Improved physicochemical pretreatment and enzymatic hydrolysis of rice straw for bioethanol production by yeast fermentation

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Abstract Lignocellulosic biomass such as agricultural and forest residues are considered as an alternative, inexpensive, renewable, and abundant source for fuel ethanol production. In the present study, three different pretreatment methods for rice straw were carried out to investigate the maximum lignin removal for subsequent bioethanol fermentation. The chemical pretreatments of rice straw were optimized under different pretreatment severity conditions in the range of 1.79–2.26. Steam explosion of rice straw at 170 °C for 10 min, sequentially treated with 2% (w/v) KOH (SEKOH) in autoclave at 121 °C for 30 min, resulted in $85 \pm 2\%$ delignification with minimum sugar loss. Combined pretreatment of steam explosion and KOH at severity factor (SF 3.10) showed improved cellulose fraction of biomass. Furthermore, enzymatic hydrolysis at 30 FPU/g enzyme loading resulted in 664.0 ± 5.39 mg/g sugar yield with $82.60 \pm 1.7\%$ saccharification efficiency. Consequently, the hydrolysate of SEKOH with 58.70 ± 1.52 g/L sugars when fermented with *Saccharomyces cerevisiae* OBC14 showed 26.12 ± 1.24 g/L ethanol, 0.44 g/g ethanol yield with $87.03 \pm 1.6\%$ fermentation efficiency.

Keywords Enzyme hydrolysis · Ethanol · Fermentation · Pretreatment · Rice straw

Introduction

Currently, bioethanol produced from renewable source is considered to be an alternative to fossil fuels (Naseeruddin et al. 2013). Environmental protection agency (EPA) aimed a goal of 36 billion gallons of green fuel to be mixed with gasoline by 2022 perhaps ethanol blended gasoline considered to be substitute fuel with decreased emission of greenhouse gases (Clairotte et al. 2013). Lignocellulosic biomass is a potential substrate for production of bioethanol fuel (Ashoor et al. 2015). Rice (*Oriza sativa*) is one of the abundant, alternative, and third most staple grain crops in the world behind wheat and corn. It is estimated that rice straw production approximately amounts to 740.95–1111.42 million tons per year globally. In India, 23% of rice straw is wasted by burning in open field causing pollution that leads to environmental issues (Abraham et al. 2016).

Pretreatment is the prerequisite step in lignocellulosic conversion process, usually aimed at improving the cellulose accessibility through thermochemical/hydrothermal treatments (Barsberg et al. 2013). Due to existence of covalent cross-linkages between lignin and hemicelluloses in biomass like rice straw, a well-organized pretreatment method must be developed and applied to surmount the recalcitrant nature of lignocelluloses for facilitating cellulase access to biomass residue (Sun et al. 2014). Therefore, to enhance the yield of sugars and reduce the inhibitors from lignocellulosic hydrolysate, a particular treatment or a group of the pretreatment techniques are required to disrupt the lignocellulosic matrix (Keshav et al. 2016a).

Among pretreatment methods, hot water at high temperature and pressure (autoclave) was used to solubilize hemicellulose fractionation of rice straw (El-Zawawya et al. 2011; Binod et al. 2012). In addition, steam explosion pretreatment (SEP) is a widely used approach which

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fractionates lignocellulosic biomass components that leads to hemicellulose solubilization and transformation of lignin at high temperature and makes the biomass easily accessible to either acid or cellulolytic enzymes (Sun et al. 2014). Alkaline chemical pretreatment methods have been shown to be a potential in dissolving lignin and increasing cellulose digestibility at different temperatures and pressures (Chaturvedi and Verma 2013). The main outcome of alkaline pretreatment on lignocellulosic material is chemical delignification by eliminating the ester bonds cross-linking lignin and hemicellulose, thus enhancing the porosity and surface area of biomass (Silverstein et al. 2007). In addition, delignification uses sodium chlorite at temperature 100–170 °C at which oxygen acts as a free radical and attacks electron-rich sites in lignin that undergo glass transition (Kafle et al. 2015).

The enzymatic hydrolysis of cellulosic biomass is carried out by group of hydrolytic enzymes highlights the prominence of cellulase enzyme system in complete hydrolysis process with improved activities remains a challenge for efficient hydrolysis to obtain ethanol. In lignocelluloses conversion for more efficient cellulase saccharification, exploration of metagenomic methods possibly open new paths for improving enzyme cocktails used in current biofuel research spike (Tiwari et al. 2017). However, the enzymatic saccharification of lignocellulose is intrinsically difficult due to the complex structures of lignocelluloses (Liu et al. 2015). For recovering better ethanol yields and efficiency, the selection of fermenting organism lies upon its resistance to stress conditions like tolerance to ethanol and potential to withstand against various inhibitors during fermentation. *Saccharomyces cerevisiae* and *Pichia stipitis* are commonly used hexose and pentose fermenting yeasts in the industries owing to their high conversion efficacy and facile adaptableness under different conditions of fermentation (Keshav et al. 2016b; Abraham et al. 2016).

Previous studies reported individual pretreatment methods such as heat, steam explosion, and chemical processes (Sun et al. 2014; Montane et al. 1998; Ibrahima et al. 2011), which eliminated lignin to a limited extent. By using combination of pretreatment methods such as steam explosion and alkali pretreatment, cellulose conversion of biomass can be improved (Keshav et al. 2016a). The overall objective of this work was to explore a combined pretreatment for effective removal of lignin and hemicellulose components from the biomass for improved cellulase access on cellulose. Alkali pretreatment using autoclave proved to be dissolving lignin, hemicelluloses, and silica from plant cell wall of straw that facilitates to enhance hydrolysis (Karuna et al. 2014). Hence, the optimization of different chemical pretreatments was carried

out in the autoclave for selection of effective chemical to remove lignin from biomass.

The pretreatments such as autoclave, steam explosion, and chemical pretreatment after steam explosion (SEKOH) using autoclave were developed for significant removal of lignin that led to improved enzymatic hydrolysis. The resulting final hydrolysates containing excessive sugars were subjected to fermentation by monoculture or co-culture using *S. cerevisiae* and *P. stipitis*.

Materials and methods

Preparation of raw material and chemical composition

Rice straw (BPT 5204) summer crop harvested from agriculture fields of Suryapet district, Telangana state, India, was air dried for 24 h before the experiment. Chopped rice straw was pulverized to the size of 0.7 ± 0.2 cm, dried in oven at 60 °C overnight, and stored for further use. The cellulose, hemicellulose, and lignin fractions of raw rice straw (RRS), steam-exploded rice straw (SERS), and steam-exploded KOH pretreated rice straw (SEKOH) were analyzed using standard NREL method (National renewable energy laboratory) (Sluiter et al. 2008).

Physico-chemical pretreatment methods of rice straw

Three different physico-chemical pretreatment strategies were employed for delignification of rice straw.

Autoclaving

Five grams of raw rice straw (RRS) was suspended in 0.05 M acetate buffer of pH 4.5 at a ratio of 1:10, the temperature was maintained at 121 °C (15 PSI) for 1 h in the autoclave, and subsequently used for enzymatic hydrolysis of sugars recovery.

Steam explosion

The steam explosion apparatus consisting of 10 L reactor designed to withhold a pressure of 4.13 Mpa, i.e. 600 psi (Spectrochem Instruments Pvt. Ltd.) was used for steam explosion of rice straw. 250 g of raw rice straw (RRS) on dry basis was taken in a reactor and operated for 10 min at 170 °C. The steam-exploded rice straw was convalesced from cyclone separator and filtered for solid residue after the content was cooled to 40 °C. The steam-exploded solid fraction (SE) of rice straw was collected and washed with

water, air dried, and used for further analysis. Subsequently, 5 g of steam-exploded rice straw was pretreated with 2% KOH at 121 °C (15 PSI) at different time intervals of 15, 30, and 45 min with solid–liquid ratio of 1:10 (w/v). The solid fraction of KOH-treated rice straw (SEKOH) was collected and neutralized with tap water, subjected to enzymatic hydrolysis, and analyzed for lignin, hemicellulose, and cellulose by NREL method.

The combination of residence time and temperature can be expressed into a single factor known as severity factor. The severities of the pretreatment conditions of steam explosion and chemical treatment at autoclave temperature with different time intervals were calculated by using the severity factor ($\log R_0$) as below (Agudelo et al. 2016):

$$SF = \log_{10} \left(t \times \exp \left(\frac{T - 100}{\omega} \right) \right),$$

where ω is the constant parameter with value of 14.75; t is the residence time of pretreatment (min), T is the process temperature (°C), and 100 is the reference temperature which is assumed to be constant.

Chemical pretreatments

Five grams of raw rice straw (RRS) was taken in each 250 mL conical flask for pretreatment with solid/liquid ratio of 1:10 (w/v) using NaOH and KOH, sodium chlorite (NaClO_2 oxidative method) individually in five different concentrations (0.5, 1, 2, 3, and 4%) at different time intervals (15, 30, and 45 min) at 121 °C (15 PSI). After cooling, filtrates were adjusted to neutral pH using acid and then used for estimation of sugar loss and phenolics. Neutralization of the substrate was carried out under tap water.

Enzymatic hydrolysis of pretreated residues

Commercial cellulase (C8546, P Code. 1001000381) from *Trichoderma reesei* ATCC26921 was purchased from Sigma-Aldrich (USA) with an activity of 60 FPU/mL. Filter-paper activity (FPA) of cellulase was calculated in proportion to the standard formula recommended by the Commission on Biotechnology, IUPAC (Ghose 1987), and expressed as filter paper units (FPU). The residues obtained from pretreatments along with raw rice straw as control were taken in the solid-liquid ratio of 1:5 with 0.05 M sodium acetate buffer and enzyme loading of 30 FPU/g was used in this study (Keshav et al. 2016a). Also xylanase (X2753 Sigma), with an activity of 592 IU/mL was used for raw rice straw (RRS), autoclaved rice straw (ARS) and steam-exploded rice straw (SERS) for hemicellulose hydrolysis using 15 FPU/g loading. Later, pH was adjusted to 4.5 and incubated at 50 °C at 150 rpm in a shaker

incubator (ORBITEK-Scigenics) for 48 h. Samples were drawn after 36 h of incubation for estimation of total and reducing sugars.

Microorganisms and their maintenance

High temperature (47 °C) and ethanol (12%) tolerant yeast isolated from the soil samples of thermal power plant, kothagudem, Telangana, India, and characterized as *S. cerevisiae* OBC14 (NCBI Gene accession no. KM873330) was selected for these studies. Yeast culture was maintained on standard yeast extract peptone dextrose medium (YEPD) consisting of 1.0% yeast extract, 2.0% peptone, 2.0% dextrose, and 2.5% agar agar. Pentose fermenting yeast *P. stipitis* NCIM 3498 was procured from NCIM culture collection center and maintained on standard yeast extract peptone xylose medium (YEPX) consisting of 1.0% yeast extract, 2.0% peptone, 3.0% xylose, and 2.5% agar agar. The pH of media was adjusted to 5.0.

Inoculum preparation

The fermentation medium used for inoculum preparation consists of 0.45% yeast extract, 0.75% peptone, and 3.0% glucose. The pH of the medium was adjusted to 5.0 and autoclaved at 121 °C for 15 min. 100 mL medium in a 250-mL conical flask was inoculated with 10% (v/v) overnight culture inoculum of each yeast. After 24 h incubation, the cell density was measured using turbidometry method at 600 nm.

Fermentation

The enzyme saccharified hydrolysates of SEKOH, SERS, and RRS (100 mL) fermentation broth containing reducing sugars were added with supplementary nutrients like 1.5 g yeast extract, 1 g peptone, 1 g $(\text{NH}_4)_2\text{SO}_4$, 0.5 g K_2HPO_4 , 0.5 g MnSO_4 , 0.5 g $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ per liter, and pH was adjusted to 5.5. After sterilization at 10 PSI for 20 min, medium was cooled and further used for fermentation. Steam-exploded (SE) and steam explosion-KOH treated enzymatic hydrolysates (SEKOH) were selected for monoculture fermentation and inoculated with 10% (v/v) *S. cerevisiae* OBC14. For co-culture fermentation of raw rice straw (RRS), hydrolysate was inoculated with *P. stipitis* NCIM 3498 initially and *S. cerevisiae* OBC14 after 24 h with inoculum concentration of 5% (v/v). Fermentation was carried out at 30 ± 0.5 °C in a shaker incubator at 150 rpm for 48 h and samples were drawn at every 12 h intervals. The samples were filtered with 0.2 μm membrane filters along with standard ethanol with appropriate dilution and analyzed by HPLC for ethanol and leftover sugars.

Analytical methods

The composition of the raw substrate, autoclaved, steam-exploded rice straw, and SEKOH rice straw residue was determined using the standard NREL methods (Sluiter et al. 2008). Folin-Ciocalteus method was used for detection of total phenols in the delignified filtrate using gallic acid as standard (Singleton et al. 1999), while furans were estimated as described by Martinez et al. (2000). Dinitrosalicylic acid method was used for determining total reducing sugars recovered after enzymatic saccharification using spectrophotometer (Systronics Double beam spectrophotometer 2203) (Miller 1959). Reducing sugars and ethanol concentration of the samples were analyzed using Shimadzu LC HPLC equipped with Rezex ROA column (300 × 15 mm) and refractive index detector (RID). The HPLC grade 0.005 N sulfuric acid was used as mobile phase and flow rate was set to 0.6 mL/min. The column and RI detector were maintained at 60 and 40 °C, respectively, and 20 µL of fermented hydrolysate was injected and the peaks for standard glucose, xylose and cellobiose were examined.

Statistical analyses

The entire experiments were performed in triplicate; mean and standard deviation (SD) values were calculated. Statistical analysis was carried out using ANOVA and *t* test by Sigma Plot software (version 12.5, Chicago, IL, USA) and the probability values ($P \leq 0.05$) were observed as statistically significant difference. Standard error values have been showed as *Y*-error bars in figures and for each treatment, means and standard errors of means were calculated.

Results and discussion

Composition of rice straw

The total carbohydrate content of rice straw was found to be 61% which is in accordance with the previous reports where the carbohydrate content was reported as 60.18% (Karuna et al. 2014; Yu et al. 2010). The raw rice straw (RRS) used for the experiment consists of cellulose ($37 \pm 0.4\%$), hemicellulose ($24 \pm 0.46\%$), and lignin ($28.8 \pm 2.6\%$) and is comparable with previous studies (Karuna et al. 2014; Yu et al. 2010). The composition of autoclaved (ARS), steam-exploded rice straw (SERS), and steam-exploded-KOH pretreated rice straw (SEKOH) along with raw rice straw is shown in Table 1. During steam explosion pretreatment of rice straw, as the severity conditions of pretreatment are increased the content of hemicellulose as well lignin is lessened in the solid residue.

Our work results are in agreement with the studies conducted by Jin and chen (2006). More lignin in substrate like rice straw affects the biomass conversion, which requires a challengeable pretreatment process. The lignin content of rice straw was found to be 28.8% and was comparable with previous studies on different agricultural biomass like wheat straw 18.55 and 13.5% (Agudelo et al. 2016; Petrik et al. 2013), cotton stalks 29.40% (Keshav et al. 2016a), bamboo stems 27.53% (Sun et al. 2014), and rice straw 18.22% (Yu et al. 2010). The heterogeneity in the lignin content of substrate is due to structural and compositional variations that depend on cultivation area and season (Binod et al. 2010). This leads to the combined pretreatment strategy of rice straw due to the structural disparities and heterogeneity of the constituents.

Physico chemical pretreatments

Pretreatment with steam under pressure (autoclaving) and steam explosion methods lessen the hemicellulose and lignin content of rice straw (arabinose and xylose) with little or no change in carbohydrate contents. Pretreatment using autoclave at 121 °C for 1 h decreased the hemicellulose with 60.8% holocellulose recovery. The autoclave pretreatment at severity factor (SF) 2.38 has showed the $63.6 \pm 1.02\%$ delignification with $3.3 \pm 0.68\%$ sugar loss. The results are in accordance to Karuna et al. (2014) and El-Zawawya et al. (2011), where the studies conducted on pretreatment at autoclave temperature. Steam explosion at 170 °C removed about 50% hemicellulose after 10 min with $61.1 \pm 1.29\%$ holocellulose recovery where the severity of steam explosion pretreatment is 3.05. Increase in lignin and relative cellulose content in filter residue due to solubilization of hemicelluloses improves enzyme accessibility of cellulose (Jin and chen 2006). The resultant products of physical pretreatments of rice straw were hydrolyzed enzymatically better than the raw substrate and produced more sugars. Two alkali, NaOH, KOH, and one oxidative chemical, NaClO₂, were used individually at different concentrations at autoclaving temperature with different time intervals for optimization of pretreatment of rice straw. With the treatment of 1% (w/v) NaOH, the maximum delignification was found to be $73.98 \pm 1.47\%$ at $p < 0.0001$ with confidence interval (CI) of 95% ($p < 0.05$, F 199.5, SE 0.517) at 45 min with $6.48 \pm 0.086\%$ sugar loss. Similarly, 2% (w/v) KOH treatment, showed the maximum delignification of $82.65 \pm 2.59\%$ at $p < 0.001$ with CI of 95% ($p < 0.05$, F 96.7, SE 0.494) at 30 min with $3.79 \pm 0.13\%$ sugar loss. However, 0.5% (w/v) NaClO₂ treatment showed a maximum delignification of $68 \pm 1.66\%$ at $p < 0.0001$ with CI of 95% ($p < 0.05$, F 60.54, SE 1.079) at 5 min with $5.26 \pm 0.14\%$ sugar loss. The higher results of NaClO₂

Table 1 Chemical composition of raw and pretreated rice straw

	Temperature (°C) and Time (min)	Severity factor (SF)	Chemical composition (%)		
			Cellulose	Hemicellulose	Total lignin
Raw rice straw (RRS)	—	—	37 ± 0.4**	24 ± 0.46*	28.8 ± 2.3**
Autoclaving	121, 60'	2.39	40 ± 0.3**	20.8 ± 0.2*	21.19 ± 0.4***
Steam exploded rice straw (SERS)	170, 10'	3.05	54 ± 0.49***	10.1 ± 0.8**	20.4 ± 0.6*
SE-KOH rice straw (SEKOH)	170, 10'	3.10	65.6 ± 0.6***	04.58 ± 1.2***	08.5 ± 1.6***
	121, 30'				

* Indicates ($p < 0.05$), ** indicates ($p < 0.01$) and *** indicates ($p < 0.001$) significant differences between the raw rice straw, steam-exploded and steam-exploded KOH-treated biomass by t test ($n = 3$)

Table 2 Severity factors (log R₀) at different experimental conditions

Chemicals	Temp. (°C)	Time (min)	Severity factor (log R ₀ , $\omega = 14.75$)
NaOH	121	15	1.79
		30	2.09
		45	2.26
KOH	121	15	1.79
		30	2.09
		45	2.26
NaClO ₂	121	15	1.79
		30	2.09
		45	2.26

All the three chemicals used at one temperature (autoclave) with different time intervals

may be due to autoclave temperature when compared with Naseeruddin et al. (2013), who studied *Prosopis juliflora* delignification at room temperature with 3% NaClO₂ showed 36.05% lignin removal. Among all the chemical pretreatments, 2% (w/v) KOH ($p \leq 0.05$) obtained maximum delignification with diminutive loss of sugars. Pretreatment at autoclave temperature showed higher delignification when compared to Naseeruddin et al. (2013), where they have observed maximum lignin removal with 0.1 M NaOH ($52.89 \pm 1.21\%$) and 0.3 M KOH ($53.89 \pm 1.35\%$) at room temperature using *P. juliflora*. They asserted that pretreatment with sodium hydroxide showed lower lignin removal than potassium hydroxide for selection of best chemical for lignocellulosic biomass pretreatment. The chemical pretreatment was carried out at constant temperature, i.e. 121 °C with varied pretreatment time intervals. The temperature and time were calculated to give a single factor of severity factor (SF) 1.79–2.26 (Table 2). A higher temperature and shorter residence time hydrolyzes more hemicelluloses than a

lower temperature and longer residence time pretreatment of the equivalent severity factor (Kim et al. 2014), whereas the temperature used for the experiments, i.e. 121 °C/15–45 min for minimized sugar loss and maximum degradation of phenols was screened at specific time interval to recover the maximum carbohydrates using chemical pretreatment methods. Further among the three chemicals used, one of the best chemical pretreatment resulted with KOH is employed after steam explosion for improvement of delignification. The results of all the three chemical treatments were statistically analyzed by ANOVA and the significant difference found is shown in Fig. 1a–f.

One of the strategies employed to increase cellulose content and enzyme accessibility to cellulose is treatment of steam-exploded rice straw with 2% KOH (SEKOH), which showed maximum lignin loss of $85 \pm 2\%$ with 4% sugar loss at 121 °C for 30 min and obtained $66.4 \pm 1.8\%$ holocellulose which is lower as compared to previous studies where the yield of holocellulose was 71.61% at 220 °C for 4 min steam explosion followed by 10% (w/w) NaOH pulping at 170 °C for 120 min (Ibrahima et al. 2011). The lower holocellulose content may be due to the lower temperature used for the pretreatment. The severity factor for the two-step process (SEKOH) was found to be 3.10 at which most hemicellulose was removed by steam explosion at 170 °C/10 min and subsequent chemical treatment with 2% KOH gave effective lignin and hemicellulose removal for excessive cellulose recovery. Samples with autoclaved rice straw (ARS) and steam-exploded rice straw (SERS) showed decreased concentrations of xylose, and post alkali-treated steam-exploded rice straw revealed almost complete removal of hemicellulose. The results are in agreement with Jin and Chen (2006), Keshav et al. (2016a). This may be due to the removal of hemicelluloses during high heat treatments (Petrik et al. 2013) and the effect of diverse pretreatments or delignification methods.

Fig. 1 Pretreatment of rice straw using different concentrations of chemicals at different time intervals at autoclave temperature **a** NaOH delignification (%), **b** NaOH sugar loss (%), **c** KOH delignification

(%), **d** KOH sugar loss, **e** NaClO₂ delignification (%), **f** NaClO₂ sugar loss (%)

Enzymatic hydrolysis

To evaluate the effect of various pretreatments like autoclaving, steam explosion, and RRS, the solid content obtained after consistent treatments was subjected to

enzymatic hydrolysis with cellulases (Cellulase—30 FPU/g cellulose and Xylanase—15 IU/g). The total sugars released were estimated and the individual sugar concentrations were investigated on HPLC. Enzymatic studies conducted for the samples of SEKOH treated with cellulase

did not show xylose while steam-exploded rice straw (SERS), raw rice straw (RRS), and autoclaved rice straw (ARS) showed xylose (Yu et al. 2010). The total sugars released after enzymatic hydrolysis in SEKOH, steam-exploded, autoclaving, and raw rice straw samples were 35.6 ± 0.34 , 34.8 ± 2.19 , 31.38 ± 0.71 , and $23.04 \pm 0.64\%$, respectively. All the samples showed good amount of glucose with a maximum of $21.51 \pm 1.08\%$ in SEKOH and a minimum of $4.18 \pm 0.13\%$ in raw rice straw (RRS). The present findings are in accordance with the earlier reports where the enzyme loading of 40 FPU/g at 140–180 °C, saccharified 43–59% by autoclaving (Yu et al. 2010) and the substrate was steam exploded at 179.9–220 °C for 4–5 min to get similar saccharification (Jin and chen 2006).

Pretreatments by autoclaving showed the saccharification efficiency of $67.2 \pm 1.62\%$ with 448.12 ± 1.48 mg/g sugars release with mixture of different enzyme loads. The present findings of autoclave pretreatment are in agreement with previous reports where the HCW (hot compressed water) pretreatment of rice straw at 140 °C for 30 min and enzymatic hydrolysis with 40 FPU/g showed only 68.21% efficiency (Yu et al. 2010). The raw rice straw yielded 351 ± 9.87 mg/g sugars with 51.78% saccharification efficiency with both enzymes. The present findings are in contrast with previous reports where the untreated rice straw with mixture of enzymes yielded 270 mg/g biomass (Wi et al. 2013). The increase in sugars from raw rice straw may be due to the plant variety, lower particle size of rice straw, surface area for higher enzyme accessibility or variations in harvest time/seasons (Sun et al. 2014).

The SEKOH (30 FPU/g) and SERS (30 FPU/g, 15 IU/G) hydrolysis using different enzymatic load to the each pretreated residues showed efficient saccharification. The rise in cellulose portion and greater lignin loss in steam-exploded rice straw treated with KOH in autoclave (SEKOH) is a prophesied reason for enhanced hydrolysis. It is agreed by attaining 664.0 ± 5.39 mg/g sugars release with $82.60 \pm 1.3\%$ efficient hydrolysis by SEKOH method and 540 ± 1.89 mg/g sugars release with $75.93 \pm 0.86\%$ by SERS method after 36 h. The high sugar release in 48 h is due to the chemical pretreatment carried out after steam explosion at autoclave temperature and pressure that facilitates the higher penetration of chemical into biomass for high lignin reduction and more cellulose accessibility to cellulase. The KOH treatment after steam explosion chiefly attributed to the efficient removal of lignin and hemicelluloses from rice straw and enhanced hydrolysis significantly. In recent study, the impact of time and pressure of steam explosion pretreatment on structural features of lignin and delignification with alkaline treatments have been examined (Sun et al. 2014). The obtained results of the present study showed that steam explosion and KOH

treatment in autoclave with a particular time (30 min) under pressure removed more lignin from SEKOH treated rice straw. Therefore, the maximum lignin loss was positively correlated with efficient hydrolysis of cellulose in 48 h. In brief, delignification by KOH treatment of rice straw after steam explosion could increase the enzymatic hydrolysis of pretreated residue.

The findings of the experiments carried were supported by recent reports where the autoclaved bamboo powder in 2% NaOH at 121 °C for 2 h, and treated with 100 MPa ultra-high pressure explosion by the homogenizer produced 93.10% reducing sugars (Jiang et al. 2016). In another study conducted for the combined pretreatment and strategic hydrolysis of rice straw for bioethanol and biopolymer (poly-3-hydroxybutyrate) production, maximum 0.374 g/g reducing sugars were released using Sonics (Vibra cell) ultrasonic cell disrupter (USA) (Sindhu et al. 2016). The pretreated miscanthus grass on enzymatic hydrolysis with 20 FPU/g cellulose at 50 °C for 72 h obtained 93.6% with 31.2 g/L glucose by combined pretreatment of ammonia and CO₂ (Cha et al. 2014). The highest cellulose saccharification (92%) of triticale straw was found with steam explosion pretreatment at 200 °C for 10 min as reported by Agudelo et al. (2016). The Maximum of 205 mg/g reducing sugars from corncobs and 100 mg/g of sugars from soybean cake were recovered with 100 IU cellulase in 48 h with 28% saccharification efficiency (Zhang et al. 2010). The rate of SEKOH treated rice straw hydrolysis is directly related to enhanced delignification as lignin effects the saccharification efficiency.

Alternatively, cellobiose was also noticed in all the physically pretreated samples. The fact is that cellulose is present in two forms, i.e. crystalline and amorphous with a cellobiose unit connected by β -1, 4 glucosidic bonds. Usually cellulase enzyme consists of three enzymes i.e. endo-glucanases, cellobiohydrolases and β -glucosidases utilized for enzymatic degradation of cellulose polymer to monomers. Endoglucanases interrupt the low-crystallinity regions of the cellulose fiber and create free chain-ends. The cellobiohydrolases promote degradation from the free chain-ends of cellulose by detaching cellobiose units (dimers of glucose). The generated cellobiose is split into mono sugar, i.e. glucose by β -glucosidase. Zhang et al. (2010) observed that the cellobiose concentrations are high in the hydrolysates due to the activity of β -1, 4-endoglucanase and β -1, 4-exoglucanase of *T. reesei* ATCC 26921. The inhibitory activities of β -1, 4-endoglucanase and β -1, 4-exoglucanase might be due to the accumulation of cellobiose which could affect the yield of fermentable sugars. The results indicate that three (RRS, ARS and SERS) pretreated residues with mixture of enzymes and cellulase treatment of SEKOH showed better saccharification. The

comparative result of enzyme hydrolysis with individual sugars and total reducing sugars have been illustrated in Fig. 2.

Fermentation

The samples of steam exploded and SEKOH rice straw hydrolysates were subjected to the mono culture fermentation by *S. cerevisiae* OBC14 along with the raw rice straw hydrolysate sample as control with co-culture by *P. stipitis* and *S. cerevisiae* OBC14 for both pentoses and hexoses fermentation. The enzymatic hydrolysis of SEKOH (5.87 ± 0.16 mg/mL), SERS (2.51 ± 0.13 mg/mL) and RRS (3.40 ± 0.19 mg/mL) sugars were produced from the respective pretreated residues. The determination of furanic compounds in fermentation of steam explosion and SEKOH hydrolysate are negligible. Samples were drawn for left over sugars and ethanol analysis at 0, 12, 24, 36 and 48 h of fermentation and were suitably diluted and analyzed by HPLC (Figs. 3 and 4). The steam exploded rice straw (SERS) hydrolysate containing 25.16 ± 2.6 g/L sugars yielded 8.28 ± 0.06 g/L (0.32 g/g) ethanol after 36 h fermentation which is comparable with previous report showed 6 g d/m³ ethanol from 15 g/L total sugars (Nakamura et al. 2001). The SEKOH hydrolysate containing 58.70 ± 1.52 g/L sugars was fermented by the same strain of yeast yielded 26.12 ± 1.24 g/L ethanol (0.44 g/g) with productivity of 0.72 g/L/h (Fig. 3). Yeast *S. cerevisiae* OBC14 was able to produce good amount of ethanol, when incubated at 30 °C at 150 rpm and maximum amount of glucose was utilized within 36–48 h of fermentation. The leftover sugars of 3.89 g/L were found in the fermented hydrolysate of SEKOH treated rice straw. The ethanol production after 36 h of fermentation was declined due to the simultaneous ingestion of sugars and hoarded ethanol

in the medium by the grooved yeast (Kuhad et al. 2010). The results were in agreement to the previous studies where (Keshav et al. 2016a), the SEKOH hydrolysate 68.20 ± 1.16 g/L sugars fermentation by *S. cerevisiae* VS3 yielded 23.17 ± 0.84 g/L ethanol or 0.44 g/g production from the cotton stalks. When co-cultures were used for raw rice straw hydrolysate fermentation, 9.6 ± 0.35 g/L ethanol was produced from 34.04 ± 0.64 g/L total sugars present in hydrolysate after 36 h (Fig. 4). Fermentation of raw rice straw hydrolysate with *S. cerevisiae* yielded 0.172 g/g ethanol after 24 h which is equivalent to 80.9% of the theoretical yield based on the glucose content of raw material has been reported by Wi et al. (2013). Thakur et al. (2013), reported fermentation of untreated wheat straw resulted in 0.31 g/g ethanol production using *S. cerevisiae*. The untreated rice straw used in this experiment yielded 0.282 g/g ethanol production is due to presence of pentose and hexose fermenting yeast strains *P. stipites* and *S. cerevisiae*. Fermentation of raw rice straw hydrolysate containing furanic compounds were detoxified as described by Martinez et al. (2000) (results not discussed). Even though there is no much amount of xylose is released, this ethanol yield may be due to the dominant growth of *S. cerevisiae* in utilizing hexoses than *P. stipites* and also utilization of glucose by *P. stipites*. Fermentation is laggard and of lesser efficiency due to conflicting oxygen requirements between two strains and/or catabolite repression on the xylose assimilation caused by glucose (Wiater and Targonski 2002). Also it may be the inability to provide optimal environmental conditions for the two strains simultaneously with efficient fermentation and ethanol production (Chandrakant and Bisaria 1998).

Under desirable conditions, theoretical yield of ethanol is between 90 and 95%, but in practical the same yield cannot be achieved because the total sugars present in the fermentation medium may not be totally converted into ethanol may also be utilized for the synthesis of cell constituents and other products such as glycerol, acetic, lactic and succinic acid (Hidalgo et al. 2013). The previous studies on ethanol production from different lignocellulosic feed stocks using enzymatic hydrolysis and fermentation by *S. cerevisiae* have been shown in Table 3.

The present results of ethanol production from lignocellulosic biomass by *S. cerevisiae* are well correspondence with the recent studies (Govumoni et al. 2013), where they reported 24.4 g/L ethanol (0.44 g/g) yield from the hydrolysate of wheat straw by *S. cerevisiae* VS3. The fermentation efficiency of yeast isolate OBC14 was found to be 87.03 ± 1.6 which is similar to the previous studies where the fermentation efficiency was reported as 88% with the hydrolysate of Chrysanthemum by *S. cerevisiae* (Hidalgo et al. 2013).

Fig. 2 Enzymatic hydrolysis of different pretreated substrates

Fig. 3 Steam-exploded (SERS) and steam-exploded, KOH-pretreated (SEKOH) cellulase saccharified hydrolysate fermentation by *S. cerevisiae* OBC14

Fig. 4 Raw rice straw (RRS) cellulase saccharified hydrolysate fermentation by co-culture *P. stipites* NCIM 3498 and *S. cerevisiae* OBC14

Conclusions

Considering the evolution and necessity of second generation biofuels, rice straw appears to be an encouraging and potential feed stock for production of bioethanol due to its

plentiful availability and attractive composition. Steam explosion (170 °C/10 min) and treatment with alkali (2% KOH) in autoclave for 30 min found as one of the suitable pretreatment methods for maximum sugar recovery by enzymatic hydrolysis of cellulosic fraction of rice straw.

Table 3 Ethanol production performance studies on different lignocellulosic feed stocks

Feedstock	Ethanol (g/L)	$Y_{p/s}$ (g/g)	Q_p (g/L h)	Enzymatic hydrolysis and fermentation using <i>S. cerevisiae</i> and other strains	Reference
Synthetic medium with glucose and xylose	9.73	0.42	2.1	<i>S. cerevisiae</i> /Scheffersomyces stipites	Ashoor et al. (2015)
Wheat straw	15.0	0.54	NA	<i>S. cerevisiae</i>	Thakur et al. (2013)
Chrysanthemum	18.82	0.45	0.76	<i>S. cerevisiae</i>	Hidalgo et al. (2013)
Cotton stalk	23.17	0.44	0.64	<i>S. cerevisiae</i>	Keshav et al. (2016a, b)
Corn cob	5.68	NA	NA	<i>Kluyveromyces marxianus</i>	Zhang et al. (2010)
Rice straw	26.12	0.44	0.72	<i>S. cerevisiae</i>	This study

NA not available

Still research on combined pretreatment is needed on sugar and ethanol yields, as this study gives knowledge on upcoming pretreatment strategies for optimization of combined sequential steam explosion and alkali treatments for maximum biomass conversion of rice straw. Yeast *S. cerevisiae* OBC14 showed ethanol fermentation efficiency of 87.03% with physico-chemical pretreated and enzyme saccharified rice straw hydrolysate.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Consent for publication This article does not contain any individual person's data.

Declaration We declare that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the final manuscript and agreed with the submission to "3 Biotech".

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