Dynamic Biochemistry, Process Biotechnology and Molecular Biology ©2012 Global Science Books

A Co-culture Process with *Pichia stipitis* NCIM 3498 and Thermotolerant *Saccharomyces cerevisiae* VS3 for Ethanol Production using Acid Hydrolysate of Delignified Sorghum Straw

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ABSTRACT

Rising oil prices has attracted the research on bioconversion of lignocellulosic biomass to ethanol as an alternative fuel. Pretreatment of lignocellulosic biomass causes release of fermentable sugars both hexoses and pentoses. An efficient conversion of these two sugars is a prerequisite for a profitable process of bioethanol production from lignocellulose. Considering the approaches available for this conversion, co-culture is a simple process, employing two different organisms for the fermentation of the two sugars. The NaOH delignified sorghum straw released maximum amount of reducing sugars (30.0 g/l) in biphasic dilute acid hydrolysis. The microbial co-cultures of *Pichia stipitis* NCIM 3498 and thermotolerant *Saccharomyces cerevisiae* VS3 were employed for efficient bioconversion of mixed sugars present in the hydrolysate into ethanol. The fermentation of detoxified acid hydrolysate with monocultures of *P. stipitis*, *S. cerevisiae* VS3 and co-culture produced 10.25 ± 0, 7.40 ± 0.07, and 12.0 ± 0.55 g/L ethanol, respectively.

Keywords: bioethanol, co-culture, lignocellulosic substrate

INTRODUCTION

With industrial development growing rapidly, depletion of petroleum-based fuels and environmental problems has stimulated the development of inexpensive production of biofuels (Demain 2009). Bioethanol is an attractive, sustainable energy source to fuel transportation as it secures reductions in environmental pollution problems due to its high oxygen content (Huang *et al.* 2008).

Lignoellulosic materials are renewable, largely unused and abundantly available sources of raw materials for the production of fuel ethanol. These materials can be obtained at low cost from variety of resources, like forest residues, municipal solid waste, waste paper and crop residues (Wyman 1996). Ethanol produced from lignocellulosic biomass provides unique environmental, economic, and strategic benefits (Wyman 2007; Chandel *et al.* 2007a; Singh and Harvey 2008).

They contain sugars polymerized in form of celluloses and hemicelluloses, which can be liberated by hydrolysis and subsequently fermented to ethanol by microorganisms (Palmqvist and Hahn-Hagerdal 2000; Millati *et al*. 2002; Chandel *et al*. 2011a).

It is critical to the environmental impact of bioethanol that agricultural wastes and waste land weedy materials can be employed for bioethanol production (Huber and Dale 2009). Andhra Pradesh is one of the agro-based states of India and produces Sorghum, as the third major crop (Reddy and Sanjana 2003). Moreover, it is drought resistant and need only limited water and can be grown in the arid zones also. All these features together with its high biomass and carbohydrate contents make, sorghum the most promising crop for ethanol production (Almodares 2008). However, efficient substrate utilization is a critical step that includes alteration of the biomass size and structure for hydrolysis of the carbohydrate fraction into monomeric sugars (Moiser *et al.* 2005).

The lignocellulosic raw materials are milled initially to sizes of a few millimeters, and pretreated with acid/enzyme to obtain fermentable sugars which are further fermented to ethanol by microorganisms (Taherzadeh and Karimi 2007). Dilute acid hydrolysis can be performed in two stages. In the first stage low acid concentration and mild process conditions are employed to obtain the sugars from hemicellulose (pentoses and hexoses at low levels) and in the second stage harsher conditions are employed for the hydrolysis to recover the hexoses from the feedstock (Farooqi *et al.* 2004; Demirbas 2007). During acid hydrolysis of lingocellulosics, in addition to the sugars, aliphatic acids (acetic, formic and levulinic acid), furan derivatives, furfural and 5-hydroxymethylfurfural (HMF), and phenolic compounds are formed. These compounds are known to affect ethanol fermentation performance (Larsson *et al.* 1999).

To make the process more economic, it is necessary to either remove these fermentation inhibitors by less expensive methods or use less severe conditions for hemicellulose breakdown degradation (Chandel *et al.* 2011a).

Several detoxification methods like neutralisation, overliming with calcium hydroxide, activated charcoal (Carvalheiro *et al*. 2005) are known for removing various inhibitory compounds from lignocellulosic hydrolysates.

Depending on the hydrolysis process, two-stage or single-stage, glucose and xylose are derived from lignocellulosic substrates. The economy of ethanol production from lignocellulosic materials is much improved by the efficient fermentation of both hexose and pentose sugars (Hinman *et al*. 1989). In this process, glucose is converted into ethanol using common glucose-fermenting microorganisms such as *Saccharomyces cerevisiae* or *Zymomonas mobilis.*

The naturally occurring xylose-fermenting yeast *Pichia*

Received: 15 April, 2011. Accepted: 19 October, 2011.

Original Research Paper

stipitis shows the ability to ferment a wide variety of sugars present in lignocellulose hydrolysates, including cellobiose (Jeffries 2006; Agbogbo and Coward-Kelly 2008; Chandel *et al.* 2011a). Moreover following the depletion of glucose, *P. stipitis* ferments xylose, galactose, mannose and cellobiose simultaneously with no noticeable preceding lag period (Du Preez *et al*. 1986).

Though *Pichia stipitis* can ferment xylose and other important hexoses with relatively high yields and rate of fermentation they have low ethanol tolerance (Laplace 1991). However it still remains a challenging issue to get the suitable strain fulfilling the requirements of ethanol production from lignocelluloses at industrial level (Zhang *et al*. 2010).

Therefore for efficient conversion of all sugars to ethanol, co-fermentation of hexoses and pentoses to ethanol is recommended. In the present study co-fermentation of hexoses and pentoses was performed for the acid hydrolysate of sorghum straw with co-cultures of *Pichia stipitis* NCIM 3498 and thermotolerant *Saccharomyces cerevisiae* VS3.

MATERIALS AND METHODS

All chemicals used in the study were procured from HiMedia laboratories, Mumbai, India.

Raw material

Sorghum bicolor [(L.) Moench] was collected from National Research Centre for Sorghum, Hyderabad, India. Dry plant was processed in a laboratory disintegrator (Bajaj Mixer Grinder, GX 21, Hyderabad, India) to attain a particle size between 4-10 mm followed by washing with tap water until the washings were clear and dust free and then oven dried at 40°C overnight.

Microorganism and maintenance

Saccharomyces cerevisiae VS3 was isolated from soil samples collected within the hot regions near Kothagudem Thermal Power Plant located in Khammam Dist, AP, India. The organism was isolated and identified as *S. cerevisiae* VS3 strain in our lab (Kiran Sree *et al*. 2000). It was maintained on yeast extract, peptone dextrose agar (YEPDA) medium consisting (g/L) of yeast extract: 10, peptone: 20, glucose 20 and agar 25, pH: 5.0 ± 0.2.

P. stipitis NCIM3498 procured from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory, Pune, India, It was maintained on MGYP (malt extract, glucose, yeast extract, peptone, xylose) medium consisting (g/L) of malt ex-tract: 5; yeast extract: 5; peptone: 20; glucose: 5; xylose: 30 and agar: 25, pH: 5.0 (Chandel *et al*. 2011a).

Inoculum preparation for *P. stipitis* and *S. cerevisiae*

Each 50 ml of modified MGYP media contained 0.5% malt extract, 1.0% glucose + xylose, 0.5% yeast extract, 0.5% peptone, pH 5.5 was inoculated with *P. stipitis* and incubated at 30°C and 150 rpm for 24 h.

Thermotolerant yeast VS3 inoculum was prepared by growing the organism on YEPD medium for 48 h at 37 ± 0.5°C and 150 rpm (Pasha *et al*. 2007).

Delignification

Dry *S. bicolor* material was soaked in 1 N NaOH solution (1:10 w/v) and kept at 28°C for 24 h. The contents were filtered with two layers of muslin cloth and the solid residue was repeatedly washed with water until the pH of the filtrate became neutral. The residue was dried at 40°C overnight and subsequently used for acid hydrolysis experiments.

Biphasic acid hydrolysis

The delignified *S. bicolor* was thermochemically saccharified with dilute sulfuric acid for biphasic sulphuric acid hydrolysis with a solid to liquid ratio of 1:10. Initially it was carried out with 1% acid at 121°C for 30 min and in second phase with 2% acid at 121°C for 45 min. Then the hydrolysates obtained from both the phases were mixed after cooling and assayed for total reducing sugars, phenolics and furans. The solid residue was washed extensively with water until neutral pH, dried at 40°C overnight. The hydrolysate was recovered and detoxified.

Detoxification

The mixed acid hydrolysate (obtained with 1 and 2% v/v H_2SO_4) was detoxified first by overliming with the addition of dried calcium hydroxide till the pH reached 10.5 ± 0.5 under rapid stirring at room temperature. Precipitation of inhibitors was allowed for 1 h by stirring. The slurry was then filtered to remove precipitates. The pH of clear filtrate was adjusted to 6.00 ± 0.5 with conc. H_2SO_4 and again filtered to remove traces of salt precipitates (Chandel *et al*. 2011a).

After overliming, 3.5% of activated charcoal was added to the hydrolysates and stirred for 1 h. The mixture was again centrifuged (3000 × *g*, 20 min) and vacuum filtered (Martínez *et al*. 2000). Sugars, phenolics and furans were estimated before and after detoxification process. The treated hydrolysate was then used for the fermentation studies.

Monoculture fermentation

For monoculture fermentation, both *P. stipitis* and *S. cerevisiae* VS3 cultures were inoculated separately in the detoxified hydrolysate of *S. bicolor.* The detoxified acid hydrolyzate was mixed along with supplementation of (g/L) of yeast extract: 1; peptone: 1; ammonium sulfate: 1; di-potassium hydrogen phosphate: 1; magnesium sulfate: 0.5; manganese sulfate: 0.5, in 250 ml Erlenmeyer flask as defined by Nigam (2002). Hydrolyzate along with supplements was sterilized at 10 PSI for 20 min. After cooling the media to 30°C, it was used for fermentation.

Ethanol fermentation by *P. stipitis* NCIM3498 and thermotolerant yeast (VS3)

The fermentation media was (prepared in the above step) aseptically inoculated with 10% of *P. stipitis* and 10% VS3. Fermentations were carried out separately at 120 rpm for *P. stipitis*, 50 rpm for VS3, for 72 h at 30 ± 0.5°C. Samples were collected after every 12 h interval up to 72 h of fermentation.

Co-culture fermentation

For co-culture fermentation, a 10% (5% *S. cerevisiae* VS3 + 5% *P. stipitis*) of inoculum containing OD_{600} = 3.0 was transferred aseptically into production medium (supplemented hydrolysate) and the fermentation was carried at 30°C, 150 rpm for first 18 h and then in static mode till the end of fermentation i.e., 72 h. Samples were collected at various intervals and centrifuged at 600 × *g* for 10 min at 4°C and analyzed for residual sugars.

Analytical methods

The total reducing sugars present in *S. bicolor* acid hydrolysate was estimated by dinitrosalicylic acid method of Miller (1959). Total content of phenolic compounds in acid hydrolysate was determined by the Folin-Ciocalteu method with vanillin as calibration standard (Tanner and Brunner 1987). Total furans were estimated by spectrophotometric method described by Martínez *et al.* (2000).

The ethanol produced was analyzed by gas chromatography using ZB-Wax column at 150°C, FID detector at 160°C and nitrogen with a 20 psi pressure carrier (Pasha *et al*. 2007). The carrier gas was nitrogen.

Experimental design

100 ml of detoxified hydrolysates along with supplements was taken in three different Erlenmeyer flasks (250 ml) and sterilized at 10 psi for 20 min. After cooling the media to 30°C, 10% of VS3

and *P. stipitis* inoculum was transferred aseptically into two different flasks separately for monoculture fermentation.

For co-culture fermentation, a 10% (5% *S. cerevisiae* VS3 + 5% *P. stipitis*) of inoculum was transferred aseptically. The fermentation was carried out at initial pH of the medium 5.5 ± 0.2, temperature 30 ± 0.2°C, and 100 rpm for 72 h. Samples were collected at 12 h intervals throughout the fermentation. The experiment was carried out in triplicates.

Statistical evaluation

To assess whether there was any significant difference among the mean values of all the data, paired or dependent *t*-tests (phenolics, furans and total reducing sugars) and Pearson correlation tests (ethanol produced and sugar utilised) were performed, using SPSS (software for windows release, 17.0, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

The complex chemical structure of lignocellulosic substrates makes the overall process of ethanol production from biomass cumbersome. The selection of suitable raw material is important for making the process economically feasible. The chemical composition of sorghum straw was determined by NREL method (Ruiz *et al*. 1996). It was found to contain, 37.51 ± 0.42% cellulose, 26.50 ± 1.5% of hemicellulose, 19.0 ± 0.27% of lignin and 10.02 ± 1.05% of ash on dry solid (DS) basis.

The total carbohydrate content (TCC) present in the sorghum straw was 63.50 mg/g of dry weight is in agreement with the report by Chandel *et al*. (2007a) and Mehmood *et al*. (2009). The level of TCC is comparable to those of other substrates (sugarcane bagasse, 67.15%; corn stover, 58.29%; and wheat straw, 54%) that are often exploited for bioethanol production (Chandel *et al*. 2007a).

Currently, the cost of feedstock, which represent more than one third of all processing cost is the most important factor in the ethanol production process (Chandel *et al*. 2007a; Wyman 2007; Lynd *et al*. 2008).

According to Kim and Dale (2005), 6% of sorghum production, is lost as waste and 10.4 million tones of sorghum biomass is available annually which can be used for the production of 4.57 giga litre ethanol and with a yield of 0.27 l/Kg of dry biomass.

Alkali delignification using dilute sodium hydroxide

Delignification of any lignocellulosic biomass is crucial before hydrolysis as the presence of lignin hinders further hydrolysis. Dilute sodium hydroxide pretreatment refers to the application of alkaline solutions such as NaOH, to remove lignin and partially some part of the hemicellulose and efficiently increase the accessibility of acid to the cellulose. Lignocelluloses are pretreated initially with alkali to dissolve the lignin caused by the breakdown of ether linkages (Lee 1997). Efficient delignifying agent should remove maximum of lignin and minimum of sugars (not > 5%) (Taherzadeh and Karimi 2007).

Chandel *et al.* (2009a) reported 79.72% removal of lignin when sodium hydroxide was used. In our study 75% lignin removal was attained (**Table 1**) with NaOH and thus making subsequent acid hydrolysis of the substrate feasible.

Table 1 Delignification of *Sorghum* straw with different alkali at 28 ± 2°C for 24 h.

Alkali (0.2M)	Delignification (%)	Loss of sugar (%)
NaOH	74.94	3.2
KOH	56.50	4.7
Sodium dithionite	48.89	6.5
Sodium sulfite	35.36	4.5
Sodium chlorite	40.42	3.8
Ammonia	54.64	5.0

Biphasic acid hydrolysis of *S. bicolor*

Acid hydrolysis of pretreated Sorghum straw was carried out for the depolymerization of cell wall carbohydrate fraction into fermentable sugars. In our study, biphasic sulphuric acid saccharification was done with 1% at 121°C for 30 min and 2% at 121°C for 45 min which generated 30 g/l sugars with a holocellulose hydrolysis efficiency of 65%. Pasha *et al.* (2008) found bi-phasic pretreatment is suitable for scale up studies for bioethanol production from *Prosopis juliflora* (mesquite). The maximum yield of pentoses and hexoses recovered from hemicelluloses in the first stage of the hydrolysis is high, while the yield of cellulose hydrolysis to glucose was low (Taherzadeh and Karimi 2007). Our results are in close comparison with their findings showing 44.8% of the sugars from the total hydrolysis were obtained in the first phase and the remaining sugars were obtained in the second phase.

Lee *et al.* (2009) reported that maximum attainable hemicellulose yield was about 80% using rice straw which was pretreated using dilute sulphuric acid at reaction conditions covering two levels of reaction temperature (140 and 150°C) and five levels of acid concentrations (1-3%).

Sánchez *et al*. (2004) carried out the two-stage dilute acid hydrolysis using Bolivian straw material, *Paja brava* with steam followed by dilute sulfuric acid (0.5 or 1.0% by weight) hydrolysis at temperatures between 170 and 230°C for a residence time between 3 and 10 min. In the first stage, the highest yield of hemicellulose derived sugars were found at a temperature of 190°C, and a reaction time of 5–10 min, whereas, in second stage hydrolysis, considerably higher temperature (230°C) was found for hydrolysis of remaining fraction of cellulose.

Sun and Cheng (2005) observed 27–33% glucan in Bermuda grass (*Cynodon dactylon*) that was converted into glucose using 1.2% acid content after 60 min treatment. The hydrolysis of the hemicellulose fraction during acid treatment involves solubilization and partial destruction of the cellulosic fraction into reducing sugars (Taherzadeh *et al*. 1997). Saha *et al*. (2005) observed 255 ± 13 mg sugars/l with 92% hemicellulose conversion from wheat straw (1% H_2SO_4, 121°C, 1 h). Roberto *et al*. (2003) reported that xylose maximum recovery was 20.5 g/l and glucose recovery was 6.3 g/1, with the use of 1.6% H_2SO_4 during 30 min. In the present study we could recover 30 g/l in the mixed hydrolyzate using 1 and 2% sulfuric acid.

Detoxification of acid hydrolysate

A critical issue in the conversion of dilute acid hydrolysates has been the ability to withstand inhibitors (Olsson and Hahn-Hagerdal 1996) and most often a detoxification step is needed to improve the fermentation efficiency.

Calcium hydroxide overliming is a prominent method for detoxification of lignocellulose hydrolysates by removing furfurals and phenolics (Martínez *et al*. 2001; Chandel *et al*. 2007c). Increasing the pH to 10.0 by $Ca(OH)_2$ and readjustment to 6.5 with H_2SO_4 caused detoxification of lignocellulosic hydrolysate (Chandel *et al*. 2010c). The detoxifying effect of overliming is due to the precipitation of toxic components and instability of some inhibitors at high pH (Martínez *et al*. 2001).

After overliming the *S. bicolor* acid hydrolysate, a decrease in furans from 0.21 to 0.048 (**Fig. 2**) and in phenolics (**Fig. 3**) from 0.79 to 0.2 g/l was observed. However, a loss of 5.61 ± 0.34% in reducing sugars (30 to 28.45 g/l) was also observed (**Fig. 1**).

Our results agreed fairly well with the data reported by Martínez *et al*. (2001) showing 51 ± 9% reduction in total furans, 41 ± 6% reduction in total phenolics and 8.7 ± 4.5% decline in sugars from lignocellulose hydrolysate. Chandel *et al*. (2007c) reported 45.8 and 35.87% reduction in furans and phenolics, respectively from sugarcane bagasse hemicellulosic hydrolysate after calcium hydroxide overliming.

The detoxification by pH alteration and active charcoal

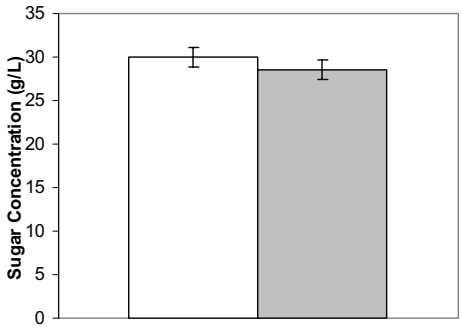

Fig. 1 Sugars present in acid hydrolysate before (white bar) and after (grey bar) detoxification. n = 3. Bar = SEm

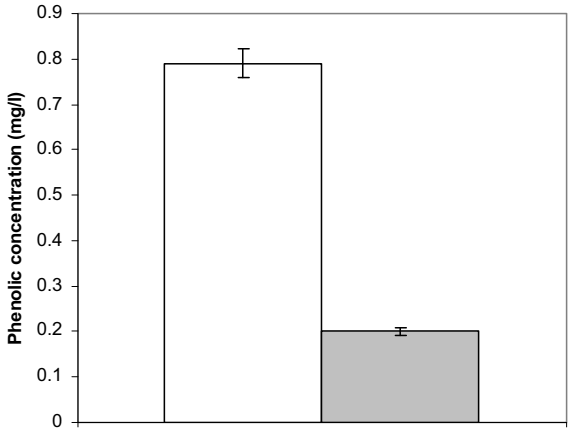

Fig. 2 Phenolics present in acid hydrolysate before (white bar) and after (grey bar) detoxification. n = 3. Bar = SEm

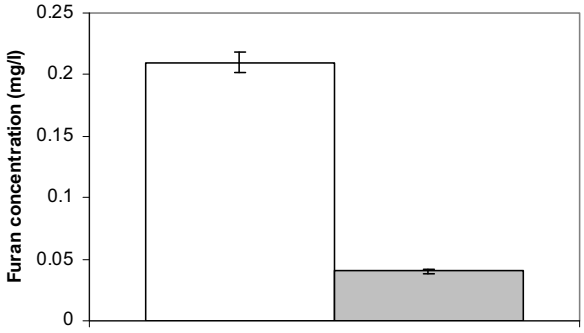

Fig. 3 Furans present in acid hydrolysate before (white bar) and after (grey bar) detoxification. n = 3. Bar = SEm

adsorption led to 6.1 g/l ethanol in 48 h, with a yield of 0.30 g/g and a productivity of 0.13 g/l/h (Larissa *et al*. 2008).

Ethanol production from monoculture of *S. cerevisiae* VS3 and *P. stipitis* NCIM 3498

Detoxified *S. bicolor* acid hydrolysate containing 28.45 ± 0.55 g/l total reducing sugars was used for ethanol production using the monocultures and co-cultures.

Monoculture (*P. stipitis* and *S. cerevisiae*) fermentation

The fermentation of acid hydrolysate was carried out using monocultures of *P. stipitis* and *S. cerevisiae* VS3. The ethanol produced was 10.25 ± 0.25 g/l, 0.39 g/g after 36 h, respectively (**Figs. 4, 5**). Our results are in close comparison with the maximum ethanol concentration of 12.08 ± 0.62 and yield of 0.42 ± 0.031 g/g reported by Chandel *et al.* (2011b) using *P. stipitis* 3498. The hemicellulosic hydrolysate of *Prosopis juliflora* containing 18.24 g/l sugars, when fermented with the same strain of *P. stipitis* NCIM 3498 produced 7.13 g/l ethanol with a yield of 0.39 g/g and productivity of 0.30 g/g after 24 h (Gupta *et al*. 2009).

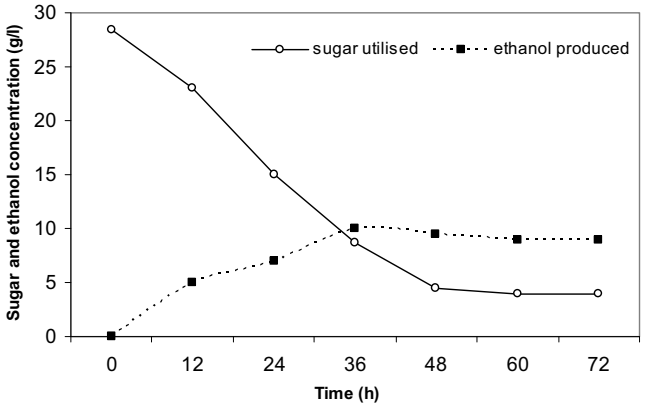

Fig. 4 Ethanol production by monoculture of *P. stipitis* NCIM3498 at 30°C, using *Sorghum straw* acid hydrolysate. n = 3. Bar = SEm

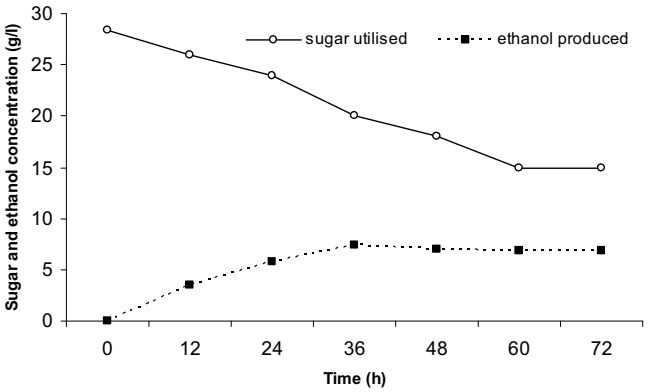

Fig. 5 Ethanol production by monoculture of *S. cerevisiae* VS3 at 30°C, using *Sorghum straw* acid hydrolysate. n = 3. Bar = SEm

On the other hand, *S. cerevisiae* VS3 produced a low amount of ethanol, 7.40 ± 0.07, yield 0.36 ± 0.05 g/g (**Fig. 5**) from detoxified acid hydrolysate of *S. bicolor*. The reason may be due to most of the xylose and some of the glucose was left unfermented by *S. cerevesiae* VS3, as the hydrolysate contains both xylose and glucose.

The mixed acid hydrolysate of *Lantana camara* when fermented with the same thermotolerant *S. cerevisiae* VS3 produced 0.431 ± 0.018 g ethanol per g sugar and productivity of 0.5 ± 0.021 g/l/h with a fermentation efficiency of 83.7% (Pasha *et al*. 2007).

Pasha *et al*. (2008) reported 30.0 g/l ethanol production with the yield of 0.431 ± 0.021 g/g of sugars with the same organism VS3 using *Prosopis juliflora* hydrolysate with a fermentation efficiency of 88%.

Co-culture fermentation of *S. bicolor* acid hydrolysate

The fermentation profile of *S. bicolor* acid hydrolysate using co-culture of *S. cerevisiae* VS3 and *P. stipitis* NCIM 3498 (28.45±0.55 g/l total reducing sugars) is shown in **Fig. 6**. The maximum ethanol produced was 12.00 ± 0.55 g/l, with a yield of 0.45 ± 0.032 g/g (**Table 2**) after 36 h of incubation with an efficiency of 90.2% and declined slowly after that. Our results are similar to the recent investigation made in our laboratory by Srilekha *et al.* (2011), with an ethanol concentration, yield, volumetric ethanol productivity and fermentation efficiency of 12 g/l, 0.33 g/l/h, 0.4 g/g and 95%, respectively by co-culture of OVB 11 (*S. cerevisiae*) and *P. stipitis* NCIM 3498 using rice straw hydrolysate. Patle and Lal (2008) studied the ethanol production using a mixed culture of *Zymomonas mobilis* and *Candida tropicalis* TERI SH 110 and reported 32 g/l ethanol from 84 g/l of total sugars obtained from mixed vegetable and fruit waste.

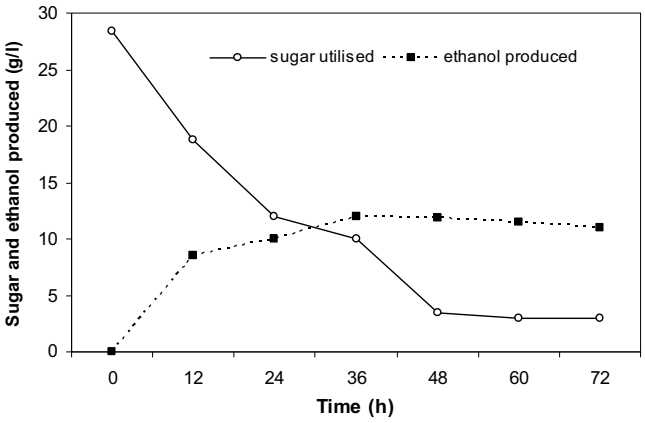

Fig. 6 Ethanol production by co-culture of *P. stipitis* NCIM 3498 and *S. cerevisiae* VS3 at 30°C, using *Sorghum bicolor* acid hydrolysate. n = 3. Bar = SEm

Table 2 Fermentation parameters of Co-culture and monocultures (*P. stipitis* NCIM 3498 and *S. cerevisiae* VS3) using *Sorghum straw* acid hydrolysate and ethanol yield.

Parameters	Co-culture	*S. cerevisiae* VS3	*P. stipitis* NCIM 3498
Initial sugar concentration (g/l)	28.45 ± 0.55	28.45 ± 0.55	28.45 ± 0.55
Sugar consumed (g/l)	26.00 ± 0.51	20.10 ± 0.22	23.4 ± 0.153
Ethanol (g/l)	12.00 ± 0.55	7.40 ± 0.07	10.25 ± 0.25
Ethanol yield (g/g)	0.45 ± 0.32	0.36 ± 0.05	0.39 ± 0.030
Incubation time: 36 h			

Rouhollah *et al*. (2007) compared the ethanol production efficiency of co-cultures of *P. stipitis* and *S. cerevisiae* with *P. stipitis* and *Kluyveromyces marxianus* which showed 29.45 g/l ethanol and productivity 0.77 g/l/h upon the mixed sugar fermentation. Qian *et al*. (2006) studied the co-culture fermentation of soft wood hemicellulose hydrolysate using co-culture of *S. cerevisiae* and *P. tannophillus* and maximum ethanol production shown was 18.2 g/l with a yield of 0.49 g/g. Recent investigations of Chandel *et al*. (2011b) found that the fermentation of hemicellulose acid hydrolysate of saccharum spontaneum with monocultures of *P. stipitis* 3498, *S. cerevisiae* VS3 and co-culture produced 12.08 ± 0.72, 1.40 ± 0.07 and 15.0 ± 0.92 g/l ethanol, respectively.

Qian (2006) also reported that fermentation of detoxified hydrolysate by adapted co-culture (*S. cerevisiae* + *Pachysolen tannophilis*) generated an exceptionally high ethanol yield on total sugar of 0.49 g/g, corresponding to 96.1% of the maximal theoretical value after 48 h of incubation.

Our studies are supported by those of Sornvoraweat *et al.* (2010), who also reported that co-culture of *S. cerevisiae* and *Candida tropicalis* produced maximum ethanol concentration of 3.39 g/l and 0.25 g/g ethanol yield in comparison to monoculture of *S. cerevisiae* using acid hydrolysate of water hyacinth.

Statistical evaluation of phenolics, furans and total reducing sugars present before and after detoxification of sorghum straw acid hydrolysate

To assess whether there is any significant difference among the mean values of phenolics, furans and reducing sugars before and after detoxification, a dependent or paired sample *t*-test was performed. The analysis showed significant difference among the mean values of three pairs, *i.e.,* pair-1, pair-2, pair-3 (**Table 3**, Sig- 0.00, 0.00 and 0.035 respectively).

The *t*-test yielded positive values for all the three pairs showing a high correlation between the pairs, before and after detoxification. This result shows that detoxification by overliming with CaO, followed by active charcoal treatment can be employed for efficient inhibitor removal without significant sugar loss, for other hydrolysates in bioethanol production.

To find out the significant correlation between ethanol produced and the sugar utilized by *P. stipitis* NCIM 3498, *S. cerevisiae* VS3 and co-culture of both strains, correlation analysis was performed. **Table 4A**, **4B** and **4C** show no significant correlation between ethanol produced and sugar utilized by *P. stipitis* NCIM 3498 and *S. cerevisiae* VS3 but there was significant correlation between ethanol produced and sugar utilized by the co-culture of both strains. Further, ethanol produced and sugar utilized in co-culture experiment was highly negatively correlated. This result shows

Table 4A Statistical evaluation (correlation) of sugar utilized and ethanol produced by monocultures of *P. stipitis* NCIM 3498.

		psep	pssu
psep	Pearson Correlation	1	-0.500
	Sig. (2-tailed)		0.667
	N	3	3
pssu	Pearson Correlation	-0.500	1
	Sig. (2-tailed)	0.667	
	N	3	3

Table 4B Statistical evaluation of sugar utilized and ethanol produced by monocultures of *S. cerevisiae* (VS3).

		vs3ep	vs3su
vs3ep	Pearson Correlation	1	-0.866
	Sig. (2-tailed)		0.333
	N	3	3
vs3su	Pearson Correlation	-0.866	1
	Sig. (2-tailed)	0.333	
	N	3	3

Table 4C Statistical evaluation of sugar utilized and ethanol produced by co-culture of *P. stipitis* NCIM 3498 and *S. cerevisiae* (VS3).

		ccep	ccsu
ccep	Pearson Correlation	1	-1.000**
	Sig. (2-tailed)		0.000
	N	3	3
ccsu	Pearson Correlation	-1.000**	1
	Sig. (2-tailed)	0.000	
	N	3	3

**. Correlation is significant at the 0.01 level (2-tailed).
ps-Pichia stipitis; cc-co-culture; ep-ethanol produced; su-sugar utilized; vs3-*S. cerevisiae* (VS3)

Table 3 Statistical evaluation (paired differences (dependent sample *t*-test) of phenolics, furans and total reducing sugars present in acid hydrolysates before and after detoxification (95% confidence interval of the differences).

Sl No.	Mean	Standard Deviation	Std. Error Mean	Lower	Upper	*t*	df	Sig (2 tailed)
Pair 1	0.58333	0.01155	0.00667	0.55465	0.61202	87.500	2	0.000**
Pair 2	0.17000	0.00200	0.00115	0.16503	0.17497	147.224	2	0.000**
Pair 3	1.50000	0.50000	0.28868	0.25793	2.74207	5.196	2	0.035**

**p < 0.05
Pair 1: Phenolics before detoxification – Phenolics after detoxification
Pair 2: Furans before detoxification - Furans after detoxification.
Pair 3: Total reducing sugars before detoxification - Total reducing sugars after detoxification.

that, for effective utilization of pentose and hexose sugars present in the hydrolysates and increased ethanol yields, co-culture fermentation can be employed over monoculture fermentations.

CONCLUSION

Sorghum bicolor straw, an abundantly available agricultural byproduct was used as a substrate for bioethanol production. A typical batch fermentation of the detoxified acid hydrolysate when fermented with co-culture of *P. stipitis* NCIM 3498 and *S. cerevisiae* VS3 resulted in high ethanol yields (90.2% fermentation efficiency) than the ethanol produced with monocultures. The results clearly demonstrate the conversion of both types of sugars present in the hydrolysate. These studies help us to understand that co-culture fermentation can be employed to a wide variety of other abundantly available lignocellulosic materials for ethanol production.

ACKNOWLEDGEMENTS

The authors are thankful to the Department of Science and Technology (DST), New Delhi, India, for the financial assistance (Project No: DST/TSG/AF/2007/48).

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