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Evaluation of different forest wastes of Northern Himalayas as a potential substrate for cellulase production by *Aspergillus niger* F7 and its partial purification

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Abstract

Biodegradation of lignocellulosic forest waste by *Aspergillus niger* F7 under solid state fermentation was explored. Different pretreatments were given to render forest wastes readily accessible to the enzymatic attack. SSF of pretreated forest biomass was found to be superior over untreated forest biomass. Pretreated forest biomass has emerged as a suitable substrate for cellulase production by *A. niger* F7 reaching upto 146.14 U/g in acid+ steam pretreated *P. roxburghii* needles. It has also been noticed that though forest lignocellulosics when used as carbon source yielded fairly good amount of cellulase. The ultimate goal of the study is the efficient utilization of wastes so as to recover cellulase in concentrations that make purification feasible. The data gathered in this study provides us a glimpse of some of the dynamics of the production of cellulase.

Keywords: Biodegradation, Cellulase, lignocellulosic forest biomass, pretreatment, *Aspergillus niger*

1. Introduction

Northern Himalayas are rich in plant diversity. Rich plant diversity is a main feature of Himachal Pradesh, a wholly mountaneous region in lap of northern Himalayas ranges from 350m to 6975m above sea level. Himachal Pradesh is situated between 300 22' 40" to 330 12' 20" north latitudes and 750 45' 55" to 790 04' 20" east longitudes. About 67% of total geographical area is covered by forests. The forests classified on ecological basis generally have coniferous as well as broad leaved trees. Coniferous trees keep on shedding needles which accumulate on earth in abundance. This generated waste generally leads to infertility of soil as well as hazardous forest fires. Because a large part of vegetation added to the soil is carbohydrates, their decomposition has a special significance in the biological cycle of carbon.

Since a prominent carbonaceous constituent of forest plants is cellulose which is the most abundant organic compound on earth. Degradation of forest waste by cellulase and subsequent fermentation of saccharified biomass to ethanol can become not only an ecofriendly solution but also a boon in an era of dwindling fossil fuels and their skyrocketing prices [1]. Besides cellulases are used in many other industries viz. in the textile industry for biobleaching of cotton fabrics and treatment of denim garments to achieve a stone wash effect, in the paper industry for de-inking , recycling of paper, in food industry for extraction of juice, in separation and isolation of starch and gluten from wheat flour and in animal food production. Cellulases are the groups of hydrolytic enzymes capable of hydrolyzing cellulose to smaller sugar components like glucose units [2].

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Cellulase enzyme system is composed of carboxymethylcellulase (CMCase) also called endoglucanase, exoglucanase and β-glucosidase. The major bottle neck of comprehensive application of cellulases in industry is its higher cost of production. Among different production methods, solid state fermentation (SSF) of lignocellulosic material holds several advantages over submerged fermentation (SmF) [3]. It has been reported that solid state fermentation (SSF) is an attractive process to produce cellulase economically due to its lower capital investment, lower operating expenses, simpler equipment and higher productivity per reactor volume [4]. The cost of cellulase production can further be reduced by using renewable lignocellulosic forest wastes which are considered as an excellent carbon source for microbial enzymes production by employing potential microbial isolates. Basically the lignocellulosic biomass comprises of cellulose, hemicellulose and lignin [5].

In the present study, hypercellulolytic fungal strain i.e., *A. niger* F7 isolated from forest soil has been explored for cellulase production by using inexpensive forest biomass with an aim to get higher yield of cellulase.

2. Materials and methods

Aspergillus niger F7 employed for cellulase potential had been isolated from forest soil of Himalayas at an altitude of 2205 meters in northern parts of India. Potato dextrose agar was used for isolation and maintenance of culture. Identification was done on the basis of cultural characteristics, texture as well as microscopic structure, septate or nonseptate hyphae, structure of hyphae and conidias.

Substrate: Forest waste i.e., softwood, hardwood and needles of following predominant local forest trees was viz. *Quercus leucotricophora, Dandracalamus strictus, Cedrus deodara, Eucalyptus sp., Acacia catechu, Populus deltoides, Pinus roxburghii, Bombax ceiba, Dalbergia sissoo, Toona ciliata,* and mixed biomass (in the ratio of 1:1 of all above mentioned species) was collected.

Physico-chemical Pretreatment of substrates Modified Microwave Irradiation pretreatment 200 g of each biomass was taken in a beaker and was irradiated in Microwave (Godrej) for 3 min at 250 V, 50 hz and kept in air tight containers for further use.

Modified Alkali (2.5 % NaOH) pretreatment. 200 g of each selected biomass was dipped in NaOH solution (2.5 %). After 4 h, it was thoroughly washed with tap water until it becomes neutral. After thorough washing it was dried at 60^0C and stored in air tight containers.

Modified Alkali (2.0 % NaOH) + steam pretreatment. 200 g of each biomass was soaked in NaOH (2 %). After 2 h, it was thoroughly washed with tap water until became neutral followed by steam pretreatment at 121 ^{0}C at 15 psi for 1 h in an autoclave. After drying at 60^0C overnight, it was stored in air tight containers.

Acid (2.5 % H_2SO_4) pretreatment. 200 g of each selected biomass was dipped in H_2SO_4 (2.5 %) solution. After 4 h, it was thoroughly washed with tap water and dried at 60^0C and stored in air tight containers [6].

Modified Acid (2.0 % H_2SO_4) + steam pretreatment. 200 g of each biomass was soaked in H_2SO_4 (2 %). After 2 h, it was thoroughly washed with tap water followed by steam pretreatment at 121 ^{0}C at 15 psi for 1 h in an autoclave. After drying at 60^0C overnight, it was stored in air tight containers.

Cellulase production. Submerged fermentation (SmF): Inoculum Production : Inoculum @ 10 % containing 1×10^7 spores/ml was added to 45 ml of modified BSM (0.6g Na_2HPO_4, 0.3g KH_2PO_4, 0.05g NaCl, 0.1g NH_4Cl, 0.2ml 1M $MgSO_4$, 0.1ml 1M $CaCl_2$, 1% urea, 1% yeast extract, 0.01% peptone, 0.02ml 1M $CoCl_2$, 0.02ml $NaNO_3$, 1g cellulose, 100 ml of distilled water, pH-4.8) in 250 ml of Erlenmeyer flask. The flasks were incubated at 25^{0}C at 120 rpm. After 7 days, the culture contents of flasks were centrifuged at 10,000 rpm for 15 min (4°C). Supernatant was collected and stored at 4°C in refrigerator for further use. The following quantitative tests were performed with crude enzyme.

Enzyme assays. The sub-enzymes of cellulase were measured by following standard assays. CMCase activity was determined by incubating 0.5 ml of culture supernatant with 4.5 ml of 1.1 % CMC in citrate buffer (0.05M, pH 5.0) at 50^{0}C or 1 h. After incubation, 1 ml was drawn 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 [7]. The reaction containing 0.5 ml of culture supernatant, 50 mg strips of filter paper (Whatmann no. 1) and 1 ml of citrate buffer (0.05 M, pH 5.0) was incubated at 50^0C for 1 h. After incubation, 1 ml was drawn 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 & 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 40^0C for 10 min. After incubation, 2 ml of 1 M Na_2CO_3 was added and the mixture was heated in boiling water bath for 15 min and OD was read at 400 nm [8].

Estimation of reducing sugars and proteins. The total reducing sugars were determined by method described by Miller [9]. and protein was determined by Lowry's method [10].

Solid state fermentation (SSF). Production: To 5 g of each untreated and pretreated biomass, 10 ml of moistening agent i.e., modified BSM was added in 250 ml Erlenmeyer flask. After autoclaving 2 ml of inoculum (1×10^7 spores /ml) was added in each flask and these were incubated at 28 ± 2 ^{0}C for 10 days in static phase and control was run without inoculum.

Extraction of Cellulase. Cellulase was extracted by repeated extraction method [11]. To 5 g of each untreated and pretreated biomass, 50 ml of Phosphate buffer (0.1 M, pH 6.9) with 0.1 % tween 20 was added in 250 ml of Erlenmeyer flask. The contents were kept in a shaker at 120 rpm for 1 h and afterwards were filtered through muslin cloth. The process was repeated twice with 25 ml of phosphate buffer each time making final volume of extracted product to 100 ml. After filtration, contents were centrifuged at 5,000 rpm for 10 min at 4oC and clear supernatant from each of the biomass was collected for further studies. Enzyme assays with crude enzyme were performed as mentioned earlier.

Partial purification of cellulose. Ammonium sulphate precipitation. The supernatant collected after centrifugation in SSF was precipitated by ammonium sulfate with an increment of 10 % till saturation attained. CMCase, FPase and β-glucosidase were precipitated at 40 to 80 %, 50 to 80 % and 50 to 70 % respectively.

Precipitates so obtained were dissolved in phosphate buffer (0.1 M, pH 6.9) and dialyzed against same buffer at 5 ^{0}C overnight. The sephadex G-100 was weighed (5g) and suspended in 500 ml of phosphate buffer (0.1 M, pH 6.9) overnight. It was swollen for 5 h in boiling water bath, and brought to room temperature before packing the column. The size of the column used had dimensions of 38.5 X 1.25 cm. The packing was done with a precaution to avoid entrapment of any air bubbles in the gel bed. The column was eluted with phosphate buffer (0.1 M, pH 6.9). 1.5 ml of dialyzed sample i.e. CMCase, FPase and β-glucosidase were loaded separately on to the sephadex G-100 packed column and 3 ml of 50 fractions were collected. In each of 50 collecting vials with a flow rate maintained at 3ml/7min.

The fractions were analysed for protein contents by taking O.D. at 280 nm. Enzyme activity and protein contents were estimated by standard colorimetric methods. Based on highest O.D. the fractions were pooled for CMCase, FPase and β-glucosidase.

SDS –PAGE (Sodium Dodecyl sulphate Polyacrylamide Gel Electrophoresis). For Minigel, bottom of both gel plates and spacer were perfectly flushed against a flat surface before tightening clamp assembly. Solutions A (Acrylamide stock solution: 30 % w/v acrylamide, 0.8% w/v bisacrylamide 100 ml), B (4X separating gel buffer : 75 ml 2M Tris HCl pH 8.8 , 4ml 10 % SDS, 21 ml distilled water) and water were combined in a small Erlenmeyer flask. Ammonium persulfate and TEMED were added, mixed and the solution was introduced into the gel sandwich. 1 cm of distilled water was layered on top of separating gel solution.

Gel was allowed to polymerize (30 min). When the gel had polymerized, a distinct interface appeared between the separating gel and the water. Water covering the separating gel was poured -off. Solution A, C (50 ml 1 M Tris HCl, 4 ml 10% SDS , 46 ml distilled water) and

water were combined in a test tube along with ammonium persulfate and TEMED and were mixed. Stacking gel solution was pipetted onto separating gel until solution reached top of front plate. Comb was inserted into gel sandwich.

Staking gel was allowed to get polymerize (about 30 min). After staking gel got polymerized, the comb was removed. The gel was placed into electrophoresis chamber. Electrophoresis buffer was added to inner and outer reservoir making sure that both top and bottom of gel were immersed in buffer. Combined protein sample and 5x sample buffer (20 µl + 5 µl) in an eppendorf tube was heated at 100°C for 2 min. Then the protein solution was spinned for 5 min in a microfuge. Sample was introduced into the well. Molecular weight marker of molecular weight 14.3-94.7 kDa was loaded in one of the well. Power supply was turned on to 200 V. The dye front was allowed to migrate to 1 cm from the bottom of the gel in 2 h for two 0.75 mm gels and power supply was turned off**.** Gel was picked up and transferred to a small container containing coomassie staining solution (1.0 g coomassie blue R-250, 450 ml methanol, 450 ml water, 100 ml glacial acetic acid).Agitated for 15 min on slow rotator shaker. The stain was poured out. 50 ml of Destain (100 ml methanol, 100 ml glacial acetic acid, 800 ml water) was added and agitated overnight.

Statistical analysis. The data recorded for different parameters were subjected to Completely Randomized Design [12]. The statistical analysis based on mean value of replications used for each treatment was made using analysis of variance (ANOVA) for Completely Randomized Design. Total cellulase production by *A .niger* F7 in hardwood and softwood substrates under SSF were predicted on the basis of CMCase, FPase and β-glucosidase by following regression models:

(i) Linear : Y=bo+b1X ;
(ii) Quadratic : Y=bo+b1X+b2X2
(iii) Logarithmic: Y=bo+ b1logX;
(iv) Compound: Y=bob1X
(v) Power: Y=boXb1;
(vi) Exponential: Y=boexpbX

3. Results and Discussion

Hypercellulolytic microbial isolate *A. niger* F7 already isolated from local forest soil had been selected for degradation of pretreated forest waste with an apparent aim to increase cellulase production alongwith an ecofriendly approach.The morphological, physiological and biochemical properties of the hypercellulolytic microbial isolate *A. niger* F7 were studied. *A.niger* F7 was showing different mycelia chacteristics and spore morphology.

Plate1.shows *A.niger* F7, long hyphae were present, septate spores bearing heads were large, globular and black [13]. Gelatin hydrolysis, MR test and fermentation of glucose were found positive [14].

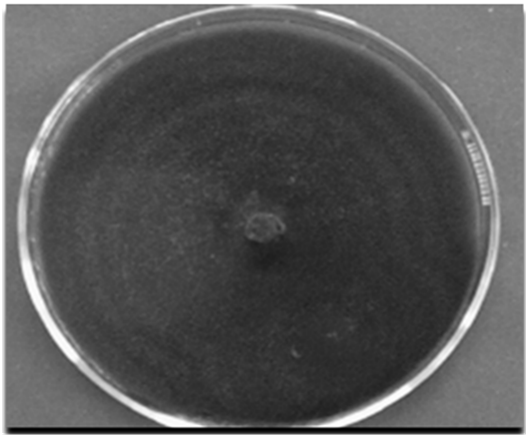

Plate 1. *Aspergillus niger* F7

The Production of enzyme was compared under Submerged and solid state fermentation, In SmF, maximum cellulase (4.997) i.e., 1.489 IU CMCase, 0.033 IU FPase and 3.479 IU β – glucosidase was measured by *A. niger* F7 when cellulose was used as sole carbon source. This fungal isolate though showed a good potential to secrete a complete set of cellulase but due to its lesser activity restricted its use for major applications. Thus an idea was conceived to enhance enzyme production by switching over to solid state mode of fermentation which generally yields higher enzyme titres with fungal inoculum. Since the main impediment to exploit the commercial potential of cellulases is cost of cellulase production and one effective approach to reduce its cost can be replacement of pure cellulose with relatively cheaper cellulosic substrates.

Therefore inexpensive lignocellulosic forest residue was selected as a substrate for cellulase production from *A. niger* F7 under SSF. SSF has additional advantages over SmF which include conditions that are similar to those of the natural habitat of the micro-organisms, lower cost, enhanced enzyme production and improved enzyme stability [15].

Since lignocellulosic forest waste is complex in nature, different pretreatments were given to the biomass to enhance its susceptibility for degradation by *A. niger* F7. Forest residue used in the present study included sawdust/ needles of different hardwood and softwood species depending upon their easy availability local abundance and least utilization. In untreated lignocellulosic materials production of cellulase ranged from 14.172 U/g in *P. deltoids* to 65.784 U/g in *P. roxburghii* needles.

Though lignocellulosic forest waste being rich in cellulose (about 60%) can serve as an attractive and inexpensive source for cellulase production but complex nature of native biomass and inaccessibility of cellulose present in it for enzymatic hydrolysis generally yields lesser enzyme by cellulolytic microorganisms. Thus, pretreatment becomes a prerequisite to increase the accessibility of cellulose in lignocellulosic forest wastes to facilitate enzyme production.

The different pretreatments viz. alkali, acid, steam and microwave irradiation given to forest biomass in the present study have resulted in a significant increase in cellulase . Table 1 and 2 revealed the data on cellulase production utilizing different pretreatments irradiated biomasses from *A. niger* F7. In case of microwave pretreatment, maximum enzyme was obtained from microwave irradiated *Eucalyptus* wood. While minimum of enzyme was yielded by *C. deodara.*

Microwave irradiation pretreatment of lignocellulosic biomass is the latest method which recently has been introduced for enzyme and biofuel production studies. Microwave technology is supposed to simplify three structural polymers along with extraneous components thus increasing enzyme yield. When the crystalline region of cellulose is exposed between electromagnetic field it gets polarized generating a charge on crystalline interphase [16].

This physical pretreatment increases the amorphous region of cellulose and thus has a dramatic effect on its decomposition because of cellulose softening [17] developed a microwave assisted low temperature decomposition process for production of high quality fuels from biomass as amorphous region of cellulose softens allowing a microwave induced arrangement.

The results in terms of cellulase production from alkali pretreated forest wastes by *A. niger* F7 revealed that *Eucalyptus* wood which was subjected to alkali pretreatment gave maximum cellulase production of 98.64 U/g (34.71 CMCase, 5.48 FPase and 58.45 β-glucosidase) while very less activity was detected in *C. deodara* giving only 32.82 U/g (12.22 CMCase, 1.12 FPase and 19.48 β-glucosidase). Effect of alkali + steam treatment on substrates is measured in terms of enzyme units released by *A. niger* F7. *P. roxburghii* and *Eucalyptus* wood produced higher amount of cellulase (146.14 U/g and 126.93 U/g respectively). Lowest yield (17.18U/g) of cellulase was obtained in case of alkali+steam pretreated *B. ceiba.* Alkaline pretreatment of lignocellulosic materials causes swelling, leading to decreased crystallinity while steam causes a fast decompression which causes the explosion of the cellular tissue and separation of components to a certain extent, enabling further hydrolysis [18]. The results revealed in case of all acid pretreated biomass, *P. roxburghii* needles supported highest cellulase yield of 104.94 U/g (33.74 CMCase, 4.40 FPase and 66.80 β-glucosidase) while *C. deodara* showed minimum value i.e. 24.70 U/g (7.17 CMCase, 0.83 FPase and 16.70 β-glucosidase).

Addition of an acid catalyst further increases cellulose digestibility and decrease the production of degradation compounds [19,20].In case of steam pretreatment along with acid pretreatment resulted in 147.84 U/g (32.27 CMCase, 2.84 FPase and 112.73 β-glucosidase) of cellulase in *P. roxburghii* needles.

Comparatively, *A.niger* F7 was not much effective in solubilising cellulose in other materials viz. *Q. leucotricophora* (43.68U/g), *D. stricus* (48.02 U/g) and *P. deltoides* (51.20U/g).

Steam explosion using an acid catalyst has been under extensive investigation in recent years and recognized as one of the effective pretreatment technologies for crystalline structure of lignocelluloses by their chemical effects and mechanical forces attributed from sudden explosive decompression [21, 22]. Addition of an acid catalyst further increases cellulose digestibility and decreases the production of degradation compounds [23]. As all pretreatments given to biomass have shown increase in cellulase titers over untreated biomass ranging from 17.18 U/g to 147.84U/g. The different carbon sources used in the present study though induced cellulase synthesis, but its amount produced in each biomass is highly variable which may be because of the influence of specific substrate on the growth of microorganism. As far pretreatments are concerned acid + steam pretreatment has emerged as the most effective method in digestion of lignocellulosic biomass. Statistically cellulase production in untreated as well as in variously pretreated biomass was significantly different from each other @ 5% level of significance.

Since acid + steam pretreated *P. roxburghii* needles have exhibited maximum cellulase production. Therefore, among all, it can be recommended as a best substrate for cellulase production by *A. niger* F7.

Cellulase produced by *A. niger* F7 on pine needles was purified by adding $(NH_4)_2SO_4$ at saturation level 80% for CMCase and FPase and 70% for β-glucosidase. Precipitates so obtained for each fraction were centrifuged at 10,000 rpm for 20 min at 4^0C and dissolved in 10 ml of phosphate buffer, pH 7.0 and their activity was measured.

After salt precipitation, gel exclusion chromatography was performed to attain next step in purification. A total of 32 fractions of 3ml were collected as shown from the elution profile of partially purified CMCase, FPase and β-glucosidase (fig 2a, 2b and 2c). Fractions showing highest enzyme activity were pooled together i.e., 5-12 for CMCase (0.237, 0.651, 0.637, 0.422, 0.415, 0.237, 0.133, 0.119 IU), 5-13 for FPase (0.027, 0.027, 0.027, 0 .027, 0.031, 0.051, 0.061, 0.027, 0.024 IU) and 4-9 for β-glucosidase (5.82, 5.76, 5.12, 4.81, 4.49, 4.36 IU). Ammonium salt precipitated CMCase, FPase and β-glucosidase were subjected to SDS-PAGE using 10 % stacking and resolving gel.

After gel exclusion chromatography total enzyme activity was increased from 1.470 IU to 9.198 IU in case of CMCase, 0.129 IU to 0.642 IU in FPase and 5.124 IU to 11.025 IU in case of β-glucosidase as given in Table 3a , 3b, 3c. While table 3d exhibited total cellulase activity reaching upto 20.865 IU of partially purified enzyme, thus turning it as a robust enzyme for industrial application.

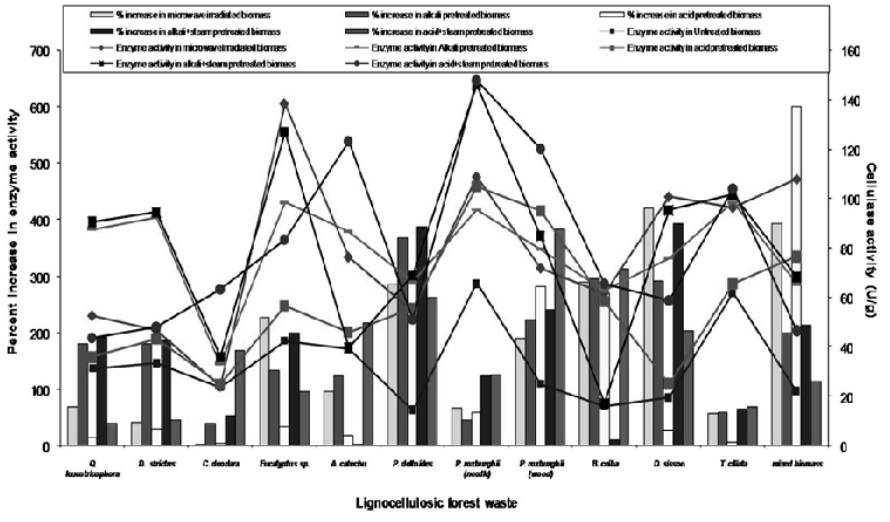

Figure 1. Overview of percent increase in enzyme activity of untreated lignocellulosic forest waste with preteated lignocellulosic forest waste by *A. niger* F7

Table 1. Optimization of extracellular cellulase production by *A. niger* F7 using hardwood biomass as substrate under solid state fermentation (SSF)

Biomass (T)		Untreated Biomass		Pre-treated biomass (I)										Mean (I)	
				Microwave		Alkali		Alkali + Steam		Acid		Acid + Steam			
		Enzyme units (U/g)	Total Cellulase (U/g)	Enzyme units (U/g)	Total Cellulase (U/g)	Enzyme units (U/g)	Total Cellulase (U/g)	Enzyme units (U/g)	Total Cellulase (U/g)	Enzyme units (U/g)	Total Cellulase (U/g)	Enzyme units (U/g)	Total Cellulase (U/g)	Enzyme units (U/g)	Total Cellulase (U/g)
1	*Quercus leucotricophora*	2.04* 0.72** 28.44***	31.20****	13.32 2.72 36.48	52.52	26.40 4.15 57.06	87.61	27.40 5.15 58.06	90.61	3.74 1.32 30.62	35.68	6.74 3.32 33.62	43.68	13.27 2.89 40.71	56.88
2	*Dandracalamus strictus*	11.73 0.85 20.52	33.10	18.84 1.44 26.28	46.56	30.31 3.76 58.45	92.52	31.31 3.76 59.45	94.52	4.56 0.88 37.58	43.02	5.56 2.88 39.58	48.02	17.05 2.26 40.31	59.62
3	*Eucalyptus sp.*	9.33 0.76 32.28	42.38	23.82 2.48 112.20	138.50	34.71 5.48 58.45	98.64	36.17 3.08 87.68	126.93	13.53 1.27 41.75	56.55	15.31 1.22 66.80	83.33	22.14 2.38 66.53	91.05
4	*Acacia catechu*	9.60 0.93 28.44	38.97	30.96 2.16 43.20	76.32	32.27 4.40 50.10	86.77	10.92 0.88 27.83	39.63	8.80 1.17 35.84	45.81	26.40 0.92 96.03	123.35	19.82 17.43 46.91	68.47
5	*Populus deltoids*	1.41 0.61 12.14	14.17	21.42 1.09 32.04	54.55	18.58 4.35 43.41	66.34	19.58 5.35 44.14	69.07	6.02 0.92 48.62	55.57	1.96 0.53 48.71	51.20	11.49 2.14 38.18	51.82
6	*Bombax ceiba*	2.76 1.68 11.38	15.82	14.40 1.92 45.36	61.68	18.25 4.05 40.36	62.66	3.58 1.07 12.52	17.18	13.85 1.47 43.14	58.46	16.85 3.47 45.14	65.46	11.61 2.27 32.98	46.88
7	*Dalbergia sissoo*	3.20 0.96 15.18	19.34	15.12 1.17 84.48	100.77	21.18 4.59 50.10	75.88	22.81 3.08 69.50	95.39	6.19 1.91 16.70	24.80	11.24 1.52 45.93	58.69	13.29 2.20 47.15	62.48
8	*Toona ciliata*	13.24 1.06 47.40	61.78	27.72 5.04 63.60	96.36	32.27 3.71 62.63	98.61	33.27 4.71 63.63	101.61	12.55 1.32 51.49	65.36	32.27 2.29 69.59	104.15	25.28 3.02 59.72	87.98
	Mean (T)	6.64 0.946 24.47	32.09	20.70 2.25 55.58	78.41	26.79 4.31 52.57	83.63	23.13 3.38 52.85	79.37	8.65 1.28 38.22	48.16	14.54 2.01 55.67	72.23		

For CMCase (T) : CD $_{0.05}$-0.0587 ,SE-0.0295 , (I):CD $_{0.05}$- 0.0508 , SE-0.0256 ,(T×I) : CD $_{0.05}$ 0.1016 - ,SE-0.0724
For FPase (T) : CD $_{0.05}$-0.014 , SE -0.0075 , (I) : CD $_{0.05}$ – 0.012 , SE – 0.006 , (T×I) : CD $_{0.05}$ – 0.036 , SE – 0.013
For β-glucosidase (T) : CD $_{0.05}$ – 0.165 , SE – 0.083 , (I) : CD $_{0.05}$ – 0.143 , SE – 0.072 , (T×I): CD $_{0.05}$ – 0.402 , SE – 0.144
For total cellulase (T) :CD $_{0.05}$ – 0.017 , SE – 0.008 , (I) : CD $_{0.05}$ – 0.015 , SE – 0.007 , (T×I) : CD $_{0.05}$ – 0.043 , SE – 0.021
* : CMCase activity , ** : FPase activity , *** β-glucosidase activity , **** : U/g= μ moles of reducing sugars released/min /g of biomass.

Table 2. Optimization of extracellular cellulase production by *A. niger* F7 using softwood biomass as substrate under solid state fermentation (SSF)

Biomass (T)		Untreated Biomass		Pretreated biomass (I)										Mean (I)	
				Microwave		Alkali		Alkali + Steam		Acid		Acid + Steam			
		Enzyme units (U/g)	Total Cellulase (U/g)	Enzyme units (U/g)	Total Cellulase (U/g)	Enzyme units (U/g)	Total Cellulase (U/g)	Enzyme units (U/g)	Total Cellulase (U/g)	Enzyme units (U/g)	Total Cellulase (U/g)	Enzyme units (U/g)	Total Cellulase (U/g)	Enzyme Units (U/g)	Total Cellulase (U/g)
1	*Cedrus deodara*	8.26* 0.76** 14.52***	23.55****	3.64 0.56 19.68	23.89	12.22 1.12 19.48	32.82	13.22 2.12 20.48	35.82	7.17 0.83 16.70	24.70	8.31 0.58 54.28	63.17	7.55 0.99 24.19	33.99
2	*Pinus roxburghii* (needles)	22.66 3.27 39.84	65.78	20.04 2.40 86.40	108.84	30.80 3.62 61.23	95.65	44.49 2.84 98.81	146.14	33.74 4.40 66.80	104.94	32.27 2.84 112.73	147.84	30.67 3.22 77.63	111.50
3	*Pinus roxburghii* (wood)	8.35 0.60 15.96	24.91	25.20 4.68 42.00	71.88	29.33 2.93 47.32	79.58	23.79 1.32 59.84	84.95	22.96 2.59 69.59	95.15	25.75 1.27 93.24	120.26	22.57 2.29 54.49	79.45
4	Mixed biomas	0.31 0.32 21.24	21.87	23.88 2.40 81.60	107.88	22.16 1.42 41.75	65.33	23.16 2.42 42.75	68.33$_5$	9.12 1.95 65.41	76.49	10.84 1.48 34.20	46.53	14.91 1.66 47.82	64.40
	Mean (T)	9.89 1.23 22.89	34.03	18.20 2.51 57.42	78.12	23.63 2.27 42.44	68.34	26.16 2.17 55.47	83.81	18.25 2.53 54.37	75.32	17.42 1.54 73.61	94.45		

For CMCase (T) : CD$_{0.05}$ – 0.009,SE – 0.004 , (I):CD $_{0.05}$- 0.011, SE- 0.005 ,(T×I) : CD$_{0.05}$- 0.022 ,SE- 0.008
For FPase (T) : CD$_{0.05}$- 0.006 , SE -0.003 , (I) : CD$_{0.05}$ – 0.008 , SE – 0.004 , (T×I) : CD$_{0.05}$ – 0.0164 , SE – 0.0057
For β-glucosidase (T) : CD $_{0.05}$ – 0.237 , SE – 0.117, (I) : CD $_{0.05}$ – 0.290 , SE – 0.144 , (T×I): CD $_{0.05}$ – 0.580 , SE – 0.204
For total cellulase (T) :CD $_{0.05}$ – 0.006 , SE – 0.003 , (I) : CD $_{0.05}$ – 0.008 , SE – 0.004 , (T×I) : CD $_{0.05}$ – 0.016 , SE – 0.005
* : CMCase activity, ** : FPase activity, *** : β-glucosidase activity, ****: U/g= μ moles of reducing sugars released/min/g of biomass

Table 3. Purification and recovery of CMCase, FPase and β-glucosidase by *A. niger* F7

Table 3a. Purification stage of CMCase

Steps	Volume	Enzyme activity	Protein (mg/ml)	Specific activity	Purification fold	Recovery %
Crude	30	1.470	0.660	2.227	1	100
$(NH4)_2SO_4$ precipitated	1.5	4.748	0.547	8.680	3.89	82.87
Partial Purified	25	9.198	0.287	32.048	14.39	43.48

Table 3b. Purification stages of FPase

Steps	Volume	Enzyme activity	Protein (mg/ml)	Specific activity	Purification fold	Recovery %
Crude	30	0.129	0.660	0.195	1	100
$(NH4)_2SO_4$ precipitated	1.5	0.220	0.446	0.493	1.56	67.57
Partial Purified	25	0.642	0.197	0.306	2.52	29.84

Table 3c: Purification stages of β-glucosidase

Steps	Volume	Enzyme activity	Protein (mg/ml)	Specific activity	Purification fold	Recovery %
Crude	30	5.124	0.660	7.763	1	100
$(NH4)_2SO_4$ precipitated	1.5	6.635	0.483	13.737	1.76	73.18
Partial Purified	25	11.025	0.508	21.702	2.79	76.96

Table 3d. Purification stages of total cellulase (CMCase + FPase + β-glucosidase)

Steps	Volume	Enzyme activity	Protein (mg/ml)	Specific activity	Purification fold	Recovery %
Crude	30	6.723	1.980	3.395	1	100
$(NH4)_2SO_4$ precipitated	1.5	11.603	1.476	7.861	2.31	74.54
Partial Purified	25	20.865	0.992	21.033	6.19	50.10

Table 4. Parameters of various models to predict total cellulase production

Table 4a. Prediction model for total cellulase on the basis of CMCase, FPase and β-glucosidase in hardwood substrates

Parameters	Prediction equations																			
	$Y=b_o+b_1X$		R^2	$Y=b_o+b_1X+b_2X^2$			R^2	$Y=b_o+b_1\log X$		R^2	$Y=b_o b_1^X$		R^2	$Y=b_o X^{b_1}$		R^2	$Y=b_o \exp b_1 X$		R^2
	b_o	b_1		b_o	b_1	b_2		b_o	b_1		b_o	b_1		b_o	b_1		b_o	b_1	
1.	24.35	2.46	0.72	18.74	3.39	-.026	0.73	-7.051	28.66	0.66	28.12	1.04	0.68	14.55	0.54	0.73	28.12	0.04	0.68
2.	41.04	10.39	0.27	25.17	26.82	-2.91	0.30	50.05	24.32	0.30	36.68	1.21	0.28	43.34	0.45	0.31	36.68	0.19	0.28
3.	2.84	1.34	0.91	-8.68	1.86	-.005	0.92	-135.14	53.94	0.85	19.91	1.02	0.81	1.30	1.02	0.94	19.91	0.02	0.81

Where X=enzyme unit(CMCase/FPase/β-glucosidase), Y=Total cellulase ,R^2=coefficient of determination

Table 4 b. Prediction model for total cellulase on the basis of CMCase, FPase and β- glucosidase in softwood substrates

Parameters	Prediction equations																			
	$Y=b_o+b_1X$		R^2	$Y=b_o+b_1X+b_2X^2$			R^2	$Y=b_o+b_1\log X$		R^2	$Y=b_o b_1^X$		R^2	$Y=b_o X^{b_1}$		R^2	$Y=b_o \exp b_1 X$		R^2
	b_o	b_1		b_o	b_1	b_2		b_o	b_1		b_o	b_1		b_o	b_1		b_o	b_1	
1.	19.26	2.80	0.69	36.18	-1.38	0.21	0.72	19.83	20.41	0.39	26.41	1.06	0.67	25.10	0.34	0.43	26.41	0.04	0.67
2.	34.82	18.34	0.34	6.52	46.30	-1.90	0.40	54.99	34.90	0.44	31.21	1.39	0.43	44.79	0.63	0.55	31.21	0.33	0.43
3.	6.14	1.29	0.95	-12.53	2.53	-.021	0.95	-143.42	57.45	0.91	22.21	1.02	0.86	1.77	0.94	0.95	22.21	0.02	0.86

Where X=enzyme unit(CMCase/FPase/β-glucosidase),Y=Total cellulase,R^2=coefficient of determination

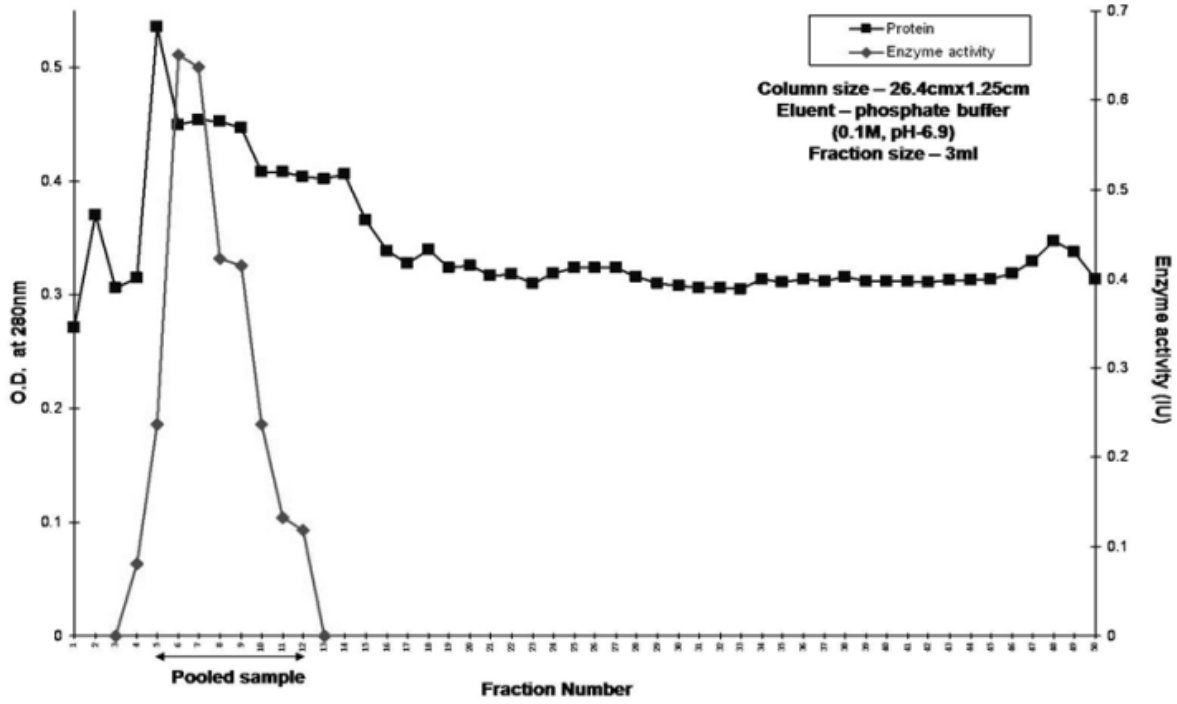

Figure 2(a). Is showing elution profile of partially purified CMCase of a *A. niger* F7 on sefadex-100 column

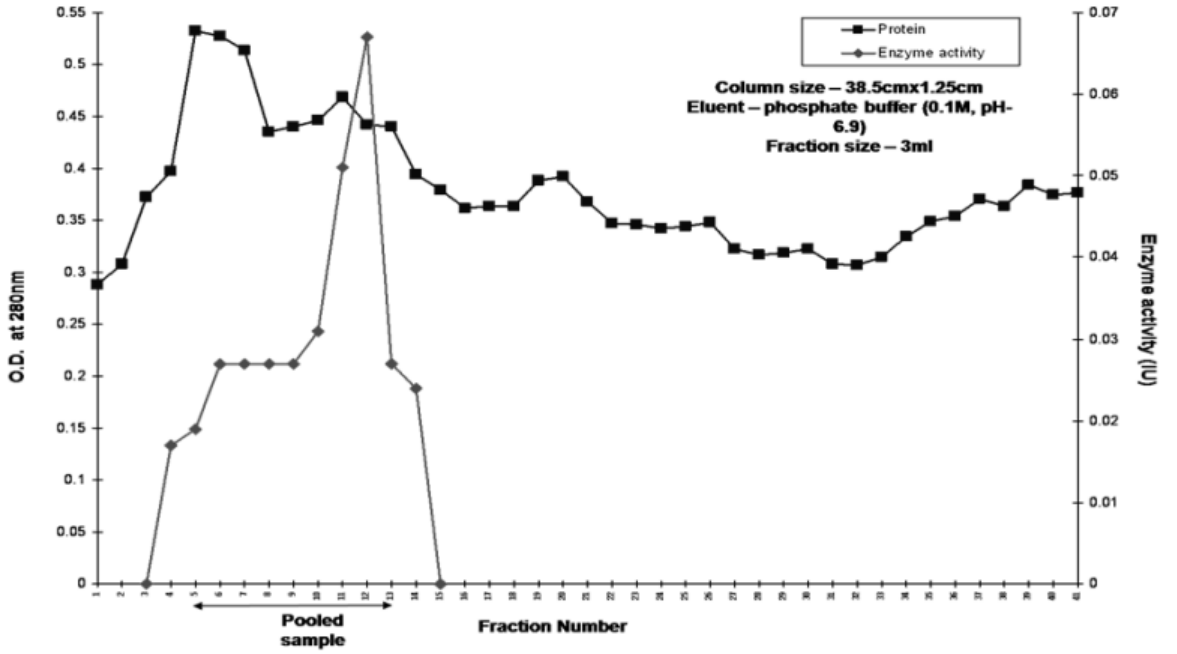

Figure 2(b). Is showing elution profile of partially purified FPase of a *A. niger* F7 on sefadex-100 column

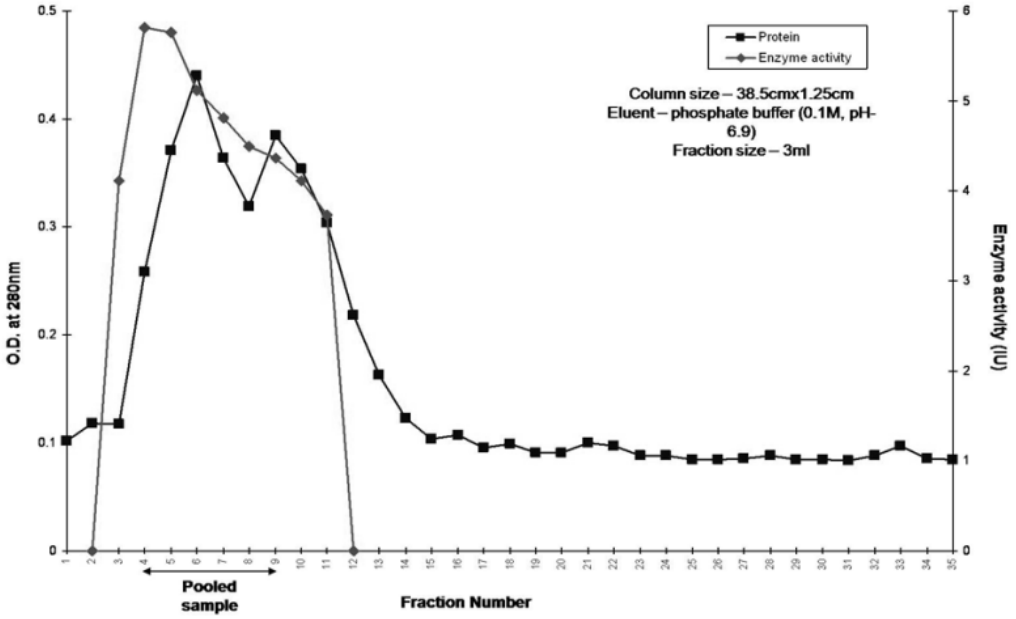

Figure 2(c). Is showing elution profile of partially purified β-glucosidase of a *A. niger* F7 on sefadex-100 column

In fig 3 a and b, for *A. niger* F7, two bands showing CMCase activity were detected on the gel corresponding to molecular mass of 20.0 kDa and 29.0 kDa and for FPase two bands were detected having molecular mass of 31.0 kDa and 17.0 kDa.

These bands were different subunits of same enzyme on electrophoresis gel. The apparent molecular mass of partially purified β-glucosidase corresponded to 23 kDa

Parameters of different regression models were fitted to predict total cellulase in hardwood and softwood substrates separately on the basis of CMCase, FPase and β-glucosidase. Values of parameters of different models viz. linear, quadratic, logarithmic, compound, power and exponential are predicted in table 4 a and b. High value of coefficient of determination (R^2) was found in quadratic and power models in case of hardwood as well as softwood substrates. Thus the total cellulase can be predicted by using the following models for CMCase, FPase and β-glucosidase in softwood as well as in hardwood substrates.

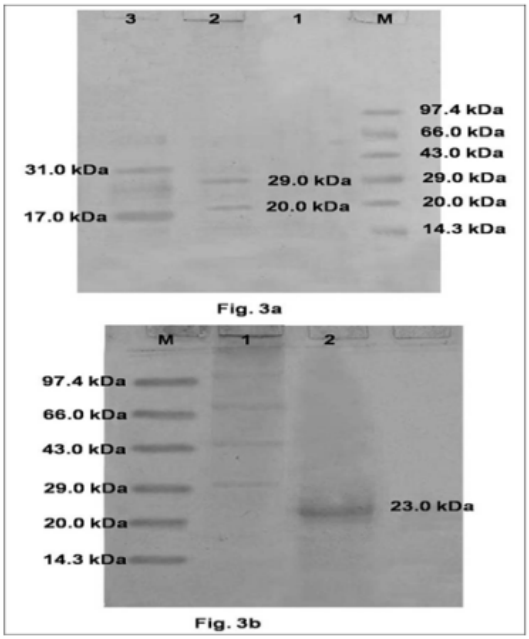

Figure 3. SDS PAGE *of A. niger* F7
Figure 3(a) is partially purified CMCase and FPase in which in lane 1 showing two bands of partially purified CMCase lane 3 showing two bands of partially purified FPase
Figure 3(b) is partially purified @-glucosidase in which lane 2 showing single band of partially purified β-glucosidase

For hardwood substrates

1. Quadratic model:

a. CMCase, $Y=18.74 +3.39X+ (-.026)X^2$

b. FPase, $Y= 25.17+26.82X + (2.91)X^2$

c. β-glucosidase, $Y= -8.68+1.86X+(-.005)X^2$

2. Power function:

$Y=14.55\ X^{0.54}$

$Y= 43.34\ X^{0.45}$

$Y=1.30\ X^{1.02}$

For softwood substrates

1.Quadratic model:

a. CMCase, $Y= -36.18 + (-1.38\ )X + 0.21X^2$

b. FPase, $Y= -12.53+ 2.53X +(-.021)X^2$

c. β-glucosidase, $Y= -12.53 +2.53\ X\ (-.021\ )X^2$

2. Power function:

$Y=25.10X^{0.34}$

$Y=44.79\ X^{0.63}$

$Y=1.77\ X^{0.94}$

In the present study, native and pretreated lignocellulosic forest wastes were used for high cellulase titres by *A. niger* F7. Production of cellulase was maximum in pretreated lignocellulosic biomass than the native ones. Among lignocellulosic wastes (soft and hard wood) and pretreatments given, results revealed that enzyme production by *A. niger* F7 was maximum in acid + steam pretreated *P. roxburghii* needles. This study proved that forest waste substrates can be used for economical production of cellulase to be used for industrial purpose.

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