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RESEARCH ARTICLE

ENZYMATIC REFINING OF PAPER CUP WASTE PULP TO PRODUCE QUALITY PAPER WITH CELLULASE FROM *TRICHODERMA LONGIBRACHIATUM* ISOLATE

[1]Dhermander Kumar, [1]Rakesh Kumar Jain and [2,*]Neelam Garg

[1]Biotechnology Division, Central Pulp and Paper Research Institute, Saharanpur, Uttar Pradesh, India
[2]Department of Microbiology, Kurukshetra University, Kurukshetra, Haryana, India

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ABSTRACT

The present paper highlights the isolation of potential fungal isolate for optimization and production of cellulase enzyme. Cellulase enzyme produced by the isolate under Solid State Fermentation (SSF) was used as refining aid to produce quality paper from paper cup waste pulp. Fifty six fungal isolates were obtianed from different sources using soil, degraded wood and paper mill waste pulp, cotton waste and screened for cellulolytic activity by congo red plate method. The most potent cellulase producing fungal isolate was identified as *Trichoderma longibrachiatum* and used in the present study to optimize environmental (initial pH, temperature, incubation period and moisture content) and nutritional (carbon and nitrogen sources) parameters for maximum cellulase production. Wheat bran an agro-industrial residue was used as carbon source to produce cellulase enzyme for economic viability of cellulase production under SSF. CMCase and FPase activities of cellulase from *Trichoderma longibrachiatum* were found to be 33.3 IU/gds and 9.4 FPU/ gds respectively. The cellulase enzyme thus produced was used as refining aid for paper cup waste pulp to convert it into quality paper. Studies were focused with an aim to find out the effect of cellulase enzyme treatment on paper cup waste pulp in reduction of energy consumption during refining and strength properties of the resultant pulp

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INTRODUCTION

Paper and its products are manufactured from either a wood based virgin fiber or from recycled fiber. Traditional pulp and paper production process is based on chemicals and mechanical industrial process, they consumes large amounts of raw materials and energy intensive, creates considerable high pressure on the environment (Bajpai, 2005; Rosenfeld and Feng, 2011). Cellulase enzyme widely used in different industries. Due to diverse applications of cellulase enzyme, industry has much more interest by adopting the biotechnological approach. The major industrial applications of cellulase enzyme are in textile industry as well as in house hold laundry detergents for improving fabric softness and brightness (Cavaco-Paulo, 1998). Cellulase also are used in food and feed for improving the nutritional quality and digestibility, while paper industries using cellulase enzyme in (de-inking and refining) as an emerging industrial application (Tolan and Foody, 1999). The cellulase enzyme are complex in nature consists of three types of enzymes (endoglucanases (EG) (EC 3.2.1.4), exoglucanases/cellobiohydrolases (CBH) (EC 3.2.1.91), and β-glucosidase (BG) (EC 3.2.1.21) act synergistically for cellulose hydrolysis (Bhat and Bhat, 1997). Combined action of the three enzymes occurs in a cascade manors, as a result of sequential and cooperative action between components in a cellulase complex, product of one enzyme reaction becomes the substrate for next one (Roussos and Raimbolt, 1982). Endoglucanases act randomly on internal glycosidic bonding of cellulose chains and release cello-oligosaccharides, exoglucanases breakdown the cellobiose units from the non reducing ends of cellulose chains and β-glucosidase converts the cellobiose into end product of hydrolysis in form of glucose (Bhat and Bhat, 1997). A wide range of bacteria and filamentous fungi produce cellulase enzyme. *Trichoderma, Humicola, Penicillium* and *Aspergillus sp.* are commonly studied for cellulolytic action (Rana and Kaur, 2012). Species of *Trichoderma* are the most extensively studied cellulolytic fungi, especially *T. reesei* and *T. longibrachiatum* which are used for enzyme production on industrial scale (Kubicek, 1992).

****Corresponding author: Neelam Garg,***
Department of Microbiology, Kurukshetra University, Kurukshetra, Haryana, India.

Pulp and paper industry is one of the energy–intensive industries. The energy consumption in the industry contributes around 25% of manufacturing cost. The required pulp properties during the refining process, requires substantial energy about 15-18% of total electrical energy consumption for producing paper from wood (Bajpai *et al*., 2006). Due to scarcity of available energy and high energy cost, energy saving becomes a necessary requirement for the pulp and paper industry. By adopting the recent advances in technologies, one of the pre-treatment steps used to produce quality paper with energy saving is the introduction of the 'Enzymatic refining' process (Singh and Bhardwaj, 2010; Liu and Hu, 2012). Application of cellulase enzyme by a route of modifying fiber properties to improve the refinability of pulps may be increased due to availability of mild and non-aggressive enzyme activities (Bajpai, 2011). Cellulase treatment has been found more effective during refining of long fiber to achieve desired freeness value (Loosvelt, 2009). Cellulase enzyme treated pulp showed a better improvement in fibrillation leading to a stronger paper (Lecourt *et al*., 2010).

Apart from the above reasons, some raw fibers materials are demanded for papermaking due to the low availability of virgin fiber and cost. Pulping of virgin fiber is pollution intensive process. Use of recycled fiber materials/ waste paper for the production of quality paper is increasing day by day along with demand of high energy during the refining process. Keeping in view the above, the present research work was focused on production of cellulase enzyme with high level in terms of CMCase and FPase from fungal isolate *Trichoderma longibrachiatum* via solid state fermentation. To explore the potential of cellulase enzyme as refining aid for paper cup waste pulp and its conversion into quality paper.

MATERIALS AND METHODS

Isolation and selection of cellulolytic fungi

Different fungi were isolated from samples of soil, degraded wood and paper mill waste pulp, cotton waste on Potato Dextrose Agar (PDA) plates. These isolates were tested for production of cellulase by solid state fermentation using Mandels and Reese (1957) medium supplemented with wheat bran at 28^{o}C for 5 days. Enzyme was extracted with citrate buffer and used both for cellulase assay. For qualitative analysis, enzyme was added to the wells on CMC agar (containing 0.5% CMC and 2.0% agar) plates and cellulolytic zone was observed after staining with 0.1% congo red dye. Isolates showing cellulolytic zone were considered as cellulase positive and were used for quantitative analysis of CMCase (Carboxy methyl cellulase) and FPase (Filter paper cellulase) using method of Ghose (1987). The isolate with highest cellulase activity was selected for further study.

Identification of selected fungal isolate

Selected fungal isolate was identified on the basis of morphology and molecular characterization. The fungus was grown on PDA plates at 28^{0}C for 3 days and colony characteristics were observed. The selected isolate was identified as *Trichoderma longibrachiatum* on the basis of colony morphology, microscopic examination and molecular characterization by NFCCI, Pune.

Production of cellulase by Solid State Fermentation (SSF)

The selected isolate, *Trichoderma longibrachiatum* was used for production of cellulase by solid state fermentation. Mandels and Reese (1957) medium containing, yeast extract (3 g/l), K_2HPO_4 (2 g/l), $CaCl_2$ (0.3 g/l), Tween-80 (2 ml/l), $MgSO_4$ (0.3 g/l), $FeSO_4$ (0.005 g/l), $MnSO_4$ (0.002 g/l), $ZnSO_4.7H_2O$ (0.0016 g/l), and $CoCl_2$ (0.0014 g/l) and supplemented with 5 g of wheat bran on dry basis was used for enzyme production in Erlenmeyer flasks. The initial moisture content was adjusted to 1:3 solid to liquid ratio and initial pH at 7.0. Each flask was inoculated with 5 disks of fungal growth from 3 days old culture plates and incubated at 28^{o}C for 3 days. Enzyme was extracted by adding 50 ml 0.05 M citrate phosphate buffer in fermented substrate, mixing in a homogenizer for 30 mins and squeezing through muslin cloth. The enzyme extract was centrifuged at 10,000 rpm for 10 min at 4°C as per (Deswal *et al*., 2011). Supernatant was used as crude cellulase enzyme and assayed for CMCase and FPase as described earlier. One International Unit of Enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of glucose from the appropriate substrate per ml per min under the standard conditions of assay.

Optimization of conditions for production of cellulase enzyme

Various physico-chemical parameters such as the environmental (pH, temperature, incubation, moisture ratio) and nutritional (lingo-cellulosic carbon and nitrogen sources) are important for cellulase production and are optimized to achieve the maximum level of cellulase enzyme production. One factor at a time (OFAT) approach was used for optimization keeping the others at constant. To optimize pH, temperature, incubation period and moisture ratio enzyme was produced under SSF at varying pH 3-10, temperatures 20-44°C, incubation period 0–168 h and initial moisture ratios (solid: liquid) 1:1.0-1:4.0. Various agro-industrial residues were used as carbon source; organic and inorganic compounds were used as nitrogen source. Crude enzyme produced under optimized conditions was used for its application in refining of paper cup waste pulp.

Refining of paper cup waste pulp

Paper cup waste pulp was procured from a local paper industry which was used for enzymatic refining with surfactant in laboratory scale Valley beater.

Enzyme treatment

Paper cup waste pulp 360 g on dry basis was taken in a poly bag and its 10% consistency was maintained with distilled water by incubating at 50°C for 30 min in water bath. Dose of enzyme was optimized at 0.05% surfactant for enzymatic refining.

Refining process

Control and enzyme treated pulp were taken in a laboratory valley beater and their consistency was maintained at 1.57%

with tap water for refining process. During the process of refining, pulp freeness was tested at different intervals by using Canadian Standard Freeness (CSF) tester to achieve target freeness level from 580 to 300 ml. The energy consumption in control and enzyme treated pulp during process of refining was also recorded and calculated in Kw/Ton. Refined pulp was also characterized for strength properties, Double fold, Tensile Index, Burst Index and Tear Index according to TAPPI standard methods.

RESULTS AND DISCUSSION

Isolation and screening of cellulose degrading fungi

In total, fifty six fungi were isolated and tested for cellulase production under solid state fermentation. Higher yield of cellulases from *T. reesei* in SSF cultures compared to liquid cultures was reported by Chahal (1985). SSF offers many advantages over SmF (Singhania *et al*., 2009). Tengerdy (1996) had indicated that there was appriximately 10-fold reduction in the production cost when SSF is employed for production as compared to SmF. SSF process are strongly recommended as systems for producing higher concentration cellulases at lower price than submerged cultures along with reducing the step in downstream processing, in turn reducing the cost of operation (Vintila *et al*., 2009). Among all the fungi isolated, the isolate DNR40 was found to produce maximum cellulolytic zone (Fig. 1) on CMC agar plate and maximum CMCase (13.8 IU/gds) and FPase (3.5 FPU/gds) activities (Table 1). The isolate DNR40 produced light green colonies with aerial filaments, white fibrous outermost concentric ring in the beginning on Rose Bengal and PDA medium plates (Fig. 2a & 2b). This isolate was identified at molecular level from NFCCI, Pune as *Trichoderma longibrachiatum* with accession no 3616 and used for cellulase production for its application in enzymatic refining of paper cup waste pulp.

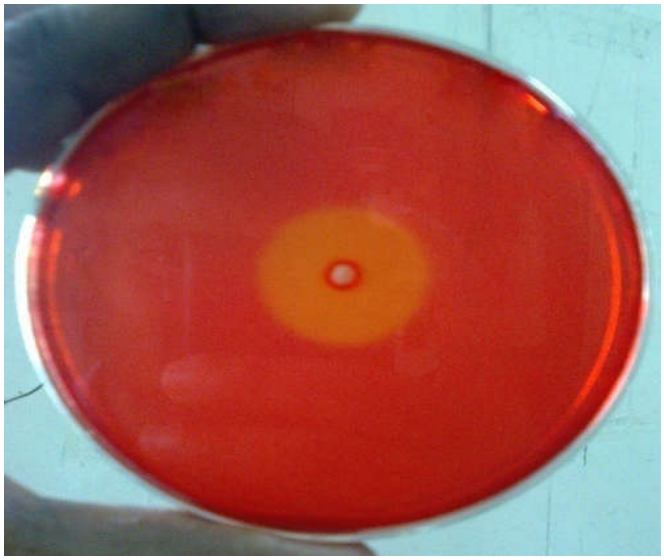
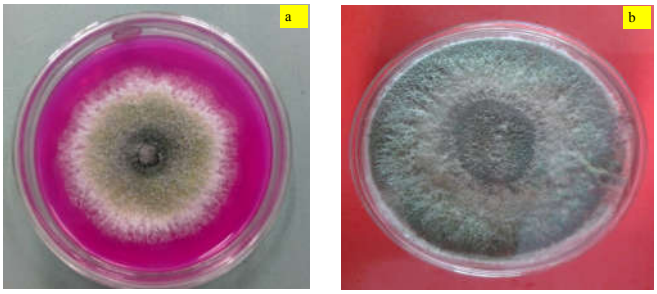


Fig. 1. Cellulolytic zone of enzyme produced from fungal isolate DNR40



a. Growth pattern on Rose Bengal Medium

b. Growth pattern on PDA Medium

Fig. 2a-b. Growth pattern of isolated fungus *Trichoderma longibrachiatum* on (a) Rose Bengal and (b) PDA medium

Table 1. Fungal isolates and cellulase enzyme production

Fugal isolates	Cellulolytic Zone	Cellulase activity (IU/gds)		Fugal isolates	Cellulolytic Zone	Cellulase activity (IU/gds	
		CMCase	FPase			CMCase	FPase
DNR1	-	NA	NA	DNR29	+	4.6	0.7
DNR2	-	NA	NA	DNR30	++	8.2	1.9
DNR3	+	4.2	0.6	DNR31	-	NA	NA
DNR4	-	NA	NA	DNR32	-	NA	NA
DNR5	-	NA	NA	DNR33	-	NA	NA
DNR6	++	7.5	1.9	DNR34	+	4.7	0.7
DNR7	+	3.7	0.6	DNR35	-	NA	NA
DNR8	-	NA	NA	DNR36	-	NA	NA
DNR9	-	NA	NA	DNR37	-	NA	NA
DNR10	+	4.3	0.7	DNR38	+	4.8	0.8
DNR11	+	4.1	0.6	DNR39	++	8.7	1.8
DNR12	-	NA	NA	DNR40	+++	13.8	3.5
DNR13	-	NA	NA	DNR41	-	NA	NA
DNR14	-	NA	NA	DNR42	+	2.8	0.4
DNR15	+	3.8	0.5	DNR43	-	NA	NA
DNR16	++	8.8	1.9	DNR44	-	NA	NA
DNR17	-	NA	NA	DNR45	-	NA	NA
DNR18	+	4.4	1.1	DNR46	+	3.1	0.4
DNR19	+	3.9	0.8	DNR47	+	2.7	0.5
DNR20	-	NA	NA	DNR48	-	NA	NA
DNR21	-	NA	NA	DNR49	-	NA	NA
DNR22	-	NA	NA	DNR50	+	3.8	0.7
DNR23	+	4.5	0.9	DNR51	-	NA	NA
DNR24	-	NA	NA	DNR52	-	NA	NA
DNR25	-	NA	NA	DNR53	+	4.5	0.9
DNR26	-	NA	NA	DNR54	+	4.1	0.8
DNR27	+	3.9	0.6	DNR55	-	NA	NA
DNR28	-	NA	NA	DNR56	+	2.6	0.3

- No cellulolytic zone; + (≤ 5mm) cellulolytic zone; ++ (≤ 10 mm) cellulolytic zone; +++ (≤ 15 mm) cellulolytic zone; NA- not attempted

Optimization of parameters for cellulase production

Fermentation parameters such as pH, temperature, moisture are very important and affect the growth and enzyme production. Studies were carried out for the optimum pH to get high level of cellulase enzyme from *Trichoderma longibrachiatum*. SSF process was used for the study to optimize the initial pH, incubation temperature, incubation period and initial moisture ratio. To optimize the initial pH, 15 different initial pH with an interval of 0.5 pH from 3-10 was analyzed. It was observed that optimum pH for fungal isolate was 6.0, with 18.3 IU/gds CMCase and 5.7 FPU/gds (Fig.3a).

The studies showed that there is gradual decrease in CMCase and FPase when initial pH is increased from 6 to 10 and gradual increase from 3 to 6. For incubation temperature, enzyme was produced at 20, 24, 26, 28, 30, 32, 34, 36, 40 and 44°C. It was observed that maximum CMCase 21.1 IU/gds and FPase 6.6 FPU/gds was produced at 30 ^{0}C (Fig.3b). To optimize the incubation period of selected fungal isolate 15 different incubation times 0- 168 hrs with the interval of 12 hrs was studied. The results showed that at 60-72 hrs (12 hrs enzyme production was almost same (Fig.3c). Moisture content plays a key role in the growth of fungi.

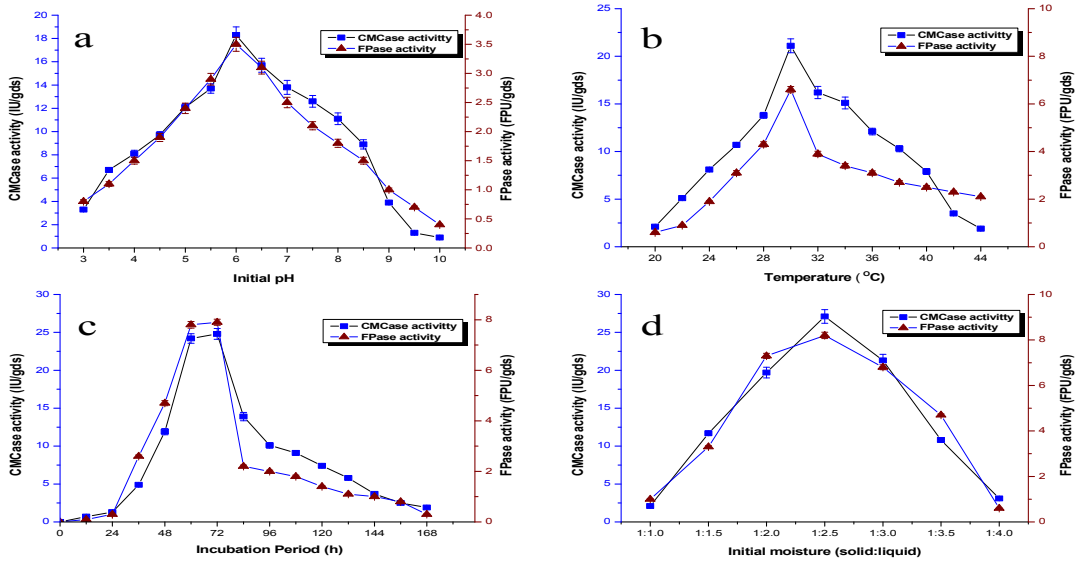


Figure 3a-d. Effect of (a) initial pH, (b) temperature, (c) incubation period and (d) initial moisture (solid: liquid) during optimization of enzyme production in terms of (CMCase and FPase activities) by *Trichoderma longibrachiatum* under SSF

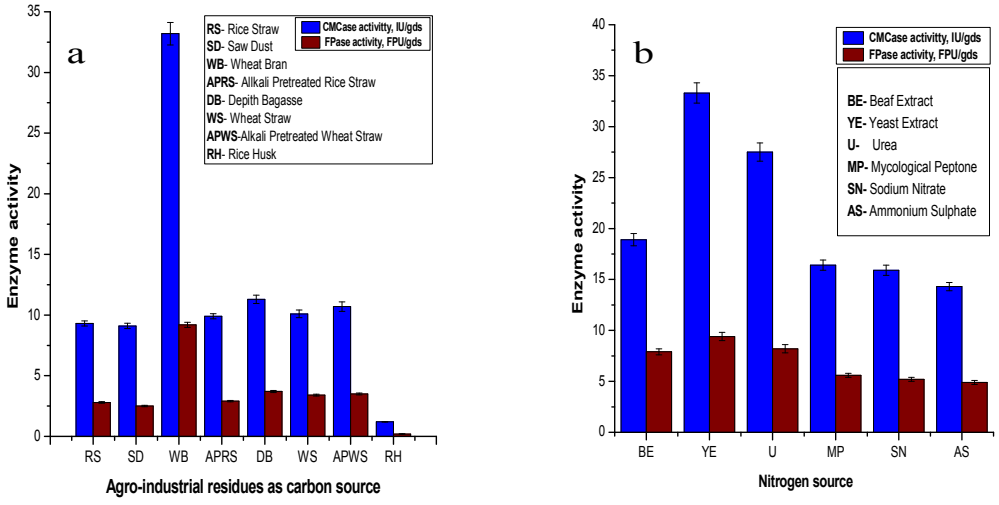


Fig.4a-b. Effect of different nutritional sources (a) agro-industrial cellulosic carbon sources, (b) organic and inorganic nitrogen sources during optimization of cellulase production (CMCase and FPase activities) by *Trichoderma longibrachiatum* under SSF

In case of initial moisture for solid state fermentation 7 different moisture (solid: liquid ratios) 1:1.0-1:4.0 with the interval of 0.5 were used for the production of cellulase enzyme. It was observed that with maintaining the 1: 2.5 initial moisture ratio produced high activity of enzyme 27.1 IU/gds for CMCase and 8.2 FPU/gds for FPase when compared to others moisture ratios (Fig.3d). At very low i.e. 1:1.0and high 1:4.0 liquid quantity for fungal growth was not favorable due to low availability of oxygen during fermentation. Thus *Trichoderma longibrachiatum* isolate was able to grow and produce high activity level of enzyme under solid state fermentation at optimum moisture ratio. Pathak *et al*. (2014); Lee *et al*. (2011) reported quite similar trend of cellulase production by *Trichoderma harzanium* via SSF.

Optimization of Carbon and Nitrogen sources

Carbon and nitrogen sources are key factors those affect the cellulase production in solid state fermentation. On the dry basis 5.0 g of 8 different (Rice straw, Alkali pretreated Rice straw, Rice husk, Wheat bran, Wheat straw, Alkali pretreated Wheat straw, Depith bagasse and Saw dust) agro-industrial residues were used in 250 ml Erlenmeyer flask as a carbon source. As results it was observed that, out of different carbon sources wheat bran produce maximum enzyme production i.e. 33.2 IU/gds and 9.2 FPU/gds for CMCase and FPase respectively (Fig.4a) which is comparable to the results of other workers e.g. Gupta *et al*., (2015) reported cellulase production by SSF from *Trichoderma sp*. With 29.04IU/gds CMCase and 4.59 FPU/gds FPase; Singhania *et al*. (2007) reported 3.8 U/gds FPU by *Trichoderma reesei* RUT C30 using wheat bran under SSF and Yang *et al*., (2004) reported Fpase activity 5.64 IU/g using Rice Chaff/Wheat Bran in the ratio of (9:1) under SSF condition from mixed culture of *Trichoderma reesei* and *Aspergillus niger*. Fujian *et al*. (2002) using *Penicillium decumbans* on wheat straw/bran as carbon sources in (8:2) ratio adopting SSF bioreactor produce the high activity level of FPase 20.4 IU/g. Use of agricultural wastes have reviewed by (Nigam & Singh, 1996). To optimize the nitrogen sources, 6 different (Beaf extact, Yeast extact, Mycological peptone, Urea and Ammonium sulphate) organic and inorganic components were used for maximum production of cellulase enzyme during this study. It was observed that maximum enzyme production i.e. 33.3 IU/gds and 9.4 FPU/gds for CMCase and FPase respectively was achieved by using yeast extract as nitrogen sources (Fig.4b).

Effect of enzymatic refining of paper cup waste pulp on energy consumption and quality of paper

Studies were carried out on refining of both control and enzyme treated pulp. The results of control (without enzyme) and enzyme treated pulp are shown in (Table 2). To optimize the enzyme dose on paper cup waste pulp, with the objective of energy consumption during refining of the control and enzyme treated pulp different doses of enzyme were used. Enzyme dose, 0.10 IU/g was found to be optimum with significant reduction in energy consumption to achieve the same freeness level i.e. 300 ml CSF as only 760 Kw/Ton of energy was consumed for refining of enzyme treated pulp and 1020 Kw/Ton with control pulp. Also, there was no significant reduction in energy with increased enzyme doses.

Table 2. Effect of enzyme dose on enzymatic refining of paper cup waste pulp in respect to energy saving

Refined pulp	Enzyme dose (IU/g)	Energy consumption (Kw/Ton)	Energy saving (%)
Control	0.0	1020	-
Enzyme treated	0.025	910	10.78
	0.050	830	18.63
	0.075	780	23.53
	0.100	760	25.49
	0.125	760	25.49
	0.150	760	25.49
	0.175	760	25.49

By adopting enzymatic refining 25.49 % energy reduction was observed with respect to control pulp. Singh and Bhardwaj, (2010) reported that cellulase based refining of the wood pulp helped in reduction of energy during refining and properties of the paper was improved. Pere *et al*. (2000) reported that energy consumption for refining can be reduced by softening the wood fibers with a cellulase enzyme prior to processing.

The strength properties of hand sheets made from control and optimized enzyme treated paper cup waste pulp after refining is shown in (Fig.5).

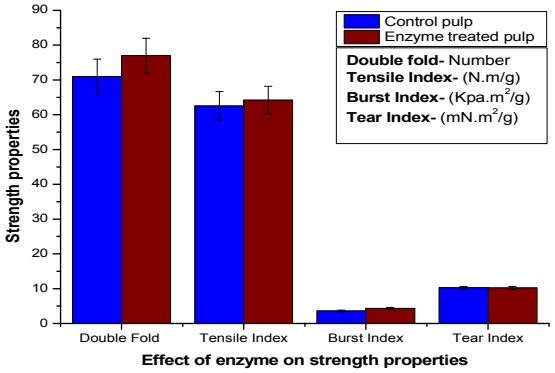


Fig. 5. Effect of enzyme on strength properties of refined pulp handsheets

It was observed that enzymatic hydrolysis during refining improved strength properties of paper cup waste pulp in respect of double fold number (7.79%), tensile index (2.57%) and burst index (17.36%) against control pulp. Increase in burst index would be highly beneficial. Since burst index is considered to be an important parameter for packaging paper.

Fiber morphology of cup waste pulp after refining

The SEM micrographs of paper cup waste pulp after refining clearly revealed increased fibrillations in enzyme treated pulp as compared to control pulp (Fig. 6a-b). Improved fibrillation in enzyme treated pulp may be responsible for increase in fiber to fiber bonding thereby resulting in improved strength properties viz: double fold, tensile index and burst index in comparison to control pulp

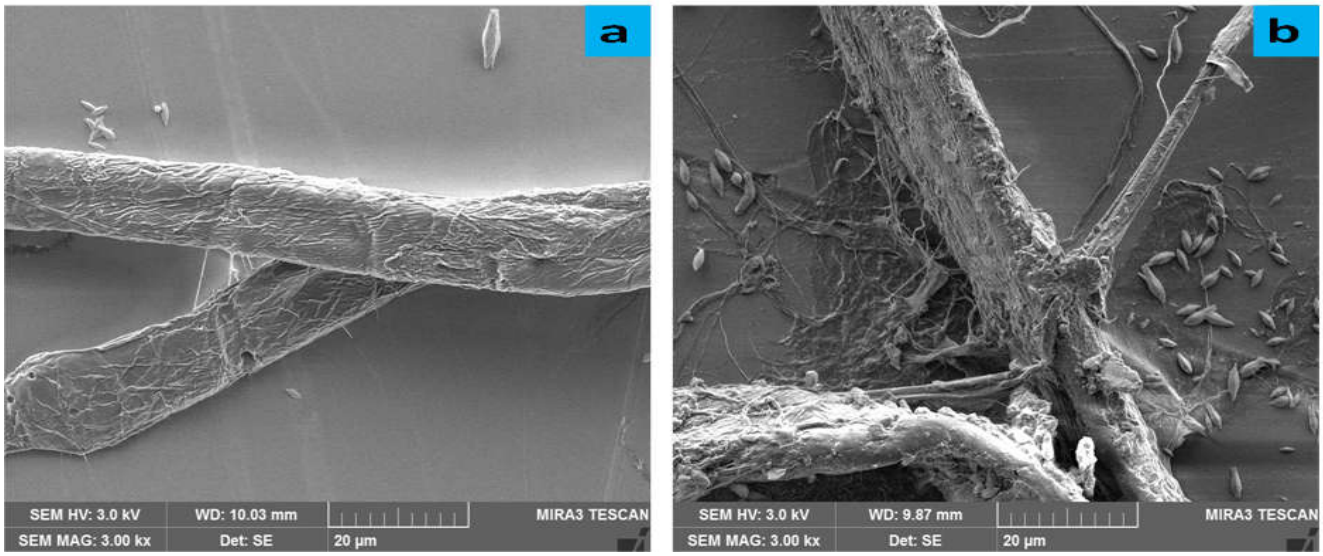


Fig. 6a-b. SEM photo micrographic images of (a) control and (b) enzyme treated refined pulp at 3.0 kx magnification

Conclusion

Keeping the objective to produce high activity level of cellulase enzyme, the fungus isolate *Trichoderma longibrachiatum* was selected from qualitative and quantitative approaches. Higher titer value of cellulase enzyme through SSF process under optimized environmental and nutritional factors may help in production of cost effective cellulase enzyme which find application as refining aid for production of quality paper from paper cup waste pulp. Enzymatic treatment of the paper cup waste pulp helped in around 25 % reduction of energy during refining with additional benefit in the form of strength properties like double fold, tensile index and burst index when compared to conventional refining process.. This showed future promotion of enzymatic refining process in Indian paper industry.

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