Potential of Xylanase from *Trichoderma viride* VKF3 in Waste Paper Pulp Characteristics Modification

Nathan Vinod Kumar, Mary Esther Rani, R. Gunaseeli, and N.D. Kannan

***Abstract*—**Lignocellulosic enzymes are usually used for pulp characteristics modification in paper and pulp industries on commercial scale. Among the prominent enzymes, xylanase is used for bio-bleaching and deinking of waste paper during paper recycling process. A potential xylanolytic fungal isolate was obtained from Valanthakad mangrove ecosystem soil which was identified as *Trichoderma viride* VKF3 based on molecular techniques. The crude enzyme produced by submerged fermentation following 7 days of incubation at room temperature was used for the pulp characteristics modification studies. Thin layer chromatography of enzyme hydrolysate confirmed xylan degradation thereby releasing of xylose. A maximum of 28 U/ml of xylanase activity was achieved using *T. viride* VKF3. Varying pulp consistency and enzyme dosage was used for understanding pulp characteristic changes. 10% enzyme per gram of oven dried pulp with 5% consistency was found to be optimal in relation with cellulose and hemi-cellulose content. FT-IR analysis of enzyme treated pulp samples had modifications in the functional groups. SEM analysis made it clear that the fiber surfaces were broken and thereby released the contaminant particles during enzymatic treatment. UV –Vis spectroscopic analysis of the effluent samples proved the release of phenolic and hydrophobic compounds into the effluent. Moreover, the physico-chemical properties of effluent were within irrigational standard limits. Low phytotoxicity was observed for the resultant effluent.

***Keywords*—**Paper pulp, xylanase, FT-IR, SEM, effluent characteristics

I. Introduction

THERE is an enormous need of energy and sustainable resource utilization to meet the emerging demands which directs a great research thrust on the ligno-cellulosic material around the world. Ligno-cellulosic materials including paper and textile are essential part of our day-to-day life. Hence there are many researches focused on the improvement of its quality and beneficial modifications. Paper is basically made up of fibers which are composed of cellulose, hemicelluloses and lignin. There are many ligno-cellulosic enzymes used in pulp characteristics modification. Xylanase is one among the major enzymes involved in the process.

Xylanase (EC 3.2.1.8) also known as hemi-cellulase, catalyzes the hydrolysis of the linear polysaccharide β-1,4 xylan to xylose. Xylan is the major constituent of hemi-cellulose and therefore xylanase along with other hydrolytic enzymes are used for hemi-cellulose hydrolysis. This enzyme is widely used in processing of food, feed, pulp and paper [1], [2]. In addition to these above applications, it is also used for bio-bleaching of wood pulp and bio-fuel preparations from lignocellulosic biomass [3]. Xylanase is produced by various microbial sources and among them certain filamentous fungi are the best producers [3], [4]. *Trichoderma* and *Aspergillus* are among the common fungi studied with xylan- degrading activity [5], [6], [7]. A prominent application of the enzyme is replacing of chlorine based bleaching of paper in the paper industries. Pulp bio-bleaching usually utilizes an enzyme stable in alkaline pH and is thermostable.

The present paper highlights the role of xylanase enzyme in modification of pulp characteristics based on its basic components. The enzyme act upon ligno-cellulosic substrate to release all contaminants and thereby contributing towards the paper quality enhancement during paper recycling process. However, the degree of fiber destruction beyond a threshold level is likely to hamper the paper quality based on its strength. Through this work an optimum pulp consistency and enzyme dosage was optimized for pulp characteristics modification during recycling of paper.

II. Materials And Methods

A. Isolation and Screening for Xylanase Producers

Soil and halophytic plant samples (leaf, stem and roots) were aseptically collected from Valanthakad mangrove ecosystem, Kerala, India (9° 55′10. 24″ N latitude and 76° 20′ 01.23″E longitude). Soil samples were serially diluted upto 10^{-2} dilution and spread plated onto Potato Dextrose Agar (PDA) plates. Similarly the plant parts were inoculated directly onto PDA and incubated at 28 ±2°C for 3-5 days. The

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colonies were purified and stored for further use. Screening of xylanolytic fungal isolates was done on PDA media supplemented with 2% brich wood xylan [8]. Xylanase producing fungi exhibit a clearing zone around the colony when counterstained with 1M NaCl solution. Iodine staining method was also used to confirm the substrate hydrolysis leading to zone formation [9].

B. Secondary Screening for Xylanase Production

The production medium was inoculated with potential fungal isolates and further incubated in shaking condition for 5-7 days at room temperature. Xylanase assay was performed on the culture filtrate to quantify the extracellular enzyme produced into the medium. Xylanase assay was performed based on DNS method using Birch wood xylan (BWX) as substrate [10]. The colour formation was measured at 540 nm and quantified by comparing D-xylose standard curve. One unit of xylanase activity was defined as the amount of enzyme releasing 1µM of reducing sugars equivalent to D-xylose per minute under standard assay condition.

C. Xylanase Enzyme Production

For the production of xylanase enzyme, culture inoculum was prepared by inoculating the fungal isolate into the fungal production medium and was incubated on a rotatory shaker at 120 rpm at room temperature for 3 days [11]. Following 3 days of incubation, 3% of fungal inoculum was added to the production medium supplemented with 1% dextrose and 1% peptone as carbon and nitrogen source respectively with 0.1% xylose as inducer. The flask was incubated similar to the above incubation conditions upto 7 days. On the 7^{th} day, culture was filtered through sterile mesh to obtain crude xylanase enzyme.

D. Kinetics of Enzyme Activity

The enzyme kinetics was studied based on the Lineweaver Burk (LB) plot using brich wood xylan as the substrate. Varying concentrations of substrate was used to determine the enzyme kinetics and LB plot was plotted to determine Km and Vmax values.

E. Validation of Xylanolytic Activity

The xylanolytic activity of the fungal isolate was validated using thin layer chromatography on silica gel plate. The standard brich wood xylan, standard enzyme treated xylan and potential fungal xylanase treated xylan was applied on TLC plate following 1 hour of incubation at 37°C. TLC was performed using acetone-ethyl acetate-acetic acid (2:1:1v/v) solvent system. The plate was developed by spraying a mixture of ethanol- sulphuric acid (95:5 v/v) and visualized by heating at 100°C until black spots become visible. D-xylose (5m/ml) was used as a standard [12].

F. Effect of Xylanase on Old Newspaper Pulp

Sorted newspaper waste was ground into fine pulp using hydrapulper and dried in an oven. Pulp was prepared by dispensing the required amount of oven dried pulp in distilled water to obtain specific consistency. Pulp was prepared with varying consistencies of 5, 7.5 and 10% and treated with constant enzyme dosage of 5% (v/w). Similarly, enzyme dosage was varied between the ranges of 5-15% (v/w) with known pulp consistency of 5%. The pulp was treated using standard commercial xylanase (SRL make) and using the crude xylanase obtained from fungal isolate for a time period of 1hr. After incubation period, the enzymes were heat inactivated by incubating at boiling water bath for 10-15 min.

G. Estimation of Cellulose, Hemi-cellulose and Lignin Content

Direct estimation of cellulose, hemi-cellulose and lignin was carried using standard method [13]. 2g of fiber was boiled in ethanol (4 times) for 15 min, washed thoroughly with distilled water and kept in oven for dry weight at 40°C overnight, then divided into two parts in which one part was considered as fraction A. Second part of residue was treated with 24% KOH for 4hrs at 25°C, washed thoroughly with distilled water, dried at 80°C overnight and the dry weight taken as fraction B. The same sample was again treated with 72% H_2SO_4 for 3hrs to hydrolyze the cellulose and refluxed with 5% H_2SO_4 for 2hrs. H_2SO_4 was removed completely by washing it with distilled water, dried at 80°C in oven for overnight and dry weight taken as fraction C.

Cellulose= B-C
Hemicellulose= A-B
Lignin= C

H. Characterization of Paper Pulp

The fungal enzyme treated and standard xylanase treated pulp were subjected to potassium bromide (KBr) pelleting and subjected to FT-IR analysis using FT-IR Thermoniocolet 385 at room temperature. Absorbance peak were assigned based on comparing with standard FT-IR spectrum. The treated paper pulp was analyzed using Scanning electron microscopy JOEL JSM 6290 for understanding the fiber rupture and changes on the fiber surface.

I. Characterization of Effluent Solution

After washing the enzyme treated pulp, the resultant wash-out solution could be considered as an effluent and it needs characterization before its disposal. Effluent characterization was performed using standard methods. Various parameters like colour, pH, electric conductivity, salinity, TDS (Multi parameter Tester), alkalinity, chlorine content, free chloride, iron, phosphate (Hi-Media Water Testing Kit), calcium, potassium and sodium (Elico CL361 Flame photometer) were estimated. Colour removal from the pulp was determined spectroscopic ally from λ_{200nm} to λ_{800nm}.

J. Phytotoxicity of Effluent Solution

For the determination of phytotoxicity of the resultant effluents, the individual wash-out solutions were pooled to make up a representative effluent sample. *Vigna radiata* L. seeds were surface sterilized for 10 min with 0.1% $HgCl_2$. The seeds were rinsed thrice with sterile distilled water and arranged in different petri-plates added with varying concentrations of effluent sample (10, 25, 50, 75 and 100%). Various parameters like germination percentage and percentage of phytotoxicity were compared with the treated and control seeds (treated with distilled water) [14].

III. Results And Discussion

A. Xyalanse Producing Fungal Isolation

Among the mangrove fungal isolates, 3 fungal isolates with potential xylanolytic activity was choosen for secondary screening based on the zone formation in primary plate screening method (Fig 1). During secondary screening, the most prominent enzyme producing fungal isolate was identified through D1/D2 region amplification and amplicon was sequenced. The obtained sequence chromatogram was analyzed and performed Blastn analysis to confirm that the isolate was *Trichoderma viride* VKF3. The enzyme activity of the *T. viride* VKF3 was found maximum of be 28 U/ml following 7 days of incubation at room temperature under shaking condition. The *T. viride* VKF3 also produces cellulase enzyme which was reported earlier [15]**.** Hence the present enzyme could be considered crude and as a consortium of cellulase and hemi-cellulase enzyme. Kinetics of the xylanase enzyme was studied by LB plot (Fig 2a). The enzyme had a K_m of 60.975 $mmolL^{-1}$ and V_{max} value of 0.0335 ms^{-1} using birch wood xylan as substrate. Enzyme activity was confirmed by performing TLC of the enzyme hydrolysate. The xylan was treated with xylanase enzyme and hydrolysate sample was used as sample for TLC. Xylose was used a standard. The release of xylose due to xylan degradation was detected on chromatogram which gave corresponding spots to the standard xylose (Fig 2b).

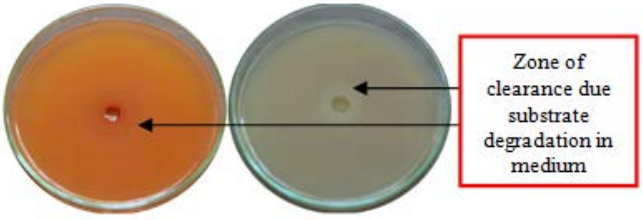


Fig 1 Plate assay showing hydrolysis of xylan substrate using crude enzyme filtrate of *T.viride* VKF3.

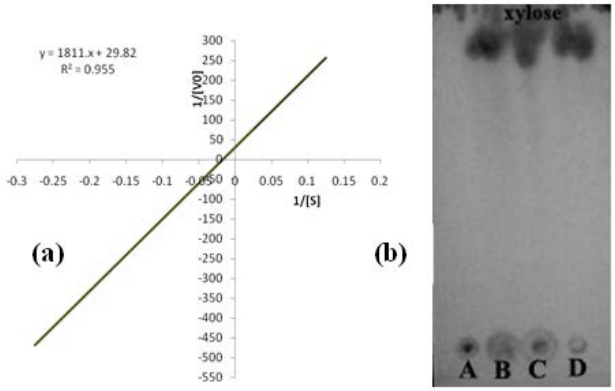


Fig 2 a) LB Plot Kinetics of xylanase enzyme produced by *T.viride* VKF3; b) Thin layer chromatography of xylanase hydrolysate-Lane A: Brich wood xylan (BWX); Lane B: BWX + standard xylanase; Lane C: BWX + *T. viride* VKF3 xylanase; Lane D: D-xylose standard

B. Estimation of Chemical Components in Pulp

A comparison of treated and untreated paper pulp was made based on the cellulose, hemi-cellulose and lignin content. Enzyme dosages (in %) and pulp consistencies were varied for analyzing the variations. At all consistencies and enzyme dosages tested, there was a decline in the hemi-cellulose and cellulose content. At highest consistency of 10%, there was a comparatively lower reduction in cellulose and hemi-cellulose content (Table 1). It could be inferred that at higher consistency, there was higher concentration of substrate and the available enzyme dosage was not sufficient to degrade the substrate. Moreover, the water activity was decreased during higher consistencies and thereby the enzymes may not be able access the available substrates in the absence of proper agitation or mixing process. It was found that 5 % pulp consistency with 10 % of enzyme could be apt for the beneficial pulp modification during paper recycling process.

C. Pulp Characterization

FT-IR spectrum of treated and un-treated pulp was compared. There were changes in peaks due to modification at functional group level during enzyme treatment (Fig 3). The changes in FT-IR spectrum was enlisted in Table 2. Fantahun *et al*., (2013) reported the FT-IR spectral changes in paper pulp during enzymatic treatment using bacterial xylanase and mannase. However, there were many bands similar to the earlier reports observed in the present work. SEM analysis of untreated pulp revealed that there was a compact network of fibers with smooth fiber surface. On enzymatic treatment, the fibers were broken and the compactness in fiber network was lost. The surface of the fiber also became rough due to degradation of fiber components mainly hemi-cellulose and cellulose. Fig 4E depicts the rough fiber surface with flake like appearance. Fig 4F and G showed total rupture of fiber

due to maximum enzyme dosage. Similar effects were also observed by many earlier workers [16],[17],[18],[19].

TABLE I
QUANTIFICATION OF PULP COMPONENTS AFTER ENZYME TREATMENT

Consistency of Pulp (%)		Enzyme Dosage (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Standard Enzyme- Xylanase					
EI	5	5	12	5.26	16.72
EII	7.5	5	14.2	18	16.31
EIII	10	5	37.8	23	16.12
EIV	5	10	47.8	12.88	16.77
EV	5	15	7.4	4.04	16.10
Enzyme consortium from *T. viride*					
EVI	5	5	7.9	7.2	8.96
EVIII	7.5	5	17.8	22.4	10.64
EIX	10	5	40	26.04	14.81
EX	5	10	11.8	11.8	7.54
EXI	5	15	6.94	1.6	6.35
CONTROL (Untreated Pulp)	5	-	48	28	16.8

TABLE II
FT-IR SPECTRUM RANGES AND FUNCTIONAL GROUP CHANGES IN THE SPECTRUM

Treatments	Ranges (cm^{-1})	Groups
IV	1429.2	Aromatic skeletal vibration in combination with $-OCH_3$
	611.9	Alkyl aldehyde, C-Cl stretch
III	1372.3	Aliphatic C-H stretching in CH_3 Phen-OH
	1508.5	Aromatic skeletal vibration
II	1040	C=S stretching
	3636.6	-OH stretching of H bonding
I	1638.9	Aromatic skeletal vibration, C=O stretch
	644.3	Alkyl aldehyde, C-Cl stretch
Control	613.1	Alkyl aldehyde, C-Cl stretch
	2924.5	Alcohol, C-H stretch
	3432.1	Alcohol, O-H Stretch

D. Physico-Chemical Characterization of Effluent

The effluents were characterized based on certain selected parameters. The analysis revealed that all the effluent samples tested were within the irrigational standard limits. There was a similar evaluation of paper mill effluent used for irrigational uses [20]**.** Phytotoxicity of the pooled effluent was checked against *V. radiata* L. and phytotoxicity of 0.1% observed only at highest effluent concentration (Fig 5). However there was a reduction in germination percentage when treated with 25% of effluent concentration and reduced further to 60% germination with 100% effluent treatment. The UV-Vis spectrum of the individual effluent samples was performed to note the colour removal (Fig 6). Maximum ink was released with highest enzyme dosage due to high degree of fiber damage which facilitates the ink release. However, during the recycling of paper, highest dosage of enzyme may cause severe fiber breakage and lower the subsequent paper quality. Hence, an optimal enzyme dosage of 10% could be used for maximal leaching of ink residues. Hydrophobic and phenolic compounds were also released into the effluent during enzymatic treatment which was also studied spectroscopically (Fig 7). There was an increase in absorbance due to release of contaminants as a result of fiber rupture at lower pulp consistency treated with maximum enzyme dosage.

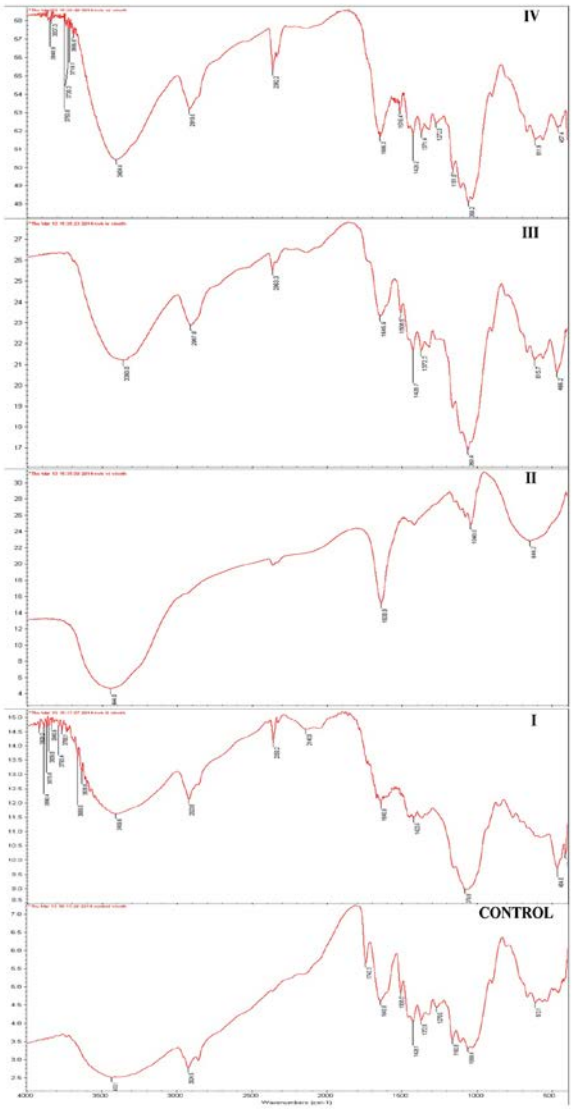


Fig 3 FT-IR profile of the *T. viride* VKF3 xylanase and standard enzyme treated paper pulp [*T.viride* VKF3 enzyme: I-5%C5%E; II-10C%15%E; Standard xylanase: III-5%C5%E; IV-10C%15%E; control: untreated pulp]

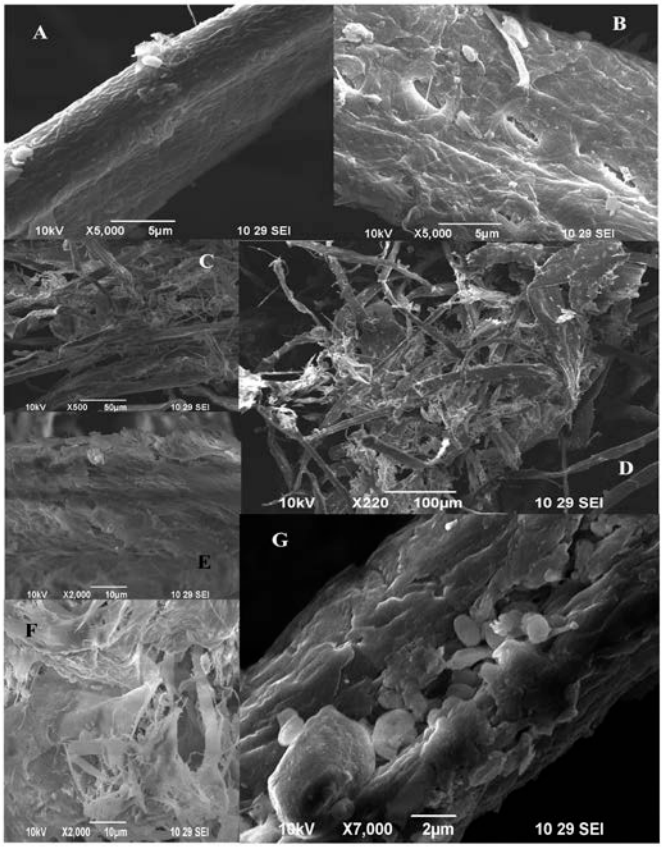


Fig 4 SEM of the paper pulp samples subjected to enzyme treatment

a) Control pulp sample without enzyme treatment
b) Pulp treated with standard xylanase enzymes showing breaking of fibers and surface modifications
c) Compactness of fibers in the control pulp samples
d) Dispersed fibers and breakage visible in *T. viride* xylanase treated pulp
e) Fiber surface toplogy changes f) Broken fibers and loss of compact network g) Total fiber damage at high enzyme dosage showing release of hydrolysates

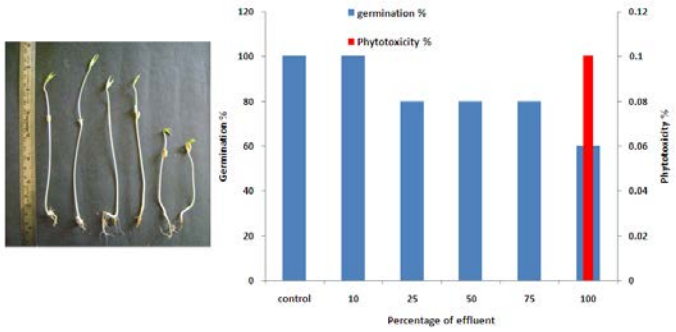
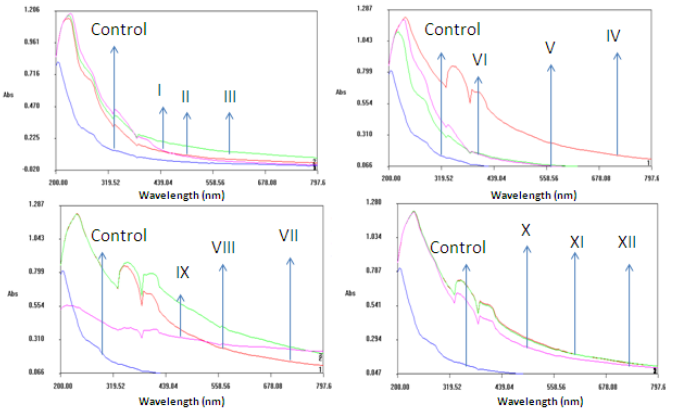


Fig 5 Phytotoxicity and germination percentage of *Vigna radiata* L. after treating with effluent sample.

TABLE III
PHYSICO-CHEMICAL CHARACTERISTICS OF RESULTANT EFFLUENT AFTER ENZYMATIC TREATMENT

Sample	Colour	pH	EC (µs)	Salinity (ppm)	TDS (ppm)	Na (ppm)	K (ppm)	Ca (ppm)
EI	TW	6.51	15.2	72.5	000	30.6	75.6	29.0
EII	TW	5.83	263	143	208	29.9	76.4	28.5
EIII	TW	5.82	000	000	24.1	43.9	76.5	22.0
EIV	TW	5.87	0.70	000	0.4	34.3	76.0	28.3
EV	TW	6.03	95.3	000	000	34.5	76.5	28.3
EVI	TW	5.51	0.10	000	000	27.4	74.5	28.4
EVII	TW	7.18	1209	292	620	40.9	55.3	24.7
EVIII	TW	7.19	1403	785	1.04	48.8	51.2	27.4
EIX	TW	7.22	4.60	14.6	2.80	39.2	76.7	25.0
EX	TW	7.58	556	362	2.50	40.6	55.5	24.3
EXI	TW	7.46	263	14.2	393	23.7	56.0	24.6
EXII	TW	7.10	1104	317	592	35.7	58.5	23.0
C	PB	7.58	2.10	14.3	154	37.5	76.3	27.4
BIS for irrigational water	-	5.5-9.0	-	-	1900 (mg/l)	-	-	200 mg/l



Control- Untreated pulp; Standard Enzyme: I- 5C%-5E%; II- 7.5C%-5E%; III-10C%-5E%; IV – 5C%-5E%; V- 5C%-10E%; VI- 5C%- 15E% ; *T.viride* :VII- 5C%-5E%; VIII-7.5C%-5E%; IX-10C%- 5E%; X – 5C%-5E%; XI- 5C%-10E%; XII- 5C%- 15E%

Fig 6 UV-Vis spectrum of effluent showing the ink released [TW- Turbid White, PB- Pale Brown]

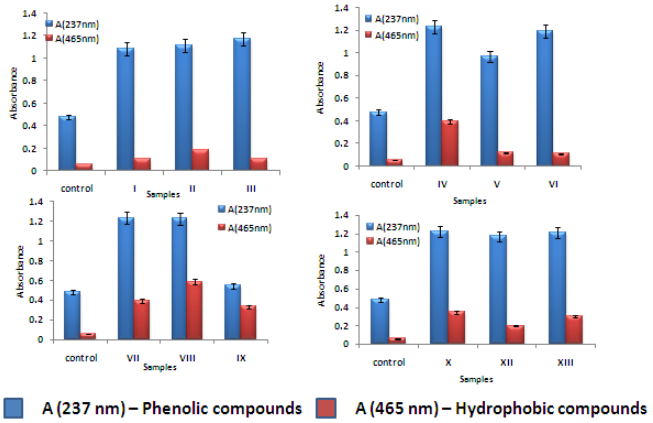


Fig 7 Release of phenolic and hydrophobic compounds into the effluents after enzymatic treatment

IV. CONCLUSION

An optimal consistency of 5% with 10% enzyme dosage could be used for paper pulp modification. There was reduction in hemi-cellulose and cellulose content which was directly related with the breakage in fiber and changes on fiber surfaces. These modifications were likely to be beneficial in maintaining the paper quality on subsequent recycling process by removal of contaminants like inks, phenolic and hydrophobic compounds. However due to the crude form of enzyme, there was a mixed action of cellulase-hemicellulase (xylanase) system acting upon and contributing to the variation in pulp characteristics. Effect of individual enzymes may not be sufficient for the above characteristic achievements and optimal dosage and consistency was critical factor for determining the final quality of recycled paper resulting from the enzyme treated paper pulp.

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