

# Saccharification of biopretreated paddy straw with indigenous holocellulase and fermentation with *Saccharomyces cerevisiae* LN1 under optimized conditions

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**Abstract** The current study was aimed at optimizing the fermentation conditions for efficient ethanol production from biologically pretreated paddy straw. The yeast strain *Saccharomyces cerevisiae* LN1 showed highest fermentation efficiency at pH 5.0 and temperature 30 °C. Paddy straw pretreated with fungus *Myrothecium roridum* LG7 was saccharified with indigenous holocellulase from *Aspergillus niger* SH3 producing total sugar yield of 26.14 mg/ml with 19.23 mg/ml of glucose. Enzymatic hydrolysate was then fermented using *S. cerevisiae* LN1 to observe the effect of nutrient supplementation (yeast extract, MgSO<sub>4</sub>·7H<sub>2</sub>O and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) on ethanol production. Higher ethanol was produced from saccharified material fermented without supplementation of any nutrient source. With the scale-up of ethanol production under optimized conditions in 7L bioreactor, 4.46 g/l of ethanol was produced with fermentation efficiency of 47.2 %. TLC of enzymatic hydrolysate confirmed the presence of p-coumaric acid, ferulic acid, vanillic acid, gallic acid and many other aromatic compounds and inhibitors in the saccharified material which limit fermentation efficiency of yeast strain. Thus, optimization of fermentation conditions can lead to development of a cost-effective process for efficient ethanol production, exploitation of which also requires removal of aromatic compounds and inhibitors which may hinder the ethanol production efficiency.

**Keywords** Bioethanol · Optimization · Fermentation efficiency · Paddy straw · *Saccharomyces cerevisiae* LN1

## 1 Introduction

The burgeoning world population and rapid industrialization have increased the global energy consumption leading to depletion of non-renewable energy resources and ever increasing oil price. India happens to be the world's fifth largest energy consumer after the USA, China, Russia and Japan accounting for 4 % of global energy consumption (FICCI 2013). India is heavily dependent on crude oil imports which during April–February, 2014–2015, were valued at US\$ 13.08 billion (Anonymous 2015). For realizing India's energy security, development of alternative fuels based on indigenous and abundant biomass feedstock to substitute or supplement petroleum-based fuels is imperative. The National Policy on Biofuels was approved by the Government of India on 24 December 2009 with the target of 20 % blending of biodiesel and bioethanol by 2017. However, as of July 2014, oil companies have been able to 'achieve' only a 1.37 % blending of ethanol with petrol. The low blending has been attributed to competing requirements of ethanol for the liquor and chemical industry and poor responses from the sugar industry.

Biofuel is a renewable and environment friendly alternative to fossil fuels (Lee et al. 2008; Thangavelu et al. 2016). In case of first-generation biofuels, the carbohydrate is usually sucrose or starch which requires the use of 'food' crops such as sugarcane, corn which can lead to shortage of food items for human consumption. Second-generation biofuels are those obtained from lignocellulosic biomass such as

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agricultural residues and woody crops. These are potential feedstock for biofuel production as they do not threaten food security and can significantly reduce carbon dioxide emission compared to fossil fuels (Dürre 2007). In spite of having all these benefits, second-generation biofuels have not been much commercialized because lignocellulosic biomass contains lignin, hemicellulose and cellulose and their conversion to reducing sugars includes two most important costly steps, i.e. pretreatment and saccharification. Pretreatment is a very complex and costly process. Also saccharification requires costly cellulase enzymes, and complete hydrolysis of cellulose and hemicellulose into fermentable sugars is extremely difficult. Thus, there is a need to develop a feasible crop-specific pretreatment and saccharification process.

Paddy straw is an abundant lignocellulosic crop residue in the world. Every kilogram of grain is accompanied by production of 1–1.5 kg of the straw when harvested. Paddy straw because of its high silica content is unsuitable for cattle feed and is therefore available for other purposes such as composting and biofuel production. On an average basis, paddy straw contains about 32–47 % of cellulose, 19–27 % of hemicellulose and 5–24 % of lignin (Saha 2003). By optimistic estimate, with annual production of paddy straw in world at about 731MT, about 205 billion litres bioethanol can be produced annually (Balat et al. 2008). In India, total biomass production is approximately 640 MT of which 234 MT is available as surplus and can be explored for ethanol production by optimizing pretreatment, saccharification and fermentation process (Singh and Nain 2014).

Pretreatment of lignocellulosic biomass is carried out to make cellulose more accessible to enzymes via removal of lignin and hemicellulose. It can be carried out physico-chemically, chemically or biologically. Biological pretreatment employs micro-organisms, mainly white and soft rot fungi, actinomycetes and bacteria which degrade lignin through the action of lignin degrading enzymes such as peroxidases and laccases (Saritha et al. 2012). The process involves low capital cost, low energy with little dependence on chemicals under mild environmental conditions. Pretreated biomass is then saccharified to convert cellulose to ethanol by either simultaneous saccharification and fermentation (SSF) process or separate hydrolysis and fermentation (SHF) process. SSF is more favoured because of its low potential cost, less time and less number of reactors. But the optimum temperature for hydrolysing enzymes and fermenting micro-organisms are different and become the major drawback of SSF process. SHF method is carried out by use of enzyme cocktail containing endo- $\beta$ -1,4 glucanase, exo- $\beta$ -1,4 glucanase, xylanase and  $\beta$ -glucosidase.

The cellulose is hydrolysed to produce glucose monomers while hydrolysate of hemicellulose is heterogeneous and includes a number of pentose and hexose sugars (Saha 2003). Among all the sugars present in saccharified biomass,

glucose and xylose are considered to be the main components (Kordowska-Wiater and Targoński 2001). Hence, to develop an economically feasible process of ethanol production from lignocellulose, complete conversion of all reducing sugars becomes obligatory, which can be achieved by selecting micro-organisms capable of fermenting sugars with greater efficiency. In our laboratory, we have already developed a biological delignification process for paddy straw by using *Myrothecium roridum* LG7 (Tiwari et al. 2013) and also developed an indigenous enzyme cocktail for saccharification of delignified substrate (Tiwari et al. 2015).

Production of ethanol using an optimization-based strategy can make the process more cost-effective and improve ethanol yield. Optimization of key process variables such as temperature, pH and nutrient supplementation during fermentation is important as they affect the growth of yeast and thus their ethanol production efficiency. An assessment of these parameters in a systematic way is thus required to economize the entire process of ethanol production. The present study was thus conducted with the objectives to select an efficient yeast strain with higher fermentation efficiency and to optimize fermentation conditions for higher ethanol production using the selected yeast strain.

## 2 Materials and methods

### 2.1 Micro-organisms

In the present investigation, a set of fifteen yeast strains (Table 1) capable of producing ethanol procured from NCL, Pune and PAU, Ludhiana were used in the study. The yeast cultures were grown on MGYB (malt extract—0.3 %, glucose—1.0 %, yeast extract—0.3 %, peptone—0.5 %) medium, and slants of cultures were stored at 4 °C. The cultures were subcultured periodically.

### 2.2 Screening of yeast strains for ethanol production

Yeast inoculums of individual strains were prepared in MGYB broth in 50-ml Erlenmeyer flasks at 30 °C with continuous shaking (150 rpm) for 3 days. The population of yeast was estimated by dilution plate technique before inoculation of fermentation media. The ability of the yeasts to ferment D-glucose was tested in 50-ml screw capped plastic bottles containing a 5 % (w/v) sugar in 25 ml minimal media containing  $\text{KH}_2\text{PO}_4$  (0.1 %),  $(\text{NH}_4)_2\text{SO}_4$  (0.5 %),  $\text{MgSO}_4$  (0.5 %) and yeast extract (0.1 %) with pH 5.0. The bottles were inoculated with 10 % v/v inoculum of yeast and incubated at 30 °C under static conditions for 48 h. Samples were withdrawn at different time intervals of 24 and 48 h. One ml of fermented broth was centrifuged at

**Table 1** Screening of yeast strains for ethanol production by glucose fermentation in minimal media under stationary condition

S. no.	Organism	Residual sugar (mg/ml)	Ethanol (mg/ml)	Yp/s (g/g)	Qp (g/l/h)	Fermentation efficiency (%)
1.	<i>Pichia stipitis</i> NCIM 3498	0.15 ± 0.00	5.99 ± 0.02	0.04 ± 0.00	0.12 ± 0.00	8.12 ± 0.01
2.	<i>Pichia stipitis</i> NCIM 3497	0.24 ± 0.01	11.88 ± 0.05	0.02 ± 0.00	0.25 ± 0.03	3.63 ± 0.01
3.	<i>Candida lusitanae</i> NCIM 3484	0.17 ± 0.00	19.83 ± 0.06	0.27 ± 0.01	0.41 ± 0.03	53.46 ± 0.22
4.	<i>Pichia stipitis</i> NCIM 3506	0.00 ± 0.00	14.02 ± 0.04	0.00 ± 0.00	0.29 ± 0.02	0.00 ± 0.00
5.	<i>Saccharomyces cerevisiae</i> LN1	0.14 ± 0.02	24.34 ± 0.11	0.49 ± 0.03	0.51 ± 0.04	95.36 ± 0.23
6.	<i>Saccharomyces cerevisiae</i> CP2	0.35 ± 0.02	23.84 ± 0.10	0.47 ± 0.02	0.50 ± 0.03	91.25 ± 0.22
7.	<i>Saccharomyces cerevisiae</i> CP4	0.76 ± 0.04	21.22 ± 0.07	0.42 ± 0.02	0.44 ± 0.03	82.04 ± 0.20
8.	<i>Rhodotorula mucilaginosa</i> YSC2	0.31 ± 0.02	19.77 ± 0.10	0.10 ± 0.00	0.41 ± 0.01	19.66 ± 0.01
9.	<i>Hanseniaspora uvarum</i> SG	0.21 ± 0.01	3.68 ± 0.01	0.06 ± 0.00	0.08 ± 0.00	12.43 ± 0.01
10.	<i>Debaryomyces hansenii</i> SF	0.34 ± 0.01	16.12 ± 0.04	0.08 ± 0.00	0.34 ± 0.02	16.59 ± 0.02
11.	<i>Candida parapsilosis</i> SC1	0.18 ± 0.00	8.42 ± 0.01	0.11 ± 0.00	0.18 ± 0.01	21.67 ± 0.03
12.	<i>Pichia stipitis</i> NCIM 3507	0.44 ± 0.02	9.32 ± 0.01	0.01 ± 0.00	0.19 ± 0.01	2.33 ± 0.01
13.	<i>Saccharomyces cerevisiae</i> DC	0.63 ± 0.03	23.32 ± 0.12	0.48 ± 0.02	0.49 ± 0.02	93.97 ± 0.21
14.	<i>Rhodotorula glutinis</i> Y1	0.54 ± 0.02	17.85 ± 0.11	0.18 ± 0.00	0.37 ± 0.02	36.15 ± 0.04
15.	<i>Candida tropicalis</i> Y6	0.19 ± 0.00	19.03 ± 0.10	0.41 ± 0.02	0.40 ± 0.03	79.72 ± 0.05

Initial glucose concentration: 5 %w/v, pH—5.0, temperature—30 °C, inoculum rate 10 %v/v, incubation—48 h, data are mean ± SD,  $n = 3$

10,000 rpm for 2 min, and supernatant was used for estimation of residual sugar and ethanol. Cell pellet was dried and weighed to calculate yeast biomass. The residual sugars and ethanol concentration were estimated by HPLC as described earlier (Pandiyani et al. 2014) (Waters 410, Milford, MA, USA) using an Aminex column with a sample injection volume of 10 µl and a mobile phase of 5 mM H<sub>2</sub>SO<sub>4</sub>. Yeast strains with higher ethanol production efficiency were selected for further experimentation.

### 2.3 Optimization of fermentation parameters (pH and temperature) for *S. cerevisiae* LN1 with one factor at a time approach

Effect of pH on ethanol production by *S. cerevisiae* LN1 was carried out by varying the initial pH of fermentation media to 4.0, 5.0, 6.0 and 7.0. Effect of temperature on ethanol production by *S. cerevisiae* LN1 was carried out by varying the incubation temperatures to 25, 30 and 35 °C. Fermentation was carried out as described in Sect. 2.2. Samples were withdrawn at intervals of 24 and 48 h and analysed for residual sugar and ethanol by HPLC.

### 2.4 Biological pretreatment of paddy straw

#### 2.4.1 Substrate and micro-organism for pretreatment

Dried paddy straw of variety Pusa Sugandh 5 with 16 % lignin, 39.3 % cellulose and 22.2 % hemicellulose content, procured from division of Agronomy, Indian Agricultural Research Institute, New Delhi (India), was used as substrate

for bioethanol production after biological pretreatment for delignification of biomass. *Myrothecium roridum* LG7, a lignolytic micromycete fungus isolated from decaying wood, was used for biological delignification of paddy straw. This fungus shows very low cellulase activity along with high lignolytic activity (Tiwari et al. 2013).

#### 2.4.2 Biological delignification of paddy straw

Sixty grams of 1-cm-sized paddy straw pieces were taken in 2000-ml Erlenmeyer flasks, and Reese's mineral medium (Reese and Mandels 1963) (10 ml/g) was added into the flasks which were autoclaved at 121 °C for 30 min. The flasks were inoculated with 15 mycelial discs (6 mm diameter) of *M. roridum* LG7 grown on PDA plates and incubated at 30 °C for 5 days. A flask containing autoclaved and uninoculated substrate was used as control. All the treatments were carried out in triplicates. The contents of the flasks were mixed on alternate days, manually for 2 min. Following incubation, the pretreated paddy straw was washed with 0.5 N NaOH (in a 1:10 ratio of biomass sample to NaOH) followed by 3 washings with distilled water in the same ratio. The washed biomass samples were stored, till used, in the refrigerator at 4 °C.

### 2.5 Saccharification of biopretreated paddy straw

#### 2.5.1 Saccharifying enzyme

Cold active holocellulolytic enzyme cocktail produced by *Aspergillus niger* SH3 was used for saccharification of bio

pretreated paddy straw. *A. niger* SH3 isolated from Himalayan region of India showed high productivity of holocellulolytic enzymes between 5 and 15 °C of growth temperatures (Tiwari et al. 2015). Filter paperase (FPase) and Carboxymethyl cellulase (CMCase) activities were assayed as described by Ghose (1987) and xylanase activity, by the method described by Ghose and Bisaria (1987). The reducing sugars released were measured by using dinitro salicylic acid (DNSA) method (Miller 1959). One unit of filter paper activity or CMCase corresponded to 1 µmol of glucose formed per minute during hydrolysis and one enzyme unit of xylanase was expressed as 1 µmol of xylose formed per minute during hydrolysis. β-Glucosidase assay was performed using p-nitrophenyl-b-D-glucopyranoside as substrate (Wood and Bhat 1988) and the activity was calculated in terms of µmoles of p-nitrophenol produced per ml of culture filtrate per minute.

### 2.5.2 Enzymatic saccharification of *M. roridum* LG7 pretreated paddy straw

Saccharification of the pretreated samples was carried out according to the NREL LAP-009 protocol (Brown and Torget 1996) with slight modifications. Five grams of *M. roridum* LG7 pretreated paddy straw was transferred in Erlenmeyer flasks to which 60 ml of 50 mM sodium citrate buffer (pH 4.8), and 5 ml of concentrated crude enzyme extract from *Aspergillus niger* SH3 was added, with supplementation of 1 ml of sodium azide (2 % w/v), to prevent microbial contamination. Another set of three flasks were added with 5 ml of commercial cellulases (Celluclast® 1.5L) for comparison of results with indigenous enzyme. The flasks were then incubated at 40 °C for 72 h under shaking conditions at 150 rpm. Samples were withdrawn from the reaction mixture at different time intervals and centrifuged at 10,000 rpm for 10 min. The supernatant was filtered through 0.20-µm nylon syringe filters and used for analysis of reducing sugar by HPLC.

## 2.6 Compositional analysis of biopretreated paddy straw hydrolysate

Enzymatic hydrolysate of bio pretreated paddy straw was also analysed for micronutrient and N, P and S contents. The hydrolysate was first added with mixture of nitric acid and perchloric acid to digest the material. The digested hydrolysate was then used for analysis of micronutrient concentrations (Fe, Zn, Cu and Mn) by using atomic absorption spectrophotometer at the most sensitive wavelengths for Zn (213.7 nm), Cu (324.6 nm) and Mn (279.5 nm) (Lindsay and Norvell 1978). Phosphorus content was measured by the method given by Jackson (Jackson 1967), and sulphur content was measured by

turbidimetric method (Hart 1961). The total nitrogen was estimated by Kjeldahl's method, and the per cent nitrogen content in the hydrolysate was recorded using N AutoAnalyzer (Jackson 1967).

## 2.7 Fermentation of enzymatic hydrolysate with yeast

### 2.7.1 Micro-organism and inoculum preparation

The yeast *S. cerevisiae* LN1 maintained in MGYB agar slants was used for fermentation of enzymatic hydrolysate. The inoculum was prepared in MGYB broth (pH 7.0 ± 0.2). Flasks were incubated at 30 °C in rotary shaker under agitation at 150 rpm for 72 h. The population of yeast was estimated by dilution plate technique before inoculation of saccharified material.

### 2.7.2 Effect of nutrient supplementation on ethanol production

Three factors (yeast extract, MgSO<sub>4</sub>·7H<sub>2</sub>O and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) were selected to observe the effect of nutrient supplementation on ethanol production by *S. cerevisiae* LN1. Factors were optimized by using one factor at a time approach. Yeast extract (0.1 %), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.5 %) and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.5 %) were added separately in three flasks each containing enzymatic hydrolysate with different pH of 4.0, 4.5 and 5.0. In control flasks, only saccharified pretreated paddy straw hydrolysate at different pH (4.0, 4.5 and 5.0) was used for fermentation at 30 °C.

### 2.7.3 Fermentation

The batch fermentation of enzymatic hydrolysate was carried out in 50-ml screw capped plastic bottles with the working volume of 20 ml. The hydrolysate was inoculated with 10 % (v/v) inoculum of *S. cerevisiae* LN1. The fermentation temperature was kept constant at 30 °C in an incubator under static condition. Samples were withdrawn at intervals of 24 h and centrifuged at 10,000 rpm for 2 min. The cell free supernatant was used to determine the ethanol content and residual sugar concentration by HPLC.

### 2.7.4 Scale-up of ethanol production in fermentor

For scale-up of production of ethanol, enzymatic hydrolysate of pretreated paddy straw (3L) was fermented with *S. cerevisiae* LN1 under optimized conditions in 7L stirred tank bioreactor (Applikon, Schiedam, Netherlands) controlled by Applikon Biocontroller, Bioconsole AD11025. The bioreactor was equipped with automatic control of temperature (30 °C), pH (5.0) and kept under static

condition for 48 h. Samples were periodically withdrawn, centrifuged at 10,000 rpm for 10 min and analysed for residual sugar and ethanol by HPLC as described earlier.

### 2.7.5 Thin-layer chromatography for the detection of inhibitors

Fermented broth (30 ml) was extracted with ethyl acetate [1:1; v/v] for 30 min in a separating funnel. The ethyl acetate fractions were evaporated to dryness and reconstituted in methanol. The methanolic solution of ethyl acetate extract was filtered through 0.45 mm Supor-450 membrane disc filter. One hundred millilitre of extract was spotted on silica gel plates (Alugram precoated TLC sheet, Camlab), and thin-layer chromatography (TLC) of plates was performed using mobile phase chloroform:methanol:formic acid (85:15:1; v/v/v) and visualized under UV light. Standards (p-coumaric acid, t-ferulic acid, vanillic acid, gallic acid and tannic acid) each with 1 mg/ml concentration were also spotted on TLC plates for identification of aromatic compounds. Total phenolic content of fermented broth was also estimated by using method of Bray and Thorpe (1954).

## 2.8 Calculation of fermentation parameters

Ethanol yield ( $Y_p/s$ , g/g) was calculated by the ratio between ethanol concentration (g/l) and sugar consumed (g/l), while the ethanol productivity ( $Q_p$ , g/l/h) was determined by the ratio between ethanol concentration (g/l) and fermentation time (h). Fermentation efficiency was calculated as per cent of theoretical ethanol yield.

## 3 Results

### 3.1 Screening of yeast strain for ethanol production

The amount of ethanol produced by the fifteen yeast strains screened for their ethanol production efficiency ranged between 3.68 and 24.34 mg/ml with ethanol yield ranging from 0.02 to 0.49 g/g of glucose utilized (Table 1). *S. cerevisiae* LN1 produced maximum ethanol concentration of  $24.34 \pm 0.11$  mg/ml (ethanol yield  $0.49 \pm 0.03$  g/g) after 48 h of fermentation under static condition. *S. cerevisiae* LN1 also showed maximum fermentation efficiency of  $95.36 \pm 0.23$  % followed by three strains *S. cerevisiae* CP4, *S. cerevisiae* CP2 and *S. cerevisiae* DC with fermentation efficiency of  $82.04 \pm 0.20$  %,  $91.25 \pm 0.22$  % and  $93.97 \pm 0.21$  %, respectively.

All the strains were able to consume glucose efficiently with production of ethanol except few strains belonging to genera *Pichia* (*P. stipitis* NCIM 3498, *P. stipitis* NCIM

3497, *P. stipitis* NCIM 3506, *P. stipitis* NCIM 3507) showing least fermentation efficiency. Maximum glucose consumption was observed with *S. cerevisiae* LN1 with minimum residual glucose of  $0.28 \pm 0.02$  mg/ml. Therefore, *S. cerevisiae* LN1 was selected for further experiments on optimization of fermentation conditions.

### 3.2 Optimization of fermentation parameters (pH and temperature) for *S. cerevisiae* LN1 with one factor at a time approach

The results indicated that maximum ethanol produced was  $24.44 \pm 0.12$  mg/ml (ethanol yield  $0.49 \pm 0.01$  g/g) at pH 5.0 with fermentation efficiency of  $95.64 \pm 0.30$  % after 48 h of incubation under static condition in minimal media with glucose as sugar source (Table 2). At pH lower or higher than 5.0, less ethanol production was observed. Since pH 5.0 was found to be optimum for ethanol production, further experiments were performed at pH 5.0. Maximum ethanol produced was  $24.39 \pm 0.12$  mg/ml (ethanol yield  $0.48 \pm 0.02$  g/g and productivity of  $0.50 \pm 0.04$  g/l/h) at temperature 30 °C with fermentation efficiency of  $95.45 \pm 0.21$  % after 48 h of incubation under static condition in minimal media with glucose as sugar source (Table 3). At temperature lower or higher than 30 °C less, ethanol production was observed. Therefore, pH 5.0 and temperature 30 °C were found to be optimum for glucose fermentation for higher ethanol production.

### 3.3 Saccharification of *Myrothecium roridum* LG7 pretreated paddy straw

#### 3.3.1 Biological delignification of paddy straw

Based on previous studies carried out in our laboratory, *M. roridum* LG7 was selected for biological delignification of paddy straw. Therefore, the delignification process was carried out under solid-state condition for 5 days at 30 °C, and subsequently, soluble lignin was washed out with mild alkali (0.5 N NaOH). Residual alkali was removed completely by subsequent washing thrice by tap water. The delignified substrate consists of cellulose (59.3 %), hemicelluloses (25.7 %) and lignin (7.9 %) (Tiwari et al. 2013).

#### 3.3.2 Enzymatic saccharification of *M. roridum* LG7 pretreated paddy straw

Indigenous enzyme preparation from *Aspergillus niger* SH3 was concentrated ten times by acetone precipitation and used for saccharification of biopretreated paddy straw. This holocellulolytic enzyme cocktail possessed endoglucanase



**Table 2** Effect of pH on ethanol production by *S. cerevisiae* LN1 in minimal media with glucose as sole carbon source

pH	24 h						48 h					
	Consumed sugar (mg/ml)	Ethanol (mg/ml)	Yp/s (g/g)	Qp (g/l/h)	Fermentation efficiency (%)		Consumed sugar (mg/ml)	Ethanol (mg/ml)	Yp/s (g/g)	Qp (g/l/h)	Fermentation efficiency (%)	
4	45.52 ± 0.03	21.98 ± 0.01	0.48 ± 0.01	0.92 ± 0.12	94.48 ± 0.11		49.99 ± 0.10	23.38 ± 0.01	0.47 ± 0.03	0.49 ± 0.10	91.50 ± 0.20	
5	45.75 ± 0.02	22.23 ± 0.00	0.49 ± 0.02	0.93 ± 0.13	95.07 ± 0.12		49.99 ± 0.10	24.44 ± 0.02	0.49 ± 0.01	0.51 ± 0.13	95.64 ± 0.22	
6	47.82 ± 0.0	20.29 ± 0.00	0.42 ± 0.01	0.85 ± 0.12	82.99 ± 0.10		49.97 ± 0.11	20.67 ± 0.01	0.41 ± 0.02	0.43 ± 0.10	80.92 ± 0.11	
7	48.78 ± 0.00	19.73 ± 0.00	0.41 ± 0.01	0.82 ± 0.10	79.15 ± 0.05		49.98 ± 0.10	21.35 ± 0.03	0.43 ± 0.02	0.44 ± 0.11	83.58 ± 0.21	

Initial glucose concentration: 5 %w/v, pH—5.0, temperature—30 °C, inoculum rate 10 %v/v, data are mean ± SD, n = 3

**Table 3** Effect of temperature on ethanol production by *S. cerevisiae* LN 1 in minimal media with glucose as sole carbon source

Temperature	24 h						48 h					
	Consumed sugar (mg/ml)	Ethanol (mg/ml)	Ethanol (g/g)	Qp (g/l/h)	Fermentation efficiency (%)		Consumed sugar (mg/ml)	Ethanol (mg/ml)	Ethanol (g/g)	Qp (g/l/h)	Fermentation efficiency (%)	
25	35.47 ± 0.12	16.83 ± 0.10	0.47 ± 0.11	0.70 ± 0.05	92.83 ± 0.12		49.46 ± 0.02	23.59 ± 0.11	0.48 ± 0.03	0.49 ± 0.03	93.32 ± 0.11	
30	44.92 ± 0.11	22.19 ± 0.03	0.49 ± 0.12	0.92 ± 0.06	96.65 ± 0.13		49.97 ± 0.02	24.39 ± 0.12	0.48 ± 0.02	0.50 ± 0.04	95.45 ± 0.21	
35	41.09 ± 0.10	19.65 ± 0.02	0.48 ± 0.10	0.82 ± 0.10	93.57 ± 0.11		48.63 ± 0.04	22.88 ± 0.10	0.47 ± 0.03	0.48 ± 0.02	92.05 ± 0.13	

Initial glucose concentration: 5 %w/v, pH—5.0, temperature—30 °C, inoculum rate 10 %v/v, data are mean ± SD, n = 3

( $65.59 \pm 2.67$  IU/ml),  $\beta$ -glucosidase ( $54.57 \pm 3.63$  IU/ml), FPase ( $33.15 \pm 0.88$  IU/ml) and xylanase ( $195.16 \pm 3.61$  IU/ml) activity. The total sugar obtained after the saccharification of *M. roridum* LG7 pretreated paddy straw with crude enzyme extract from *A. niger* SH3 was  $26.14 \pm 0.21$  mg/ml or  $522.81 \pm 3.02$  mg/gds. The saccharified hydrolysate contained 19.28 mg/ml glucose, 6.91 mg/ml xylose and 1.49 mg/ml of arabinose (Fig. 1). Total sugar yield obtained from commercial cellulases (Celluclast@ 1.5L) was  $19.23 \pm 0.16$  mg/ml or  $385.53 \pm 2.01$  mg/gds (Table 4).

### 3.4 Fermentation of enzymatic hydrolysate with *S. cerevisiae* LN1

#### 3.4.1 Effect of nutrient supplementation on ethanol production from saccharified substrate

Higher ethanol was produced without supplementation at optimized pH of 5.0 than at other pH values (Table 5). The effect of any of the factor (yeast extract,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and  $(\text{NH}_4)_2\text{SO}_4$ ) over ethanol yield was found insignificant as compared to that of control (enzymatic hydrolysate without any nutrient supplementation). Maximum ethanol of  $4.49 \pm 0.03$  mg/ml was observed in control flask at pH 5.0 (ethanol yield  $0.23 \pm 0.02$  g/g and productivity  $0.09$  g/l/h) from 19.28 mg/ml of glucose.

#### 3.4.2 Composition of enzymatic hydrolysate from biopretreated paddy straw

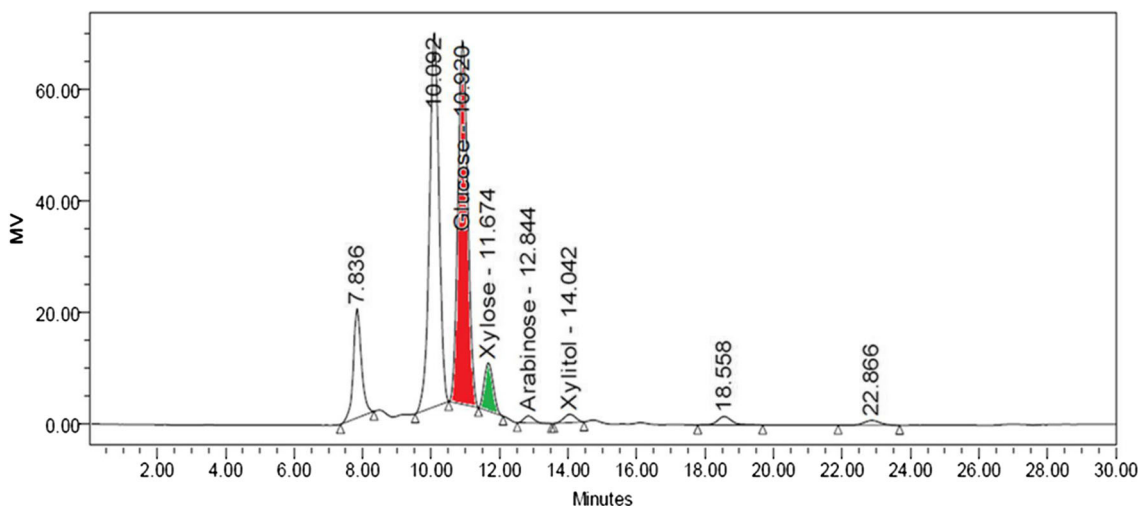
Enzymatic hydrolysate obtained from bio pretreated paddy straw was found to have high level of macro- and micronutrients apart from sugars (Table 6). Nitrogen, phosphorus and sulphur was recorded to be 400, 1200 and 2300  $\mu\text{g/ml}$  and micronutrient (Cu, Mn and Zn) concentration of 20, 40 and 10  $\mu\text{g/ml}$ , respectively.

#### 3.4.3 Scale-up of ethanol production in fermentor

Fermentation of enzymatic hydrolysate of bio pretreated paddy straw with *S. cerevisiae* LN1 under optimized conditions of pH (5.0) and temperature ( $30^\circ\text{C}$ ) in stirred tank bioreactor produced 4.64 g/l of ethanol (ethanol yield 0.241 g/g and productivity of 0.09 g/l/h) with fermentation efficiency of 47.20 % under static condition for 48 h. The yeast biomass increased continuously up to 48 h with complete utilization of glucose (data not shown).

#### 3.4.4 Thin-layer chromatography for detection of inhibitors

As compared to fermentation of pure glucose, only 47 % fermentation efficiency was observed during fermentation of saccharified hydrolysate. Therefore, saccharified



**Fig. 1** HPLC chromatogram of saccharified bio pretreated paddy straw

**Table 4** Sugar yield after saccharification of *Myrothecium roridum* LG7 pretreated paddy straw with indigenous enzyme cocktail

S. no.	Organism (25 FPU Cellulase)	Sugar yield (mg/ml)	Sugar yield (mg/gds)
1.	<i>Trichoderma reesei</i> ATCC 26921 (Celluclast @1.5 l)	$19.23 \pm 0.16$	$385.53 \pm 2.0$
2.	<i>Aspergillus niger</i> SH3	$26.14 \pm 0.21$	$522.81 \pm 3.0$

Substrate loading: 5 %w/v, incubation temperature:  $40^\circ\text{C}$ , enzyme loading:  $25\text{FPUg}^{-1}$ , incubation time: 72 h, data are mean  $\pm$  SD,  $n = 3$

**Table 5** Effect of nutrient supplementation on ethanol production by *S. cerevisiae* LN1 from bio pretreated paddy straw hydrolysate

S. no.	Nutritional sources			pH	Responses				
	Yeast Extract (g/l)	MgSO <sub>4</sub> ·7H <sub>2</sub> O (g/l)	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (g/l)		Consumed sugar (mg/ml)	Ethanol (mg/ml)	Yp/s (g/g)	Qp (g/l/h)	Fermentation efficiency (%)
1.	1.0	0.0	0.0	4.0	17.69 ± 0.10	3.40 ± 0.00	0.19 ± 0.00	0.07 ± 0.00	37.58 ± 0.10
2.	1.0	0.0	0.0	4.5	19.25 ± 0.11	4.33 ± 0.01	0.22 ± 0.01	0.09 ± 0.00	43.94 ± 0.21
3.	1.0	0.0	0.0	5.0	19.27 ± 0.11	4.41 ± 0.02	0.23 ± 0.02	0.09 ± 0.00	44.75 ± 0.20
4.	0.0	5.0	0.0	4.0	19.26 ± 0.12	4.05 ± 0.01	0.21 ± 0.01	0.08 ± 0.00	41.10 ± 0.10
5.	0.0	5.0	0.0	4.5	19.26 ± 0.12	4.13 ± 0.01	0.21 ± 0.01	0.09 ± 0.00	41.91 ± 0.10
6.	0.0	5.0	0.0	5.0	19.27 ± 0.12	4.38 ± 0.02	0.23 ± 0.02	0.09 ± 0.00	44.42 ± 0.22
7.	0.0	0.0	5.0	4.0	16.71 ± 0.12	2.77 ± 0.01	0.17 ± 0.00	0.06 ± 0.00	32.45 ± 0.10
8.	0.0	0.0	5.0	4.5	18.83 ± 0.07	3.95 ± 0.01	0.21 ± 0.00	0.08 ± 0.00	41.07 ± 0.14
9.	0.0	0.0	5.0	5.0	19.25 ± 0.12	4.32 ± 0.02	0.22 ± 0.01	0.09 ± 0.00	43.85 ± 0.13
10.	0.0	0.0	0.0	4.0	17.07 ± 0.11	2.95 ± 0.01	0.17 ± 0.00	0.06 ± 0.00	33.82 ± 0.11
11.	0.0	0.0	0.0	4.5	19.25 ± 0.13	4.21 ± 0.02	0.22 ± 0.01	0.09 ± 0.00	42.72 ± 0.15
12.	0.0	0.0	0.0	5.0	19.26 ± 0.13	4.49 ± 0.03	0.23 ± 0.02	0.09 ± 0.00	45.53 ± 0.23
13.	0.0	0.0	0.0	5.6	19.27 ± 0.11	4.44 ± 0.02	0.23 ± 0.02	0.09 ± 0.00	45.07 ± 0.12

Data are mean ± SD, *n* = 3

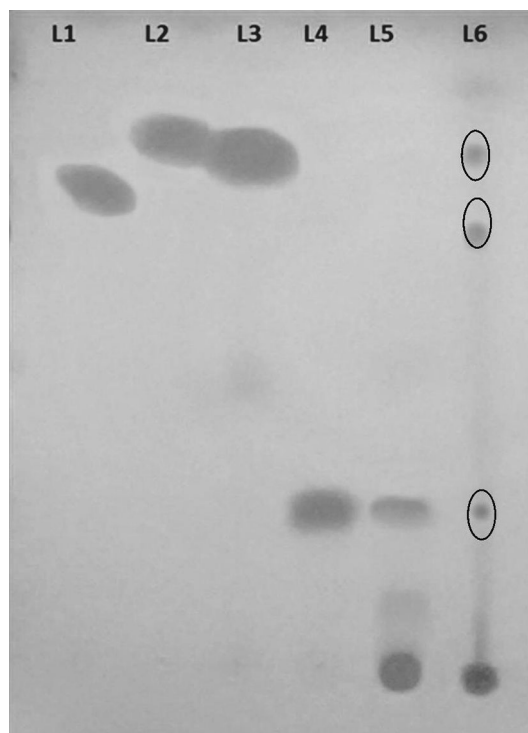
**Table 6** Composition of enzymatic hydrolysate from bio pretreated paddy straw

Components	Concentration
Total sugar	26.14 ± 0.32 mg/ml
Glucose	19.28 ± 0.2 mg/ml
Xylose	6.91 ± 0.1 mg/ml
Arabinose	1.49 ± 0.04 mg/ml
Elements	µg/ml
Nitrogen	400 ± 10
Phosphorus	1200 ± 18
Sulphur	2300 ± 42
Copper	20 ± 0.3
Manganese	40 ± 0.5
Zinc	10 ± 0.01

Data are mean ± SD, *n* = 3

hydrolysate of biopretreated paddy straw was analysed for the presence of aromatic compounds, lignin monomers and inhibitors.

TLC analysis of the ethyl acetate extract of fermented broth indicated that at least six aromatic compounds were present in the saccharified sample (Fig. 2). Analysis of the R<sub>f</sub> values (retardation value that is the ratio of the distance travelled by solute/distance travelled by mobile phase) of the compounds and the standards separated by TLC confirmed the presence of p-coumaric acid, ferulic acid, vanillic acid and gallic acid in the sample. The rest of spot were not identified but aromaticity of compound was confirmed because all the spots were visible under UV light.

**Fig. 2** Thin-layer chromatography profile of methanolic extract of fermented broth of biopretreated paddy straw hydrolysate under UV light. L1 p-coumaric acid; L2 t-ferulic acid; L3 vanillic acid; L4 gallic acid; L5 tannic acid; L6 concentrated fermented broth (20 µl)

### 3.4.5 Estimation of total phenols

Total phenolic content of fermented broth and its methanolic extract was 0.44 and 0.40 mg/ml, respectively,



indicating the presence of phenolic lignin monomers in the hydrolysate.

#### 4 Discussion

Energy security is the most important prerequisite for the economic growth and sustainable development of any country. Keeping in view the depleting fossil fuels, bio-fuels are considered the only foreseeable, feasible and sustainable energy resource. Production of bioethanol using an optimization-based strategy with efficient pretreatment, enzymes and yeast can make the process economical viable.

In the present study, *S. cerevisiae* LN1 was found to be the best strain in terms of ethanol production and fermentation efficiency. *S. cerevisiae* is the most commonly used micro-organism for ethanol production among various ethanol producers. Also, most of the strains of *S. cerevisiae* are well-established choice for large-scale production of ethanol from hexose sugars (Fernandes and Murray 2010).

As yeasts are acidophilic in nature, the optimum pH range for yeast growth can vary from 4.0 to 6.0, depending on the level of aeration and temperature. The optimum pH for *S. cerevisiae* LN1 in the present study was found to be 5.0 on minimal media with 5 % glucose (w/v). These results are in accordance with a study by Júnior et al. (2009). In the present investigation, maximum ethanol concentration was achieved at temperature 30 °C. Reed (1982) and Sedha and Verma (2002) have also recommended 30 °C as optimal temperature for ethanol production. Most of the yeasts capable of producing ethanol are not thermotolerant and their growth and fermentation efficiency decreases at 40 °C (Murata et al. 2015; Antil et al. 2015).

All the lignocellulosic substrates need pretreatment for delignification. In the present study, biological delignification was carried out using *M. roridum* LG7 which produces negligible amount of cellulase that resulted in cellulose enrichment during biodelignification (Tiwari et al. 2013). Saccharification of biologically pretreated paddy straw resulted in better yield of total reducing sugars (455.81–509.65 mg/gds) after enzymatic hydrolysis. This was supported by a study of Wanderley et al. (2013) which showed that enzymatic hydrolysis of delignified sugarcane bagasse produced total reducing sugar of 39 g/l as compared with non-delignified substrate (17 g/l).

Commercial cellulases are very costly and their use in saccharification makes the entire process of bioethanol production economically unfeasible. Thus, the use of indigenous enzyme cocktail from *A. niger* SH3 was used which can reduce the total cost of production. While the maximum activity for most cellulases has been observed at

50 ± 5 °C (Taherzadeh and Karimi 2007), *A. niger* SH3 cellulase function over a broad temperature range between 30 and 50 °C with maximum activity at 40 °C (Tiwari et al. 2015). Lower optimum temperature would make the hydrolysis process more cost-effective and thus economically feasible.

Experiment on effect of nutrient supplementation on ethanol production showed that the maximum ethanol was produced in the hydrolysate without any nutrient supplementation at optimized pH of 5.0 and temperature 30 °C. Similar results were obtained in a study by Castro and Roberto (2015) during saccharification and fermentation of dilute acid pretreated rice straw by thermotolerant yeast *Kluyveromyces marxianus* NRRL Y-6860. Among the evaluated strategies, similar ethanol yields were recorded with or without nutrient addition confirming the observation that hydrolysate must be containing sufficient nutrients to sustain metabolic activity of yeast during fermentation (Akao et al. 2015). Analysis of hydrolysate also reveals the presence of sufficient macro and micro nutrients. Thus, the present study can lead to a development of a more cost-effective method of ethanol production as nutrient supplements are not required for high ethanol yield.

Based on these findings, fermentation of biopretreated paddy straw hydrolysate was carried out in 7L bioreactor (Applikon, Schiedam, Netherlands) under optimized conditions of pH (5.0) and temperature (30 °C); 4.64 g/l (ethanol yield 0.241 g/g and productivity of 0.09 g/l/h) ethanol was produced with fermentation efficiency of 47.2 % after 48 h. Similar results were observed in a study by Sindhu et al. (2014) by fermentation of the enzymatically hydrolysed liquid from the pretreated Indian bamboo biomass using *S. cerevisiae* which showed bioethanol yield of 1.76 % (v/v) with fermentation efficiency of 41.69 %. Choice of pretreatment may also affect the ethanol yield due to variation in cellulose content. Low fermentation efficiency was obtained by fermentation of enzymatic hydrolysate of biopretreated paddy straw by *S. cerevisiae* LN1 as compared to that obtained through fermentation of minimal media with glucose as sole source of carbon. It was hypothesized that this could be due to the presence of some kind of inhibitors such as lignin monomers or aromatic compounds such as phenols in the saccharified and pretreated paddy straw. The analysis of saccharified hydrolysate revealed the presence of at least four aromatic compounds including p-coumaric acid, ferulic acid, vanillic acid and gallic acid. Saccharified material also contained phenolic compounds which might have been inhibitory for glycolytic enzymes resulting in decreased ethanol yield.

In a study by Ko et al. (2015), it was found that the insoluble lignin which predominantly remains within the pretreated solids act as a significant inhibitor of cellulases

during hydrolysis of cellulose. Similarly, lignin-derived phenolics inhibit cellulase and  $\beta$ -glucosidase by irreversible binding or precipitation. The performance of fermenting micro-organisms is also negatively affected by phenolics. Several other researchers (Jönsson et al. 1998; Kim et al. 2013) also confirmed the presence of phenolic compounds in the pretreatment slurry derived from lignin pretreatment.

## 5 Conclusion

This study therefore unveils the development of a bioprocess for efficient ethanol production under optimized conditions of fermentation. However, the aromatic compounds and lignin monomers present in biopretreated substrate may inhibit the fermentation and thus reduce ethanol yield. Efforts can be made to develop the yeast strains which may be tolerant to multiple inhibitors. Bioabatement and metabolic engineering of micro-organisms can prove to be the most promising approaches for overcoming the inhibitory effects of aromatic compounds on microbial performance and to enhance the fermentation efficiency.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that there is no conflict of interest regarding the publication of this paper.

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