Development of cyclic shifting of temperature strategy for simultaneous saccharification and fermentation for lignocellulosic bioethanol production

A Thesis

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DOCTOR OF PHILOSOPHY

by

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STATEMENT

I do hereby declare that the content embodied in this thesis is the result of investigations carried out by me in the Department of Biosciences & Bioengineering, Indian Institute of Technology, Guwahati, Assam, India under the supervision of Dr. Soumen Kumar Maiti.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date:12/07/2024 **Suraj Kumar Panda**

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CERTIFICATE

It is certified that the work described in this thesis entitled, **"Development of cyclic shifting of temperature strategy for simultaneous saccharification and fermentation for lignocellulosic bioethanol production"** by Mr. Suraj Kumar Panda, submitted for the partial fulfilment of Degree of Doctor of Philosophy is an authentic record of the results obtained from his research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India. The work described in this thesis has been not submitted elsewhere for a degree.

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Abstract

The usage of second-generation bioethanol as an eco-friendly, cleaner transport fuel has gained momentum due to its potential to mitigate environmental pollution and global warming. However, challenges persist in the multistep biochemical conversion process in lignocellulosic bioethanol production from agricultural wastes like rice straw. These challenges exist in the stages of pretreatment, enzymatic hydrolysis, and fermentation, demanding solutions to enhance economic viability and efficiency. To tackle these challenges, the objective the studies were framed to develop an acid pretreatment strategy for the low-cost agricultural waste i.e., rice straw. Rather than relying on commercial cellulase for enzymatic hydrolysis, cellulase production was carried out by using cellulolytic fungus using rice straw. This was aimed to optimize the bioethanol production by an integrated process in a single vessel system through an *in situ* cellulase production, saccharification, and fermentation (ICPSF) strategy employing fungus and yeast culture. Additionally, a novel cyclic shifting of temperature strategy (CSTS) was developed and implemented throughout the simultaneous saccharification and fermentation (SSF) process to improve the bioethanol titre. This strategy aimed to replace the conventional approaches like prolong prehydrolysis followed by fermentation or SSF at mutual optimum temperatures. Moreover, to utilise the sugars present in the hemicellulosic fraction, co-fermentation of yeast *Saccharomyces cerevisiae* and *Pichia stipitis* was applied in the pretreated rice straw hydrolysate aimed to utilise both glucose and xylose for bioethanol production.

A dilute acid pretreatment method for rice straw was optimised, aiming for two primary goals: maximizing the sugar yield and minimizing the generation of inhibitors in the pretreated hydrolysate. From optimisation studies, best pretreatment condition was obtained at 2% H_2SO_4 and 30 min autoclave time (121ºC, 15 psi pressure). This condition have been used further for

all the pretreatment methods for cellulase production. For secretion of diverse range of cellulase enzyme complexes, the fungi *Trichoderma reesei* and *Penicillium janthinellum* were utilised for cellulase production in the acid pretreated rice straw whole slurry. Various factors such as substrate loading, incubation temperature and medium pH were optimised for both individual cultures as well as the co-culture in the shake flask medium. It was further applied in the bioreactor under controlled conditions. The maximum cellulase production in the co-culture under optimal conditions (3% w/v rice straw loading, medium pH 5, 30ºC, agitation speed: 250 rpm, air flow rate: 1VVM) in bioreactor yielded: FPase: 1.16 IU/ml, CMCase: 27.68 IU/ml, Beta-glucosidase: 4.63 IU/ml, and Xylanase: 45.60 IU/ml, respectively. Furthermore, the study also presents a lesser studied aspect, concomitant biodetoxification and cellulase production. Both *T. reesei* and *P. janthinellum* were able to metabolise the acid pretreatment by-products such as formic acid, acetic acid, HMF and furfural. This study presents a scope for one step process for fungal cellulase production and biodetoxification of the lignocellulose pretreated hydrolysate in order to avail an inhibitor free medium for subsequent yeast co-culture for bioethanol production.

Further, a novel strategy using cyclic shifting of temperature was developed for simultaneous saccharification and fermentation (SSF) for bioethanol production from rice straw. The *in-situ* cellulase production, saccharification and fermentation (ICPSF) was carried out using *P. janthinellum* and *S. cerevisiae*. The thermotolerance of *S. cerevisiae* was studied in pure glucose medium (10 g/l) both at constant incubation temperatures and cyclic shifting of temperature to further apply for bioethanol production using rice straw. Bioethanol titre of 14.98 g/l was obtained using base followed by acid pretreated rice straw (BAP-RS) by employing the cyclic shifting of temperature strategy “30 °C for 2 h to 40 °C for 2 h”. The holding time was further tuned to increase the productivity and the tuned condition 30°C (1.7 h)-40°C (2 h) improved the bioethanol titre to 15.9 g/l. Using this strategy, resulted 5.1-fold

and 2.8-fold increment of bioethanol production compared to known approaches, SSF at mutual optimum temperature and prolong prehydrolysis followed by fermentation respectively.

To maximise the enzyme activity in the crude cellulase for SSF process and fermentation of both pentose, hexose sugars present in the solid pretreated rice straw fibres, a poly-culture system of fungus and yeast was employed for the ICPSF process. Apart from *S. cerevisiae*, *P. stipitis* was introduced for its capability to metabolise both glucose and xylose. The thermotolerance of *P. stipitis* was studied in pure xylose medium (10 g/l) both at constant incubation temperatures and cyclic shifting of temperature prior to the application for SSF of rice straw. Bioethanol titre of 11.81 g/l was obtained from BAP-RS (10% w/v) by ICPSF through a tri-culture system comprising *P. janthinellum*, *T. reesei* and *P. stipitis* using CSTS 30ºC(2h)-37ºC(3h). Similarly, the application of tri-culture system involving *P. janthinellum*, *T. reesei* and *S. cerevisiae* improved the bioethanol titre to 17.05 g/l from BAP-RS (10% w/v) by application of CSTS 30ºC(1.7h)-40ºC(2h) in the SSF process.

The primary fraction of hemicellulosic sugars undergoes solubilisation in the acid pretreated hydrolysate during acid pretreatment and does not have any contribution for bioethanol fermentation during SSF process using solid pretreated rice straw fibres. However, for direct fermentation of acid pretreated hydrolysate, it must be concentrated to augment the soluble sugar fraction, thereby enhancing the fermentation yield. In this study, the hemicelluloses fraction in pretreated rice straw hydrolysate was concentrated by re-cycling the obtained acid pretreated hydrolysate after initial pretreatment, achieved by further pre-treatment of fresh delignified rice straw. This process yielded a maximum glucose:10.4 g/l and xylose: 44.5 g/l in the base followed by third recycled acid pretreated hydrolysate. For both glucose and xylose conversion strategies, a co-culture of hexose fermenting yeast *S. cerevisiae* and the pentose fermenting yeast *P. stipitis*, capable of fermenting both pentoses and hexoses was employed. The maximum bioethanol titre achieved from the co-culture of *S. cerevisiae*

and *P. stipitis* was 11.29 g/l. However, by employing a sequential culture approach with *S. cerevisiae* followed by *P. stipitis* with an intermediate heat inactivation at 50ºC, resulted in an improvement of bioethanol titre to 12.39 g/l. This resulted in an improvement of 2.9-fold and 1.2-fold bioethanol titre as compared to sole fermentation by *S. cerevisiae* and *P. stipitis* respectively. The application of reuse of acid pretreated hydrolysate for high recovery of soluble sugar and subsequent fermentation by sequential culture strategy demonstrates a potential to maximize lignocellulose for sustainable bioethanol production.

Contents

Abbreviations/Notations

Term	Description
ICPSF	*In situ* cellulase production, saccharification, and fermentation
CSTS	Cyclic shifting of temperature strategy
SSF	Simultaneous saccharification and fermentation
SI engines	Spark ignition engines
FFVs	Flexible fuel vehicles
OMCs	Oil marketing companies
EBPs	Ethanol blended petrol
NOx	Oxides of nitrogen
FAO	Food and Agricultural Organisation
HMF	5-Hydroxy methyl furfural
AFEX	Ammonia fibre explosion
NAD	Nicotinamide adenine dinucleotide
ATP	Adenosine triphosphate
PMF	Proton motive force
CBD	Cellulose binding domain
CD	Catalytic domain
MFS	Major facilitator superfamily
ADP	Adenosine di-phosphate
PPP	Pentose Phosphate Pathway
SHF	Separate hydrolysis and fermentation
CB	Consolidated bioprocessing
RS	Rice straw

NCIM	National Collection of Industrial Microorganisms
NCL	National Chemical Laboratory
NDF	Neutral detergent fibre
ADF	Acid detergent fibre
NDS	Neutral detergent solution
ADS	Acid detergent solution
IUPAC	International Union of Pure and Applied Chemistry
DNS	Dinitrosalicylic acid
CMC	Carboxymethyl cellulose
pNPG	p-nitrophenol-β-D-glucopyranoside
OD	Optical density
CrI	Crystallinity index
FESEM	Field emission-scanning electron microscopy
EDX	Energy Dispersive X-ray
RID	Refractive Index Detector
HPLC	High-performance liquid chromatography
TS	Total soluble sugar
TI	Total inhibitor
dO_2	dissolved oxygen
AP-RS	Acid pretreated rice straw
BP-RS	Base pretreated rice straw
BAP-RS	Base followed by acid pretreated rice straw
MSM	Mineral salt medium
E_{max}	Maximum ethanol concentration
$Y_{E/G}$	Glucose to ethanol yield

R	Ratio of glucose released to glucose uptake
BAP-RS hydrolysates	Base followed by acid pretreated hydrolysate
LCA	Life Cycle Analysis
D.F.	Dilution factor

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Chapter-1

Introduction

1.1. Background of the study

The extensive use of technology to propagate industrialisation, economic development, human and social welfare to uplift lifestyle has led to a surge in per capita energy consumption (Wang et al., 2019). A dominant part of energy requirement comes through utilization of fossil fuels e.g., coal and petroleum sources. However, these fossil fuel reserves are limited across the globe and its sustainability is estimated to last for next few decades. Another factor of concern is global warming, as the exhaust emission from industrial chimney and automobiles causing accumulation of a majority greenhouse gases into the environment (Chien et al., 2023). In 2021, the carbon emission into the atmosphere has reached to 37.12 billion metric tons, an increment of 63% was observed since the rapid industrialisation in 1990s in the Asian continent (Friedlingstein et al., 2022). To decrease the global carbon foot prints and tackle the serious implications of the climate change, the international community has significantly increased the adoption of bioethanol within the transportation sector over the past decade to promote it as an environment friendly cleaner alternative to fossil fuel (Debnath & Whistance, 2023; Kaur, 2023).

The utilization of bioethanol-gasoline blend such as E5, E20 and E85 enhances fuel quality due to the elevated octane rating of ethanol (Iodice et al., 2018). This, in turn, aids in reducing engine knocking, promoting complete fuel combustion and minimizing the production of greenhouse gases through exhaust emission (Sakai & Rothamer, 2019). The substrate for bioethanol production is primarily sourced from first-generation feedstock, which are mostly comprised of starch-based sources (e.g., corn, sugarcane, rice, and wheat grains etc.). However, use of starch-based sources remained questionable due to the food

security concern, focus has been drawn towards the production of second-generation bioethanol from the lignocellulosic wastes. Lignocellulose biomass mostly comprises of forestry residues (e.g., wood chips, wood saw dust), agricultural by-products (e.g., rice straw, sugarcane bagasse, wheat straw etc.) and other wastes (e.g., fruit peels, dried leaves etc.). These feedstocks are widely available worldwide, provide a sustainable source of biomass, do not compete with the food supply, and contribute to waste mitigation (Anwar et al., 2023; Guimarães et al., 2023). Rice straw as a feedstock for bioethanol production is of great interest in Asian countries, because the geography has extensive rice cultivation (Binod et al., 2010).

Lignocellulose biomass is biochemically converted into ethanol in three main steps: pretreatment of biomass, enzymatic saccharification utilising cellulase and followed by fermentation (Lamichhane et al., 2021). A pretreatment step is applied to dismantle complex structure (Cellulose, hemicelluloses, and lignin) of lignocellulose biomass. This process makes cellulose fibres more available for enzymatic hydrolysis (Akhtar et al., 2016). Cellulases are used for saccharification of cellulose for converting into the end product glucose. The next step fermentation is carried out by the fermenting yeasts which converts sugars into ethanol. While hexose fermenting yeasts can ferment glucose which is released from enzymatic hydrolysis of cellulose, pentose fermenting yeasts are also used to maximise the sugar convertibility from the available pentose sugars (mainly xylose) from the pretreatment of hemicelluloses. While most of the pilot and large-scale studies utilises the commercial cellulases, the enzyme cost hugely lowers the profit margins and restricting the commerciality of second-generation bioethanol. The challenges that exist in the stages of pretreatment, enzymatic hydrolysis, and fermentation, demands solutions to enhance economic viability and efficiency of the bioethanol production. Therefore, the present study focused on developing an *in situ* cellulase production, saccharification and fermentation

(ICPSF) process by employing the culture of fungus and yeast. It also emphasised on a novel cyclic shifting of temperature approach for SSF processes to improve the bioethanol yield.

1.2. Problem statements

To address the limitations in SSF process at a single mutual optimum temperature, a novel approach involving phase-wise adjustments of temperature for saccharification and fermentation within a thermotolerant range of yeast was developed to maximize bioethanol yield. One way to achieve this by operating the process with higher temperature for a short period to facilitate the enzymatic hydrolysis of cellulose, subsequently, the process can be shifted to lower temperature for a specific period to promote growth and fermentation of yeast. This shifting of temperature in cyclic manner can improve the sugar release and sugar utilization throughout the bioethanol production process. This approach is novel and prior to this research work no studies have been conducted on any of lignocellulosic feedstock for bioethanol production.

1.3. Objectives of the study

The following objectives are framed based upon the current bottlenecks in lignocellulosic bioethanol production.

I. Development of an acid pretreatment strategy for rice straw and optimisation of cellulase production by the coculture of cellulolytic fungi *Trichoderma reesei* and *Penicillium janthinellum*.
II. Process optimisation for *in situ* cellulase production, saccharification and fermentation by co-culture of *Penicillium janthinellum* and *Saccharomyces cerevisiae* through novel approach of cyclic shifting of temperature.

III. Establishment of a poly-culture system of fungus *Penicillium janthinellum*, *Trichoderma reesei* and yeast *Saccharomyces cerevisiae*, *Pichia stipitis* for bioethanol production through cyclic shifting of temperature.

IV. Bioethanol production from pretreated rice straw hemicellulosic hydrolysate by co-fermentation using *Saccharomyces cerevisiae* and *Pichia stipitis*.

1.4. Uniqueness of the study

- The study highlights the optimisation of process parameters for bioethanol production using rice straw. Instead of using commercial cellulase for the enzymatic hydrolysis, a cost effective *in situ* cellulase production was implemented by using cellulolytic fungus using a cheaper substrate i.e., acid pretreated rice straw whole slurry mixture.
- The process of pretreatment followed by integrated cellulase production, saccharification, and fermentation processes in a single vessel system. For bioethanol production from rice straw, an *in situ* cellulase production, saccharification and fermentation (ICPSF) process was established by employing the culture of fungus and yeast.
- To address the temperature ambiguity that is inherent in saccharification and fermentation process for bioethanol production through the SSF process, the conventional approaches such as prolong prehydrolysis at high temperature followed by fermentation or the SSF at mutual incubation temperature were replaced by novel cyclic shifting of temperature strategy (CSTS). This strategy effectively resolved the issue encountered in the conventional methods such as feedback inhibition of cellulase enzyme during prolong prehydrolysis. This also helped in providing optimum temperature to maximize both the process of saccharification as well as fermentation in

SSF. The developed cyclic shifting of temperature strategy (CSTS) for SSF process has not been previously reported in the literature.

- To enhance the fermentation yield from the hemicellulose fraction, the soluble sugar present in the pretreated rice straw hydrolysate was concentrated by re-cycling the hydrolysate stream obtained after initial pretreatment through further pre-treating fresh delignified rice straw. Here, the conventional methods for concentrating the soluble sugars, such as evaporation and nano-filtration were replaced with the above-mentioned innovative approaches.

1.5. Thesis outline

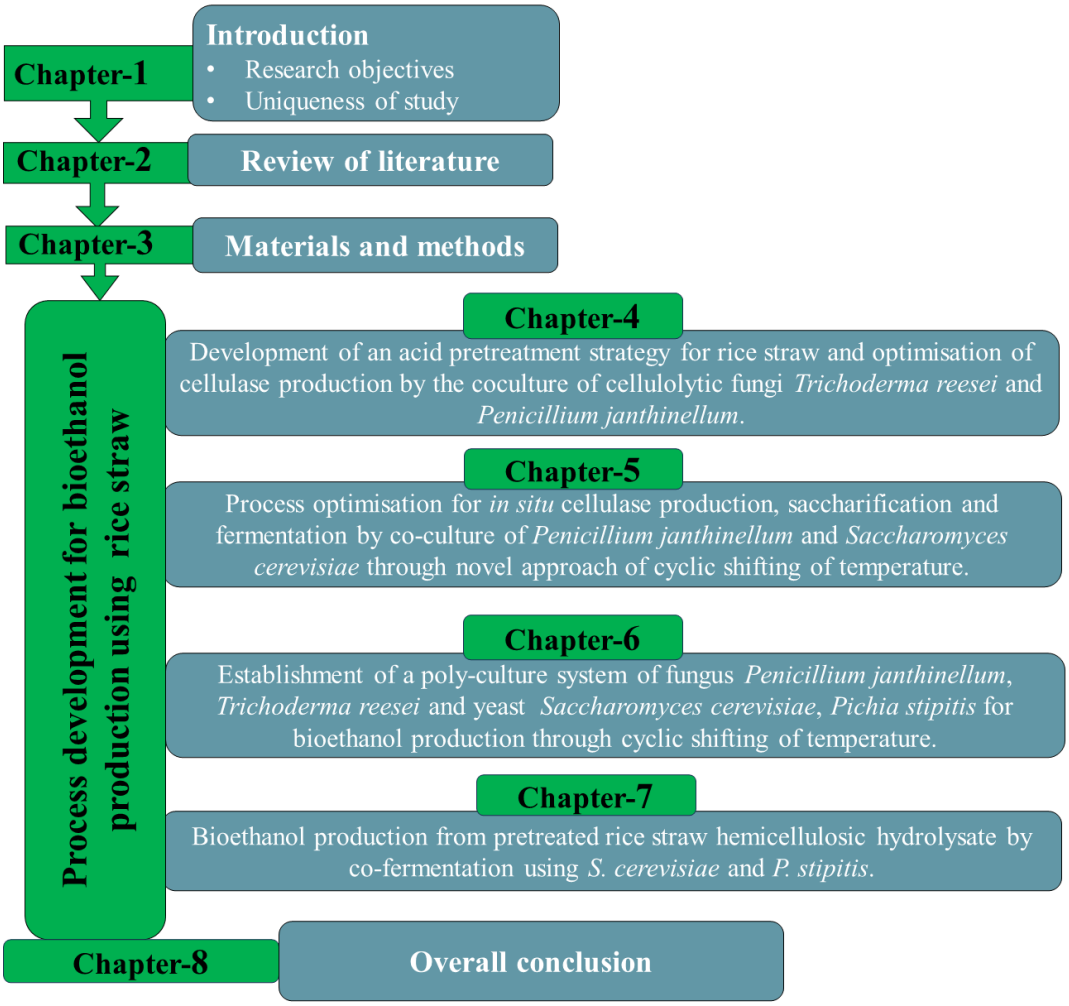

Fig.1.1: Outline of the thesis

Chapter 1 provides an overview of the background and motivation behind the research, outlining the problem statement by identifying the bottlenecks present in current lignocellulosic bioethanol production. It also states the objectives of the study and presents the thesis outline **(Fig. 1.1).** Additionally, the chapter details the experimental approaches undertaken to fulfill the project's aims.

Chapter 2 provides a comprehensive review of recent scientific literature to provide context for the study. It emphasizes the significance of bioethanol as a viable transport fuel, exploring the diverse feedstocks utilized for bioethanol production. Additionally, the chapter discusses various pretreatment methods employed in the bioethanol production process, as well as the production of cellulase through fungal utilization. Factors influencing cellulase production and enzymatic hydrolysis are thoroughly examined. Furthermore, the chapter elucidates the ethanol production mechanism by yeast and delves into process strategies for the biochemical conversion of lignocellulosic biomass into bioethanol.

Chapter 3 outlines the materials and methods employed to conduct various experiments and achieve the specified research objectives.

Chapter 4 details development of an acid pretreatment strategy for rice straw and optimisation of cellulase production by the coculture of cellulolytic fungi *Trichoderma reesei* and *Penicillium janthinellum*.

Chapter 5 deals with process optimisation for *in situ* cellulase production, saccharification and fermentation by co-culture of *Penicillium janthinellum* and *Saccharomyces cerevisiae* through novel approach of cyclic shifting of temperature.

Chapter 6 outlines the establishment of a poly-culture system of fungus *Penicillium janthinellum*, *Trichoderma reesei* and yeast *Saccharomyces cerevisiae*, *Pichia stipitis* for bioethanol production through cyclic shifting of temperature.

Chapter 7 details with bioethanol production from pretreated rice straw hemicellulosic hydrolysate by co-fermentation using *Saccharomyces cerevisiae* and *Pichia stipitis*.

Chapter 8 presents the overall conclusions derived from all the studies conducted to fulfill the designated objectives.

Chapter-2

Review of literature

2.1. Bioethanol as a transport fuel

The use of ethanol has gained momentum as an alternative to gasoline due to escalating oil prices, rise in demand of fuel-powered vehicles, efforts to provide national energy security and to combat climate crisis. The majority of ethanol is produced via fermentation, while the synthetic ethanol is produced from ethylene through catalytic hydration (Hidzir et al., 2014). Based on the fraction of ethanol blending with gasoline, different annotations were given to the blends such as E10 (10% v/v ethanol in gasoline). The lower blends such as E5, E10 are currently used in the Spark ignition engines (SI engines) without modifications. While higher blends such as E85 and above, uses special flex engines in the flexible fuel vehicles (FFVs) (Kroyan et al., 2022). In India, the oil marketing companies (OMCs) supply achieved the current ethanol blended petrol (EBPs) of E10. The National policy on biofuel, Amendment of 2022 revised the target of E20 blending by 2025-26 (Morimoto et al., 2021).

The quality of fuel is indicated by its physicochemical properties described in **(Table 2.1)** for both gasoline and bioethanol (Yusoff et al., 2015). The higher-octane rating of bioethanol makes it an excellent blend with gasoline (Alhassan et al., 2021). It restricts the premature ignition and improves the thermal efficiency of engine. The blending also produces higher torque output. Torque is determined by the higher turning force derived from the pressure exerted from the crankshaft of piston.

The burning of fossil fuel in the internal combustion engine releases the non-toxic combustion gases into the atmosphere i.e., carbon dioxide (CO_2), water vapour and Nitrogen (N_2). The incomplete combustion gives rise to the production of more toxic gas i.e., carbon

monoxide (CO). At high combustion temperature, oxides of nitrogen (NO_X) are released and other gases includes Ozone (O_3) and particulate matters. All these gases is the causative factor for greenhouse effect. The higher oxygen content (~34.73%) of ethanol, when blended with gasoline produces a mixture having higher air-fuel equivalence ratio. It enhances fuel combustion efficiency and releases lower level of greenhouse gases through exhaust emissions. The low heating value of bioethanol reduces the exhaust gas temperature and thereby reduces the concentration of NO_X in the whole exhaust gas mixture (Yusoff et al., 2015).

Table 2.1: Physiochemical properties of gasoline and bioethanol (Yusoff et al., 2015).

Physicochemical properties	Gasoline	Bioethanol
Chemical formula	~$C_8H_{15.6}$	C_2H_5OH
Molecular weight	100-105	46
Oxygen content (wt %)	0	34.73
Octane number	95	106-130
Specific gravity	0.739	0.789
Flash point (ºC)	-43	8
Boiling point (ºC)	30-215	78.3
Auto ignition temperature (ºC)	257	423
Adiabatic flame temperature (ºC)	1970	1923
Stoichiometric air–fuel ratio	14.8	9
Low heating value (MJ kg^{-1})	44.4	28.9
Density (kg m^{-3})	720-750	794
Heat of vaporization, (MJ kg^{-1})	0.32	0.92
Kinematic viscosity at 20 ºC (mm^2 s^{-1})	0.4-0.8	1.5

2.2. Feedstock for bioethanol production

Based on the substrate utilised for bioethanol production, it is classified into first-generation and second-generation feedstock (Ayodele et al., 2020). The first-generation sources are mainly comprised of edible sources having high sucrose or starch content. The sucrose-based sources includes sweet sorghum, sugar cane juice and molasses, sugar beets etc. The sugars

available in these feedstocks can be directly utilised for fermentation by yeast into bioethanol. The starch-based feedstock are mainly grains i.e., wheat, corn, rice, barley etc. These starch contains amylose structures, which are the polymers of glucose and needs to breakdown into monomers D-glucose known as dextrose. This reaction is catalysed by amylase enzymes, and the emulsion obtained known as dextrose syrup and it is further fermented into bioethanol (Bušić et al., 2018).

The second-generation bioethanol is produced by utilising non-edible feedstocks such as lignocellulose biomass. It constitutes forestry products such as wood chips, leftover agricultural wastes e.g., rice straw, wheat straw etc., agro-industrial residues such as corn fibre, sugarcane bagasse etc., forestry-industrial residues such as wood saw dust etc. and other energy crops e.g., poplar, switch grass, miscanthus etc. It is rich in cellulose and a promising source of fermentable sugars. It's potential can be unleashed for the production of bioethanol (Isikgor & Becer, 2015; Lamichhane et al., 2021).

Bioethanol production from lignocellulose biomass is mainly carried out in three stages, which includes pretreatment, enzymatic hydrolysis, followed by fermentation. The objective of the pretreatment is to expose cellulose microfibres from lignocellulose biomass, or to make the biomass more porous in nature for permeability of cellulase enzyme. Cellulases are utilised for breaking down the cellulose into the final soluble sugar glucose. Xylanase can also be utilised to cleave the hemicelluloses into pentose sugar i.e., xylose. Enzymatic hydrolysis is mostly a temperature dependent process and optimum activity happened to be at 50°C. Onsite cellulase is being produced using cellulolytic bacteria and fungus or fetched commercially. Further, fermentation of sugar into bioethanol is carried out by ethanol-fermenting yeasts (Saini et al., 2015).

2.2.1. Composition of lignocellulose biomass

The chemical composition of lignocellulose biomass determines its structural rigidity and also responsible for the efficiency of biofuel production (Das et al., 2021). The composition primarily varies with the plant species **(Table 2.2)**. Lignocellulose biomass is primarily composed of cellulose, which is shielded by hemicelluloses matrix and the outer layer contains lignin as a branched structures and embedded with hemicelluloses.

Cellulose is a polysaccharide molecule comprised of glucose monomers, which is associated by the β-1,4- glycosidic linkages. The major part is composed of microfibrils which are highly crystalline in nature and resistive to chemical hydrolysis because of strong hydrogen bonds. The minor parts comprised of amorphous cellulose and play the role of linkers between two crystalline celluloses. Hemicelluloses are the branched heteropolymers which includes xylan, glucuronoxylan, arabinoxylan, glucomannan, xyloglucan. Xylose is present in the dominant form in hardwoods and agricultural residues. In annual plants like rice straw, hydrolysis of acetyl moieties present in hemicellulose forms the acetic acid. Lignin is the branched structure of phenyl propanoids, responsible for providing compressive strength, rigidity and stiffness to the cell wall and prevents the plants from attack against pathogens. The lignin precursors (coniferyl alcohol, p-coumaryl alcohol, and sinapyl alcohol) or the lignin structural units (guaiacyl, syringyl and p-hydroxyphenyl units). The inorganic components like the ash which have silica content which measures the fraction after complete incineration of lignocellulose (Bajpai & Bajpai, 2016; Cai et al., 2017).

Table 2.2: Composition of different lignocellulose biomass.

Lignocellulose biomass	Cellulose	Hemicelluloses	Lignin	Ash	References
Rice straw	46.33	31.09	10.17	15.1	(Zhao et al., 2023)
Corn straw	30.81	25.52	16.76	7	(Zhao et al., 2023)
Soyabean straw	42.39	22.05	18.93	4.43	(Zhao et al., 2023)
Peanut straw	36.56	20.27	18.36	13.05	(Zhao et al., 2023)
Wheat straw	35.7	29.7	18.8	6.1	(Ziaei-Rad et al., 2023)
Sugarcane bagasse	45	32	19	5	(Hamin et al., 2023)
Corn stover	39.12	30.95	10.73	-	(Li et al., 2022)
Switch grass	44.4	22.8	29.8	1	(Rodrigues et al., 2016)
Miscanthus	45.7	19.6	32.2	2.9	(Rodrigues et al., 2016)
White poplar saw dust	55.78	14.94	20.2	2.55	(Gökkaya et al., 2020)
Sugarcane bagasse	39.84	17.19	22.25	4.54	(Gomes et al., 2020)

2.2.2. Rice straw: a potential lignocellulosic source

Among the cultivated grain crops in the world, rice is the third largest harvest after wheat and corn, producing rice straw as the one of the potential agriculture generated waste. As per FAO statistics in 2022-23, the estimated net rice grain production was estimated at 519.8 million tons (FAO, 2022). While rice production is predominated in Asian countries, China has highest production i.e., 146 million metric tons, followed by India i.e., 135 million metric tons in the crop year 2022-23 (Statista, 2024). Based on the average cutting height, the paddy to straw ratio varies from 0.74-0.79 (Nguyen et al., 2016), 0.5-0.7 (Van Hung et al., 2020). So, the paddy cultivation generates a huge amount of rice straw as waste. The part of produced rice straw was used to fed cattle and rest were being disposed causing slow degradation in soil, rice stem disease or incinerated at the field which causes air pollution. As an agricultural waste, rice straw is a rich source of cellulose. So, it can be explored for the production of bioethanol.

2.3. Lignocellulosic bioethanol production

There are various factors such as severity of pretreatment, solid loadings, fermentation temperature and enzyme loading etc. effects the final bioethanol titre. The high pretreatment severity results in the formation of higher amount of inhibitors (Panagiotopoulos et al., 2011). At the same time low pretreatment severity arises the need for high cellulase loading for a better recovery of sugar from saccharification. Providing an optimal pretreatment condition is necessity to keep a balance between recovery of high sugar and low by-product formation. The pretreatment conditions also varies with different species of lignocellulose biomass and complexity of their recalcitrance (Zhao et al., 2012b). Another important factor is the optimisation of solid biomass loadings, as lower biomass loadings often leads to the lower ethanol titre, however high solid loadings creates hinderance in mixing and oxygen mass transfer operations. Fermentation temperature plays a vital role for cell viability and bioethanol yield. A temperature optimum has to be maintained for saccharification and fermentation, as lower temperature responsible for incomplete saccharification of biomass and higher temperature causes reduced cell viability (Azhar et al., 2017). Enzyme concentrations in the fermentation medium is a critical step for the process of bioethanol production. The lower enzyme concentrations can create carbon starvation for yeast cell, higher enzyme loadings makes the process expensive. So, an optimum enzyme loading can give the best outcome for high titre end product as well as can make the process economically viable (Rastogi & Shrivastava, 2017). Multiple strategies are utilised for lignocellulosic bioethanol production but the principal classification is based on the number of step applied or the strategy involved to ferment both the soluble sugar i.e., glucose and xylose. The following classification of lignocellulosic bioethanol production are primarily used.

2.3.1. Separate hydrolysis and fermentation (SHF)

In SHF, post pretreatment of lignocellulose biomass, saccharification and fermentation step is employed in two separate steps in two separate vessels. The pretreated slurry is first neutralised, then the solid cellulosic fibres are collected for enzymatic hydrolysis. Here, the cellulase enzyme is added with cellulose and incubated at a relatively higher temperature (optimum ~50ºC). After a specific incubation time, the hydrolysate are collected in a separate vessel and fermentation is carried out by adding yeast at normal incubation temperature (~30ºC) (Peng & Chen, 2011; Taherzadeh & Karimi, 2007). Although, this process qualifies to provide optimum incubation temperature for saccharification and fermentation, it has several disadvantages. During the saccharification, the accumulation of reducing sugars exerts the feedback inhibition on cellulase enzyme and thereby reduces the maximised conversion cellulose. Although feedback inhibition can be minimised by increasing the enzyme loading but it will be detrimental to the economics of bioethanol production. The use of separate steps in this process, makes it relatively more prone to contamination as well as makes the whole process cumbersome and expensive (Axelsson, 2011).

2.3.2. Simultaneous saccharification and fermentation (SSF)

In SSF, after the pretreatment of lignocellulose biomass, both the saccharification and fermentation is done simultaneously in a single vessel (Ojeda et al., 2011). Here, after the pretreatment of lignocellulose biomass, the solid cellulosic fraction is incubated with cellulase enzyme and fermenting yeast at an optimum temperature which favours saccharification, growth and ethanol production by yeast. Usually, the incubation temperature varies based on the thermotolerance of the yeast strain utilised for the process. Although, this process is widely accepted both in research and industrial scale. Still certain challenges are encountered, such as inefficient enzymatic hydrolysis at lower temperature reduces the

glucose yield, arise of carbon starvation condition in the medium that may trigger utilisation of ethanol for growth and lipid metabolism by the yeast and thereby reducing the final ethanol titre (Sudiyani et al., 2019).

2.3.3. Consolidated bioprocessing (CB)

Consolidated bioprocessing utilises the genetic engineering tools to express the cellulases-producing gene in the fermenting microorganisms or vice versa (den Haan et al., 2021; Du Plessis, 2008). A study conducted on recombinant *S. cerevisiae* by Hasunuma et al., 2014, where they used a multi-gene integration system (GIN11/FRT) to introduce the cellulases-producing genes such as endoxylanase gene (source: *T. reesei*), β-glucosidase gene (source: *A. aculeatus*). For xylose assimilating genes xylitol dehydrogenase and xylose reductase genes (*P. stipitis*), to increase inhibitor tolerance TAL1, XKS1, ADH1 and FDH1 genes (Source: *S. cerevisiae*) and found a 2.7-fold increment in bioethanol production as compared to a normal xylose assimilating yeast (Hasunuma et al., 2014). However, consolidated bioprocessing has variety of limitation that is restricting its usage at industrial scale such as lower enzymatic efficiency for saccharification which reduces the sugar yield, substrate compatibility factor for different lignocellulosic biomass, product inhibition of CBP microorganisms, arising of genetic instability of CBP system etc (Fan, 2014; Xu et al., 2009).

2.4. Pretreatment of lignocellulosic biomass

The typical complex nature of lignin and shielding effect by hemicelluloses restricts the cellulase enzymes to interact with the cellulose. Pretreatment is an important step in lignocellulose processing where the cellulose is made more available for enhancing enzymatic conversion to monosaccharide glucose by the disrupting of the compact structure outer matrixes.

2.4.1. Physical pretreatment

Physical pretreatment methods are conventional techniques that are widely used for processing of agricultural and forestry residues for decreasing the particle densification, increasing the surface area, flow properties, porosity which would be helpful for effective enzymatic accessibility (Barakat et al., 2014).

2.4.1.1. Milling

Milling process effectively reduces the crystalline nature of cellulose, particle size and the degree of polymerization of the lignocellulose feedstocks. The most frequently used mills includes a ball mill, centrifugal mill, knife mill, vibratory mill etc. (Cheng & Timilsina, 2011). It mostly includes chipping which leads to the reduction in the biomass size from 10mm to 30mm. It helps in further enhancement of the heat and mass transfer rate during biochemical conversion. Grinding can reduce the particle size up to 0.2 mm which greatly helps in reduction of crystallinity. However, grinding is an energy intensive process. The energy requirement is greatly influenced by initial particle size, material feeding rate and the moisture content of the biomass (Amin et al., 2017).

2.4.1.2. Microwave Treatment

Microwave pretreatment disrupts the organized cellulose structure with minimal production of inhibitors. It is widely used because of its easy operation, shorter biomass incubation time, high heating capacity, and low energy requirement. The study by Chen et al., 2011, shows that high power treatment resulted in increased release of glucose but the pretreatment time has no significant effect (Chen et al., 2011).

2.4.1.3. Sonication treatment

The treatment with ultrasonic sound has both physical and chemical changes in lignocellulose biomass. The formation of cavitation bubbles in this process, disrupts the compact structure of cellulose and hemicelluloses. It also helps in cleaving the C-C bonds between hemicelluloses and lignin, making the biomass more susceptible to enzymatic attack (Yachmenev et al., 2009). The maximum effectiveness was reported at 50°C and likely it is the optimum temperature for cellulase enzyme activity. The efficiency of pretreatment is dependent on the characteristics of biomass, reactor configuration, ultrasonic frequency, duration and type of solvent used (Bussemaker & Zhang, 2013).

2.4.1.4. Pyrolysis

Pyrolysis is the treatment of lignocellulose biomass, operated at a temperature of 500–800 °C without the presence of any oxidizing agent. This has been widely applied for bio-oil production but not has been utilized for bioethanol perspective. This high-temperature treatment generally converts the lignocellulose into the end products like pyrolysis oil, charcoal, and gaseous substance.

2.4.1.5. Pulsed electric field

Electroporation by the application of pulsed electric field exposes the cellulose fibers by making pores and helping in the entry of the agents like enzyme that will help in degradation of cellulose into constituent monosaccharide. The application of sudden voltages from 5-20 KV/cm for nano/mili second duration was applied to treat the lignocellulose; the short duration treatment and less electricity application makes it an energy-efficient pretreatment method (Salerno et al., 2009).

2.4.2. Chemical pretreatment

2.4.2.1. Dilute acid pretreatment

The dilute acid treatment is a conventional method of pretreatment of lignocellulose biomass, widely applied both at lab and industrial scale. The application of dilute acids like H_2SO_4, HCl, HNO_3 and H_3PO_4 has been studied earlier for pretreatment (Marzialetti et al., 2008). The application of dil. H_2SO_4 along with hydrothermal processing is widely applied because of high impregnation efficiency, dissolving the hemicellulosic fraction and maximum recovery of the dissolved hemicellulosic sugars. The dilute acid pretreatment often generates by-products like furfural, 5-Hydroxy methyl furfural (HMF), formic acid, acetic acid due to further degradation of soluble sugars in the hemicellulosic hydrolysate. The pretreated hydrolysate needs another detoxification step for further use of soluble sugars in the fermentation process (Larsson et al., 1999).

2.4.2.2. Dilute alkali pretreatment

The hydroxyl derivatives of sodium, calcium, potassium, and ammonium salts are the most commonly used alkali reagents. However, sodium hydroxide found to be most effective for biomass pretreatment (Kumar & Wyman, 2009).It effectively breaks the side chains of the glycosides and esters, causing cellulose decrystalization, lignin degradation, swelling of cellulose (Ibrahim et al., 2011; Sun et al., 1995). The degradation efficiency cellulose and hemicellulose by alkali pretreatment methods is less effective than that of acid pretreatment.

2.4.2.3. Ozonolysis

This pretreatment is mainly done for removal of lignin. It has negligible effect on hemicelluloses and cellulose content of the lignocellulose biomass (Kumar et al., 2009).This process is carried out at normal temperature conditions ~30°C and doesn't produce any

fermentation inhibitors (Quesada et al., 1999).The high quantity requirement of ozone for pretreatment makes it infeasible for use at industrial scale.

2.4.2.4. Ionic liquids

Ionic liquids are the classes of solvents whose main constituents are cations or anions which competes for hydrogen bonding with the components of lignocellulose biomass and simultaneously works by breaking the branches of polymer (Moulthrop et al., 2005). Ionic liquids are highly polar, thermally stable, have low melting points (<100°C) and has lower vapor pressure (Behera et al., 2014; Zavrel et al., 2009). Cholinium acetate, cholinium amino acids, 1-Butyl-3-methylimidazolium-acetate, Ethyl-3-methylimidazolium diethyl phosphate-acetate are some ionic liquids that has been used for pretreatment of rice straw, rice husk, sugarcane bagasse, switch grass (Bajpai, 2016).

2.4.3. Physicochemical pretreatment methods

2.4.3.1. Steam explosion

The application of super saturated steam has explosive effect on the lignocellulose substrate and leads to the biomass deconstruction. The acetyl groups present in the hemicelluloses are being converted into acetic acid. It further promotes the hydrolysis of hemicellulose fractions into the glucose and xylose. Hence, the process is also called as autohydrolysis (Mosier et al., 2005).

2.4.3.2. Liquid hot water

Liquid hot water at relative high temperature and pressure forms hydronium ions which helps in penetration, dissociation of lignocellulose network. The autoclave temperature and pressure i.e.,121°C and 15 psi pressure with different holding time has been highly utilized for the pretreatment at lab scale. This process along with acid catalyst is mainly utilized for

the pretreatment of agricultural byproducts, because of efficient recovery of soluble sugars from hemicellulosic fraction. The disadvantages of this method includes formation of fermentation inhibitors, loss of sugars during neutralization process and also it uses high amount of water (Agbor et al., 2011).

2.4.3.3. AFEX (Ammonia Fibre explosion)

In AFEX method, the lignocellulose biomass treatment with liquid ammonia is carried out at an equal ratio (1:1 w/w), higher temperature (70-200ºC) and high pressure (100-400 psi) (Bals et al., 2010). Thereafter, the reactor valve is opened to release the pressure. This creates an abrupt pressure change, which causes swelling, reduction in cellulose crystallinity and breaking of lignin network (Uppugundla et al., 2014).

2.4.3.4. Oxidative pretreatment

The oxidative pretreatment involves the use of oxidizing agents such as ozone hydrogen peroxide for pretreatment of lignocellulose by means of certain chemical reactions like oxidative cleavage, side chain displacement, electrophilic substitution of aromatic compounds. This process mainly causes delignification of biomass but it also produces some acidic compounds which acts as inhibitors for fermentation process (Alvira et al., 2010).

2.4.4. Biological pretreatment method

Certain microbial secretions can act on lignocellulose biomass to disintegrate its recalcitrance. Specific group of fungi such as brown rot fungi, white rot fungi and soft rot fungi effectively carry out the degradation of lignin and hemicelluloses. The commonly utilized white rot fungus for biological pretreatment such as *Cyathus stercolerus, Pleurotus ostreaus* which produces laccases and peroxidases enzymes which degrades the lignin (Sánchez, 2009).The common basidiomycetes species used are *Fomes fomentarius, , Lepista*

nuda and *Trametes versicolor* are extensively studied which have high delignification efficiency (Shi et al., 2008).

2.5. Acid pretreatment and by-product formation

Dilute Sulfuric acid pretreatment along with hydrothermal processing have been studied widely for pretreatment lignocellulose biomass. Owing to its high impregnation efficiency, high solubilisation hemicellulosic fraction into soluble sugars in the hydrolysate fraction and the high recovery of solid cellulose with high enzymatic convertibility makes it a preferable pretreatment method (Hu & Ragauskas, 2012; Yang & Wyman, 2008). This process possess certain drawbacks such as high cost of construction materials for the reactors, formation gypsum after neutralization and accumulation of inhibitory by-products in hydrolysate fraction during pretreatment.

The acid pretreatment solubilises hemicelluloses into sugars, pentoses (e.g., xylose, arabinose) and hexoses (e.g., glucose, galactose, mannose and rhamnose). During the pretreatment, pentoses are degraded into 2- furfuraldehyde by dehydration process, which is also known as furfural. Similarly, hexoses are further degraded in to 5-hydroxymethyl-2-furaldehyde, also termed as HMF (Gairola & Smirnova, 2012; LEE & NAGY, 1990). HMF is degraded into weak acids such as formic acids and levulinic acid at severe pretreatment conditions such as higher temperature, longer pretreatment time or increased acid concentration (Fengel & Wegener, 1983). The acetyl moieties attached to the xylan also converts into acetic acid. The amorphous fraction of cellulose gets hydrolysed into glucose and pretreatment by-products are HMF, HMF further gets converted into formic acid and levulinic acid and. During acid pretreatment, breakdown of beta-O-4 ether linkages from lignin macromolecule results in different phenolic compounds (Larsson et al., 2000; Mitchell et al., 2014).The most common phenolic compounds formed during acidic pretreatment are 4-

hydroxybenzaldehyde, vanillin, 4-hydroxybenzoic acid etc. Besides, acid pretreatment of annual plants produces phenolic acids such as ferulic acid, p-coumaric acid etc. (Martín et al., 2007).

2.5.1. Inhibitory effect of acid pretreatment by-products

The presence of sugar degradation by-products in the hydrolysate formed post lignocellulose pretreatment has the potential to hinder the growth of yeast cell as well the final ethanol yield **(Fig. 2.1)** (van der Pol et al., 2014). The weak acids e.g., acetic acid or formic acid are liposoluble. They travel across the plasma membrane and further dissociates into its anions and cations. As the inflow of weak acid begins, the yeast cytoplasm starts buffering to neutralize the intracellular pH. At high acidic concentration, the cell activates the plasma membrane ATPase to pump out the H^+ ions at the expense of ATP hydrolysis to maintain the intracellular pH. But this process leads to dissipation of proton motive force (PMF), exhaustion of the cell, further acidification of cytoplasmic fluid and eventually occurring of cell death. this enzyme. Enolase, one of the glycolytic enzyme which converts 2-phosphoglycerate into phosphoenolpyruvate, is highly sensitive to the cytoplasmic presence of weak acids. These weak acid either directly inhibits the enzyme, or the activity is interfered by cytoplasmic acidification (Cola et al., 2020; Palmqvist & Hahn-Hägerdal, 2000).

The conversion of acetaldehyde into ethanol is catalysed by alcohol dehydrogenase enzyme. In the presence of furans (furfural and HMF), alcohol dehydrogenase preferably catalyses furans into their respective furyl alcohols. This process leads to accumulation of excess acetaldehydes, ceased reduction of NADH to NAD^+, gradually ceased the glucose consumption by the cell and the restrict the ATP yield in the glycolytic pathway. The presence of furans in the medium also leads to a prolong lag phase in the cell, lower biomass

yield and reduction in the specific growth rate. The phenolic compounds generated from lignin degradation, protrude into the lipid membrane, disrupt the membrane integrity. Thus, phenolic compounds effect the ability of cell membrane to act as a selective barrier (Stagge et al., 2015). The soluble aromatic compounds have strong inhibitory effects for the cellulase enzyme. The hydrophobic interaction between aromatic compounds and cellulase enzymes imposes its inhibitory action. The critical tolerance concentration of by-products varies cell to cell in the different strain of yeast (Jönsson & Martín, 2016).

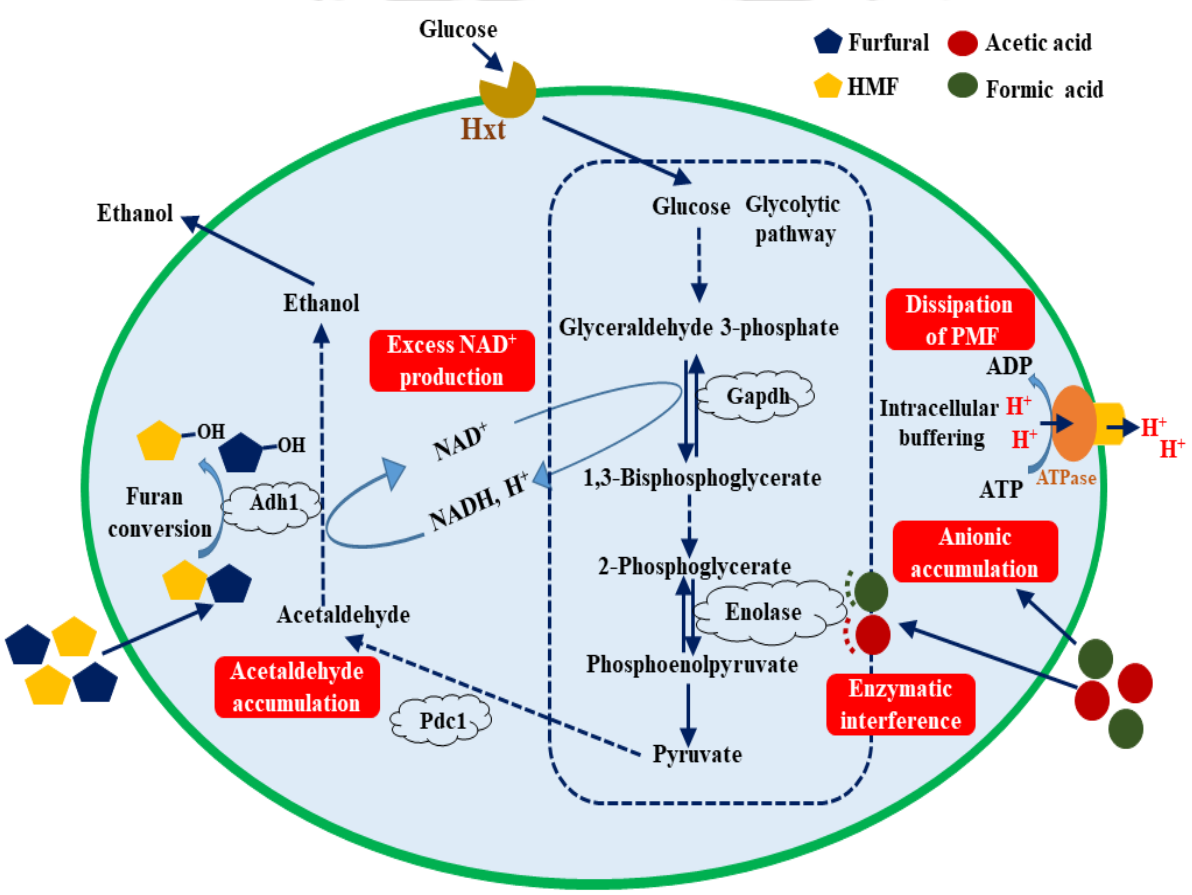

Fig.2.1: Mechanism of inhibition in yeast cell by acid pretreatment by-products furans and weak acids.

2.5.2. Detoxification processes for acid pretreated hydrolysate

All form of acid pretreatment by-products present in the acid pretreated hydrolysate synergistically inhibit the cellular metabolism and bioethanol fermentation in the yeast cell. A

detoxification step helps in decreasing the toxicity of pretreatment liquid by reducing the concentration of inhibitors (Jönsson & Martín, 2016). There are physical methods (e.g., vacuum evaporation, membrane-based separations), physicochemical detoxification processes (e.g., neutralisation, overliming, activated charcoal, Ion exchangers, solvent extraction), biological detoxification process (Lacases / peroxidase mediated, *in situ* microbial detoxification) are widely utilised.

2.5.2.1. Physical detoxification methods

The vacuum rotary evaporators are used to concentrate the hydrolysate fraction and remove volatile inhibitory components (e.g., furfural, acetic acid, and vanillin). The drawback of this process is accumulation of non-volatile inhibitors that may further inhibit the process of fermentation. Membrane based separations techniques include nanofiltration, can be applied to retain the soluble sugars and simultaneously separate the inhibitors from the hydrolysate. This is a less energy intensive process and has unique separation properties for all the inhibitors. However, certain drawbacks include choking of membrane and high cost of membrane filters (Maiti et al., 2012).

2.5.2.2. Physicochemical detoxification methods

The use of alkali such as NaOH to neutralise the acidic hydrolysate mostly precipitate the acid soluble lignin. While overliming used to precipitate both the phenolics and furans, is a cheaper and promising detoxification process. Activated charcoal-based adsorption effectively adsorbs the phenolic compounds from the acid pretreated hydrolysate and at the same time keeps the sugar fraction intact in the solution. The ion exchange resins are utilised to remove phenolics, acetic acid and furans at small scale but slower diffusion across the pore, longer operational time restricts the usage at large scale. The organic solvents such as chloroform, ethyl acetate, trichloroethylene etc. also used in extraction of furfural, acetic

acid, vanillin and other phenolics (Cantarella et al., 2004). Liquid-liquid extraction carried out with solvents isobutyl acetate, methyl isobutyl ketone, 1-butanol efficiently removed inhibitors from the sulphuric acid pretreated hemicellulosic hydrolysate (Roque et al., 2019).

2.5.2.3. Biological detoxification methods

The biological detoxification methods include use of laccase and peroxidase enzyme which oxidises the anilines, aromatic thiols, phenols at the expense of oxygen molecule. Certain drawbacks associated with this process are longer incubation period and higher cost of enzyme. *In situ* microbial detoxification was also reported, where *Amorphotheca resinae* ZN1 was able to grow in toxins present in pretreated hydrolysate and assimilated the furans, weak acids and lignin derived inhibitors as part of its carbon source (Zhang et al., 2010a). Microbial assisted biodetoxification has advantage of reduced incubation period as compared to the enzyme-based strategy.

2.6. Cellulase enzyme: the essential catalyst for lignocellulose hydrolysis

2.6.1. Mode of action of cellulase

Cellulases are mainly classified into EG: Endoglucanases (EC 3.2.1.4), CBH: Exoglucanses or cellobiohydrolases (EC 3.2.1.91) and BGL: beta-glucosidases (EC 3.2.1.21). All the three domains of cellulase synergistically act for the degradation of cellulose **(Fig.2.2)** (Hamid et al., 2015). Endoglucanases non-specifically cleaves at the amorphous site of the cellulose polymer and reduces to oligosaccharides with different monomeric chain lengths. Exoglucanases acts at both the reducing end and non-reducing end of the cellulose chain creating disaccharides i.e., cellobiose. The further hydrolysis of cellobiose into monosaccharide glucose units is carried out by beta-glucosidases enzyme by cleaving the beta- 1,4 glycosidic linkages (Rabinovich et al., 2002; Velleste et al., 2010; Yamada et al., 2005).

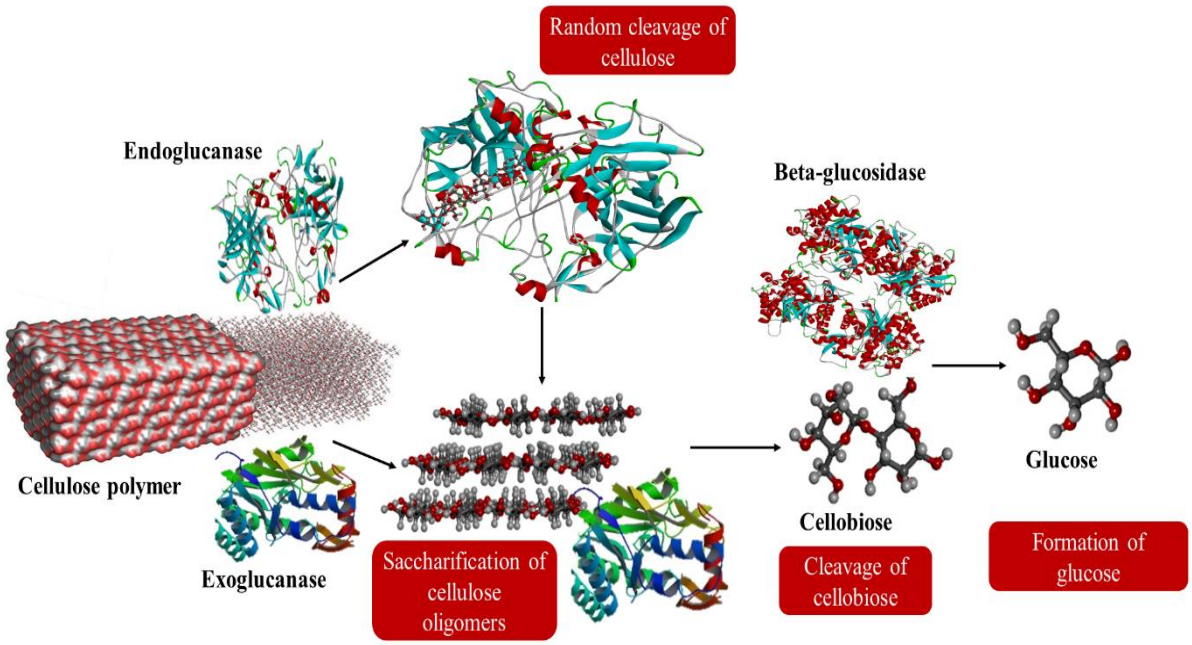

Fig.2.2: Mechanism of enzymatic hydrolysis of cellulose into glucose by cellulase enzyme.

The enzymatic hydrolysis of cellulose does not follow the Michaelis-Menten kinetics, due to the limitations in enzyme diffusion and insoluble nature of cellulosic substrate (Gan et al., 2003). The mode of action of cellulases differs from other enzyme groups, because the interacting substrate present in insoluble form. The cellulose binding domain (CBD) present in exoglucanases and endoglucanases facilitates catalytic domain (CD) to provide adequate contact to interact between the cellulase enzyme and the substrate. Xylanase enzyme cleaves the xylan into monomeric pentose and hexose sugars from hemicellulose fraction, which hydrolyses both the beta- 1,4 glycosidic linkages and beta- 1,6 glycosidic linkages (Motta et al., 2013).

2.6.2. Feedback inhibition by end products from enzymatic hydrolysis

The higher concentration of products derived from cellulolytic saccharification imposes feedback inhibition on cellulase enzyme itself. Higher accumulation of cellobiose in the hydrolysis media tend to inhibit the both endoglucanase and exoglucanase enzyme. A higher concentration of oligomers released from cleavage of cellulose inhibits the endoglucanase

enzyme. The increased in the concentration of end product glucose inhibits the activity of beta-glucosidase enzyme. To overcome this, usually higher dosage of beta-glucosidase is added for efficient saccharification(Razzaq et al., 2018; Singhania, 2009).

2.6.3. Fungal cellulase production

Many species of bacteria and fungi are capable of cellulase production (Enari & Markkanen, 2005; Maki et al., 2009). Fungal cellulase production is widely applied because of its property of extracellular enzyme production that can be utilised directly and production of high enzyme titre as compared to bacteria (Coughlan, 1985; Singh et al., 2021). The cellulase production in fungi is induced by the cellulosic substrate present in the medium. The gene expression for cellulase production in fungi is controlled by a number of transcription regulators **(Fig.2.3)**. The global activator complexes (Hap 2/3/5), key activator (Xyr1), additional activator (Ace2, Ace3) are responsible for expression of both cellulases and xylanases. Pac1 activates alkali expressed genes by repressing acid expressed genes and henceforth responsible for pH based protein induction (Shida et al., 2016). The Beta-glucosidase gene is activated by BglR. The repression of all the cellulolytic enzyme is carried by the Ace1 repressor. The carbon catabolite repression is controlled by Cre1 gene, which is pathway specific and represses the expression of cellulase enzyme if the growth of fungi is occurring in glucose medium (Portnoy et al., 2011).

Cellulase production using fungi can be carried out by both solid-state fermentation and also through submerged fermentation (Singhania et al., 2010). Solid state fermentation has advantages such as high yield of enzyme production, higher cellulase activity of crude enzyme, reduced requirement of water and lower chances of contamination. But there are several limitations of this process such as difficulty in controlling the process parameters for

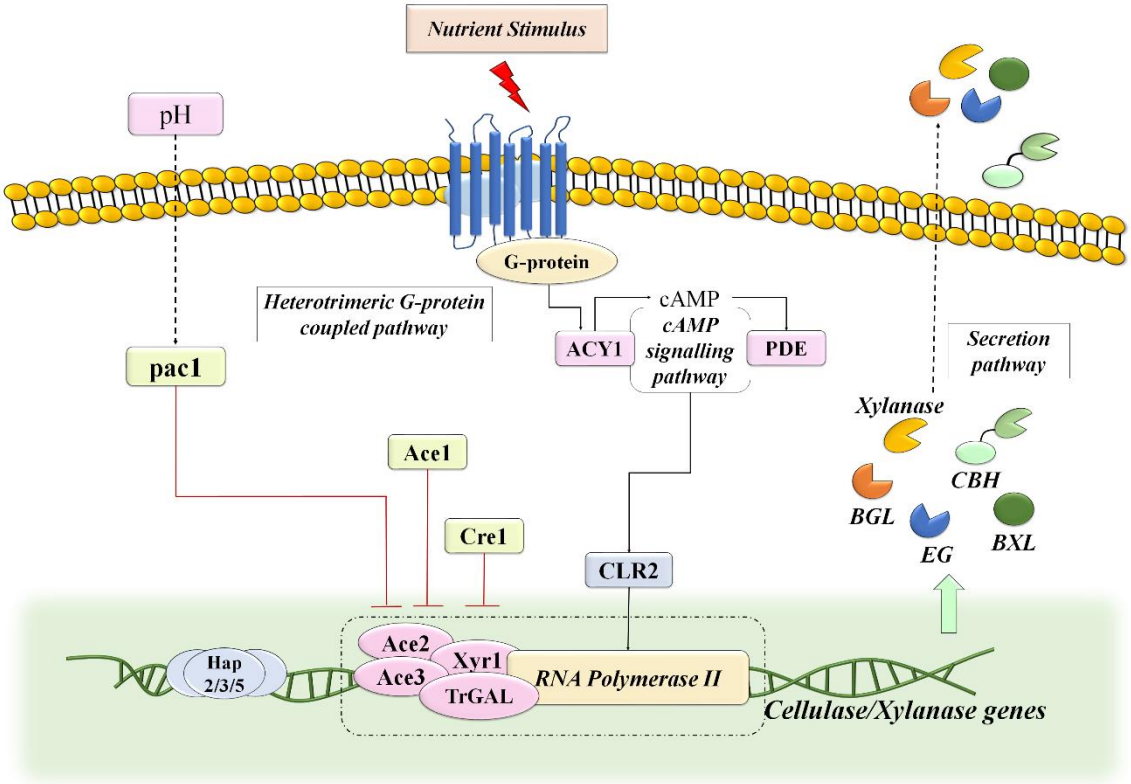

Fig.2.3: The gene expression system for cellulase production in fungus.

inoculums at large scale, heat transfer limitations causes building up of heat, scaling up difficulties, labour and cost intensive extraction of enzyme from the enzyme-substrate complex (Yoon et al., 2014). While submerged fermentation is a choice of operation at both lab and industrial scale of cellulase production. This process provides a better heat transfer throughout the medium, higher oxygen mass transfer to the highly viscous fungal mycelial broth, and easy to maintain the process parameters such as dissolved oxygen, pH etc., and the crude enzyme can be directly utilised after separation from the broth mixture. The drawbacks includes higher amount of effluent at the end of fermentation, consumption of high energy in the process, dilution of enzyme present in the culture broth (Chakraborty et al., 2019).

Many of the available commercial cellulosic substrates are utilised for cellulase production e.g., Avicel (Han et al., 2020; Zhang et al., 2018), Solka floc (Liu et al., 2020; Wang et al., 2020). While commercial substrates are costly, lignocellulosic substrates along with a

specific pretreatment process can be used as a cheaper alternative **(Table 2.3)**. The cellulase production is influenced by the type of substrate used, as the crystallinity nature of lignocellulose biomass varies with variation in plant species (Zhao et al., 2012a). Certain lignocellulose biomass may need more extensive pretreatment if they have higher rigidity in the structural complex(Leu & Zhu, 2013).

The fungal cellulase production is also affected by variety of physiological factors such as carbon sources, nitrogen sources, incubation temperature, pH, agitation, and aeration (Akula & Golla, 2020). The presence of cellulosic substrate promotes cellulase expression, while addition of certain inducers such as lactose increases the production (Amore et al., 2013). The fungal cells requires nitrogen source for growth and metabolism. Certain nitrogen sources, such as yeast extracts, peptone, or complex protein-based nitrogen sources, have been reported to enhance cellulase production in some fungi (Yoon et al., 2014). However, excessive nitrogen can repress cellulase production (Gautam et al., 2010). The temperature plays a crucial role for growth and enzyme production. Most of the cellulolytic fungi grows and produce cellulase at mesophilic temperature (between 25-35°C) conditions, while extreme temperature have inhibitory effects (Srivastava et al., 2018). One of the critical parameter is medium pH, which is essential for maintaining the cytoplasmic pH as well as ionic fluxes across the cell membrane. Cellulase production in fungi is mostly performed at slightly acidic pH condition (Barapatre et al., 2020; Helal et al., 2022). Most of the fungi are aerobic in nature, so maintaining a critical oxygen concentration is a necessity and influences the cellulase production. Agitation helps in proper mixing of nutrients as well as a requisite for a better oxygen mass transfer (Dey et al., 2021). The optimisation in agitation speed is a necessity to maintain a balance between shear stress to the cell and optimal mixing. All the above physiological parameters impact the fungal morphology such as pellet size, hyphal growth, branching characteristics, mycelial density etc., which in turn influences the amount

Table 2.3: Cellulase production by fungi using different lignocellulose biomass as substrate.

Fungus	Substrate	FPase (IU/ml)	CMCase (IU/ml)	Beta-glucosidase (IU/ml)	Reference
A. flavus KUB2	Sugarcane bagasse	0.72	1.27	-	(Namnuch et al., 2021)
T. reesei MTCC 164	rice straw	9.3	3.87	2.65	(Dey et al., 2023)
P. oxalicum GZ-2	rice straw	1.4	2	2.7	(Liao et al., 2015)
Penicillium sp.TG2	empty palm fruit bunches	0.31	1.27	0.54	(Jung et al., 2015)
P. decumbens	Rice straw	3.06	-	3.23	(Hou et al., 2020)
T. reesei Rut C-30	Rice straw	1.07	-	-	(Sun et al., 2008)
T. reesei	Sugarcane bagasse	1.01	1.5	-	(Maibam & Maiti, 2020)
T. viridae	Sugarcane bagasse	0.88	33.8	0.33	(Adsul et al., 2004)
N. crassa	Wheat straw	1.33	19.7	0.58	(Romero et al., 1999)

of cellulase production. So, tuning the physiological factors can be utilised to develop an efficient bioprocess to maximize the cellulase production.

2.7. Ethanol production using yeast

A variety of yeasts are utilised for ethanol production from sugar sources (glucose or xylose). However, specific process requirements necessitate selecting particular strains based on properties like tolerance to high ethanol concentrations, thermotolerance, substrate consumption capabilities, and the type of substrate they can utilize. A several well studied yeasts for ethanol production are *Saccharomyces cerevisiae* (Parapouli et al., 2020; Varize et al., 2022), *Pichia stipitis* (Germec et al., 2016; Okonkwo et al., 2016), *Kluyveromyces*

marxianus (Sene et al., 2023; Tinôco et al., 2021), *Pichia pastoris*(Yang & Zhang, 2018), *Zygosaccharomyces rouxii* (De Francesco et al., 2015) etc.

S. cerevisiae is a widely used yeast for its higher fermentative ability to convert glucose into ethanol with high yield and also have a great ability to tolerate stress to higher ethanol titres (Ma & Liu, 2010; Pereira et al., 2011). While *P. stipitis* has the ability to consume both glucose, xylose and convert it into ethanol (Agbogbo & Coward-Kelly, 2008). So, this yeast is of high importance to maximise the lignocellulosic bioethanol by utilising the hemicellulose derived sugars. Certain yeast such as *K. marxianus* exhibit thermotolerant properties and can be employed at elevated temperatures which is useful for second generation bioethanol production(Lane et al., 2011). For the purpose of protein expression, the yeast *P. pastoris* is widely used and it also has application in ethanol production from glucose with a higher yield of biomass (Karbalaei et al., 2020). To operate with waste streams containing high salt and sugar conditions, *Z. rouxii* was suitable for production of ethanol even at low pH (Magan, 2007).

Certain yeasts exhibit a phenomenon called Crabtree effect, where yeast prefers fermentation over respiration even in the presence of oxygen (De Deken, 1966). The resultant end product is ethanol from glucose. Yeasts which displays Crabtree effects are *Saccharomyces cerevisiae, Kluyveromyces lactis, Candida utilis, Pichia pastoris.* This phenomenon is advantageous to the bioprocess of ethanol production, as the glucose is utilised for ethanol production metabolism with lower biomass yields (Thierie & Penninckx, 2010).

2.7.1. Glucose metabolism in *S. cerevisiae*

The glucose transportation activity into the cell membrane in *S. cerevisae* is carried by specific proteins known as hexose transporter proteins or Hxt proteins (Özcan & Johnston,

1999).There are seventeen Hxt proteins responsible for glucose transportaion mechanism, encoded by gene HXT1 to HXT17 (Boles & Hollenberg, 1997). They are assigned to family of transporters known as major facilitator superfamily (MFS), and they transport glucose across concentration gradient by facilitated diffusion which is passive and energy independent (Kruckeberg, 1996). Besides Hxt proteins, Snf3 and Rgt2 are other two glucose transporter proteins responsible for low affinity and high affinity hexose sensing and transportaion mechanism repsectively (Özcan et al., 1998; Rolland et al., 2002).

After transportation of glucose inside the cell, it is further metabolised through glycolytic pathway explained in **Fig.2.4** (Van den Brink et al., 2008).The initial step of glucose metabolism is phosphorylation of glucose by hexokinase enzyme and formation of glucose-6-phosphate. It further isomerised into fructose-6-phosphate by phosphoglucose isomerase. The next step is conversion into fructose 1,6-biphosphate by phosphofructokinase enzyme at the expense of one ATP. The further catalysis is carried out by aldolase enzyme, which is also known as Fbap (fructose 1,6-bisphosphate aldolase). This enzyme cleaves the fructose 1,6-biphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. These two components undergoes reversible conversion between them which is catalysed by triosephosphate isomerase. The next step is the oxidation of glyceraldehyde 3-phosphate by NAD^+ and the further production of 1,3-diphosphoglycerate and reduced equivalent NADH, H^+ catalysed by the enzyme glyceraldehyde-3-phosphate dehydrogenase enzyme. Then 1,3-diphosphoglycerate converted by action phosphoglycerate kinase enzyme into 3-phosphoglycerate, this action leads to a phosphate group donation to an ADP molecule and thereby release of an ATP. The next reaction is formation of 2-phosphoglycerate, catalysed by phosphoglycerate mutase where phosphate group relocation takes place from position-3 to position-2. In the next step, enolase enzyme catalysed the dehydration of 2-phosphoglycerate and forms the phosphoenol pyruvate. Then, the last product of glycolysis, pyruvate is formed

by relesae of an ATP and this reaction is catalysed by pyruvate kinase enzyme. For ethanol fermentation, the decarboxylation of pyruvate is carried out by pyruvate decarboxylase enzyme and forms the acetaldehyde by release of a CO_2 molecule (Pronk et al., 1996). The alcohol dehydrogenase catalyses the final reaction to from ethanol from acetaldehyde. This is a reversible reaction and promotes the oxidation of NADH (released formed during the oxidation of glyceraldehyde-3-phosphate) into NAD^+. The overall reaction of alcoholic fermentation, where two molecules of ethanol is formed from one glucose molecule. It has a maximum theoritical yield of 0.51g ethanol /g glucose (Wills, 1990).

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$$

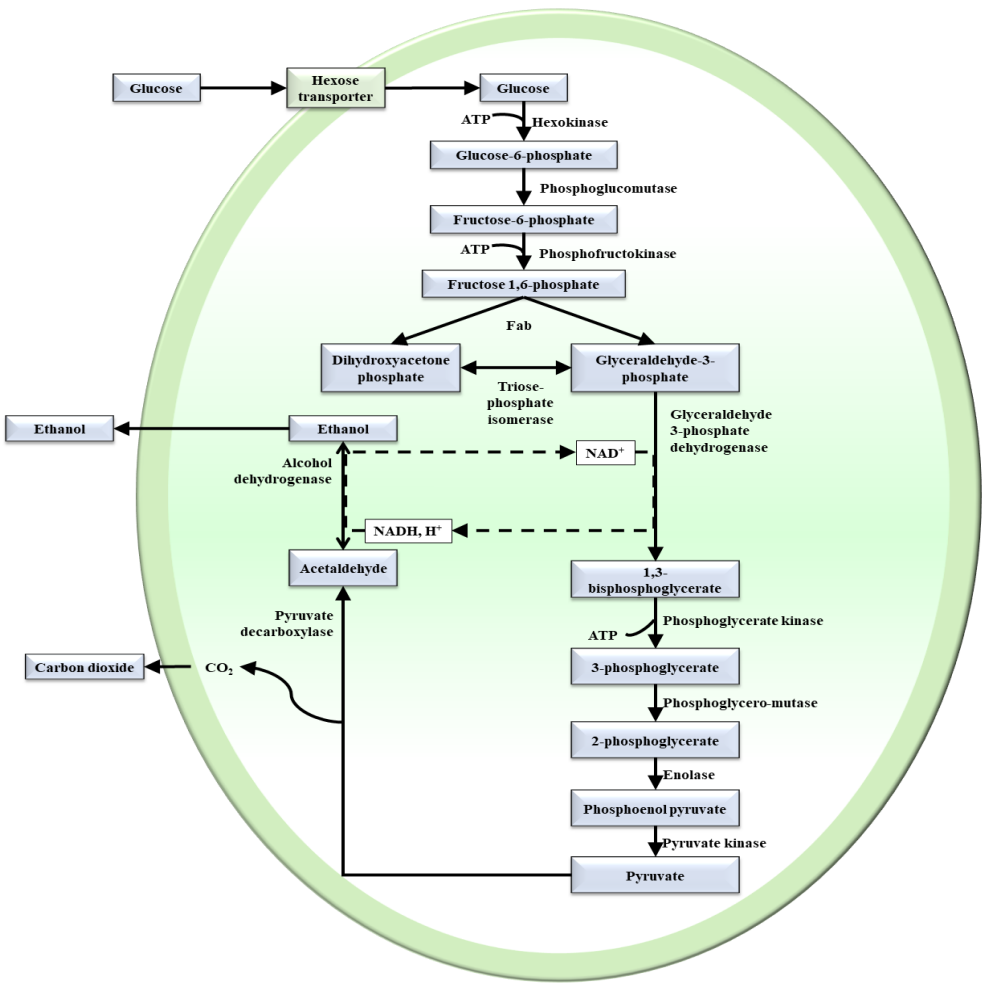

Fig.2.4: The pathway for ethanol production from glucose in *S. cerevisiae*.

2.7.2. Xylose metabolism in *P. stipitis*

Pichia stipitis is capable of producing ethanol by utilising both glucose and xylose **(Fig. 2.5)**. It has both hexose and pentose transporters to transport glucose and xylose across the cell membrane. However, in a co-fermentation process, it exhibits a diauxic growth pattern, preferentially the uptake of glucose takes place prior to that of xylose, and so is the preferential transport behaviour of the substrates (Grootjen et al., 1991). The hexose transportation was mediated by transporter proteins Sut1, Sut2 and Sut3, encoded by genes SUT1, SUT2 and SUT3 respectively. These proteins showed a heterologous transportation mechanism with minor affinity for xylose transportation. The induction of SUT1 transcription is caused by presence of glucose in the medium and independent of aerobic conditions. However, SUT2 and SUT3 expression occurs in the presence of oxygen but independent of glucose availability (Weierstall et al., 1999). For Xylose transport, 7 genes (XUT1-XUT7) transcribe for xylose transport proteins. It was reported that Xut1 and Xut3 proteins which are encoded by genes XUT1 and XUT3 respectively, demonstrated a significant difference in their affinity for xylose, with Xut1 showing high affinity and Xut3low affinity for xylose respectively. Xylose transport in *P. stipitis* is energy dependant and facilitated by Xyl/H^+ proton symports (Does & Bisson, 1989). Although, in presence of glucose and xylose, glucose non-competitively inhibits the high-affinity symporters, while direct substrate inhibition or competitive inhibition takes place with low affinity symporters (Kilian & Van Uden, 1988).

After glucose intake in *P. stipitis*, it metabolises through glycolysis to form pyruvate, then converts to acetaldehyde and yields the final product ethanol (similar to the ethanol production mechanism in *S. cerevisiae*). However, glucose-6-phosphate (a glycolytic pathway intermediate formed after the phosphorylation of glucose) may enter into the oxidative phase of Pentose Phosphate Pathway (PPP) to form the final PPP-intermediate

ribulose-5-phosphate. Here, glucose-6-phosphate is transformed into 6-phosphogluconolactone through the action of glucose-6-phosphate dehydrogenase (G6PDH) enzyme. This enzymatic reaction results in the release of a NADPH and a CO_2 molecule. Subsequent hydrolysis of 6-phosphogluconolactone is facilitated by phosphogluconolactonase enzyme, leading to the formation of 6-phosphogluconate. The final decarboxylation of 6-phosphogluconate is carried out by phosphogluconate dehydrogenase enzyme to form ribulose-5-phosphate, this process releases one NADPH and a CO_2. Ribulose-5-phosphate acts as a precursor for formation of glyceraldehyde-3-phosphate (glycolytic pathway intermediate) through a series of reversible reactions.

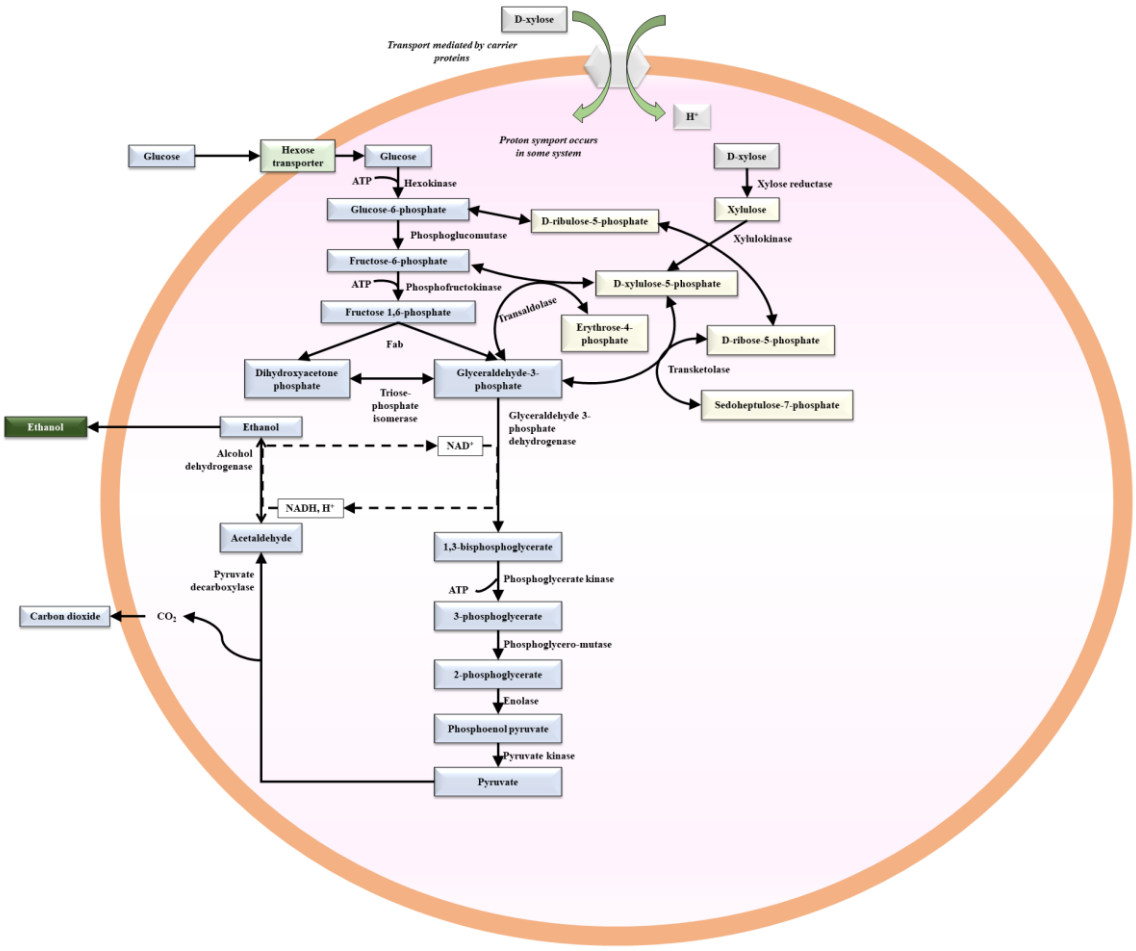

Fig.2.5: The pathway of ethanol production from both glucose and xylose by *P. stipitis*.

After xylose intake, it is catalysed by the xylose reductase (XR) to form xylulose. Then, xylulose is further phosphorylated by xylulokinase (XK) enzyme to form xylulose-5-phosphate. The further metabolism of xylulose-5-phosphate takes place in the non-oxidative phase of PPP, where two enzymes i.e., transaldolase and transketolase mediates a series of interconvertible reactions between different sugar phosphates (i.e., ribulose-5-phosphate, ribose-5-phosphate, erythrose-4-phosphate, seduheptolose-7-phosphate) to form the final glycolytic pathway intermediates i.e., fructose-6-phosphate or glyceraldehyde-3-phosphate. Through glycolytic pathway, the end product pyruvate is formed and it further converted into acetaldehyde and final product ethanol. The theoretical yield of ethanol from xylose is 0.51 g ethanol/g of xylose (Delgenès et al., 1991; Skoog & Hahn-Hägerdal, 1990).

$$3C_5H_{10}O_5 \longrightarrow 5C_2H_5OH+5CO_2$$

Chapter 3

Material and methods

3.1. Lignocellulose biomass processing

Rice straw (RS) was collected from Kamrup region of the state of Assam, India. Initially, it was washed with tap water to remove the soil debris and dried overnight in a hot air oven at 80°C. Then, it was cut, grinded, and screened through a standard 1-mm sieve prior to the pretreatment.

3.2. Fungal culture collection and maintenance

The pure culture of *Penicilium janthinellum* NCIM 1171 and *Trichoderma reesei* NCIM 1186 was obtained from the National collection of Industrial microorganisms, National Chemical Laboratory, Pune, India. The fungal culture was maintained in Yeast Potato Dextrose Agar (HiMedia Laboratories, India) at pH 5.6 and incubated at 30°C till good sporulation. The spores were then harvested using 1% Triton X and spore counting was carried out using hemocytometer under an inverted microscope. To prepare the fungal preculture, approximately $5*10^7$ spore count volume was inoculated in 250 ml Erlenmeyer flask containing 100 ml of Mandel's mineral salt medium described in **Table 3.1** (Mandels & Reese, 1957). It was supplemented with 10 g/l glucose and 1 g/l yeast extract, an initial pH was set to 5.5, temperature 30 °C and maintained at a constant agitation of 180 rpm.

Table 3.1: Mandel's mineral salt medium for fungus growth.

Media component	Composition (g/l)
$(NH_4)_2SO_4$	1.4
KH_2PO_4	2
$MgSO_4.7H_2O$	0.3
$CaCl_2.2H_2O$	0.4
$CoCl_2.6H_2O$	0.0037
$MnSO_4.H_2O$	0.0016
$ZnSO_4.7H_2O$	0.0014
$FeSO_4.7H_2O$	0.005

3.3. Yeast culture collection and maintenance

Saccharomyces cerevisiae NCIM 3594 and *Pichia stipitis* NCIM 3497 were obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India. It was maintained in YPD Agar medium having composition yeast extract (10 g/l), peptone (20 g/l), dextrose (20 g/l), agar 1.5 % at pH 6.5 and at 30 ºC for 48h. The seed culture was prepared in 10 ml YPD broth by picking a single colony from YPD agar plate and incubated at 30 ºC, 180 rpm for 24 h. The seed culture was subsequently transferred to 50 ml volume preculture media with following composition in g/l: Glucose:10, yeast extract: 5, yeast mineral salt medium described in **Table 3.2**, agitation speed of 180 rpm for 16 h. The pre-culture was further used for inoculation in the bioethanol production media.

Table 3.2: Mineral salt medium utilised for yeast growth.

Media component	Composition (g/l)
$(NH_4)_2SO_4$	5
KH_2PO_4	1
$MgSO_4$	0.5
$CaCl_2$	0.1
NaCl	0.1
$ZnSO_4$	0.0004
$FeCl_3$	0.0002
$CuSO_4.5H_2O$	0.0005

3.4. Analytical methods

3.4.1. Compositional analysis of lignocellulose biomass

3.4.1.1. Holocellulose content measurement

The holocellulose content was measured by incubating 0.5 g of rice straw sample at 70°C water bath with regular interval shaking for 4 h in a 100-ml Erlenmeyer flask containing 1.25 ml of 10% acetic acid, 0.375 g of sodium chlorite, and 25 ml distilled water (Leavitt & Danzer, 1993). The mixture of sodium chlorite and 1.25 ml of 10% acetic acid was added to the reaction mixture at every 1-h interval of time during the process of incubation. The whole reaction mixture was filtered through filtering crucibles and the residue was washed two times with distilled water and acetone, respectively, and left for overnight drying. Then, the dry weight of the residue was measured which constitutes the total holocellulose content.

3.4.1.2. Hemicelluloses and cellulose measurement

The hemicellulose content was measured from the difference of neutral detergent fibre (NDF) and acid detergent fibre (ADF) (Rinne et al., 1997). NDF and ADF were measured according to the methods of Van Soest (Soest, 1963; Van Soest et al., 1991). The NDF content was determined by taking 0.5 g of rice straw in a solution of 50 ml neutral detergent solution (NDS), 1 ml of decahydronaphthalene, and 0.25 g sodium sulfite in a reflux flask and boiled and refluxed for 1 h. The NDS was prepared by mixing 9.3 g of disodium Ethylene diamine tetra acetic acid (EDTA) and 3.4 g sodium borate decahydrate with 100 ml distilled water in a beaker and heated for complete mixing. Then, 100 ml solution containing 15 g of Na-lauryl sulphate and 5 ml of 2-ethoxy ethanol is added to it. Another 50 ml solution containing 2.25 g of disodium hydrogen phosphate was added and the volume was made up to 500 ml with distilled water. Then, the residue was filtered through a glass fibre filter, washed twice with distilled water, subsequently twice with acetone, and transferred to a crucible and dried

overnight in a hot air oven at 80°C. Then, the weight of the NDF was measured prior to cooling inside the desiccator. The ADF content was measured by taking 0.5 g of rice straw in a solution of 50 ml acid detergent solution (ADS) and 1 ml of decahydronaphthalene in a reflux flask and boiled and refluxed for 1 h. The ADS was prepared by dissolving 1 g of cetyl trimethylammonium bromide in 50 ml of 1(N) H_2SO_4. After cooling, the residue was filtered through a glass fibre filter, washed twice with distilled water, subsequently twice with acetone, and transferred to a crucible and dried overnight in a hot air oven at 80°C. Then, the ADF content was measured prior to cooling inside the desiccator. The cellulose content of rice straw was determined by subtracting the holocellulose content to that of hemicellulose content.

3.4.1.3. Lignin and ash content measurement

The lignin content was measured by taking 0.3 g of rice straw, allowed for complete hydrolysis by adding 3 ml of 72% H_2SO_4 for 2 h, and then 84 ml of distilled water was added (Rabemanolontsoa et al., 2011). The whole slurry was autoclaved at 121°C for 1 h for carrying out the second hydrolysis step and then vacuum filtered with filtering crucible. The filtered residue was oven-dried at 80°C and the acid-insoluble lignin content was measured. The acid-soluble lignin was quantified from the resulted liquid hydrolysate by taking absorbance at 205 nm. The total lignin content accounts for the comprehensive mixture of total acid insoluble and acid soluble lignin. The ash content was analysed by incinerating the hydrolyzed rice straw residue at a muffle furnace temperature of 575°C (Sluiter et al., 2008).

3.4.2.EDX for elemental analysis

The elemental analysis of rice straw was performed by energy dispersive X-ray spectrometer (Oxford instrument) coupled to FESEM (Sigma 300). The elements were determined by mapping the relative surface of rice straw and quantified in terms of w/w %.

3.4.3. Measurement of enzyme activity

The filter paper activity (FPase) and carboxymethyl cellulase activity (CMCase) were analysed following the procedures outlined by Ghose et al., as per the guidelines by Commission of Biotechnology, IUPAC (Ghose, 1987) . FPase activity was estimated by taking 100 µl of appropriately diluted crude enzyme and 10 mg of Whatman no.1 filter paper as substrate. For estimation of CMCase activity, appropriately diluted 100 µl of crude enzyme incubated with 100 µl substrate carboxymethyl cellulose (2% w/v) mixed homogeneously with citrate buffer (0.05 M, pH 4.8). The xylanase activity was carried out with appropriately diluted 100 µl of crude enzyme incubated with 100 µl substrate 1% (w/v) birchwood xylan. For FPase, CMCase and xylanase activity, the incubation temp. was 50° C with the reaction time 20 min, 10 min, 10 min respectively. The reducing sugar released was analysed by dinitrosalicylic acid assay (DNS assay) and the activity was represented in IU/ml i.e., amount of reducing sugar released in micromole per unit time. Beta-glucosidase activity was measured by pNPG assay, where 100 µl of app. Diluted crude enzyme ,100 µl p-nitrophenol-β-D-glucopyranoside (pNPG) substrate (1mg/ml) incubated for 10 min. at 50° C. The release of p-nitrophenol was measured by adding 400 µl of sodium carbonate (2%) which gave a yellowish colour at highly basic pH and the O.D. was measured at 410 nm. All enzyme dilutions were made with citrate buffer (0.05 M, pH 4.8). Beta-glucosidase activities were calculated as amount of p-nitrophenol released in micromole per unit time and represented in IU/ml.

3.4.4. Determination of biomass growth and biomass productivity The yeast cell biomass of both *S. cerevisiae* and *P. stipitis* were quantified by measuring the optical density (OD) at 600 nm in a visible spectrophotometer. The absorbance values were

converted into dry cell weight using the slope obtained from calibration curve (Fig. 3.10). For *S. cerevisiae*, one cell OD = 0.59 g dry cells/l **(Fig. 3.1 A)** and for *P. stipitis*, one cell OD = 0.45 g dry cells/l **(Fig. 3.1 B).** The calibration curve were performed with the medium containing glucose: 10 g/l, yeast extract: 5 g/l and yeast mineral salt medium **(Table 3.2)**.

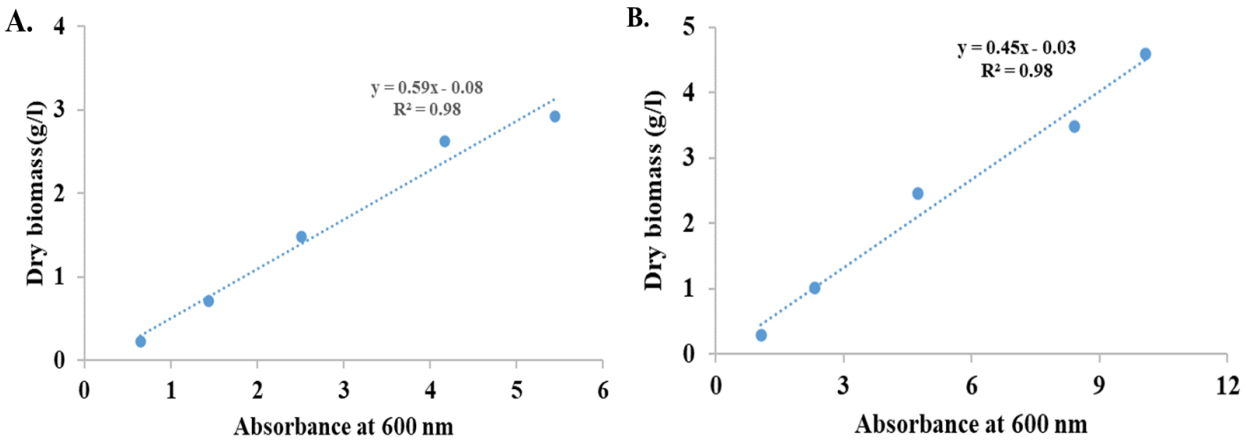

Fig. 3.1: Calibration curve for biomass estimation **A.** for *S. cerevisiae* **B.** for *P. stipites*.

$$\text{Biomass productivity, } PB = \frac{X_{fg} - X_0}{t_{fg} - t_0} \qquad \text{.....Eq.3.1}$$

Where X_{fg} is final biomass titre at the end of growth phase at time, t_{fg} and X_0 is initial biomass concentration at time, t_0.

3.4.5. Measurement of crystallinity index

The crystallinity index of the untreated, AP and BAP RS samples was performed by X-ray diffractometer (Rigaku smart lab ,9KW) using Cu Kα radiation (λ=1.54 A^0). The scanning was carried out with intensity in 2θ range from 10^0 to 80^0. The crystallinity index (CrI) of biomass samples were calculated with following formula (Segal et al., 1959).

$$CrI\ (\%) = \frac{I_{Crystalline} - I_{Amorphous}}{I_{Crystalline}} * 100 \qquad \text{.....Eq.3.2}$$

Where $I_{Crystalline}$ is intensity at $2\theta=22^0$ and $I_{Amorphous}$ is intensity at $2\theta = 18^0$.

3.4.6. Surface morphology study by FESEM

The surface morphology of untreated rice straw, treated rice straw by fresh H_2SO_4, and acidic hydrolysate was examined by field emission-scanning electron microscopy (FESEM). The samples were processed by drying, double coated by sputtering with gold particles. The FESEM was carried out with an operating beam voltage of 2 kV.

3.4.7.Analysis of Soluble sugars, ethanol, and pretreatment by-products

The concentration of sugars (e.g., glucose, xylose), ethanol and inhibitors (i.e., formic acid, acetic acid, 5-HMF, furfural) were determined through high-performance liquid chromatography (Shimadzu Corporation, Japan) using Aminex HPX- 87H column (Bio-Rad Laboratories, USA). H_2SO_4 (5 mM) was used as a mobile phase with a flowrate of 0.6 ml/min and the column oven temperature maintained at 50 °C. The sugar and ethanol concentrations were estimated through RID detector. However, the concentration of formic acid, acetic acid, HMF and furfural were estimated by UV-detector at a wavelength of 210 nm.

Chapter 4

Development of an acid pretreatment strategy for rice straw and optimisation of cellulase production by the coculture of cellulolytic fungi *Trichoderma reesei* and *Penicillium janthinellum*.

4.1. Background of the study

Rice cultivation across the Asian continent generates a significant amount of rice straw as a by-product. This surplus holds substantial promise as a feedstock for sustainable biofuel production. Typically, a pretreatment step is required to break the shielding effect of hemicellulose and lignin to have more access to cellulolytic enzymes for efficient enzymatic hydrolysis into sugars from cellulose (Bhatia et al., 2020; Mupondwa et al., 2017). The application of acid pretreatment along with hydrothermal processing effectively solubilizes the hemicellulosic fraction into soluble sugars (Scapini et al., 2021). The use of both soluble sugars of hemicellulose and solid cellulosic part can make the bioconversion process cost-effective.

During acid pretreatment, inhibitors such as formic acid, acetic acid, hydroxymethyl furfural (HMF), furfural, and phenolic compound are generated from sugar breakdown in hydrolysate. It can hamper the growth of yeast cell biomass as well as the yield of final product in fermentation (Pienkos & Zhang, 2009; van der Pol et al., 2014). There are many detoxification strategies that have been applied earlier, such as use of chemical additives, precipitation with lime, liquid–liquid extraction, liquid–solid extraction, and enzymatic treatments to counteract the inhibitors from the liquid hydrolysate (Jönsson & Martín, 2016). But the limitations of these processes are the requirement of an extra separation process step apart from the detoxification operation, and the loss of sugar components from hydrolysate has made it infeasible for techno-economic aspects at industrial scale. Detoxification through

biological route using several fungi has also been proven to be an effective process (Nichols et al., 2008). *Trichoderma spp.* have also shown the capability of acetic acid consumption and are well known for secretion of exoglucanase and endoglucanase, making them superior over other strain for simultaneous detoxification and enzyme production (Palmqvist et al., 1997).

Typically, the cellulolytic enzymes used for hydrolysis are produced using pure cellulose, and therefore, the process of bioethanol production becomes expensive. Enzyme production using lignocellulosic agricultural residues such as rice straw (Khan et al., 2007; Kogo et al., 2017), sugarcane bagasse (Rocha et al., 2013), wheat straw (Romero et al., 1999) etc. have shown great potential. Onsite cellulase production is critically important from both economic and scalability perspective for lignocellulosic bioethanol production (Siqueira et al., 2020). Numerous cellulolytic fungi such as *Trichoderma sp.*, *Aspergillus sp.*, *Neurospora sp.*, *Penicilium sp.* etc. have been extensively studied for extracellular cellulase production using various lignocellulose feedstocks (Shruthi et al., 2019; Vaishnav et al., 2018; Verma & Kumar, 2020; Xiang et al., 2021). While some fungi are hyperproducers of endoglucanase and exoglucanase enzyme e.g., *T. reesei*, some species mainly produces betaglucosidase enzyme e.g., *A. niger*. Some organisms have the ability to produce the optimum cocktail e.g., *P. janthinellum* (Adsul et al., 2004). When a coculture of more than one cellulolytic fungus were applied, superior enzyme production was reported (Lodha et al., 2020; Ming et al., 2019).

In the present work, an acid pretreatment strategy for rice straw was developed and optimised. Furthermore, cellulase production was optimised for co-culture of cellulolytic fungi *T. reesei* NCIM 1186 and *P. janthinellum* NCIM 1171 using lignocellulosic biomass i.e., rice straw as a substrate. The study focuses on optimizing the various process parameters for both individual cultures and the co-culture to maximize cellulase production in a

submerged culture setup. Additionally, the research explores the ability of these fungal strains to metabolize the inhibitors present in acid-pretreated hydrolysate, thereby facilitating the detoxification of acid-pretreated biomass slurry for potential use in biofuel production. To further enhance enzyme production, bioreactor experiments were conducted under the controlled set ups by employing the optimum conditions determined in the shake flask experiments.

4.2. Materials and methods

4.2.1. Optimization of pretreatment

Prior to the pretreatment, cellulose, hemicelluloses, lignin and ash content of untreated rice straw (RS) were analysed by the procedure described in Material and methods section **(section 3.4.1)**. The elemental analysis was carried by EDX analysis **(Section 3.4.2).** For optimization of pretreatment process, acid hydrolysis was carried out by thermal treatment of rice straw (5%, w/v) with different combinations of H_2SO_4 amount (0.1%, 0.5%, 1%, 2%, 3%, 4%) and autoclave holding time (10, 30, and 60 min) at 121°C and 15psi. The "compromising programming approach" has been used for choosing the best pretreatment method.

4.2.2. Preculture media preparation for fungi

The fungal preculture media was prepared by inoculating 1% v/v of individual spores of *T. reesei* and *P. janthinellum* separately in media containing 10 g/l glucose (HiMedia Laboratories, India), 1 g/l yeast extract (HiMedia Laboratories, India) and Mandel's mineral salt medium **(Table. 3.1).** The initial pH of the medium was set to pH 5.5, temperature at 30 °C and a constant agitation of 180 rpm was maintained throughout the culture period. Preculture (10% v/v) of 48 h age was inoculated in the production media and incubated at 30°C and 180 rpm for cellulase production.

4.2.3. Optimisation of cellulase production

The cellulase production for both the individual cultures *T. reesei* and *P. janthinellum* was optimized by one factor at a time approach and the parameters taken into consideration were feedstock loading, incubation temperature and medium pH respectively. The production media volume was kept 100 ml in a 250 ml Erlenmeyer flask. The initial rice straw loading was varied 1% w/v, 3% w/v, 5% w/v, 7.5% w/v, and 10% w/v respectively. The pretreatment was carried out with 2% H_2SO_4 with an autoclave holding time of 30 min at 121ºC. The experiments were continued for a period of 14 days at 30°C, 180 rpm with initial pH 5.5. The substrate loading with maximum activity were considered for temperature optimisation studies. Further cellulase production was carried out at four different incubation temperature i.e., 25°C, 30°C, 33°C and 37°C respectively for 14 days at 180 rpm and by keeping the initial pH 5.5. The ideal conditions for substrate loading and incubation temperature were identified. Subsequently, cellulase production was conducted in production media at a wide range of pH. In pH variation experiments, the initial pH of the cellulase production mediums were set at pH 3, pH 4, pH 5, pH 6 and pH 7 respectively. To maintain the initial pH, adjustments were made at every 24h by adding 1M NaOH or 1M HCl as needed. The cellulase productions experiments were carried out for 14 days and samples were collected at regular intervals to conduct the cellulase activity assay (FPase, CMCase, Beta- glucosidase and Xylanase), measurement of sugar and inhibitors in the hydrolysate.

4.2.4. Coculture of fungi for cellulase production

In the case of coculture involving *T. reesei* and *P. janthinellum* for cellulase production, both preculture (10% v/v) each of 48h age were inoculated into cellulase production media containing acid pretreated rice straw whole slurry (3% w/v) in 250 ml Erlenmeyer flask with 100 ml media volume. The media were supplemented with Mandel's mineral salt solution

(Table 3.1) and 1g/l yeast extract and the initial pH was set to pH 5 and further maintained at every 24 h interval. The cellulase production were carried out at 30ºC, 180 rpm and over a time period of 14 days. The samples were collected at specific time interval and cellulase activity assays were performed.

4.2.5. Cellulase production in stirred tank bioreactor

The batch cultures were carried out in a 5 l volume autoclavable stirred tank bioreactor (Model: Minifors 2, Make: Infors HT, Switzerland) equipped with 6 blade Rushton impeller, automatic controlled system for temperature, pH, agitation, antifoam etc. The production media composed of 2% H_2SO_4 pretreated rice straw whole slurry (3% w/v) with an operating volume of 1.2 L. The initial pH was maintained at pH 5 (optimised from shake flask studies) with 25M NaOH and further pH control during cellulase production was carried out with 2M NaOH or 2M HCl during the whole enzyme production process. The agitation speed of the medium was maintained at 300 rpm, air sparging was carried out with a flow rate of 1VVM and the operational temperature was maintained at 30ºC. The foaming was controlled by addition of 1% v/v diluted silicone antifoaming agent (HiMedia, India). In individual experiments aimed at cellulase production from *T. reesei* and *P. janthinellum*, the culture inoculum volume was kept at 10% v/v. However, in coculture studies, the total inoculum volume was kept at 20% v/v, with an equal ratio of 1:1 of *T. reesei* and *P. janthinellum*. The culture broth was supplemented with 1 g/l yeast extract and Mandel's mineral salt medium. The culture period for cellulase production was kept as 14 days and samples were collected at regular interval for estimation of enzyme activities, sugar, and inhibitors.

4.3. Results and discussion

4.3.1. Characterization of rice straw

Rice straw is a potential lignocellulosic waste containing a huge source of sugar and can be processed into different biochemical products. The characteristics of rice straw differ based on the variety of rice production and geographical location. The composition of rice straw obtained from the state of Assam, India, is presented in **Table 4.1.** The dry biomass compositional analysis shows that the cellulose and hemicellulose fractions are 32.8 ± 2.3% and 28.8 ± 2.8%, respectively. It has 16.2 ± 4.2% lignin, 16.8 ± 4.4%. ash, and 4.8 ± 1.8% Si.

Table 4.1: Composition analysis of untreated rice straw.

Composition	% (w/w)	Elemental analysis (% w/w)			
Cellulose	32.8 ± 2.3	C	49.9 ± 2.9	Al	0.13 ± 0.05
Hemicellulose	28.8 ± 2.8	O	43.4 ± 1.9	Mg	0.12 ± 0.08
Total lignin	16.2 ± 4.2	Si	4.8 ± 1.8	Na	0.08 ± 0.04
Acid soluble lignin	1.5 ± 0.2	F	0.5 ± 0.3	Ti	0.06 ± 0.05
Acid insoluble lignin	14.7 ± 3.4	K	0.3 ± 0.1	P	0.01 ± 0.00
Ash	16.8 ± 4.4	Cu	0.2 ± 0.1	Fe	0.01 ± 0.00
		Ca	0.2 ± 0.1		

4.3.2. Selection of pretreatment method

The viability of the lignocellulosic-based fermentative enzyme or ethanol production process depends on the amount of sugar released as well as the toxic inhibitors released in hydrolysate during pretreatment of lignocellulosic materials. Here, two objective functions were considered for choosing the acid pretreatment method where both are going to be maximized. These are (i) total sugars released during pretreatment (g/l) and (ii) total sugars

released (g/l) per unit released of inhibitors in hydrolysate (g/l) during pretreatment as expressed follows:

F1= Total sugar released (g/l) = Glucose + xylose ……(Eq. 3.1)

$$\mathbf{F2} = \frac{\text{Total sugar released (g/l)}}{\text{Total inhibitor released (g/l)}} = \frac{\text{(Glucose+Xylose) released}}{\text{(acetic acid+formic acid+furfural+HMF)}} \quad \text{.....(Eq. 3.2)}$$

The releasing of individual sugar and inhibitors during pretreatment with different percentages of H_2SO_4 (0.1-4%) and different autoclaving time (10–60 min) are shown in **Fig. 4.1 (A, B).** The optimal percentage of H_2SO_4 and autoclaving time are characterized by the

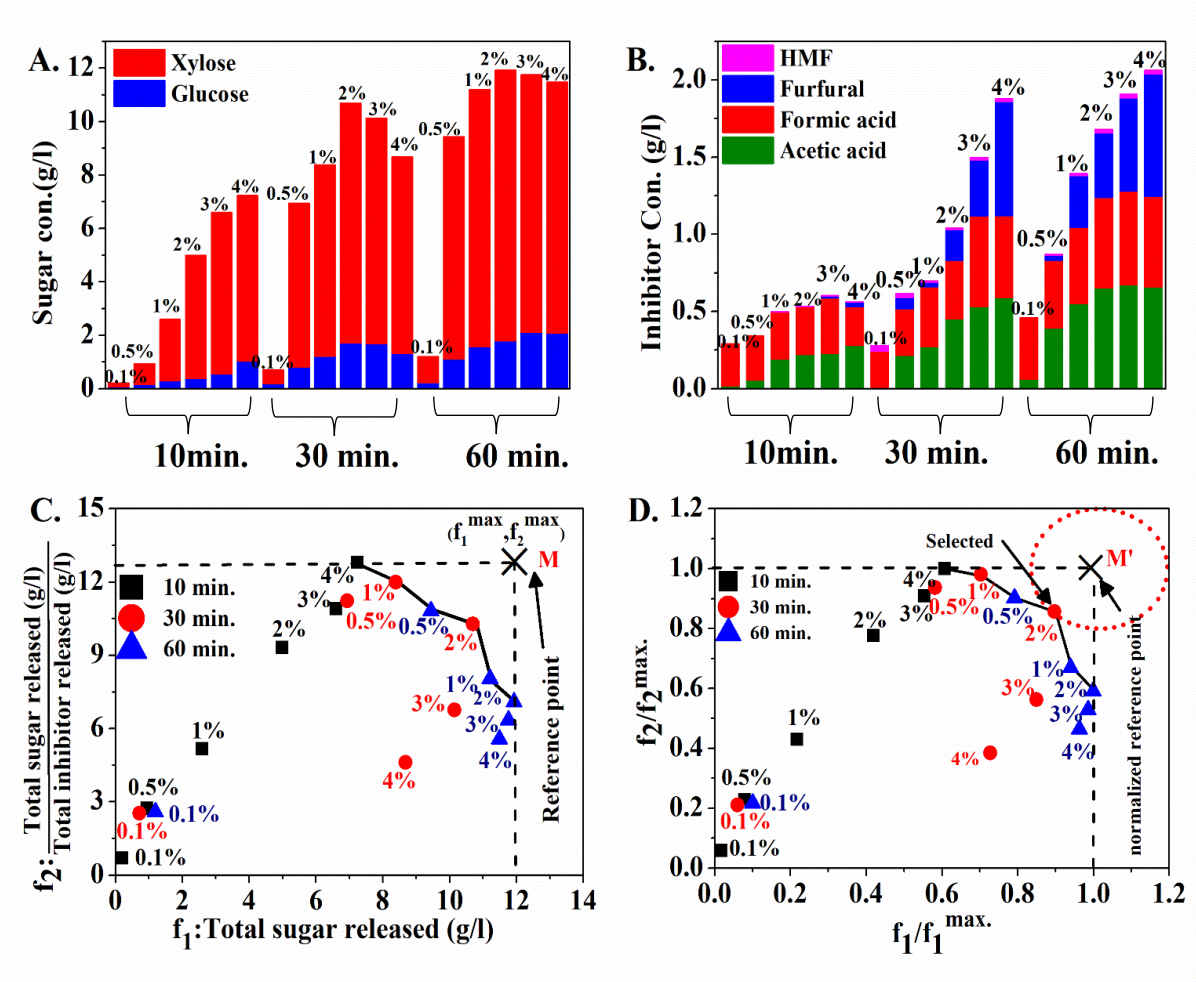

Fig.4.1: Selection of optimum pretreatment method based on sugar release and inhibitors formation in rice straw hydrolysate.

pareto front: a collection of solutions which indicates the best possible trade-offs between the competing objectives.

In the current study, a pareto front was generated using all the available sugars and inhibitors released data at different percentages of H_2SO_4 and autoclaving time **(Fig. 4.1 (C)).** Based on additional practical considerations, the operating point (% H_2SO_4 and holding time) from this pareto set can be selected. Here, we have selected one pareto optimal solution using the "Compromising Programming Approach" (also termed as the "global criteria method") (Deb & Algorithms, 2003). Using this method, the point M **(Fig. 4.1 (C))** was fixed as the reference point as it corresponds to the maximum possible values of both the objectives. In this approach, the pareto optimal solution closest to this reference point (M', using the normalized Euclidean distance) is then chosen as best. This solution (indicated by the arrow in **Fig. 4.1 (D)**) corresponds to 2% H_2SO_4 and 30 min autoclaving time. So, 2% H_2SO_4 and 30 min autoclaving time have been used for all the pretreatment methods used for feeding experiments.

4.3.3. Surface morphology

The FESEM analysis of untreated rice straw fibres show the smoother surface **(Fig. 4.2. A).** while the pretreated fibres after acid pretreatment **(Fig. 4.2. B)** shows that the fibres are more

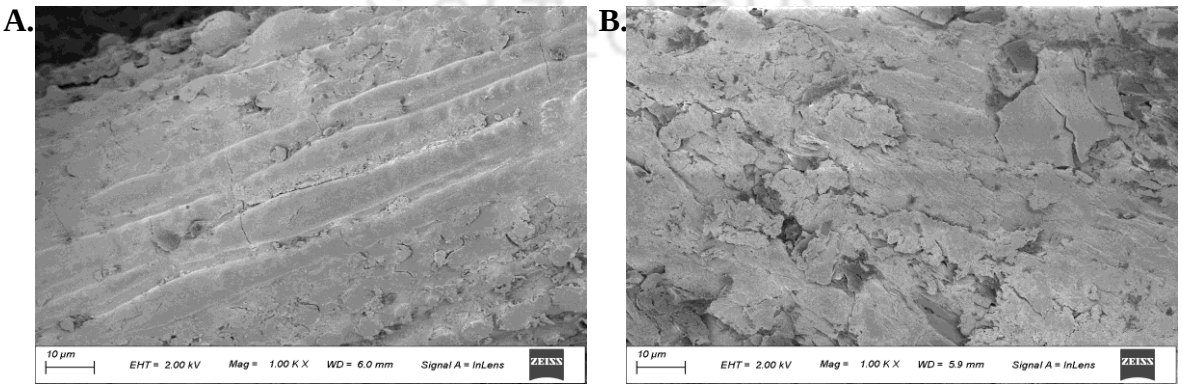

Fig.4.2. FESEM of rice straw samples **A.** Before pretreatment **B.** after acid pretreatment.

amorphous accompanied by development of cracks and formation of pores over the surface showing the decrease in crystalline nature after pretreatment.

4.3.4. Effect of feedstock loading on cellulase production

4.3.4.1. Optimisation of Substrate loading for enhanced enzyme production

The lignocellulose feedstock loading in the production media is one of the key factors that modulate the amount of cellulase induction in the fungus. In both the case of *T. reesei* and *P. janthinellum,* 3% w/v rice straw loading resulted in a maximum cellulase enzyme production **(Fig. 4.3 A, Fig. 4.4 A and Table 4.4).** In *T. reesei* culture with 3% w/v rice straw loading, the maximum FPase, CMCase, beta-glucosidase and xylanase activity obtained were 0.59 IU/ml, 0.67 IU/ml, 0.04 IU/ml, 3.56 IU/ml respectively **(Fig. 4.3 A, Table 4.4).** While in *P. janthinellum*, the maximum FPase, CMCase, Beta-glucosidase and xylanase activity were 0.65 IU/ml, 14.12 IU/ml, 3.84 IU/ml, 11.64 IU/ml respectively **(Fig. 4.4 A, Table 4.4).** Notably, *P. janthinellum* is able to produce a superior cocktail with a 1.1-fold higher FPase, 21-fold higher CMCase and 3.3-fold higher xylanase activity as compared to *T. reesei.* Note that, a negligible amount of beta-glucosidase production was observed in *T. reesei.* When 1% w/v rice straw loading was used, cellulase production was lower as compared to the 3% loading. This could be attributed to gradual carbon starvation condition in the production media, which limited the growth and cellulolytic enzymic production. The rice straw loading above 3% w/v caused gradual decline in cellulase production **(Fig. 4.3 A, Fig. 4.4 A),** with lowest activity observed in the 10% w/v substrate loading. The decline in cellulase yield in submerged cultures at higher solid loading may be due to the limitation in the oxygen mass transfer to the fungus. Additionally, higher solid loading during pretreatment not only decreases the efficiency of pretreatment, but also releases the higher amount of inhibitors.

These inhibitors at high concentrations hinders both the growth as well as cellulase production.

4.3.4.2. Concomitant cellulase production and biodetoxification

Both *T. reesei* and *P. janthinellum* demonstrated the ability to assimilate the inhibitors present in the liquid fraction of acid pretreated whole slurry as a part of their carbon source along with soluble sugars **(Fig. 4.5).** In both the fungal culture medium, at 1% (w/v) substrate loading, both *T. reesei* and *P. janthinellum* completely consumed the total inhibitor within 48h at rate of 0.49 g/l/day and 0.12 g/l/day respectively **(Fig. 4.5, Table 4.2, Table 4.3).** When the substrate loading was increased to 3% w/v, corresponding to the loading where cellulase activity was maximum, the total soluble sugar and total inhibitor were consumed at a rate of 2.17 g/l/day, 0.65 g/l/day respectively in *T. reesei* **(Table 4.2)** and 2.16 g/l/day, 0.25 g/l/day respectively in *P. janthinellum* **(Table 4.3).** At lower substrate loading up to 5% w/v, soluble sugars glucose and xylose were consumed simultaneously along with inhibitors **(Fig. 4.5).** However, at higher substrate loading, specifically at 7.5% and 10% w/v, where the hydrolysates contained elevated level of inhibitors, it impacted the consumption of soluble sugars **(Fig. 4.5).** While consumption of formic acid commenced once the concentration of acetic acid decreased in the medium.

The process of biodetoxification by fungus *Amorphotheca resinae* ZN1 was extensively studied and characterized by Zhang et. al, 2010. They have reported the detoxification of supplemented furfural, HMF, acetic acid and formic acid from pretreated food crop residues such as e.g., corn stover, wheat straw and rice straw and oil and fibre crop residues such as e.g., corn stover, cotton stalk and rape straw (Zhang et al., 2010b). Another study by Zhang et al., 2022, dilute acid pretreated corn stover hydrolysate treated with fungus strain *Paecilomyces variotii* FN89, resulted in the detoxification of acetate (8.8g/l), HMF (3.6

g/l), furfural (3.4 g/l) from the medium (Zhang et al., 2022). Simultaneous cellulase production and biodetoxification carried out by Yu et al., 2011, they employed *Aspergillus nidulans* FLZ10 in steam exploded corn stover resulted in detoxification of 53.6% acetic acid, 75.2% formic acid, complete removal of 5-HMF and furfural after 72 h cultivation. Additionally, cellulase was produced simultaneously with final FPase activity of 0.49 IU/ml (Yu et al., 2011). In our previous study, we have also reported the concomitant consumption of total inhibitor by *T. reesei* NCIM 1171 at a rate of 0.5 g/l/day from acid pretreated rice straw hydrolysate, along with cellulase production with maximum FPase and CMCase activity of 0.83 IU/ml and 0.88 IU/ml respectively (Panda & Maiti, 2019).

Table 4.2: Sugar and inhibitor consumption profile by *T. reesei* in acid pretreated rice straw whole slurry at various substrate loading.

Rice straw loading (% w/v)	Sugar consumption profile ($gL^{-1}day^{-1}$)			Inhibitor consumption profile ($gL^{-1}day^{-1}$)				
	Glucose ($gL^{-1}day^{-1}$)	Xylose ($gL^{-1}day^{-1}$)	Total sugar consumption rate ($gL^{-1}day^{-1}$)	Formic acid ($gL^{-1}day^{-1}$)	Acetic acid ($gL^{-1}day^{-1}$)	HMF ($gL^{-1}day^{-1}$)	Furfural ($gL^{-1}day^{-1}$)	Total inhibitor consumption rate ($gL^{-1}day^{-1}$)
1	0.21	0.77	0.88	0.195	0.29	0.001	0.01	0.496
3	0.67	1.83	2.17	0.35	0.29	0.003	0.007	0.65
5	0.59	1.63	1.86	0.706	0.135	0.0048	0.1	0.294
7.5	0.49	1.68	1.89	0.438	0.128	0.0028	0.053	0.621
10	0.25	1.33	1.53	0.289	0.151	0.003	0.045	0.488

Table 4.3: Sugar and inhibitor consumption profile by *P. janthinellum* in acid pretreated rice straw whole slurry at various substrate loading.

	Sugar consumption profile ($gL^{-1}day^{-1}$)			Inhibitor consumption profile ($gL^{-1}day^{-1}$)				
	Glucose ($gL^{-1}day^{-1}$)	Xylose ($gL^{-1}day^{-1}$)	Total sugar consumption rate ($gL^{-1}day^{-1}$)	Formic acid ($gL^{-1}day^{-1}$)	Acetic acid ($gL^{-1}day^{-1}$)	HMF ($gL^{-1}day^{-1}$)	Furfural ($gL^{-1}day^{-1}$)	Total inhibitor consumption rate ($gL^{-1}day^{-1}$)
1	0.04	0.6	0.626	0.095	0.003	0.015	0.01	0.118
3	0.515	1.823	2.166	0.156	0.12	0.010	0.01	0.253
5	1.215	2.182	2.79	0.276	0.25	0.015	0.014	0.2862
7.5	0.618	1.822	2.264	0.167	0.206	0.010	0.106	0.367
10	0.7083	2.078	2.61	0.222	0.218	0.011	0.131	0.462

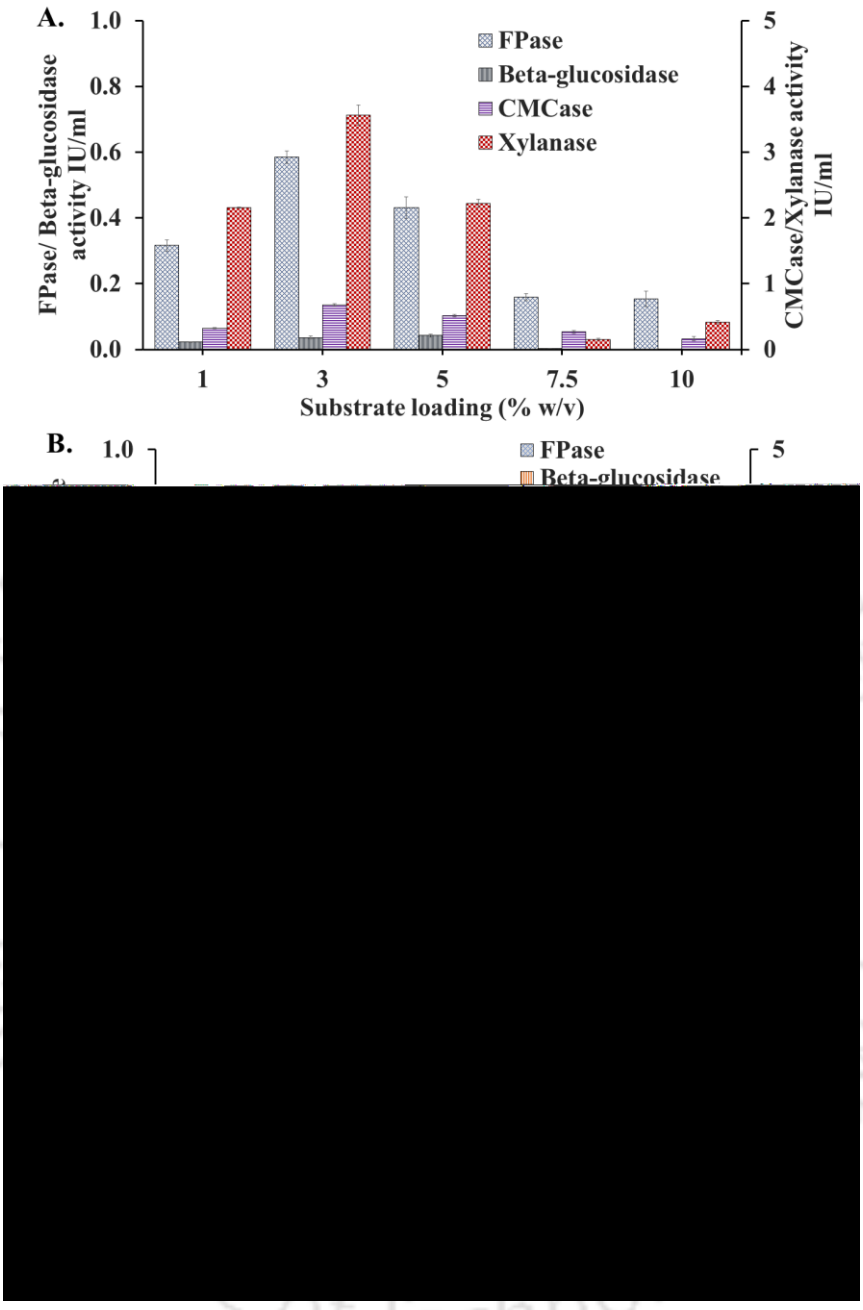

Fig.4.3: Cellulase production by *T. reesei* using rice straw. **A.** at various substrate loading, **B.** at different incubation temperature **C.** at different medium pH.

4.3.5. Impact of temperature on enzyme production

The cellulase production was maximum at 30ºC in both *T. reesei* and *P. janthinellum.* Notably, there were no significant variations in enzyme production between 25ºC and 33ºC, while it was significantly declined at incubation temperature of 37ºC. At 30ºC, *T. reesei* exhibited the highest cellulase activity with FPase: 0.67 IU/ml, CMCase 0.75 IU/ml and xylanase activity 3.62 IU/ml respectively, although beta-glucosidase production was minimal with activity 0.08 IU/ml **(Fig. 4.3 B).** Similarly, *P. janthinellum* also has optimal cellulase production at 30ºC, where FPase, CMCase, Beta-glucosidase and xylanase activity were 0.41 IU/ml, 15.37 IU/ml, 2.53 IU/ml and 24.29 IU/ml respectively **(Fig. 4.4 B).**

4.3.6. Medium pH: a key factor that drives the fungal cellulase induction The medium pH plays a crucial role in influencing the amount of cellulase production. In the present study, the optimum pH for cellulase enzyme production was obtained at pH 5 for both *T. reesei* and *P. janthinellum*. However, cellulase production was minimal at acidic condition pH 3 and pH 4, and it also decreased significantly at pH 7. At pH 5, the obtained FPase, CMCase, beta-glucosidase and xylanase activity in *T. reesei* were 0.77 IU/ml, 0.92 IU/ml, 0.1 IU/ml, 4.07 IU/ml respectively **(Fig. 4.3.C).** Notably, an increment of 1.1-fold, 1.3-fold, 2.5-fold and 1.8-fold as compared to medium with initial pH 5 followed by no intermediate pH maintenance, Similarly, in *P. janthinellum*, the maximum cellulase activities were observed at pH 5 with FPase: 0.94 IU/ml, CMCase:16.34 IU/ml, beta-glucosidase: 4.27 IU/ml and xylanase: 30.49 IU/ml respectively **(Fig.4.4.C).** This corresponds to an increment of 1.4-fold, 1.1-fold, 1.1-fold and 2.6-fold respectively as compared to cellulase production with initial pH 5 followed by no intermediate pH maintenance (data not shown). pH maintenance measures were effective in fungal cellulase production. In a study by Li et al., 2013 in *T. reesei*, an

improvement of 17.6% FPase activity and 22% increment in Beta-glucosidase activity were obtained by pH maintenance approach (Li et al., 2013).

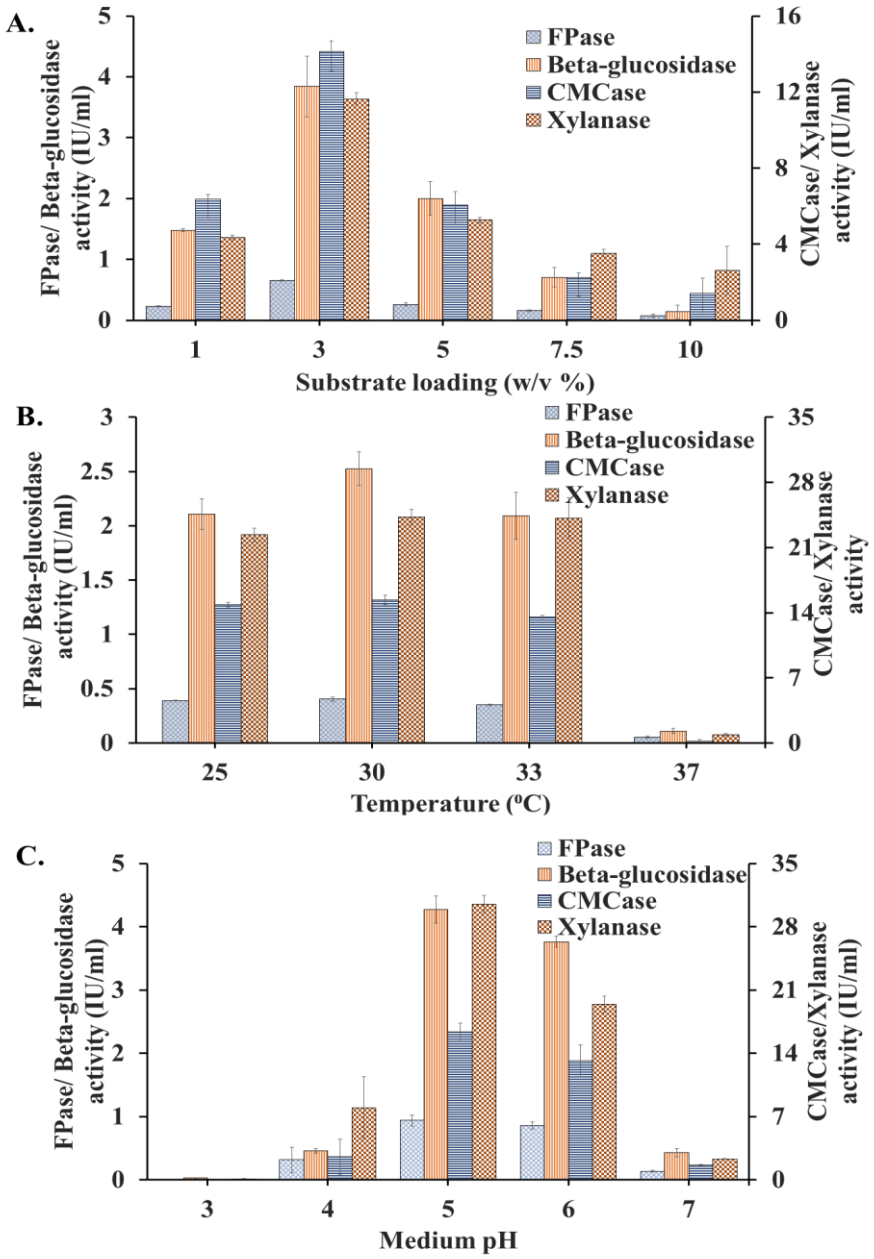

Fig.4.4: Cellulase production by *P. janthinellum* using rice straw. **A.** at various substrate loading, **B.** at different incubation temperature and **C.** at different medium pH.

Table 4.4: Comparison of cellulase production by *T. reesei*, *P. janthinellum* and coculture in shake flask experiments at 3% w/v rice straw loading, 30ºC and pH 5.

Enzyme activity (IU/ml)	*T. reesei*	*P. janthinellum*	Coculture of *T. reesei* and *P. janthinellum*	Fold increment in co-culture	
				Compared to *T. reesei*	Compared to *P. janthinellum*
FPase	0.77±0.05	0.94±0.004	1.09±0.004	1.41	1.15
CMCase	0.92±0.03	16.34±1.01	24.47±1.47	26.59	1.49
Beta-glucosidase	0.1±0.01	4.27±0.21	4.74±0.43	47.4	1.11
Xylanase	4.07±0.04	30.49±0.21	36.74±0.98	9.02	1.2

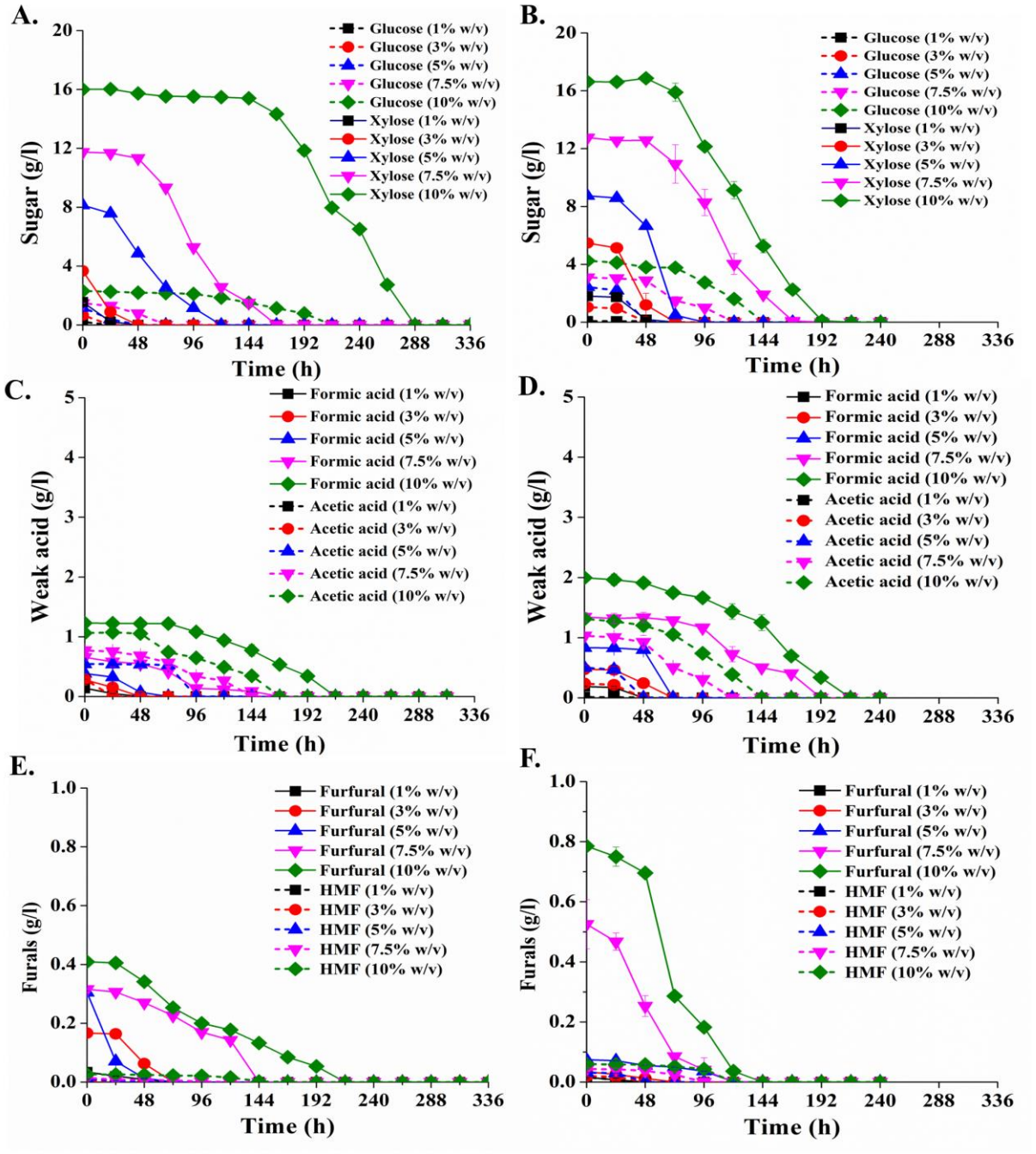

Fig.4.5: Sugar and inhibitors consumption by fungi. **A.** sugar consumption by *T. reesei*, **B.** sugar consumption by *P. janthinellum*, **C.** weak acid consumption by *T. reesei*, **D.** weak acid consumption by *P. janthinellum*, **E**. fural consumption by *T. reesei* and **F.** fural consumption by *P. janthinellum.*

4.3.7. Increment of cellulase production in fungal co-culture system

In the current study, the maximum enzyme activity obtained from co-culture of *T. reesei* and *P. janthinellum* at 3% w/v rice straw loading, 30ºC and pH 5 were as followed FPase: 1.09 IU/ml, CMCase: 24.47 IU/ml, beta-glucosidase: 4.74 IU/ml and xylanse: 36.74 IU/ml respectively **(Fig. 4.6).** In co-culture, an improvement of 15.9% FPase, 49.7% CMCase, 11% Beta-glucosidase and 20.5% Xylanase activity was achieved as compared to activity obtained at optimum condition in *P. janthinellum.* Furthermore, the co-culture system consumed the pretreatment inhibitors within 48 h, with an assimilation rate of 0.37 g/(l/day) **(Fig. 4.6 F).** Both acetic acid and HMF were consumed at faster rate within 24 h, in contrast to the individual culture where it took 48 h. The phenomenon of symbiotic co-existence among fungi is commonly found in the natural environment (Alizadeh, 2011). Such co-cultures, when established in the lignocellulose substrates, has the potential to enhance cellulase enzyme production with improved cellulolytic activities (Lodha et al., 2020; Zhao et al., 2018). A study conducted on the effect of monocultures, co-cultures, tricultures and polycultures on cellulase production, using organisms *F. oxisporum* C02-E06, *T. harzianum* PS1-G06, *T. harzianum* PS3, *A. niger* Asp546 with sugarcane bagasse (2% w/v) as substrate, stated the superiority of co-cultures over others. The co-culture of *F. oxisporum* and *A. niger* showed higher endoglucanase activity: 7.5 IU/ml and beta-glucosidase activity: 6.3 IU/ml than the monoculture of *A. niger* (endoglucanase activity: 6.3 IU/ml and beta-glucosidase activity: 5.9 IU/ml). They also established the fact that in polyculture produced fewer protein isoforms, decrease in enzyme production and also the competition for substrate in the polycultures led to lower enzyme yields (Hernández et al., 2018).

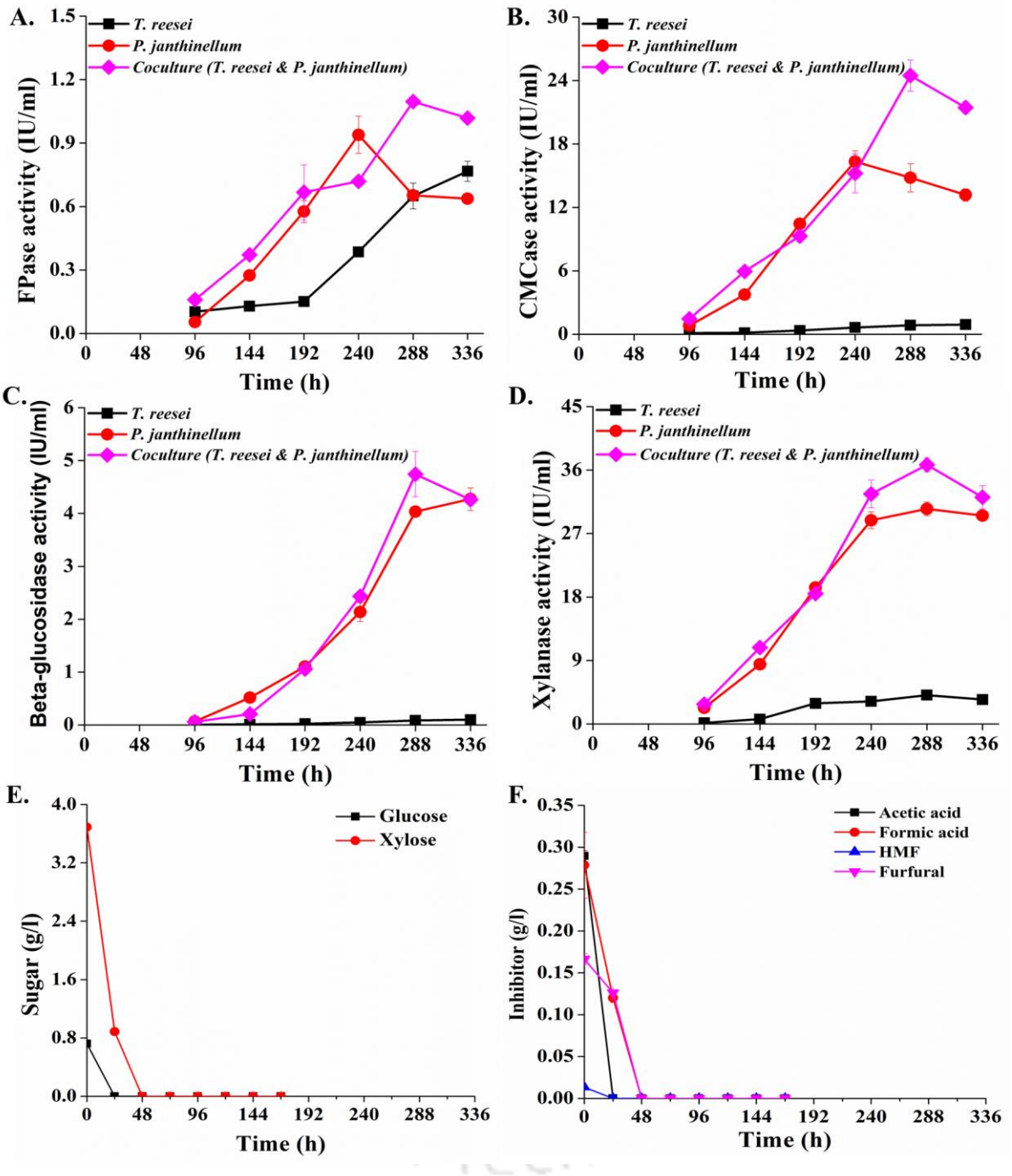

Fig.4.6: Cellulase production profile by individual cultures of *T. reesei* , *P. janthinellum* and co-culture at optimised condition **A.** FPase activity **B.** CMCase activity **C.** Beta-glucosidase activity **D.** Xylanase activity **E.**Sugar consumption profile **F.** Inhibitor consumption profile.

4.3.8. Enhanced cellulase production in bioreactor

In bioreactor study for cellulase production by *T. reesei*, the maximum enzyme activities were obtained as follows, FPase: 0.82 IU/ml, CMCase: 6.99 IU/ml, beta-glucosidase: 0.55 IU/ml and Xylanase: 12.36 IU/ml respectively **(Fig. 4.7 A).** There was a significant increment in 7.6-fold CMCase, 5.5-fold Beta-glucosidase, 3-fold xylanase activity in the bioreactor studies as compared to the shake flask experiment at optimum condition. In *P. janthinellum*, the maximum FPase: 1.16 IU/ml, CMCase: 23.05 IU/ml, beta-glucosidase: 4.63 IU/ml and xylanase: 45.6 IU/ml respectively **(Fig. 4.7 B).** Similarly, the cellulase production in the co-culture system yielded a maximum FPase: 1.16 IU/ml, CMCase: 27.68 IU/ml, Beta-glucosidase: 4.63 IU/ml and Xylanse: 45.6 IU/ml respectively **(Fig. 4.7 C).** This represents an increase of 1.1- fold FPase, 1.1-fold CMCase and 1.2- fold xylanase activity with respect to shake flask-based enzyme production using co-culture. In bioreactor, total inhibitors released from acid pretreatment of 3% w/v rice straw consumed with in 24 h by both individual fungi and in co-culture **(Fig. 4.7 F).** This biodetoxification process was faster in compared to the shake flask studies, where it typically took 48h. The faster biodetoxification in bioreactor might be attributed to aeration factor, which accelerated the assimilation of inhibitor by fungus. A study by He et al., 2016 supports this observation, where they reported that well mixing condition and aeration accelerated the biodetoxification by more than 2-fold faster using *A. resinae,* reducing the detoxification time from 96 h to 36 h in dry dilute acid pretreated corn stover hydrolysate (He et al., 2016). In the current study, the morphology of *T. reesei* strain observed to be in the free filamentous form while *P. janthinellum* exhibited pellet form. During cellulase production in all the bioreactor experiment, a two stage drop in dissolved oxygen (dO_2) was observed, with brief intermediary increase of dO_2 levels in *T. reesei, P. janthinellum,* Co-culture **(Fig.4.7 D).** The second stage of drop in dissolved oxygen is attributed by high viscous visual growth and mycelium movement slackening around

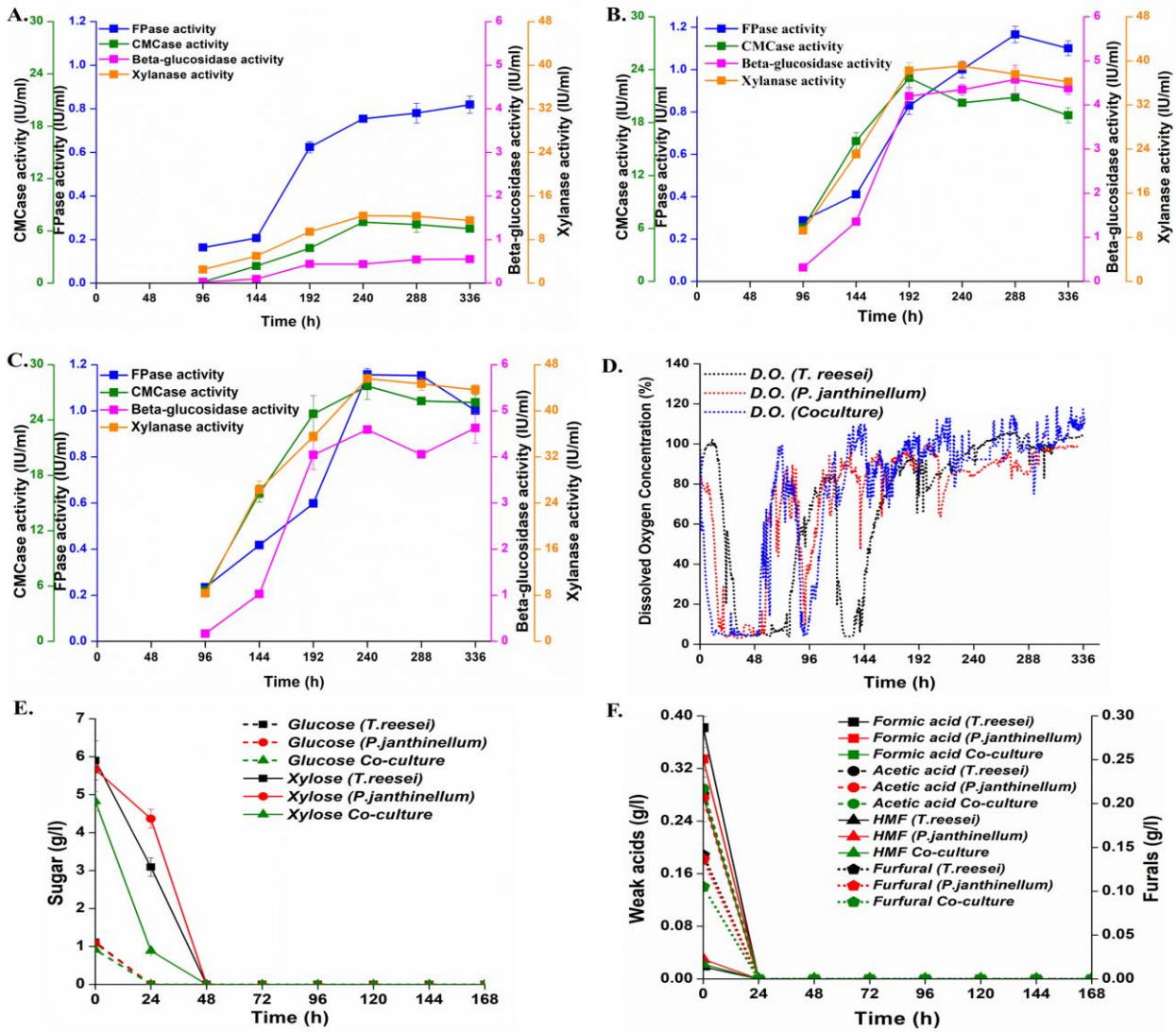

Fig.4.7: Cellulase production in bioreactor **A.** by *T.reesei* **B.** *P. janthinellum* **C.** Co-culture **D.** Dissolved Oxygen concentration **E.** Sugar consumption profile **F.** Inhibitor consumption profile.

reactors wall. This aspect of biphasic growth in fungus was explained by an initial rapid growth, primarily induce by easily metabolised components such as soluble sugars, yeast extract. This is followed by a second phase of slower growth, which is driven by gradual hydrolysis of cellulose components and further increasing in cellulase concentrations in the medium (Ahamed & Vermette, 2008; Marten et al., 1996). In a bioreactor study by da Silva Delabona et al., 2012, cellulase production using *T. harzianum* using steam pretreated sugarcane bagasse at pH 5, 29ºC produced enzyme with FPase activity: 0.73 IU/ml, beta-

glucosidase activity: 5.22 IU/ml and Xylanase activity: 29.69 IU/ml (da Silva Delabona et al., 2012). The current study of cellulase production from *T. reesei* in bioreactor has 1.1-fold higher FPase activity, while xylanase activity is 58.3% lower and insignificant amount of beta-glucosidase activity. In bioreactor studies using *P. funiculosum* with substrate loading of 20 g/L (w/v) acid pretreated sugar cane bagasse under submerged fermentation produced cellulase enzyme with activity FPase: 1.135 U/mL, CMCase: 10.252 U/mL and Beta-glucosidase: 2.260 U/mL respectively (Maeda et al., 2013). This study is in accordance with FPase activity from *P. janthinellum* in our current study but there is an increment of 2.2-fold CMCase activity and 2-fold beta-glucosidase activity.

4.4. Conclusion

In the current study, based on multi-objective optimization with pareto front analysis, autoclaving time of 30 min and 2% H_2SO_4 have been chosen as optimal levels for pretreatment of rice straw. The agricultural byproduct rice straw used as a viable alternative to commercial cellulosic substrate for cellulase enzyme production using *T. reesei* and *P. janthinellum*. The optimum conditions (3% w/v rice straw, pH 5, Temperature: 30ºC) for cellulase production determined from shake flask studies were applied in the controlled condition in bioreactor. It enhanced the enzyme production with maximum cellulase activities obtained in co-culture were as followed, FPase: 1.16 IU/ml, CMCase: 27.68 IU/ml, beta-glucosidase: 4.63 IU/ml and xylanse: 45.60 IU/ml respectively. The simultaneous cellulase production and biodetoxification by both the fungus is a notable advantage and eliminates the requirement for additional physical or chemical detoxification of hydrolysate from the medium. The current approach provides a synergy for onsite cellulase production and also provides an insight to maximise the yeast growth for bioethanol production within a medium free from potential inhibitors.

Chapter 5

Process optimisation for *in situ* cellulase production, saccharification and fermentation by co-culture of *Penicillium janthinellum* and *Saccharomyces cerevisiae* through novel approach of cyclic shifting of temperature.

5.1. Background of the study

Bioethanol production from rice straw involves pretreatment to break down the biomass matrix of hemicellulose and lignin, followed by enzymatic saccharification with cellulase to convert cellulose into fermentable glucose, and subsequent fermentation of the reducing sugar by yeast to produce ethanol (Lamichhane et al., 2021). Since enzymatic saccharification operates most efficiently at higher temperatures, typically around 45-50^{0}C, these conditions are not conducive to yeast growth and fermentation, which prefer normal temperature conditions around 30^{0}C (de Souza et al., 2012). Therefore, a separate hydrolysis and fermentation process (SHF) is used, with each step taking place at their respective optimal temperature (Sarbishei et al., 2021). Despite the use of the two-step process with different temperature for saccharification and fermentation, there is a notable challenge of glucose feedback inhibition arises. It occurs due to the accumulation of certain glucose concentrations in the medium, which hinders the rate of cellulose hydrolysis by the cellulase enzyme (Gusakov & Sinitsyn, 1992). The current approach in lignocellulose bioethanol production revolves around a technique called Simultaneous Saccharification and Fermentation (SSF). This method optimizes the temperature conditions to an intermediate level that is conducive to both saccharification and fermentation processes (Jugwanth et al., 2020). However, this approach often faces challenges such as relatively poor enzymatic hydrolysis, inadequate yeast growth or lower fermentation yield at these intermediate temperatures (Sarkar et al., 2012).

To address the limitations in SSF process at a single mutual optimum temperature, a novel approach involving phase-wise adjustments of temperature for saccharification and fermentation within a thermotolerant range of yeast was developed to maximize bioethanol yield. One way to achieve this by operating the process with higher temperature for a short period to facilitate the enzymatic hydrolysis of cellulose, subsequently, the process can be shifted to lower temperature for a specific period to promote growth and fermentation of yeast. This shifting of temperature in cyclic manner can improve the sugar release and sugar utilization throughout the bioethanol production process. This approach is novel and prior to this research work no studies have been conducted on any of lignocellulosic feedstock for bioethanol production.

The current research work focuses on development of a novel cyclic shifting of temperature strategy for SSF to improve the bioethanol production using rice straw. The optimum cyclic shifting of temperature strategy has been developed using pure glucose medium and then implemented for rice straw to improve the lignocellulosic bioethanol production. Additionally, an *in situ* cellulase production and simultaneous saccharification and fermentation (ICPSF) using optimum cyclic shifting of temperature strategy has been developed using rice straw by sequential inoculation of *P. janthinellum* NCIM 1171 and *S. cerevisiae* NCIM 3594.

5.2. Materials and methods

5.2.1. Lignocellulose pretreatment

For cellulase production, 3% w/v RS was further subjected to dil. acid pretreatment with previously optimized pretreatment condition i.e. 2% H_2SO_4 at 121^0C, 15 psi for 30 minutes. Two types of pretreated substrate were utilized for saccharification i.e., acid pretreated rice straw (AP-RS), base followed by acid pretreated rice straw (BAP-RS). The acid pretreatment

mostly solubilizes the hemicellulose fraction, while a base pretreatment is used to remove the lignin fraction from the biomass. The acid pretreatment of rice straw (10% w/v) for saccharification carried out with above mentioned acid pretreatment condition. While for base followed by acid pretreatment, initially 2% NaOH was used at 121^0C, 15 psi for 30 minutes, the black liquor was separated by filtration through muslin cloth, further the solid fibers were washed with tap water and further subjected to acid pretreatment as mentioned above. In both the cases, the AP-RS and BAP-RS fibres were thoroughly washed with tap water to neutralize the purported acidity prior to use for saccharification. The composition (described in **section 3.4.1**) and crystallinity index (described in **section 3.4.5**) of untreated RS, AP-RS and BAP-RS was analysed.

5.2.2. Cellulase enzyme production using rice straw

The *P. janthinellum* preculture (10% v/v) of 48 h age was inoculated into the cellulase production media (volume :100 ml), which contains acid pretreated rice straw (3% w/v) whole slurry with initial pH 5. It was supplemented with Mandel's mineral salt solution **(Table 3.1)** with same concentration of preculture and 1g/l yeast extract, and incubated at 30°C with180 rpm for 10 days. The samples were collected at specific interval to measure the enzyme activities. Temperature dependent cellulase activities (FPase, CMCase and beta-glucosidase) were determined by performing the cellulase assay at different incubation temperature from 30°C to 60°C. Thermostability of enzyme was analyzed by incubating the enzyme at 30°C, 40°C, 45°C, 50°C for 120 hours and the enzyme activities were measured periodically.

5.2.3. Ethanol production in pure glucose

5.2.3.1. Identification of thermotolerant range of at constant incubation temperature

Growth and bioethanol production capability of *S. cerevisiae* at different incubation temperature were studied in pure glucose medium. The precultures (5% v/v) were inoculated into 100 ml culture medium containing glucose:10 g/l, yeast extract: 5 g/l, yeast mineral salt medium **(Table 3.2)** with initial pH 6.5. The culture period was kept for 32 h and the agitation speed was kept at 180 rpm. Four set of flasks were incubated at 30 °C, 33°C, 37°C and 40°C separately. The absorbance was analyzed at every 4 h interval to measure the cell growth in UV-vis spectrophotometer at 600nm wavelength. The samples were centrifuged at 10,000 rpm for 5 minutes and stored at 4°C for measurement of glucose and ethanol.

5.2.3.2. Identification of thermotolerant range cyclic shifting of temperature

Prior to the implementation of cyclic shifting of temperature strategy (CSTS) on lignocellulosic bioethanol production, CSTS has been applied with pure glucose media to check thermotolerance and ethanol production capability of *S. cerevisiae*. Based on the optimum growth and ethanol production at different incubation temperature, the lower temperature was fixed at 30°C for cyclic shifting of temperature strategy (CSTS). To choose the optimum upper limit of temperature and holding period for CSTS, experiments with different upper temperature have been conducted with varying holding time where 30°C was kept as lower limit having different holding period. The following experiments were considered for cyclic shifting of temperature.

i. ***Cyclic shifting of temperature strategy (CSTS) from 30°C-40°C*:** Three sets of experiment were carried out where the temperature cyclically shifted with different incubation time. a) 30°C kept for 1 h and then shifted to 40 °C for 1 h which is represented as "30 °C(1h)- 40 °C(1h)" b) 30°C kept for 2 h and then shifted to 40 °C for 2 h which is represented as 30°C(2h)-40°C(2h) and c) 30°C kept for 3 h and then shifted to 40 °C for 3 h which is represented as 30°C(3h)-40°C(3h). Generally, temperature

ramp up time from 30°C to 40°C and ramp down time from 40°C to 30°C took 10 minutes each in the incubator used for experiments.

ii. ***Cyclic shifting of temperature strategy (CSTS) from 30°C-43°C*:** Two set of experiments were carried out where the upper temperature limit was raised to 43°C. The lower and upper temperature with different incubation time shifted cyclically as following a) 30°C kept for 2 h and then shifted to 43 °C for 2 h which is represented as "30 °C(2h)- 43 °C(2h)" and b) 30°C kept for 2 h and then shifted to 43 °C for 30 min which is represented as "30 °C(2h)- 43 °C (30 min)". Temperature ramp up time and ramp down time took 15 minutes each in the incubator.

iii. ***Cyclic shifting of temperature strategy (CSTS) from 30°C-46°C*:** By observing the growth and fermentation from previous study, further experiments were carried out by increasing the upper holding temperature to 46°C. Two sets of experiment were carried out with different incubation time. a) 30°C kept for 2 h and then shifted to 46 °C for 30 min which is represented as "30 °C(2h)- 46 °C (30 min)" and b) 30°C kept for 2 h and then shifted to 46 °C for 10 min which is represented as "30 °C(2h)- 46 °C (10 min)". Temperature ramp up and ramp down time took 20 minutes each.

iv. ***Cyclic shifting of temperature strategy (CSTS) from 30°C-50°C*:** Only one set of experiment was carried out with cyclic shifting of temperature. 30°C kept for 2 h and then shifted to 50 °C for 10 min which is represented as "30 °C(2h)- 50 °C (10 min)". Temperature ramp up and ramp down time took 23 minutes each in the incubator.

All experiment with different cyclic shifting of temperature strategy (CSTS) were carried out for 32 h and the sampling was done at 4 h interval. The optical density of yeast samples was measured at 600 nm. The supernatant was collected by centrifugation at 10,000 rpm for 5 minutes and stored at 4°C for estimation of glucose and ethanol by HPLC.

5.2.4. *In situ* cellulase production, saccharification and fermentation (ICPSF) using rice straw

To study ICPSF, two kinds of substrate AP-RS or BAP-RS fibers are fed separately into the 10 days old *P. janthinellum* culture broth containing cellulase enzyme and then three types of studies were conducted i.e., at different incubation temperatures, prolong prehydrolysis and fermentation, and with cyclic shifting of temperature (CSTS) for bioethanol production **(Fig. 5.1).**

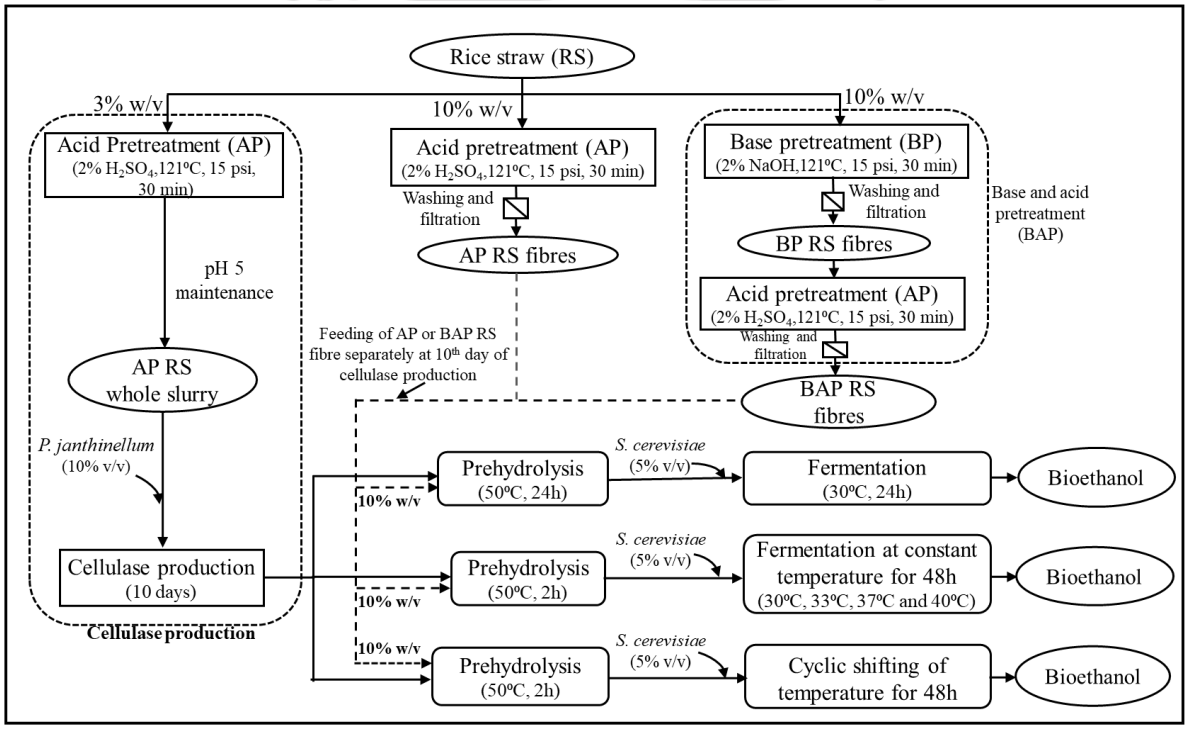

Fig. 5.1: Flowchart for *in situ* cellulase production, saccharification and fermentation (ICPSF) via co-culture of *P. janthinellum* and *S. cerevisiae* using rice straw as a feedstock.

5.2.4.1. Saccharification and fermentation at different incubation temperature using rice straw

To evaluate a mutual optimum temperature that favors saccharification and fermentation, both AP-RS (10% w/v) and BAP-RS (10%w/v) were fed separately into the 10 days old *P.*

janthinellum broth. 10 days has been chosen based on enzyme production by *P. janthinellum*. A short prehydrolysis step at 50^0C for 2h was given, further the temperature brought down to 30^0C and *S. cerevisiae* (5% v/v) inoculated along with yeast MSM, yeast extract (5g/l) supplementation. The medium pH was set to pH 6 and four different sets of experiment were carried out at four different incubation temperature 30 °C, 33°C, 37°C and 40°C respectively for 48h. The samples were collected at 6h interval, centrifuged at 10000 rpm for 5 minutes and supernatants were collected for HPLC analysis.

5.2.4.2. Prolong prehydrolysis and fermentation using rice straw

This study includes a prolong enzymatic prehydrolysis step at elevated temperature followed by fermentation at normal temperature. After cellulase production by *P. janthinellum,* pretreated rice straw was added into crude cellulase media. Two separate experiments were carried out by adding two different pretreated rice straw: AP-RS (10% w/v) and BAP-RS (10% w/v) separately. The temperature for enzymatic prehydrolysis was kept at 50°C for 24 h, further the temperature was brought down to 30 °C for inoculation of *S. cerevisiae* (5% v/v). The initial pH and media supplementation were same as previous section. The fermentation phase was continued for next 24 h at 30 °C and the samples were collected at every 4 h interval, centrifuged at 10000 rpm for 5 minutes. The supernatants were collected for measurement of glucose and ethanol.

5.2.4.3. SSF of rice straw using cyclic shifting of temperature strategy (CSTS)

AP-RS (10% w/v) and BAP-RS fibers (10% w/v) were added separately into the two different flasks containing *P. janthinellum* cellulase broth. Then, a short prehydrolysis was carried out at 50°C for 2 h duration and further the temperature was brought down to 30°C, and *S. cerevisiae* (5% v/v) was inoculated along with previously described Yeast MSM and 5g/l yeast extract. The starting incubation temperature was kept at 30°C for 4 h prior to the

applying of cyclic shifting of temperature strategy (CSTS). Based on thermotolerance of *S. cerevisiae* using CSTS with pure glucose medium following cyclic shifting of temperature strategy; 30°C(2h)-46°C(10min), 30°C(2h)-43°C(30min), 30°C(1h)-40°C(1h), 30°C(2h)-40°C(2h), 30°C(3h)-40°C(3h) were applied for SSF with rice straw. The sampling was done at every 6 h interval, centrifuged at 10000 rpm for 5 minutes and HPLC analysis were further carried out for measurement of glucose and ethanol concentration.

5.2.4.4 Tuning of cyclic shifting of temperature strategy for improvement of productivity:

The highest bioethanol titer achieved under the aforementioned cyclic shifting of temperature conditions was further tuned to increase the ethanol titer. The holding time were tuned to balance the rate of glucose release at upper holding time with the rate of glucose consumed in the lower holding time within a cycle, constituting cyclic shifting of temperature process.

5.3. Results and Discussion

5.3.1. Pretreatment of rice straw for enzyme and bioethanol production

The initial sugars and pretreatment byproducts present in the hydrolysate after acid pretreatment of rice straw (3% w/v) for cellulase enzyme production were as followed, glucose: 1.03±0.04 g/l, xylose: 5.47±0.03 g/l, acetic acid: 0.24±0.006 g/l, formic acid:0.47±0.06 g/l, furfural:0.03±0.001 g/l, HMF 0.02±0.001 g/l respectively. The rice straw (10% w/v) used for bioethanol production, there was 56 % recovery in AP-RS fibers and 30% recovery in BAP-RS fibers of initial rice straw used for pretreatment. The composition (w/w %) as follows for untreated RS (cellulose: 35.8±2.88, hemicelluloses: 36.38±4.02, lignin: 17.01±1.32), AP-RS (cellulose: 51.49±3.34, hemicelluloses: 20.66±1.88, lignin: 20.04±2.06), BAP-RS (cellulose: 78.18±2.16, hemicelluloses: 13.46±0.83, lignin: 0.59±0.04) respectively. The crystallinity index (%) measured by X-ray diffractometer of untreated RS, AP-RS fibers,

BAP-RS fibers were 53.2%, 58.3%, and 68%, respectively. The higher crystallinity index in base and acid pretreatment shows that the BAP-RS fibers have greater exposure of cellulose fibrils for enzymatic hydrolysis.

5.3.2. *In-situ* cellulase production by *P. janthinellum* using acid pretreated rice straw

In current study, activity of enzymes produced by *P. janthinellum* in acid pretreated rice straw were measured at specific time interval and production of enzyme was maximum after 10^{th} days. The FPase, CMCase and beta-glucosidase activity of crude filtrate after 10 days were 0.77±0.002 IU/ml, 20.73±0.45 IU/ml and 3.16±0.01 respectively **(Fig. 5.2 A).** Optimum cellulolytic activity is highly temperature dependent and the evaluation of enzymatic hydrolysis at various temperature ranging from 30^0C-60^0C showed that the activity is optimum at 50^0C **(Fig. 5.2 B).** The relative activity at 50^0C is maximum and 117% higher FPase, 88% higher CMCase and 4950% higher beta-glucosidase respectively as compared to enzyme activity at 30^0C. While there is insignificant difference in FPase and CMCase activity between 45^0C to 60^0C, there is a decrement of 33% beta glucosidase activity at 60^0C as compared to the activity at 50^0C and negligible activity at 30^0C **(Fig. 5.2.B).** The reduction of CMCase and beta-glucosidase activity at 60^0C may be attributed to gradual thermal deactivation of the cellulase at this temperature range.

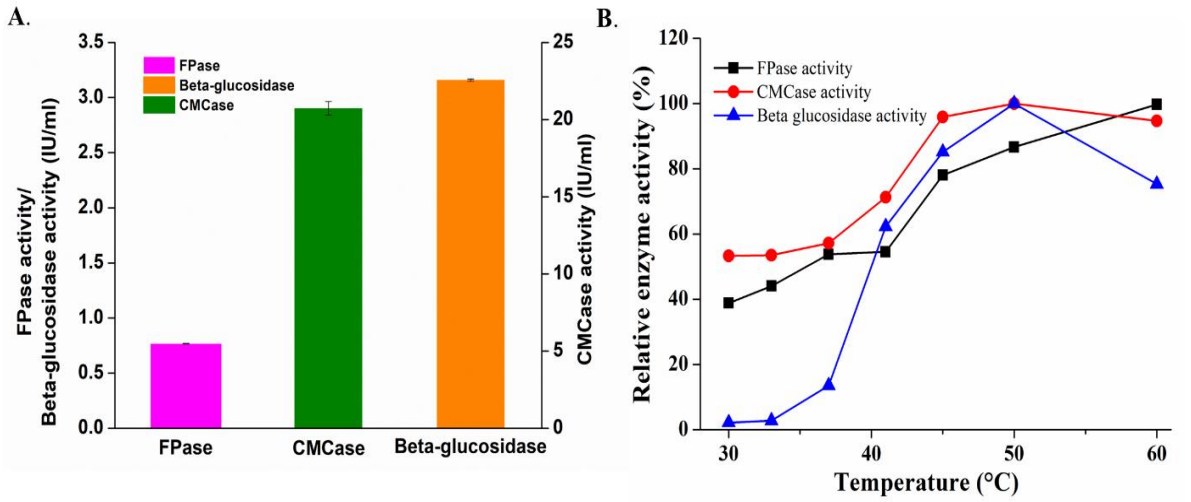

Fig. 5.2: Cellulase production from *P. janthinellum* **A.** Enzyme activity (FPase, CMCase, beta glucosidase, xylanase) on 10th day of cellulase production **B.** Relative enzyme activity profile at different temperature ranging from 30ºC to 60ºC.

The thermostability study of enzyme showed that the both the FPase and beta-glucosidase enzyme retained complete activity at 50^{0}C till 96 h, further reduction of 35% and 26% residual activity observed at 120 h of incubation, while there is no loss of activity observed at 30^{0}C **(Fig. 5.3).**

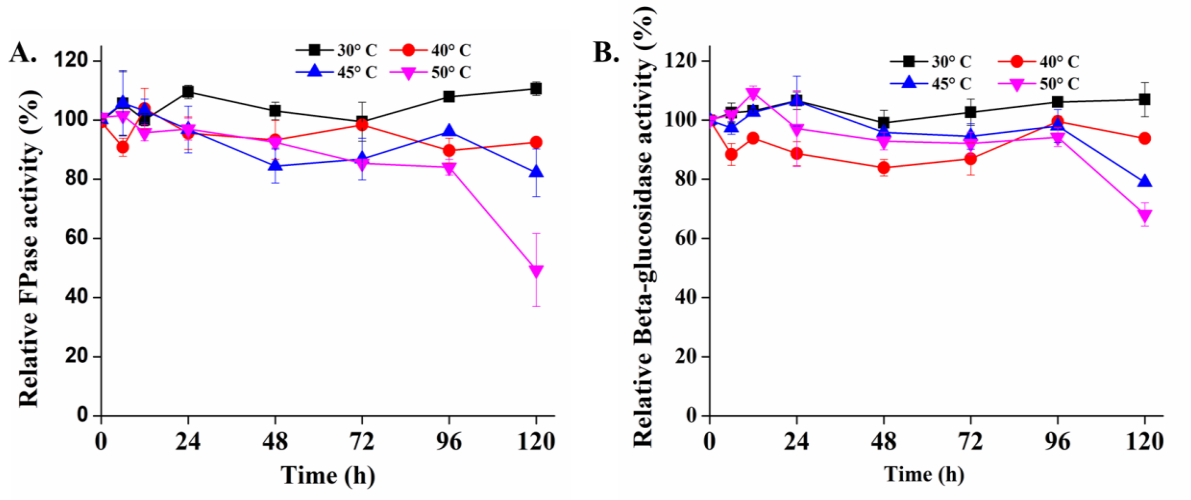

Fig. 5.3: Thermostability analysis of cellulase enzyme at different temperature (30ºC, 40ºC, 45ºC, 50ºC).

P. janthinellum was chosen for the current research as it can produce endoglucanase, exoglucanase and high level of β-glucosidase enzyme. Traditional strains such as *Trichoderma spp*. generally produce mainly endoglucanase and exoglucanase (Christopher et al., 2023; Singhania et al., 2021). A study conducted on cellulase production by *P. janthinellum* NCIM 1366 using cellulose powder (1% w/v), Mendel's mineral salt medium has reported the filter paper activity: 0.83 FPU/ml, CMCase activity: 21.72 IU/ml on the 10^{th} day and 12^{th} day respectively (Sreeja-Raju et al., 2020). The cellulase enzyme produced in the above study is as par with the current study.

5.3.3. Yeast thermotolerance and viability in pure glucose medium

5.3.3.1. Viability at different incubation temperature with pure glucose

To study the yeast viability, *S. cerevisiae* was grown in pure glucose with initial concentration of 10 g/l at various incubation temperatures from 30 ^{0}C to 40 ^{0}C **(Fig. 5.4A).** The highest ethanol concentration (E_{max}) of 4.11 g/l with glucose to ethanol yield ($Y_{E/G}$) of 0.43 was achieved at 30 ^{0}C, while 3.44 g/l ($Y_{E/G}$ =0.38) and 3.34 g/L ($Y_{E/G}$ =0.35) were obtained at 33^{0}C and 37^{0}C respectively (Table 5.1). The ethanol production was very low at 40^{0}C, with maximum ethanol concentration of 0.55 g/l at 12 h and thereafter glucose (8 g/l) remained unutilized in the medium and biomass growth also completely ceased. The maximum thermotolerance of yeast was observed up to 37^{0}C, while prolong incubation at 40^{0}C proved to be lethal for cell growth. The fermentation rate of glucose was optimum at 30^{0}C, with glucose uptake rate of 1.2 g/l/h with ethanol productivity of 0.49 g/l/h. The ethanol productivity deaccelerated to 1.8-fold and 4.9-fold at incubation temperature of 33^{0}C and 37^{0}C respectively. Noted that after complete depletion of glucose, the growth of *S. cerevisiae* persisted, as the yeast utilized the product ethanol as an alternative carbon source. The consumption of product ethanol took place at a rate of 0.109 g/(l h), 0.107 g/(l h) and

0.003 g/(l h) at 30^0C, 33^0C and 37^0C incubation temperature respectively after glucose exhaustion **(Fig. 5.4 A).** The process of ethanol consumption in the absence of glucose in the medium due to the enzyme alcohol dehydrogenase which catalyzes the reversible conversion of acetaldehyde to ethanol (Azhar et al., 2017). The overall biomass growth at 30^0C, 33^0C and 37^0C were 2.51 g/l, 2.38 g/l and 1.06 g/l respectively **(Fig. 5.4 A).** Biomass productivity from the primary substrate glucose were 0.07 g/(l h) for both the temperature 30^0C and 33^0C, it decreased to 2.3-fold at 37^0C **(Fig. 5.4 A).**

5.3.3.2. Thermotolerance at cyclic shifting of temperature with pure glucose

The maximum thermotolerance shown by *S. cerevisiae* was 37^0C with prolong incubation, whereas growth was restricted at incubation temperature 40^0C. Further, cycling shifting of temperature was applied from optimal fermentation condition i.e., 30^0C to different elevated temperatures ranging from 40^0C to 50^0C to check the thermotolerance and cell viability. Yeast can manifest thermotolerance property for a short duration because of their growth and adaptability to diurnal temperature condition in their natural habitats, which is exceptionally high at day time and minimum at night time (Kalyuzhin, 2011).The underlying biochemical operation behind heat resistance in the yeast is mainly due to the production of specific heat shock protein and regulation of chemiosmotic channel present over the plasma membrane (Huang et al., 2018).

Initially the first set of experiments carried out by cyclically shifting the temperature from 30^0C to the near growth inhibition temperature 40^0C **(Fig. 5.4 B).** The yeast was able to sustain this cyclic shifting of temperature and the maximum ethanol concentration (E_{max}) obtained were 3.98 g/l, 3.6 g/l and 3.93 g/l with following cyclic shifting of temperature conditions i.e., 30°C(1h)-40°C (1h), 30°C(2h)-40°C(2h) and 30°C(3h)-40°C(3h) respectively. A higher rate of glucose consumption of 0.93 g/l/h was observed within 8 h of incubation

using 30°C(2h)-40°C(2h) cycle compared to other two shifting cycles with ethanol yield ($Y_{E/G}$) of 0.39 and biomass productivity of 0.12 g/(l h) **(Fig. 5.4 B and Table 5.1).** Due to the observed heat resistance of *S. cerevisiae* resulting from cyclic shifting of temperature between 30°C and 40°C, the upper limit for elevated temperature conditions was extended to 43°C. Additionally, the exposure time was adjusted to assess the yeast's survivability under these conditions **(Fig. 5.4 C).** The first set of cyclic shifting of temperature experiment conducted was 30°C(2h)-43°C(2h), but no significant biomass growth was observed till 16 h of incubation period. Two-hour incubation at 43 ^{0}C might be growth limiting. Hence, the upper holding time for the shifting cycle was decreased to 30 minutes i.e., 30°C(2h)-43°C(30min). Here, the glucose was consumed at the rate of 0.75 g/(l h) and the maximum ethanol produced was 3.95 g/l with a yield of 0.39 g/g **(Fig. 5.4C).** A maximum biomass of 0.91 g/l was achieved at 16 h and a stationary phase was observed further with ethanol consumption at the rate of 0.08 g/(l h) **(Fig. 5.4C).** In order to assess the further thermotolerance of *S. cerevisiae*, the upper temperature limit was increased to 46°C and additional adjustments to the holding time were made to evaluate cell viability under these conditions **(Fig. 5.4D).** The maximum ethanol obtained in 30°C(2h)-46°C(30min) cycle was 3.02 g/l, 23% lower as compared to ethanol concentration obtained in 30°C(2h)-43°C(30min) cycle. The biomass growth was ceased after 16h and henceforth 2.85 g/l residual glucose remained unutilized in the medium. So, in next experiment the upper temperature holding period was decreased from 30 minutes to 10 minutes, the E_{max} obtained were 4.27 g/l with a yield ($Y_{E/G}$) and productivity of 0.43, 0.25 g/(l h) respectively in 30°C(2h)-46°C(10 min) cycle **(Fig. 5.4 D).** Higher thermotolerance was observed with 10 minutes at 46^{0}C, a total biomass yield of 1.95 g/l was achieved, 2.1-fold higher as compared to the total biomass obtained in 30°C(2h)-46°C(30min) cycle **(Fig. 5.4 D and Table 5.1).** When the upper temperature range was elevated to 50°C, no cell growth was observed in the temperature

shifting of 30°C(2h)-50°C(10 min) cycle **(Fig. 5.4 D).** The current study explored the effectiveness of controlled, intermittent exposure to high temperatures as a means to foster thermotolerance in the organism.

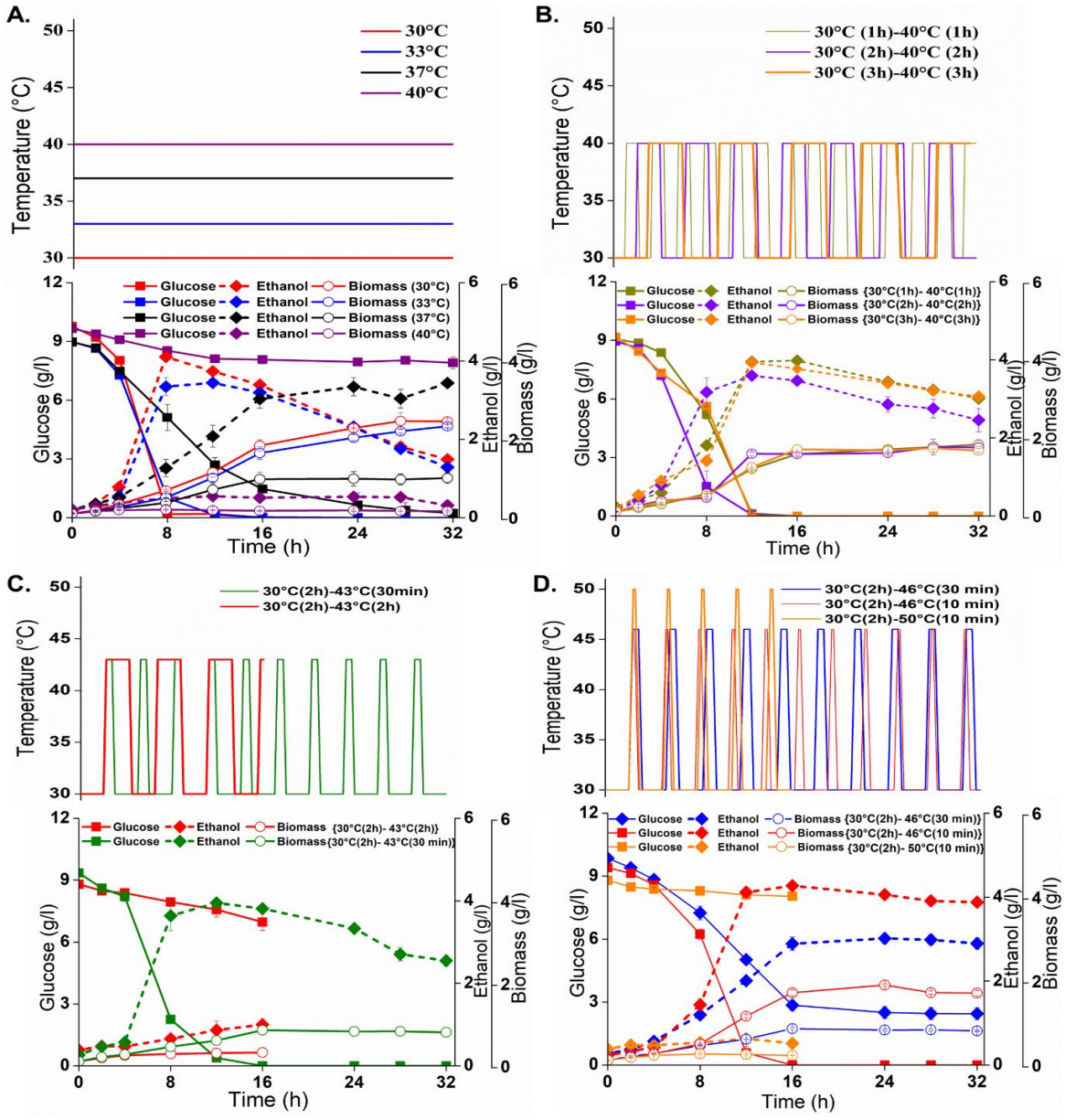

Fig. 5.4: Growth, glucose consumption and ethanol production profile of *S. cerevisiae* in pure glucose medium. **A.** At different incubation temperature. **B.** Cyclic shifting of temperature from 30 °C to 40 °C. **C.** Cyclic shifting of temperature from 30 °C to 43 °C. **D.** Cyclic shifting of temperature from 30 °C to 46 °C and 30 °C to 50 °C.

Table 5.1: Ethanol production profile of *S. cerevisiae* at constant incubation temperatures and cyclic shifting of temperature in pure glucose. medium.

	Control				Shifting from 30°C to 40°C			Shifting from 30°C to 43°C		Shifting from 30°C to 46°C		Shifting from 30°C to 50°C
	30°C	33°C	37°C	40°C	30°C(1h)-40°C(1h)	30°C (2h)-40°C(2h)	30°C (3h)-40°C(3h)	30°C(2h)-43°C(2h)	30°C (2h)-43°C (30min)	30°C(2h)-46°C (30min)	30°C(2h)-46°C(10min)	30°C(2h)-50°C(10min)
$E_{max.}$ (g/l)	4.11±0.05	3.44±0.1	3.34±0.2	0.5±0.1	3.98±0.04	3.60±0.01	3.93±0.01	0.9±0.1	3.95±0.1	3.02±0.12	4.27±0.02	0.63±0.01
$Y_{E/G}$ (g/g)*	0.43	0.38	0.35	0.35	0.43	0.39	0.43	0.34	0.39	0.36	0.43	0.3
Glucose Consumption rate (g/l/h)	1.2	0.73	0.27	0.05	0.57	0.74	0.76	0.11	0.75	0.31	0.59	0.04
Ethanol consumption rate (g/l/h)	0.109	0.107	0.003	-	0.080	0.057	0.043	-	0.07	0.016	0.024	-
Ethanol Productivity (g/l/h)**	0.49	0.27	0.10	0.04	0.23	0.29	0.31	0.06	0.31	0.11	0.25	0.04
Biomass productivity from Glucose (g/l/h)	0.07	0.07	0.03	0.007	0.09	0.12	0.09	0.02	0.08	0.05	0.10	0.01
Overall biomass (g/l)	2.51	2.38	1.05	0.25	1.88	1.81	1.78	0.37	0.91	0.91	1.95	0.27

Note: Experimental data is presented as mean ± standard deviation of duplicate experiment.

*$Y_{E/G}$ (g/g) calculated based on time of maximum ethanol production.

**Ethanol Productivity (g/(l h)) calculated based on time of maximum ethanol production.

5.3.4. Bioethanol production from rice straw

5.3.4.1. SSF at different incubation temperature with rice straw

To identify the mutual optimum temperature for saccharification and fermentation, experiments at different temperature have been conducted. The impact of incubation temperature within the yeast thermotolerance range of 30°C to 40°C was studied for bioethanol production using pretreated RS as the cellulosic feedstock **(Fig. 5.5).** Ethanol production at 30°C and 33°C exhibited a notable decrease for both AP-RS and BAP-RS, with the maximum concentration of 0.58 g/l using BAP-RS at 33°C **(Fig. 5.5 A, Fig. 5.5 B, Table 5.2).** Incubation at 37°C, led to a relative increase in ethanol concentration reaching up to 0.94 g/l in AP-RS and 3.27 g/l in BAP-RS **(Fig. 5.5 and Table 5.2).** The 3.5-fold increment observed in BAP-RS can be attributed to the enhanced enzymatic saccharification efficiency for BAP-RS fibers compared to that of AP-RS fibers. Elevating the temperature to 40 ^{0}C resulted in an increment of glucose level due to higher saccharification rate, leading to a maximum glucose concentration of 5.5 g/l and 5.9 g/l in both AP- RS and BAP-RS respectively **(Fig. 5.5 and Table 5.2).** At 40 ^{0}C maximum ethanol concentration of 1.02 g/l and 1.58 g/l were achieved within 12 h with AP-RS and BAP-RS respectively **(Fig.5.5).** However, after this point, no further production was observed due to lack of thermotolerance of yeast, similar to observation at 40°C in pure glucose medium **(Fig. 5.4 A and Fig. 5.5).** In a similar study, it was observed that the maximum bioethanol yield and productivities occurred at 35°C with *S. cerevisiae* CAT-1 using Carnauba straw. After 12 h, no further production of ethanol was occurred in that study also (da Silva et al., 2018). As observed in the current study, the lower bioethanol productivities at constant incubation temperatures due to reduced enzymatic saccharification at lower temperatures and the heat stress to the yeast at elevated temperatures.

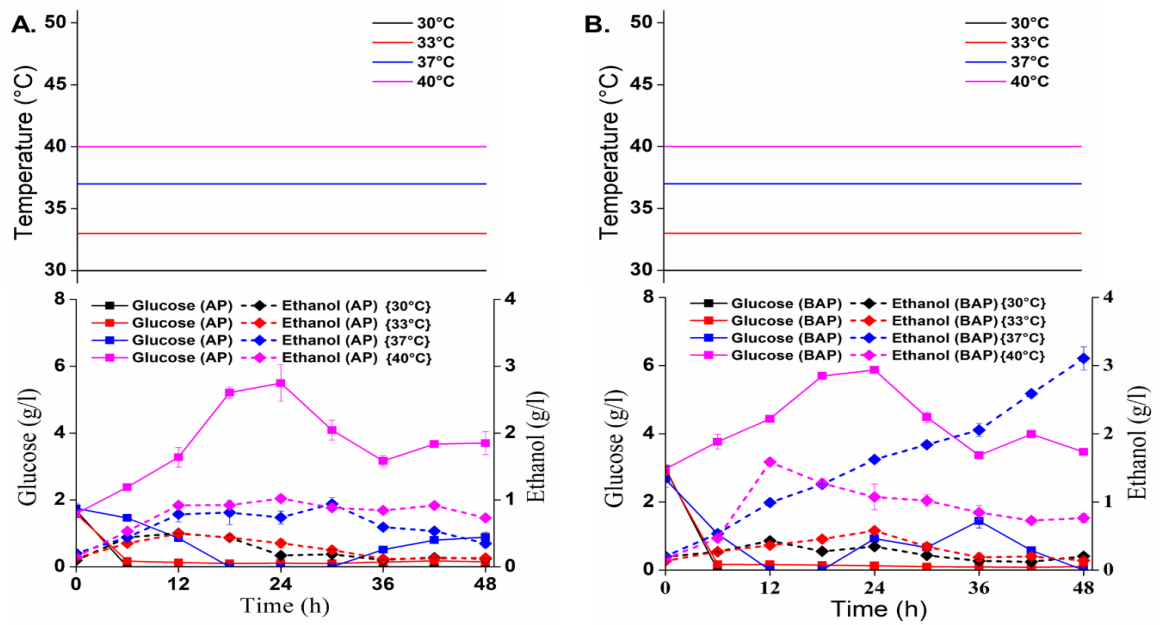

Fig. 5.5: Bioethanol production by sequential coculture of *P. janthinellum* and *S. cerevisiae.* Glucose and bioethanol profile *after* feeding of pretreated rice straw (10% w/v) on 10th day, simultaneous inoculation of *S. cerevisiae* and culture at constant temperature (30°C, 33°C, 37°C, and 40°C). **A.** Feeding of Acid pretreated (AP) rice straw (10% w/v) **B.** Feeding of Base and acid pretreated (BAP) rice straw (10% w/v).

5.3.4.2. Fermentation with prolong enzymatic prehydrolysis

Another known approach, prolonged enzymatic prehydrolysis (24 h) at higher temperature (50 ^{0}C) to increase initial glucose availability for yeast followed by fermentation at 30 °C to provide independent optimum for saccharification of lignocellulose biomass and yeast fermentation in a single vessel system, was also applied. The prehydrolysis of AP-RS (10% w/v) and BAP-RS (10% w/v) fibers at 50 ^{0}C for 24 h separately using cellulase enzyme from *P. janthinellum* yielded glucose of 8.44 g/l and 16.83 g/l respectively (Fig.5.6). After 24 h, temperature was brought down to 30^{0}C and *S. cerevisiae* was inoculated to perform the fermentation process. The maximum bioethanol (E_{max}) produced in the fermentation stage were 2.89 g/l and 5.66 g/l in AP-RS and BAP-RS respectively (Fig.5.6). The rice straw added after base and acid pretreatment (BAP-RS) yielded 2-fold more ethanol compared to

the only acid pretreated rice straw (AP-RS). A similar study carried out with NaOH-pretreated sugarcane bagasse, where cellulase from *P. sanguineus* was utilized for prehydrolysis at 50^{0}C for 48h and further fermentation by sequential addition of *S. cerevisiae* yielded 4.5 g ethanol/100 g of feedstock (Yoon et al., 2019). Sharma et al., 2019 achieved 4.03 g/l ethanol from alkali-pretreated rice straw (10% w/v) with 14.5 FPU/gds Accellerase enzyme loading using prehydrolysis (24 h at 50 °C) and fermentation by *S. cerevisiae* (Sharma et al., 2019).

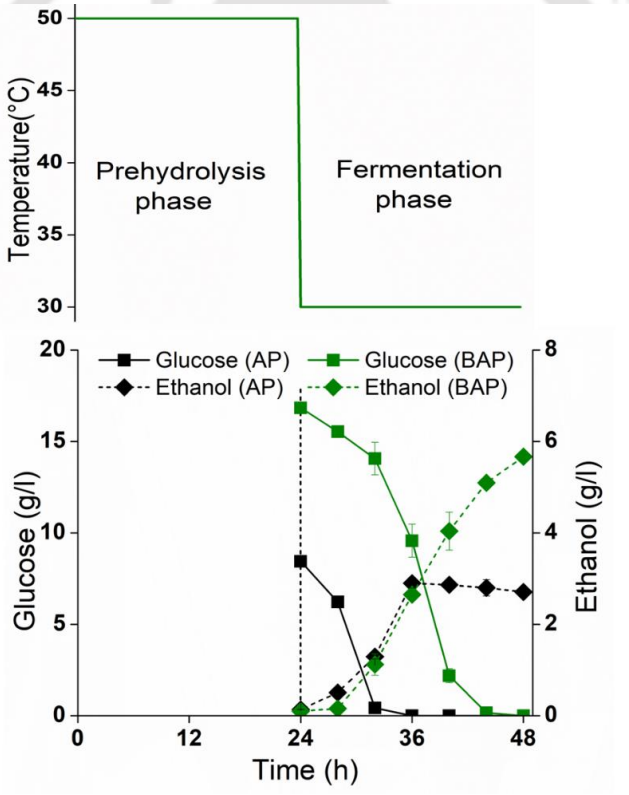

Fig. 5.6: Prolong prehydrolysis by feeding of acid pretreated (AP) rice straw, base and acid pretreated (BAP) RS in crude cellulase produced by *P. janthinellum* and subsequent fermentation by *S. cerevisiae.*

5.3.4.3. SSF using cyclic shifting of temperature strategy (CSTS) with rice straw

A novel strategy of cyclic shifting of temperature was developed to overcome the limitations encountered in conventional bioethanol production processes such as feedback inhibition caused by end product in case of prolonged high-temperature saccharification and inefficient sugar release in case of SSF at mutual optimum temperature. To overcome these drawbacks cyclic shifting of temperature strategy was implemented to maintain both sugar release by enzymatic saccharification at elevated temperature and glucose uptake by yeast fermentation at lower temperature with specific holding time. Based on the survivability and ethanol production of *S. cerevisiae* in pure glucose medium with CSTS **(Fig. 5.4 B, 5.4 C, 5.4 D)** five combinations; 30°C(2h)-46°C(10min), 30°C(2h)-43°C(30min), 30°C(1h)-40°C(1h), 30°C(2h)-40°C(2h), 30°C(3h)-40°C(3h) have been selected for ethanol production using rice straw with cyclic temperature shifting strategy. Using 30°C(2h)-46°C (10min) cyclic shifting of temperature, the maximum bioethanol titer of 4.05g/l at 36 h was achieved with AP-RS feeding, while for BAP-RS feeding, it reached 4.92 g/l at 24 h **(Fig. 5.7 A).** By employing cyclic shifting of temperature 30°C(2h)-43°C(30min) showed a notable improvement as compared to 30°C(2h)-46°C (10min) cycle. This resulted in a 1.6-fold increment in AP-RS feeding reaching ethanol titer of 6.55 g/l and a 1.75-fold increment in BAP-RS feeding achieving a maximum ethanol titer of 8.63 g/l **(Fig. 5.7 B).** Among the different cyclic shifting of temperature strategies tested between 30°C to 40°C, 30°C(2h)-40°C(2h) cycle resulted in highest bioethanol concentration of 14.98 g/l, with productivity of 0.31 g/(l h) in BAP-RS feeding **(Fig. 5.7 D).** This represented a significant 3.3-fold and 3-fold increment as compared to the bioethanol concentrations achieved in the 30°C(1h)-40°C(1h) and 30°C(3h)-40°C(3h) cyclic shifting of temperature for BAP-RS feeding respectively **(Table 5.2).** The lower ethanol titer observed in 30°C(2h)-46°C(10min) and 30°C(2h)-43°C (30 min) cyclic shifting of temperature conditions was attributed to the shorter upper holding time available

for enzymatic hydrolysis throughout the entire cycle. The extended saccharification time given in 30°C(2h)-40°C(2h) cyclic shifting of temperature helped in a significant improvement of bioethanol titer.

However, lower ethanol titers in 30°C(1h)-40°C(1h) and 30°C(3h)-40°C(3h) can be attributed to the phenomenon that the frequent exposure or longer exposure of yeast to elevated temperature may limit the fermentation capability of yeast **(Fig. 5.7 C and 5.7 E)**. Note that ethanol production was higher with BAP-RS than AP-RS for all the cyclic temperature shifting conditions. This could be due to the inefficient delignification in pretreatment of rice straw using acid only. The presence of lignin in biomass restricts the enzyme access to the cellulose fibrils, limiting the efficiency of enzymatic hydrolysis. Additionally, lignin tends to absorb cellulase enzyme to a great extent, further reducing the rate of enzymatic hydrolysis of biomass. As a result, AP-RS feeding did not show substantial improvements in bioethanol production. The ICPSF used by application of cyclic shifting of temperature (CSTS) at 30°C(2h)-40°C(2h), resulted in an increment of 2.6-fold and 4.8-fold bioethanol production as compared the approach involving at prolong prehydrolysis followed by fermentation and constant optimum temperature at 37^0C respectively **(Table 5.2).**

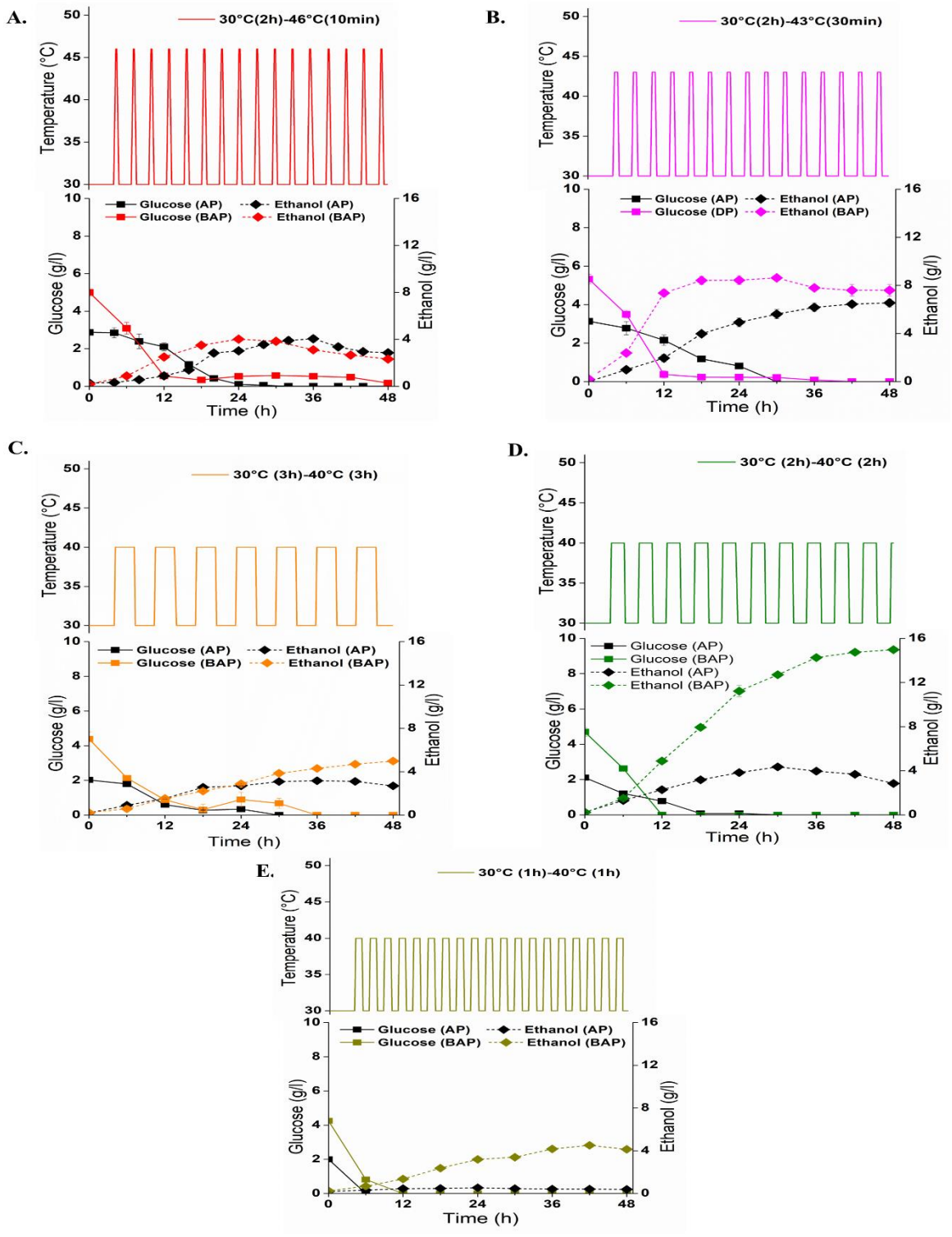

Fig. 5.7: Bioethanol production by co-culture of *P.janthinellum* and *S.cerevisiae* by cyclic shifting of temperature by feeding of acid pretreated (AP) rice straw, Base and acid pretreated (BAP) rice straw separately **A.** Cyclic shifting from 30ºC(2h)-46ºC(10 min) **B.** Cyclic shifting from 30ºC(2h)-43ºC(30 min) **C.** Cyclic shifting from 30ºC(3h)40ºC(3h) **D.** Cyclic shifting from 30ºC(2h)-40ºC(2h) **E.** Cyclic shifting from 30ºC(1h)-40ºC(1h).

5.3.4.4 Tuning the holding time of cyclic shifting of temperature strategy (CSTS) for improvement of productivity:

Ethanol production was highest using rice straw with cyclic shifting strategy of 30 °C as lower level with 2 h holding time and 40 °C as upper level with 2 h holding time. To improve the ethanol productivity carbon starvation phase (see **Fig. 5.7D**) can be eliminated by tuning the holding times and combination of lower and upper temperature. For tuning the cyclic shifting strategy, 30 °C and 40 °C have been chosen as lower and upper temperature and kept fixed, and only holding time has been tuned. The holding time was tuned based on glucose release from pretreated rice straw by enzyme activity and glucose consumption by yeast for ethanol production. The theoretical glucose release was estimated from the enzyme activity at specific temperature. At lower temperature (30 °C) glucose release from pretreated rice straw was insignificant due to negligible β-glucosidase activity **(Fig. 5.2B).** At upper temperature (40 °C) glucose release has been determined based on FPase activity. The FPase activity has been considered over β-glucosidase as it is observed to be the limiting at 40 °C in current study **(Fig. 5.2).** Glucose uptake by yeast was estimated from the experiments with pure glucose at different temperature **(Fig.5.4A).** Noted that glucose uptake by yeast was negligible at 40 °C and maximum at 30 °C **(Fig 5.4A).** It has been seen that glucose starvation period was present in the cyclic shifting strategy of 30 °C (2h) -40 °C(2h) as the ratio of glucose released to glucose uptake (R) is 0.78 calculated using equation 2 **(Fig. 5.7D).**

$$R = \frac{\text{Rate of sugar release by enzymatic hydrolysis at } 40^0\text{C} \times \text{holding time at } 40^0\text{C}}{\text{Rate of glucose uptake by yeast at } 30^0\text{C} \times \text{holding time at } 30^0\text{C}} --(2)$$

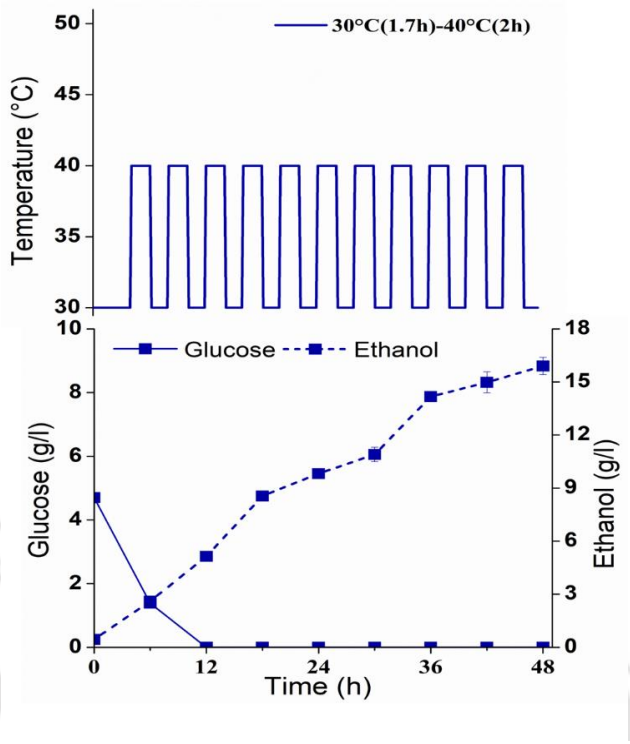

Fig. 5.8: *In-situ* cellulase production followed by simultaneous saccharification and fermentation (ICPSF) for bioethanol production by *S. cerevisiae* using BAP-RS (10 % w/v) by tuned cyclic shifting of temperature strategy (CSTS) 30°C(1.7h)- 40°C(2h). Glucose and bioethanol profile after adding of pretreated rice straw on 10 days old P. janthinellum culture broth containing cellulase enzyme.

Starvation period can be removed by fixing R value as 1 and was achieved by decreasing the holding time to 1.7 h from 2 h at 30 °C. For validation of this tuned cyclic temperature shifting strategy "30°C(1.7h)-40°C(2h)", experiment has been conducted using rice straw (RS). Final ethanol titer of 15.9 g/l with an ethanol productivity of 0.331 g/(l h) was obtained using BAP-RS **(Fig. 5.8).** Both the titer and productivity have been improved compared to 30°C(2h)-40°C(2h) cyclic shifting strategy **(Table 5.2).** By tuning of the holding time of cyclic shifting of temperature strategy, the new condition 30°C(1.7h)-40°C(2h) resulted a significant increase of 5.1-fold bioethanol titer compared to the bioethanol production achieved by maintaining a constant incubation temperature of 37°C using BAP-RS feeding **(Table 5.2).**

Table 5.2: ICPSF for bioethanol production from rice straw using prolong prehydrolysis followed by fermentation, different incubation temperatures and cyclic shifting of temperature.

	Using AP-RS		Using BAP-RS	
	Max. Ethanol titre (E_{max}) g/l	**Ethanol productivity g/(l h)**	**Max. Ethanol titre (E_{max}) g/l**	**Ethanol productivity g/(l h)**
Prolong prehydrolysis followed by fermentation				
	2.9±0.09	0.241	5.66±0.03	0.235
Different Incubation temperatures				
30^0C	0.5±0.003	0.041	0.43±0.01	0.035
33^0C	0.5±0.01	0.041	0.58±0.02	0.024
37^0C	0.94±0.09	0.031	3.11±0.169	0.064
40^0C	1.02±0.05	0.042	1.59±0.03	0.132
Cyclic shifting of temperature strategy (CSTS)				
30^0C (2h)-46^0C (10 min)	4.05±0.28	0.112	4.01±0.01	0.167
30^0C (2h)-43^0C (30 min)	6.55±0.33	0.204	8.63±0.09	0.287
30^0C (1h)-40^0C (1h)	0.54±0.01	0.0225	4.51±0.18	0.107
30^0C (2h)-40^0C (2h)	4.35±0.13	0.145	14.98±0.01	0.312
30^0C (3h)-40^0C (3h)	3.17±0.01	0.088	4.98±0.03	0.104
Tuned cyclic shifting of temperature strategy				
30^0C (1.7h)-40^0C (2h)	-	-	15.9±0.01	0.331

5.4. Conclusion

A novel cyclic shifting of temperature approach was developed for SSF for lignocellulosic bioethanol production and implemented in this study. Using the optimum cyclic shifting of temperature strategy the maximum bioethanol production was 15.9 g/l with a productivity of 0.331 g/l/h. By applying this strategy resulted a 5.1-fold and 2.8-fold increment of bioethanol production compared to mutual optimum temperature strategy and prolong prehydrolysis followed by fermentation strategy respectively. This study underlines the potential of the cyclic shifting of temperature technique in advancing lignocellulosic bioethanol synthesis, while also spotlighting scopes for future research aimed at elevating bioethanol titers. The subsequent chapter introduces an advancement in bioethanol production by employing a mixed culture consisting of the fungi *T. reesei* and *P. janthinellum*. This approach aims to enhance cellulase production and facilitate enzymatic hydrolysis during the SSF process. Furthermore, the yeast species *S. cerevisiae* and *P. stipitis* are utilized for SSF process to ferment both glucose and xylose found in the solid pretreated rice straw fibers for bioethanol production.

Chapter 6

Establishment of a poly-culture system of fungi *Penicillium janthinellum, Trichoderma reesei* and yeast *Saccharomyces cerevisiae, Pichia stipitis* for bioethanol production through cyclic shifting of temperature.

6.1. Background of the study

Various species of cellulolytic fungi e.g., *Trichoderma sps.*, *Aspergillus sps.*, *Penicillium sps.*, *Humicola sps.*, *Fusarium sps.*, *Acremonium sps.* etc. possess the enzymatic machinery for cellulase production (Dakshayani et al., 2019; Dwiarti et al., 2012; Jing et al., 2015; Kogo et al., 2017; Kuhad et al., 2016). However, the diversity of cellulase expression varies among fungal species, some have higher level of endoglucanase and exoglucanase expression e.g., *Trichoderma* but has minimal beta-glucosidase expression (Ng et al., 2011). Certain species have only higher beta-glucosidase expression system e.g., *Aspergillus sps.*(Narasimha et al., 2016), certain species produces a blend constituting above three enzyme e.g., *Penicillium sps.*(Jørgensen & Olsson, 2006). To maximise the enzyme yields and saccharification efficiency, a single cultivation system employing two or more fungus can be utilised, where each organism specializes in the production of a specific cellulase component (Juwaied et al., 2010; Lodha et al., 2020). Here, the microorganisms can coevolve synergistically, adapt to optimize enzyme production and ultimately facilitating efficient cellulose degradation (Fatma et al., 2021).

The solid pretreated rice straw fibres utilised in the current study for SSF process in bioethanol production contains both glucose from cellulose and minor xylose fraction from hemicellulose respectively. To maximise the sugar utilisation and bioethanol production, it is necessary to utilise both glucose and xylose for fermentation processes. *S. cerevisiae* is known to have superior ability to ferment glucose into ethanol but lack the ability to

assimilate xylose. However, *P. stipitis* have the ability to consume both the glucose and xylose and ferment into bioethanol. To achieve cost-effective bioethanol production, it is necessary to establish a poly-culture system of fungus and yeast. The co-culture system of fungus can facilitates optimal cellulase production pre-requisite for saccharification, further SSF process is carried out using yeast within a single vessel system. One significant aspect during the SSF process is the temperature requirement for enzymatic hydrolysis of cellulase, which is optimal at elevated temperatures (~50ºC). However, the growth and fermentation of yeast optimum under normal atmospheric temperatures(~30ºC). The traditional methods mostly wrap the process with an intermediate mutual temperature as optimum. It gives rise to lower saccharification efficiency creating carbon starvation conditions for yeast in the medium and further effecting ethanol production.

The objective of the present study is to implement an *in situ* cellulase production, saccharification, and fermentation (ICPSF) process using poly-cultures of the fungi *P. janthinellum* and *T. reesei*, along with the yeasts *S. cerevisiae* and *P. stipitis* to produce bioethanol from rice straw. To address the optimum temperature required for SSF process, a cyclic shifting of temperature strategy was employed. The maximum thermotolerance of *P. stipitis* was investigated in a pure sugar medium, both under constant temperature and cyclic shifting of temperature conditions, prior to its application for bioethanol production for ICPSF. Various combinations of consortia were assessed to examine their impact on the end-product yield via cyclic shifting of temperature.

6.2. Material and methods

6.2.1. Ethanol production by *P. stipitis* in pure xylose medium

6.2.1.1. Identification of thermotolerant range at constant incubation temperature

P. stipitis was cultivated in 100 ml volume pure xylose medium to assess its growth and bioethanol production at various incubation temperatures. The precultures of *P. stipitis* (5% v/v) were inoculated into the medium containing xylose: 10 g/l, yeast extract: 5 g/l, yeast mineral salt medium (Table 3.2) with initial pH 6.5. Five set of experiments were carried out at different incubation temperature 25°C, 30 °C, 33 °C, 37 °C and 40 °C separately at agitation speed 180 rpm and a culture period kept for 32 h. To measure the yeast growth, the absorbance of the cell was carried out at 600 nm wavelength at every 4 h interval. The collected samples were further centrifuged at 10,000 rpm for 5 minutes. The supernatants were collected and stored at 4°C for measurement of xylose and ethanol.

6.2.1.2. Identification of thermotolerant range at cyclic shifting of temperature

Prior to the application of CSTS for SSF process for bioethanol production using pretreated rice straw, the optimal CSTS designated for *S. cerevisiae* in pure glucose medium i.e., CSTS 30ºC(2h)-40ºC(2h) was applied for *P. stipitis* in pure xylose medium (10 g/l). After inoculation of *P. stipitis* (5% v/v) into the xylose medium, containing xylose:10 g/l, yeast extract:5 g/l, yeast mineral salt medium (Table 3.2) with initial pH 6.5, agitation speed 180 rpm and a CSTS 30ºC(2h)-40ºC(2h) was applied for 32 h culture period. Typically, temperature ramp up time from 30 °C to 40 °C and ramp down time from 40 °C to 30 °C took 10 min each in the incubator used for experiments. Sampling was done at every 4h interval to measure biomass growth by taking O.D. at 600 nm. The samples were centrifuged at 10,000 rpm and the supernatants were collected to measure the concentrations of xylose and ethanol through HPLC.

6.2.2. *In situ* cellulase production, saccharification and fermentation (ICPSF) by employing *P. janthinellum* and *P. stipites*

The process of cellulase production was carried out by using *P. janthinellum* in acid pretreated rice straw (3% w/v) whole slurry for 10 days. It is followed by addition of solid pretreated rice straw fibres (10% w/v) and three kind of experiment were carried out separately i.e., SSF at mutual incubation temperature, prolong prehydrolysis and fermentation and SSF by employing cyclic shifting of temperature strategy (CSTS).

6.2.2.1. SSF at mutual incubation temperature

After addition 10% w/v of base followed by acid pretreated rice straw fibres (BAP-RS) to the 10 days old crude cellulase broth produced by *P. janthinellum*. Initially, a prehydrolysis was given at 50℃ for 2 h and subsequently, the temperature was brought down to 37℃. *S. cerevisiae* (5% v/v) was inoculated into the production media, supplemented with yeast extract: 5g/l and yeast mineral salt medium **(Table 3.2).** Subsequent to inoculation, the SSF process was conducted at an incubation temperature of 37℃ for next 48 h. Sampling was performed out at every 6 h interval and centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected and stored at 4℃ for further estimation sugars and ethanol using HPLC.

6.2.2.2. Prolong prehydrolysis and fermentation using rice straw

Three separate experiments were carried out by adding three kind of pretreated rice straw i.e., acid pretreated rice straw; AP-RS (10% w/v), base pretreated rice straw; BP-RS (10% w/v), base followed by acid pretreated rice straw; BAP-RS (10% w/v) separately into the 10 days old *P. janthinellum* culture broth containing crude cellulase. The prolonged enzymatic hydrolysis of pretreated rice straw was carried out at 50 °C for 24 h. After prolong prehydrolysis, the medium temperature was brought down to 30 °C and followed by

inoculation of *P. stipitis* (5% v/v). The medium was set at pH 6, supplemented with yeast extract: 5 g/l and yeast mineral salt solution **(Table 3.2)** respectively. Further, the fermentation was carried out at 30 °C for next 24 h. The sampling was carried out at every 6 h interval, centrifuged at 10000 rpm for 5 min, supernatants were collected for measurement glucose, xylose and ethanol concentration using HPLC.

6.2.2.3. SSF of rice straw by using cyclic shifting of temperature strategy (CSTS)

To carry out the SSF process, only BAP-RS fibers (10% w/v) are added as substrate for bioethanol production in the 10 days old crude cellulase broth produced by *P. janthinellum*. A short prehydrolysis was applied at 50°C for 2 h duration for release of initial sugar to support initial growth of yeast and further the media was cooled down to 30°C. It was followed by inoculation of *P. stipitis* (5% v/v), supplemented with yeast extract: 5 g/l and yeast mineral salt medium (described in section). The following cyclic shifting of temperature strategy (CSTS) were applied for SSF process i.e. 30°C(1h)-37°C(1h), 30°C(2h)-37°C(2h), 30°C(3h)-37°C(3h), 30°C(1h)-37°C(3h), 30°C(2h)-37°C(3h) respectively for bioethanol production. The sampling was carried out at every 6 h interval, centrifuged at 10000 rpm for 5 min and HPLC analysis was carried out for measuring the concentration of glucose, xylose, and ethanol.

6.2.3. ICPSF by employing the tri-culture system of *P. janthinellum, T. reesei and P. stipitis*

Initially, cellulase production was carried out by inoculating each pre-culture of *P. janthinellum* (10% v/v) and *T. reesei* (10% v/v) into the acid pretreated rice straw whole slurry (3% w/v). It was also supplemented with yeast extract: 1 g/l, fungal mineral salt medium (Table 3.1). The process of enzyme production was carried out for twelve days at an incubation temperature 30°C, medium was set to pH 5 and an agitation speed of 180 rpm.

After twelve days of cellulase production, the BAP-RS (10% w/v) was added to the crude cellulase broth and a prehydrolysis was carried out at 50°C for 2 h. Further, the temperature was brought down to 30°C, *P. stipitis* (5% v/v) was inoculated and also supplemented with yeast extract: 5 g/l and yeast mineral salt medium **(Table 3.2)**. Further, in one set of experiments, SSF process was carried out by applying a mutual incubation temperature of 37°C for next 48h. In another sets of experiment using cyclic shifting of temperature strategy, wherein the condition that resulted high ethanol titres from the ICPSF using *P. janthinellum* and *P. stipitis* were selected and applied further. Two cyclic shifting of temperatures strategies i.e., 30°C(1h)-37°C(3h) and 30°C(2h)-37°C(3h) were applied further for SSF using *P. stipitis.* The sampling was carried out at every 6h interval, and further it was centrifuged at 10,000 rpm and the supernatant were collected for measurement of glucose, xylose and ethanol through HPLC.

6.2.4. ICPSF through the tri-culture system of *P. janthinellum, T. reesei and S. cerevisiae*

The process of cellulase production was carried out by using acid pretreated rice straw whole slurry (3% w/v). The pre-culture of *P. janthinellum* (10% v/v) and *T. reesei* (10% v/v) was inoculated into the cellulase production media along with yeast extract: 1 g/l, fungal mineral salt medium **(Table 3.1).** After twelve days of cellulase production, BAP-RS (10% w/v) was added into the culture broth containing crude cellulase and a prehydrolysis was carried out at 50°C for 2 h. After prehydrolysis, the media was cooled down to 30°C followed by inoculation of *S. cerevisiae* (5% v/v). The media was also supplemented with yeast extract: 5 g/l and yeast mineral salt medium **(Table 3.2).** Two separate studies were conducted for SSF process for bioethanol production. In the first experimental set, SSF process was conducted at a mutual incubation temperature i.e., 37°C and the experiment was carried out for 48 h. In other set of experiments, SSF process was conducted using the cyclic shifting of temperature

strategy. It was subjected to a cyclic shifting of temperature condition, previously optimised condition i.e., 30°C(2h)-40°C(1.7h) for bi-culture system of *P. janthinellum* and *S. cerevisiae* (see section 5.3.4.4). The experiment was continued for 48 h and the samples were collected at every 6 h interval to measure the concentrations of glucose and ethanol.

6.3. Result and discussion

6.3.1. Thermotolerance and viability of *P. stipitis* in pure xylose medium

6.3.1.1. Viability at different incubation temperature with pure xylose

To study the thermotolerance and viability of *P. stipitis* in pure xylose medium (10 g/l), it was grown at various temperature ranging from 25°C to 40 °C **(Fig. 6.1).** The highest ethanol titre (E_{max}) was obtained at 30 °C, with xylose to ethanol yield ($Y_{E/X}$) of 0.24, while 1.96 g/l ($Y_{E/X}$=0.20) and 2.28 g/l ($Y_{E/X}$=0.23) at 25°C and 33°C respectively. However, ethanol production was decreased to 1.14 g/l at 37°C with an $Y_{E/X}$ of 0.11. *P. stipitis* was sensitive to incubation temperature of 40 °C and the ethanol production was restricted to 0.12 g/l in 32 h of incubation period, xylose remained unutilized in the medium and biomass growth also completely ceased. The fermentation of xylose was optimum at 30°C with the maximum xylose uptake rate of 0.411 g/(l h) **(Fig. 6.1A, Table 6.1).** Similarly, the maximum bioethanol productivity obtained was at 30°C with a productivity of 0.114 g/l, with increase in incubation temperature to 37°C, the ethanol productivity also decreased to 0.071 g/(l h). The overall biomass yield at 30°C was maximum with 6.11 g/l, followed by 5.59 g/l at 25°C, 4.97 g/l at 37°C, 4.66 g/l at 33°C respectively **(Fig.6, Table 6.1).** After xylose depletion in the medium, the ethanol was consumed as a part of carbon source at a rate of 0.047 g/(l h), 0.099 g/(l h), 0.066 g/(l h), 0.036 g/(l h) at incubation temperature of 25°C, 30°C, 33°C, and 37°C respectively.

Table 6.1: Ethanol production profile of *P. stipitis* at constant incubation temperatures and cyclic shifting of temperature in pure xylose medium.

	25°C	**30°C**	**33°C**	**37°C**	**40°C**	**CSTS 30°C (2h)-40°C(2h)**
E_{max}	1.96±0.05	2.40±0.06	2.28±0.04	1.14±0.30	0.12±0.01	0.31±0.004
$Y_{E/G}$ (g/g)*	0.20	0.24	0.23	0.11	NA	NA
Xylose Consumption rate (g/(l h))	0.353	0.411	0.402	0.309	0.003	0.018
Ethanol consumption rate (g/(l h))	0.047	0.099	0.066	0.036	0.008	0.003
Ethanol Productivity (g/(l h))**	0.082	0.114	0.108	0.071	0.005	0.019
Biomass productivity from xylose (g/(l h))	0.166	0.182	0.136	0.146	0.038	0.11
Overall biomass (g/l)	5.59±0.01	6.11±0.02	4.66±0.04	4.97±0.02	1.53±0.06	0.44±0.01

Note: Experimental data is presented as mean ± standard deviation of duplicate experiment.

*$Y_{E/G}$ (g/g) calculated based on time of maximum ethanol production.

**Ethanol Productivity (g/(l h)) calculated based on time of maximum ethanol production.

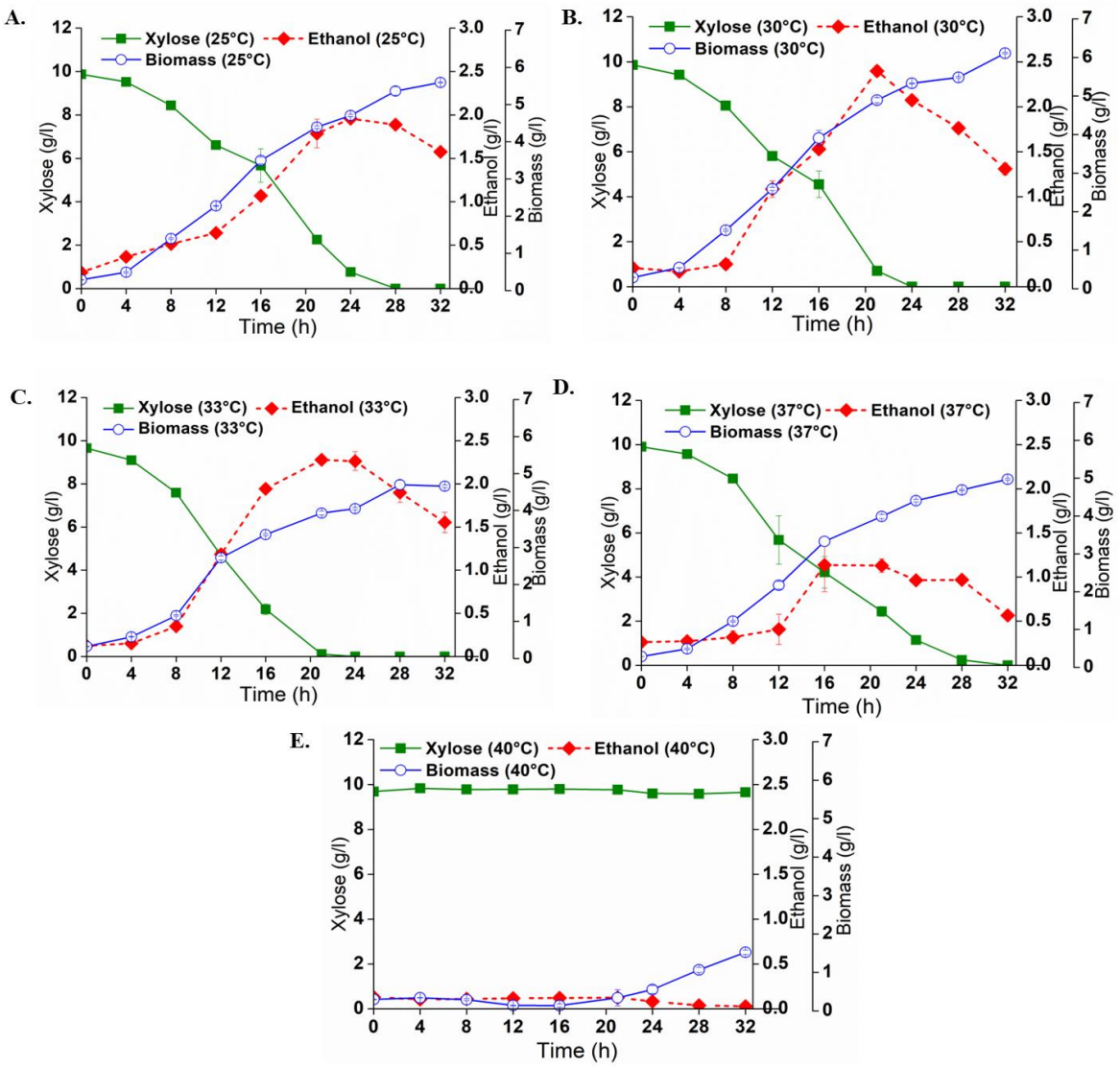

Fig.6.1: Ethanol production from xylose (10 g/l) by *P. stipitis* at different incubation temperature.

6.3.1.2. Viability at cyclic shifting of temperature

The culture of *P. stipitis* was sensitive to cyclic shifting of temperature at 30ºC (2h)-40ºC (2h). The maximum biomass growth was restricted to 0.44 g/l **(Fig. 6.2).** There was no consumption of xylose throughout the culture period of 32 h and therefore the ethanol production also restricted to 0.31 g/l. Since, cyclic shifting of temperature strategy 30ºC (2h)-40ºC (2h) did not offer any thermotolerance to *P. stipitis*, all the SSF process for bioethanol production using *P. stipitis* were carried out by applying CSTS from 30ºC to 37ºC for further experiments.

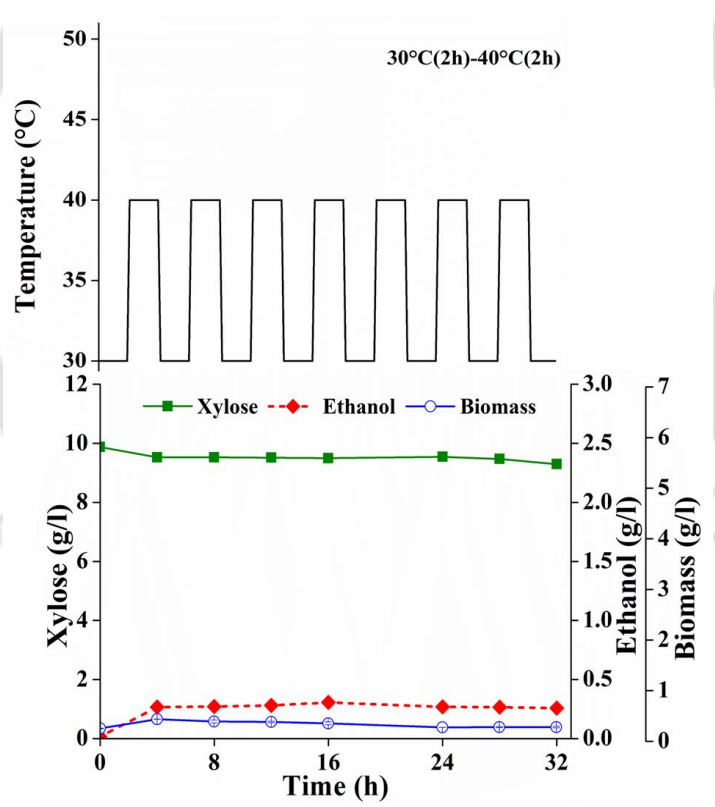

Fig.6.2: Growth, xylose consumption and ethanol production profile of *P. stipitis* in pure xylose medium at cyclic shifting of temperature from 30ºC (2h)-40ºC (2h).

6.3.2. ICPSF by employing the bi-culture system of *P. janthinellum* and *P. stipitis* using rice straw

6.3.2.1. SSF at mutual incubation temperature for bioethanol production

Initially, in situ cellulase production was conducted using *P. janthinellum* over a span of 10 days. The resultant crude cellulase broth was then utilized for simultaneous saccharification and fermentation (SSF) of BAP-RS fibers employing *P. stipitis* at an incubation temperature of 37°C. This process resulted in a maximum bioethanol titre of 2.71 g/l, with a productivity of 0.056 g/(l h) **(Fig. 6.3).** Throughout the SSF process, *P. stipitis* preferentially consumed glucose, leaving xylose unused. Consequently, the maximum accumulated xylose concentration reached 3.36 g/l by the end of 48 h.

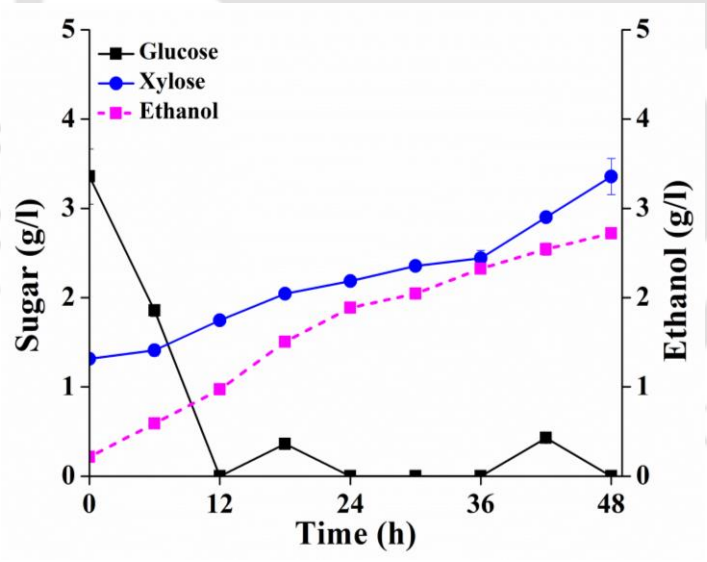

Fig.6.3: In-situ cellulase production by *P. janthinellum* followed by simultaneous saccharification and fermentation (ICPSF) for bioethanol production by *P. stipitis* using BAP-RS (10 % w/v) at 37°C incubation temperature.

6.3.2.2. Fermentation with prolong enzymatic prehydrolysis

The prolong prehydrolysis for 24 h at 50ºC by utilising the crude cellulase produced by the *P. janthinellum* yielded 11.8 g/l of glucose, 4.3 g/l of xylose from BAP-RS respectively **(Fig.6.4).** The maximum bioethanol (E_{max}) produced in the fermentation stage (24 h) at 30℃ was 5.27g/l from BAP-RS fibres **(Fig.6.4).** The bioethanol titre in prolong prehydrolysis and fermentation was improved by 1.9-fold as compared to the bioethanol titres obtained in SSF process at 37℃.

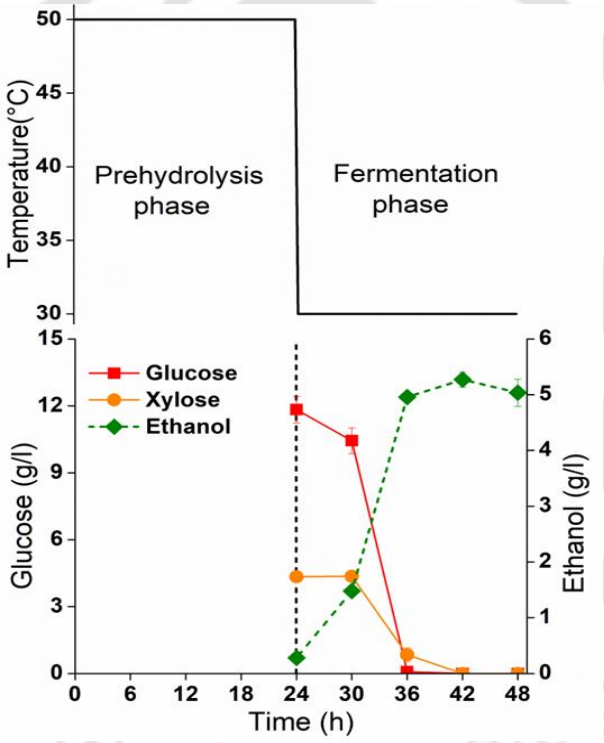

Fig.6.4: Prolong prehydrolysis and fermentation for bioethanol production by ICPSF of *P. janthinellum* and *P. stipitis* using BAP-RS fibres.

6.3.2.3. Cyclic shifting of temperature by *P. janthinellum* and *P. stipitis*

As *P. stipitis* is sensitive to constant incubation temperature of 40°C and also to cyclic shifting of temperature (CSTS) 30°C(2h)-40°C(2h) in pure xylose medium. All the SSF process for bioethanol production using BAP-RS were carried out by cyclic shifting of

temperature from 30°C to 37°C. Following five combinations of CSTS were applied 30°C(1h)-37°C(1h), 30°C(2h)-37°C(2h), 30°C(3h)-37°C(3h), 30°C(1h)-37°C(3h), 30°C(2h)-37°C(3h) respectively for SSF process. The maximum bioethanol titre resulted in CSTS 30°C(1h)-37°C(1h) was 2.19 g/l **(Fig. 6.5 A, Table 6.2).** By applying CSTS 30°C(2h)-37°C(2h), resulted in a concentration of 2.01 g/l of bioethanol (Fig. 6.5B, Table 6.2). and through application of 30°C(3h)-37°C(3h) restricted to a maximum of bioethanol concentration of 2.42 g/l **(Fig. 6.5 C, Table 6.2).** However, by applying the CSTS 30°C(1h)-37°C(3h) resulted in the bioethanol titre of 5.26 g/l with a productivity of 0.146 g/(l h) **(Fig. 6.5 D, Table 6.2).** Further application of CSTS 30°C(2h)-37°C(3h) resulted in the highest bioethanol titre of 8.76 g/l with a productivity of 0.208 g/(l h) **(Fig. 6.5 E, Table 6.2).** The improvement in bioethanol titre obtained in CSTS 30°C(2h)-37°C(3h) is 1.6-fold, 4.3-fold as compared to bioethanol titre obtained in CSTS 30°C(1h)-37°C(3h) and CSTS 30°C(2h)-37°C(2h) respectively. Similarly, the maximum bioethanol titre obtained by using cyclic shifting of temperature strategy at 30°C(2h)-37°C(3h) is 3.2-fold and 1.6-fold higher as compared to SSF at 37°C and SSF by prolong prehydrolysis and fermentation respectively.

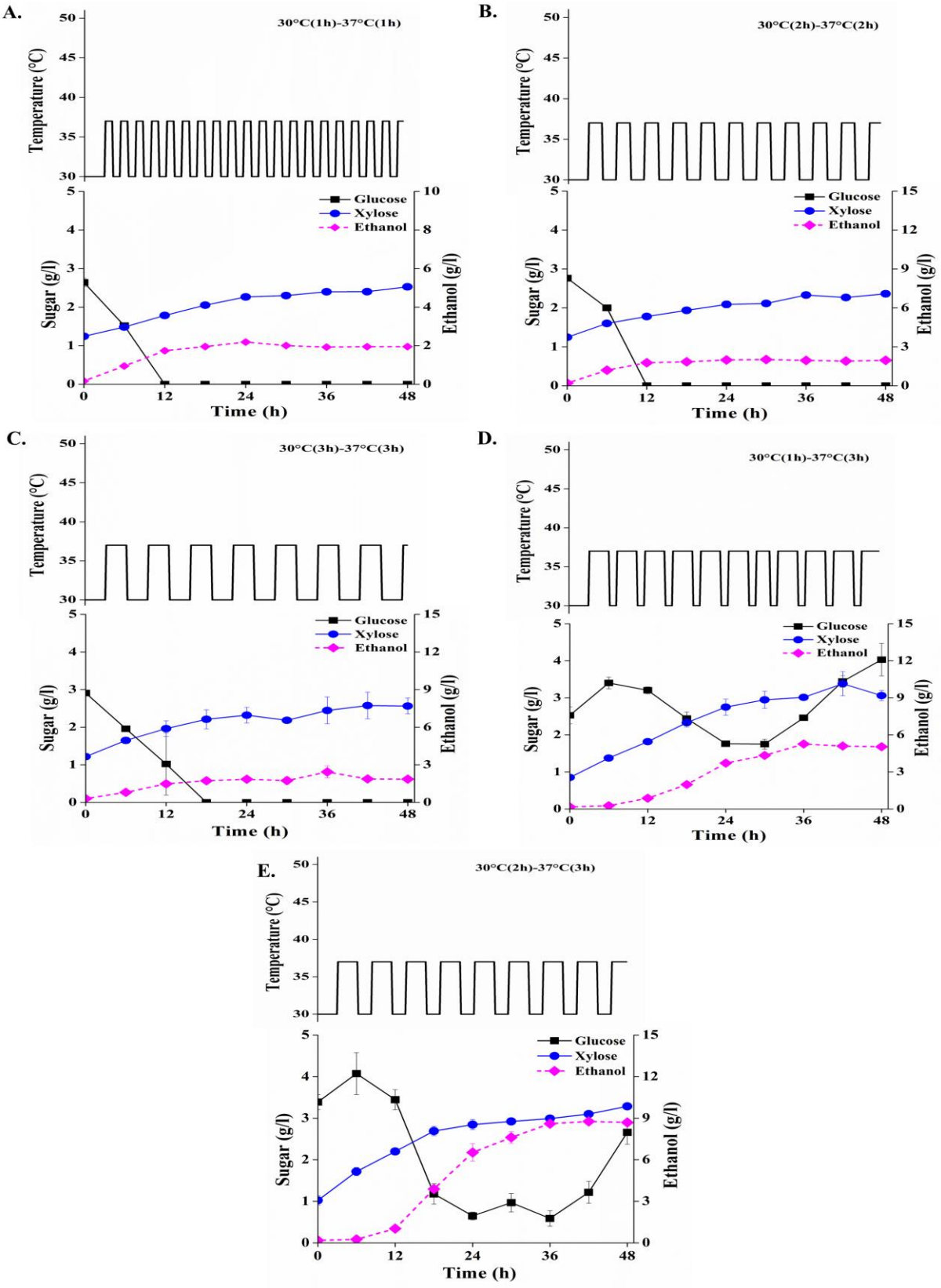

Fig.6.5: Bioethanol production using *P. janthinellum* and *P. stipitis* by cyclic shifting of temperature **A.** 30°C(1h)-37°C(1h) **B.**30°C(2h)-37°C(2h) **C.** 30°C(3h)-37°C(3h) **D.** 30°C(1h)-37°C(3h) **E.** 30°C(2h)-37°C(3h).

6.3.3. ICPSF through the tri-culture system of *P. janthinellum, T. reesei and P. stipitis* for bioethanol production

To improve the cellulase activity and enhance the enzymatic hydrolysis of pretreated rice straw, cellulase production was carried out by coculture of fungus *T.reesei* and *P. janthinellum*. After twelve days of cellulase production, BAP-RS fibres (10% w/v) was added to the the crude cellulase broth and two kind of SSF experiments were carried out separately. In the first set of experiment, SSF was carried out at a mutual incubation temperature and second sets SSF was carried out by applying cyclic shifting of temperature strategy.

6.3.3.1. SSF at mutual incubation temperature using rice straw

The SSF process carried out using BAP-RS fibers by employing *P. stipitis* at an incubation temperature of 37℃ resulted in a maximum bioethanol titre of 3.72 g/l, with a productivity of 0.077 g/(l h) **(Fig. 6.6, Table 6.2).** Throughout the SSF process, *P. stipitis* preferentially consumed glucose, leaving xylose unused. Consequently, the maximum accumulated xylose concentration reached 3.67 g/l by the end of 48 h.

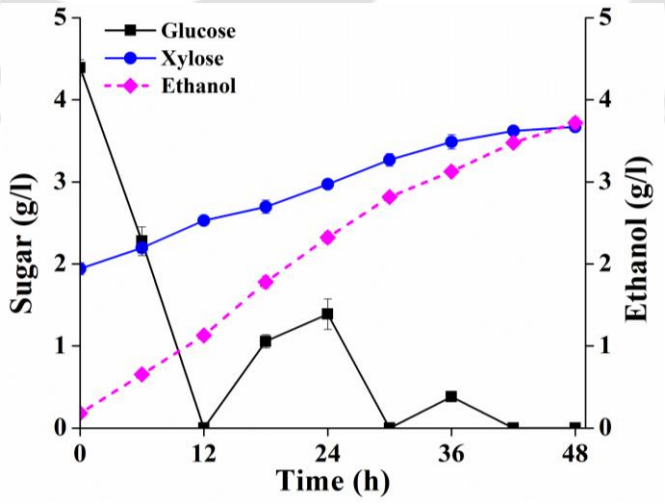

Fig.6.6: *In-situ* cellulase production by co-culture of *P. janthinellum* and *T. reesei* followed by simultaneous saccharification and fermentation (ICPSF) for bioethanol production by *P. stipitis* using BAP-RS (10 % w/v) at 37℃ incubation

6.3.3.2. SSF by cyclic shifting of temperature strategy using rice straw

The SSF process was carried out by applying cyclic shifting of temperature strategy (CSTS). The maximum bioethanol titre obtained in CSTS 30°C(1h)-37°C(3h) was 9.3 g/l at 48 h with a productivity of 0.193 g/(l h) (Fig. 6.7A, Table 6.2). However, when CSTS 30°C(2h)-37°C(3h) was applied, the final bioethanol titre improved by 1.3-fold to 11.81 g/l, with a productivity of 0.246 g/(l h) **(Fig. 6.7B).**

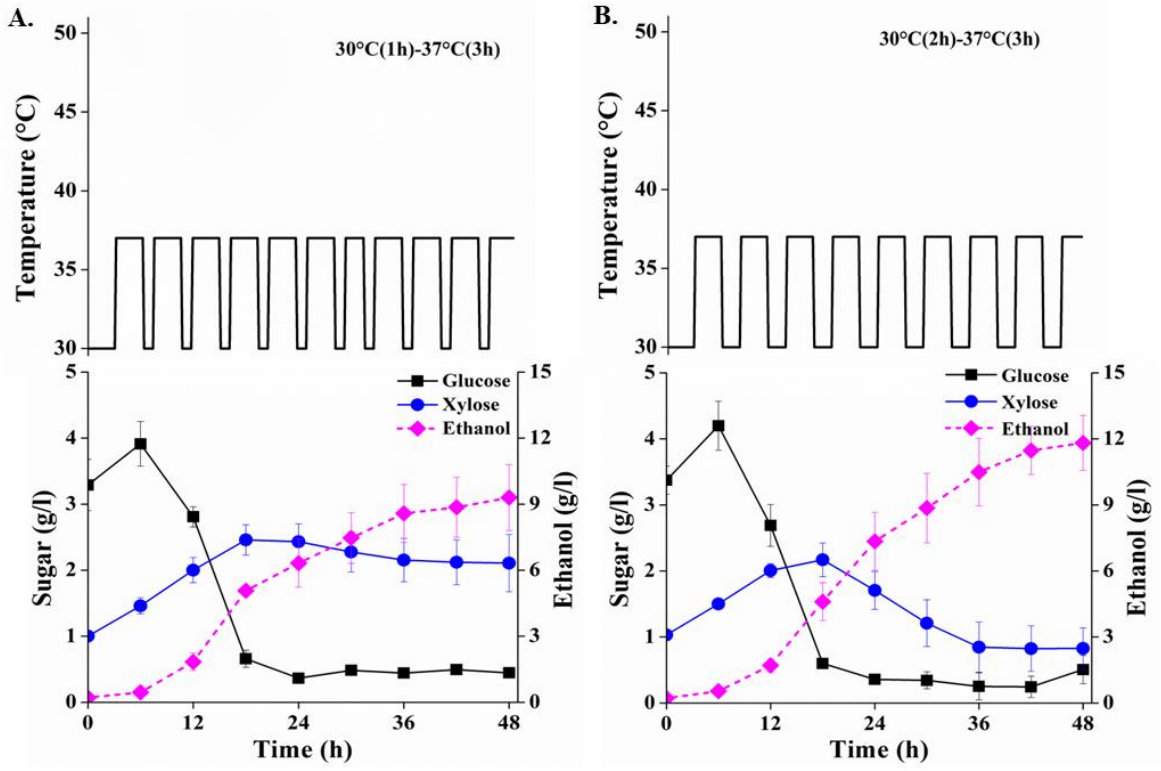

Fig.6.7: Bioethanol production by ICPSF process using tri-culture system of *T. reesei, P. janthinellum* and *P. stipitis* by cyclic shifting of temperature **A.** 30°C(1h)-37°C(3h) **B.** 30°C(2h)-37°C(3h).

Similarly, the maximum bioethanol titre obtained by using cyclic shifting of temperature strategy at 30°C(2h)-37°C(3h) is 3.2-fold as compared to SSF at 37°C. The application of cyclic shifting of temperature for SSF at 30°C(2h)-37°C(3h), with ICPSF by tri-culture system (*P. janthinellum, T. reesei* and *P. stipitis*) improved by 1.3-fold final

bioethanol titre as compared to ICPSF by the bi-culture system (*P. janthinellum* and *P. stipitis*). In a study by Suriyachai et al., 2013, the SSF process by applying commercial enzyme Accellerase1500 and *P. stipitis* reported a maximum bioethanol titre of 12.7 g/l using rice straw (Suriyachai et al., 2013).

6.3.4. ICPSF through the tri-culture system of *P. janthinellum, T. reesei and S. cerevisiae* for bioethanol production

A tri-culture system of *T.reesei* , *P. janthinellum* and *S.cerevisae* was utilised for ICPSF for bioethanol production. After twelve days of cellulase production by co-culture of *T.reesei* and *P. janthinellum*, BAP-RS fibres (10% w/v) was added to the the crude cellulase broth and two kind of SSF experiments were carried out separately. In the first set of experiment, SSF was carried out at a mutual incubation temperature and second sets SSF was carried out by applying cyclic shifting of temperature strategy.

6.3.4.1.SSF at mutual incubation temperature

The SSF process carried out by employing *S. cerevisiae* at an incubation temperature of 37℃ using BAP-RS fibers resulted in a maximum bioethanol titre of 4.59 g/l, with a productivity of 0.095 g/(l h) **(Fig. 6.8, Table 6.2).** Notably, the current study represents an increment of 1.4-fold as compared to previous study which employed a single fungus *P. janthinellum* and followed by SSF at 37℃ using *S. cerevisiae*.

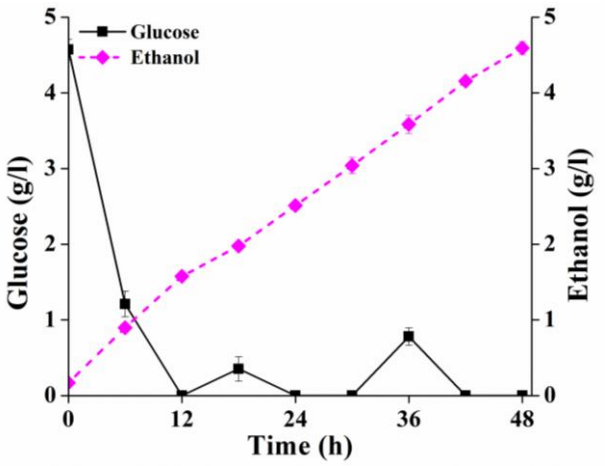

Fig.6.8: SSF for bioethanol production using BAP-RS (10% w/v) at 37℃ incubation temperature by employing *S. cerevisiae*.

6.3.4.2. Bioethanol production from rice straw through cyclic shifting of temperature strategy using SSF

In the current study using the tri-culture system where cellulase produced by co-culture of *T. reesei* and *P. janthinellum* followed by SSF using *S. cerevisiae*, the maximum bioethanol titre achieved was 17.05 g/l using BAP-RS at cyclic shifting of temperature strategy 30°C(1.7h)-40°C(2h), with a productivity of 0.405 g/(l h) **(Fig. 6.9, Table 6.2).** Notably, this represented an enhancement from 15.9 g/l of bioethanol titre obtained in previous study, which employed a single fungus *P. janthinellum* and followed by SSF using *S. cerevisiae* through application of cyclic shifting of temperature. The application of cyclic shifting of temperature for SSF process also increased the bioethanol titre by 3.7-fold as compared to the SSF at mutual incubation temperature of 37°C.

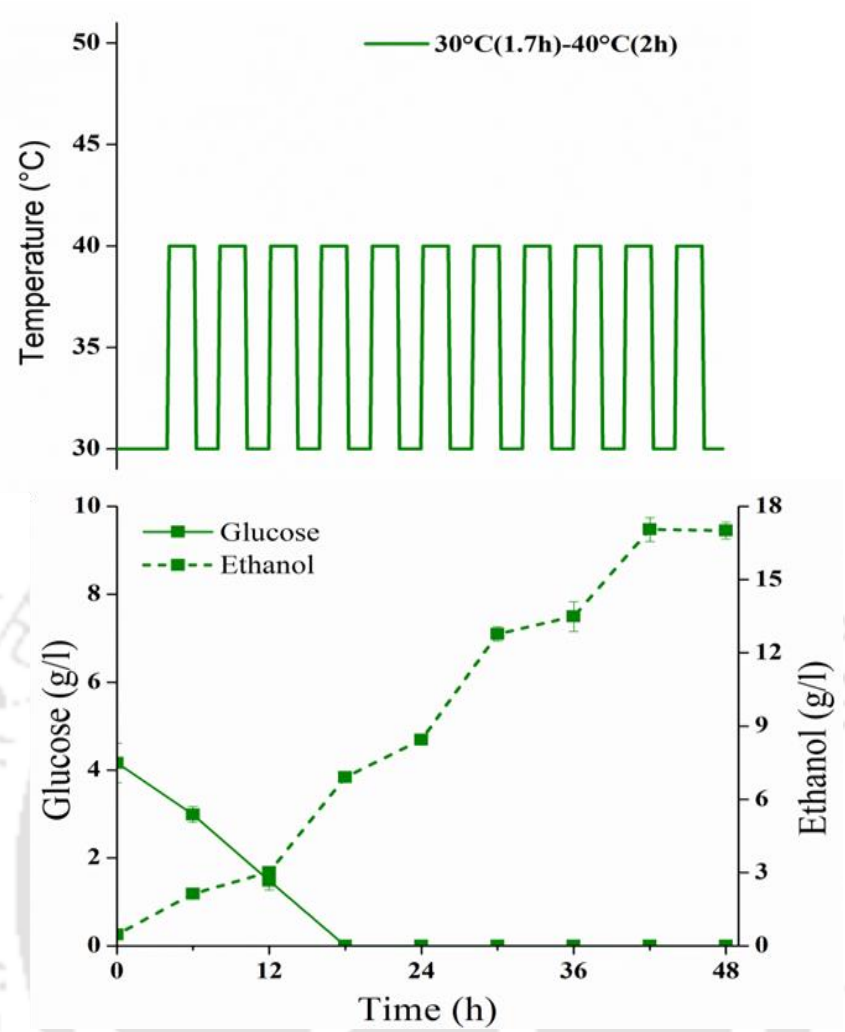

Fig.6.9: ICPSF by tri-culture of *T. reesei, P. janthinellum* and *S. cerevisiae* by cyclic shifting of temperature at optimised conditions at 10% w/v BAP RS feeding by cyclic shifting from 30ºC(1.7h)-40ºC(2h).

Table 6.2: *In situ* cellulase production, saccharification and fermentation (ICPSF) for bioethanol production from BAP-RS using poly-culture system of fungus and yeast.

Cyclic shifting of temperature	Ethanol titre (g/l)	Productivity(g/(l h))
Bi-culture of *P. janthinellum* & *P. stipitis*		
37°C	2.71±0.039	0.056
Prolong prehydrolysis and fermentation	5.27±0.136	0.219
Cyclic shifting of temperature strategy		
30°C(1h) 37°C(1h)	2.19 ±0.115	0.091
30°C(2h) 37°C(2h)	2.01±0.033	0.067
30°C(3h) 37°C(3h)	2.42±0.476	0.067
30°C(1h) 37°C(3h)	5.26±0.221	0.146
30°C(2h) 37°C(3h)	8.76±0.056	0.208
Tri-culture of *P. janthinellum, T. reesei* & *P. stipitis*		
37°C	3.72±0.068	0.077
Cyclic shifting of temperature strategy		
30°C(1h)-37°C(3h)	9.30±1.498	0.193
30°C(2h)-37°C(3h)	11.81±1.242	0.246
Tri-culture of *P. janthinellum, T. reesei* & *S. cerevisiae*		
37°C	4.59±0.083	0.095
Cyclic shifting of temperature strategy		
30°C (1.7 h)- 40°C(2h)	17.05±0.493	0.405

6.4. Conclusion

In the current study, a poly-culture system of both cellulolytic fungi and fermenting yeast were employed in order to maximise the *in situ* cellulase production, saccharification and fermentation (ICPSF) by utilising the pentoses and hexoses present in both cellulose and hemicellulose fraction of pretreated solid rice straw fibres. The ICPSF process carried out by employing the bi-culture system of *P. janthinellum* and *P. stipitis* by CSTS 30°C(2h)-37°C(3h) produced a maximum bioethanol titre of 8.76 g/l using BAP-RS (10% w/v). This was an increment of 1.6-fold bioethanol titre as compared to the prolong prehydrolysis and fermentation by above bi-culture system. The ICPSF by employing tri-culture system of *T. reesei*, *P. janthinellum* and *P. stipitis* further increased the bioethanol concentration by 1.3-fold to 11.8 g/l by applying CSTS 30°C(2h)-37°C(3h). However, the application of CSTS 30ºC(1.7h)-40ºC(2h) by utilising the tri-culture system of *T. reesei*, *P. janthinellum* and *S. cerevisiae* resulted in a maximum bioethanol titre of 17.05 g/l by utilising BAP-RS (10% w/v). In the present study, *P. stipitis* exhibits lower fermentation yields for both glucose and xylose compared to *S. cerevisiae*, the simultaneous use of both yeasts in the SSF process of pretreated rice straw fibers could reduce overall bioethanol yield. Furthermore, the xylose fraction released during enzymatic hydrolysis constitutes a minor portion. Even if a sequential fermentation approach were adopted, with *S. cerevisiae* utilized first followed by *P. stipitis* to metabolize glucose and xylose respectively, the impact on xylose to ethanol yield would likely be insignificant. The subsequent chapter details the implementation of a sequential fermentation strategy aimed at effectively utilizing the predominant fraction of xylose present in the pretreated rice straw hydrolysate.

Chapter 7

Bioethanol production from pretreated rice straw hemicellulosic hydrolysate by co-fermentation using *Saccharomyces cerevisiae* and *Pichia stipitis*

7.1. Background of the study

The major fraction of hemicellulosic sugars undergoes solubilisation in the acid pretreated hydrolysate during acid pretreatment (Vancov & McIntosh, 2011). However, for direct fermentation of acid pretreated hydrolysate, it must be concentrated to augment the soluble sugar fraction, thereby enhancing the bioethanol titre after yeast fermentation. To enhance ethanol titer, soluble sugars were concentrated by various processes such as evaporation method or membrane filtration (Dehkhoda, 2008; Pan et al., 2019). Evaporation methods takes longer processing time. While membrane filtration is costlier, and certain issues pertain such as membrane scaling, clogging or fouling. Another approach involves elevating biomass loading during dilute acid pretreatment to boost the recovery of sugar in the hydrolysate. Yet, surpassing a 10% w/v biomass loading makes it impractical to maintain a submerged condition in the pretreatment medium. It is crucial to maintain a submerged condition in order to obtain higher efficiency of pretreatment and for release of soluble sugars into the pretreated hydrolysate. This problem can be addressed by recycling the acid pretreated hydrolysate obtained after first pretreatment for subsequent pretreatment of fresh lignocellulose biomass without addition of further dilute acid solution. This process can concentrate the sugar in final hydrolysate, will limit the extra usage of acid and water for further pretreatment and will also help in cutting the cost of pretreatment.

A variety of yeast are utilised for bioethanol production from sugar sources i.e., glucose or xylose (Azhar et al., 2017). But different processes specifications need specific strain based on their properties such as tolerance to higher concentration of ethanol, inhibitors

release in pretreatment, temperature tolerance, type of sugar utilisation etc. *Pichia stipitis* is a yeast species that possesses the ability to ferment both glucose and xylose present in hydrolysate into ethanol (Buaban et al., 2010). In contrast, *Saccharomyces cerevisiae*, which is widely used for its high ethanol yield, can only ferment glucose (da Silva Fernandes et al., 2022). The ethanol yield from glucose is lower with *P. stipitis* compared to with *S. cerevisiae*. Therefore, to increase the ethanol yield co-culture of *S. cerevisiae* and *P. stipitis* is utilized in fermentation of hemicellulosic hydrolysate where both glucose and pentose are available (Shrivastava & Sharma, 2022; Wu et al., 2023). In yeast co-culture both the yeasts consume glucose in first phase and later when glucose is exhausted, P. *stipitis* can utilized the xylose. However, *S. cerevisiae* starts consuming ethanol as a carbon source during glucose starvation condition in the medium, thereby reducing the final ethanol titre (Panda & Maiti, 2024; Pham et al., 1998). To increase the ethanol yield, it is important to halt the growth of *S. cerevisiae* after glucose depletion in the medium. Also, in yeast co culture process both the yeasts consume glucose competitively in first phase and as the ethanol yield generally low with *P. stipitis*, it decreases the total ethanol yield. Sequential culture where first fermentation by only *S. cerevisiae* followed by deactivation of *S. cerevisiae* and fermentation of xylose by *P. stipitis* can improve the ethanol yield.

The current study focuses on concentrating the hemicellulosic sugar in the rice straw pretreated hydrolysate by recycling the acid stream for the subsequent pretreatment of fresh delignified rice straw. The bioethanol fermentation was carried out using the base followed by acid pretreated hydrolysate for primary, secondary and tertiary recycled stream. The glucose fermentation was carried out using *S. cerevisiae* NCIM 3594, while both glucose and xylose fermentation in the pretreated rice straw hydrolysate stream was carried out by *P. stipitis* NCIM 3497 separately. Additionally, a co-culture approach was utilised to study the co-fermentation of both pentose and hexose sugars. For further improvement of bioethanol

production, a sequential culture approach was applied to initially metabolise glucose using *S. cerevisiae* followed by an intermediate heat inactivation and further xylose fermentation using *P. stipitis*.

7.2. Materials and methods

7.2.1. Pretreatment of rice straw

For delignification of the rice straw, base pretreatment was carried for biomass loading (10% w/v) with 2% NaOH at 121 °C, 15 psi for 30 minutes. Further, muslin cloth was used to filter out the black liquor by hand pressing. For neutralisation purpose, the solid fibers were washed with tap water until the substituted black liquor was removed. For extraction of hemicellulosic sugar, the base pretreated solid rice straw fibres (BP-RS) with 10% w/v solid loading were then subjected to acid pretreatment with 2% H_2SO_4 at 121 °C, 15 psi for 30 minutes. After acid pretreatment the liquid hydrolysate was filtered out by using muslin cloth and pH was adjusted to pH 6 prior to the fermentation.

7.2.2. Recycling of acid pretreated hydrolysate to concentrate hemicellulosic sugar

To concentrate the sugar in the hydrolysate, dil. H_2SO_4 stream was recycled for acid pretreatment of rice straw **(Fig. 7.1).** Initially, the BP-RS fibre (10% w/v) was hydrothermally pretreated with 2% H_2SO_4 at 121 °C, 15 psi for 30 minutes. Then, the hydrolysate was filtered out by hand press through muslin cloth. The collected pretreated hydrolysate after initial pretreatment was annotated as BAP-1 RS hydrolysate. This BAP-1 RS hydrolysate was further reused for the pretreatment of fresh delignified rice straw fibres (10% w/v) without adding fresh acid. The liquid pretreated hydrolysate was filtered through muslin cloth, collected, and termed as BAP-2 RS hydrolysate. The process was repeated, where BAP-2 RS hydrolysate was utilised for pretreatment of fresh BP-RS fibres (10% w/v).

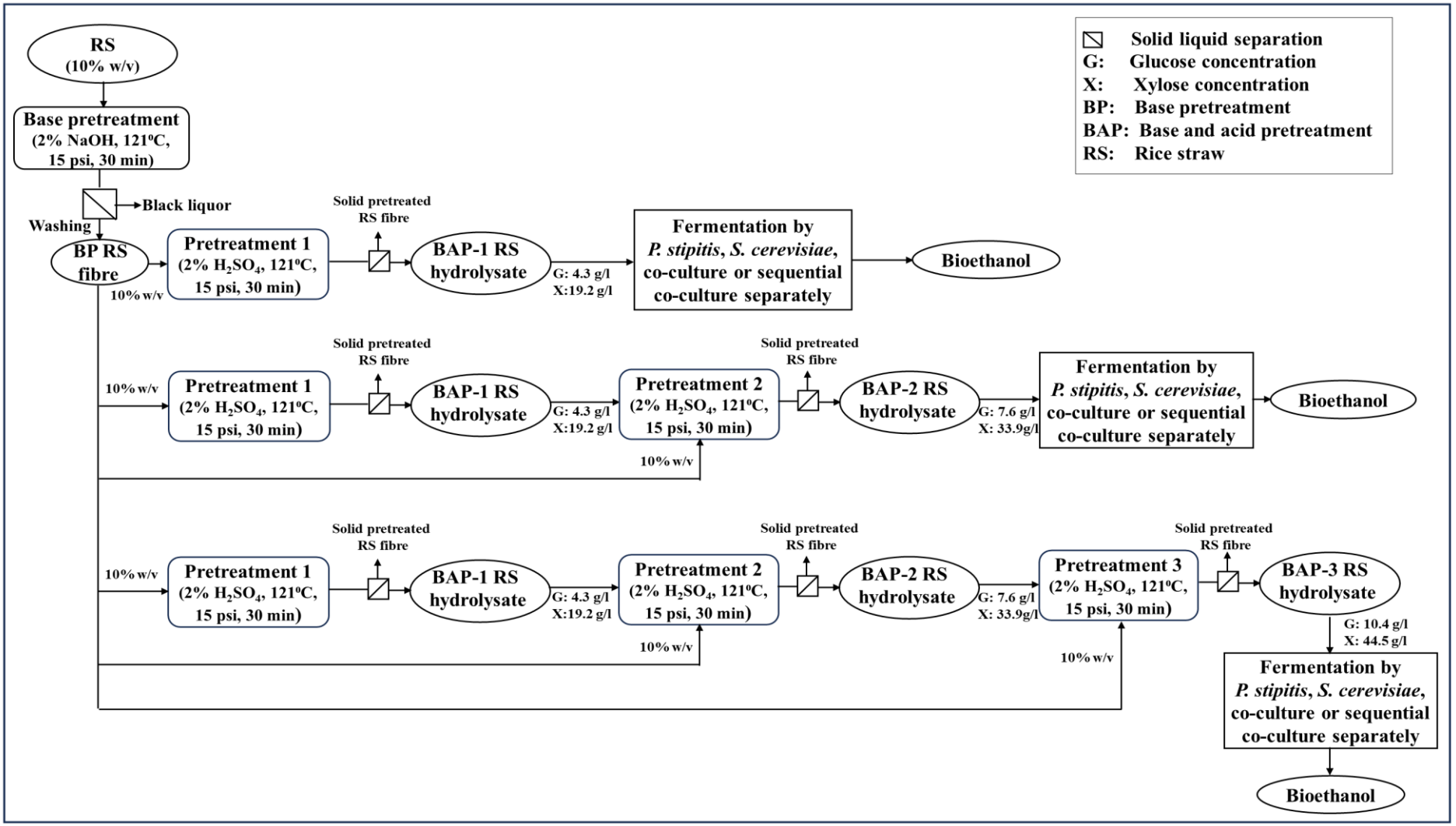

Fig.7.1: Process flowchart for reuse of BAP-RS hemicellulosic hydrolysate for bioethanol production.

The pretreated hydrolysate was filtered out using muslin cloth and named as BAP-3 RS hydrolysate. The reuse of acidic-hydrolysate for pretreatment of BP-RS fibres helped in accumulation of sugar in each pretreatment step. The pH of the collected BAP RS hydrolysates was adjusted to pH 6 prior to the fermentation both the yeast *S. cerevisiae* and *P. stipitis*.

7.2.3. Growth of yeast in pure sugar medium

Both the yeast *S. cerevisiae* and *P. stipitis* was grown in pure sugar medium to study the growth and ethanol production capabilities. Initially, preculture of *S. cerevisiae* (5 % v/v) was inoculated into 100 ml of pure glucose medium containing glucose: 20 g/l, yeast extract: 5 g/l, and yeast mineral salt medium containing $MgSO_4$: 0.51 g/l, $CaCl_2$: 0.11 g/l, KH_2PO_4:1 g/l, NaCl:0.11 g/l, $ZnSO_4$:0.00041 g/l, $FeCl_3$:0.00021 g/l, $CuSO_4 \cdot 5H_2O$: 0.00051 g/l. Similarly, the preculture of *P stipitis* (5 % v/v) was inoculated into three different cultures media separately containing glucose: 20 g/l, xylose: 20 g/l and a mixed sugar medium containing both glucose: 20 g/l and xylose: 20 g/l each. Each medium was supplemented with yeast extract: 5 g/l, and yeast mineral salt medium as described above. The initial pH of all the culture mediums were adjusted to pH 6.5, culture temperature was maintained at 30 °C with an agitation speed of 180 rpm. The culture period was kept for 32h while for mixed sugar medium 44h until the complete sugar depletion in the medium. The samples were collected at various time interval to measure the cell growth via an UV- Vis spectrophotometer at an absorbance of 600 nm. Further, samples were centrifuged at 10,000 rpm for 5 min, the collected supernatants were analysed for quantification of sugar and ethanol using HPLC.

7.2.4. Fermentation of pretreated rice straw hydrolysate for bioethanol production

To carry out the bioethanol production from pretreated RS hydrolysates i.e., BAP-1 RS hydrolysate and recycled hydrolysate streams i.e., BAP-2 RS hydrolysate, BAP-3 RS hydrolysate were supplemented with yeast extract: 5 g/l and yeast mineral salt medium mentioned above. To evaluate the fermentation by individual yeasts, *S. cerevisiae* (5% v/v), *P. stipitis* (5% v/v) were inoculated separately into the all three streams of BAP RS hydrolysates. Additionally, to evaluate the bioethanol production in co-culture, both the yeast *S. cerevisiae* (5% v/v) and *P. stipitis* (5% v/v) were inoculated simultaneously into the primary pretreated hydrolysates as well as recycled pretreated RS-hydrolysates. Furthermore, a sequential culture was studied, where the fermentation process was initiated by inoculating *S. cerevisiae* (5% v/v) into the BAP RS hydrolysates. After the depletion of glucose in the medium, the shake flasks were kept at 50 °C for 30 minutes to halt the growth of *S. cerevisiae*. Then, it was cooled down to the normal temperature and further *P. stipitis* (5% v/v) was inoculated into the medium for bioethanol production from xylose. The samples were collected at different time interval, centrifuged at 10,000 rpm, the supernatant was collected and stored at 4 °C for further estimation of sugar and ethanol by HPLC.

7.3. Results and discussion

7.3.1. High sugar recovery in recycled acid pretreated hydrolysate

After the base pretreatment, the delignification of rice straw (10% w/v) resulted in the recovery of approximately 51.7% w/w solid base-pretreated rice straw (BP-RS). The further acid pretreatment of BP-RS (10% w/v) with 2% H_2SO_4, where 78.5% v/v BAP-1 RS hydrolysate was recovered. The reuse of BAP-1 RS hydrolysate for the successive pretreatment of BP-RS (10% w/v) resulted in the recovery of 72.8% v/v BAP-2 RS hydrolysate. In the next pretreatment by recycling the BAP-2 RS hydrolysate stream for pre-

treating the BP-RS (10% w/v) resulted in the recovery of approximately 67.5 % BAP-3 RS hydrolysate. The total sugar released in BAP-1 RS hydrolysate after first pretreatment of rice straw is 23.5 g/l (Glucose: 4.3 g/l, Xylose: 19.2 g/l), after second pretreatment it was increased to 1.8-fold to 41.5 g/l (Glucose: 7.6 g/l, Xylose: 33.9 g/l) in BAP-2 RS hydrolysate, and after third pretreatment the total sugar increased to 54.9 g/l (Glucose: 10.4 g/l, Xylose: 44.5 g/l) in BAP-3 RS hydrolysate **(Fig. 7.2 A).** Similarly, the total inhibitor concentration released as a byproduct of acid pretreatment in BAP-1 RS hydrolysate present is 1.89 g/l (Formic acid:0.64 g/l, Acetic acid: 0.83 g/l, HMF: 0.02 g/l and Furfural: 0.4 g/l) **(Fig.7.2 B).** In successive secondary pretreatment, the total inhibitor concentration increased by 98.4% in BAP-2 RS hydrolysate to 3.75 g/l (Formic acid: 1.2 g/l, Acetic acid: 1.76 g/l, HMF: 0.04 g/l

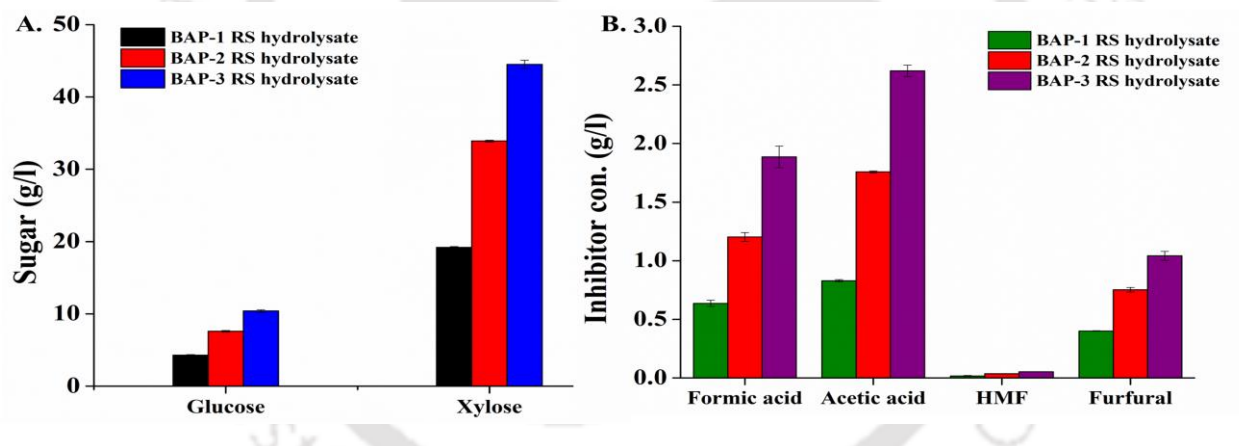

Fig.7.2: The concentration of **A.** sugar (i.e., glucose and xylose) **B.** inhibitory by-products (i.e., formic acid, acetic acid, HMF, furfural) in the pretreated hydrolysate BAP-1 RS hydrolysate, BAP-2 RS hydrolysate, BAP-3 RS hydrolysate.

and Furfural: 0.75 g/l) **(Fig.7.2 B).** In the tertiary pretreatment using BAP-2 RS hydrolysate, the total inhibitor concentration increased by 49.3 % in BAP-3 RS hydrolysate to 5.6 g/l (Formic acid: 1.89 g/l, Acetic acid: 2.62 g/l, HMF: 0.05 g/l and Furfural: 1.04 g/l) **(Fig.7.2 B).**

7.3.2. Ethanol production by *S. cerevisiae* and *P. stipitis* in pure sugar medium

In the case of *S. cerevisiae* cultured in glucose medium (20 g/l), a maximum bioethanol titre of 8.84 g/l was achieved with a glucose to ethanol yield of 0.46 and a productivity of 0.736 g/(l h) **(Fig.7.3 A).** The glucose depletion occurred within 12 h, after which ethanol was consumed as a part of carbon source at a rate of 0.165 g/(l h). The overall biomass yield was 6.15 g/l with a biomass productivity of 0.256 g/(l h) **(Fig.7.3 A).** For *P. stipitis*, cultivation in pure glucose medium (20 g/l), resulted in a maximum bioethanol titre of 6.37 g/l with glucose to ethanol yield of 0.31 and productivity of 0.531 g/(l h) **(Fig. 7.3 B).** Comparing the sugar to ethanol conversion efficiency, the ethanol yield of *S. cerevisiae* from glucose was 1.5-fold higher than that of *P. stipitis* in glucose. When xylose medium (20 g/l) was used, the resulted

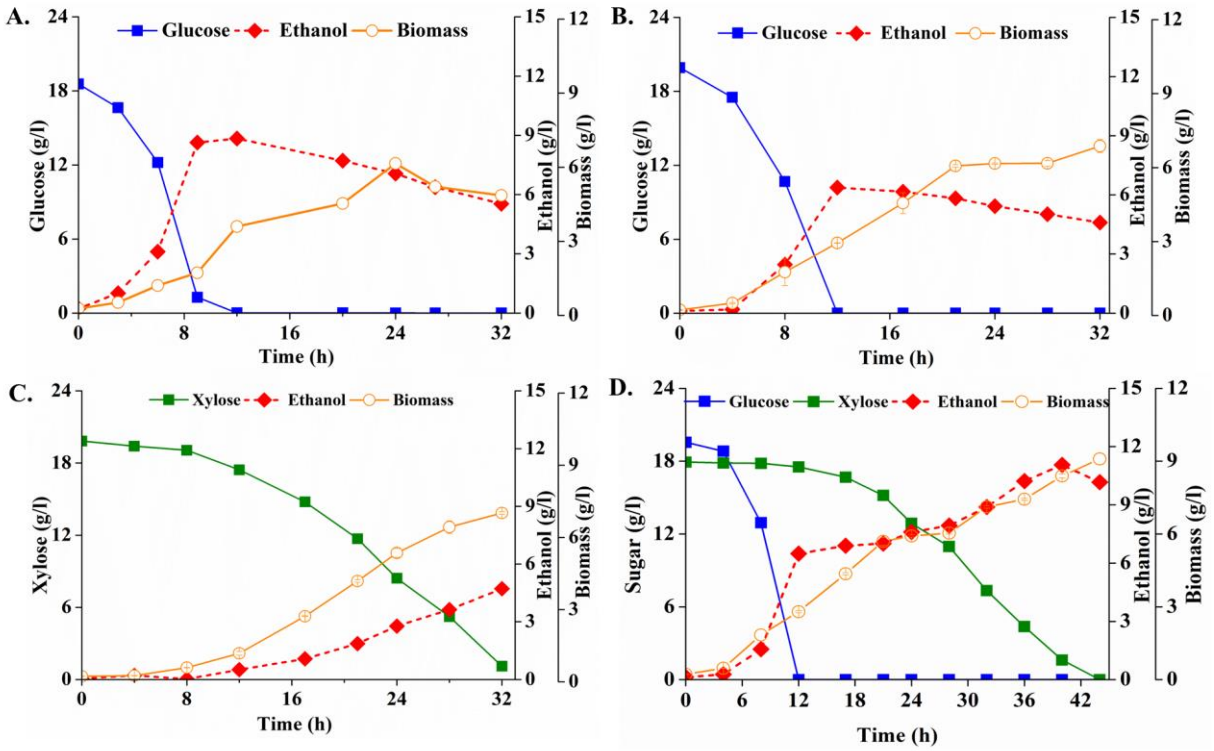

Fig.7.3: Yeast ethanol production using pure sugar medium **A.** *S. cerevisiae* growth and ethanol production in pure glucose medium. *P. stipitis* growth and ethanol production in **B.** pure glucose medium, **C.** pure xylose medium, **D.** mixed sugar medium containing both glucose and xylose.

maximum bioethanol titre was decreased to 4.72 g/l with xylose to ethanol yield of 0.24 and a productivity of 0.147 g/(l h) **(Fig. 7.3 C).** Cultivation in a mixed sugar medium containing glucose and xylose (each at 20 g/l) yielded a maximum bioethanol titre of 11.06 g/l with a total sugar to ethanol yield of 0.29 and a productivity of 0.276 g/(l h) **(Fig. 7.3 D).** The overall biomass yield for *P. stipitis* in glucose, xylose and mixed sugar medium was 6.87 g/l, 7.01 g/l, 9.09 g/l respectively, with corresponding productivity of 0.214 g/(l h), 0.212 g/(l h), 0.222 g/(l h) respectively.

7.3.3. Fermentation of glucose present in pretreated rice straw hydrolysate by *S. cerevisiae*

The fermentation by *S. cerevisiae* in all the pretreated rice straw hydrolysate shows that only glucose was being consumed, while xylose remain unutilised throughout the fermentation period **(Fig.7.4).** The maximum bioethanol titre obtained by fermentation of BAP-1 RS hydrolysate was 1.71 g/l from 4.3 g/l of glucose, with glucose to ethanol yield ($Y_{E/S}$) of 0.39 and a productivity of 0.285 g/(l h) **(Table 7.1).**

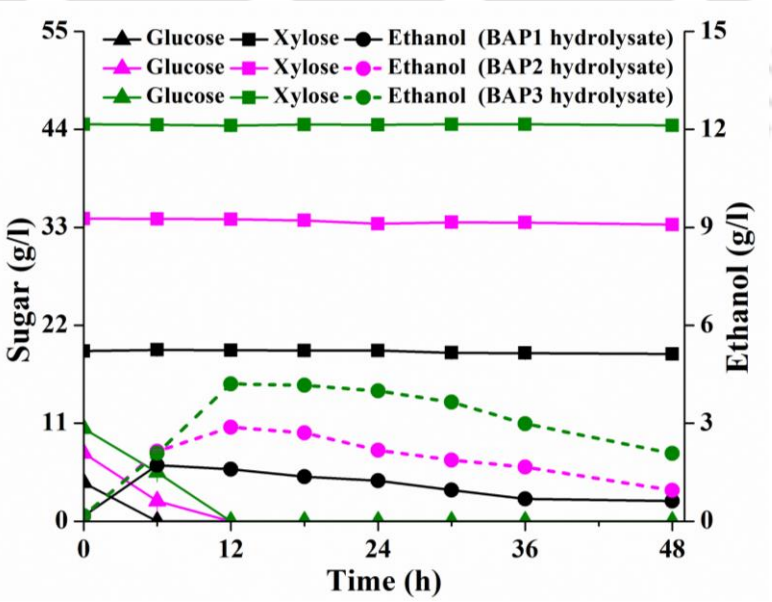

Fig.7.4: Bioethanol production from BAP-RS hemicellulosic hydrolysate by using *S. cerevisiae.*

Fermentation in BAP-2 RS hydrolysate resulted in 1.7-fold increment of ethanol titre to 2.88 g/l with an $Y_{E/S}$ of 0.38 and productivity of 0.24 g/(l h) respectively. Further utilisation of BAP-3 RS hydrolysate for fermentation led to a final bioethanol titre of 4.21 g/l by utilising the 7.6 g/l of glucose present in the medium, with an $Y_{E/S}$ of 0.40 and productivity of 0.35 g/(l h) respectively. There are no significant changes in the glucose-to-ethanol yield resulting from increased inhibitor levels due to the reuse of pretreated hydrolysates.

The fermentation of BAP-3 RS hydrolysate improved the bioethanol titre by 2.5-fold and 1.5-fold as compared to BAP-1 and BAP-2 RS hydrolysate respectively. After the depletion of glucose in the medium, ethanol was consumed as a part of carbon source by *S. cerevisiae* at a rate of 0.026 g/(l h), 0.053 g/(l h), 0.059 g/(l h) in BAP-1, BAP-2 and BAP-3 RS hydrolysates respectively. The diauxic growth pattern of *S. cerevisiae* for consumption of glucose and subsequent utilisation of the product ethanol in a glucose starvation condition with an intermediate lag phase was observed earlier (Beck & Von Meyenburg, 1968; Jones & Kompala, 1999; Woehrer & Roehr, 1981).

7.3.4. Bioethanol production from glucose and xylose by *P. stipitis* using pretreated rice hydrolysate

It was observed that *P. stipitis* was able to ferment both glucose and xylose present in the pretreated rice straw hydrolysates. Preferentially, the fermentation initiated with the consumption of glucose, and once glucose was depleted from the medium, xylose was subsequently utilized **(Fig. 7.5).** The fermentation of hemicellulosic sugars present in the BAP-1 RS hydrolysate i.e., glucose: 4.3 g/l, xylose: 19.2 g/l, the maximum bioethanol titre reached was 4.86 g/l with a total sugar (TS) to ethanol yield ($Y_{E/TS}$) of 0.21 and a productivity of 0.162 g/(l h). While the fermentation of BAP-2 RS hydrolysate resulted in a 1.6-fold improvement in the bioethanol titre to 7.62 g/l, maintaining a $Y_{E/TS}$ of 0.18 and a productivity

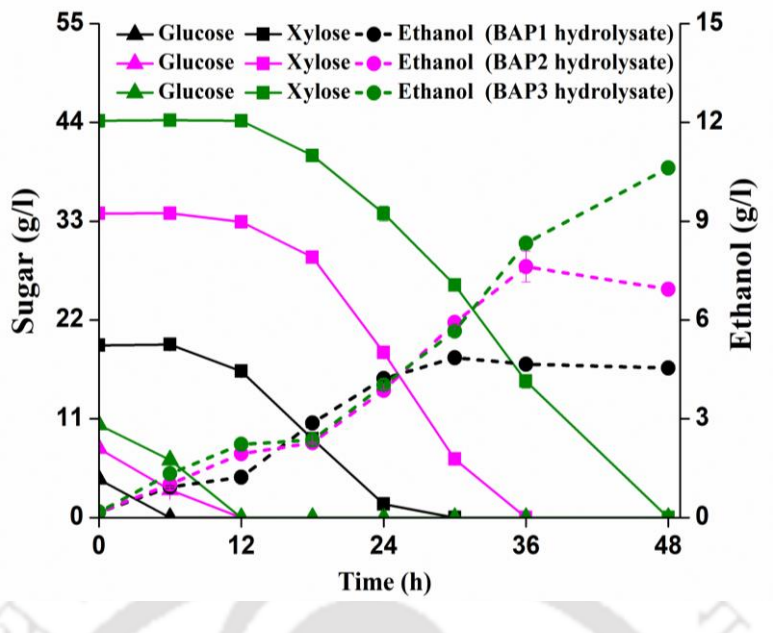

Fig. 7.5: Bioethanol production from BAP-RS hemicellulosic hydrolysate by using *P. stipitis.*

of 0.211 g/(l h). Subsequent fermentation of BAP-3 RS hydrolysate, increased the bioethanol titre to 10.62 g/l, achieving a $Y_{E/TS}$ of 0.19 and a productivity of 0.221 g/(l h) **(Table 7.1, Fig.7.5)**. Notably, the final bioethanol titre in BAP-3 RS hydrolysate showed a 2.2-fold increment as compared to ethanol titre obtained in BAP-1 RS hydrolysate and a 1.4-fold increment as compared to ethanol titre obtained by fermentation of BAP-2 RS hydrolysate. The bioethanol yield from glucose ($Y_{E/S}$) at the time of glucose depletion in the medium 0.22, 0.25 and 0.21 in BAP-1, BAP-2, and BAP-3 RS hydrolysates, respectively **(Fig.7.5).** Note that, the increase in the inhibitor concentration in the BAP- RS hydrolysates due to successive pretreatment has no significant effect on the sugar to bioethanol yield by *P. stipitis* across all three fermentation experiments. The glucose conversion into bioethanol by *P. stipitis* is significantly lower as compared to the ethanol yield obtained by fermentation through *S. cerevisiae* in all the BAP RS hydrolysates. In a study by Kaur et. al, 2019, a fermentation process carried out using acid pretreated rice straw hydrolysate by employing *P. stipitis* NCIM 3499, resulted in a bioethanol titre of 9.4 g/l (Kaur & Kuhad, 2019).

7.3.5. Co-fermentation of pretreated rice straw hydrolysates

The fermentation by *S. cerevisiae* has high glucose to ethanol yield as compared to fermentation by *P. stipitis*, a co-culture approach was applied to increase sugar conversion to ethanol from the BAP-RS hydrolysate. The maximum bioethanol titre obtained by co-fermentation were 5.23 g/l, 9 g/l, 11.28 g/l in BAP-1, BAP-2 and BAP-3 RS hydrolysates respectively, an increment of 1.2-fold, 1.2-fold and 1.1-fold respectively as compared to the sole fermentation by *P. stipitis* **(Table 7.1, Fig.7.6).** The ethanol yield from total sugar ($Y_{E/TS}$) were as followed 0.22, 0.23, 0.21 with a bioethanol productivity of 0.217 g/(l h), 0.25 g/(l h), 0.235 g/(l h) by co-fermentation utilising BAP-1, BAP-2 and BAP-3 RS hydrolysates respectively. Following the exhaustion of total sugars in the medium, the bioethanol concentration started declining at a rate of 0.104 g/(l h) and 0.145 g/(l h) in BAP-1 and BAP-2 RS hydrolysates respectively **(Fig.7.6).** This decrease could be attributed to the ethanol consumption by *S. cerevisiae* after depletion of glucose. Notably, this decline became evident only after the depletion of total sugars in the medium, as *P. stipitis* continued producing

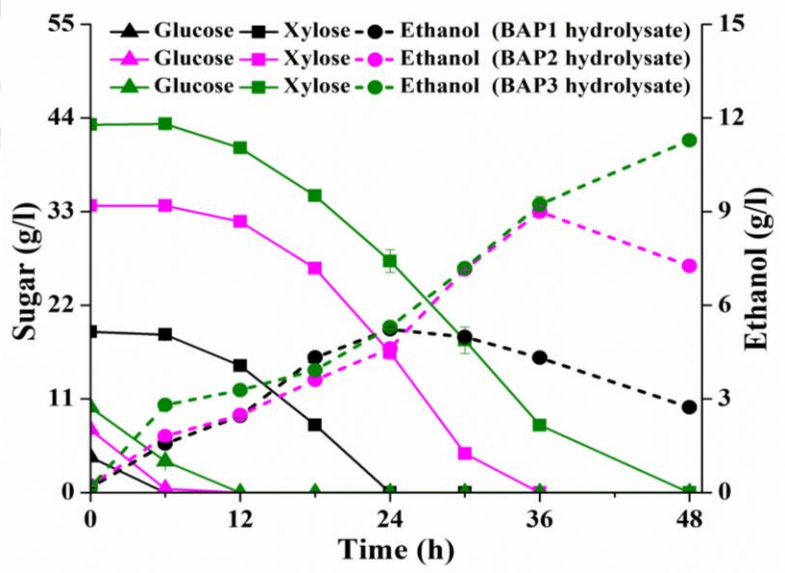

Fig.7.6: Co-fermentation of glucose and xylose present in BAP-RS hemicellulosic hydrolysate by *P. stipitis* and *S. cerevisiae.*

bioethanol from xylose even under glucose-starvation conditions in the medium. Therefore, it is important to devise a cultivation strategy to further enhance bioethanol titres in co-fermentation systems. In a study conducted by Yadav et al., 2011, the sugar recovered from dilute sulphuric acid-pretreated rice straw hydrolysate was concentrated through vacuum distillation followed by detoxification and co-fermentation of detoxified hydrolysate by *S. cerevisiae* and *P. stipitis* resulted in a maximum bioethanol titre of 12 g/l (Yadav et al., 2011).

7.3.6. Sequential co-fermentation with an intermediate heat inactivation to improve bioethanol production

The tendency of *S. cerevisiae* to consume bioethanol during glucose scarcity in the culture medium prompted the need to halt its growth post-glucose depletion in the co-culture study. At the same time, *P. stipitis* has lower glucose to ethanol yield. Therefore, to maximise the sugar conversion and reduce the competition for glucose in a co-fermentation approach using

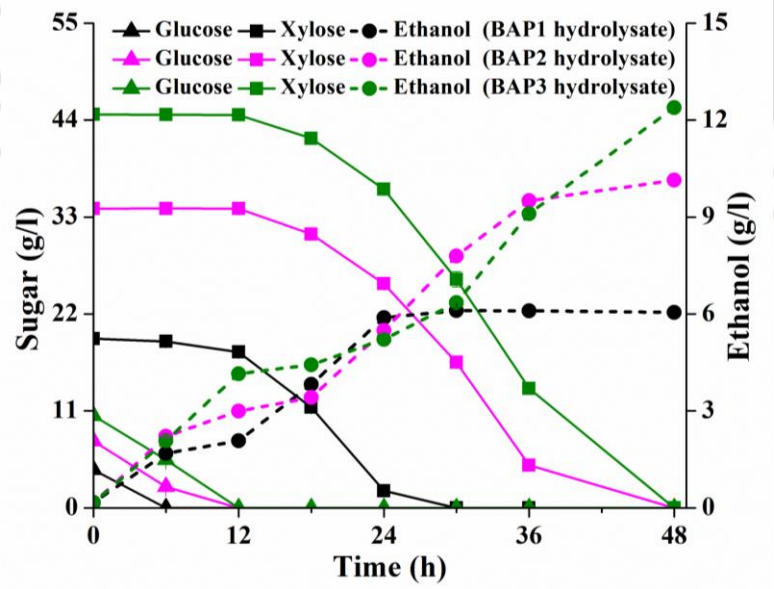

Fig.7.7: Sequential co-fermentation where glucose fermentation by *S. cerevisiae* followed by *P. stipitis* fermentation of xylose with an intermediate heat inactivation at 50 °C.

Table 7.1: Bioethanol production from acid pretreated rice straw hemicellulosic hydrolysate using *S. cerevisiae*, *P. stipitis*, co-culture and sequential co-fermentation.

	BAP1 RS hydrolysate			BAP2 RS hydrolysate			BAP3 RS hydrolysate		
	Ethanol titre (g/l)	**Ethanol Yield**	**Ethanol Productivity (g/(l h))**	**Ethanol titre (g/l)**	**Ethanol Yield**	**Ethanol Productivity (g/(l h))**	**Ethanol titre (g/l)**	**Ethanol Yield**	**Ethanol Productivity (g/(l h))**
S. cerevisiae	1.71±0.04	0.39	0.285	2.88±0.03	0.38	0.24	4.21±0.07	0.40	0.35
P. stipitis	4.86±0.13	0.21	0.162	7.62±0.47	0.18	0.211	10.62±0.07	0.19	0.221
Co-culture	5.23±0.07	0.23	0.217	9±0.19	0.22	0.25	11.28±0.14	0.21	0.235
Sequential co-fermentation	6.10±0.09	0.26	0.203	10.14±0.1	0.24	0.211	12.39±0.14	0.22	0.258

* The ethanol yield from S. cerevisiae fermentation is determined based on the ethanol-to-glucose yield, while in other experiments, the yield is calculated as ethanol obtained from the total consumed sugars (both glucose and xylose).

Note: Experimental data is presented as mean ± standard deviation of duplicate experiment.

S. cerevisiae and *P. stipitis*, a sequential culture method was applied. In this approach, initially *S. cerevisiae* was inoculated for glucose fermentation. After glucose depletion in the pretreated rice straw hydrolysate medium, growth was arrested through a 30-minute heat treatment at 50 °C and then after temperature was brought down to 30 °C for further fermentation of xylose by *P. Stipitis*. In BAP-1 RS hydrolysate, the initial glucose concentration of 4.3 g/l was completely consumed by *S. cerevisiae* within 6 h of cultivation period. After the thermal treatment, *P. stipitis* was inoculated to ferment the remaining 19.2 g/l of xylose **(Fig.7.7).** This process resulted in a maximum bioethanol titre of 6.1 g/l at 30 h, with $Y_{E/TS}$ of 0.26 and productivity of 0.203 g/(l h) respectively **(Table 7.1, Fig. 7.7).** Similarly, the initial fermentation by *S. cerevisiae* in BAP-2 and BAP-3 RS hydrolysate medium, glucose was completely utilised within 12 h. Subsequently, an intermediate heat treatment was applied, followed by fermentation of remaining xylose by *P. stipitis*. The above sequential culture approach resulted in a maximum bioethanol 10.14 g/l, with a yield of 0.24 and a productivity of 0.211 g/(l h) in BAP-2 RS hydrolysate and 12.39 g/l bioethanol titre with an ethanol yield from total sugar of 0.22 and a productivity of 0.258 g/(l h) in BAP-3 RS hydrolysate **(Table 7.1, Fig. 7.7).** When comparing the maximum bioethanol yield achieved in BAP-3 RS hydrolysates, the sequential culture with intermediate heat inactivation led to a 2.9-fold improvement in bioethanol titre compared to individual yeast fermentation using *S. cerevisiae*. Additionally, compared to sole fermentation by *P. stipitis*, it resulted in a 1.2-fold increase in bioethanol titre.

7.4. Conclusion

The utilisation of acidic hydrolysate to augment hemicellulose sugar fraction from rice straw presents a promising method to maximize whole sugar conversion potential from lignocellulosic biomass. In the current study, the reuse of acidic hydrolysate after initial

pretreatment for subsequent pretreatment of delignified rice straw helped in recovery of glucose up to 10.4 g/l and xylose 44.5 g/l in BAP-3 RS hydrolysate. By employing a sequential co-fermentation process using *S. cerevisiae* and *P. stipitis* with an intermediate heat inactivation at 50 °C resulted in a maximum bioethanol titre of 12.39 g/l with $Y_{E/TS}$ of 0.22, productivity of 0.258 g/(l h). The resultant bioethanol titre improved by 2.9-fold and 1.2-fold as compared to the compared to sole *S. cerevisiae* and *P. stipitis* fermentation respectively. The study emphasizes the viability of recycling acidic hydrolysate for multiple pretreatment and concentrating hemicellulosic sugar at each stage, thereby boosting fermentation yield from whole sugar. Moreover, the sequential culture approach for selectively fermenting glucose and xylose from the concentrated hemicellulosic sugars offers a promising avenue for sustainable bioethanol production.

Chapter-8

Conclusion

The use of lignocellulosic bioethanol as a second-generation biofuel remains promising for environmental safety, and sustainability. However, challenges persist in the multistep biochemical conversion process, comprising pretreatment, enzymatic hydrolysis, and fermentation, demanding solutions to enhance economic viability and efficiency. Addressing these challenges involves achieving low-cost, efficient pretreatment while minimizing by-product generation and its subsequent detoxification. Optimizing temperature specifications in simultaneous saccharification and fermentation (SSF) processes stands critical for maximizing both saccharification and fermentation rates. The current research work focused on the development of an acid pretreatment strategy for rice straw aiming at cellulase production using fungus. Further, an *in situ* cellulase production, saccharification, and fermentation (ICPSF) was employed with a combination of fungal cultures and yeast. To enhance bioethanol production, a novel cyclic shifting of temperature strategy has been developed and implemented throughout the SSF process. Moreover, to fully utilize both pentose and hexose sugars present in the hemicellulose fraction, a co-fermentation strategy using specific yeast species in the pretreated hydrolysate has been devised. This comprehensive approach ensured the optimal utilization of the entire sugar content found in rice straw for bioethanol production.

The acid pretreatment strategy for rice straw was aimed at maximising sugar yield while minimising inhibitor production in the resulting pretreated hydrolysate. From pretreatment optimisation, 2% H_2SO_4 with 30 min of autoclave time at 121ºC, 15 psi was chosen as best condition for cellulase production experiments. Two fungal cultures, *T. reesei* and *P. janthinellum*, were selected for their diverse enzymatic profiles i.e., endoglucanase,

exoglucanase, and beta-glucosidase enzymes crucial for hydrolysis of cellulose. The cellulase production process were optimised for individual cultures as well co-cultures in shake flasks by considering the key factors such as substrate loading, incubation temperature and medium pH. The optimum conditions obtained in shake flask studies were further applied for cellulase production in the controlled bioreactor condition. Additionally, the study revealed the potential of these fungi for detoxifying pretreatment by-products, utilizing them as part of their carbon source. In the bioreactor studies, the maximum cellulase production in the co-culture under optimal conditions (3% w/v rice straw loading, medium pH 5, 30ºC, agitation speed: 250 rpm, air flow rate: 1VVM) yielded: FPase: 1.16 IU/ml, CMCase: 27.68 IU/ml, beta-glucosidase: 4.63 IU/ml, and xylanase: 45.60 IU/ml, respectively.

Post cellulase production optimisation, bioethanol production were carried out by *in situ* cellulase production, saccharification and fermentation (ICPSF) process by utilising the fungus *P. janthinellum* and yeast *S. cerevisiae*. Prior to SSF process, the thermotolerance of yeast were explored prior both at constant incubation temperature (ranging from 30ºC to 40ºC) and cyclic shifting of temperature between 30ºC to 50ºC in the pure glucose medium. The optimum cyclic shifting of temperature strategies (CSTS) which supported growth and ethanol fermentation were further applied to perform the SSF process. The cyclic shifting of temperature at 30ºC(2h)- 40ºC(2h) resulted in a maximum bioethanol titre of 14.98 g/l using BAP-RS (10% w/v). To increase productivity, the holding time were tuned further and tuned CSTS 30ºC(1.7h)- 40ºC(2h) resulted in increment of bioethanol titre to 15.9 g/l.

Further, to increase the bioethanol production from solid pretreated rice straw fibres in the ICPSF process, a poly-culture system of fungus and yeast were employed by utilising the cyclic shifting of temperature strategy. The co-culture of fungi *P. janthinellum* and *T. reesei* was employed to increase the cellulase activities in the crude cellulase broth. To maximise both pentose and hexose fermentation as well as to utilise the sugar conversion

efficiency both *S. cerevisiae* and *P. stipitis* was utilised. The yeast *P. stipitis* was introduced for the SSF process to utilise both the glucose and xylose present in the pretreated rice straw fibres. However, the lower sugar to ethanol yield of *P. stipitis* restricted the maximum bioethanol titre to 11.81 g/l in the ICPSF using tri-culture system of *P. janthinellum*, *T. reesei* and *P. stipitis* using CSTS 30ºC(2h)-37ºC(3h). Similarly, the application of CSTS 30ºC(1.7h)-40ºC(2h) for SSF process using *S. cerevisiae* in the ICPSF using tri-culture system of *P. janthinellum*, *T. reesei* and *S. cerevisiae* resulted in the maximum bioethanol titre of 17.05 g/l from BAP-RS (10% w/v).

The xylose fraction present in solid pretreated rice straw fibres (BAP-RS) in minor amount, the rest of the hemicellulosic sugar fraction which mostly contain majority of xylose released in the hydrolysate during acid pretreatment and left unutilised. However, lower sugar content in the acid pretreated hydrolysate resulted in low ethanol titres. Current methods to concentrate hemicellulosic sugars in the hydrolysate involves time-consuming evaporation techniques that result in water wastage. Additionally, using rice straw hydrolysate in nano-filtration processes can lead to clogging issues. In the current research, the acid pretreated hydrolysate obtained after pretreatment of delignified rice straw were further recycled to do the further acid-pretreatment of fresh delignified rice straw. The acid pretreated hydrolysate obtained after successive pretreatment concentrated the hemicellulosic sugar. A sequential co-fermentation of *S. cerevisiae* and *P. stipitis* with an intermediate heat inactivation in the acid pretreated hydrolysates (BAP-3 hydrolysate) resulted in a maximum bioethanol titre of 12.39 g/l of bioethanol. This strategy facilitated a high initial glucose conversion rate by *S. cerevisiae*, with the subsequent heat inactivation preventing *S. cerevisiae* from consuming ethanol during glucose scarcity. *P. stipitis* was then able to ferment the remaining xylose, contributing to the overall ethanol production.

Future prospects

The cyclic shifting of temperature strategy (CSTS) for simultaneous saccharification and fermentation (SSF) process, as developed and implemented in the present study, can be applied with commercial cellulase for enhanced saccharification. The CSTS exhibits potential for significantly increase the bioethanol titres, necessitating further optimization for upscaling operations from laboratory to pilot or industrial scale, thereby assessing its feasibility for large-scale bioethanol production. The prospect of introducing an *in-situ* bioethanol removal process serves as a plausible extension to the current investigation. This extension aims to optimize for production of high bioethanol titres by reducing ethanol toxicity in batch process, thereby enhancing process efficiency. Furthermore, the implementation of a fed-batch study, holds potential for significantly improving productivity.

A comprehensive Life Cycle Analysis (LCA) and economic assessment of the *in situ* cellulase production, saccharification, and fermentation (ICPSF) utilizing the CSTS for SSF can be explored. Such analyses will provide an evaluation of the overall sustainability of the process, especially in the context of its potential application at an industrial scale. Additionally, the conceptualization and development of a lignocellulose biorefinery on a broader scale represent an avenue for future research. This approach will be helpful for the utilization of waste streams generated during bioethanol production, such as lignin extraction and the remaining waste slurry post-bioethanol extraction for purposes like biogas production. The comprehensive assessment of the economic and environmental viability of such a biorefinery model will not only validate the current research but also offer valuable insights into its commercial potential.

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Appendices

1. Preparation of glucose standards for estimation of Filter paper (FPase) activity

A stock solution of anhydrous glucose (10 mg/ml) was prepared and diluted further with following dilutions.

Dilutions

100 µl glucose standard +50 µl citrate buffer =1:1.5 (0.67 mg/100 µl)

100 µl glucose standard+ 100 µl citrate buffer =1:2 (0.5 mg/100 µl)

100 µl glucose standard+ 200 µl citrate buffer =1:3 (0.33 mg/100 µl)

100 µl glucose standard+ 400 µl citrate buffer =1:5 (0.20 mg/100 µl)

Preparation of Spectro zero

300 µl citrate buffer
600 µl DNS reagent
Incubate for 5 minutes in boiling water bath and measure against spec 0.

Preparation of standards

100 µl standard
200 µl citrate buffer
600 µl DNS reagent
Incubate for 5 minutes in boiling water bath and measure against spec 0.

Unit calculation

1. A linear glucose standard was constructed using the absolute amount of glucose (mg/100 µl) against A_{540}.
2. Using the standards, the absorbance value of standards in the sample tube (after subtraction of spectro zero) were translated into glucose (= mg glucose produced during the reaction).

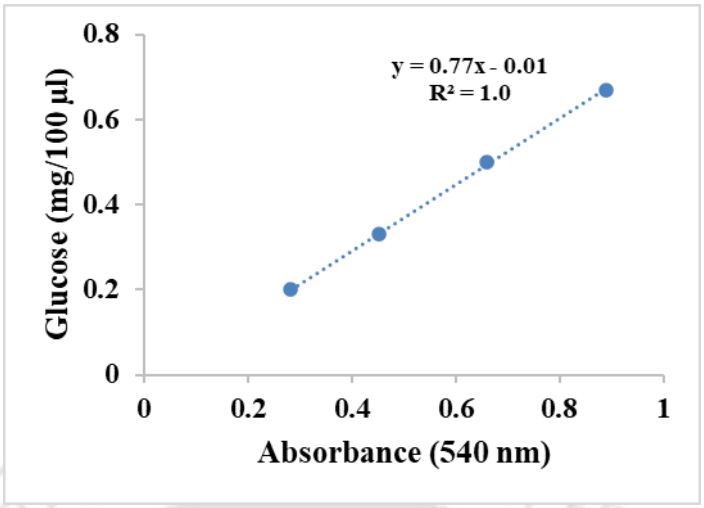

Fig.A1: Absorbance of absolute glucose standards at 540 nm.

Calculation of FPU unit (IU/ml)

1 IU = 1µmol/ min of substrate (filter paper) converted.

=1µmol/ min of glucose formed during the hydrolysis reaction.

$$\text{Enzyme activity (IU/ml)} = \frac{\text{Con. of glucose release in fiter paper assay (g/l)}}{\text{M.W. of glucose} * \text{incubation time (min)}}$$

$$= \frac{((A_{540}\ (\text{sample}) - A_{540}\ (\text{blank})) * \text{slope} * \text{D.F.} * 10 * 1000}{\text{M.W. of glucose} * \text{incubation time (min)}}$$

$$= \frac{((A_{540}\ (\text{sample}) - A_{540}\ (\text{blank})) * 0.77 * \text{D.F.} * 10 * 1000}{180 * 20}$$

Note:

A_{540} (Sample) = Absorbance of sample (enzyme + filter paper) after DNS assay at 540 nm.

A_{540} (Blank) = Absorbance of blank (enzyme) after DNS assay at 540 nm.

Slope = slope obtained from absolute glucose standards.

D.F. = dilution factor for cellulase used in enzyme activity assay.

2. Preparation of glucose standards for estimation of Carboxymethyl cellulase (CMCase) activity

A stock solution of anhydrous glucose (2 mg/ml) was prepared and diluted further with following dilutions.

Dilutions

Undiluted = 1:1.5 (0.2 mg/100 µl)

100 µl glucose standard+ 50 µl citrate buffer = 1:1.5 (0.133 mg/100 µl)

100 µl glucose standard+ 100 µl citrate buffer = 1:2 (0.1 mg/100 µl)

100 µl glucose standard+ 300 µl citrate buffer = 1:4 (0.05 mg/100 µl

Preparation of Spectro zero	**Preparation of standards**
200 µl citrate buffer	100 µl standard
600 µl DNS reagent	100 µl citrate buffer
Incubate for 5 minutes in boiling water bath and measure against spec 0.	600 µl DNS reagent Incubate for 5 minutes in boiling water bath and measure against spec 0.

Unit calculation

1. A linear glucose standard was constructed using the absolute amount of glucose (mg/100 µl) against A_{540}.
2. Using the standards, the absorbance value of standards in the sample tube (after subtraction of spectro zero) were translated into glucose (= mg glucose produced during the reaction).

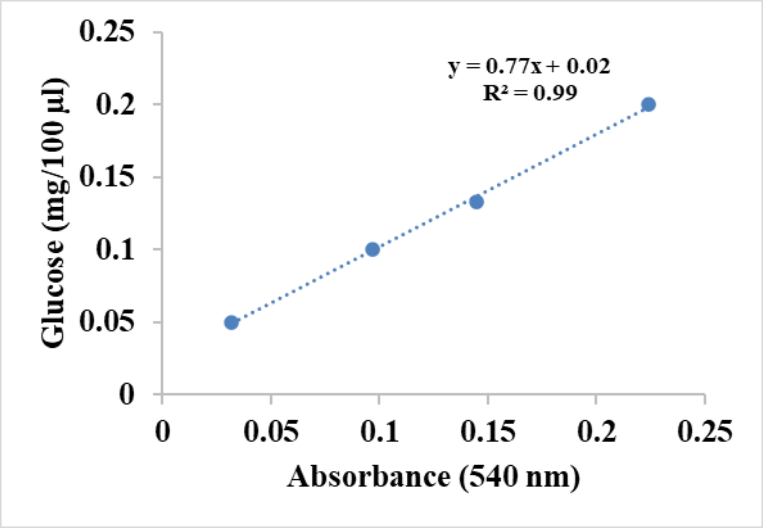

Fig.A2: Absorbance of absolute glucose standards at 540 nm.

Calculation of CMCase activity unit (IU/ml)

1 IU = 1µmol/ min of substrate (CMC) converted.

= 1µmol/ min of glucose formed during the hydrolysis reaction.

$$\text{Enzyme activity (IU/ml)} = \frac{\text{Con. of glucose release in Carboxymethyl cellulase assay (g/l)}}{\text{M.W. of glucose} * \text{incubation time (min)}}$$

$$= \frac{((A_{540}\ (\text{sample}) - A_{540}\ (\text{blank})) * \text{slope} * \text{D.F.} * 10 * 1000}{\text{M.W. of glucose} * \text{incubation time (min)}}$$

$$= \frac{((A_{540}\ (\text{sample}) - A_{540}\ (\text{blank})) * 0.77 * \text{D.F.} * 10 * 1000}{180 * 10}$$

Note:

A_{540} (Sample) = Absorbance of sample (enzyme + CMC) after DNS assay at 540 nm.

A_{540} (Blank) = Absorbance of blank (enzyme) after DNS assay at 540 nm.

Slope = slope obtained from absolute glucose standards.

D.F. = dilution factor for cellulase used in enzyme activity assay.

3. Preparation of xylose standards for estimation of Xylanase activity

A stock solution of xylose (2 mg/ml) was prepared and diluted further with following dilutions.

Dilutions

Undiluted =1:1.5 (0.2 mg/100 µl)

100 µl xylose standard+ 50 µl citrate buffer =1:1.5 (0.133 mg/100 µl)

100 µl xylose standard+ 100 µl citrate buffer =1:2 (0.1 mg/100 µl)

100 µl xylose standard+ 300 µl citrate buffer =1:4 (0.05 mg/100 µl)

Preparation of Spectro zero

200 µl citrate buffer
600 µl DNS reagent
Incubate for 5 minutes in boiling water bath and measure against spec 0.

Preparation of standards

100 µl standard
100 µl citrate buffer
600 µl DNS reagent
Incubate for 5 minutes in boiling water bath and measure against spec 0.

Unit calculation

1. A linear xylose standard was constructed using the absolute amount of xylose (mg/100 µl) against A_{540}.
2. Using the standards, the absorbance value of standards in the sample tube (after subtraction of spectro zero) were translated into xylose (= mg xylose produced during the reaction).

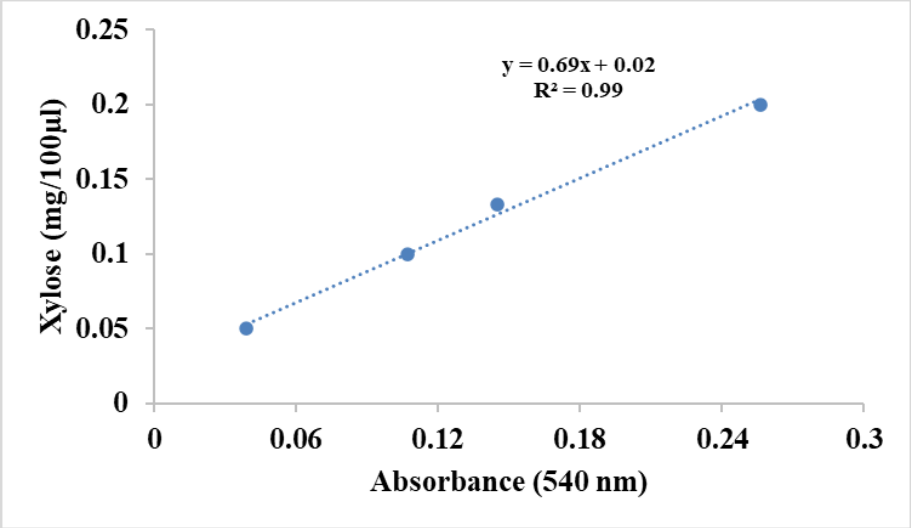

Fig.A3: Absorbance of absolute xylose standards at 540 nm.

Calculation of Xylanase activity unit (IU/ml)

IU = 1µmol/ min of substrate (xylan) converted.

= 1µmol/ min of glucose formed during the hydrolysis reaction.

$$\text{Enzyme activity (IU/ml)} = \frac{\text{Con. of glucose release in xylanase assay (g/l)}}{\text{M.W. of xylose} * \text{incubation time (min)}}$$

$$= \frac{((A_{540}\ (\text{sample}) - A_{540}\ (\text{blank})) * \text{slope} * \text{D.F.} * 10 * 1000}{\text{M.W. of xylose} * \text{incubation time (min)}}$$

$$= \frac{((A_{540}\ (\text{sample}) - A_{540}\ (\text{blank})) * 0.69 * \text{D.F.} * 10 * 1000}{150 * 10}$$

Note:

A_{540} (Sample) = Absorbance of sample (enzyme + xylan) after DNS assay at 540 nm.

A_{540} (Blank) = Absorbance of blank (enzyme) after DNS assay at 540 nm.

Slope = slope obtained from absolute xylose standards.

D.F. = dilution factor for cellulase used in enzyme activity assay.

4. Preparation of p-nitrophenol standards for estimation of Beta-glucosidase activity.

A stock solution of p-nitrophenol (200 µg/ml) was prepared and further dilutions were made to obtain the following absolute standards i.e., 40 µg/ml, 20 µg/ml, 10 µg/ml, 6 µg/ml, 2 µg/ml. All the dilutions to prepare standards were made with citrate buffer. The Spectro zero was prepared by adding 200 µl citrate buffer with 400 µl of sodium carbonate (2 % w/v). All the absolute standards of p-nitrophenol (200 µl) were added with 400 µl of sodium carbonate (2 % w/v). A linear p-nitrophenol standard was constructed using the absolute amount of p-nitrophenol (µg/ml) against A_{410}. Using the standards, the absorbance value of standards in the sample tube (after subtraction of spectro zero) were translated into p-nitrophenol (=µg p-nitrophenol produced during the reaction).

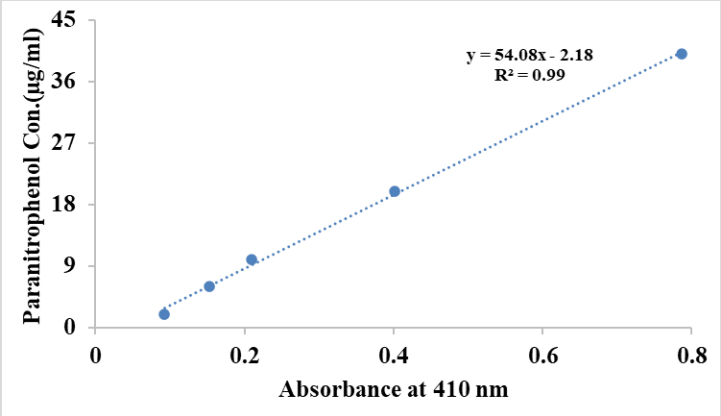

Fig.A4: Absorbance of absolute p-nitrophenol standards at 410 nm.

Calculation of Beta-glucosidase activity unit (IU/ml)

1 IU = 1µmol/ min of substrate (p-nitrophenol-β-D-glucopyranoside (pNPG) substrate) converted.

= 1µmol/ min of p-nitrophenol formed during the hydrolysis reaction.

$$\text{Enzyme activity (IU/ml)} = \frac{\text{Con. of p-nitrophenol release in pNPG assay (g/l)}}{\text{M.W. of p-nitrophenol} * \text{incubation time (min)}}$$

$$= \frac{((A_{410}\ (\text{sample}) - A_{410}\ (\text{blank})) * \text{slope} * \text{D.F.}}{\text{M.W. of p-nitrophenol} * \text{incubation time (min)}}$$

$$= \frac{((A_{410}\ (\text{sample}) - A_{410}\ (\text{blank})) * 54.08 * \text{D.F.} * 10 * 1000}{139 * 10}$$

Note:

A_{410} (Sample) = Absorbance of sample (enzyme + pNPG) after p-NPG assay at 410 nm.

A_{410} (Blank) = Absorbance of blank (enzyme) after p-NPG assay at 410 nm.

Slope = slope obtained from absolute p-nitrophenol standards.

D.F. = dilution factor for cellulase used in enzyme activity assay.

Tables

Table A1: Composition of DNS reagent for reducing sugar estimation for measurement of enzyme activity

Mix:	Distilled Water	1000 ml
	3,5-Dinitrosalicylic acid	7.5 g
	NaOH	14 g
Dissolve above, then add:		
	Rochelle salts (Na-K tartarate)	216 g

Table A2: Preparation of citrate buffer.

Add:	Distilled Water	950 ml
	Sodium citrate tribasic dihydrate	14.7 g
Adjust pH to 4.8 by adding 5M HCl solution. Then, make up the final volume to 1000 ml by adding distilled water.		

Table A3: Comparison of bioethanol production using various lignocellulose feedstock.

Biomass used	Pretreatment method	Biomass loading	Enzyme source	Enzyme loading	Fermenting micro-organism	Process used	Bioethanol titre	Reference
Rice straw	Base pretreatment (2% NaOH, 121°C, 30 min) followed by acid pretreatment (2% H_2SO_4, 121°C, 30 min)	10% w/v	*P. janthinellum*	9.4 FPU/gds	*S. cerevisiae*	Prehydrolysis at 50ºC (2h) followed by SSF at cyclic shifting of temperature 30ºC (1.7h)-40ºC (2h) for 48h.	15.9 g/l	Present study
Rice straw	Base pretreatment (2% NaOH, 121°C, 30 min) followed by acid pretreatment (2% H_2SO_4, 121°C, 30 min)	10% w/v	*P. janthinellum*	9.4 FPU/gds	*P. stipitis*	Prehydrolysis at 50ºC (2h) followed by SSF at cyclic shifting of temperature 30ºC (2h)-37ºC (3h) for 48h.	8.76 g/l	Present study
Rice straw	Base pretreatment (2% NaOH, 121°C, 30 min) followed by acid pretreatment (2% H_2SO_4, 121°C, 30 min)	10% w/v	*T. reesei and P. janthinellum*	10.9 FPU/gds	*S. cerevisiae*	Prehydrolysis at 50ºC (2h) followed by SSF at cyclic shifting of temperature 30ºC (1.7h)-40ºC (2h) for 48h.	17.05 g/l	Present study
Rice straw	Base pretreatment (2% NaOH, 121°C, 30 min) followed by acid pretreatment (2% H_2SO_4, 121°C, 30 min)	10% w/v	*T. reesei and P. janthinellum*	10.9 FPU/gds	*P. stipitis*	Prehydrolysis at 50ºC (2h) followed by SSF at cyclic shifting of temperature 30ºC (2h)-37ºC (3h) for 48h.	11.81 g/l	Present study

Rice straw	Ensilation	10% w/v	*Acremonium sps.*	8.6 FPU/gds	*S. cerevisiae*	SSF at 30ºC (16 days)	13.5g/l	(Shinozaki & Kitamoto, 2011)
Rice straw	thermoalkaline-treatment	10% w/v	*Aspergillus niger P-19*	5 FPU/gds	*S. cerevisiae*	Prehydrolysis at 50ºC for 5 days followed by fermentation	15.6 g/l	(Kaur et al., 2020)
Rice straw	1% NaOH,121 °C, 15 psi , 30 min	10% w/v	Accellerase®1500 *(Genencor, Denmark)*	14.5 FPU/gds	*S. cerevisiae*	Prehydrolysis at 50ºC for 24h followed by fermentation at 30ºC for 24 h	4.03 g/l	(Sharma et al., 2019)
Rice straw	2% NaOH,121 °C, 15 psi , 60 min	10% w/v	*Palkosoft super 700*	7.5 FPU/gds	*S. cerevisiae*	SSF at constant incubation temp. of 35ºC for 96h	19.24 g/l	(Goel & Wati, 2016)
Rice straw	Aqueous ammonia soaking at room temperature for 14 days	10% w/v	Cellulase (Cellic Ctec2) and xylanase (Cellic Htec2)	15 FPU/gds and 100 XU/gds	*S. cerevisiae*	SSF at 37ºC for 96 h.	21.7 g/l	(Phitsuwan et al., 2016)
Sugarcane bagasse	Ball milling (2h)	5% w/v	*P. chrysogenum and A. flavus*	5 FPU/gds	*Pichia stipitis BCC15191*	Prehydrolysis at 45ºC for 72h followed by fermentation at 30ºC for 24h	8.4 g/l	(Buaban et al., 2010)

Wheat straw	Steam pretreatment	10% w/v	*P. janthinellum*	20 FPU/gds	*K. marxianus*	Prehydrolysis at 50ºC (72h) followed by SSF at 42ºC for 72h	6.45 g/l	(Singhania et al., 2015)
Carnauba straw	4% NaOH, 121 °C, 15 psi , 30 min	4% (w/v)	Cellulase (Sigma-Aldrich, USA), Beta-glucosidase and xylanase (Novozymes, Denmark)	20 FPU/gds, 20 CBU/gds	*K. marxianus*	SSF at 45ºC	7.53 g/l	(da Silva et al., 2018)

Publications

Research paper

1. **Panda, S.K.** and Maiti, S.K., 2019. An approach for simultaneous detoxification and increment of cellulase enzyme production by Trichoderma reesei using rice straw. Energy Sources, Part A: Recovery, Utilization, and Environmental Effects, 41(22), pp.2691-2703.
2. **Panda, S. K.**, and Maiti, S. K. ,2024. Novel cyclic shifting of temperature strategy for simultaneous saccharification and fermentation for lignocellulosic bioethanol production. Bioresource Technology, 391, pp. 129975.
3. **Panda, S. K.**, and Maiti, S. K., Fungus-yeast tri-culture system for in situ cellulase production, biodetoxification and bioethanol production using rice straw with cyclic shifting of temperature strategy. (Under communication in Bioenergy Research).
4. **Panda, S. K.**, and Maiti, S. K., Recycling of acid-pretreatment hydrolysates of rice straw for high bioethanol production by *S. cerevisiae* and *P. stipitis* fermentation. (Accepted in Energy Sources, Part A: Recovery, Utilization, and Environmental Effects).

Book chapters

1. Naira, V.R., Mahesh, R., **Panda, S.K.** and Maiti, S.K., 2020. Biorefinery Approaches for the Production of Fuels and Chemicals from Lignocellulosic and Algal Feedstocks. In Biorefinery of Alternative Resources: Targeting Green Fuels and Platform Chemicals, pp. 141-170.
2. Mahesh, R., **Panda, S.K.**, Das, M., Yashavanth, P.R., Dhull, S., Negi, B.B., Jakhwal, P. and Maiti, S.K., 2021. Advances in biotechnological tools for bioremediation of wastewater using bacterial–algal symbiotic system. In Wastewater Treatment, pp. 385-411, Elsevier.

Workshops/Conferences attended

1. Poster presentation entitled, 'An approach for simultaneous detoxification and cellulase enzyme production by *Trichoderma reesei* NCIM 1186 using rice straw' *Bioprocess India* conference, 2018 held at IIT Delhi.
2. Oral presentation entitled, '*In situ* cellulase production, saccharification and fermentation for lignocellulosic bioethanol production via coculture of *Penicillium janthinellum* and *Saccharomyces cerevisiae* by cyclic shifting of temperature. 3rd International Conference BIORESTEC, 2020, by ELSEVIER.