INTERNATIONAL JOURNAL OF BIOASSAYS
ISSN: 2278-778X
CODEN: IJBNHY
OPEN ACCESS

ORIGINAL RESEARCH ARTICLE

Bioethanol production from alkaline hydrogen peroxide pretreated *Populus deltoides* wood using hydrolytic enzymes of *Bacillus stratosphericus* N_{12}(M) and *Bacillus altitudinis* Kd_1(M) under different modes of separate hydrolysis and fermentation by monoculture and co-culture combinations of ethanologens

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Received for publication: November 22, 2015; **Revised**: December 12, 2015; **Accepted**: January 13, 2016

Abstract: An integrated approach was studied for in-house cellulase and xylanase production, from novel hyper hydrolytic enzyme producers and enzymatic hydrolysis of pretreated *Populus deltoides* wood into bioethanol. A xylanase producer *Bacillus altitudinis* Kd_1 (M) and cellulase producer *Bacillus stratosphericus* N_{12} (M) was isolated from soil. Optimization of process parameters led to an optimal xylanase activity of 96.25 IU at 30°C and pH 5.5 and cellulase activity of 5.98 IU at 30°C and pH 8.0. The $NaOH+H_2O_2$ pretreated biomass was hydrolysed using cellulase and xylanase producing 12.45 mg/g of reducing sugars. Further fermentation of lignocellulosic hydrolysate was performed using different yeasts viz. *Saccharomyces cerevisiae* I, *Saccharomyces cerevisiae* II, *Pichia stipitis*, *Candida shehatae* and *Zymomonas mobilis* and maximum 11.10 g/l ethanol yield achieved with co-culture of *S. cerevisiae II* + *P. stipitis* with fermentation efficiency of 43.52% under method IV of SHF. The results have significant implications and further applications regarding production of fuel ethanol from agricultural lignocellulosic waste.

Key words: Bioethanol; Cellulose; Pretreatment; Xylanase.

Introduction

Today fossil fuels take up 80% of the primary energy consumed in the world, of which 58% alone is consumed by the transport sector. Continuous depletion of conventional fossil fuel reserves with increasing energy demands and climate change have led to a move towards alternative, renewable, sustainable, efficient and cost-effective energy sources with smaller emissions (Nigam & Singh, 2011). Bioethanol from lignocellulosic biomass is one of the important alternatives being considered due to the easy adaptability of this fuel to existing engines and because this is a cleaner fuel with higher octane rating than gasoline. Lignocellulosic wastes are the largest group of wastes present on this plant causing environmental pollution (Rani and N and, 2000). Bioconversion of lignocellulosic wastes could make a significant contribution to the production of organic chemicals and biofuels. Cellulases and hemicellulases have numerous applications and biotechnological potential for various industries including chemicals, fuel, food, brewery and wine, animal feed, textile, pulp and paper, laundry and agriculture (Sun and Cheng, 2002). Once an adequate feedstock has been selected the most important factor for the economic outcome of the bioethanol process is the overall ethanol yield. As a consequence, it is essential to maximize the overall sugar yield in the process, that is, to obtain high yield of both glucose and hemicelluloses sugars from the biomass feedstock selected.

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Ethanol from renewable resources has been of interest in recent decades as an alternative fuel or oxygenate additive to the current fossil fuels. Lignocellulosic materials are cheap renewable resources, available in large quantities (Millati *et al.,* 2002). *P. deltoides* is one of the abundant lignocellulosic materials in the world. A pretreatment process is therefore essential in order to remove lignin, reduce cellulose crystallinity and increase the porosity of the materials (Sun and Cheng, 2002). Alkaline hydrogen peroxide has been successfully developed for pretreatment of lignocellulosic materials. Alkaline solutions of H_2O_2 can also solubilize a part of the hemicellulose present in the plant material which makes cellulose more accessible for the microorganisms taking part in the fermentation process. Moreover, using H_2O_2 leads to breaking bonds between carbohydrates and lignin (Kim *et al.,* 2001) and to reducing cellulose crystallinity (Yu *et al.,* 2009). The main advantage of this method of chemical pretreatment is the degradation of the major agent into oxygen and water, so the residual H_2O_2 should not be present in pretreated biomass. In SHF, hydrolysis and fermentation can be carried out in different conditions and reactors and there is no need to compromise between different optimal hydrolysis and fermentation conditions. Separate reactors have also been suggested to improve process control (Hamelinck *et al.,* 2005). However, the accumulation of end products can reduce the efficiency of hydrolysis in SHF. Improved SHF can potentially be obtained by the

use of higher temperatures in the hydrolysis and by selecting ù-glucosidases with decreased product inhibition. The sugars generated from lignocellulose are a mixture of hexoses and pentoses. The hexose sugars can be 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. The aim of the present study was to assess the potential of the lignocellulosic forest waste as a substrate for bioethanol production by using monoculture and co-culture combinations of ethanologens. For the analysis of bioethanol production efficiency, different pre-treatment methods as well as SHF was carried out in four different modes.

Materials and Methods

Microorganisms and cultivation

The bacterial strains used in the present study were isolated by enrichment culture technique from soil by using Riviere's medium (Riviere, 1961). Plates were then incubated at 35 ±2 °C for 5 days. The pure line cultures were maintained and stored in the laboratory for further study at 4°C on nutrient agar medium supplemented with 0.1% (w/v) cellulose/ oat spelt xylan. From the isolated strains some of them are good cellulase and xylanase producers which were subjected to further improvements by physical as well as chemical mutations. Out of them N_{12} (EtBr mutated) and Kd_1 (UV mutated) were selected as hypercellulase and hyperxylanase producers respectively. The strains (wild as well as mutant) were then characterized on the basis of morphological, physiological, biochemical characteristics and genotyping was done by 16S rRNA sequencing. The sequences obtained were compared with 16S rRNA gene sequences available in the GenBank databases by BLAST search. Multiple sequence alignments of partial 16S rRNA gene sequences were aligned using CLUSTAL W, version 1.8. Tree generated was analyzed with the TREEVIEW program. Sequences obtained were submitted to NCBI for accession number.

Substrate used and pretreatments:

Lignocellulosic materials are renewable, low cost and are abundantly available. So here we used forestry waste viz. *Dandracalamus strictus*, *Eucalyptus sp.*, *Populus deltoides*, *Pinus roxburghii*, *Pinus wallichiana and Cedrus deodara*. Various physical and chemical pretreatments were used to make the substrate accessible for enzymatic hydrolysis as steam, 2.0% $NaOH+H_2O_2$, 2.5% H_2SO_4, 2.5% HCl, 5.0% Ammonia (NH_3).

Production of extracellular hydrolytic enzymes

Production of cellulase under SmF by *Bacillus stratosphericus* N_{12} (M): Cellulase production was studied in a 250 ml Erlenmeyer flask containing 45 ml of PYC medium (CMC - 10.0g; peptone-5.0g; Yeast extract - 5.0g; K_2HPO_4 - 1.0g; Mg $SO_4.7H_2O$-0.2g; NaCl - 5.0g; Cellulose - 10.0 g, Distilled water -1000 ml, pH – 7.0) (Kim *et al.,* 2005). 5 ml of the inoculum at 1.0 OD was added to each 45 ml of PYC broth containing 2% lactose in 250 ml Erlenmeyer flasks and the flasks were incubated at 30±2^{0}C for 3 days at 120 rpm. The samples were withdrawn at specific intervals, were centrifuged at 10,000 rpm for 15 min at 4 ^{0}C and the supernatant was used as crude enzyme for enzyme assay.

Cellulase assay

The sub-enzymes of cellulase were measured by following standard assays. CMCase activity was determined by incubating 0.5ml of culture supernatant with 0.5ml of 1.1% CMC in citrate buffer (0.05M, pH 5.0) at 50 °C for 1h. After incubation, 3 ml of 3,5 – dinitrosalicylic acid (DNS) reagent was added. The tubes were immersed in boiling water bath and removed after 15min. The optical density was read at 540 nm. FPase activity was measured by Reese and Mandel (1963) method. The reaction containing 0.5ml of culture supernatant, 50 mg strips of filter paper (Whatmann no. 1) and 0.5ml of citrate buffer (0.05M, pH-5.0) was incubated at 500C for 1h. After incubation and 3ml of DNS reagent was added. The tubes were boiled in boiling water bath and removed after 15min. The OD was read at 540nm (Reese and Mandel, 1963). For β-glucosidase activity the reaction mixture containing 1ml of 1mM p-nitrophenol β-D-glucopyranoside in 0.05M acetate buffer (pH-5.0) and 100µl of enzyme solution was incubated at 450C for 10 min. After incubation, 2ml of 1M Na_2CO_3 was added and the mixture was heated in boiling water bath for 15min and OD was read at 400nm (Berghem and Petterson, 1973).

Production of xylanase from *B. altitudinis* Kd_1 (M) under SmF

Xylanase production was studied in a 250 ml Erlenmeyer flasks containing 45 ml of TGY medium (Yeast extract - 5.0g; Tryptone - 5.0g; K_2HPO_4 - 1.0g; Xylose - 10.0g, Distilled water-1000 ml, pH-7.0) (Garg *et al.,* 2009). 5 ml of the inoculum at 1.0 OD was added to each 45 ml of TGY broth containing 1% xylan in 250 ml Erlenmeyer flasks and the flasks were incubated at 35±2^{0}C for 5 days at 120 rpm. The samples were withdrawn at specific intervals, were centrifuged at 10,000 rpm for 15 min at 4 ^{0}C and the supernatant was used as crude enzyme for enzyme assay.

Xylanase assay

To 0.5 ml of xylan solution centrifuged and clear supernatant was added along with 0.3ml citrate buffer (pH 5) and 0.2ml 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 3ml of DNSA reagent was added and the mixture was then heated on boiling water bath for 15min, after cooling down at room temperature, absorbance of reaction mixture was read at 540nm (Miller, 1959).

Partial Purification of cellulase and xylanase

Crude enzymes produced were partially purified by using ammonium sulphate precipitation. The crude supernatant was fractionated by precipitation with ammonium sulphate between 50-80% of saturation for cellulase and 30-50% of saturation for xylanase. The protein pellet obtained after ammonium sulphate precipitation was suspended in 0.05M (pH 5.0) phosphate buffer and dialyzed against the same buffer. All these subsequent steps were carried out at 4°C.

Hydrolysis

Lignocellulosic forest wood *Populus deltoides* was used as a carbon source for ethanol production. 5g of *P. deltoides* sawdust was pretreated by 2% NaOH+ H_2O_2 (9:1) kept at 65°C (Sharma and Sharma, 2013) in water bath for 3h and the pyrolysate was filtered with muslin cloth and reducing sugars were estimated in supernatant. Enzymatic saccharification of the alkaline hydrogen peroxide pretreated biomass was done by adding hydrolytic enzymes cellulase of *B. stratosphericus* and *B. altitudinis* xylanase (1ml/g) and incubated for 72h. The wood hydrolysate so prepared was used for fermentation in four different ways as given below:

Method 1: The saccharified syrup was pooled together with supernatant obtained after NaOH+ H_2O_2 pretreatment making the total volume to 100 ml

Method 2: The saccharified syrup was pooled together with supernatant of NaOH+ H_2O_2 pretreated wood, followed by detoxification by overliming with Ca $(OH)_2$.

Method 3: The supernatant from NaOH+ H_2O_2 pretreatment was decanted off, only enzymatic wood hydrolysate was used for fermentation without detoxification.

Method 4: The supernatant from NaOH+ H_2O_2 pretreatment was decanted off and detoxified enzymatic wood hydrolysate was used for fermentation.

Fermentation

To the detoxified as well as nondetoxified supernatants, 0.5 % yeast extract and 0.5% peptone were added followed by autoclaving at 121°C, 15 lbs for 20 min. To the fermentation medium, *Saccharomyces cerevisiae*-I, *Saccharomyces cerevisiae*-II, *Pichia stipitis*, *Candida shehatae*, *Zymomonas mobilis*, *S. cerevisiae*-I + *P. stipitis*, *S. cerevisiae*-I + *C. shehatae*, *S. cerevisiae*-II + *P. stipitis* and *S. cerevisiae*-II + *C. shehatae* were added @10% (1 OD) and kept for 72 h (3 days) at 25°C.

Ethanol estimation

34.0g of potassium dichromate was dissolved in 500ml of distilled water. To this 375ml 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 29ml of distilled water and 1 ml of sample was added. On the other side to the 50 ml volumetric flask 25ml of potassium dichromate was added. Distillation was set at 60°C and tap water was turned ON. To the 25ml of potassium dichromate, 20ml 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 (Caputi and Wright, 1969).

Results and Discussion

Enzyme production

Cellulase and xylanase producing microorganisms were isolated from soil using serial dilution method. Isolates were identified using various morphological and biochemical criteria and genotyping was done by using 16S rRNA gene technique. Sequences of the isolates thus obtained were submitted to NCBI database and matched with already existing sequences. 16S rRNA sequences of these isolates have been registered to GenBank databases. Isolate Kd_1 showed 99% species specific alignment with *Bacillus altitudinis* strain MD02 and isolate N_{12} showed 99% species similarity with *Bacillus stratosphericus* strain SH150. These isolates have been registered with accession numbers as *Bacillus altitudinis* Kd_1 (M) [KC995117] and *Bacillus stratosphericus* N12 (M) [KC995118]. Strain improvement was done by UV irradiations (physical) and ethidium bromide (chemical). Kd_1 (M) showed good increase in xylanase production upon irradiation of UV rays while N_{12} (M) gave increased cellulase production when treated (chemically improved) with ethidium bromide. These mutants were used for further enzyme production study. The different process parameters i.e. media, pH, temperature, incubation time, inoculum size, concentration of substrate for cellulase and xylanase production from *B. stratosphericus* N_{12} (M) and *B. altitudinis* Kd_1 (M), respectively were optimized using Classical approach One Factor at a Time (OFAT). After optimization of different process parameters, PYC media, 8.0 pH, 72 h of incubation days, 10% inoculum size with substrate @ 2.0% was found the best. Cellulase titers were increased from 3.230 IU to 5.983 IU after optimization, attaining

85.23% increase in its production. For xylanase production, TGY media, 5.5 pH 72 h of incubation period, 12.5% inoculum size, with substrate @ 2.5% were found the best. During optimization of these process parameters, enzyme titers increased from 56.83 IU to 96.25 IU exhibiting 85.60% increase in xylanase production.

Partial purification of cellulase and xylanase

To strengthen the enzymes concentration, partial purification of cellulase and xylanase was done by ammonium sulphate precipitation. After ammonium sulphate precipitation, between 30-60% of saturation level maximum CM Case and FPase activity was recovered, whereas β-glucosidase activity was found in the range of 0-30%, while the maximum xylanase was noticed between 0-70% of saturation level. Similarly, xylanase enzyme was produced *Trichoderma viride* in solid state fermentation using sugar cane as a substrate. The enzyme was partially purified by ammonium sulphate (60%) fractionation (Irfan and Syed, 2011). Shanmugapriya *et al.,* 2012 partially purified cellulase at 80% saturation by ammonium sulfate precipitation. The molecular weight was found to be 32.5 kilodaltons by SDS-PAGE method.

Bioconversion of NaOH H_2O_2 pretreated *P. deltoides* into bioethanol under four different modes of separate hydrolysis and fermentation (SHF)

A sequential process where the hydrolysis of cellulose + hemicellulose and the fermentation of lignocellulosic biomass is 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. The hydrolytic enzymes produced during this study i.e. cellulase from *B. stratosphericus* N_{12} (M) and xylanase from *B. altitudinis* Kd_1 (M) were used for enzymatic degradation of wood into fermentable sugars. The inhouse enzymes with their total dose @ 1ml/g are mixed in the ratio of 1:1 i.e. 0.5 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) were used with an aim of complete hydrolysis of alkali pretreated wood. Fermentation of the hydrolysed sugars into bioethanol was done 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.

In the first method of SHF, where pooling of supernatant of alkali pretreatment with wood hydrolysate was done (without detoxification), the maximum ethanol i.e 7.15 g/l was fermented by mono-culture of *S. cerevisiae* I followed by 6.34 g/l by *S. cerevisiae* I + *C. shehatae*, while the minimum ethanol production i.e 3.96 g/l was noticed in monoculture of *C. shehatae* and co-culture of *S. cerevisiae* II + *C. shehatae.* The maximum fermentation efficiency (27.99%) was obtained in *S. cerevisiae* I (Table 1).

Table 1: (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	0.90	7.15	0.14	27.99	8.82
Saccharomyces cerevisiae-II	0.56	4.44	0.088	17.41	8.82
Pichia stipitis	0.52	4.12	0.082	16.17	8.82
Candida shehatae	0.50	3.96	0.079	15.55	8.82
Zymomonas mobilis	0.68	5.39	0.107	21.14	8.82
S. cerevisiae-I + P. stipitis	0.78	6.18	0.123	24.25	8.82
S. cerevisiae-I + C. shehatae	0.80	6.34	0.126	24.88	8.82
S. cerevisiae-II + P. stipitis	0.60	4.75	0.095	18.66	8.82
S. cerevisiae-II + C. shehatae	0.50	3.96	0.079	15.55	8.82
Mean	0.64	4.69	0.12	24.57	-

Table 2 exhibited the 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 10.38 g/l was observed in co-culture of *S. cerevisiae* I+ *P. stipitis* followed by *S. cerevisiae* II + *P. stipitis* i.e. 9.99 g/l while the least ethanol yield was found in monoculture of *Z. mobilis* (6.66g/l). Higher yield of ethanol after detoxification of pooled liquor is due to the removal of inhibitors formed during pretreatment, thus facilitating fermentation of sugar by ethanologenic microorganisms.

Table 2: (Method-II): SHF of $NaOH+H_2O_2$ pretreated *P. deltoides* wood by pooling supernatant + solid residue followed by detoxification with Ca $(OH)_2$

Strains	Ethanol(%)	Ethanol(g/l)	Ethanol(g/g)	Fermentation Efficiency (%)	Sugars(mg/g)
Saccharomyces cerevisiae-I	1.10	8.72	0.17	34.27	11.96
Saccharomyces cerevisiae-II	1.20	9.51	0.19	37.32	11.96
Pichia stipitis	1.10	8.72	0.17	34.27	11.96
Candida shehatae	0.94	7.45	0.14	29.23	11.96
Zymomonas mobilis	0.84	6.66	0.13	26.12	11.96
S. cerevisiae-I + P. stipitis	1.31	10.38	0.20	40.74	11.96
S. cerevisiae-I + C. shehatae	1.20	9.51	0.19	37.25	11.96
S. cerevisiae-II + P. stipitis	1.26	9.99	0.19	39.18	11.96
S. cerevisiae-II + C. shehatae	1.20	9.51	0.19	37.25	11.96
Mean	1.12	8.93	0.17	34.64	-

The maximum ethanol of 6.42 g/l was produced by co-culture of *S. cerevisiae* I+ *P. stipitis* with highest fermentation efficiency of 25.09% while the least ethanol of 2.37 g/l was found by monoculture of *P. stipitis* (Table 3) following the third method of SHF. The alkaline hydrogen peroxide pretreatment of *P. deltoides* causes delignification of biomass thus most of lignin products i.e. Vanillin, levulinic acid, furfurals, 5-HMF etc. are removed with supernatant in turn minimizing the total load of inhibitors in the wood hydrolysate. 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. Yah *et al.,* 2010 compared two methods used for production of bioethanol i.e. acid hydrolysis and enzyme hydrolysis; corncobs are treated with 0.3 M HCl solution at 121C for 1 h. acid hydrolysis of corncobs giving maximum bioethanol production 24.4g/l by *S. cerevisiae* and *P. stipitis.*

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	0.41	3.27	0.06	12.74	8.82
Saccharomyces cerevisiae-II	0.70	5.51	0.11	21.76	8.82
Pichia stipitis	0.30	2.37	0.04	9.33	8.82
Candida shehatae	0.31	2.49	0.04	9.60	8.82
Zymomonas mobilis	0.34	2.69	0.05	10.39	8.82
S. cerevisiae-I + P. stipitis	0.81	6.42	0.12	25.09	8.82
S. cerevisiae-I + C. shehatae	0.71	5.63	0.11	22.08	8.82
S. cerevisiae-II + P. stipitis	0.71	5.63	0.11	22.08	8.82
S. cerevisiae-II + C. shehatae	0.71	5.67	0.11	22.15	8.82
Mean	0.54	4.52	0.08	17.20	

Table 4 revealed the results in case of fourth method 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 11.10 g/l was observed in co-culture *S. cerevisiae* I + *P. stipitis* followed by *S. cerevisiae* II + *P. stipitis* i.e. 10.38 g/l. The least ethanol of 7.53 g/l was observed in mono-culture *Z. mobilis*. The maximum fermentation efficiency of *S. cerevisiae* I + *P. stipitis* was observed as 43.52 %. 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	1.20	9.51	0.19	37.25	12.45
Saccharomyces cerevisiae-II	1.20	9.51	0.19	37.25	12.45
Pichia stipitis	1.10	8.72	0.17	34.11	12.45
Candida shehatae	1.10	8.72	0.17	34.11	12.45
Zymomonas mobilis	0.95	7.53	0.15	29.54	12.45
S. cerevisiae-I + P. stipitis	1.40	11.10	0.22	43.52	12.45
S. cerevisiae-I + C. shehatae	1.20	9.51	0.19	37.25	12.45
S. cerevisiae-II + P. stipitis	1.31	10.38	0.20	40.58	12.45
S. cerevisiae-II + C. shehatae	1.25	9.91	0.19	38.87	12.45
Mean	1.20	9.51	0.19	37.27	

Figure 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 (Wan *et al.,* 2012). Furfurals have been shown to decrease the ethanol productivity of yeast considerably. Larsson *et al.,* (1999) and Modig *et al.,* (2002) 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.

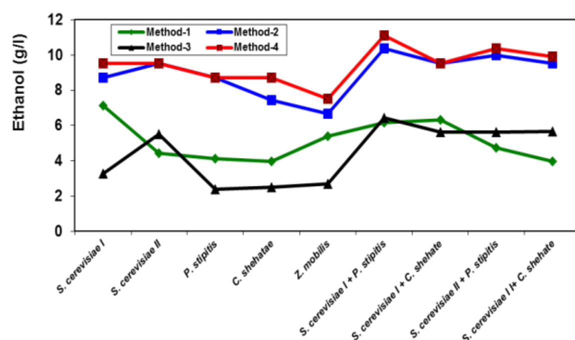

Figure 1: Overall comparison of bioethanol production under four different modes of SHF

Conclusion

In the present study, development of a technology for effectively converting alkaline hydrogen peroxide pre-treated *P. deltoides* wood to simple sugars by potential in-house enzymes produced from isolated potential microorganisms i.e. *Bacillus altitudinis* Kd_1 (M) xylanase producer and cellulase producer *Bacillus stratosphericus* N_{12} (M) and intern fermenting them to appreciable concentration of ethanol by mono culture and co-culture combinations of ethanologens in which the best combination were *S. cerevisiae-I* + *P. stipitis* and *S. cerevisiae-II* + *P. stipitis* that fulfills the main aim of our study, thus envisaging sustainable energy production and improved environmental quality.

Acknowledgement

This study was financially supported by Department of Science and Technology (DST), New Delhi in the form of "INSPIRE FELLOWSHIP", JRF [IF-110299] and authors gratefully acknowledge the financial support given by DST, New Delhi- India.

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Cite this article as:
Nisha Sharma and Nivedita Sharma. Bioethanol production from alkaline hydrogen peroxide pretreated *Populus deltoides* wood using hydrolytic enzymes of *Bacillus stratosphericus* N_{12}(M) and *Bacillus altitudinis* Kd_1(M) under different modes of separate hydrolysis and fermentation by monoculture and co-culture combinations of ethanologens. *International Journal of Bioassays* 5.2 (2016): 4810-4816.

Source of support: Department of Science and Technology (DST), New Delhi, India.
Conflict of interest: None Declared