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Cost effective production of cellulase and xylanase enzymes by *Myceliophthora thermophila* SH1 using lignocellulosic forest waste and bioconversion of alkaline hydrogen peroxide pretreated *P. deltoides* wood to bioethanol under SHF

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Abstract

We studied the bioconversion of untreated/ pretreated lignocellulosic biomass by fungus *Myceliophthora thermophila* SH1 under solid state fermentation. Pretreated lignocellulosic biomass has emerged as a suitable substrate for cellulase and xylanase production by *M. thermophila* SH1 reaching upto 35.32 U/g cellulase and 203.20U/g of xylanase in $NaOH+H_2O_2$ pretreated *Populus deltoides* wood. 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 13.40 g/l was obtained in method IV by monoculture of *S. cerevisiae* II.

Keywords: *Myceliophthora thermophila* SH1, *Populus deltoides*, Cellulase, Xylanase, Bioethanol

1. Introduction

Lignocellulosic wastes are the largest group of wastes present on this plant causing environmental pollution [1]. 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 [2].

To date, the production of cellulase and xylanase has been widely studied in submerged culture processes, but the relatively high cost of enzyme production has hindered the industrial application of cellulose bioconversion [3]. It has been reported that solid-state fermentation is an attractive process to produce cellulase economically due to its lower capital investment and lower operating expenses [4]. Another approach to reduce the cost of cellulase production is the use of lignocellulosic materials as substrates rather than expensive pure cellulose [5].

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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 [6]. *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 [2]. 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 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.

In the present study, hypercellulolytic fungal strain i.e. *M. thermophila SH1* was explored for cellulase and xylanase production by using pretreated and untreated inexpensive forest biomass with an aim to get higher yield of cellulase and xylanase. 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

R *M. thermophila SH1* explored for cellulase and xylanase potential had been isolated previously from hot springs of Northern Himalayas region of Himachal Pradesh and maintained on Malt extract agar slants at 4ºC in a refrigerator were taken for the present study.

2.1 Substrate

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.*

2.2 Pretreatment of substrate

2.2.1 Steam pretreatment. 200g of each selected biomass was taken in beaker and moistened with distilled water, followed by steam pretreatment at 121^0C at 15 psi for 1 h in an autoclave. After drying at 60^0C overnight, it was stored in air tight containers.

2.2.2 Alkali (2.0% NaOH+H_2O_2) pretreatment. 200 g of each selected biomass was dipped in 2.0% NaOH+H_2O_2 (in the ratio 9:1). After 2 h, it was washed until it becomes neutral. After drying at 60ºC overnight, it was stored in air tight containers.

2.2.3 Acid (2.5% H_2SO_4) pretreatment. 200 g of each selected biomass was dipped in 2.5% H_2SO_4. After 2h it was washed until it becomes neutral, dried at 60 ºC and kept in air tight containers.

2.2.4 Acid (2.5% HCl) pretreatment. 200g of each selected biomass was dipped in 2.5% HCl. After 2h it was washed until it becomes neutral, dried at 60ºC and kept in air tight containers.

2.2.5 5.0% Ammonia (NH_3) pretreatment. 200 g of each selected biomass was dipped in 5.0%NH_3. After 2 h it was washed until it becomes neutral, dried at 60ºC and kept in air tight containers.

2.3 Cellulase and xylanase production

2.3.1 Solid state fermentation (SSF). Production: To 5g of each untreated and pretreated biomass, 20ml of moistening agent i.e. Vogel's medium was added in 250ml Erlenmeyer flask. After autoclaving 2ml of inoculum (1×10^7 spores /ml) was added in each flask and these were incubated at 45 ± 2^0C for 8 days in static phase and control was run without inoculum.

2.3.2 Extraction of Cellulase and xylanase. Enzymes were extracted by repeated extraction method [7]. To 5g of each untreated and pretreated biomass, 50ml 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 10,000rpm for 15min at 4°C and clear supernatant from each of the biomass was collected for further studies. Enzyme assays with crude enzyme were performed.

2.3.3 Enzyme assays

2.3.3.1 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^{0}C or 1h. 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 15min. The optical density was read at 540 nm. FPase activity was measured by Reese and Mandel 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 50^{0}C 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 [8]. 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 45^{0}C 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 [9].

2.3.3.2 Xylanase assay. To 0.5 ml of xylan solution (which is incubated overnight at 37°C), centrifuged and clear supernatant was used, 0.3ml of citrate buffer (pH 5) was added 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.

2.3.3.3 Estimation of reducing sugars and proteins. The total reducing sugars were determined by method described by Miller [10] and protein was determined by Lowry's method [11].

2.4 Hydrolysis

5 g of untreated *P. deltoides* sawdust 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°C in water bath for 3 h. After 3 h supernatant was separated out from solid biomass and reducing sugars were estimated in supernatant [10]. Enzymatic saccharification of the alkaline hydrogen peroxide pretreated biomass was done by adding hydrolytic enzymes. To the solid fraction 50 ml of phosphate buffer (0.5 M, pH-5.0) and enzymes were added at 1 ml/g of residue. After 72 h of enzymatic saccharification centrifugation at 10000 rpm for 20 min was done, the saccharified syrup and supernatant obtained after alkaline hydrogen peroxide pretreatment were pooled together making the total volume to 100 ml. In second method the supernatant after pretreatment was discarded, while only saccharified enzymatic hydrolysate was used for fermentation. Reducing sugars so produced were estimated.

2.5 Detoxification with Calcium hydroxide

Calcium hydroxide was added to the alkaline peroxide pretreated and enzyme saccharified hydrolyzate to increase the pH upto 10.5. At high pH inhibitors were precipitated out with calcium hydroxide. The whole mixture was stirred for 30 min at 90°C, allowed to cool slowly to room temperature and then adjusted back to pH 6.0 with HCl. It was then centrifuged (15,000 × g, 30 min) to remove precipitate formed before using as substrate for fermentation. After removing the precipitates sugar estimation was done [10].

2.6 Fermentation

2.6.1 Ethanologens used. *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) procured from NCIM- Pune (India) were used for fermentation in the experiments. Inoculum was prepared by growing cells aerobically in 250 ml flask containing 100 ml of the growth medium in a rotary shaker incubator for 24 h at 25±2°C to make 1.O.D. culture.

2.6.2 Growth Medium. For *Saccharomyces cerevisiae* MTCC 3089: YEPD media**,** for *Pichia stipitis* NCIM 3498 and *Candida shehatae* NCIM

3500: MGYP and for *Zymomonas mobilis* NCIM 5134: Nutrient agar with 2% glucose were used.

2.6.3 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°C, 15 lbs for 20 min. To the fermentation media inoculum *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 @10% (1 O D) added and kept for 72 h (3 days) at 25°C.

2.7 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 [12].

3.Results and Discussion

Hypercellulolytic microbial isolate *M. thermophila* SH1 already isolated from hot springs of Northern Himalayas region of Himachal Pradesh [13], had been selected for degradation of pretreated forest waste with an apparent aim to increase cellulase and xylanase production alongwith an ecofriendly approach.

Therefore inexpensive lignocellulosic forest residue was selected as a substrate for enzyme production from *M. thermophila* SH1 under SSF. Forest residue used in the present study included sawdust of different hardwood and softwood species depending upon their easy availability local abundance and least utilization.

In untreated lignocellulosic biomass production of cellulase ranged from 2.86 U/g in *D. strictus* to 35.32 U/g in NaOH +H_2O_2 pretreated *P. deltoides*. In case of xylanase it ranged from 29.82U/g in *P. roxburghii* to 203.20 U/g in NaOH +H_2O_2 pretreated *P. deltoides*.

The different pretreatments viz. alkali, acid, steam and ammonia given to forest biomass in the present study have resulted in a significant increase in enzyme production.

Table 1 and 2 revealed the data on cellulase and xylanase production utilizing different pretreatments irradiated biomasses from *M. thermophila* SH1. In case of steam pretreatment, maximum cellulase was obtained from *Eucalyptus* wood i.e. 29.00 U/g and xylanase from *C. deodara* i.e. 167.60 U/g. While minimum of cellulase and xylanase was yielded by *P. roxburghii* i.e. 9.30 U/g and 38.64 U/g respectively. Steam pretreatment is an attractive pretreatment process as it makes limited use of chemicals, requires relatively low levels of energy and, depending on the conditions employed, results in the recovery of most of the original cellulose and hemicellulose-derived carbohydrates in a fermentable form [2].

The results in terms of enzyme production from alkaline hydrogen peroxide pretreated forest wastes by *M. thermophila* SH1 revealed that *P. deltoides* which was subjected to alkali pretreatment gave maximum cellulase production of 35.32 U/g and *P. deltoides* also gave the maximum xylanase i.e. 203.20U/g, while very less activity of cellulase and xylanase giving only 9.40 U/g and 48.56 U/g in *P. roxburghii*. Effect of alkali treatment on substrates is measured in terms of enzyme units released by *M. thermophila* SH1.

Alkaline pretreatment of lignocellulosics digests the lignin matrix and makes cellulose and hemicellulose available for enzymatic degradation [3].

Alkali treatment of lignocellulose disrupts the cell wall by dissolving hemicelluloses, lignin and silica by hydrolyzing uronic and acetic esters and by swelling cellulose, so crystallinity of cellulose is decreased due to swelling [14]. The results revealed in case of all 2.5% H_2SO_4 pretreated biomass, *P. deltoides* supported highest cellulase yield of 34.56 U/g while *P. wallichiana* showed minimum value i.e. 11.12 U/g. In case of xylanase production

maximum was also observed in *P. deltoides* i.e. 192.52 U/g and minimum was in *P. roxburghii.*

In case of 2.5% HCl the maximum cellulase was observed in *C. deodara* i.e. 30.98 U/g and minimum of cellulase was in *P. wallichiana* i.e. 9.40 U/g. In case of xylanase production maximum was observed in *D. Strictus* i.e. 195.58 U/g and minimum was in *P. roxburghii* i.e. 40.50 U/g. Pretreatment with dilute acid shows the advantage of hemicellulose solubilization, but also of the conversion of the solubilized hemicellulose in fermentable sugars [15].

In case of ammonia pretreated biomass the maximum of cellulase was found in *Eucalyptus* sp. wood i.e. 31.06 U/g and minimum was in *P. roxburghii* i.e. 9.56U/g. Where as the maximum of xylanase was observed in *D. Strictus* i.e. 200.00 U/g and minimum was in *P. roxburghii* i.e. 43.64 U/g. One of the known reaction of aqueous ammonia with lignin is the cleavage of C-O-C bonds in lignin as well as ether and ester bonds in the lignin-carbohydrate complex (LCC).

Ammonia pretreatment selectively reduces the lignin content in the biomass.

As shown in figure 1, the overall percent increase in cellulase activity i.e. 143.82 was achieved by ammonia pretreated *D. strictus* wood, while the highest percent increase in xylanase activity i.e. 73.71 was found in alkaline hydrogen peroxide pretreated *Eucalyptus sp*. wood (figure 2). Therefore statistically cellulase and xylanase production in untreated and pretreated biomass was significantly different from each other.

Cellulase and xylanase produced by *M. thermophila* SH1 on *P. deltoides* was partially purified by adding $(NH_4)_2SO_4$ at saturation level 30- 60% for CMCase and FPase and 0-30% for β-glucosidase and for xylanase is 0-70%. As the tables 3 revealed the results it showed 2.780, 1.893 and 4.079 specific activity of CMCase, FPase and β-glucosidase activity respectively. As the Table 4 depicted the results of partial purification of xylanase by *M. thermophila* SH1 under SSF, Crude xylanase units of 10.16 IU were enhanced to 12.59 IU after ammonium sulphate precipitation. The partially purified xylanase depicted 1.23 fold increase in xylanase activity with the specific activity of 11.44 and final recovery with 72.36% yield.

Table 1. Optimization of extracellular cellulase production by M. thermophila SH1 using untreated and pretreated lignocellulosic forest waste as substrate under SSF

PRETREATMENTS	**BIOMASS**											
	Dandracalamus strictus		***Populus deltoides***		***Eucalyptus sp.***		***Cedrus deodara***		***Pinus roxburghii***		***Pinus wallichiana***	
	Enzyme units (U/g)*	**Total cellulase (U/g)**	**Enzyme units**	**Total cellulase (U/g)**	**Enzyme units**	**Total cellulase (U/g)**	**Enzyme units**	**Total cellulase (U/g)**	**Enzyme units**	**Total cellulase (U/g)**	**Enzyme units**	**Total cellulase (U/g)**
Untreated	0.680** 2.180*** 8.96****	11.82 (0.543)	1.280 0.300 16.36	17.94 (0.897)	2.860 5.600 18.18	26.64 (1.332)	5.740 5.660 10.72	22.12 (1.106)	6.520 2.900 0.000	9.42 (0.471)	6.300 2.900 0.000	9.200 (0.460)
Steam	1.380 2.00 13.00	16.380 (0.969)	1.800 0.560 23.46	25.82 (1.291)	6.460 6.460 16.08	29.00 (1.450)	6.760 6.500 20.96	22.82 (1.711)	5.720 3.580 0.000	9.80 (0.465)	6.520 2.500 0.000	9.320 (0.467)
$NaOH+H_2O_2$	3.120 6.880 12.820	22.82 (1.141)	4.020 3.220 23.16	30.40 (1.52)	1.680 6.360 27.28	35.32 (1.766)	6.700 6.700 13.40	26.80 (1.340)	5.060 3.480 0.860	9.50 (0.470)	5.600 2.540 1.020	9.260 (0.465)
2.5%H_2SO_4	2.300 5.840 3.820	11.96 (0.598)	2.520 2.360 29.68	34.56 (1.711)	2.880 3.960 2.240	28.08 (1.154)	7.140 7.780 28.04	32.96 (1.800)	6.520 3.340 3.820	13.68 (0.684)	5.060 3.480 0.860	9.400 (0.470)
2.5%HCl	2.680 7.760 17.320	27.76 (1.388)	2.800 2.280 21.82	26.90 (1.34)	2.740 5.880 14.36	27.15 (1.049)	6.720 7.220 17.04	30.98 (1.549)	6.300 3.680 1.140	11.12 (0.556)	3.340 3.820 3.820	10.98 (0.549)
5%NH_3	2.400 6.040 20.380	28.82 (1.441)	3.180 2.460 11.48	18.12 (0.862)	5.140 6.400 19.520	31.06 (1.553)	6.100 5.720 11.00	22.82 (1.141)	5.600 2.540 1.420	9.56 (0.478)	6.300 2.540 0.000	9.840 (0.530)
S.E.	0.115		0.0831		0.333		0.333		0.0115		0.004	
C.D	0.251		0.181		0.726		0.726		0.0251		0.0103	

* : U/g= μ moles of reducing sugars released/min/g of biomass; **: CMCase activity: ***:FPase activity : ****:β-glucosidase activity

Table 2. Optimization of extracellular xylanase production *by M. thermophila* SH1 using untreated and pretreated lignocellulosic forest waste as substrate under SSF

Pretreatments	Biomass											
	D. strictus		*P. deltoides*		*Eucalyptus sp.*		*C. deodara*		*P. roxburghii*		*P. wallichiana*	
	Xylanase (U/g)*	Protein (mg/g)	Xylanase (U/g)	Protein (mg/g)	Xylanase (U/g)	Protein (mg/g)	Xylanase (U/g)	Protein (mg/g)	Xylanase (U/g)	Protein (mg/g)	Xylanase (U/g)	Protein (mg/g)
Untreated	145.84	38.08	88.10	17.40	126.48	41.60	168.00	16.00	29.82	17.40	43.64	10.64
Steam	157.56	27.18	115.86	10.64	142.02	42.60	167.60	34.00	38.60	10.62	48.56	10.68
NaOH+H_2O_2	153.04	37.20	203.20	46.38	197.74	47.00	85.34	18.60	48.56	16.00	40.51	10.70
2.5%H_2SO_4	192.52	43.56	142.22	26.80	150.42	46.00	178.44	35.80	56.46	18.60	85.34	17.40
2.5%HCl	195.58	44.28	136.12	32.00	175.80	46.40	113.64	28.40	40.50	17.40	43.64	16.00
5%NH_3	200.00	47.00	122.10	38.20	166.02	44.80	175.80	17.40	43.64	10.64	56.46	10.62
S.E.	0.333	0.333	0.008	0.333	0.007	0.471	0.333	0.471	0.009	0.333	0.008	0.333
C.D	0.726	0.726	0.0177	0.726	0.0167	1.027	0.726	1.027	0.019	0.726	0.017	0.726

* : U/g= µ moles of reducing sugars released/min/g of biomass

Table 3. Partial purification of cellulase by M. thermophila SH1 using NaOH +H2O2 pretreated P. deltoides wood under SSF

Steps	Volume (ml)	Protein (mg/ml)**	CMCase activity		FPase activity		β-glucosidase activity		Total cellulase		Total activity (IU)*	Purification fold ****	Recovery %*****
			CMCase (IU)	Specific activity (U/mg)	FPase (IU)	Specific activity (U/mg)	β-glucosidase (IU)	Specific activity (U/mg)	Total cellulase	Specific activity***			
Crude culture supernatant	100	0.75	0.084	0.112	0.318	0.424	1.364	1.818	1.766	2.35	176.6	1.000	100
$(NH_4)_2$ SO_4 precipitated	50	0.63	1.752	2.780	1.193	1.893	2.571	4.079	5.516	8.75	275.8	3.72	84.00

*Total activity was determined by multiplication of volume and activity; ** Protein concentration was determined by Lowry's method; ***Specific activity is the activity unit/ protein ****Purification fold is increase in specific activity; *****Recovery % is remaining protein concentration as % of the initial protein

Table 4. Partial purification of xylanase by M. thermophila SH1 using NaOH +H2O2 pretreated P. deltoides wood under SSF

Steps	Volume (ml)	Xylanase activity (IU)	Protein concentration (mg/ml)**	Specific activity (U/mg)***	Purification fold****	Total activity*	Recovery %*****
Crude culture supernatant	100	10.16	1.52	6.684	1.000	101.60	100
$(NH_4)_2SO_4$ precipitated	50	12.59	1.10	11.44	1.712	629.5	72.36

*} same as table 3

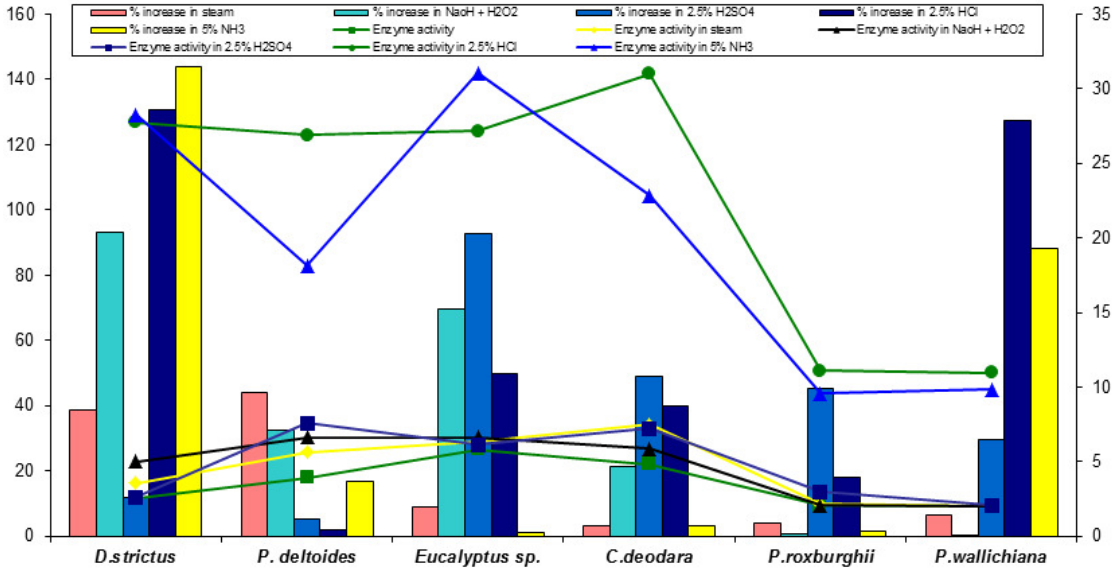

Figure 1. A comparison of percent increase in cellulase activity of pretreated lignocellulosic forest waste over untreated lignocellulosic forest waste by M. thermophila SH1

Table 5 (Method-I): SHF of alkaline hydrogen peroxide pretreated *P. deltoides* wood by pooling supernatant + solid residue without detoxification

Strains used	Sugars (mg/g)	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)
Saccharomyces cerevisiae-I	12.88	1.30	10.31	0.206	40.39
Saccharomyces cerevisiae-II	12.88	1.42	11.26	0.220	43.13
Pichia stipitis	12.88	1.10	8.72	0.174	34.21
Candida shehatae	12.88	1.10	8.78	0.170	33.33
Zymomonas mobilis	12.88	0.79	6.32	0.125	24.57
S. cerevisiae-I + P. stipitis	12.88	1.20	9.51	0.190	37.32
S. cerevisiae-I + C. shehatae	12.88	1.20	9.54	0.194	37.32
S. cerevisiae-II + P. stipitis	12.88	1.30	10.31	0.200	40.43
S. cerevisiae-II + C. shehatae	12.88	1.42	11.26	0.225	44.16
S.E.		0.04	0.27	0.01	1.30
$C.D._{0.05}$		0.03	0.18	0.01	0.89

Table 6 (Method-II): SHF of alkaline hydrogen peroxide pretreated *P. deltoides* wood by pooling supernatant + solid residue followed by detoxification with calcium hydroxide

Strains used	Sugars (mg/g)	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)
Saccharomyces cerevisiae-I	7.08	1.10	8.72	0.174	34.21
Saccharomyces cerevisiae-II	7.08	1.26	9.99	0.199	39.18
Pichia stipitis	7.08	0.97	7.69	0.153	30.16
Candida shehatae	7.08	0.86	6.82	0.136	26.74
Zymomonas mobilis	7.08	0.86	6.82	0.136	26.66
S. cerevisiae-I + P. stipitis	7.08	1.30	10.31	0.206	40.43
S. cerevisiae-I + C. shehatae	7.08	1.10	8.72	0.174	34.21
S. cerevisiae-II + P. stipitis	7.08	0.99	7.85	0.157	30.78
S. cerevisiae-II + C. shehatae	7.08	1.10	8.72	0.174	34.11
S.E.		0.137	0.182	0.022	1.610
$C.D._{0.05}$		0.094	0.125	0.015	1.108

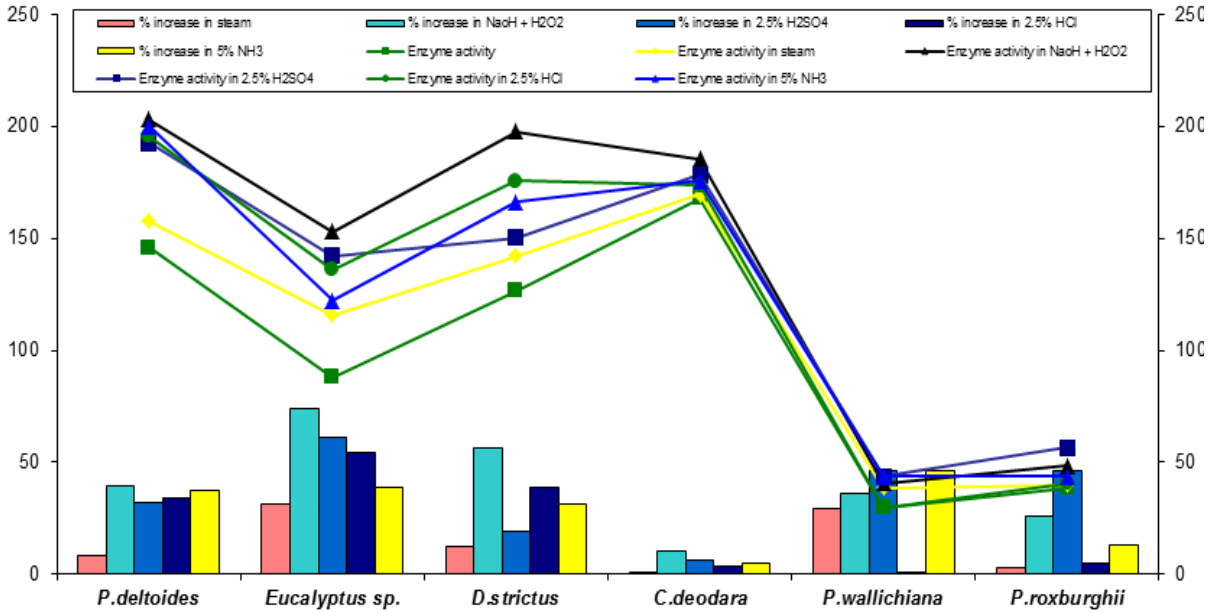

Figure 2. A comparison of percent increase in xylanase activity of pretreated lignocellulosic forest waste over untreated lignocellulosic forest waste by *M. thermophila* SH1

Table 7 (Method-III): SHF of solid residue of P. deltoides wood after alkaline hydrogen peroxide pretreatment by discarding supernatant without detoxification

Strains used	Sugars (mg/g)	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)
Saccharomyces cerevisiae-I	9.00	0.71	5.65	0.112	22.08
Saccharomyces cerevisiae-II	9.00	1.31	10.38	0.207	40.74
Pichia stipitis	9.00	0.158	1.25	0.025	4.91
Candida shehatae	9.00	0.079	0.626	0.012	2.45
Zymomonas mobilis	9.00	0.61	4.83	0.096	18.97
S. cerevisiae-I + P. stipitis	9.00	0.55	4.36	0.087	17.10
S. cerevisiae-I + C. shehatae	9.00	0.79	6.26	0.125	24.47
S. cerevisiae-II + P. stipitis	9.00	0.63	4.99	0.099	19.59
S. cerevisiae-II + C. shehatae	9.00	0.39	3.14	0.061	12.12
S.E.		0.016	0.222	0.002	0.405
$C.D._{0.05}$		0.011	0.153	0.001	0.279

Table 8 (Method-IV) SHF of solid residue of *P. deltoides* wood after alkaline hydrogen peroxide pretreatment by discarding supernatant followed by detoxification with calcium hydroxide

Strains used	Sugars (mg/g)	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)
Saccharomyces cerevisiae-I	9.55	1.26	9.99	0.19	39.18
Saccharomyces cerevisiae-II	9.55	1.70	13.40	0.26	52.87
Pichia stipitis	9.55	0.99	7.85	0.15	30.79
Candida shehatae	9.55	1.10	8.72	0.17	34.21
Zymomonas mobilis	9.55	1.20	9.51	0.19	37.32
S. cerevisiae-I + P. stipitis	9.55	1.29	10.23	0.20	40.12
S. cerevisiae-I + C. shehatae	9.55	1.26	9.99	0.19	39.18
S. cerevisiae-II + P. stipitis	9.55	1.40	11.10	0.22	43.54
S. cerevisiae-II + C. shehatae	9.55	1.30	10.31	0.20	40.43s
S.E.		0.109	0.317	0.024	0.981
$C.D._{0.05}$		0.075	0.218	0.017	0.675

As the tables 5, 6, 7 and 8 depicted the results in the present study, different strategies of Separate Hydrolysis and Fermentation (SHF) were used and compared for bioethanol production. Hydrolysis of NaOH + H_2O_2 pretreated *P. deltoides* wood was done by using cellulase and xylanase produced from potential fungus *M. thermophila* SH1 i.e. 5ml/5g biomass dose of these enzymes was used with an apparent aim to degrade most of cellulose and xylan components of *P. deltoides* wood.

The enzymatic hydrolysate was further fermented 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*.

In method I, where pooling of liquid fraction with enzyme hydrolysate was done without detoxification, the highest ethanol yield of 11.26 g/l was obtained by mono-culture of *S. cerevisiae* II followed by mono culture *S. cerevisiae* I i.e 10.31 g/ l, while the least ethanol production (6.32 g/l) was noticed in mono-culture *Z. mobilis*. The highest fermentation efficiency i.e 43.13 % was shown by *S. cerevisiae* II (Table 5). Results in second method (Table 6) in which pooling of supernatant with enzyme hydrolysate and detoxification with Ca $(OH)_2$ at high pH of 10.5 was done because at high pH degradation of sugars can be minimized [16]. The highest ethanol concentration of 10.31 g/l and fermentation efficiency of 40.43 % was noticed by co culture of *S. cerevisiae* II + *P. stipitis* in detoxified liquor which was followed by monoculture *S. cerevisiae* II i.e 9.99 g/l, while the minimum ethanol production of 6.82 g/l was observed in *Z. mobilis* and *C. shehatae*.

Over-liming by Ca $(OH)_2$ with a combination of high pH and temperature has for a long time been considered as a promising detoxification method for chemically pretreated hydrolysate of lignocellulosic biomass [17].

Inhibitors are generated due to sugar/ lignin degradation and hinder the overall process of ethanol fermentation by adsorbing sugars/ binding sugars and also by excerting lethal effect on fermenting yeasts. Therefore, detoxification or removal of inhibitors from hydrolysate becomes very important for favourable fermentation of lignocellulosic biomass and thus increasing the yield of ethanol. In a similar study ethanol production was evaluated from wheat straw acid hydrolysate using an adapted and parent strain of *Pichia stipitis* NRRL Y-7124. The treatment by boiling and overliming with Ca $(OH)_2$ significantly improved the fermentability of the hydrolysate. The suspension was then centrifuged to remove the unhydrolysed residue and was washed with water at 80 ± 0.5°C to extract the sugars. The centrifugate and washing were pooled together and overlimed with Ca $(OH)_2$ upto pH 10. Ethanol yield and productivity were increased 2.4± 0.10 and 5.7± 0.24 folds, respectively [16].

Results revealed in method III in which supernatant of the slurry after pretreatment was discarded to minimize the inhibitors of hydrolysate and without detoxification only solid residue was subjected to enzymatic saccharification and then fermentation was performed with different monoculture and co-culture combinations, The highest ethanol yield of 10.38 g/l was observed in monoculture of *S. cerevisiae* II, while the minimum ethanol production i.e. 0.626 g/l was observed in monoculture *C. shehatae*. The maximum fermentation efficiency shown by *S. cerevisiae* II was 40.74% (Table 7).

In case of IV method where supernatant was discarded and enzymatic hydrolysate was subjected to detoxification with Ca $(OH)_2$ to remove the degradative inhibitors. The highest ethanol yield of 13.40 g/l and maximum fermentation efficiency of 52.87 % was noticed by mono-culture of *S. cerevisiae II* which was followed by co-culture of *S. cerevisiae II* + *P. stipitis* i.e 11.10 g/l.

Statistically overall it was observed the monoculture *S. cerevisiae* II was the best strain and method IV of SHF mode for the highest ethanol production. The four different strategies used for bioconversion of lignocellulosic *P. deltoides* wood into ethanol clearly reflect the need of pretreatment of substrate to simplify its complex chemical bonding and making it accessible for hydrolysate by prepare enzymes i.e. cellulase and xylanase to saccharify lignocellulosic biomass into soluble sugars. Detoxification of hydrolysate is a prerequisite to facilitate their fermentation by maintaining the vigour and agility of ethanologens, thus improving the yield of ethanol.

4.Conclusion

In the present study different untreated/ pretreated softwood and hardwood biomass were used for bioconversion by *M. thermophila* SH1 under SSF, reaching upto 35.32 U/g of cellulase and 203.20 U/g of xylanase in alkaline hydrogen peroxide pretreated *P. deltoides* wood. Four different strategies of SHF i.e. by non detoxification as well as detoxification were used for bioethanol production. Different ethanologens in monoculture and co-culture combinations were used. The highest ethanol concentration of 13.40 g/l was obtained in method IV by monoculture of *S. cerevisiae* II. Thus in the present study, development of a technology for effectively converting alkaline hydrogen peroxide pretreated *P. deltoides* wood to simple sugars by potential inhouse enzymes produced from isolated microorganisms and intern fermenting them to appreciable concentration of ethanol fulfills the main aim of our study, thus envisaging sustainable energy production and improved environmental quality.

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Compliance with Ethics Requirements:

Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human and/or animal subjects (if exists) respect the specific regulations and standards.

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