Upgradation of Recycled Pulp Using Endoglucanase Enzyme Produced by *Pycnoporus sanguineus* NFCCI-3628

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ABSTRACT: *Importance of recycling and utilization of recycled fiber has recently been recognized because of concerns about environmental issues and economical aspects. In spite of several advantages offered by paper recycling such as wood savings through substitution of virgin pulp and reduction in chemical usage, recycled pulp also contains great amount of fines having high relative surface area and high amount of amorphous cellulose. These fines adsorb water to a large extent and dewatering rate is lower compared to virgin pulp. Selective hydrolysis of these excess ultra-fines through eco-friendly cellulase enzymes can be utilized for enhancing the drainage rate leading to increased paper production. The present study deals with the isolation, production, biochemical characterization of alkali stable endoglucanase enzyme from Pycnoporus sanguineus NFCCI-3628 and its potential application for the improvement of pulp drainage. The enzyme produced by the fungus was stable even in neutral to alkaline pH range 7-8. In the experiments carried out, significant improvement of ~9-14% in drainability of pulp treated with endoglucanase enzyme was achieved along with better paper properties such as tensile index & smoothness.*

KEYWORDS: *Amorphous cellulose; Drainability; Endoglucanase production; Enzyme purification; Recycled pulp.*

INTRODUCTION

The term cellulase actually refers to a complex of three hydrolytic components, recognized on the basis of substrate specificity or mode of action. Endoglucanase component randomly cleaves internal glucosidic bonds

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along the length of the cellulose chain in the amorphous regions, decreasing the degree of polymerization (DP) of the substrate in the process. Cellobiohydrolases (exoglucanases) are processive enzymes that initiate their action from the ends of the cellulose chains, producing primarily cellobiose that is then released to solution. They attack the crystalline parts of the substrate and decrease the DP of the substrate, albeit slowly. The β-D-glucosidase or cellobiase converts cellooligosaccharides and cellobiose to glucose. Cellulases that are active and stable in an alkaline pH range are in demand because of their potential applications in textile [1], detergents [2] and pulp and paper industries [3, 4]. To our knowledge, this *Trametes/Pyconoporus* strain has not been already reported in the open literature for the production of alkali stable cellulases, thus our findings can contribute to the advancement of science in this field.

Paper recycling offers several advantages over virgin fibers such as savings of wood for making pulp and reduced chemical usage, but several problems are also associated with the recycling of waste paper such as deinking of different types of post-consumer papers, drainability of recycled pulp, stickies contamination and lower strength. One of the main problems with recycled fiber is that due to high relative surface area of fines, dewatering rate is lower compared to virgin pulp. Thus, the productivity of the paper making process is considerably decreased, compared to operation using virgin pulp. Dewatering properties of the pulp strongly affect the energy efficiency of paper machine and thus the cost efficiency of papermaking. Enhanced water removal in former, press, and dryer sections enables lower energy consumption or increased production capacity. Alternately, due to improved drainage, a shorter drying section would decrease investment costs. Cellulase enzymes are reported to modify the interfacial properties of recycled fibres, increasing the water affinity, which in turn change the properties of pulp and paper, such as drainability and strength [5].

Enzymes can either flocculate or hydrolyze fines and remove fibrils from the surface of large fines [6]. The flocculation aided by enzyme occurs when a low enzyme dosage is used. In this case, fines and small fiber particles aggregate with each other or with larger fibers, decreasing the amount of small particles in pulp and consequently improving pulp drainage. On the other hand, at higher enzyme concentration, hydrolysis of fines begins to predominate and flocculation becomes less significant.

Several commercial cellulase enzymes are available which claim to improve the drainage of secondary fibers. But using mixtures of cellulases can be harmful for certain pulp properties. When applying cellulase enzyme mixtures, identification of the key component, responsible for the required effect on pulp and paper properties, is required. The rate of hydrolysis of amorphous cellulose has been reported to be five to thirty times higher than that of crystalline cellulose [7]. In the present study, the concept of monocomponent cellulase treatment of recycled pulp for improvement in drainage as a result of selective and controlled hydrolysis is described.

EXPERIMENTAL SECTION

Isolation and screening of cellulase producing fungi

The fungal strains were isolated from the samples collected from soil, decaying wood and sludge from various sites of Northern India, using enrichment technique. Serially diluted samples prepared from different sources were spread on surface of potato dextrose agar and incubated for 7 days at 30ºC. Colonies were picked and sub-cultured to obtain a pure culture. The cellulolytic nature of 31 fungal strains obtained as above was confirmed by cultivation on CMC agar culture plate and by subsequent staining using Congo red staining method [8]. The strains that showed a clearing zone around the colony were isolated as potential alkaline cellulase producing fungi. The experiments were done in triplicates.

Microorganism & culture conditions

Among 96 different strains of fungi, the selected fungal isolate PVYA 07 selected by primary (congo red) and secondary screening (CMCase activities) was sent to National Fungal Culture Collection of India (NFCCI), Pune, India, for further identification. The culture was identified as *Trametes sanguinea* based on sequencing of the genomic DNA, and was deposited at NFCCI, Pune, India with accession number PVYA07 NFCCI-3628. The said strain displayed strong cellulase activity at pH 7.0 - 8.0. Currently *Trametes sanguinea* is known as *Pycnoporus sanguineus,* also mentioned in the identification report. After the molecular identification

the images of the two fungal strains were found to be similar in appearance, to the respective images of the two strains available on internet (Fig. 1a, b, c).

Pycnoporus sanguineus (L.) Murrill is a basidiomycete and an efficient producer of polyphenol oxidase that acts on variety of aromatic hydrogen donors. It has been reported for fermentation of agro-industrial waste, decolourization of Kraft effluent, and in various dyes. Despite the limited references on this fungus, there is a growing trend to employ it in biotechnological processes. It is a white rot fungus that has been found to produce a wide range of enzymes, such as cellulases, cellobiose dehydrogenase, laccase, manganese peroxidase [9]. The other fungal strains showed average, weak and no activity of the enzymes.

The fungus was grown in M-1 medium [10]. The basal M-1 medium contained (in g/L of distilled water) KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; $CaCl_2$. $2H_2O$, 0.3; $MgSO_4.7H_2O$, 0.3; Urea, 0.3; Proteose peptone, 0.25; Yeast extract, 0.2; and trace metal solution, 1mL [$FeSO_4$. $7H_2O$, 5 mg/L; $MnSO_4.7H_2O$, 5.6 mg/L; $ZnSO_4.7H_2O$, 3.34 mg/L; $CoCl_2.2H_2O$, 2 mg/L], Tween 80, 1mL. The pH of the medium was adjusted to 7.0 after autoclaving by separately sterilized 1M Na_2CO_3. The culture was grown for 3 days and used as the inoculum. The fungus was grown in M-1 basal medium supplemented with different cellulosic substrates, inorganic nitrogen sources & surfactants.

Enzyme production

Enzyme production was carried out in 500 mL Erlenmeyer flask containing 100 ml M-1 medium. The culture was incubated at 30°C on a rotary shaker at 200 rpm. At regular intervals, the samples were withdrawn. The mycelium was removed by centrifugation at 6000 rpm. This enzyme preparation after 8 days of incubation was used for measurement of enzyme activities. Results given here are the mean of at least duplicate experiments. Protein concentration was measured using bovine serum albumin standard as described by *Lowry et al.*, 1951[11].

Purification of Endoglucanase

Ultrafiltration and Gel filtration on Sephadex G-200

The harvesting of mycelia was done by centrifugation at 6000 rpm for 15 min and clear culture filtrate was concentrated in Amicon ultrafiltration unit with PM 10 membranes (molecular weight cut off 10,000D). The enzyme concentrate from ultrafiltration step was then fractionated on a Sephadex G-200 column equilibrated at 4°C with 0.05M sodium phosphate buffer at pH 7.0. The eluted fractions, each of 5.0 ml, were collected at a flow rate of 10 mL/ h and assayed for protein and/ or enzymatic activity. In this step the specific activity of Endoglucanase got increased to 11.3 IU/mg of protein (Table 1).

Preparative isoelectric focusing (IEF)

The pooled endoglucanase fraction obtained from gel filtration column was concentrated in an Amicon ultrafitration (PM 10) unit. Finally the concentrated enzyme was purified further by using miniscale isoelectric focusing (IEF) unit. The glycerol density gradient was used as the stabilizing medium. Isoelectric focusing using Biolyte, carrier electrolytes in the pH range of (3-10) was carried out for 6 h at 450 volts giving a current of 2 mA. The fraction of 0.5 mL were collected from the side arm of the unit and immediately processed for the determination of endoglucanase, CBH & filter paper activities. A single sharp peak around pH 3.8 was observed after isoelectric focusing. The purified enzyme thus obtained has the specific activity of 17.4 IU / mg (Table 1).

Molecular weight determination on SDS –PAGE

Molecular weight of purified endoglucanase was estimated by its migration in 10% SDS polyacrylamide gel as described by *Laemmli*, 1970 [12]. After electrophoresis, the protein bands were stained by 0.25% Coomassie Brilliant Blue R-250 in methanol: acetic acid: water (40:10:50) and destained in the same solution without dye. The molecular weight markers used included β- galactosidase (120 kDa), Bovine Serum Albumin (91 kDa), Serum Albumin (66 kDa), Glutamic dehydrogenase (56 kDa), Ovalbumin (48 kDa) Carbonic anhydrase (34 kDa) and Myoglobin (26 kDa). The plot of log molecular weight versus relative mobilities (Rf) of standard proteins and endoglucanase was prepared. The molecular weight of purified endoglucanase enzyme was determined to be 46.5 kDa.

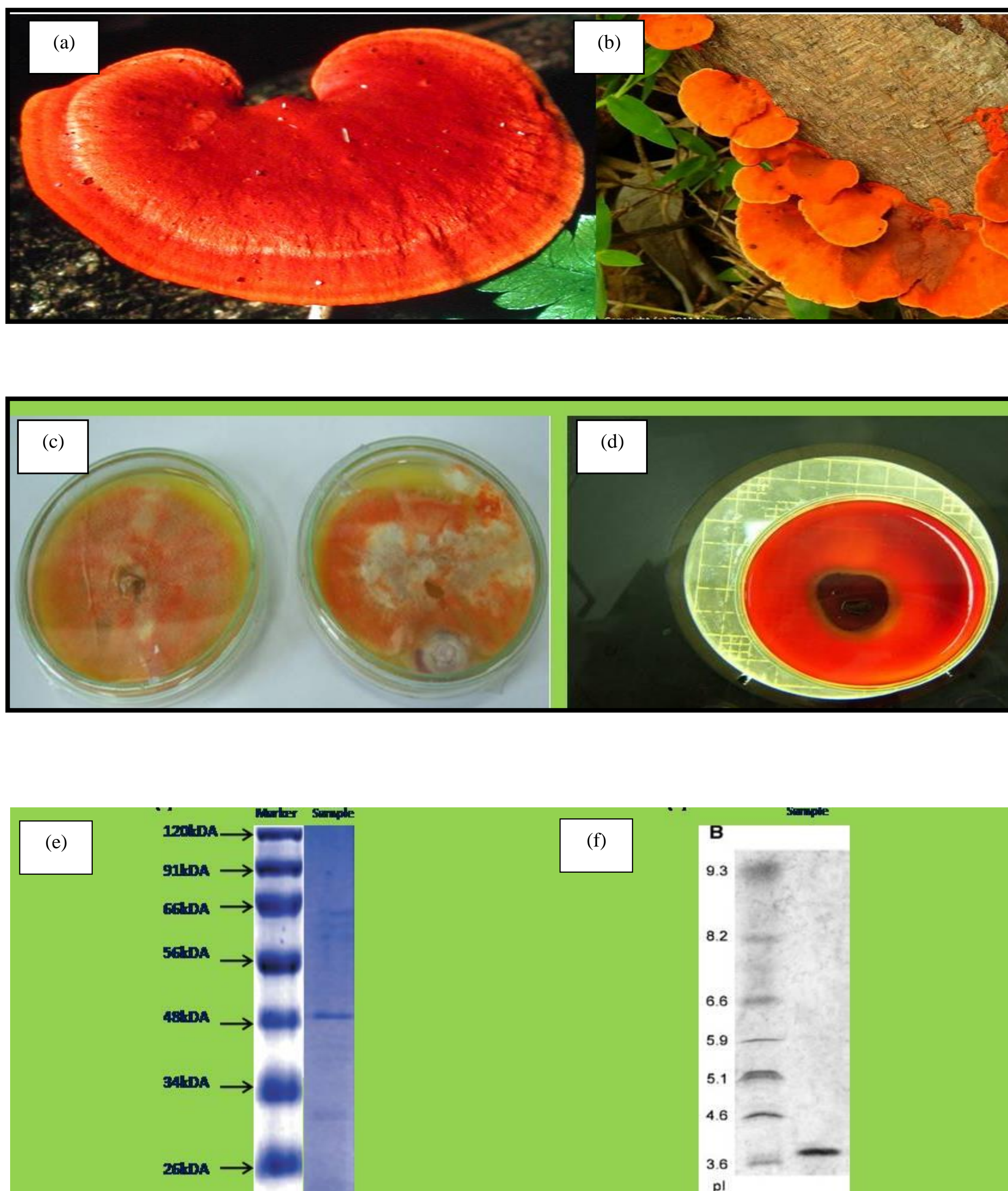

Fig. 1: Images of Pycnoporus sanguineus. a) http://www.diark.org/diark/species_list?species_id=5149, b) (http://www.mycodb.fr/fiche.php?genre=Pycnoporus&espece=sanguineus), c) Pycnoporus sanguineus (research work), d) Zone of clearance produced by Pycnoporus sanguineus (research work), (e) SDS-PAGE (f) Isoelectric focusing of purified endoglucanase from Pycnoporus sanguineus NFCCI-3628.

Table 1: Summary of purification steps of endoglucanase enzyme from Pycnoporus sanguineus NFCCI-3628.

Purification steps	Total Activity (IU)	Total Proteins (mg)	Specific Activity (IU/mg)	Fold Purification	Enzyme Yield (%)
Culture filtrate	660	440	1.5	--	100
PM 10 & Sephadex G-200	380	33.7	11.3	7.5	58.7
Isoelectric focusing	68	3.9	17.4	11.6	10.2

Effect of pH on enzyme activity and stability

The optimum pH of the endoglucanase enzyme was determined by incubating it at different pH values of the reaction mixtures for 30 min and determining the activity. Sodium phosphate buffer (pH 5-8) was used for observing the effect of pH on the enzyme activity.

Effect of temperature on enzyme activity and stability

For determining the effect of temperature on endoglucanase enzyme, enzyme activities were estimated in a temperature range of 40-70°C. The temperature stability of the enzyme was detected by incubating suitably diluted enzyme at 40-70°C for different time intervals. The samples were removed periodically and assayed for different enzyme activities under standard assay conditions.

Enzyme assays

The endoglucanase activity of enzyme preparations was determined using sodium Carboxy Methyl Cellulose (CMC) as a substrate [13]. The reducing sugar formed was determined using Di Nitrosalicylic Acid (DNS) assay [14]. The activity was expressed in IU/ml. The exocellulase (cellobiohydrolase, CBH) was determined with p-Nitrophenyl-β-D-Glucoside (pNPG) (Sigma, USA) as a substrate [15]. Quantity of p-Nitrophenol released was determined using its extinction coefficient (18,300 M^{-1} cm^{-1}), and then the enzyme activity was calculated. The FPase activity was determined by incubating 0.5 mL of suitably diluted enzyme with 50 mg of Whatman No. 1 filter paper of 1 cm *6 cm size [13]. After 60 minutes of incubation at 50 ^{0}C, the reducing sugars were measured. The activity was expressed in FPU/mL.

Enzyme Treatment of pulp

The recycled pulp consisting of sorted office pack, old newsprint and old record was procured from an indigenous paper mill in Eastern India. All enzyme treatments were done at 4% consistency, 45°C & pH~7. Pulp suspension was warmed up to desired temperature and the pH was adjusted by addition of aluminum sulfate, which is used as retention aid and flocculating agent in pulp and paper industry. The purified endoglucanase enzyme was added at varying dosage ranging from 0.05 to 0.125%.

Drainability of Pulp

The drainage time of the pulp slurry was measured on modified °SR tester using the method used by previous researchers [16-19]. The time required to drain the water from pulp slurry for collection of 800 mL filtrate from the front orifice was measured and reported as the drainage time in seconds.

Water Retention Value

The measurement of the WRV used a slightly modified version of the standard method proposed by Tappi Useful Method 256. WRV was calculated according to the following formula [20],

$$WRV = \frac{(m2 - m1)100}{m1}$$

where *m1* equals the mass of dry sample, and *m2* equals the mass of moist sample. Four *WRV* measuring were conducted in each experimental series to calculate the average value.

Hand-sheet Preparation and Testing

The treated and filtered pulp was diluted to a consistency of 0.3% and hand sheets were prepared according to TAPPI test method T 205 sp-06. The hand sheets were prepared immediately following enzyme treatment to avoid extended contact time with the cellulase enzyme. The standard hand sheets were conditioned at 27^{0}C and 65% relative humidity. The strength properties were tested according to TAPPI test methods.

RESULTS AND DISCUSSION

Characteristics of the fungal isolate

On the basis of morphology, microscopic and molecular observations (at NFCCI, Pune, India), the fungus was identified as an alkalotolerent *Pycnoporus sanguineus* NFCCI-3628 (Fig. 1a). The cultural studies showed that the fungus grows best at 30°C. However, the optimum growth was observed around pH 7.0, the culture grows well at pH 7.0-8.0, indicating its alkalotolerant nature. The cellulolytic nature of the culture was confirmed by clearance zone formation on CMC agar plate as CMC was hydrolyzed with cellulases produced during fungal growth (Fig.1d).

Effect of different culture conditions on enzyme production

The optimum temperature and pH for growth of the fungus was 30°C and 7.0, respectively. The fungus grew well around pH 7.0-8.0 and produced high amounts of enzyme in neutral to alkaline medium confirming alkalotolerant nature of the fungal isolate (Fig.2a). The endoglucanase enzyme retained around 85% activity after incubation at pH 8.0 while 50% residual activity was detected at pH 9.0.

The fungus was grown on various cellulosic substrates and different enzyme activities were analyzed (Fig.2b). The highest amount of cellulase activities were induced on refined cellulosic substrate such as Avicel PH 101 as well as carboxy methyl cellulose. The enzyme can also be produced on agricultural residues like bagasse pith and rice bran. However, yields obtained were quite moderate when compared to refined cellulosic substrates (Fig. 2b).

Yeast extract was found to be the suitable inorganic nitrogen source for enzyme production amongst inorganic nitrogen sources, added at a concentration of 0.25 g/l, (Fig. 2c). Addition of surfactant (0.1%, v/v) was found to be essential in order to facilitate the release of cellulases in the medium. Tween 80 was found to be the best surfactant (Fig. 2d). It was also demonstrated by *Sukan et al.*, 1989 [21] that emulsification with Tween 80 led to higher cellulase activities. The reason might be the increased permeability of cell membranes and/or enhanced release of cell-bound enzymes. The maximum enzyme production was observed with the inoculum size of 6 discs (for 0.5% CMC) in 8 to 9 days for *Pycnoporous. sanguineus.* Maximum endoglucanase and cellobiohydrolase activities i.e. 7.1±0.4 IU/mL and 2.6±0.4 IU/mL, respectively were observed after inoculation of 6 discs (Fig. 2e).

The effect of incubation days was also assessed from 1 to 12 d for the two fungal strains. *P. sanguineus* PVYA07 NFCCI-3628 exhibited the maximum CMCase (7.6±0.8 IU/mL) and CBH (2.5±0.4 IU/mL) activity at 8 days incubation time. After 10 days, the production of enzymes started to decrease (Fig.2f).

The summary of the purification scheme for Endoglucanase is presented in Table 1. The data indicated that the purified Endoglucanase shows 11.6 fold higher specific activity with 10.2 % overall yields.

Purified endoglucanase enzyme: pH and temperature profile

The purified endoglucanase preparation was active in a broad pH range of 5 to 8 with optimum pH at 7.0 at 50°C (Fig.3a). The enzyme showed maximum activity at 50°C and retained 70% of the maximum enzyme activity at 60°C (Fig. 3b). The enzyme got rapidly inactivated at 70°C. It has been reported previously that although certain strains of *Trichoderma* sp and *Penicillium* sp produce high amounts of cellulases, their activities at alkaline pH are negligible [22]. Whereas, the extracellular enzyme produced by alkalotolerant *Pycnoporus sanguineus* NFCCI-3628 strain reported in this work was active and stable under alkaline conditions indicating their potential for commercial use.

Effect of enzyme treatments on pulp drainability:

Endoglucanase enzyme displayed significant improvement in pulp drainage ranging from 8.7 to 13.9 % at various dosages (Fig. 4a). It is in agreement with the published data that the endoglucanases significantly improve secondary pulp drainage [23]. Significant improvement in drainage rate along with improvement in smoothness & tensile index using endoglucanase enzymes was also reported by *Oksanen et al.*, 2009 [24]. Since the amorphous cellulosic fines are held responsible for the deteriorated drainability of recycled pulps, decreasing the amount of excess fines could be the key solution for improving drainage. The pulp freeness as measured by CSF also increased with increasing enzyme dose (Fig. 4b).

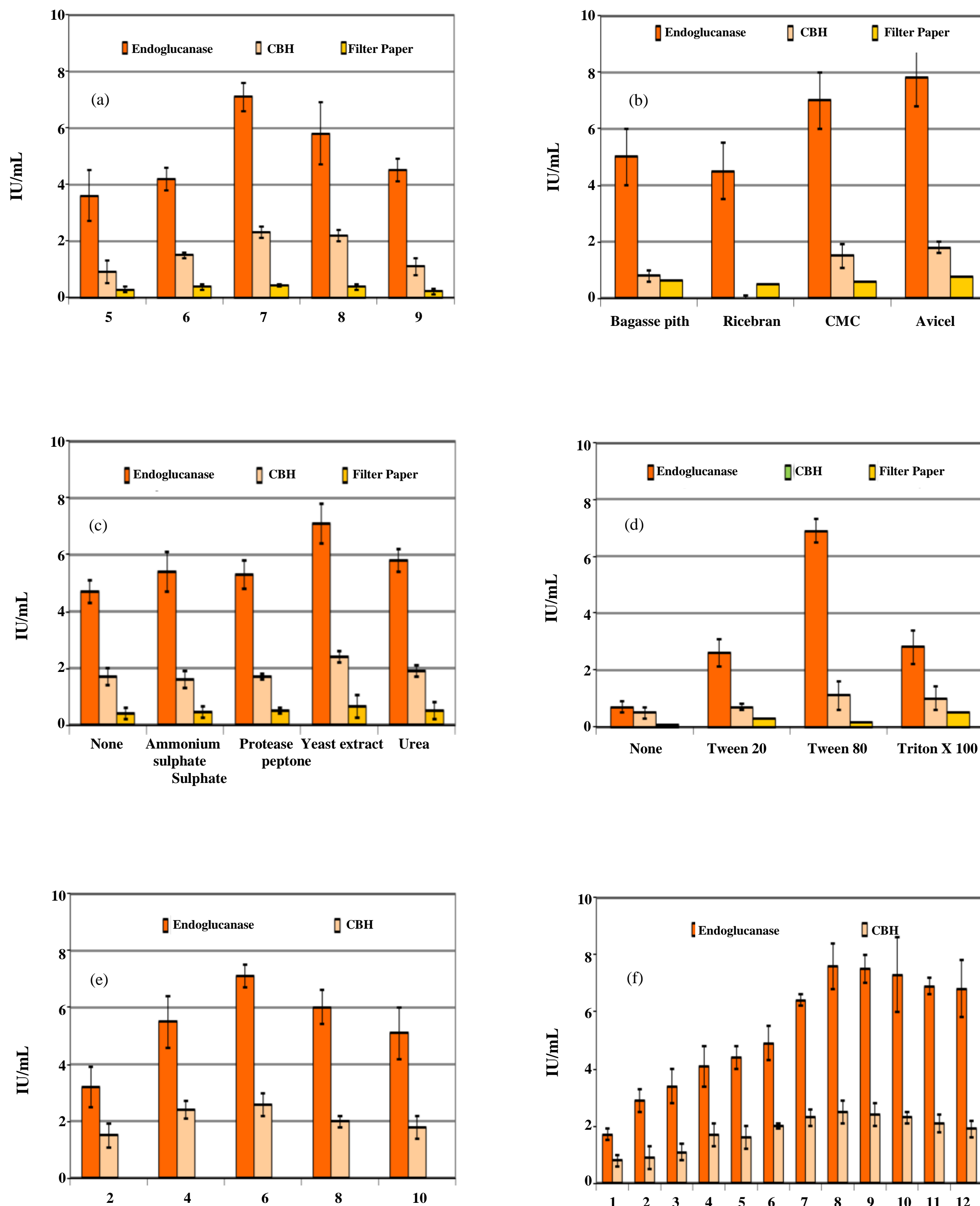

Fig. 2: Effect of different culture conditions on enzyme production (a) cellulosic substrates (b) Initial pH (c) inorganic / organic nitrogen sources (d) surfactants (e) inoculum size (no. of discs) (f) incubation days.

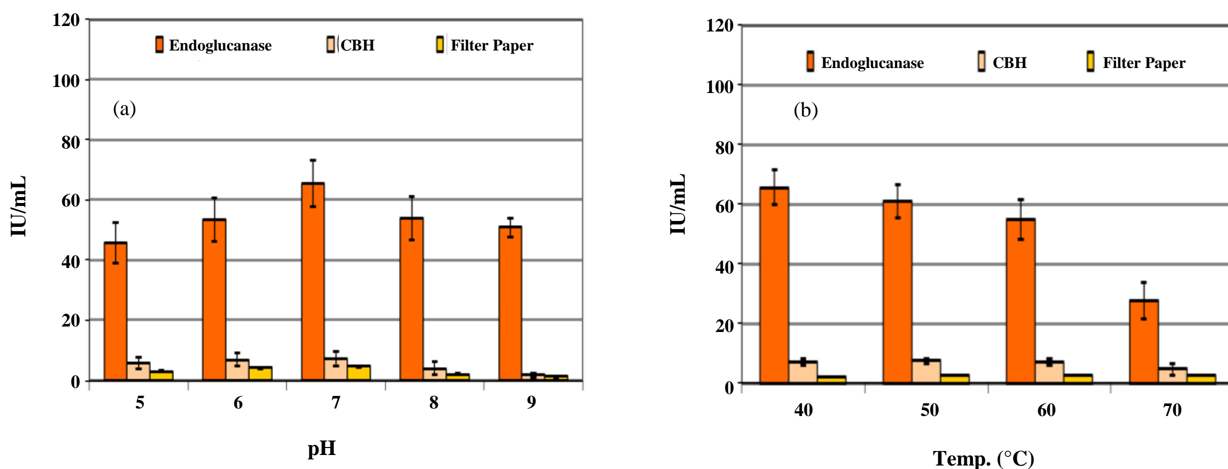

Fig. 3: pH and temperature profile of endoglucanase enzyme produced from P. sanguineu.

(a) Decrease in drainage time (%) — P. sanguineus — Enzyme dose (%): 0.050, 0.075, 0.100, 0.125

(b) CSF (mL) — Untreated — P. sanguineus — Enzyme dose (%): 0.050, 0.075, 0.100, 0.125

(c) WRV (%) — Untreated — P. sanguineus — Enzyme dose (%): 0.050, 0.075, 0.100, 0.125

(d) Viscosity (cp) — Untreated — P. sanguineus — Enzyme dose (%): 0.050, 0.075, 0.100, 0.125

Fig. 4: Effect of different enzymes on pulp properties at various doses (a) Drainage time (b) Canadian Standard Freeness (c) Water retention value (d) Viscosity.

Effect of Enzyme Treatments on Water Retention Value of Pulp

Effect of enzymatic treatment was also investigated on the Water Retention Value (WRV) as well. Water Retention Value (WRV) showed that pulps treated with endoglucanase enzyme retain more water (higher WRV by 24%) compared to control pulp (Fig. 4c). The reason may be the hydrolysis of lower molecular weight carbohydrates and partial deterioration of crystalline structure of cellulose initiating the formation of more hydrogen bonds between cellulose and water molecules.

Effect of Enzyme treatments on Pulp Viscosity

Analysis of pulp viscosity was based on TAPPI standard test method T230 om-99. The pulp viscosity was somewhat reduced in enzyme treated pulp. But the reduction in pulp viscosity was not that much significant (Fig. 4d).

Effect of Enzyme Treatments on Paper Properties

Purified endoglucanase enzyme showed significant increase in tensile index (about 17 %) and smoothness while the tear index was found to be slightly decreased (Fig. 5). Probably the endoglucanase action at low enzyme charge contributes to an improvement of the paper structure independent of degradation process of dissolved colloidal substances. Therefore this study reflects that applying purified endoglucanase enzymes, specific regions of the cellulose fibers can be attacked and the desired part of the pulp could be modified in a controlled manner to get expected benefits. In the literature also, it has been reported by *Ivo V. Valchev & Petar Y. Bikov*, 2011 [25] that the effect of endoglucanase treatment on the pulp strength properties showed increase in tensile index, while the tear index and the burst index decreased.

Scanning Electron Microscopic Studies

Examination of fiber surface, treated with higher enzyme dosage was performed by field emission scanning electron microscope. The untreated recycled fibers displayed minimal amounts of fibrillation that would contribute to inter fiber bonding (Fig. 6a). There were limited examples of fibrils interconnecting adjacent fibers.

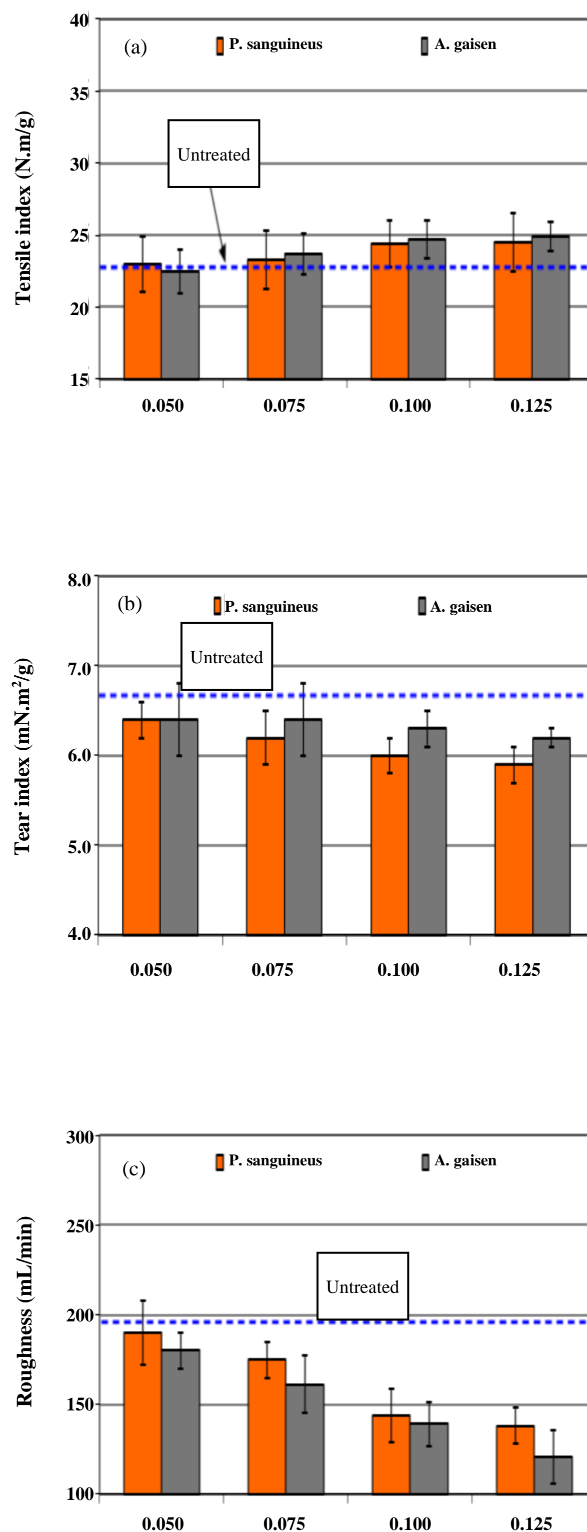

Fig. 5: Effect of different enzymes on paper properties at various doses (a) tensile index (b) tear index (c) roughnes

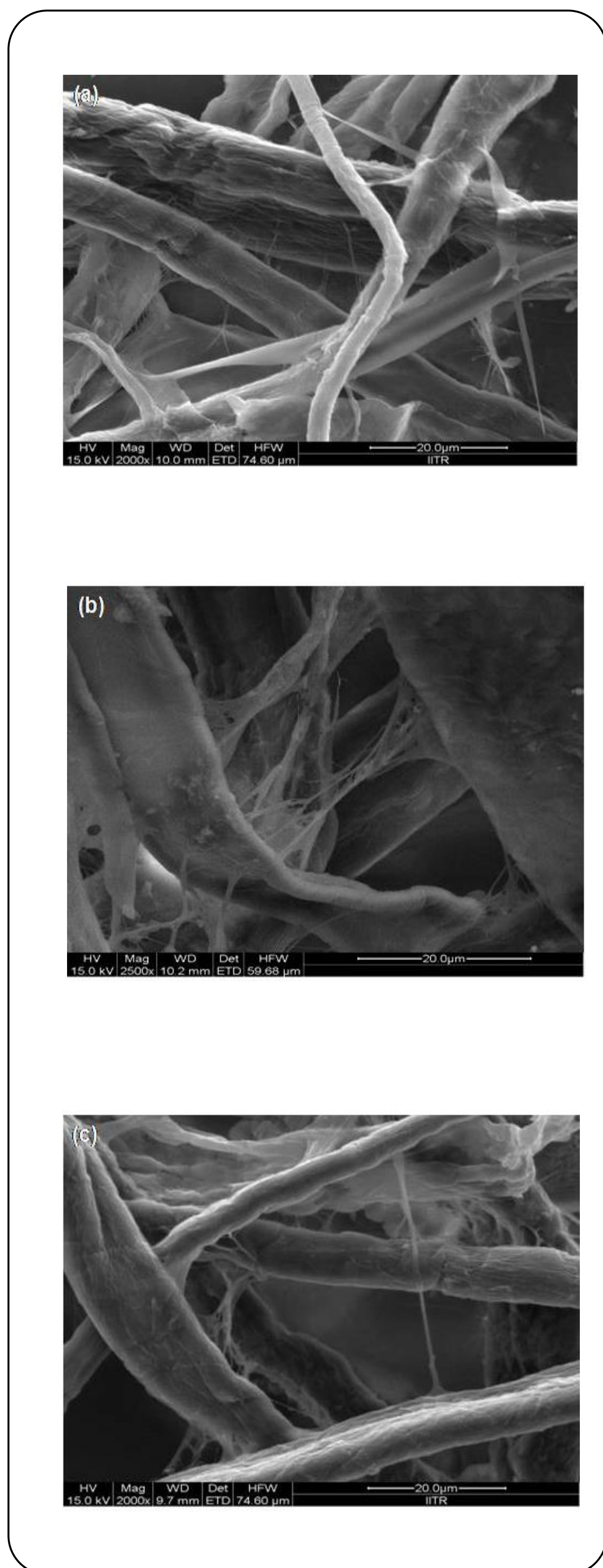

Fig. 6: FE-SEM micrographs (at 15kV and 2000X) of untreated and enzyme treated recycled fibers.

and enzyme treated recycled fibers.

FE-SEM images of fibers treated with high dose of endoglucanase enzyme were visually quite different. In case of endoglucanase treated samples; the adjacent fibers appear to be connected by relatively large patches of outer cell wall material (Fig.6b, c). Treatment with the endoglucanase enzyme at higher dose resulted in extensive fiber wall peeling, fiber collapse and increased fiber flexibility. The high degree of fiber collapse contributed to an increase in Relative Bonded Area (RBA) which may be the reason of increased inter fiber bonding, improved strength properties, decreased fiber-water interactions and improvement in pulp drainage.

CONCLUSIONS

The present research work demonstrates that enhanced dewatering along with better tensile strength and sheet smoothness could be achieved by controlled modification of carbohydrates through alkaline active endoglucanases isolated from *Pycnoporus sanguineus* NFCCI-3628. Advantage of higher freeness achieved by selective hydrolysis of excess ultra-fines and other dissolved colloids using endoglucanase can be utilized for enhancing the operation rate and/or greater dilution can be applied in the head box of the paper machine.

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