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RESEARCH ARTICLE

EVALUATION OF DIFFERENT PRETREATMENTS FOR ENZYMATIC DIGESTIBILITY OF FOREST RESIDUES AND CELLULASE PRODUCTION BY *BACILLUS STRATOSPHERICUS* N_{12} (M) UNDER SUBMERGED FERMENTATION

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ABSTRACT

Lignocellulosic biomass which is rich in cellulose and hemicellulose can be used in submerged fermentation for the production of industrially important enzyme i.e., cellulase. The present work deals with standardization of pretreatments of forestry waste for enhancing the suitability of this abundant waste for cellulase production. Among different pretreatments alkali peroxide pretreatment to *Populus deltoides* has emerged as a cost effective substrate for cellulase production by *Bacillus stratosphericus* N_{12} (M) yielded maximum cellulase activity of 41.73 U/g. The maximum cellulase production for pretreated *P. deltoides* was carried out in the PYC, pH of the medium was 8 and incubated at 30°C for the production and incubation period of 72 h and partial purification of cellulase was done by ammonium sulphate precipitation method leads to increased cellulase activity. It has also been noticed that though forest lignocellulosics when used as carbon source yielded fairly good amount of cellulase but its production is directly dependent upon type of biomass used versus physicochemical treatment given to it prior to biodegradation.

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INTRODUCTION

Cellulose abounds in nature as is the abundant natural product in the biosphere. It is the primary product of photosynthesis in terrestrial environments, and the most abundant renewable bioresource produced in the biosphere i.e 100 billion dry tons/year (Zhang and Lynd, 2004). A number of bioconversion methods have been proposed and employed ranging from direct chemical methods like acid hydrolysis to biological methods such as application of cellulase enzymes. Acid hydrolysis of cellulosic materials though appear to be cheaper than cellulase hydrolysis but the former often requires high temperature and pressure; it is highly corrosive and leads to the accumulation of objectionable by-products thus leading to serious pollution. Enzymatic hydrolysis is an ecofriendly approach. The conversion is carried out under mild conditions thus greatly reducing the cost of hydrolysis equipment. The cost of high activity cellulolytic enzymes solution is at present very high. This enzyme can be produced industrially or by micro-organisms. The industrial production of cellulase is however very expensive but considerable cost reduction may be possible by exploring novel hypercellulolytic microorganisms. Cellulase is a synergistic enzyme and degradation of the cellulose to glucose or other oligosaccharide compounds (Chellapandi and Himanshu, 2008) requires a combined and cooperative action of at least 3 enzymes namely an endo-1, 4-β-glucanase, exo-1, 4-β-glucanase and β-glucosidase. Cellulase has several applications in various industries. Potential applications are in food, animal feed, textile, fuel, chemical industries, paper and pulp industry, waste management, medical/pharmaceutical industry, protoplast production, genetic engineering and pollution treatment (Tarek and Nagwa, 2007). Due to their vast applications and ever increasing demand, novel cellulases with better process suitability, high specificity and stability are being discovered from new lineages of cellulolytic organisms (Bajaj *et al*., 2009). Thus utilization of cellulosic biomass by saccharification to induce cellulase synthesis derived from cellulolytic organisms has been suggested as a feasible process. One effective approach to reduce the cost of enzyme production is to replace pure cellulose by relatively cheaper substrates such as lignocelluloses materials (Sridevi *et al*., 2009). One of the primary challenges for process commercialization is the development of cost effective

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pretreatment technologies for lignocellulosic feed stocks (Wyman *et al*., 2005). Pretreatment is necessary to increase the accessibility of cellulose in lignocellulosic biomass to facilitate enzymatic hydrolysis because unlike traditional sources of fermentable sugars, such as starch and sucrose, the cellulose component of lignocelluloses is a structural polymer and is protected against enzymatic attack by surrounding matrix of lignin and hemicellulose (Sridevi *et al*., 2009). The pretreatment can be classified into physiochemical, chemical and biological processes. This is a main processing challenge in the ethanol production from lignocellulosic biomass. Inefficient pre-treatment processes might result in non-hydrolysable residue formation and incomplete hydrolysis that affect the fermentation process adversely. In the present study an attempt has been made to reduce the cost of cellulase production from hypercellulolytic bacterial *B. stratosphericus* N_{12} (M) by using inexpensive carbon sources viz. forestry wastes which are rich in hemicellulose.

MATERIALS AND METHOD

Substrate used: *Dandracalamus strictus*, *Eucalyptus* sp., *Populus deltoides*, *Pinus roxburghii*, *Pinus wallichiana* and *Cedrus deodara*.

Pretreatments

Each of the selected biomass was followed by steam pretreatment at 121^{0}C at 15 psi for 1 h in an autoclave and dried at 60^{0}C. For each pretreatment, 200 g of each selected biomass was dipped in 2.0% NaOH+H_2O_2 (in the ratio 9:1), 2.5% H_2SO_4, 2.5% HCl, 5.0% NH_3 separately for 2 hrs.

Bacterial culture and inoculum preparation

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.

Fermentation protocol

To 5 g of each untreated and pretreated biomass, 100 ml of PYC medium was added in 250 ml Erlenmeyer flask and was autoclaved, followed by 10% of inoculum and incubated at kept 30±2°C for 3 days.

Enzyme analysis

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^{0}C 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^{0}C 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 (Reese and Mandel, 1963). 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^{0}C 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 (Berghem and Petterson,1973).

Sugar estimation

Reducing sugars in untreated and pretreated raw materials in the culture filtrate were determined by dinitrosalicylic acid (DNS) method with glucose as standard (Miller, 1959).

Partial purification of extracellular cellulase by Ammonium sulfate precipitation

Different concentrations of ammonium sulfate i.e 0-10%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90% were evaluated to attain saturation point for each of cellulase subunits i.e CMCase, FPase and β-glucosidase. The preparations were kept at 4°C for overnight and then centrifuged which resulted in separation of pellets and supernatants. CMCase and FPase were precipitated at 30-60% and β-glucosidase at 0-30% level of saturation of ammonium sulfate. Precipitates of each subunit so obtained were dissolved in phosphate buffer (0.1 M, pH 6.9) separately. Dissolved precipitates of CMCase, FPase and β-glucosidase were dialyzed against same buffer overnight at 4°C using dialysis membrane of cut-off 14 kDa.

Proximate chemical compositional analysis of substrate

Analytical studies of different components of lignocellulosic biomass by standard methods of technical association of pulp and paper industry (TAPPI).

Alcohol-benzene extractives (T 6M-59- Anonymous, 1974)

2 g of oven dried wood meal placed in a porous thimble. The thimble was placed in soxhlets apparatus and extracted with alcohol: benzene mixture in ratio of 1:2 for 6 h. The porous thimble was taken out and allowed to dry in open air and finally in oven at 50^{0}C till constant weight. The solubility was determined by calculating the loss in weight of the sample and expressed in percentage.

Lignin extraction (T12 M-59-ANONYMOUS, 1988)

2 g of oven dried wood meal pre-extracted with alcohol benzene was treated with 15 ml of 72% sulphuric acid for 2 h at 18-20^{0}C with constant stirring and transferred to 1 litre flask and the acid concentration was brought down to 3% by adding 545 ml of distilled water. The solution was refluxed for 4 h and allowed to settle. The material was filtered and washed with hot water, allowed to dried in an oven at 50^{0}C till constant weight. The lignin content was expressed in percentage.

Holocellulose extraction (T 9M-54-Anonymous, 1993)

4g of oven dried wood meal pre-extracted with alcohol-benzene was taken in a conical flask of 250 ml and 160 ml of distilled water was added. The contents were treated with 15 g of sodium chlorite ($NaClO_2$) and 10 drops of acetic acid at 70-80^{0}C on a water bath for 1 h. The process was repeated 4 times till wood sample becomes white. The contents were filtered, washed with water and finally with acetone. The

sample was dried at 50^0C till constant weight. The holocellulose content was expressed in percentage.

RESULTS AND DISCUSSION

Extracellular cellulase production from hypercellulolytic *B. stratosphericus* N_{12} (M) was evaluated under submerged fermentation (SmF) utilizing lignocellulosic forest waste as carbon source. Forest residue used in the present study include sawdust of *Dandracalamus strictus*, *Populus deltoides*, *Eucalyptus* sp., *Cedrus deodara*, *Pinus roxburghii* and *Pinus wallichiana*. As the major impediments to exploit the commercial potential of cellulases is the cost of cellulase production and yield stability. One effective approach to reduce the cost of enzyme production is to replace conventional carbon source i.e. pure cellulose by relatively cheaper substrates such as lignocellulosic forest waste. Several pretreatments had been tried to make forest waste readily accessible for enzyme production by hypercellulolytic microorganisms. Thus keeping in view the above facts, cost effective production of cellulases by *B. stratosphericus* N_{12} (M) under SmF using untreated/ pretreated inexpensive forest waste had been done. Maximum cellulase i.e. 31.88 U/g was observed in *P. deltoides* wood by *B. stratosphericus* N_{12} (M), while minimum cellulase production 5.76 U/g was produced in *C. deodara* wood. Rest of the values for cellulase production from different untreated forest wastes varied between these two extremes (Table 1). Maximum reducing sugars released during the submerged fermentation (SmF) process was 6.46 mg/g by *P. deltoides* wood. In case of acid (H_2SO_4) pretreatment as the Table 2 depicted, the maximum cellulase units 39.70 U/g and maximum reducing sugars 7.46 mg/g from *B. stratosphericus* N_{12} (M) were in *P. deltoides* wood followed by *C. deodara* wood (20.26 U/g) in comparison to other substrate. The least cellulase i.e. 9.930 U/g were expressed in *P. roxburghii* wood. The highest percent increase (39.27) was observed in *P. roxburghii* wood while the least percent increase was seen in *P. wallichiana* wood i.e. 7.63 %. An appraisal of table 3 revealed that in case of HCl pretreated substrates the maximum cellulase of 31.93 U/g and reducing sugars i.e. 6.87 mg/g by *B. stratosphericus* N_{12} (M) were secreted in *P. deltoides* wood. On the other hand the minimum cellulase 10.15 U/g titers were present in *Eucalyptus sp* wood.

Table 1. Extracellular cellulase production by *B. stratosphericus* N_{12} (M) using untreated lignocellulosic forest waste

Biomass	#Final pH	Specific activity	CMCase	FPase	β-glucosidase	Total enzyme (U/g)	Reducing sugars mg/g
P. deltoides	9.00	1.91 (16.88)	10.24 (1.02)	10.92 (1.09)	10.72 (1.07)	31.88* (3.18)**	6.46*** (0.64)
D. strictus	9.14	1.28 (6.80)	3.36 (0.33)	2.40 (0.24)	2.96 (0.29)	8.72 (0.87)	1.65 (0.16)
Eucalyptus sp.	8.20	0.86 (10.60)	3.36 (0.33)	3.27 (0.32)	2.58 (0.25)	9.21 (0.92)	1.79 (0.17)
P. roxburghii	8.22	6.72 (10.60)	1.25 (0.12)	3.25 (0.32)	2.63 (0.26)	7.13 (0.71)	1.35 (0.13)
P. wallichiana	9.00	1.25 (12.90)	3.88 (0.38)	4.92 (0.49)	7.45 (0.74)	16.25 (1.62)	3.21 (0.32)
C. deodara	9.16	6.40 (0.90)	0.001 (0.01)	0.16 (0.01)	5.60 (0.56)	5.76 (0.57)	1.10 (0.11)
$CD_{0.05}$		0.01 (1.01)	0.01 (0.16)	0.02 (0.01)	0.72 (0.01)	0.01 (0.02)	1.03 (0.02)
S.E.		0.01 (0.46)	0.07 (0.01)	0.01 (0.08)	0.33 (0.01)	0.07 (0.10)	0.47 (0.01)

#Initial pH- 8.0
* U/g = µmoles of reducing sugars released / min / g of biomass
** IU =µ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)
***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)
Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 2. Extracellular cellulase production by *B. stratosphericus* N_{12} (M) using H_2SO_4 pretreated lignocellulosic forest waste

Biomass	#Final pH	Specific activity	CMCase	FPase	β-glucosidase	Total enzyme (U/g)	Reducing sugars mg/g	% Increase
P. deltoides	8.9	1.79 (22.10)	11.82 (1.182)	11.75 (1.17)	16.13 (1.61)	39.70* (3.97) **	7.46*** (0.74)	24.52
D. strictus	8.6	0.91 (11.10)	3.70 (0.37)	2.86 (0.28)	3.59 (0.35)	10.15 (1.01)	1.69 (0.16)	16.39
Eucalyptus sp.	8.2	0.67 (16.70)	3.53 (0.35)	3.55 (0.35)	4.69 (0.46)	11.77 (1.17)	2.00 (0.20)	27.79
P. roxburghii	8.5	13.22 (0.85)	2.21 (0.22)	3.89 (0.38)	3.83 (0.38)	9.93 (0.99)	1.65 (0.16)	39.27
P. wallichiana	84	0.92 (18.10)	4.99 (0.49)	6.25 (0.62)	6.25 (0.62)	17.49 (1.74)	3.17 (0.31)	7.63
C. deodara	8.8	4.74 (4.39)	4.39 (0.43)	0.36 (0.03)	19.15 (1.91)	20.26 (2.02)	3.68 (0.36)	25.17
$CD_{0.05}$		0.12 (0.17)	0.17 (0.01)	0.16 (0.12)	0.17 (0.01)	0.162 (0.01)	1.20 (0.01)	
S.E.		0.05 (0.08)	0.08 (0.01)	0.07 (0.05)	0.08 (0.01)	0.07 (0.07)	0.05 (0.01)	

#Initial pH- 8.0
* U/g = µmoles of reducing sugars released / min / g of biomass
** IU =µ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)
***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)
Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 3. Extracellular cellulase production by *B. stratosphericus* N_{12} (M) using HCl pretreated lignocellulosic forest waste

Biomass	#Final pH	Specific activity	CMCase	FPase	β-glucosidase	Total enzyme (U/g)	Reducing sugars mg/g	% Increase
P. deltoides	8.3	1.376 (23.20)	10.50 (1.05)	11.00 (1.10)	10.43 (1.04)	31.93* (3.19)**	6.87*** (0.68)	0.15
D. strictus	8.9	1.15 (11.1)	3.79 (0.37)	2.96 (0.29)	6.12 (0.61)	12.87 (1.28)	2.66 (0.26)	47.59
Eucalyptus sp.	8.6	0.714 (14.20)	3.53 (0.35)	3.51 (0.35)	3.11 (0.31)	10.15 (1.01)	2.78 (0.27)	10.20
P. roxburghii	8.6	3.25 (4.06)	2.03 (0.20)	3.52 (0.35)	7.66 (0.76)	13.21 (1.32)	2.76 (0.27)	85.27
P. wallichiana	8.4	1.41 (13.91)	3.53 (0.35)	7.43 (0.74)	8.71 (0.87)	19.67 (1.96)	4.34 (0.43)	21.04
C. deodara	8.9	3.72 (3.99)	0.87 (0.08)	0.83 (0.08)	13.16 (1.31)	14.86 (1.48)	3.15 (0.31)	157.98
$CD_{0.05}$		0.74 (1.62)	1.25 (0.73)	1.45 (0.74)	1.77 (0.14)	1.77 (0.12)	1.25 (0.01)	
S.E.		0.33 (0.74)	0.57 (0.33)	0.66 (0.33)	0.81 (0.06)	0.81 (0.05)	0.57 (0.01)	

#Initial pH- 8.0
* U/g = µmoles of reducing sugars released / min / g of biomass
** IU =µ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)
***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)
Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 4. Extracellular cellulase by *B. stratosphericus* N_{12} (M) using NAOH+H_2O_2 pretreated lignocellulosic forest waste

Biomass	#Final pH	Specific activity	CMCase	FPase	β-glucosidase	Total enzyme (U/g)	Reducing sugars mg/g	% Increase
P. deltoides	8.6	1.57 (26.50)	11.99 (1.19)	12.03 (1.20)	17.71 (1.77)	**41.73*** **(4.17)****	7.46*** (0.74)	30.89
D. strictus	8.5	1.02 (11.1)	3.59 (0.35)	2.74 (0.27)	5.07 (0.50)	11.40 (1.14)	2.12 (0.21)	30.73
Eucalyptus sp.	8.5	0.72 (19.9)	3.79 (0.37)	4.17 (0.41)	6.46 (0.64)	14.42 (1.44)	2.45 (0.24)	56.56
P. roxburghii	8.7	3.66 (2.75)	2.03 (0.20)	3.80 (0.38)	4.26 (0.42)	10.09 (1.00)	1.79 (0.17)	41.51
P. wallichiana	8.5	1.14 (19.90)	4.95 (0.49)	7.48 (0.74)	10.43 (1.04)	22.86 (2.28)	4.16 (0.41)	40.67
C. deodara	8.9	4.50 (3.75)	1.02 (0.10)	0.79 (0.07)	15.08 (1.50)	16.89 (1.68)	3.10 (0.31)	193.22
$CD_{0.05}$		0.16 (1.26)	0.17 (0.01)	0.17 (0.07)	0.177 (0.01)	0.74 (0.07)	1.67 (0.01)	
S.E.		(0.07) (0.58)	0.08 (0.08)	0.08 (0.03)	0.081 (0.01)	0.34 (0.03)	0.76 (0.01)	

#Initial pH- 8.0
* U/g = µmoles of reducing sugars released / min / g of biomass
** IU =µ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)
***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)
Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 5. Extracellular cellulase by *B. stratosphericus* N_{12} (M) using NH_3 pretreated lignocellulosic forest waste

Biomass	#Final pH	Specific activity	CMCase	FPase	β-glucosidase	Total enzyme (U/g)	Reducing sugars mg/g	% Increase
P. deltoides	8.6	1.57 (22.00)	11.36 (1.13)	11.42 (1.14)	11.97 (1.19)	**34.75*** **(3.47)****	6.94*** (0.69)	9.00
D. strictus	8.5	2.27 (5.30)	4.11 (0.41)	3.17 (0.31)	4.78 (0.47)	12.06 (1.20)	2.23 (0.22)	38.30
Eucalyptus sp.	8.9	0.94 (16.30)	4.07 (0.40)	4.29 (0.42)	6.99 (0.69)	15.35 (1.53)	3.15 (0.31)	66.66
P. roxburghii	8.6	9.25 (0.86)	2.08 (0.20)	3.49 (0.34)	2.39 (0.23)	7.96 (0.79)	1.49 (0.14)	11.64
P. wallichiana	8.8	0.96 (19.70)	4.62 (0.46)	6.04 (0.60)	8.42 (0.84)	19.08 (1.90)	3.89 (0.38)	17.41
C. deodara	8.9	1.74 (3.50)	0.45 (0.04)	0.40 (0.04)	5.26 (0.52)	6.11 (0.61)	1.26 (0.12)	6.07
$CD_{0.05}$		0.72 (0.81)	1.45 (0.14)	1.62 (0.16)	0.72 (0.01)	0.01 (0.07)	1.25 (0.01)	
S.E.		0.33 (0.37)	0.66 (0.06)	0.74 (0.07)	0.33 (0.09)	0.07 (0.03)	0.57 (0.01)	

#Initial pH- 8.0
* U/g = µmoles of reducing sugars released / min / g of biomass
** IU =µ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)
***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)
Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 6. Extracellular cellulase by *B. stratosphericus* N_{12} (M) using steam pretreated lignocellulosic forest waste

Biomass	#Final pH	Specific activity	CMCase	FPase	β-glucosidase	Total enzyme (U/g)	Reducing sugars mg/g	% Increase
P. deltoides	8.6	1.607	10.45	11.17	11.97	33.59*	6.54***	5.36
		(20.09)	(1.04)	(1.11)	(1.19)	(3.35)**	(0.65)	
D. strictus	8.6	1.81	3.54	2.68	5.41	11.63	2.35	33.37
		(6.40)	(0.35)	(0.26)	(0.54)	(1.16)	(0.23)	
Eucalyptus sp.	8.5	0.73	3.67	3.45	2.58	9.70	1.78	5.32
		(13.20)	(0.36)	(0.34)	(0.25)	(0.97)	(0.17)	
P. roxburghii	8.9	7.85	1.94	3.62	7.56	13.12	2.45	84.01
		(1.67)	(0.19)	(0.36)	(0.75)	(1.31)	(0.24)	
P. wallichiana	8.6	1.16	5.11	6.70	7.24	19.05	3.59	17.23
		(16.31)	(0.51)	(0.67)	(0.72)	(1.90)	(0.35)	
C. deodara	8.5	2.66	0.66	0.31	8.33	9.30	1.75	61.45
		(3.49)	(0.06)	(0.03)	(0.83)	(0.93)	(0.17)	
$CD_{0.05}$		1.02	1.62	1.45	1.77	1.62	1.02	
		(1.77)	(0.12)	(0.14)	(0.16)	(0.72)	(0.01)	
S.E.		0.47	0.74	0.66	0.81	0.74	0.47	
		(0.81)	(0.05)	(0.06)	(0.07)	(0.33)	(0.01)	

#Initial pH- 8.0
* U/g = µmoles of reducing sugars released / min / g of biomass
** IU =µ moles of reducing sugar released /min/ml of enzyme (Values in parentheses)
***Reducing sugars released mg/g (Value in parentheses depicted sugars mg/ml)
Specific activity = activity/mg protein concentration (Values in parentheses depicted protein mg/g)

Table 7. Partial purification of cellulase by *Bacillus stratosphericus* N_{12} (M) using NaOH +H_2O_2 pretreated *P. deltoids*

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	2.03	2.202	1.084	1.852	0.912	1.929	0.950	5.983	2.94	598.3	1.000	100
$(NH_4)_2SO_4$ precipitated	50	1.50	2.384	1.589	2.249	1.499	2.451	1.634	7.084	4.72	354.2	1.605	73.89

*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 8. Analytical studies for estimation of Holocellulose (cellulose +hemicellulose) and lignin in lignocellulosic (hardwood and softwood) forest waste

Pretreatments	Biomasses																		
	D. strictus			*Eucalyptus* spp.			*P. deltoides*			*C. deodara*			*P. roxburghii*			*P. wallichiana*		
	H.C. (%)	L (%)	E (%)	H.C. (%)	L (%)	E (%)	H.C. (%)	L (%)	E (%)	H.C. (%)	L (%)	E (%)	H.C. (%)	L (%)	E (%)	H.C (%)	L (%)	E (%)
2.5%H_2SO_4	72.8	19.33	1.4	69.6	19.5	3.5	72.5	20.0	1.5	69.0	26.0	0.4	69.2	23.5	0.7	68.8	28.0	0.5
2.5%HCl	72.8	18.50	1.0	68.4	18.5	2.7	71.4	20.0	1.2	69.2	26.0	0.2	70.4	24.0	2.8	68.6	28.5	0.2
NaOH+H_2O_2	68.2	19.50	0.5	69.2	19.0	0.5	74.0	17.5	1.4	68.8	27.0	0.5	72.0	22.5	0.2	68.8	28.0	0.2
5%NH_3	72.0	19.0	0.5	70.0	18.5	1.1	69.2	20.5	0.5	69.2	26.5	0.2	71.4	23.0	1.7	68.6	28.5	1.1
Steam	72.2	19.5	1.7	71.5	20.5	1.6	69.4	21.0	0.5	69.4	28.0	0.6	72.33	24.5	1.4	69.0	29.0	2.1
Untreated	78.0	20.5	1.5	77.6	20.5	1.0	75.8	22.5	0.7	70.4	29.0	0.2	72.0	26.0	1.2	69.2	29.5	1.0
$C.D_{.0.05}$	1.148	0.202	0.096	1.129	0.202	0.003	1.13	0.197	0.100	1.10	0.171	0.17	1.126	0.18	0.24	1.10	1.12	0.144
S.E.	0.527	0.092	0.044	0.518	0.092	0.141	0.52	0.090	0.046	0.50	0.078	0.07	0.517	0.08	0.11	0.50	0.51	0.066

H.C: Holocellulose; L: Lignin; E: Extractives in percent

The highest percent increase of HCl pretreated substrate over untreated was 157.98 in *C. deodara* wood and the lowest percent increase (0.156) was observed in *P. deltoides* wood over untreated substrates. The highest percent increase was noticed in *C. deodara* wood and least percent increase (0.156%) was found in *P. deltoides* wood. Acid pretreatment involves the use of concentrated and diluted acids to break the rigid structure of the lignocellulosic material. The most commonly used acid is dilute sulphuric acid, which has been commercially used to pretreat a variety of lignocellulosic biomass. The action mode of dilute acid is to solubilize hemicellulose and remains lignin and cellulose intact so that the enzymatic digestibility of cellulose enhanced. Marzialetti and coworkers (2008) studied the acid hydrolysis of pine wood using a number of different acids (Tri-fluoro acetic acid, Hydrochloric acid and sulphuric acid). The effect of type of acid, pH, reaction temperature and reaction time on hydrolysis products such as monosaccharides and subsequent degradation products, HMF and furfurals is reported. A perusal of Table 4 revealed that the significant difference in cellulase production was observed when different lignocellulosic substrates and pretreatments were used by *B. stratosphericus* N_{12} (M) under submerged fermentation (SmF). Maximum cellulase titers 41.73U/g and reducing sugars i.e. 7.46 mg/g were estimated in *P. deltoides* wood, followed by *P. wallichiana* wood (22.86 U/g). The minimum cellulase titers 10.09 U/g was observed in *P. roxburghii* wood. Maximum percent increase (193.22) was observed in *C. deodara* wood over untreated, while minimum (30.73) was observed in *D. strictus* wood. Alkaline peroxide is an effective method for pretreatment of biomass. It is basically a delignification process, in which a significant amount of hemicellulose is solubilized as well. In this method, the lignocelluloses are soaked in pH-adjusted water (e.g. to pH 11-12 using NaOH) containing H_2O_2 at room temperatures for a period of time (e.g. 2-3 h).

The process can improve the enzymatic hydrolysis by delignification. The additional advantages of alkaline hydrogen peroxide pretreatments are the use of reagents with low environmental impact (Banerjee *et al*., 2011). In this process the pH is one of the most important parameters for efficient application of peroxide. Depending on pH adopted during lignin oxidation, no significant changes in chemical structure might be observed since the oxidizing agent acts only in the aliphatic part of the macromolecule. It is generally accepted that the hydro peroxide anion formed in alkaline media, is the principal active species in hydrogen peroxide bleaching systems. Cellulase production by utilizing ammonia (NH_3) treated substrate from *B. stratosphericus* N_{12} (M) under SmF. Among all the six forest waste used, maximum cellulase units 34.75 U/g and 6.94 mg/g of reducing sugars had been depicted in *P. deltoides* wood, while the minimum cellulase i.e. 6.110 U/g was noticed in *C. deodara* wood and other substrates showed cellulase production between these two ranges. The maximum percent increase 66.66 was noticed in *Eucalyptus sp.* wood, while minimum was observed in *C. deodara* wood i.e. 6.07 (Table 5). Pretreatment of biomass with aqueous ammonia at elevated temperatures reduces lignin content and removes some hemicellulose while decrystallising cellulose. Ammonia pretreatment techniques include the ammonia fibre explosion-method (AFEX), ammonia recycle percolation (ARP) and soaking in aqueous ammonia (SAA). Soaking in aqueous ammonia (SAA) at low temperature removes efficiently the lignin in the raw material by minimizing the interaction with hemicellulose. As a result an increase of surface area and pore size of biomass is achieved. Thus, retained hemicellulose and cellulose can be hydrolyzed to fermentable sugars by most xylanase and cellulase enzymes mixtures. Table 6 showed significant differences in cellulase production when different substrate and steam pretreatment was used for the study in *B. stratosphericus* N_{12} (M) under SmF. Maximum cellulase titers 33.59 U/g and reducing sugars i.e 6.54 mg/g were produced in *P. deltoides* wood and the least cellulase titers 9.30 U/g were observed in *C. deodara* wood, while other substrates showed cellulase production between these two ranges. Maximum percent increase 84.01 was observed in *P. roxburghii* wood over untreated, while least percent increase found in *Eucalyptus sp.* wood i.e. 5.32.

The steam explosion pretreatment process has been a proven technique for the pretreatment of different biomass feedstocks. It is able to generate complete sugar recovery while utilizing a low capital investment and low environmental impacts concerning the chemicals and conditions being implemented and has a higher potential for optimization and efficiency. Steam pretreatment destruct a part of the xylan in biomass, incompletely solubilization of lignin- carbohydrate matrix and generate inhibitory compounds that may affect the growth of microorganisms used in downstream processes. Therefore, various pretreatment technologies have been devised to increase its hydrolysis rate using hyper cellulase producing *B. stratosphericus* N_{12} (M) with an aim to reduce size, degree of polymerization, to preserve cellulose and hemicellulose fractions, to increase their structural porosity, to limit the formation of inhibitors and to turn it a cost effective process (Hu *et al*., 2008). These screening variables which vary from biomass to biomass play a vital role to assess the effect of pretreatments on individual biomass. The relationship among structural and compositional factors reflects the complexity of lignocellulosic biomass matrix. The variability of these characteristics explains the change of enzymatic digestibility among different biomass (Zheng *et al*., 2009). Thus each pretreatment acts specifically from wood to wood of forest species. It strongly proves that cellulases production from enzymatic hydrolysis of forest biomass is substrate and pretreatment specific.

Partial purification of cellulase by ammonium sulphate precipitation

To strengthen the enzyme activity, the enzymes cellulases were partially purified by ammonium sulphate precipitation. The titers of crude cellulases of *B. stratosphericus* N12 (M) i.e CMCase: 2.202 IU, FPase: 1.852 IU, β- glucosidase was 1.929 IU were strengthened after ammonium sulphate precipitation as shown in Table 7 and reached to : CMCase: 2.384 IU, FPase: 2.249 IU and β- glucosidase: 2.451 IU within an increase of 1.08, 1.21 and 1.27 fold respectively. The final recovery of partially purified cellulase was 1.605 fold with 73.89% yield and final specific activity of 1.63 was observed. As is well known Ammonium sulfate precipitation is a method used to purify proteins by altering their solubility. It is a specific case of a more general technique known as salting out. Ammonium sulfate is commonly used as its solubility is so high that salt solutions with high ionic strength are allowed. The solubility of proteins varies according to the ionic strength of the solution, and hence according to the salt concentration. Two distinct effects are observed in the process: at low salt concentrations, the solubility of the protein increases with increasing salt concentration (i.e. increasing ionic strength), an effect termed salting in. As the salt concentration (ionic strength) is increased further, the solubility of the protein begins to decrease. At sufficiently high ionic strength, the protein will be almost completely precipitated from the solution (salting out). Since proteins differ markedly in their solubilities at high ionic strength, salting-out is a very useful procedure to assist in the purification of a given protein (Bollag and Edelstein, 1993).

Analytical studies for estimation of holocellulose (cellulose + hemicellulose) and lignin by TAPPI method

Lignocellulose is an abundant material created from solar energy and renewable resources on earth which makes them attractive feedstocks for their bioconversion into ethanol.

It is composed of three main fractions like cellulose (~45% of dry weight), hemicellulose (~ 30% of dry weight) and lignin (~ 25% of dry weight). In the present study analytical studies for estimation of holocellulose (cellulose + hemicellulose), lignin and extractives were performed by using TAPPI (Technical Association of Paper and Pulp Industry) method. Table 8 exhibited the percentage of holocellulose (cellulose+ hemicellulose), lignin and extractives in different untreated and pretreated hardwood and softwood biomass. Extractives estimated in the present study vary between 0.2% to 3.5%. Extractives mainly contained alkaloids, proteins, simple and complex phenolics, pectin, gums, resins, terpenes, essential oils etc. The amount of holocellulose was more in hardwoods i.e 78.0% in *D. strictus*, 77.6% in *Eucalyptus* sp. and 75.8% in *P. deltoides* wood in the untreated biomass as compared to softwood biomass. The softwood contains higher content of lignin i.e. 29.5% in *P. wallichiana*, 29.0% in *C. deodara* and 26.00% in *Pinus roxburghii* wood in untreated biomass as compared to hardwoods i.e 20.5% in *D. strictus*, 20.5% in *Eucalyptus sp.* and 22.5% in *P. deltoides* wood. Different physico-chemical pretreatments were given to the hardwood and softwood biomass. Among the all pretreated lignocellulosic biomass with the main aim to extract maximum

lignin out of biomass and simplify the complex structure of cellulose and hemicellulose present in them. Maximum holocellulose content (74.0%) and minimum lignin content i.e. 17.5% were estimated in NaOH + H_2O_2 pretreated *P. deltoides* wood, while 1.4% of extractives were also present. On the other hand minimum holocellulose 68.2 % in NaOH + H_2O_2 pretreated *D. strictus* wood and the maximum lignin i.e. 29.0 % was found in steam pretreated *P. wallichiana* wood.

In lignocellulosic waste products, cellulose and hemicelluloses are closely associated with lignin in the plant cell wall. Cellulose the most abundant polymer on earth is composed of thousands of molecules of anhydroglucose linked by β (1,4) glycosidic bonds. Hemicellulose is a complex carbohydrate structure that consists of different polymers like pentoses, hexoses and sugar acids (Hendriks and Zeeman, 2009). Hemicellulose makes hydrogen bonds to cellulose micro-fibrils, forming a network that provides the structural backbone to the material (Mosier *et al*., 2005). Softwood hemicelluloses have a higher proportion of mannose and glucose units than hardwood hemicelluloses, which usually contain higher amount of xylose units (Palmquist and Hagerdal, 2006). In hardwood the main hemicelluloses is O-acety-4-O-methylglucuronxylan in which xylan chain is linked at irregular intervals with acetic acid and 4-O-methyl glucuronic acid units. Lignin concentration was maximum in case of the native soft and hardwood biomass which had been reduced to a varying range significantly after pretreatments due to delignification of biomass. Though some of the holocellulose had also washed out with lignin. But pretreated holocellulose had become more accessible for enzymatic hydrolysis because of breaking of lignin shield, complex inter and intra bonds and crystalline structure of cellulose.

Conclusion

Overall, it has been observed that lignocellulosic forest waste can act as a suitable substrate for cellulases production, though; different physico-chemical pretreatments are prerequisite to enhance the yield of enzyme. Forest wastes are believed to be as one of the abundant resource of sugars although limited work has been reported on them because of their complex structure for biodegradation. Thus each pretreatment acts specifically for agricultural wastes and wood to wood of forest species. It strongly proves that cellulase production from enzymatic hydrolysis of forest biomass is substrate and pretreatment specific. In principle, an effective pretreatment method is expected to disrupt multiple barriers so that cellulose and hemicellulose can be hydrolysed easily and more of extracellular cellulase enzymes can be secreted.

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