International Journal of Advanced Biotechnology and Research (IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol 6, Issue1, 2015, pp133-143
http://www.bipublication.com

Research Article

Two Step Enzymatic Hydrolysis and Fermentation Strategy for Conversion of Acid Treated Hydrolysate of Corn Cob to Ethanol

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[Received-01/03/2015, Published- 12/03/2015]

ABSTRACT:

For economic production of ethanol from corncobs, it is very crucial that lignocellulosic fractions containing glucose and xylose are simultaneously fermented to ethanol in a single operation i.e. cofermentation. Cofermentation of mixed acid treated corn cob hydrolysate containing mixed sugars at low concentration (<50 g L^{-1}) using *Pichia stipitis* showed 92.35% ethanol fermentation efficiency. However cofermentation of corn cob hydrolysate comprising sugar above 50 g L^{-1} showed only 46.71% ethanol fermentation efficiency. Therefore a two step enzymatic hydrolysis and fermentation strategy was designed for cofermentation of corn cob hydrolysate containing high concentration of sugars which resulted in 86.74% enzymatic hydrolysis efficiency and 75.32% ethanol fermentation efficiency. Using two step hydrolysis and fermentation, there was 1.1 fold improvement in the enzymatic hydrolysis efficiency. Final ethanol concentration, ethanol yield and volumetric productivity were improved by 1.7, 1.66 and 2.6 fold respectively as compared to single step hydrolysis and fermentation process.

Keywords: Corncob hydrolysate, Cellulosic ethanol, *Pichia stipitis*, Cofermentation

[I] INTRODUCTION

Rising energy consumption, depletion of fossil fuels and increased environmental concerns has shifted the focus of energy generation towards the use of biofuels [5]. Burning of fossil fuels is a big contributor to increasing the level of CO_2 in the atmosphere which has led to search for a suitable alternative of fossil fuel [8].

Ethanol is a sustainable and renewable fuel which has high octane number i.e. oxygenating factor that can completely replace MTBE for

oxygenating fuel. Blending ethanol in gasoline is the greenest way which will reduce dependency on fossil fuels and reduce the emission of green house gases, thereby controlling the environment pollution [10].

The production of ethanol from sugars or starch which comes under 1st generation biofuels impacts negatively on the economics of the process, thus making ethanol more expensive compared with fossil fuels [9]. 'First generation' biofuels can offer some CO_2 benefits and can help to improve domestic energy security. But concerns exist about the sourcing of feedstocks, including the impact it may have on biodiversity, land use and competition with food crops [16].

Lignocellulose is the most abundant unutilized biomass that can be successfully utilized for second generation bioethanol production. Majority of ethanol technologies in the United States use corn as a feedstock due to its high carbohydrate content and it is the least cost feedstock available for ethanol production [23]. USDA's World Agricultural Outlook Board estimated that 0.127 billion tonnes of corn were used to produce ethanol and co-products during the 2012-13. Every part of corn has been used extensively to produce ethanol and other co-products. According to the data published in the forbes magazine (April, 2014), 40% of corn crops went to produce ethanol, 45% was used to feed livestock, and only 15% was used for food and beverage in 2013 Corn-based ethanol accounts for approximately 97 % of the total ethanol produced in the United States [23]. Corn cobs thus can be utilized as sustainable feedstock for cellulosic ethanol production since it does not interfere with the food chain of the ecosystem. Average composition of corn cobs represents 45% cellulose, 35% hemicelluloses and 15% lignin on dry basis [22]. Low lignin and high carbohydrate content make corncobs susceptible for enzymatic hydrolysis after suitable pretreatment [11].

The fermentable sugars produced after saccharification can be fermented with suitable ethanologenic microbe to ethanol. However, acid treated corn cob hydrolysate cannot be fermented efficiently due to presence of inhibitors viz; acetic acid, furans and phenolic compounds arising out of pretreatment. Sugar degradation products—like, furfural (from pentoses) and hydroxymethyl furfural (HMF) (from hexoses) are formed in high concentrations during severe acidic pre-treatment conditions [13]. Acetic acid is ubiquitous in hemicellulosic hydrolysates from all lignocellulosics, where the hemicelluloses and to some extent lignin is acetylated [13]. Phenolic compounds are formed from lignin degradation during acid hydrolysis [7]. Phenols, furans, carboxylic acids, and salts, are potential fermentation inhibitors that have a negative effect on cell membrane function, growth, and glycolytic enzymes in ethanol-producing yeast and bacteria [13].

Non-glucose sugars including pentose sugars like xylose and arabinose present in hemicellulosic fraction are not easily amenable to *Saccharomyces cerevisiae* yeast used in ethanol industry, due to lack of necessary enzymes for pentose utilization [25,26]. Also naturally occurring xylose-fermenting yeasts are not effective fermentative microorganisms due to relatively low ethanol and inhibitor tolerance [24, 6]. For economic production of ethanol it is necessary that the cofermentation of mixed sugar is converted to ethanol simultaneously by a single micro-organism. Substantially greater savings can be obtained in capital and operating expenditure by cofermentation strategy due to high ethanol titer, yield and productivity. Simultaneous cofermentation of glucose, xylose, and cellobiose is challenging for most microbes because glucose represses utilization of the other sugars [15]. Economical bioconversion of acid treated corncob hydrolysate to ethanol presents several challenges viz; inefficient enzymatic

hydrolysis, incomplete xylose fermentation in presence of pretreatment inhibitors and lack of suitable microbe for fermentation of sugars. The present study addresses these challenges involved in the economic bioconversion of corncob hydrolysate to ethanol. *Pichia stipitis* was the organism of choice for cofermentation as it can ferment glucose, mannose, galactose, cellobiose, mannan and xylan oligomers present in the lignocellulosic hydrolysate. It ferments mixture of glucose and xylose with a high ethanol yield as compared to other pentose fermenting micro-organisms. It shows no absolute requirement for vitamins and no apparent xylitol production [20, 17]. An integrated two step enzymatic hydrolysis and fermentation process has been proposed for acid treated corn cob hydrolysate to ethanol wherein non-detoxified mixed acid (Sulfuric acid + oxalic acid) treated corncob hydrolysate fermentation was carried out using yeast *Pichia stipitis* and *Saccharomyces cerevisiae.*

[2] MATERIALS AND METHODS

2.1 Substrate

Corn plants were grown in Abhyudaya Biotech, Nashik (18.33° N 73.16° E), Maharashtra, India. Corn cobs (moisture content of 10%) were harvested in November 2012. Corncobs were stored in the dry place at room temperature in the storage room until use.

2.2 Preparation of acid hydrolysate of Corncob

Corn cobs having total solids of about 92% by weight, containing cellulose (33.20%) hemicelluloses (27.32%) and lignin (12.90%) was used as a feedstock. It was subjected to mechanical shearing for size reduction to about 20-40 mm particle size. This particulate material was soaked in water for about 30 min. The pretreatment was done in a continuous high pressure digester with constant supplies of acid and steam to maintain desired temperature of 140-200ºC. The feed containing about 30% total solids was prepared and mixed with the mixture of 2% sulphuric acid and 1% oxalic Acid on solids basis. The resultant reaction mixture was then subjected to hydrolysis in digester at a temperature 160 º C and pressure of about 6 bars for a period of 20 minutes. Due to the addition of steam and acid the total solids of pretreated slurry is diluted to about 18-20% w/v. The pretreated slurry (18-20% solids) is diluted further with process water to obtain final slurry solids of about 14-15%. Composition of the slurry is as shown in Table-1.

2.3 Yeast strains and Media:

Pichia stipitis ATCC 58784 was adapted by serial propagation in non-detoxified xylose rich supernatant arising from mixed acid treatment of corncob. The adapted strain was designated as *Pichia stipitis* PSA_{30}. It was maintained at -80 º C in the form of glycerol stock in filter sterilized non-detoxified xylose rich supernatant in order to maintain selection pressure.

Inoculum medium was prepared using 50% non detoxified xylose rich supernatant. Xylose rich supernatant was prepared by centrifugation of corn cob slurry obtained after pretreatment. Inoculum medium contained filter sterilized 50% non-detoxified xylose rich supernatant, 5 g L^{-1} Corn steep liquor and 2 g L^{-1} Urea. CSL and urea were separately sterilized and added to filter sterilized xylose rich supernatant. The pH of the inoculum medium was adjusted to 6.0 with 40% NaOH and the flask was inoculated with single colony of *Pichia stipitis* aseptically. Lab Inoculum was prepared in 250 mL shake flasks with 100 mL medium, and incubated on a rotary shaker at 2.5 Hz and temperature of 30ºC. After 24 h, inoculum flasks were analyzed for culture purity and Packed Cell Weight (PCW). Fermenter seed was prepared in BioFlo®-310 fermenter of capacity 2.5 L containing 1L inoculum medium. The fermenter was pre-sterilized at 121ºC for 20 min before addition of

the medium. After pre-sterilization, medium was added to the fermenter and pasteurized at 110ºC for 5 min. The pH was adjusted to 6.0 before inoculation. Lab inoculum was transferred at 10% to the seed fermenter aseptically. Aeration and agitation were set to 0.5 L min^{-1} and 3.33 Hz respectively. Temperature was set to 30ºC. After 24 h, fermenter seed was analyzed for culture purity and packed cell weight (PCW) succinic acid, lactic acid, HMF, furfural and Ethanol by HPLC.

2.4 Cofermentation of mixed acid treated corncob hydrolysate

Mixed acid treated corncob slurry of 10 L containing 14% total solids was added to the pre-seterilized BioFlo® 415 fermenter of 14 L capacity. The pH of the slurry was adjusted to 5.0 using 40% NaOH. Commercial cellulase preparation was added to the slurry at a dose of 8 $FPUg^{-1}$ of cellulose. Temperature was adjusted to 55ºC and agitation was set to 6.66 Hz. After incubation of 120 h, enzymatic hydrolysate was analyzed for glucose, xylose, acetic acid, HMF and furfural. Enzymatic hydrolysate was pasteurized by heating at 110ºC for 5 min in the fermenter itself. Cofermentation medium was prepared to a final volume of 10 L in the same fermenter using enzymatic hydrolysate containing mixed sugars, corn steep liquor and urea. Corn steep liquor and urea were sterilized separately and added in the fermentation medium at final concentration 5 g L^{-1} and 2 g L^{-1} respectively. The fermenter medium was cooled to 30ºC and pH of the fermentation medium was adjusted to 6.0. Fermenter seed (10%) of *Pichia stipitis* PSA_{30} was transferred aseptically to the fermenter. Agitation was set to 3.33 Hz and aeration was kept at 1 L min^{-1} for 120 h. Every 24 h, samples were withdrawn and analyzed for glucose, xylose, acetic acid,

2.5 Two step hydrolysis and cofermentation of mixed acid treated corncob slurry

Step-1

Mixed acid treated corncob slurry of 10 L containing 14% total solids was added to the pre-seterilized BioFlo® 415 fermenter of 14 L capacity. The pH of the slurry was adjusted to 5.0 using 40% NaOH. Commercial cellulase preparation was added to the slurry at a dose of 3 FPU g^{-1} of cellulose. Temperature was adjusted to 55ºC for 24 h and agitation was set to 6.66 Hz.

After pre-hydrolysis of 24 h, fermenter medium was cooled to 30ºC and pH was adjusted to 6.0 using 40%NaOH. Separately sterilized CSL and urea were added to the fermenter at the final concentration 5 g L^{-1} and 2 g L^{-1} respectively. Fermenter seed (10%) of *Pichia stipitis* PSA_{30} was transferred aseptically to the fermenter containing pre-hydrolyzed corncob slurry. Agitation was set to 3.33 Hz and aeration was kept at 1 L min^{-1} for 50 h. Every 24 h samples were withdrawn and analyzed for glucose, xylose, acetic acid, succinic acid, lactic acid, HMF, furfural and ethanol by HPLC.

Step-2

After 50 h incubation, pH of the broth was adjusted to 5.0 using 40% NaOH and fresh cellulase enzyme dose of 5 $FPUg^{-1}$ of cellulose was added. Temperature of the fermenter was adjusted to 55ºC for 72 h to hydrolyze unconverted cellulose. After maximum release of glucose at 72 h, fermenter was cooled to 30ºC and urea was added to the broth at a final concentration of 0.5 g L^{-1} for fermentation using *Saccharomyces cerevisiae* (Active Dry Yeast at the final concentration 0.8 g L^{-1}). Samples were withdrawn at initial and 24 h of fermentation using *Saccharomyces cerevisiae*. The two step hydrolysis and cofermentation process has been further illustrated in Fig-1.

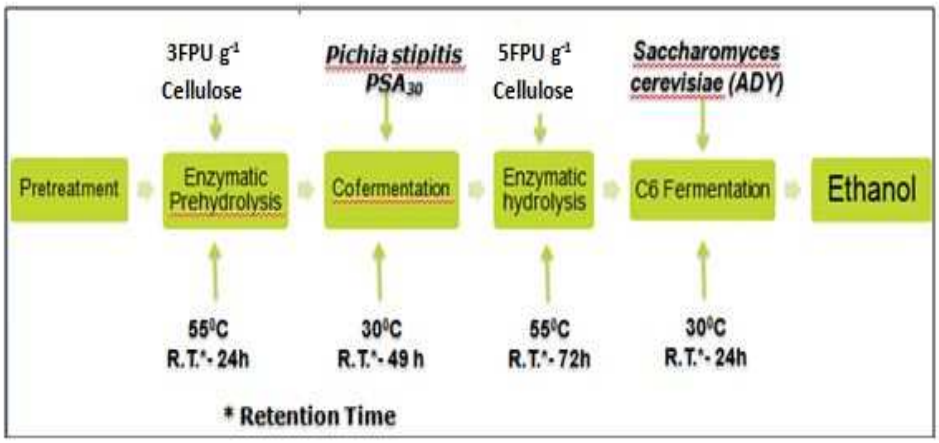


Fig-1: Process Flow for two step hydrolysis and fermentation of acid treated corn cob

2.6 Analysis

Sugars (cellobiose, glucose, xylose, and arabinose), fermentation products (ethanol, xylitol, glycerol) and inhibitors (formic acid, acetic acid, levulinic acid, 5-hydroxymethylfurfural, and furfural) were analyzed by high performance liquid chromatography (HPLC) using an Agilent 1100 system, refractive index detector and Bio-Rad Aminex HPX-87H column (300×7.8mm i.d.) for separation of compounds at 55°C. Sulfuric acid (5 mol m^{-3}) was used as the mobile phase at flow rate of 0.6 ml min^{-1}. Cellulose and lignin content of the biomass was determined by NREL's laboratory analytical procedure.

Calculations

Theoretical glucose was calculated on the basis of cellulose content (g cellulose in 100 g dry solids) of the feedstock and multiplying it by factor 1.11 (Equation-1).

$$\%\text{Glucose Content} = \frac{[\%\text{Cellulose Contentx1.11}] \times [\%\text{total insoluble Solids}]}{100} \quad \text{Eq.1}$$

For determination of hydrolysis efficiency of enzyme, glucose released after mixed acid pretreatment was subtracted from the final glucose concentration achieved after enzymatic hydrolysis. For calculation of fermentation efficiency, initial ethanol was subtracted from final ethanol produced.

RESULTS AND DISCUSSION

3.1. Single step hydrolysis and cofermentation of mixed acid treated corncob hydrolysate using *Pichia stipitis*

3.1.1. Enzymatic hydrolysis of mixed acid treated corn cob hydrolysate

Pretreatment of corn cob slurry with mixed acid resulted in breakdown of xylan into monomeric xylose along with some inhibitors viz; acetic acid, HMF, furfural and phenolics which are known to inhibit microbial growth and fermentation [13, 4]. Cellulose remained intact after pretreatment. Composition of slurry after mixed acid pretreatment is given in **[Table –1]**.

Component	Concentration (g L^{-1})
Total Insoluble Solids (TIS)*	89.2
Glucose	5.9
Xylose	37.0
Furfural	0.2
HMF	0.2
Acetic Acid	4.0
Phenolic Compounds	3.5

*Cellulose content of the TIS was 54.05%.

Table – 1: Composition of mixed acid treated corncob slurry

Enzyme hydrolysis profile of acid treated corn cob slurry is shown in fig – 2. Initial rate of glucose release in first 24 h was found to be 1.08 g $L^{-1}h^{-1}$ which gradually decreased with time with the release of monomeric glucose. About 50 % of cellulose in the corn cob slurry was found to be hydrolyzed in first 24 h.

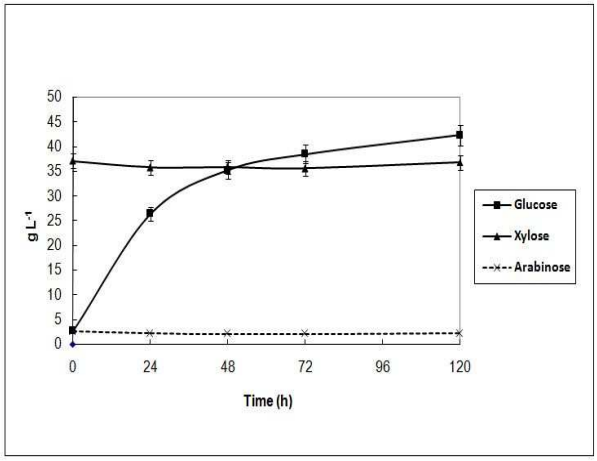


Fig – 2: Enzymatic hydrolysis of mixed acid treated Corn cob slurry using 8 FPU g^{-1} enzyme.

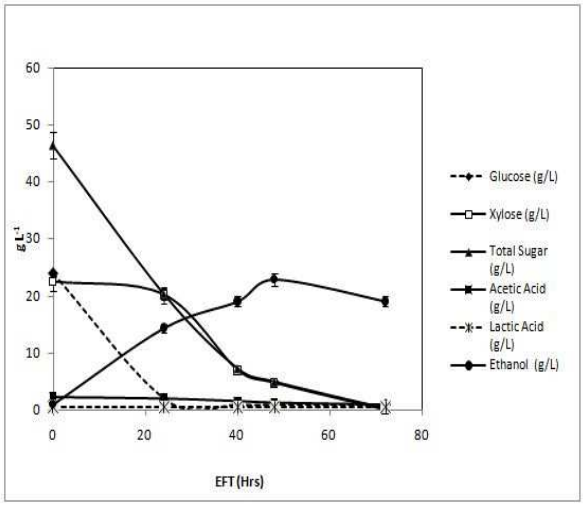
Monomeric glucose released after 120 h was found to be 42.2 g L^{-1} while xylose, arabinose and acetic acid did not change significantly in the hydrolysis reaction. Glucose release rate was observed to decrease continuously after 24 h. Efficiency of cellulose to glucose during single step enzymatic hydrolysis was found to be 79.51% of theoretical maximum. Composition of corn cob slurry after enzymatic hydrolysis is shown in

Sr. No.	Component	Concentration (g L^{-1})
1	Cellobiose	0
2	Glucose	42.2
3	Xylose	36.7
4	Acetic acid	3.7
5	HMF	0
6	Furfural	0

Table – 2: Composition of enzymatic hydrolysate of mixed acid treated Corn cob slurry.

3.1.2 Cofermentation of corn cob enzymatic hydrolysate using *Pichia stipitis*

Three independent trials were taken in shake flasks containing enzymatic hydrolysate of corncob. Cofermentation was started with 46.4 g L^{-1} total sugar comprising 23.9 g L^{-1} glucose and 22.5 g L^{-1} xylose. The pH of slurry was adjusted to 6.0 to overcome inhibition of *Pichia stipitis* by un-dissociated form of acetic acid [13, 3].



Fig– 3a: Cofermentation of Mixed acid treated Corncob Slurry containing 46.4 g L^{-1} mixed sugar using *Pichia stipitis*

From profiles for sugar consumption, ethanol production (Fig – 3a) it could be observed that the glucose and xylose consumption by *Pichia stipitis* was not simultaneous and glucose was preferred sugar over xylose. Xylose assimilation was observed only after complete exhaustion of glucose (24 h) in the fermentation medium [22]. The final ethanol concentration reached 22.9 g L^{-1} equivalent to 92.35% of the theoretical maximum and the volumetric productivity was found to be 0.477 g L^{-1} h^{-1}. Re-assimilation of the ethanol was observed before all the xylose was completely consumed. Ethanol re-assimilation was also studied by Skoog et al. who reported increase in ethanol oxidation with increase in oxygenated conditions. No ethanol

was metabolized anaerobically which suggested requirement of controlled aeration for maximum ethanol production from xylose using *Pichia stipitis* [19]. It could be observed that the glucose fermentation rate was not affected by pretreatment inhibitors present in the hydrolysate. Although rate of xylose consumption was slower than glucose, xylose was completely consumed resulting in maximum cofermentation efficiency by *Pichia stipitis.* Cofermentation using *Pichia stipitis* with 46.4 g L^{-1} (Fig-3a) total sugar was not economical at higher scale due to relatively low ethanol titer upto 21.9 g L^{-1}, leading to high capital and operational expenditure. To increase the final concentration of ethanol, concentration of mixed sugar was increased to 71.2 g L^{-1} containing glucose upto 36.3 g L^{-1} and Xylose upto 34.9 g L^{-1}. Cofermentation was carried out under same operating conditions using *Pichia stipitis.*

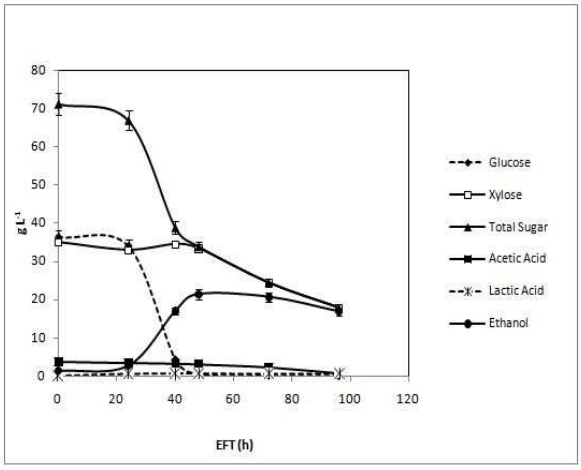


Fig – 3b: Cofermentation of mixed acid treated corncob slurry containing 71.2 g L^{-1} mixed sugar

As shown in fig – 3b, at higher sugar concentration, lag of 24 h was observed even for glucose consumption due to increase in concentration of associated inhibitors arising out of pretreatment. Glucose was completely consumed in 40 h and xylose consumption initiated thereafter with partial conversion of xylose to ethanol. As the total sugar concentration was increased, inhibitors viz; acetic acid (3.7 g L^{-1}), HMF (0.1 g L^{-1}), furfural (0.05 g L^{-1}) were found to be increased as compared to corn cob slurry containing less than 50 g L^{-1} total sugar. The final ethanol concentration reached 16.8 g L^{-1}, equivalent to 46.17% of the theoretical maximum and the volumetric productivity was found to be 0.16 g $L^{-1}h^{-1}$. Residual xylose concentration at the end of fermentation was found to be 17.8 g L^{-1}. With increase in initial sugar concentration to 71.2 g L^{-1} decrease in cofermentation efficiency was observed. Xylose consumption was found to be ceased due to synergistic inhibitory effect of glucose, xylose, pretreatment inhibitors and ethanol [2]. Inhibition of xylose consumption in the presence of glucose was also studied by young et al who reported low specific activity of enzymes involved in xylose utilization pathway; xylose reductase (XR) and xylitol dehydrogenase (XDH) in the glucose containing medium as compared to medium containing only xylose [14]. A two step enzymatic and fermentation process was designed to improve the overall fermentation efficiency and xylose consumption rate in cofermentation of corn cob slurry containing more than 50 g L^{-1} of mixed sugars.

3.2 Two step hydrolysis and Co-fermentation of mixed acid treated corncob slurry

Mixed acid treated corncob slurry containing monomeric xylose, pretreatment inhibitors and unhydrolyzed cellulose was subjected to two step hydrolysis and cofermentation process. In Step – 1 of enzymatic hydrolysis, cellulose was partially hydrolyzed to release upto 15 g L^{-1} glucose. At this concentration of glucose, *Pichia stipitis* was found to coferment xylose sugar present in the corncob slurry (data not shown). Composition of corncob slurry after Step – 1 of enzymatic hydrolysis is shown in Table-3.

No.	Component	Concentration (g L^{-1})
1	Cellobiose	0.01
2	Glucose	14.7
3	Xylose	32.9
4	Acetic acid	3.4
5	HMF	0.2
6	Furfural	0.2

Table-3: Composition of enzymatic hydrolysate of mixed acid treated Corn cob slurry after step – 1 prehydrolysis

Total sugar after step – 1 of enzymatic hydrolysis (Table – 3) did not exceed 50 g L^{-1} which was completely consumed by *Pichia stipitis* in 50 h. The final ethanol concentration at the end of fermentation by *Pichia stipitis* reached upto 19.82 g L^{-1}; equivalent to 80.25% of theoretical maximum on total released sugar in step – 1 of enzyme hydrolysis. The volumetric productivity was found to be 0.44 g $L^{-1}h^{-1}$ with xylose consumption rate of 0.68 g $L^{-1}h^{-1}$ (Fig – 4). Ethanol fermentation efficiency and the volumetric productivity were found to be lower as compared to the single step hydrolysis and cofermentation of mixed sugar at 46.4 g L^{-1} concentration. This could be attributed to the presence of insoluble solid content of the slurry as cellulose was partially hydrolyzed in prehydrolysis step and also high concentration of inhibitors viz; acetic acid (3.4 g L^{-1}), HMF (0.2 g L^{-1}) and furfural (0.2 g L^{-1}).

The viscosity of fermentation broth was found to be higher due to the presence of undigested cellulose in the form of insoluble solids leading to oxygen limited conditions in the fermentation broth. Re-assimilation of ethanol by *Pichia stipitis* was found to be negligible under oxygen limited conditions as compared to aerobic conditions during single step hydrolysis and cofermentation.

After fermentation by *Pichia stipitis*, broth was subjected to second step of enzymatic hydrolysis resulting in further release of 28.5 g L^{-1} glucose from the unhydrolyzed cellulose. Overall glucose released in two step enzymatic hydrolysis process was upto 43.2 g L^{-1}, equivalent to 86.74% of theoretical maximum. An improvement of 9% in the efficiency of enzymatic hydrolysis was observed in two step hydrolysis reaction as compared to single step hydrolysis. Improvement in the enzymatic hydrolysis could be achieved due to removal of xylose and the pretreatment inhibitors present in the hydrolysate by *Pichia stipitis* fermentation [18]. The inhibitory effect of the xylose on enzymatic hydrolysis was also investigated by *Qing et al* [18]. A higher concentration of xylose compounds was reported to have significant inhibitory effect on final sugar yields and initial hydrolysis rates. It was also reported that the concentration about 12.5 mg/ml of xylose, lowered the initial rates of enzymatic hydrolysis by 37.8% [18]. The strong inhibition of xylose on cellulose hydrolysis suggested that removal of xylose or xylo-oligomers from the reaction mixture not only increase the enzyme accessibility but also reduce cellulase inhibition by xylose. Qing et al also concluded that the hemicellulose removal before enzymatic hydrolysis was more beneficial to hydrolysis rates and yields than adding a higher dose of enzyme.

The inhibitory effect of xylose on enzymatic hydrolysis of cellulose was further investigated by addition of different concentrations of xylose rich supernatant (10 – 50 g L^{-1}) during enzymatic hydrolysis of mixed acid treated corn cob cake. The addition of xylose rich supernatant to enzymatic hydrolysis of corn cob wet cake lowered the final hydrolysis yields by 10 – 20% as compared to control (data not shown). The limitation of xylose inhibition on enzymatic hydrolysis process was eliminated by employing two step hydrolysis and cofermentation by *Pichia stipitis*. Addition of cellulase enzyme in two steps led to minimum exposure of active enzyme molecules to high

initial concentration of xylose. Cofermentation by *Pichia stipitis* after step – 1 hydrolysis led to complete consumption of xylose, which in turn avoided exposure of active enzyme molecules added in second step of hydrolysis to xylose.

The glucose released after second hydrolysis step was fermented by ADY preparation of *Saccharomyces cerevisiae* in 24 h and final ethanol concentration increased up to 29.66 g L^{-1}, equivalent to 75.32% of theoretical maximum and volumetric productivity was found to be 0.43 g $L^{-1}h^{-1}$ (Fig – 4).

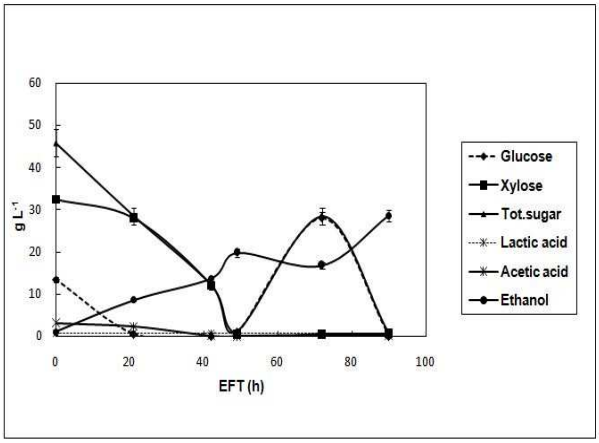


Fig-4: Two step enzymatic hydrolysis and fermentation of acid treated corn cob

There was improvement in the overall cofermentation efficiency of *Pichia stipitis* at xylose concentration of 32.5 g L^{-1} in two step hydrolysis and fermentation process due to non repressible initial glucose concentration (14.7 g L^{-1}) as compared to single step hydrolysis and cofermentation of 71.2 g L^{-1} mixed sugar. In this strategy, repression of enzymes involved in xylose metabolism by glucose was overcome by avoiding exposure of *Pichia stipitis* cells to high concentration of glucose.Cost of nutrients should be minimum for production of high volume and low cost product like ethanol.

Two step hydrolysis and fermentation process also allowed effective utilization of nutrients. Fermentation by *Sachharomyces cerevisiae* was achieved with minimal nutrients due to presence of unutilized CSL and urea from *Pichia stipitis* fermentation. Using two step hydrolysis and fermentation process, there was 1.1 fold increase in the enzymatic efficiency of cellulose hydrolysis over single step hydrolysis process due to removal of inhibitors like xylose and glucose.

Final ethanol concentration, ethanol yield and volumetric productivity were improved by 1.7, 1.66 and 2.6 fold respectively as compared to one step enzymatic and fermentation process (Table-4).

Table-4: Comparison of cofermentation processes

Process	Total Sugar (g L^{-1})	Enzymatic hydrolysis efficiency (%)	Ethanol (g L^{-1})	Fermentation efficiency (%)	Residual Sugar (g L^{-1})	Yield (g g^{-1})	Volumetric Productivity (g $L^{-1}h^{-1}$)
Co fermentation at lower sugar concentration	46.2	79.5	22.9	92.35	0	0.471	0.477
Co fermentation at higher sugar concentration	71.2	79.5	16.8	46.71	17.8	0.23	0.1604
Two step enzymatic and fermentation at higher sugar concentration	74.4	86.74	28.66	75.32	0.2	0.384	0.4277

CONCLUSIONS

Cofermentation offers several benefits over separate glucose and xylose fermentation using *Pichia stipitis*. Therefore two step enzymatic

and fermentation process turns out to be potential strategy to increase the hydrolysis efficiency, overall ethanol yield, titer and volumetric productivity. It reduces glucose repression in mixed sugar fermentation, requires low cost industrial media ingredients like CSL and urea in the seed and fermentation media which increases economic viability of the process.

This strategy provides potential prospects for scale up of lignocellulose to ethanol process using corn cob as feedstock. Although cellulose to ethanol process has been improved over the years using genetically engineered microbes (GEMs), it is not yet clear how easily GM yeasts might be accepted for use on an industrial scale. Also there are few concerns associated with the process in terms of environmental safety at large scale of ethanol production using GEMs, like effluent management containing GMs and maintaining a stable performance of GEMs in commercial scale fermentation operations.

Two step hydrolysis and fermentation process which employs naturally occurring yeasts provides suitable alternative for economic bioconversion of lignocellulose to ethanol. The developed process addressed major challenges associated with enzymatic hydrolysis and cofermentation. This study successfully demonstrated conversion of corn cob slurry containing high concentration of mixed sugars with significant improvement in the fermentation efficiency using low cost industrial media ingredients.

ACKNOWLEDGEMENT

We gratefully acknowledge the analytical division, Praj Matrix for providing analytical support. We also acknowledge the efforts put in by Ms. Sonali Bhadra and Mr. Mandar Deshpande during experimentation. We thank Praj Industries Ltd for the financial support.

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