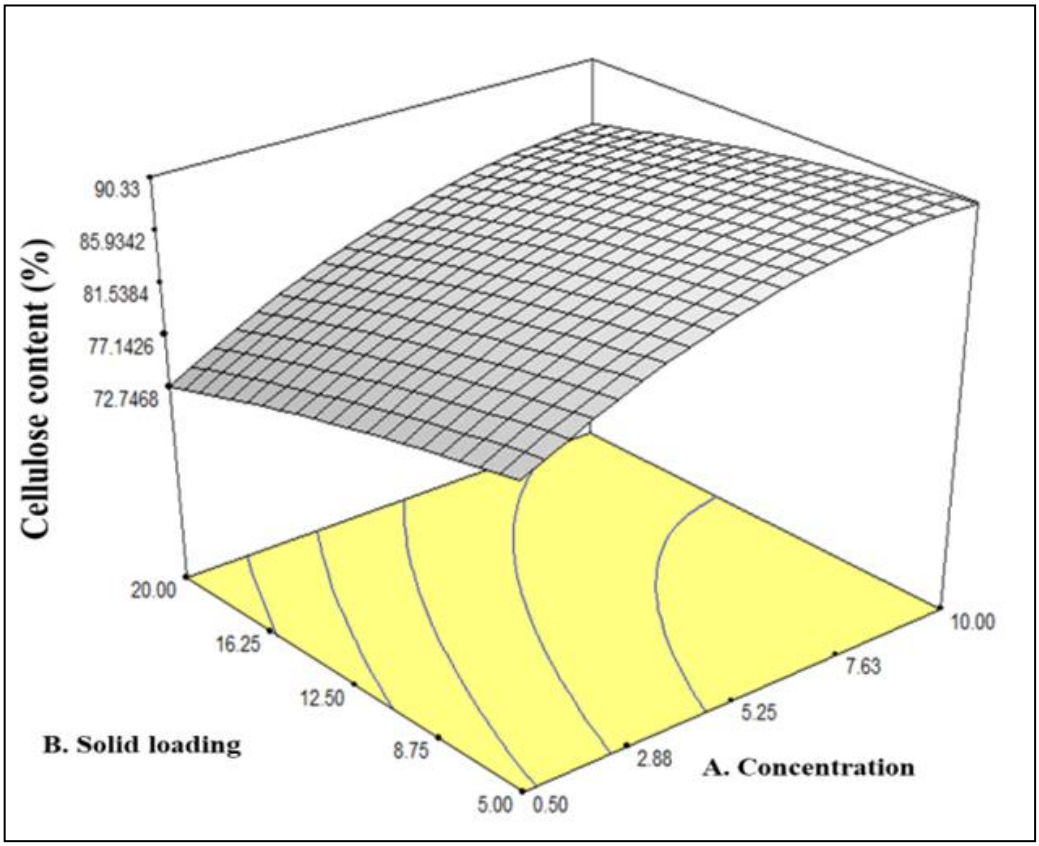


Figure 4.20: Three dimensional response surface plot showing influence of alkali concentration and solid loading on the response cellulose content for optimization of sequential pretreatment of sugarcane bagasse through D-optimal design

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**).

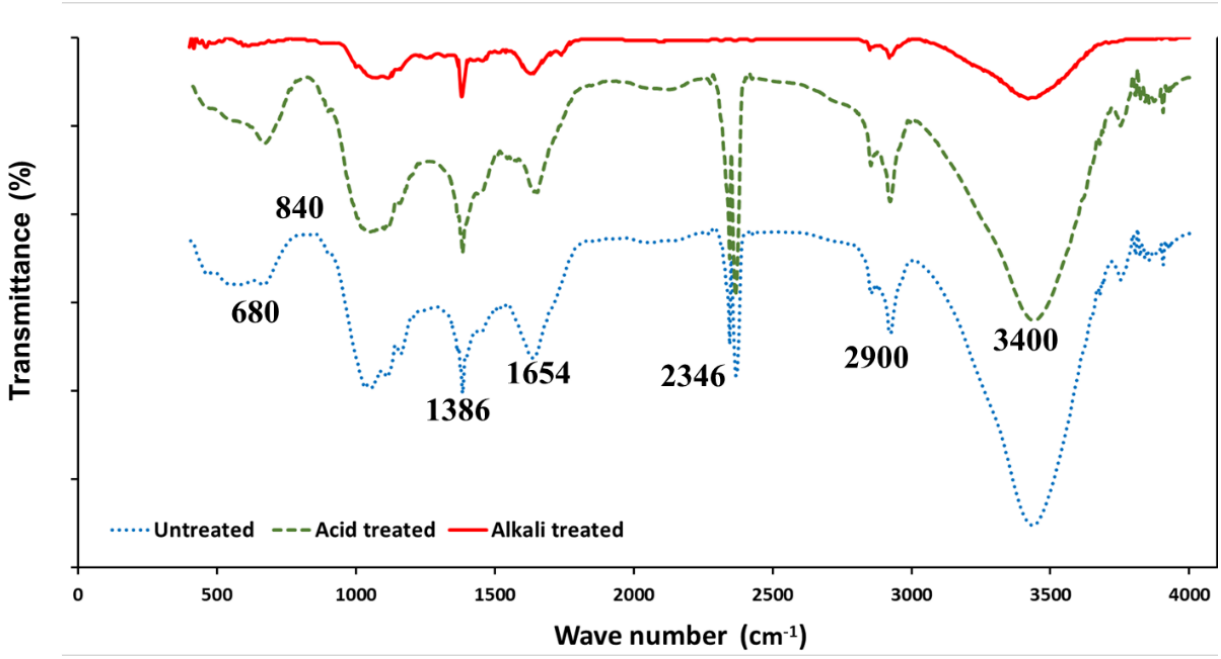


Figure 4.21: FTIR spectra of untreated and pretreated sugarcane bagasse

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.

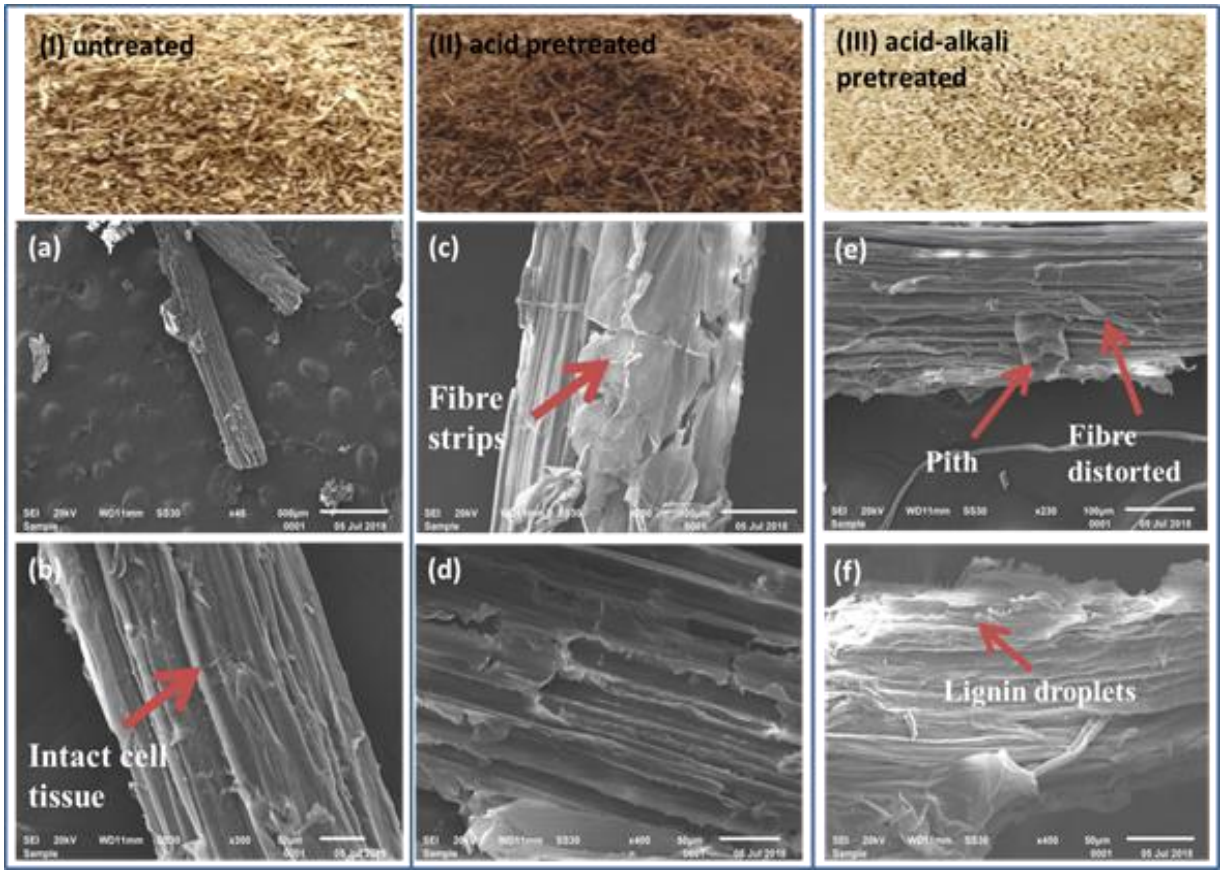


Figure 4.22: Scanning electron micrographs of (i) untreated, (ii) dilute acid pretreated and (iii) sequential dilute acid-alkali pretreated sugarcane bagasse

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.

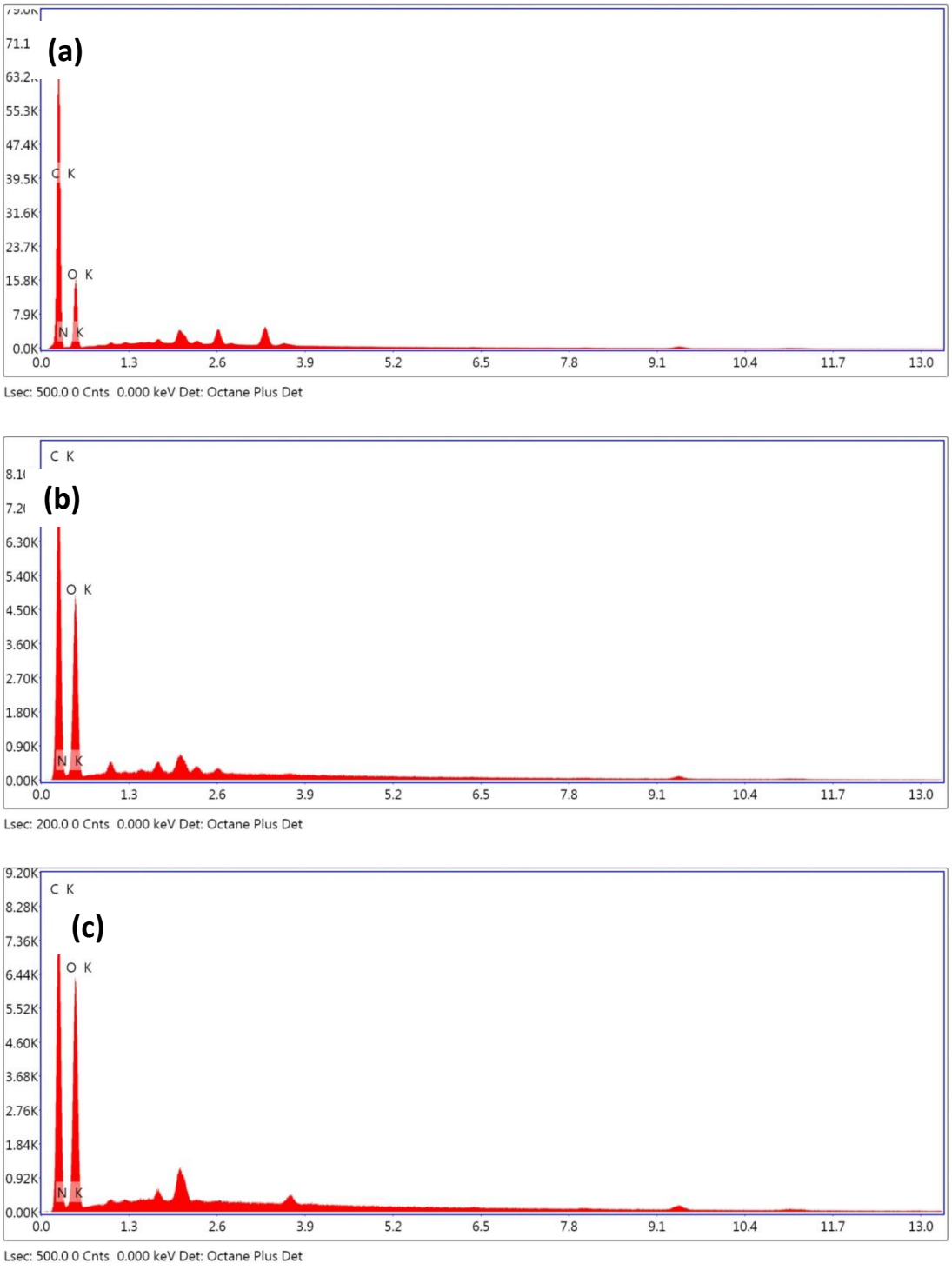


Figure 4.23: EDX spectrum of (a) untreated, (b) dilute acid pretreated and (c) dilute acid- alkali pretreated sugarcane bagasse.

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.

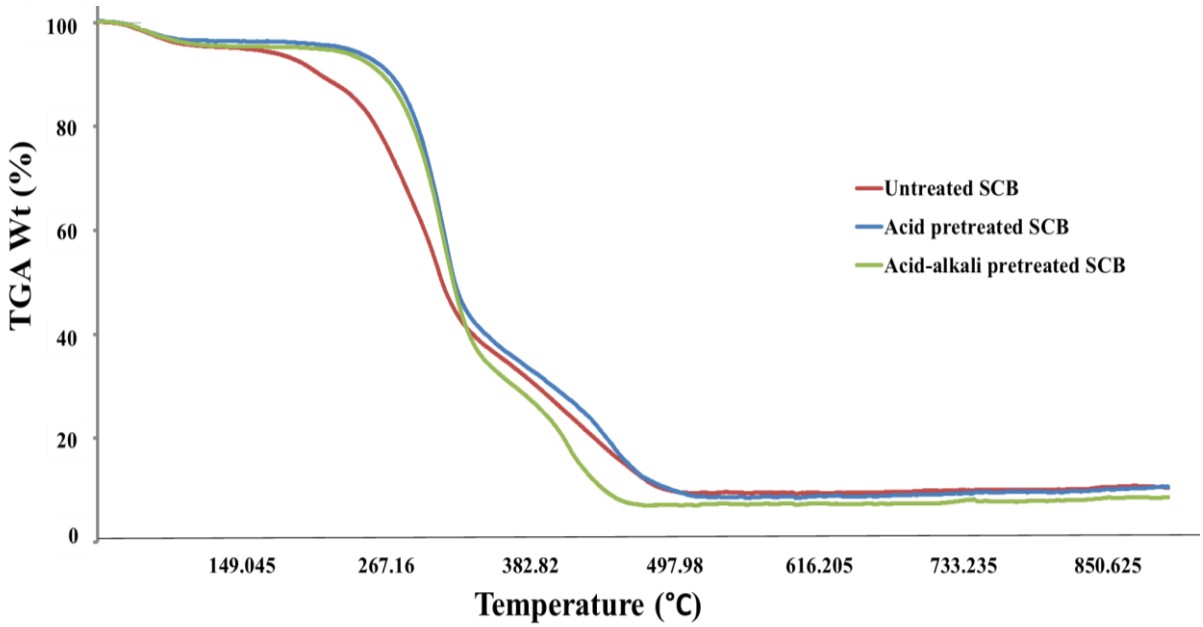


Figure 4.24: Thermal degradation curves of untreated, dilute acid pretreated and sequential dilute acid-alkali pretreated sugarcane bagasse

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.

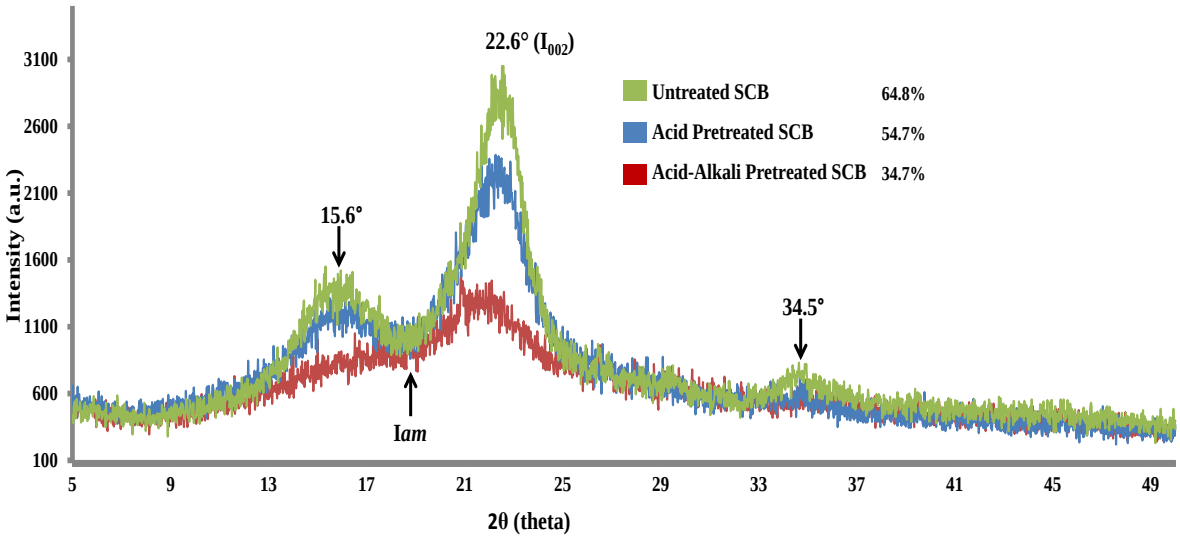


Figure 4.25: X-ray diffraction analysis of untreated and pretreated sugarcane bagasse

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.

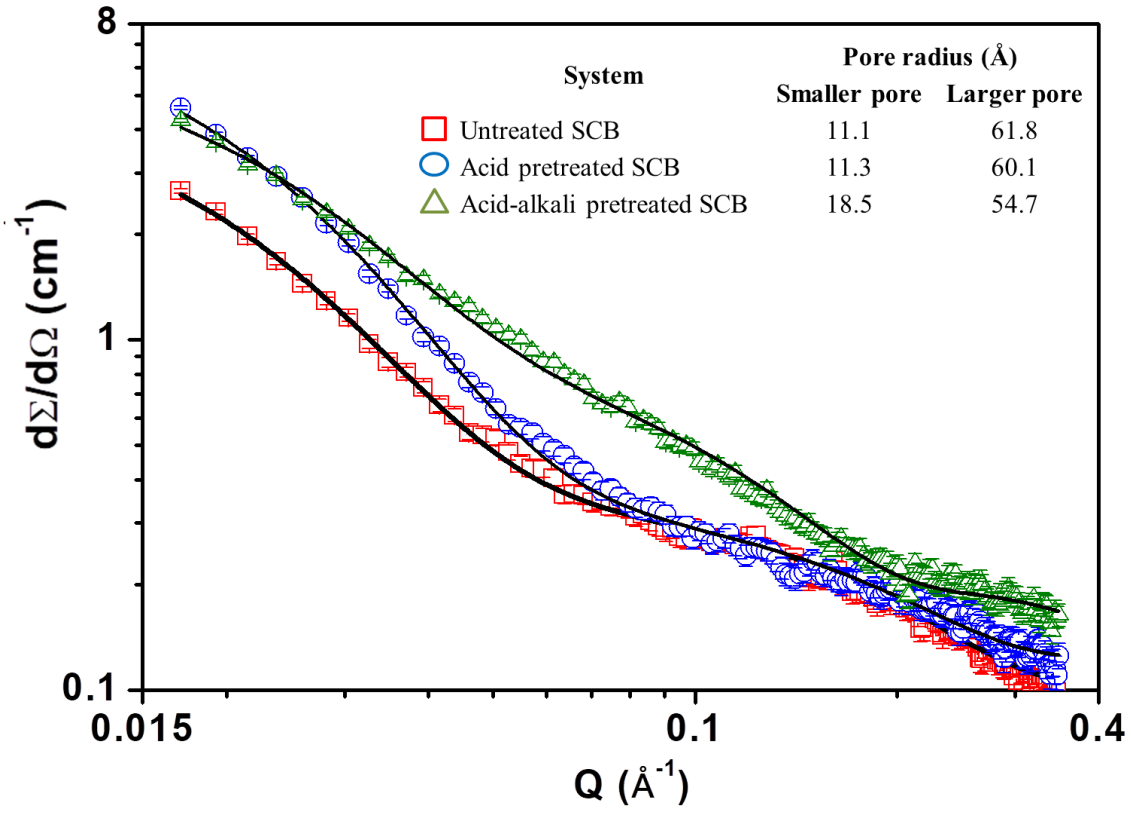


Figure 4.26: SANS analysis of untreated and pretreated sugarcane bagasse

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**).

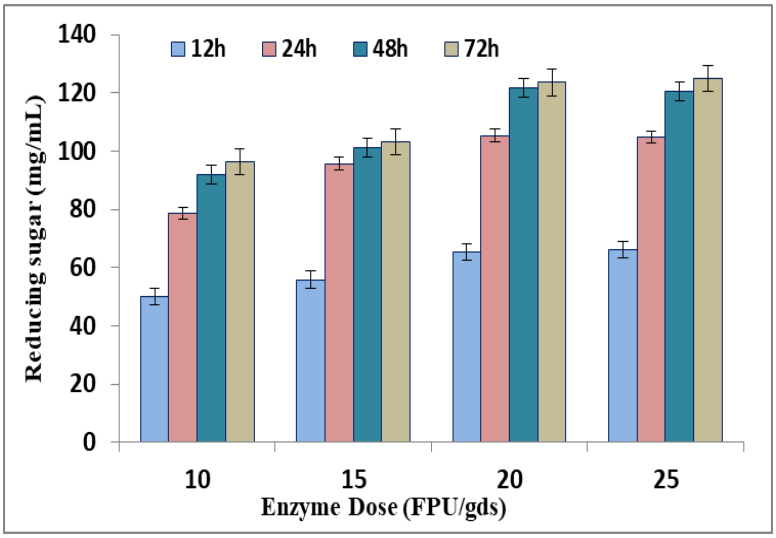


Figure 4.27: Effect of enzyme dosage on enzymatic saccharification of sequentially pretreated sugarcane bagasse

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).

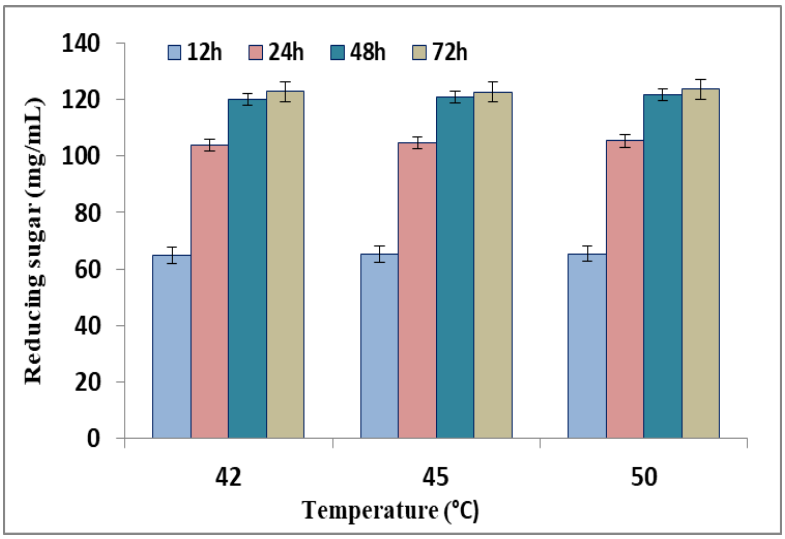


Figure 4.28: Effect of temperature on enzymatic saccharification of sequentially pretreated sugarcane bagasse

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).

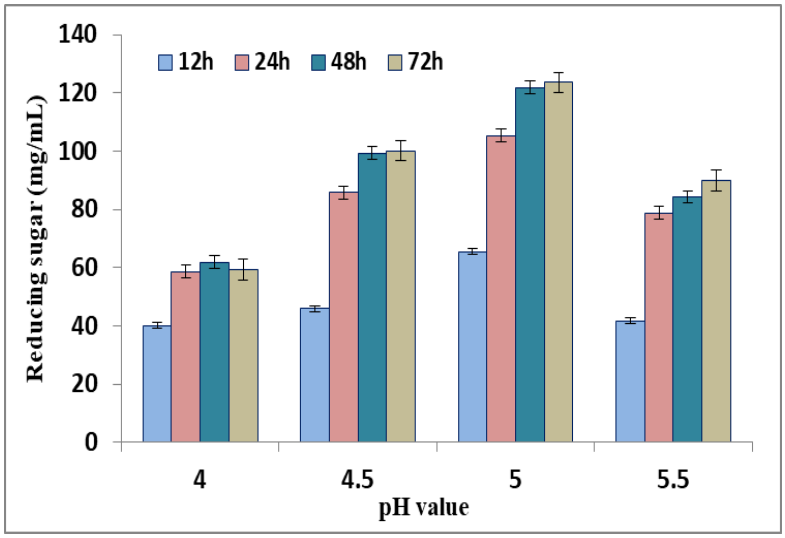


Figure 4.29 Effect of initial pH on enzymatic saccharification of sequentially pretreated sugarcane bagasse

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).**

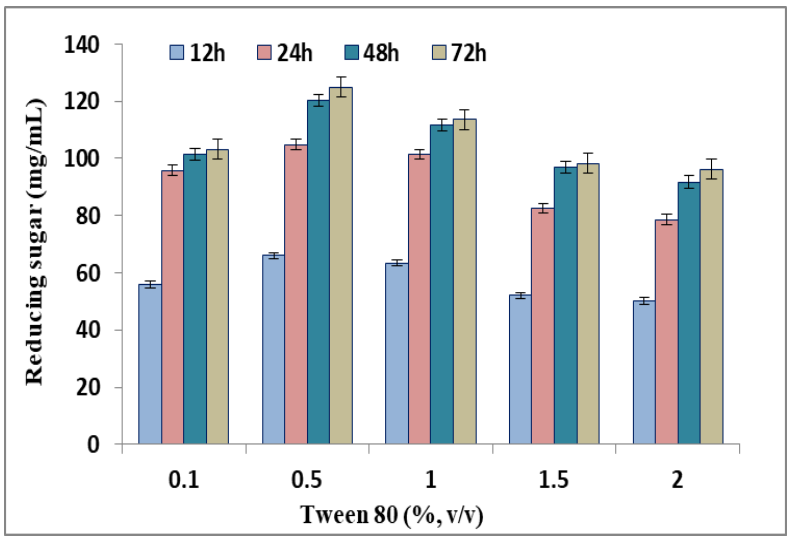


Figure 4.30: Effect of Tween 80 concentration on enzymatic saccharification of sequentially pretreated sugarcane bagasse

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).

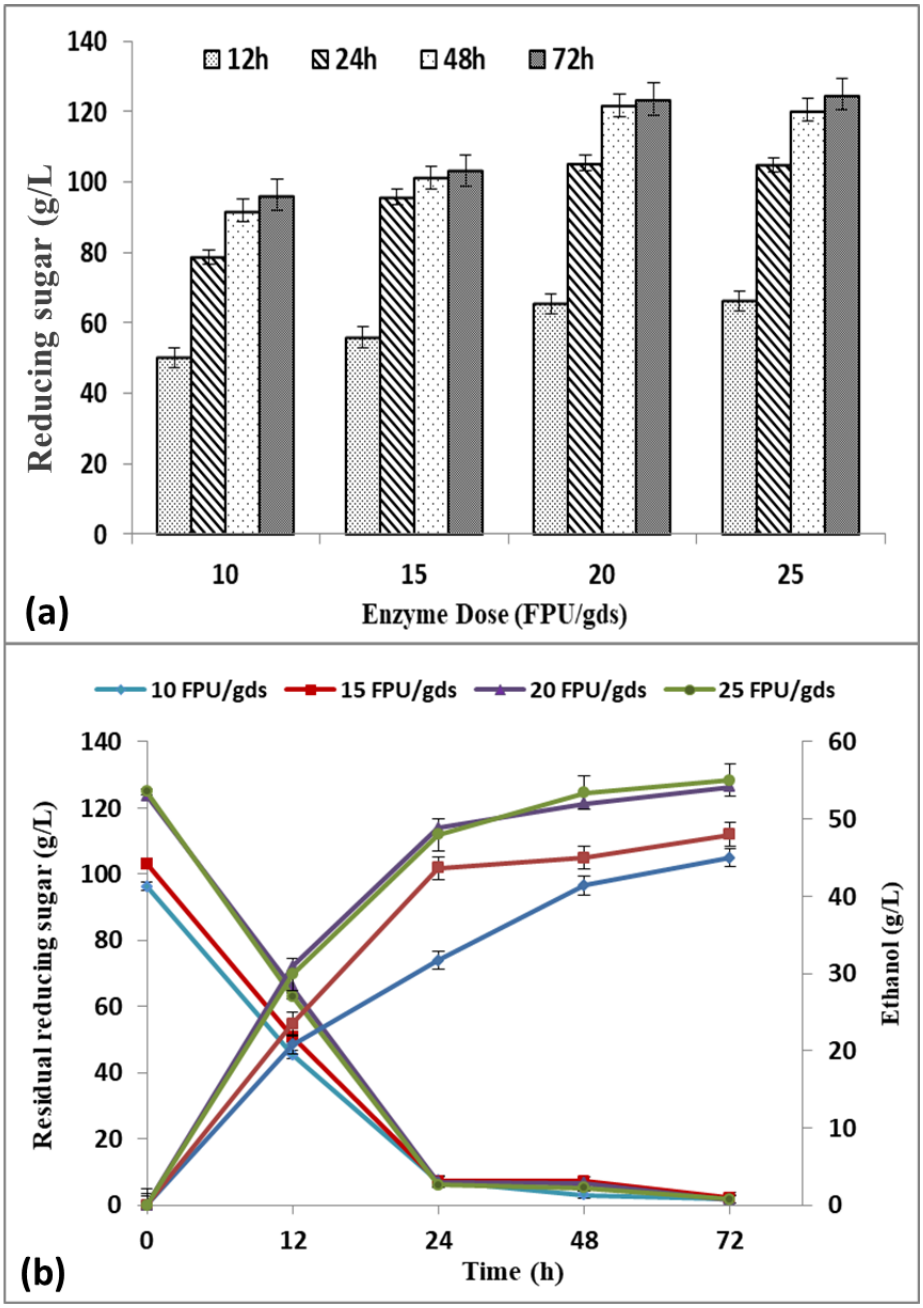


Figure 4.31: Separate hydrolysis and fermentation of sequential pretreated sugarcane bagasse by adapted yeast *K. marxianus* JKH5 C60 (a) enzymatic hydrolysis at different enzyme doses and (b) ethanol fermentation

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.

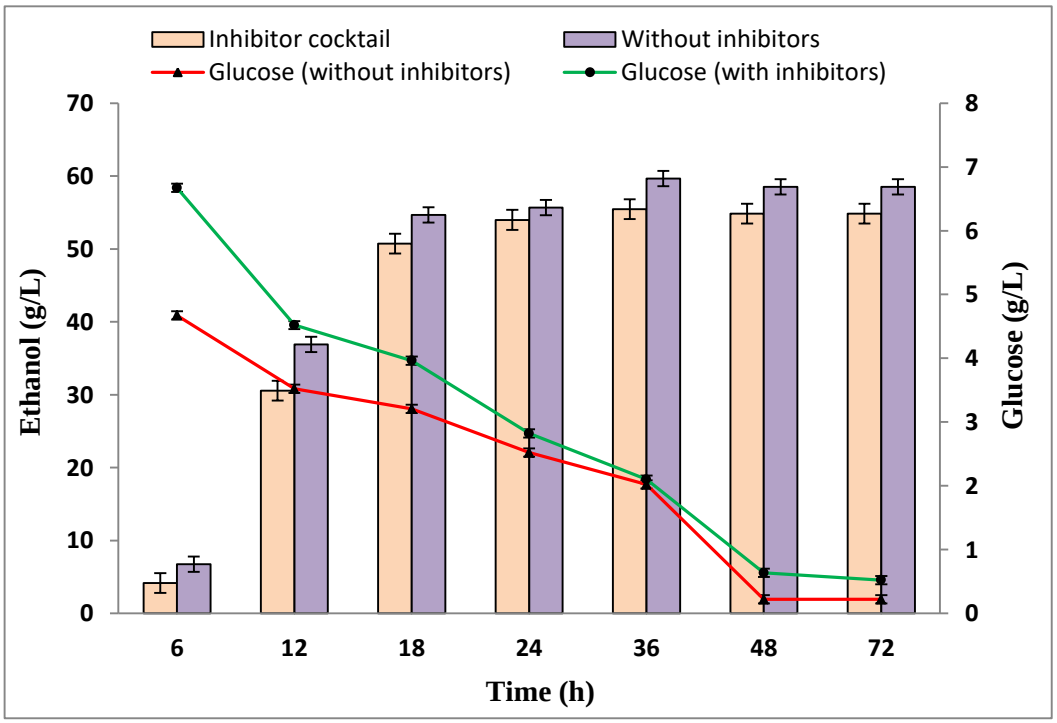


Figure 4.32: Batch simultaneous saccharification and fermentation of sequentially pretreated sugarcane bagasse in the presence of fermentation inhibitors by *K. marxianus* JKH5 C60 under shake flask. The solid loading of 15% (dry wt.), enzyme dosage of 20 FPU/g and inhibitor concentrations: acetic acid+furfural+vanillin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

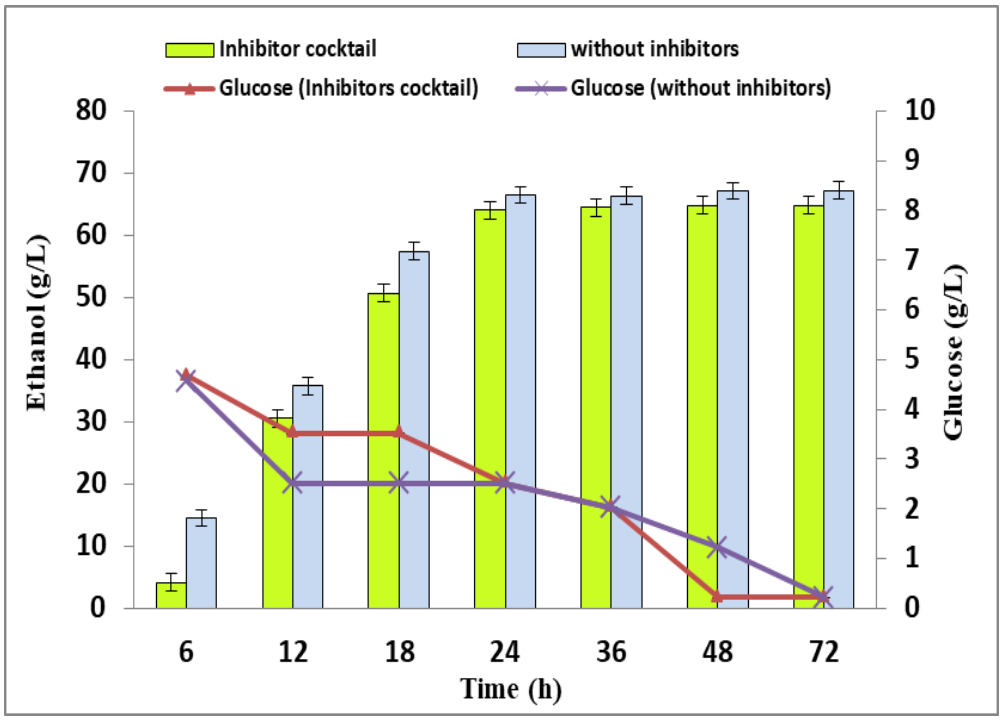


Figure 4.33: Batch simultaneous saccharification and fermentation of sequentially pretreated sugarcane bagasse by the adapted yeast *K. marxianus* JKH5 C60 at lab-scale fermenter in presence and absence of inhibitors. The solid loading was 15% (dry wt.) and 20 FPU/g enzyme dosage

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.

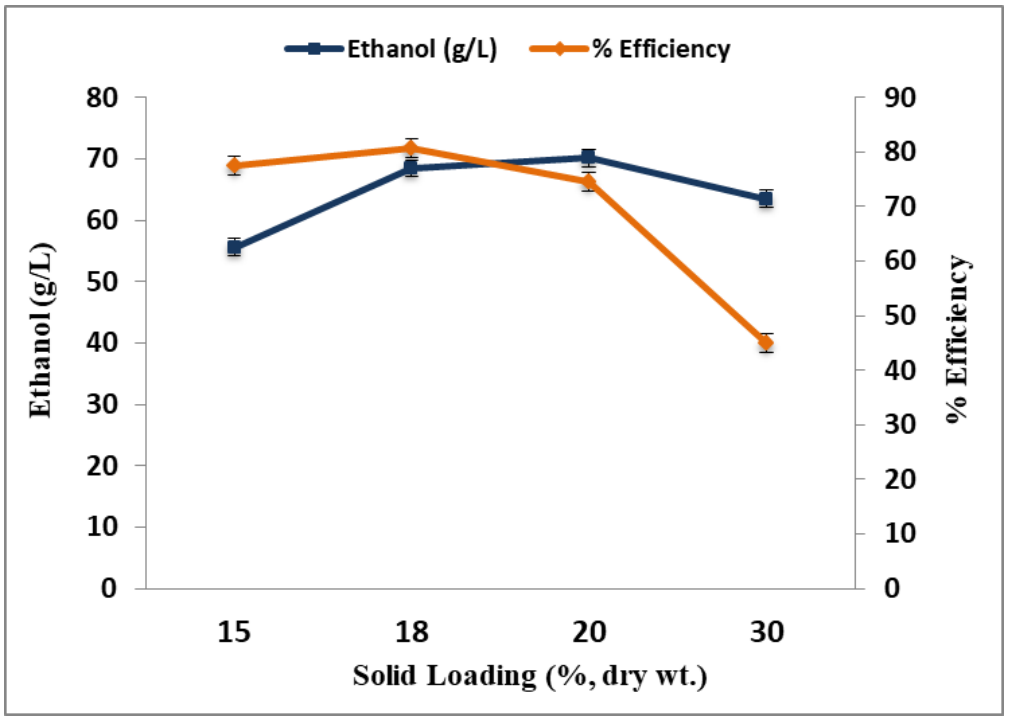


Figure 4.34: Batch simultaneous saccharification and fermentation of sequentially pretreated sugarcane bagasse at high solid loadings by adapted yeast *K. marxianus* JKH5 C60 under shake-flask.

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.

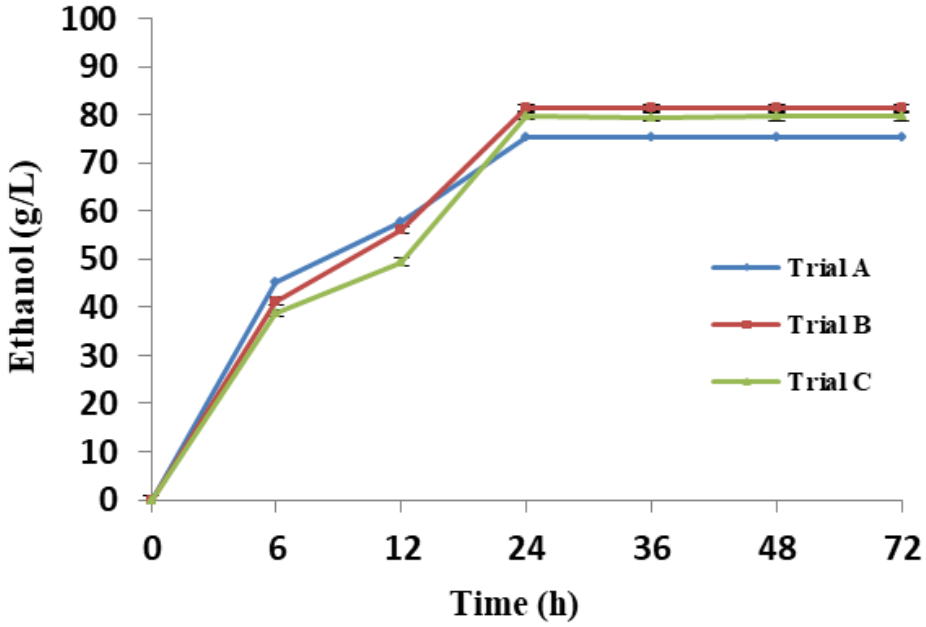


Figure 4.35: Fed-batch simultaneous saccharification and fermentation of sequential pretreated sugarcane bagasse by *K. marxianus* JKH5 C60 under shake flask employing biomass feeding strategy. Trial A, B and C were conducted at different solid loadings 18, 20 and 30 %, respectively

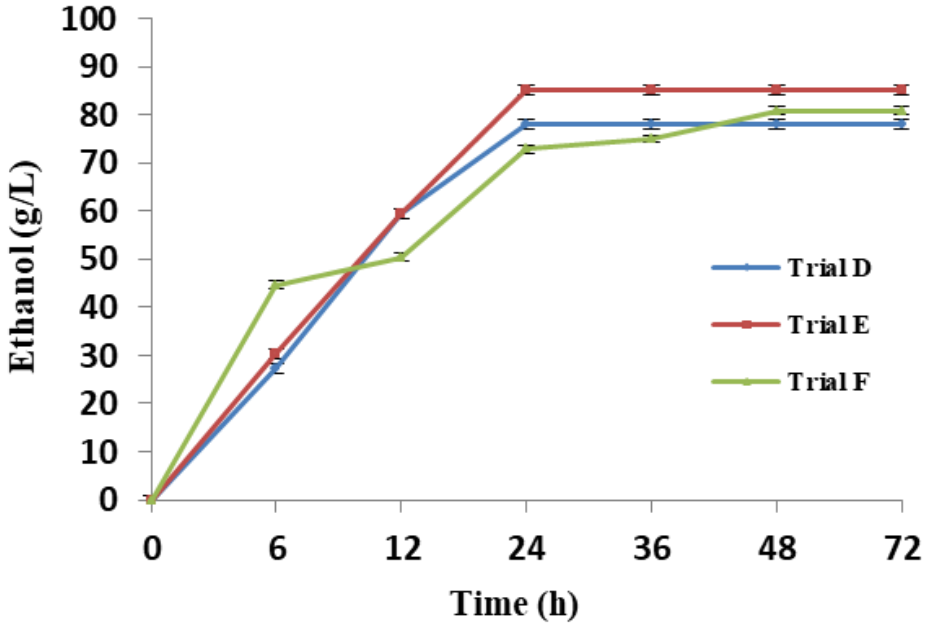


Figure 4.36: Fed-batch simultaneous saccharification and fermentation of sequential pretreated sugarcane bagasse by *K. marxianus* JKH5 C60 under shake flask employing biomass and enzyme feeding strategy. Trial D, E and F were conducted at different solid loadings 18, 20 and 30 %, respectively

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.

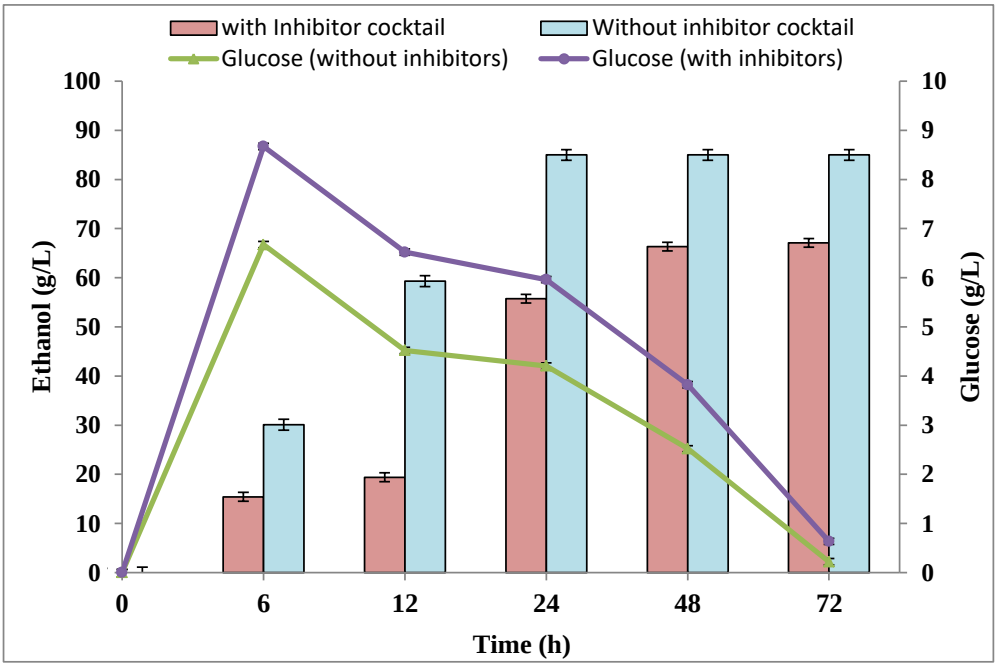


Figure 4.37: Fed-batch simultaneous saccharification and fermentation of sequentially pretreated sugarcane bagasse by adapted yeast *K. marxianus* JKH5 C60 under shake flask in presence and absence of inhibitory compounds. The solid loading was 20% (dry wt.) and 20 FPU/g enzyme dosage were employed

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.

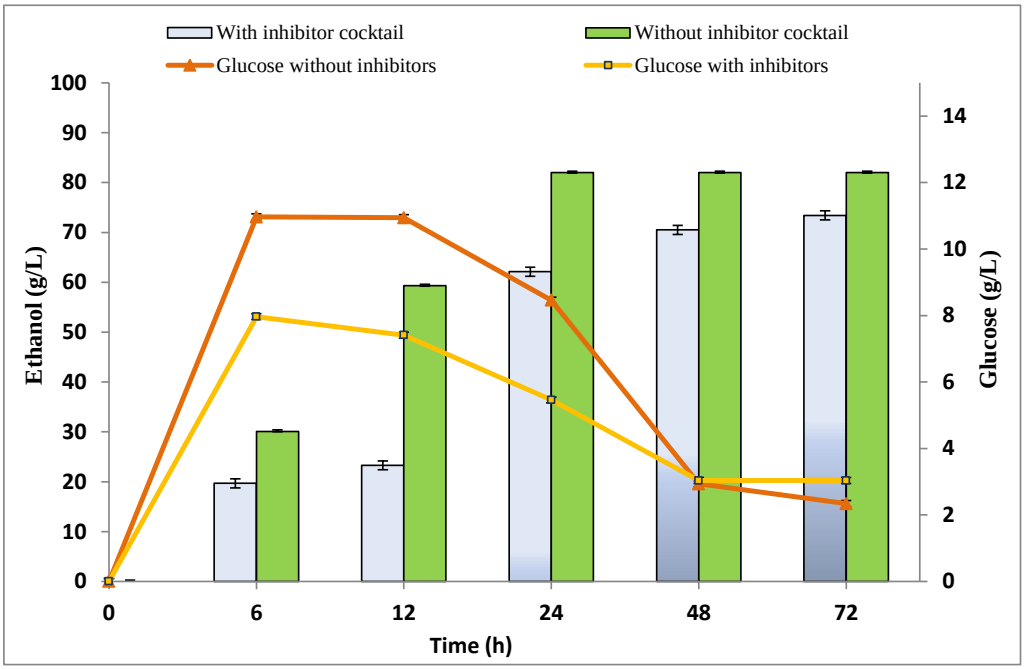


Figure 4.38: Fed-batch simultaneous saccharification and fermentation of pretreated sugarcane bagasse in the presence of inhibitors by *K. marxianus* JKH5 C60 at bench-top fermenter. The solid loading was 20% (dry wt.) and 20 FPU/g enzyme dosage were employed

(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).

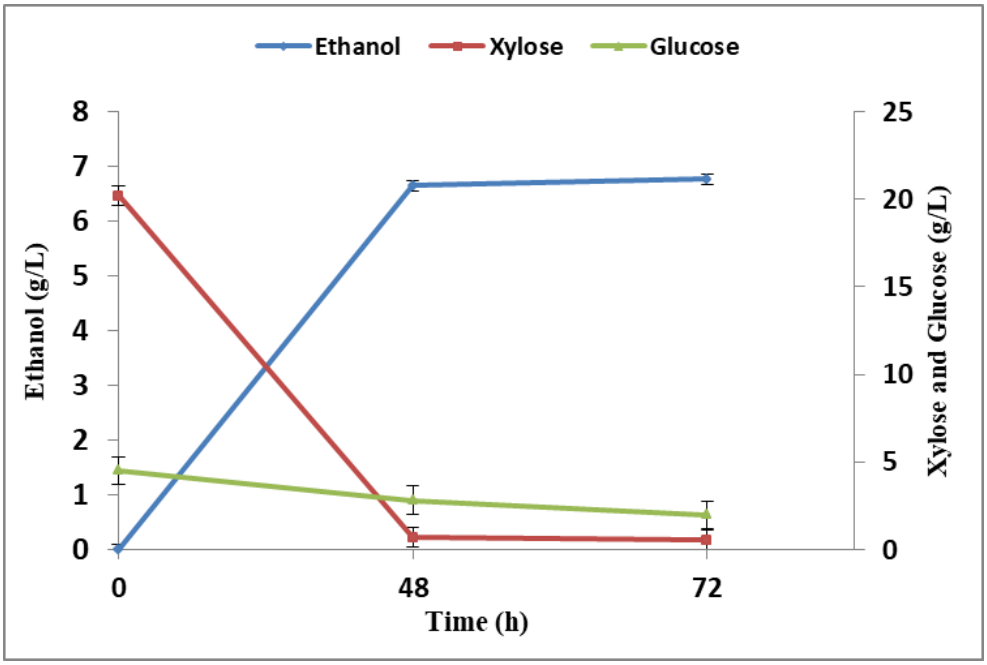


Figure 4.39: Fermentation of acid hydrolysate by *Pichia stipitis* NCIM 3499 for ethanol production under shake flask

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.

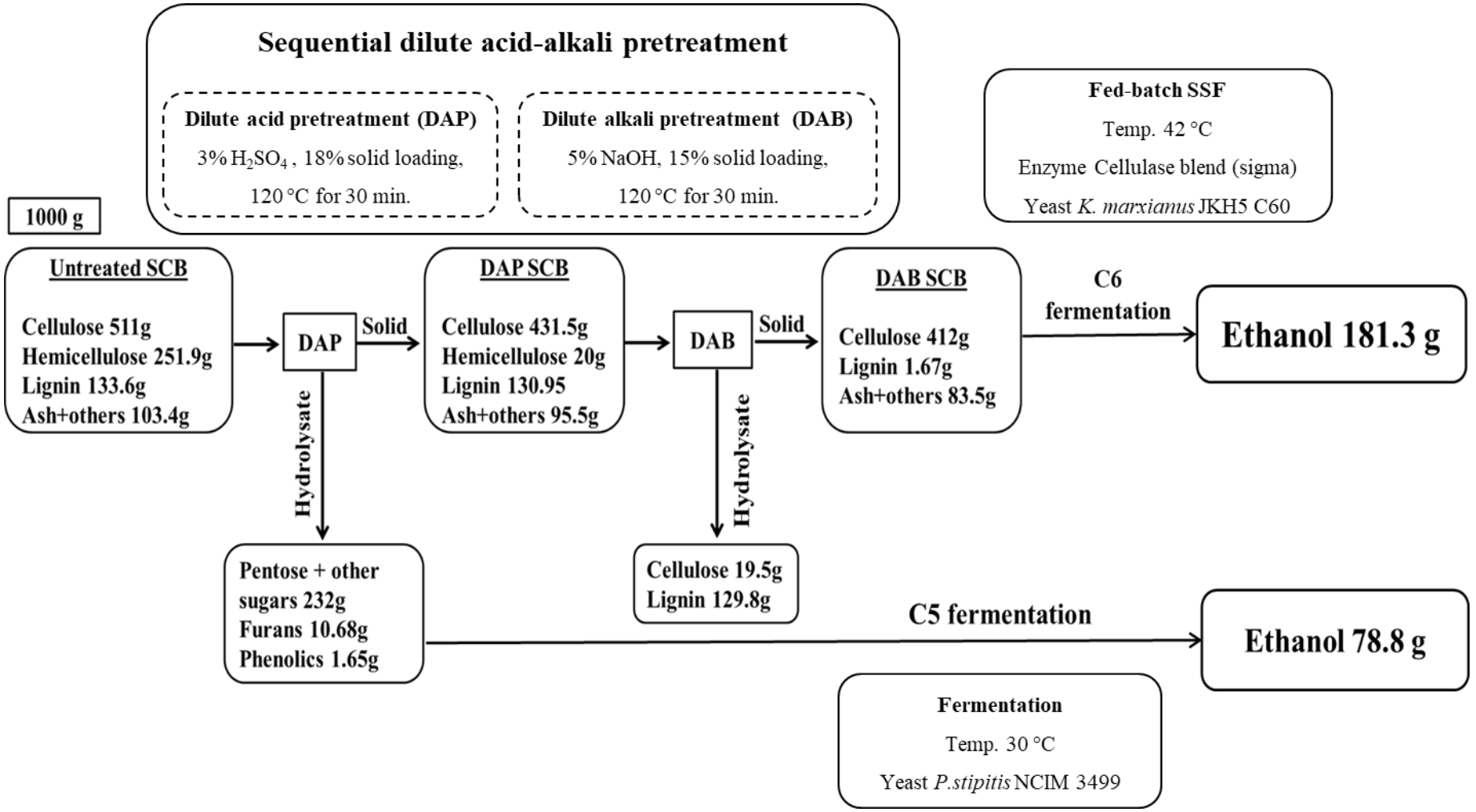


Figure 4.40: Mass Balance for bioconversion of sequential dilute acid-pretreated sugarcane bagasse to ethanol

DISCUSSION