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Research paper

Effect of cultivar variation and *Pichia stipitis* NCIM 3498 on cellulosic ethanol production from rice straw

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A R T I C L E I N F O

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A B S T R A C T

Lignocellulosic wastes such as rice straw gain importance in ensuring sustainable production of biofuels, being one of the most abundant agricultural residues worldwide. This study points the need for characterization and selection of substrate, optimizing the pretreatment method, as well as employing suitable fermenting organism, which can be of immense help in the path towards a more efficient ethanol production process. Microwave assisted alkali, acid, or water pretreated rice straw of five cultivars (PRH-10, Pusa Basmati 1121, Pusa 44, Taraori basmati and IR-36) were subjected to enzymatic hydrolysis, followed by fermentation with *Pichia stipitis* 3498. Saccharified hydrolysate of pretreated rice straw gave 4–5 times higher sugar recovery compared to untreated straw. Total sugar recovery from different cultivars after pretreatment and saccharification ranged between 20 and 59%. Among the cultivars, Taraori basmati gave significantly higher sugar recovery and ethanol yield of 39.65% ± 3.70 and 5.16% respectively, through microwave/alkali pretreatment which was found best. Kinetic studies done in biofermentor were able to produce the maximum yield of 6.63% (v/v) ethanol, equivalent to 92.9% of the theoretical yield, with an ethanol yield coefficient of $0.47 g g^{-1}$. This study confirmed the importance of considering varietal differences of substrate, highlighted the higher efficiency of microwave/alkali pretreatment in enhancing straw digestibility and also the potential of the strain *P. stipitis* 3498 for ethanol production from lignocellulosic biomass.

1. Introduction

Emphasis and focus on producing a cost-competitive sustainable liquid transportation fuel such as ethanol is growing worldwide as the demands for renewable and non-petroleum based fuels boom. Lignocellulosic substrates are the most suitable candidates for this purpose globally. Rice is extensively cultivated in South and South-East Asia, leaving a sizable amount of straw in the paddy field. Of the 352 Mt of crop residues available in India, 70% is contributed by grain crops, of which paddy straw constitutes 34% [1]. The fact that surplus amounts (44.5 Mt) of rice straw is burned annually and also contributing 0.05% of greenhouse gas emissions [2], makes rice straw a promising agricultural residue for ethanol production in India. The average composition of rice straw comprises of 35–40% cellulose, 20–25% hemicellulose, 15–20% lignin and ~8% silica [3]. However, the composition of cellulose, hemicelluloses, as well as those of inhibitory compounds such as lignin and silica varies with the variety of rice. Hence, the selection of high fermentable sugar yielding varieties from amongst the multitude of rice varieties grown throughout India assumes vital importance.

The typical process for lignocellulosic ethanol production consists of biomass pretreatment (which breaks down the complex lignocellelosic structure) and detoxification followed by enzymatic hydrolysis (saccharification) using cellulolytic enzymes (cellulases), subsequent fermentation of the sugar rich hydrolysate using yeasts into ethanol. In the biomass, long chain polymers of cellulose are packed into micro fibrils covered by hemicellulose and lignin. This complex crystalline structure is broken down during pretreatment, leaving it accessible to hydrolytic enzymes. Efficient pretreatment and sequential enzymatic hydrolysis is equally necessary to maximize sugar productivity and minimize loss of sugar [4]. Studies have shown that microwave irradiation could change the ultrastructure of cellulose, degrade lignin and hemicellulose in lignocellulosic materials, and increase the enzymatic susceptibility of lignocellulosic materials. Microwave irradiation may be easily combined with chemicals to accelerate the reaction rate. Numerous studies have reported microwave-assisted alkali treatment to be highly suitable

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for enhancing the digestibility of paddy straw [5,6]. The cellulases, which mainly consist of three major enzyme groups, which synergistically help in the successful process of enzymatic hydrolysis or saccharification, include endoglucanases, exoglucanases, and β-glucosidases. Consequently, the main end hydrolysis products include glucose (from cellulose) and other pentose (e.g.xylose) and hexose sugars (from hemicelluloses) [7].

The next step involves the utilisation of the pentose and hexose sugars from hydrolysis through fermentation process. Economical production of ethanol requires that the major carbohydrate polymers (cellulose and hemicellulose) present in straw, are utilized during fermentation. As utilisation of pentoses, which form a significant portion of sugars generated from paddy straw, is beyond the ability of standard industrial microorganisms, we need to use organisms which can utilize all sugar types for improved fermentation efficiency. The promising yeasts species with the ability to use pentose and hexose sugars include *Pichia stipitis,* and *Pachysolan tannophilus* and *Candida shehatae* [8]. A study by Dhanya [9] gave a list of yeast strains (*Saccharomyces cerevisiae, Pichia stipitis)* showing more than 80% sugar conversion efficiency, out of which one strain (*P. stipitis* 3498) was used in the current study. The present study was carried out to assess the ethanol production potential of five common rice varieties grown in northern India. Comparative analysis of the sugar recovery and ethanol generation from microwave-assisted alkali/acid/hot water pretreated rice straw subjected to enzymatic saccharification, subsequently to fermentation with the strain, *Pichia stipitis* NCIM 3498, was done. Further confirmation of the ethanol yield of the best performing variety and treatment was done through kinetic study for better understanding of the process.

2. Materials and methods

2.1. Raw material

The rice straw of five different varieties PRH-10, Pusa basmati 1121, Pusa 44, Taraori basmati and IR-36 were collected randomly in triplicates from research farm of CESCRA, ICAR-Indian Agricultural Research Institute, New Delhi. Collected samples were air dried, cleaned, cut into smaller pieces and then powdered using a laboratory grinder so that it passes through 20–40 mesh sieve, sealed in plastic containers, and stored at room temperature. Table 1 gives the compositional analysis of rice straw of different varieties [10]. The cellulose content in the selected varieties varied between 35 and 41%, hemicellulose content from 15 to 23%, lignin contents from 3.75 to 6% and silica contents from 6.5 to 13.6%. The variety Taraori basmati was observed to have lower quantity of silica and lignin, the inhibitory factors to efficient sugar and subsequently ethanol recovery.

2.2. Rice straw hydrolysate preparation

Pretreatment was done by microwave assisted acid and alkali in a domestic microwave-convection oven (LG Electronics Inc., South Korea) at 850W power. One gram of oven dry, powdered rice straw sample was taken in glass tubes and immersed in 10 mL of 1% sulphuric acid (H_2SO_4), or 1% sodium hydroxide (NaOH), or hot water (control). The glass tubes were placed at the centre of the microwave oven and treated at 160 °C for 15 min. After treatment, the slurry was filtered with Whatman no.42 filter paper, to separate residues and filtrate. Weight loss in the pretreated samples was also determined, by measuring dry weight before and after pretreatment using a laboratory weighing balance (AD-180, Adair Dutt & Company (India) Pvt. Ltd). The residue after washing and neutralization was used for enzymatic hydrolysis while the filtrate was stored in a refrigerator till further fermentation, after the determination of soluble sugar yields by the Anthrone method [11]. All of the treatments were performed in triplicates.

The pretreated rice straw of the different selected cultivars was subjected to enzymatic saccharification as per the protocol described by NREL LAP-009 [12]. The cellulase and β-glucosidase enzymes used in the study were obtained from Merck, India. Pretreated rice straw, equal to the equivalent of 0.1g of cellulose, was taken in 250 mL Erlenmeyer flasks. Then, 5 mL of 0.05 M sodium citrate buffer (pH 4.8) was added to all flasks. Cellulase and β-glucosidase enzyme equivalent to approximately 60 FPU/g cellulose and 64p NPGU/g cellulose, respectively and 20 mg/L sodium azide as an antibiotic was also added [12]. Then the volume of each flask was made up to 10 mL with distilled water. Separate untreated rice straw was taken in 250 mL Erlenmeyer flasks as control and subjected to saccharification. The flasks were plugged with cotton and incubated at 50 °C in a BOD incubator-shaker (Orbitek Orbital Laboratory Incubator shaker) at 68 rpm for 96 h. Residues were filtered from the saccharified slurry by through Whatman no. 42 filter paper. The filtrate after sugar analysis was used for fermentation.

2.3. Fermentation

The yeast strain, *Pichia stipitis* NCIM 3498 obtained from NCIM, National Chemical Laboratory, Pune was used for ethanol fermentation. Malt extract glucose yeast extract-peptone (MYGP) agar slants were used to maintain the strain. A loopful culture from 24 h old yeast culture maintained on slants was inoculated in100 mL MGYP broth and incubated at 30 °C on the gyratory shaker.

Sterile 250 mL Erlenmeyer flasks with 125 mL of neutralised, autoclaved growth media, containing equal proportions of the pretreated filtrate and the hydrolyzed filtrate of rice straw were used to carry out fermentation. A day old culture of *Pichia stipitis* NCIM 3498 was inoculated into the media at10% v/v. The flasks were incubated at 100 rpm at a temperature of 35 °C and controlled pH of 4.8, and fermentation was carried out for 72 h. Samples were collected at 6 h intervals. The samples collected were analyzed for residual sugar using the Anthrone method and ethanol by gas chromatography. The fermentation parameters, i.e., pH, temperature, and inoculum rate for the fermentation studies were followed as optimized by Prasad et al. [13].

The concentration of ethanol in fermented liquor samples were analyzed by gas chromatography (Model: Shimadzu GC-14B, Japan, injection temperature: 200 °C, carrier gas: N_2, solid phase: Polyethylene

Table 1
Quality attributes of rice straw varieties (Adapted from Sheetal et al. [10]).

Characters (% oven dry weight basis)	Varieties				
	Pusa 44	IR 36	Pusa basmati 1121	PRH 10	Taraori basmati
Total solids	92.54 ± 1.81	94.47 ± 1.98	90.68 ± 3.25	93.89 ± 1.95	88.97 ± 2.61
Silica	9.70 ± 0.14	12.35 ± 1.51	13.57 ± 0.12	10.55 ± 0.35	6.50 ± 0.11
Cellulose	41.76 ± 0.34	35.51 ± 0.35	40.7 ± 0.7	39.7 ± 0.36	36.21 ± 1.35
Hemicellulose	15.25 ± 0.15	22.74 ± 0.8	15.8 ± 0.4	20.15 ± 0.25	22.95 ± 1.19
Lignin	5.54 ± 0.64	6.15 ± 0.45	4.95 ± 0.45	4.50 ± 0.17	3.75 ± 0.65
Nitrogen	0.45 ± 1.00	0.46 ± 0.76	0.49 ± 0.25	0.55 ± 0.18	0.46 ± 0.52
Phosphorus	0.12 ± 0.005	0.16 ± 0.004	0.11 ± 0.002	0.14 ± 0.006	0.17 ± 0.012
Potassium	1.16 ± 0.05	1.58 ± 0.11	0.97 ± 0.05	1.15 ± 0.08	1.19 ± 0.17

Glycol-20 M, oven temperature: 180 °C, FID temperature: 230 °C; and internal standard: isopropanol). Theoretical ethanol yield was calculated using the formula given below:

Theoritical ethanol yield = Total sugar used*92/180

where, 92 is the mass of 2 mol of ethanol, formed from 1 mol of hexose, and 180 is the molar mass of hexose.

Yield efficiency (Ey) or conversion efficiency percentage was calculated as:

$$Ey = Yps^{*}\frac{100}{0.51}$$

where, Yps is ethanol yield expressed as gram of ethanol per gram sugar utilized (gg^{-1}), and the maximum theoretical ethanol yield of glucose consumption is 0.51 [13,14].

The pooled results of the three replicates (n = 3) were expressed as mean ± standard deviation. Statistical evaluation of the data was carried out with the help of analysis of variance (ANOVA) technique for completely randomised design using Statistical Analysis System (SAS) software, Windows version 6.11. The comparisons of means were carried out using least significant difference (LSD) at the 5% level of significance.

3. Result and discussion

3.1. Total sugar recovery of rice straw as affected by pretreatment

The choice of chemical used in association with microwave treatment was found to be a significant factor determining the recovery of fermentable sugars and solid recovery; this defines the efficiency of pretreatment. Fig. 1 shows the changes in sugar recovery with the use of acid, alkali and water; which has been discussed in detail previously [6]. It was precisely observable that the rice straw had more sugar yield when treated with microwave/alkali than by other combinations. This may be due to the solubilisation of impeding components such as lignin, disruption of the straws' silicified waxy surface and disruption of lignin-hemicellulose complex by microwave irradiation. Similar results have also been reported before [15,16]. Weight loss of the rice straw is a relevant index for the effectiveness of the pretreatment. Treatment with microwave/alkali evidently resulted in more weight loss from rice straw [6], which may be due to elimination of more lignin and hemicellulose from paddy straw as indicated by Zhu et al. [17]. Ma et al. [15] also demonstrated the importance of microwave irradiation on biomass digestion. Zhu et al. [18] indicated that lignocellulosic biomass had greater cellulose content and weight loss as well as lower lignin, hemicellulose and moisture content when treated with microwave-alkali combination than by alkali only.

Upon comparing the total sugar recovery and weight loss, it can be observed that sugar recovery showed an increase with the increase in the weight loss which indicates a direct relation between sugar recovery and weight loss. This may be due to the degradation of lignin and efficient conversion of sugars from fibers as evidenced by their weight loss [6].

Table 2
Total soluble sugar recovery by enzymatic hydrolysis of pretreated rice straw.

Pretreated with	Sugar recovery %				
	Pusa 44	IR 36	Pusa Basmati 1121	PRH-10	Taraori basmati
Microwave/H_2SO_4	10.09[b]	13.90[b]	15.60[b]	12.89[b]	19.05[b]
Microwave/NaOH	17.89[a]	18.98[a]	31.15[a]	23.93[a]	39.65[a]
Microwave/Hot Water	9.18[b]	9.53[c]	11.67[c]	11.50[b]	14.18[c]
Control	4.08[c]	4.13[d]	6.66[d]	4.40[c]	7.97[d]

Values with different superscripts within column are significantly different at 0.05 level.

3.2. Saccharification of pretreated rice straw for sugar recovery

Saccharification was employed for the recovery of fermentable sugar, from all the sets of microwave/acid, microwave/alkali and microwave/water pretreated rice straw. In order to assess the impact of pretreatment on digestibility of paddy straw, a control was also kept with unpretreated rice straw. Significant differences (p = 0.05) in sugar recovery were obtained with the treatments in the different varieties (Table 2). Microwave assisted alkali treatment was seen to give significantly higher sugar recovery in all the five varieties. Total sugar recovery after 72-hr enzymatic hydrolysis was found the maximum in hydrolysate from microwave assisted alkali pretreated Taraori basmati, i.e., 39.65% ± 3.70, followed by Pusa basmati 1121(31.15% ± 0.93). Saccharification yields (of sugar) from rice straw pretreated with microwave assisted alkali showed the highest sugar recovery; almost twice the sugar yields obtained from saccharification of acid or hot water pretreated straw. The current result is also consistent with other research findings [19,20], in which the superiority of pretreatment with NaOH over that with H_2SO_4 or liquid-hot-water for obtaining greater enzymatic conversion ratio of cellulose pretreatment. Even though acid treatment can solubilize hemicelluloses, by-product (e.g., acetic acid, furfural, and HMF) generation [21] can inhibit enzyme activity, which might be reasons for lower sugar yields obtained in this study with microwave/H_2SO_4 pretreatment. With microwave/H_2SO_4 pretreatment, the maximum sugar yield obtained was 19.05% ± 0.39 in Taraori basmati. The control with untreated straw gave insignificant sugar which must be due to the high resistance of the untreated

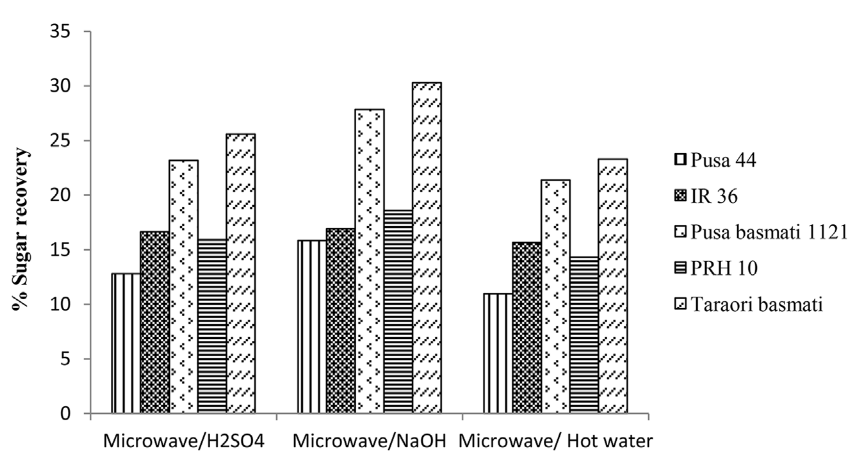

Fig. 1. Effect of microwave assisted acid, alkali, and water treatment on sugar recovery (Adapted from Sheetal et al. [6]).

Table 3
Ethanol production by fermentation with *Pichia stipitis* 3498.

Varieties	Microwave/H_2SO_4			Microwave/NaOH			Microwave/Hot Water		
	Sugar (%)	Ethanol % (v/v)	EYC (gg^{-1})	Sugar (%)	Ethanol % (v/v)	EYC (gg^{-1})	Sugar (%)	Ethanol % (v/v)	EYC (gg^{-1})
Pusa 44	5.91[d]	2.30[e]	0.39[a]	5.62[d]	2.33[e]	0.41[a]	5.04[d]	2.11[d]	0.42[a]
IR 36	7.55[c]	3.17[c]	0.42[a]	7.98[c]	3.49[c]	0.44[a]	6.30[c]	2.86[c]	0.45[a]
P. basmati 1121	9.90[b]	4.06[b]	0.41[a]	9.83[b]	4.12[b]	0.42[a]	8.26[b]	3.72[b]	0.45[a]
PRH-10	7.32[c]	2.78[d]	0.38[b]	7.40[c]	3.11[d]	0.42[a]	6.45[c]	2.69[c]	0.42[a]
T. basmati	11.15[a]	4.35[a]	0.39[a]	11.76[a]	5.16[a]	0.44[a]	9.36[a]	4.19[a]	0.45[a]

Values with different superscripts within column are significantly different at 0.05 level.

lignocelluloses to enzymatic attacks as has been also found by Taherzadeh and Karimi [22] in their studies. This result justifies the assumption that microwave irradiation enhances the saccharification by lignin removal and increasing accessibility to enzymes, in confirmation with other similar studies [5,17,19]. Microwave pretreatment has also been reported by Rezanka and Sigler [23] and Ma et al. [15] to improve biomass digestibility by reducing silica content.

3.3. Ethanol yield by fermentation

The filtrate obtained after pretreatment and saccharification under all three treatments was subjected to fermentation by strain *Pichia stipitis* NCIM 3498 in 250 mL flasks. The results for total sugar content, ethanol yield, and ethanol yield coefficient obtained after fermentation of the saccharified straw are given in Table 3. Maximum ethanol yield and productivity was 5.16%, and 0.44gg^{-1} respectively, from the microwave assisted alkali pretreated and hydrolyzed straw. Ethanol yields varied from 2.1 to 5.16% (v/v) in rice varieties, giving maximum yields. Among the cultivars, ethanol yield was highest in Taraori basmati (4.19–5.16%), followed by Pusa basmati 1121 (3.72–4.12%). The straw pretreated with microwave assisted alkali and further saccharified, exhibited higher ethanol yield compared to the other treatments given. Zhu et al. [17] also reported that microwave/alkali pretreated rice enhanced the rate of enzymatic saccharification and generated a hydrolysate consisting of greater glucose and reduced xylose with favourable subsequent fermentation. The high value of ethanol yield conversion and fermentation efficiency also proves the efficiency of the yeast *Pichia stipitis* NCIM 3498 to ferment biomass, which contains pentose sugars too. Yields of 0.44 gg^{-1} or 87% of the maximum possible ethanol conversion have also been found by Huang et al. [24] using an adapted strain of *P. stipitis* with enhanced inhibitor tolerance on NaOH-neutralised rice straw hydrolysate.

Biomass is a huge resource with global availability. Understanding why the variation in sugar and ethanol yield occurs will also help to extend the study to other biomass resources also. Lindedam et al. [25] in their study on wheat straw stated that understanding the biological variation of feedstock could strongly help optimize cellulosic ethanol yield. Hence, to recognize the contribution of different components of biomass to the ethanol yield, simple correlation coefficients were calculated among the ethanol yield and chemical characteristics of straw of different cultivars (Table 4). As evident from Table 4, ethanol yield and total sugar content of straw showed a highly significant positive correlation (r = 0.67**), while significant negative correlation was found between straw silica content and total sugar content (r = −0.70**) and naturally ethanol yield (r = −0.66**). This might also be the reason for the higher ethanol recovery reported from the variety Taraori basmati as it had the lowest silica and lignin contents among all tested varieties, as explained in Table 1. Several studies [15–18] have reported the inhibitory effects of lignin and silica on sugar recovery and ethanol yield from biomass. Lindedam et al. [25] reported less efficient conversion rates to sugar with increasing cellulose content in studied wheat straw cultivars. This study also observed a slight negative correlation between sugar and cellulose contents. Lesser sugar conversion indirectly leads to lower ethanol yields. The cellulose and hemicellulose were obviously found to be negatively correlated. Higher content of hemicellulose is also observed to restrict cellulose access to cellulases [26]. This is again proved by the negative correlations observed between cellulose digestibility with silica and lignin contents, observed in our study. On the other hand, Pereira et al. [27] emphasized the importance of the distribution of the different components, rather than on the individual content, when considering yields respective to the chemical composition of the feedstock.

3.4. Kinetic studies in the bioreactor

Maximum ethanol yield was obtained from the hydrolyzed and saccharified straw of rice variety Taraori basmati. Hence, this particular variety was further subjected to fermentation to confirm the high ethanol potential in a 7-L capacity Aplikon fermentor. Fig. 2 shows the time course of ethanol production during fermentation. It was found that saccharified sugar-rich hydrolysate from rice straw fermented by *Pichia stipitis* NCIM 3498, was able to produce a maximum yield of 6.63% (v/v) ethanol. Theoretically, every gram of glucose utilized yields 0.51 g ethanol, and thus the ethanol yield obtained in our study is equivalent to 92.9% of the attainable yield. The ethanol yield coefficient (Yps) was found to be 0.47 g ethanol per gram sugar used. The kinetic parameters were in agreement with that reported by other works [13,28].

The results convincingly justify our above conclusions that microwave assisted alkali pretreatment could indeed enhance enzymatic

Table 4
Correlation coefficient of ethanol yield and rice straw characteristics.

	Ethanol yield	Cellulose	Hemicellulose	Lignin	Total sugar	Silica content
Ethanol yield	1					
Cellulose	−0.19	1				
Hemicellulose	0.07	−0.93[a]	1			
Lignin	−0.34	−0.05	−0.17	1		
Total sugar	0.67[a]	−0.42	0.41	−0.45	1	
Silica content	−0.66[a]	0.26	−0.36	0.54[b]	−0.70[a]	1

[a] Correlation is significant at the 0.01 level.
[b] Correlation is significant at the 0.05 level.

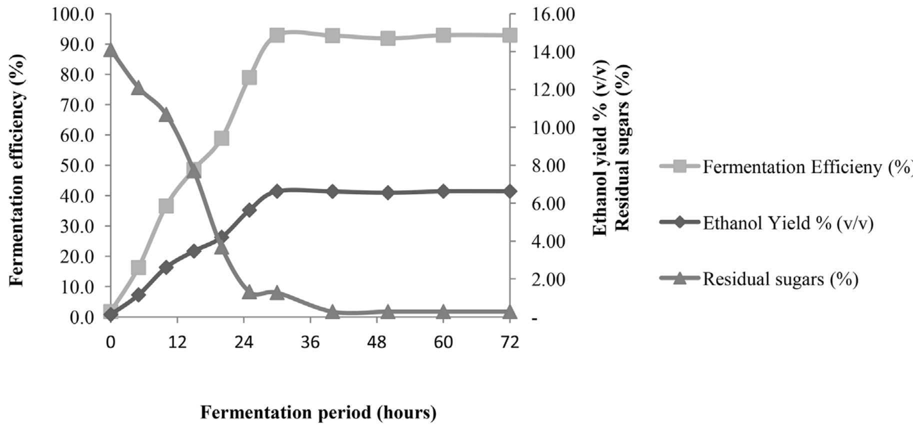

Fig. 2. Kinetics of ethanol production in batch fermentation.

hydrolysis of recalcitrant lignocellulosic materials like rice straw. It also highlights the excellent potential of the strain *Pichia stipitis* 3498 in utilizing both pentoses and hexoses for the conversion to ethanol.

4. Conclusions

From the data gathered in the current investigation, it could be summarised that the pretreatment method, choice of fermenting organism as well as the varietal differences are significant factors affecting ethanol production capacity and efficiency. A thorough understanding of feedstock variability will help optimize ethanol yield. The study confirmed that the use of microwave assisted alkali pretreated straw could be useful in increasing the enzymatic digestibility of rice straw. The study also illustrated the importance of pretreatment to enhance the susceptibility of attack by enzymes or microorganisms, giving an increase of 4–5 times the sugar recovery after hydrolysis of pretreated paddy straw over the untreated straw. Fermentation of pretreated and enzymatically hydrolyzed straw gave ethanol yield coefficients ranging from 0.38 to 0.45 gg^{-1} of sugar. Thus, microwave assisted alkali pretreatment followed by hydrolysis and fermentation was found to be the best process among the methods compared in this study. Also, *Pichia stipitis* NCIM 3498 was seen to have high lignocellulose fermenting efficiency. Taraori basmati showed highest yields for ethanol production and hence was selected for further kinetic studies. Maximum ethanol obtained was 6.63% (v/v), equivalent to 92.9% of the theoretical yield, further confirming the potential of the rice straw. An attempt to understand sugar and ethanol recovery from biomass, led to the observations that those lignocellulosic biomass having lesser contents of lignin and silica, and higher contents of total soluble sugars are more suitable feedstocks for biofuel production. Our results may suggest that selection of feedstock (crops or cultivars within crops) which contain more of favourable components and less of the hindering components may help to obtain more sugar recovery and subsequently higher biofuel productions. Further studies may be carried out to find out the effect of inhibitors and possible improvements that may be made in production efficiencies by the use of modified strains of microorganisms.

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