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BIOETHANOL PRODUCTION FROM NAOH+H_2O_2 PRETREATED *POPULUS DELTOIDES* WOOD USING COCKTAIL OF INHOUSE AND COMMERCIAL ENZYMES UNDER FOUR DIFFERENT MODES OF SEPARATE HYDROLYSIS AND FERMENTATION

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ABSTRACT

In the present study the bioconversion of alkaline hydrogen peroxide pretreated *P. deltoides* wood by using cocktail of inhouse enzymes from *Bacillus stratosphericus* N_{12} (M), *Myceliophthora thermophila* SH1 and *Bacillus altitudinis* Kd_1 (M) under submerged as well as solidstate fermentation was done which was compared with commercial enzymes. Comparative study of bioethanol production from *P. deltoides* wood was carried out using different methods of non-detoxification/detoxification, enzymatic hydrolysis and fermentation. In method I and II supernatant of alkaline hydrogen peroxide pretreated *P. deltoides* wood was pooled together with enzyme saccharified residue fraction, while in method III and IV supernatant after pretreatment was discarded and only enzymatic hydrolysate containing simple sugars was used for the fermentation. Different ethanologenic yeasts and bacteria were employed under monoculture and co-culture combinations for fermentation. The highest ethanol yield of 18.47 g/l was obtained in method IV as compared to others and 21.41g/l in case of commercial enzymes. The co-culture of *Saccharomyces cerevisiae* II+ *Pichia stipitis* was found best combination of ethanologens in both cases i.e. in inhouse enzyme saccharified hydrolysate as well as by using commercial enzymes.

KEYWORDS: *Populus deltoides*, Bioethanol, Monoculture, Co-culture.

1. INTRODUCTION

Although CO_2 is the most important greenhouse gas (GHG), several studies show that it is important to consider other GHGs as well. The continued use of fossil fuels to meet the majority of the world's energy demand is threatened by increasing concentrations of CO_2 in the atmosphere and concerns over global warming.[1] The combustion of fossil fuels is responsible for 73% of the CO_2 production.[2] The heightened awareness of the global warming issue has increased interest in the development of methods to mitigate GHG emissions.[3] Much of the current effort to control such emissions focuses on advancing technologies that: (i) reduce energy consumption, (ii) increase the efficiency of energy conversion or utilization, (iii) switch to lower carbon content fuels, (iv) enhance natural sinks for CO_2 and (v) capture and store CO_2. Reducing use of fossil fuels would considerably reduce the amount of CO_2 produced, as well as reduce the levels of pollutants.[4] As concern about global warming and dependence on fossil fuels grows, the search for renewable energy sources that reduce CO_2 emissions becomes a matter of widespread attention.[5] To reduce the net contribution of GHGs to the atmosphere, bioethanol has been recognized as a potential alternative to petroleum-derived transportation fuels.[6] A high number of research projects to produce biofuels in the first generation were concentrated on the production of ethanol by fermentation of corn and sugar. This way of only using corn and sugar for producing biofuels, faces a big problem of not replacing the 5.4×1020 J consumed worldwide every year and need a large quantity of food crops. Nowadays, in second generation bioethanol, lignocellulosic biomass play a great role in the production of bioenergy (especially biofuels) which is organic in composition and has the same property and energy content like petroleum based energy.[7] Lignocellulose have been taken into consideration as one form of biomass suitable to be a renewable energy source because, it is obtained from non-food biomass which can avoid the conflict between food and fuel.[8] Lignocellulosic wastes are composed of cellulose, hemicellulose and lignin. Other components such as ash, proteins, pectin etc. are also found in the lignocellulosic residues in different proportions.[9] The production of energy of this way depends on the type, abundance and cost of biomass feedstocks, efficiency of the available processing technologies and the pattern of energy demand.[10] The closeness of those main components (cellulose, hemicellulose and lignin) in the lignocellulosic wastes, induces the necessity of pretreatment process in order to make these carbohydrates available for enzymatic hydrolysis and fermentation during biofuels production.[11] Alkaline hydrogen

peroxide has been successfully developed for pretreatment of lignocellulosic materials. The sugars generated from lignocellulose are a mixture of hexoses and pentoses. The hexose sugars can readily fermented into ethanol using industrial strains of the yeast, *Saccharomyces cerevisiae*. However, yeast varieties of the genus *Saccharomyces* have not been found that can ferment pentose sugar such as xylose into ethanol, other organisms are there which can ferment xylose.[12] In the present study, hypercellulolytic bacteria i.e. *B. stratosphericus* N_{12} (M) and fungus *M. thermophila SH1* were explored for cellulase and *B. altitudinis* Kd_1 (M) *for* xylanase production. Alkaline hydrogen peroxide pretreated *P. deltoides* wood biomass was used as the substrate for bioethanol production under different four methods of separate hydrolysis and fermentation (SHF) and deals with the different monoculture and co-culture combinations of yeasts and bacteria. `

2. MATERIALS AND METHODS

2.1 Substrate

Alkaline hydrogen peroxide pretreated *Populus deltoides* wood.

2.2 Cellulase production by *B. stratosphericus* N_{12} (M) under submerged fermentation (SmF)

100 ml nutrient broth was seeded with 10% *B. stratosphericus* N_{12} (M) (O.D. 1.0) culture in 250 ml Erlenmeyer flasks and was kept at 30 ±2°C at 120 rpm for 24 h. To 5 g of each untreated and pretreated biomass, 100 ml of PYC (Peptone yeast extract) medium was added in 250 ml Erlenmeyer flask and was autoclaved. After autoclaving, the flasks were inoculated with 10% of inoculum and incubated at kept 30±2°C for 3 days.

2.3 Xylanase production by *B. altitudinis* Kd_1 (M) under submerged fermentation (SmF)

100 ml nutrient broth was seeded with 12.5% *B. altitudinis* Kd_1 (M) (O.D. 1.0) culture in 250 ml Erlenmeyer flasks and was kept at 30±2°C at 120 rpm for 24 h. To 5 g of each untreated and pretreated biomass, 100 ml of TGY was added in 250 ml Erlenmeyer flask and was autoclaved. After autoclaving, the flasks were inoculated with 12.5% of inoculum and incubated at kept 30±2°C for 3 days.

2.4 Cellulase and xylanase production by *M. thermophila* SH1 under solidstate fermentation (SSF)

To 5 g of each untreated and pretreated biomass, 20 ml of moistening agent i.e. Vogel's medium was added in 250 ml Erlenmeyer flask. After autoclaving 2 ml of inoculum (1×10^7

spores/ml) was added in each flask and these were incubated at 45± 2^0Cfor 8 days in static phase and control was run without inoculum. Enzymes were extracted by repeated extraction method.[13] To 5 g of each untreated and pretreated biomass, 50 ml of Phosphate buffer (0.1 M, pH 6.9) with 0.1% tween 20 was added in 250 ml of Erlenmeyer flask. The contents were kept in a shaker at 120 rpm for 1 h and afterwards were filtered through muslin cloth. The process was repeated twice with 25 ml of phosphate buffer each time making final volume of extracted product to 100 ml. After filtration, contents were centrifuged at 10,000 rpm for 15 min at 4^oC and clear supernatant from each of the biomass was collected for further studies. Enzyme assays with crude enzyme were performed.

2.5 Cellulase assay

The sub-enzymes of cellulase were measured by following standard assays. CMCase activity was determined by incubating 0.5 ml of culture supernatant with 0.5 ml of 1.1% CMC in citrate buffer (0.05M, pH 5.0) at 50^0C or 1 h. After incubation and 3 ml of 3,5 – dinitrosalicylic acid (DNS) reagent was added. The tubes were immersed in boiling water bath and removed after 15 min. The optical density was read at 540 nm. FPase activity was measured by Reese and Mandel method. The reaction containing 0.5 ml of culture supernatant, 50 mg strips of filter paper (Whatmann no. 1) and 0.5 ml of citrate buffer (0.05 M, pH 5.0) was incubated at 50^0C for 1 h. After incubation and 3 ml of DNS reagent was added. The tubes were boiled in boiling water bath and removed after 15 min. The OD was read at 540 nm.[14] For β-glucosidase activity the reaction mixture containing 1 ml of 1mM p-nitrophenol β-D-glucopyranoside in 0.05 M acetate buffer (pH 5.0) and 100 µl of enzyme solution was incubated at 45^0C for 10 min. After incubation, 2 ml of 1 M Na_2CO_3 was added and the mixture was heated in boiling water bath for 15 min and OD was read at 400 nm.[15]

2.6 Xylanase assay

To 0.5 ml of xylan solution (which is incubated overnight at 37°C), centrifuged and clear supernatant was used, 0.3 ml of citrate buffer (pH 5) was added and 0.2 ml of enzyme. The control was run with all components except the enzyme. The reaction mixture was incubated at 45°C for 10 min and then 3 ml of DNSA reagent was added and the mixture was then heated on boiling water bath for 15 min, after cooling down at room temperature, absorbance of reaction mixture was read at 540 nm.

2.7 Inhouse enzyme cocktail

The inhouse enzymes which were prepared had been mixed in the ratio of (2:2:1) i.e 2 ml of cellulase from *M. thermophila* SH1 (CMCase: 1.752 IU, FPase: 1.193 IU and β-glucosidase: 2.571 IU) + 2 ml of cellulase from *B. stratosphericus* N_{12}(M) (CMCase: 2.384 IU, FPase: 2.249 IU and β-glucosidase: 2.451 IU) and 0.5 ml of xylanase from *B. altitudinis* Kd_1 (M) (71.49 IU) + 0.5 ml of xylanase from *M. thermophila* SH1 (12.59 IU) and enzymatic dose was adjusted @ 1ml/g of biomass for hydrolysis.

2.8 Commercial enzymes as control

Commercial enzymes i.e. celluclast 1.5 L (Cellulase from *Trichoderma reesei*, C2730, Sigma Aldrich), Novozyme 188 (Cellobiase from *Aspergillus niger*, C6105, Sigma Aldrich) and Pantopan Mono BG (Xylanase from *Thermomyces lanuginosus*, X2753, Sigma Aldrich) were used as control to compare the results of hydrolysis of biomass with inhouse enzyme preparation. Commercial enzymes were mixed in the ratio of 1:1:1 i.e. 1ml celluclast 1.5L (CMCase: 29.75 IU, FPase: 29.75 IU), 1ml Novozyme 188 (β-glucosidase: 20.51 IU) + 1ml Pantopan Mono BG (xylanase: 22.64 IU) and used @ 1 ml/g of biomass.

2.9 Separate hydrolysis and fermentation (SHF)

SHF was carried out under four different strategies in order to ascertain the effect of pretreatment and inhibitors on final ethanol concentration.

2.9.1 Hydrolysis

5 g of untreated *P. deltoides* wood biomass was taken in 250 ml of flask. To this 50 ml of 2% NaOH +H_2O_2 (9:1) was added and kept at 65^0C in water bath for 3 h. After 3 h supernatant was separated out from solid biomass by centrifugation at 10,000 rpm for 20 min and reducing sugars were estimated in the supernatant.[16] Enzymatic saccharification of the alkaline peroxide pretreated left over solid biomass was done by adding hydrolytic enzymes at 1ml/g of residue. After 72 h saccharified biomas was centrifuged at 10,000 rpm for 20 min and clear hydrolysate was collected. The wood hydrolysate so prepared was used for fermentation in four different ways as given below.

2.9.2 Method 1

The saccharified syrup was pooled together with supernatant obtained after alkaline hydrogen peroxide pretreatment making the total volume to 100 ml. Reducing sugars so obtained were analysed.

2.9.3 Method 2

The saccharified syrup was pooled together with supernatant of alkali pretreated wood, followed by detoxification by overliming with $Ca(OH)_2$.

2.9.4 Method 3

The supernatant from alkaline hydrogen peroxide pretreatment was decanted off, only enzymatic wood hydrolysate was used for fermentation without detoxification.

2.9.5 Method 4

The supernatant from alkaline hydrogen peroxide pretreatment was decanted off and detoxified enzymatic wood hydrolysate was used for fermentation.

2.10 Detoxification by overliming with Ca $(OH)_2$

Calcium hydroxide was added to the alkaline peroxide pretreated and enzyme saccharified hydrolyzate (50^{o}C, pH-5.0, 72 h) to increase the pH to10.5. At high pH inhibitors were precipitated out with calcium hydroxide. The whole mixture was stirred for 30 min at 90^{o}C, allowed to cool slowly to room temperature and then adjusted back to pH 6.0 with HCl. It was then centrifuged (15,000g ×, 30 min) to remove precipitate formed before using as substrate for fermentation. After removing the precipitates sugar estimation was done.

2.11 Ethanologens used

Fermenting microbes i.e. *Saccharomyces cerevisiae* I (MTCC-3089) procured from MTCC Chandigarh -India, *Saccharomyces cerevisiae* II (DSM-1334) procured from DSM-Germany, *Pichia stipitis* (NCIM 3498), *Candida shehatae* (NCIM 3500) and *Zymomonas mobilis* (NCIM 5134) were procured from NCIM- Pune, India were used for fermentation in the experiments.

2.12 Fermentation Process

To the detoxified as well as non-detoxified supernatants, 0.5% yeast extract and 0.5% peptone were added followed by autoclaving at 121^{o}C, 15 lbs for 20 min. To the fermentation media inoculum *S. cerevisiae-I*, *S. cerevisiae-II*, *P. stipitis*, *C. shehatae*, *Z. mobilis*, *S. cerevisiae-I* + *P. stipitis*, *S. cerevisiae-I* + *C. shehatae*, *S. cerevisiae-II* + *P. stipitis* and *S. cerevisiae-II* + *C. shehatae* were @10% (1 O D) added and kept for 72 h (3 days) at 25^{o}C.

2.13 Ethanol estimation

34.0 g of potassium dichromate was dissolved in 500ml of distilled water. To this 375 ml of concentrated sulphuric acid was added, mix thoroughly and allowed to cool. Final volume was made 1000ml by adding distilled water. To the distillation flask 29 ml of distilled water and 1 ml of sample was added. On the other side to the 50 ml volumetric flask 25 ml of potassium dichromate was added. Distillation was set at 60°C and tap water was turned ON. To the 25 ml of potassium dichromate, 20 ml of distilled sample was collected and it became total 45 ml. To this 45 ml solution 5 ml of distilled water was added and total volume became 50 ml and was at 60°C for 20 min. After that O.D. was measure at 600 nm against blank.[17]

3. RESULTS AND DISCUSSION

A sequential process where the hydrolysis of cellulose + hemicellulose and the fermentation of lignocellulosic biomass are carried out as two separate units, this configuration is known as separate hydrolysis and fermentation (SHF). In the present study, different methods of separate hydrolysis and fermentation (SHF) have been devised in order to maximize the final yield of bioethanol production. Tables depicted bioethanol production by using different ethanologenic microorganisms (monoculture and co-culture) i.e. *S. cerevisiae I*, *S. cerevisiae II*, *P. stipitis*, *C. shehatae*, *Z. mobilis*, *S. cerevisiae I* + *P. stipitis*, *S. cerevisiae I* + *C. shehatae*, *S. cerevisiae II* + *P. stipitis* and *S. cerevisiae II* + *C. shehatae* under SHF using alkaline hydrogen peroxide pretreated *P. deltoides* wood as carbon source. The hydrolytic enzymes produced during this study i.e cellulase from *B. stratosphericus* N_{12} (M) + *M. thermophila* SH1 and xylanase from *B. altitudinis* Kd_1 (M) + *M. thermophila* SH1 were used as a cocktail for enzymatic degradation of wood into fermentable sugars. The inhouse enzymes with their total dose @ 5ml/5g, where cellulases and xylanases are mixed in the ratio of 4:1 i.e. 2 ml of cellulase from *M. thermophila* SH1 (with CMCase: 1.752 IU, FPase: 1.193 IU, β-glucosidase: 2.571 IU + 2 ml of cellulase from *B. stratosphericus* N_{12} (M) (with CMCase: 2.384 IU, FPase: 2.249 IU, β- glucosidase: 2.451 IU) and 0.5 ml of xylanase from *B. altitudinis* Kd_1 (M) (71.49 IU) with an aim of complete hydrolysis of pretreated wood.

In case of the first method (Table1) where pooling of supernatant of alkaline hydrogen peroxide pretreatment with wood hydrolysate was done (without detoxification), the maximum ethanol i.e 7.93 g/l was fermented by co-culture of *S. cerevisiae* II + *C. shehatae* closely followed by 7.85 g/l by *S. cerevisiae II* + *P. stipitis*, while the minimum ethanol

production i.e 3.48 g/l was noticed in monoculture of *P. stipitis*. The maximum fermentation efficiency (31.10%) was obtained in co-culture of *S. cerevisiae* II and *C. shehatae*.

Table1. (Method-I): SHF of NaOH+H_2O_2 pretreated *P. deltoides* wood by pooling supernatant + solid residue without detoxification.

Strains	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)	Sugars (mg/g)
Saccharomyces cerevisiae-I	0.82	6.50	0.13	25.49	6.40
Saccharomyces cerevisiae-II	0.92	7.28	0.143	28.61	6.40
Pichia stipitis	0.44	3.48	0.06	13.52	6.40
Candida shehatae	0.52	4.12	0.08	16.07	6.40
Zymomonas mobilis	0.82	6.50	0.13	25.49	6.40
S. cerevisiae-I + P. stipitis	0.80	6.34	0.12	24.70	6.40
S. cerevisiae-I + C. shehatae	0.80	6.34	0.12	24.70	6.40
S. cerevisiae-II + P. stipitis	0.99	7.85	0.15	30.78	6.40
S. cerevisiae-II + C. shehatae	1.00	7.93	0.15	31.10	6.40
S.E..	0.04	0.27	0.01	1.30	
C. $D._{0.05}$	0.03	0.18	0.01	0.89	

The different ethanologens were used in the present study in monoculture as well as co-culture combinations for fermenting sugars produced from enzymatic saccharification of lignocellulosic wood. Co- culture of *S. cerevisiae* II + *C. shehatae* and *S. cerevisiae* II+ *P. stipitis* produced highest ethanol as compared to other co-culture and monoculture. Since *S. cerevisiae* is widely known for hexose utilization whereas *P. stipitis* can ferment pentose sugars thus this co-culture combination would have utilized most of fermentable sugars formed in the wood hydrolysate to yield highest ethanol concentration. Table 2 exhibited the results of second modified method in which pooling of supernatant after NaOH + H_2O_2 pretreatment was done with enzyme hydrolysate along with detoxification using Ca $(OH)_2$ and the highest ethanol production of 18.24 g/l was noticed in co-culture *S. cerevisiae* II + *P. stipitis* followed by *S. cerevisiae* I + *P. stipitis* i.e. 17.44 g/l while the least ethanol yield was formed in monoculture of *P. stipitis* (8.72g/l). The maximum fermentation efficiency of co-culture *S. cerevisiae* II + *P. stipitis* was 71.53%. Since during the pretreatment several inhibitors like furfurals, 5-hydroxy methyl furfurals, acetic acid and levulinic acid and vanillin are generated due to sugar and lignin degradation that hinder the overall process of ethanol fermentation by adsorbing binding sugars and also by excerting lethal effect on fermenting yeasts. Therefore, detoxification or removal of inhibitors from hydrolysate becomes very important for efficient fermentation of sugars formed from lignocellulosic biomass and thus eventually increasing the yield of ethanol. Higher ethanol production after

detoxification of pooled liquor is due to removal of most of the inhibitors formed during pretreatment and hydrolysis, thus facilitating the fermentation of sugars smoothly by ethanologenic microorganisms. Over-liming with a combination of high pH and temperature has for a long time been considered as a promising detoxification method for pretreated hydrolysate of lignocellulosic biomass.[18] This process has been demonstrated to help in the removal of volatile inhibitory compounds such as furfurals and hydroxyl methyl furfurals from the hydrolysate.[19] The only disadvantage of this method is that it not only affects inhibitors, but to an extent also the sugars, which affect the ethanol yield.[20]

In case of third method in which supernatant of the alkaline hydrogen peroxide pretreated slurry was discarded and only solid mass after thorough washing was subjected to enzymatic hydrolysis without any detoxification. The results presented in Table 3 showed that the maximum ethanol of 7.63 g/l was produced by co-culture of *S. cerevisiae* II + *P. stipitis* with highest fermentation efficiency of 29.80%, while the least ethanol of 3.33 g/l was found by mono-culture of *P. stipitis*.

Table 2. (Method-II): SHF of NaOH+H_2O_2 pretreated *P. deltoides* wood by pooling supernatant + solid residue followed by detoxification with calcium hydroxide.

Strains	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)	Sugars (mg/g)
Saccharomyces cerevisiae-I	1.29	10.23	0.204	40.00	7.81
Saccharomyces cerevisiae-II	1.30	10.31	0.206	40.43	7.81
Pichia stipitis	1.10	8.72	0.174	34.11	7.81
Candida shehatae	1.19	9.43	0.188	37.01	7.81
Zymomonas mobilis	1.23	9.75	0.195	38.25	7.81
S. cerevisiae-I + P. stipitis	2.20	17.44	0.348	68.42	7.81
S. cerevisiae-I + C. shehatae	1.31	10.38	0.207	40.58	7.81
S. cerevisiae-II + P. stipitis	2.30	18.24	0.364	71.53	7.81
S. cerevisiae-II + C. shehatae	1.40	11.10	0.222	43.52	7.81
S. E.	0.137	0.182	0.022	1.610	
$C.D._{0.05}$	0.094	0.125	0.015	1.108	

The alkaline hydrogen peroxide pretreatment of *P. deltoides* causes delignification of biomass thus most of lignin along with lignin degrading products viz. Vanillin, levulinic acid, furfurals, 5 HMF etc. are removed with supernatant in turn minimizing the total load of inhibitors in the wood hydrolysate. Therefore with an idea to minimize the inhibitors from the hydrolysate in the present experiment, the supernatant after pretreatment had been discarded, solid wood biomass was subjected to enzymatic saccharification and it was used as sugary substrate for fermentation with different monoculture and co-culture combinations without

any overliming. In a study, the non-detoxified dilute acid hydrolysate was used to produce ethanol by co-culture *S. cerevisiae* Y5 + *P. stipitis* CBS6054. The co-culture consumed glucose along with furfurals and HMF completely in 12 h and all xylose within 96 h, resulting in a final ethanol concentration of 24.4 g/l and ethanol yield of 0.43 g ethanol/g sugar corresponding to 85.1% of the maximal theoretical value.[21]

Table 3. (Method-III): SHF of solid residue of *P. deltoides* wood after NaOH+H_2O_2 pretreatment by discarding supernatant without detoxification.

Strains	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)	Sugars (mg/g)
Saccharomyces cerevisiae-I	0.90	7.13	0.142	27.84	11.26
Saccharomyces cerevisiae-II	0.89	7.05	0.141	27.64	11.26
Pichia stipitis	0.42	3.33	0.066	13.06	11.26
Candida shehatae	0.51	4.04	0.082	15.86	11.26
Zymomonas mobilis	0.78	6.18	0.123	24.25	11.26
S. cerevisiae-I + P. stipitis	0.79	6.26	0.125	24.50	11.26
S. cerevisiae-I + C. shehatae	0.83	6.58	0.131	25.68	11.26
S. cerevisiae-II + P. stipitis	0.96	7.63	0.152	29.80	11.26
S. cerevisiae-II + C. shehatae	0.86	6.82	0.136	26.66	11.26
S.E.	0.016	0.222	0.002	0.405	
C.D. $_{0.05}$	0.011	0.153	0.001	0.279	

As the results revealed in case of fourth method (Table 4) in which supernatant obtained after alkaline hydrogen peroxide pretreatment was discarded and detoxification of woody mass with calcium hydroxide was done followed by enzymatic hydrolysis with inhouse enzymes. Highest yield of 18.47 g/l was observed in co-culture *S. cerevisiae* II + *P. stipitis* followed by *S. cerevisiae* I + *P. stipitis* i.e. 18.24 g/l. The least ethanol of 9.51 g/l was observed in mono-culture *C. shehatae*. The maximum fermentation efficiency of *S. cerevisiae* II + *P. stipitis* was observed as 72.46%. Here, the supernatant of the hydrolysate was decanted off after delignification to remove lignin and its degraded compounds and the overliming was done additionally in this scheme with an apparent aim to eliminate most of the toxic inhibitors generated during enzymatic hydrolysis from hydrolysate, thus resulting in the highest yield of ethanol.

Table 4. (Method-IV) SHF of solid residue of *P. deltoides* wood after NaOH+H_2O_2 pretreatment by discarding supernatant followed by detoxification with calcium hydroxide.

Strains	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)	Sugars (mg/g)
Saccharomyces cerevisiae-I	1.39	11.02	0.220	43.13	12.45
Saccharomyces cerevisiae-II	1.40	11.10	0.225	43.54	12.45
Pichia stipitis	1.23	9.75	0.195	38.23	12.45
Candida shehatae	1.20	9.51	0.190	37.25	12.45
Zymomonas mobilis	1.29	10.23	0.204	40.12	12.45
S. cerevisiae-I + P. stipitis	2.30	18.24	0.364	71.53	12.45
S. cerevisiae-I + C. shehatae	1.40	11.10	0.222	43.54	12.45
S. cerevisiae-II + P. stipitis	2.33	18.47	0.369	72.46	12.45
S. cerevisiae-II + C. shehatae	1.48	11.73	0.234	46.03	12.45
S.E.	0.109	0.317	0.024	0.981	
$C.D._{0.05}$	0.075	0.218	0.017	0.675	

Fig 1 showed a comparative bioethanol yield by different methods i.e. I, II, III and IV of SHF using inhouse enzymes for hydrolysis of *P. deltoides* wood. Among all these methods, the highest ethanol yield was obtained in case of method IV. Among different ethanologenic strains used as monoculture and co-culture combinations, *S. cerevisiae II + P. stipitis* and *S. cerevisiae I + P. stipitis* had emerged as the best co-culture combinations for improved bioethanol production. This combination seem to have an edge over others probably due to conversion of both glucose and xylose in fermentation liquor to ethanol as well as degrading inhibitors in the hydrolysate effectively, thus increasing their fermentation efficiency.[21]

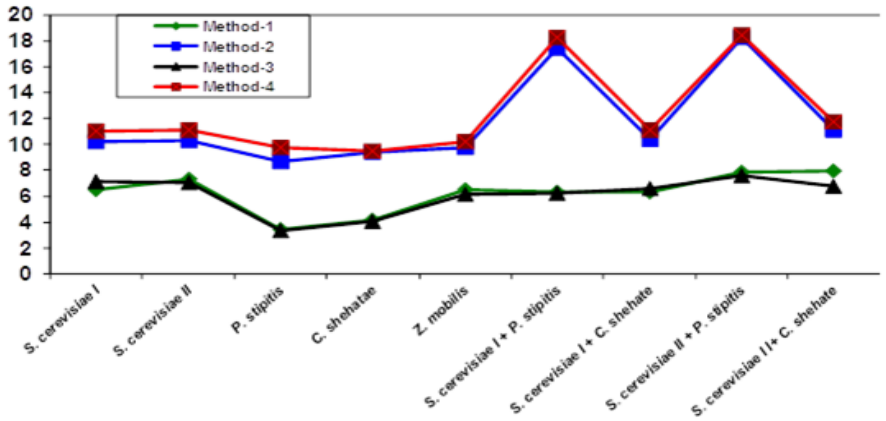

Fig1. Overall comparison of bioethanol production under four methods of SHF using cocktail of inhouse enzymes.

Furfurals have been shown to decrease the ethanol productivity of yeast considerably. Larsson *et al* and Modig *et al* suggested that furfural inhibits several glycolytic enzymes. But adaptation of some of fermenting microorganisms on high furfural concentration has been found a successful option to decrease the furfural effect on growth to an extent. It may be due to the synthesis of new enzymes or co-enzymes by fermenting microorganisms for furfural reduction.[22,23] The commercial enzymes i.e. Celluclast 1.5L, Novozyme188 and Pantopan Mono BG have been used as control to compare the degree of robustness of inhouse enzymes prepared as part of this study for lignocellulosic biomass hydrolysis. The reducing sugars formed during saccharification with commercial enzymes were 531.50 mg/g yielding 21.41 g/l of ethanol and fermentation efficiency of 83.97% *S. cerevisiae* II + *P. stipitis*, while the minimum ethanol production was 15.86 g/l (62.15% of its fermentation efficiency) with mono-culture of *P. stipitis* (Table 5).

Table 5. Bioconversion of $NaOH+H_2O_2$ pretreated *P. deltoides* wood using commercial enzymes under Separate hydrolysis and fermentation (SHF).

Strains used	Sugars (mg/g)	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)
Saccharomyces cerevisiae-I	531.50	2.3	18.24	0.364	71.37
Saccharomyces cerevisiae-II	531.50	2.4	19.03	0.380	74.50
Pichia stipitis	531.50	2.0	15.86	0.317	62.15
Candida shehatae	531.50	2.1	16.65	0.333	65.29
Zymomonas mobilis	531.50	2.5	19.82	0.396	77.64
S. cerevisiae-I + P. stipitis	531.50	2.3	18.24	0.364	71.37
S. cerevisiae-I + C. shehatae	531.50	2.5	19.82	0.396	77.64
S. cerevisiae-II + P. stipitis	531.50	2.7	21.41	0.428	83.97
S. cerevisiae-II + C. shehatae	531.50	2.6	20.62	0.412	80.70
S. cerevisiae-I + C. shehatae + P. stipitis	531.50	2.1	16.65	0.333	65.29
S. cerevisiae-II + C. shehatae+ P. stipitis	531.50	2.3	18.24	0.364	71.37
S.E.		0.85	4.38	0.069	2.62
C.D. $_{0.05}$		1.77	9.09	0.144	5.45

The variation in ethanol yield is dependent upon sugars formed from biomass. The variation in the concentration of sugars hydrolysed with enzymes is due to several factors viz. substrate concentration, type of substrate, enzyme dose, titers and inhibitors which can influence the enzymatic hydrolysis of cellulose. A low substrate concentration would result in a low overall glucose yield. An increase in the substrate concentration would lead to an increased glucose yield as well as an increased rate of reaction.[24] However, a high substrate concentration can cause substrate inhibition, which would substantially decrease the rate of the hydrolysis and the extent of substrate inhibition depends on the ratio of total substrate to total enzyme. A

high cellulase dosage would also significantly raise process costs.[25] The susceptibility of cellulosic substrates to enzymatic hydrolysis is another important factor.

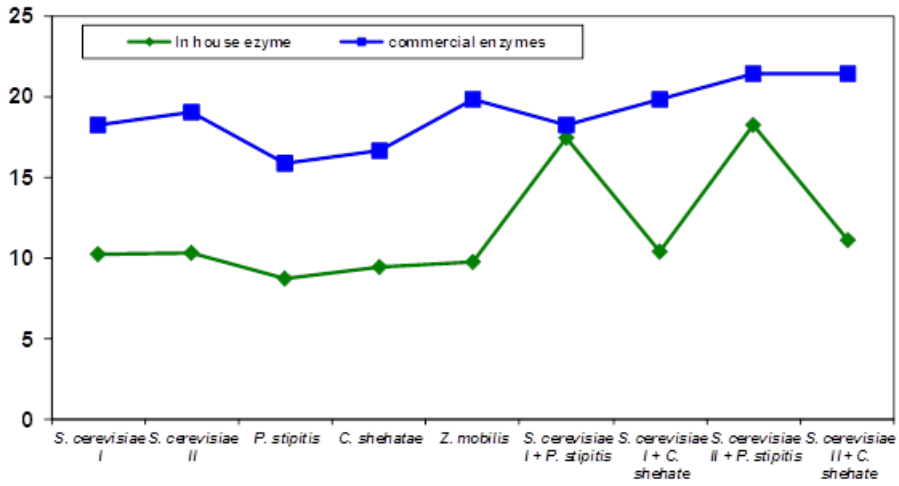

Fig2. Comparison of bioethanol production by using inhouse enzyme hydrolysate (method-IV) with commercial enzyme hydrolysate under SHF.

As shown in fig 2, an attempt to use inhouse enzymes for hydrolysis of pretreated *P. deltoides* wood to ferment to ethanol with co-culture of *S. cerevisiae* II and *P. stipitis* according to method IV was appreciable as compared to control, thus proving their efficiency. This strongly advocates the use of inhouse enzymes prepared during this study to replace commercial enzymes for hydrolysis of lignocellulosic substrates turning these bioconversion to ethanol as a cost effective process as compared to highly expensive commercial enzymes.

In SHF, hydrolysis and fermentation are carried out as completely separate steps, whereby enzymes are added and saccharification is executed to completion, only after which, yeasts are added. The main advantage of this process that is it allows for each step to be performed at its optimum conditions for temperature and pH i.e. hydrolysis at 50^0C and pH 4.8 where cellulase enzymes are maximally active, where as for fermentation 30^0C, pH 6.0 for fermenting microbes is provided. However, a major disadvantage of SHF is that the accumulation of glucose and cellobiose during hydrolysis may lead to end-product inhibition.[26] In the present study, with the modification of SHF, method-IV was considered the best method as compared to others. The co-culture combination of *Saccharomyces cerevisiae II* and *Pichia stipitis* was an appreciable combination of ethanologens which gave

the highest ethanol yield in case of inhouse enzyme (18.47g/l) as well as by using commercial enzymes (21.41g/l).

4. ACKNOWLEDGEMENT

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5. REFERENCES

1. Yu J, Corripio AB, Harrison OP, Copeland RJ. Analysis of the sorbent energy transfer system (SETS) for power generation and CO_2 capture. Adv Environ, 2003; 7: 335–45.
2. Wildenborg T, Lokhorst A. Introduction on CO_2 Geological storage-classification of storage options. Oil Gas Sci Technol Rev IFP, 2005; 60: 513–5.
3. Lombardi L. Life cycle assessment comparison of technical solutions for CO_2 emissions reduction in power generation. Energy Convers Manage, 2003; 44: 93–108.
4. Demirbas A. Hazardous emissions, global climate change and environmental precautions. Energy Sources B, 2006; 1: 75–84.
5. de Oliveria MED, Vaughan BE, Rykiel Jr EJ. Ethanol as fuel: energy, carbon dioxide balances and ecological footprint. BioScience, 2005; 55: 593–602.
6. Govindaswamy S, Vane LM. Kinetics of growth and ethanol production on different carbon substrates using genetically engineered xylose-fermenting yeast. Bioresource Technol, 2007; 98: 677–85.
7. Limayema A, Ricke SC. Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects. Prog Energ Eombust Sci, 2012; 38: 449-467.
8. Tan IS, Lee KT. Enzymatic hydrolysis and fermentation of seaweed solid wastes for bioethanol production: An optimization study. Energy, 2014; 78: 53-62.
9. Behera S, Arora R, Nandhagopal N, Kumar S. Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. Renew Sust Energ Rev, 2014; 36: 91-106.
10. Moe ST, Janga KK, Hertzberg T, Hagg MB, Oyaas K, Dyrset N. Saccharification of lignocellulosic biomass for biofuel and biorefinery applications-A renaissance for the concentrated acid hydrolysis. Energ Proc, 2012; 20: 50-58.

11. Kumar P, Barrett DM, Delwiche MJ, Stroeve P. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Engg Chem Res, 2009; 48(8): 3713-3729.
12. Sharma N, Sharma N. Cost effective production of cellulase and xylanase enzymes by Myceliophthora thermophila SH1 using lignocellulosic forest waste and bioconversion of alkaline peroxide pretreated P. deltoides wood to bioethanol under SHF. J Agro Process Technol, 2013; 19(4): 419-428.
13. Bollag DM, Edelstein SJ. Protein methods. Wiley Liss, John Wiley and Sons, Inc. New York, 1993.
14. Reese ET, Mandel M. Enzymatic hydrolysis of cellulose and its derivatives. In: Methods Carbohydrate Chemistry (ed Whistler R L) 3rd edn. Academic Press, London, 1963.
15. Berghem LER, Petterson LG. Mechanism of enzymatic cellulose degradation and purification of a cellulolytic enzyme from T. viride active on highly ordered cellulose. J Biochem, 1973; 37: 21-30.
16. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Ann Chem, 1959; 3: 426-428.
17. Caputi A, Wright D. Collaborative study of the determination of ethanol in wine by chemical oxidation. J Assoc off Ann Chem, 1969; 52: 85-88.
18. Chandel AK, Chan ES, Rudravaram R, Narasu M L, Rao VR. Economics and environmental impact of bioethanol production technologies: an appraisal. Biotechnol Mol Biol, 2007; 2(1): 014-032.
19. Chandel AK, Singh OV. Weedy lignocellulosic feedstocks and microbial metabolic engineering: Advancing the generation of biofuel. Appl Microbiol Biot, 2011; 89: 1289-1303.
20. Nilvebrant NO, Reimann A, Larsson S, Jonsson LJ. Detoxification of lignocellulose hydrolysates with ion exchange resins. Appl Biochem Biotech, 2003; 91: 35-49.
21. Wan P, Zhai D, Wang Z, Yang X, Tian S. Ethanol production from nondetoxified dilute acid lignocellulosic hydrolysate by co-culture of Saccharomyces cerevisiae Y5 and Pichia stipitis CBS6054. Biotechnol Research Int, 2012; 1-6.
22. Larsson S, Palmqvist E, Hahn- Hagerdal B, Tengborg C, Sterberg K, Zacchi G, Nilvebrant NO. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme Microb Tech, 1999; 24: 151-159.

23. Modig T, Liden G, Taherzadeh M. Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. J Biochem, 2002; 363: 769-776.
24. Hamelinck CN, Hooijdonk GV, Faaij APC. Ethanol from lignocellulosic biomass: techno-economic performance in short, middle and long term. Biomass Bioenerg, 2005; 28: 384-410.
25. Prasad S, Singh A, Jain N, Joshi HC. Ethanol production from sweet sorghum syrup for utilization as automotive fuel in India. Energ Fuel, 2007; 21(4): 2415-2420.
26. Galbe M, Zacchi G. A review of the production of ethanol from softwood. Appl Microbiol Biot, 2002; 59(6): 618-628.