Low Molecular Weight Xylanase from *Trichoderma viride* VKF3 for Bio-bleaching of Newspaper Pulp

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Xylanase is a major enzyme used in the paper and pulp industries for bio-bleaching applications. There are possibilities for xylanase with better properties suitable for industrial applications. This paper focused on a potential xylanolytic fungus, *Trichoderma viride* VKF-3, obtained from a mangrove soil sample. Optimum conditions for xylanase production were tested by culturing *T. viride* VKF3 under varying carbon and nitrogen sources, medium pH, and incubation temperature. The isolate *T. viride* VKF3 achieved a maximum of 3.045 IU/mL of xylanase activity by utilizing coconut oil cake as a substrate. During purification, 84% yield was obtained with 40% ammonium sulphate. The enzyme activity was confirmed through zymogram analysis, and a band was observed at 14 kDa. The xylanase facilitated maximum hexenuronic acid release with a 30% enzyme dosage following 4 h of incubation. Moreover, the Kappa number tended to decrease with increased enzyme dosage and incubation time. There was a Δ brightness of 11% following 4 h of enzymatic treatment. Strength properties, such as the tensile, burst indices, and folding endurance, was improved during the xylanase assisted deinking of pulp. Hence, the present xylanase was found to be suitable for the bio-bleaching of newspaper waste *via* an eco-friendly process.

Keywords: Xylanase; Deinking; Trichoderma viride; Solid state fermentation, bio-bleaching

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INTRODUCTION

Microbial enzymes are extensively studied for various applications. Among these enzymes, lignocellulosic enzymes are widely utilized in many industrial processes. Fungal isolates are prominent enzyme producers and are easily cultivable. Filamentous fungi are potential producers of enzymes, like cellulase and xylanase, on a commercial scale that can easily be obtained from lignocellulolytic biomass (Olanbiwoninu and Odunfa 2016).

Researchers have identified the bio-bleaching potential and pulp modification properties of certain microbial enzymes, such as cellulase, xylanase, pectinase, mannase, laccase, and lipase (Raghukumar *et al*. 2004; Nathan *et al*. 2014; Chutani and Sharma 2015; Chutani and Sharma 2016). Their mode of action in bio-bleaching is to facilitate the residual ink removal by fiber breakage. Though there have been many reports on the bio-bleaching applications of such enzymes, they have not been widely used. Paper and pulp industries still prefer chemical deinking methods in spite of their negative effects on the environment. The biological deinking method using microbial enzymes is quite promising and eco-friendly, but highly challenging when compared to chemical deinking. Although

the combined use of the chemical and biological deinking methods has the potential to reduce the chemical consumption, the toxicity of the resultant effluent is to be resolved and can be achieved only by the biological method.

The current deinking process depends on the usage of many environmentally hazardous chemicals, namely sodium hydroxide, sodium silicate, sodium carbonate, hydrogen peroxide, chelating agents, and surfactants (Zhang *et al*. 2008; Pathak *et al*. 2011). The chemical-based deinking methods produce toxic effluents that increase the COD values of water and hence result in costly wastewater treatment (Zhang *et al*. 2008). Enzymes are used as alternative agents to overcome the problems of the polluting technologies (Srinivasan and Rele 1999). Enzymatic deinking has had to overcome the disadvantages of chemical treatment through its high efficiency and low environmental impact (Thomas 1994; Bajpai and Bajpai 1998; Pala *et al*. 2006). Bio-bleaching using enzymes could replace the use of chlorine and chloride compounds in the bleaching process **(**Raghukumar *et al*. 2004). Casimir *et al.* (1995) reported the application of white-rot fungi to degrade residual lignin in the pulp *via* lignolytic enzymes, such as manganese peroxidase and laccase, or by hemi-cellulolytic enzymes such as xylanase. The major problem of paper bleaching is the removal of lignin and its derivatives, which are linked to xylan and cellulose. Xylanases are more suitable in the paper and pulp industry than the lignin-degrading systems such as laccase (Subramaniyan and Prema 2002). Marques *et al.* (2003) studied the application of endoglucanases and endo-xylanases in the deinking of mixed office wastepaper. When the crude enzyme of *Trichoderma viride* was used, a 24% increase in ink removal was observed. Both enzymes contributed to the enhancement of the paper's strength properties and were found effective for the deinking of mixed office wastepaper.

In the enzyme-assisted biobleaching, large amounts of octachlorodibenzo-p-dioxin were formed that are toxic compounds. This dioxin production might be the result of promotion of the release of organic molecules bound to lignin and thus accelerating the formation of octachlorodibenzo-p-dioxin through organic molecular precursors. Previously, the enzyme-aided Cl_2 bleaching was considered to be efficient, but recent findings on dioxin compound formation have limited the enzyme usage for the bio-bleaching applications. However, the xylanase enzyme in the present work was used individually and not in combination with chloride bleaching and hence, the dioxin compound formation is limited.

Though many xylanase-producing strains have been reported from multiple microbial sources, certain enzymes were unstable and had poor efficiency in the deinking of paper waste. There is a need for prospecting a better enzyme for the application in the paper and pulp industry, as the paper pulp is rich in carbohydrates, especially cellulose. There is a possible feedback mechanism that would inhibit the possible enzyme action. This study evaluated the efficacy of the xylanase obtained from *Trichoderma viride* VKF3 in the deinking of old newspaper waste pulp and depicts the pattern of HexA and Kappa number changes during the enzymatic treatment. *Trichoderma viride* is a promising plant growth promoting fungus; after the enzyme production, residues of this fungus could potentially be used in agricultural fields. Moreover, no phyto-pathogenicity was detected for the fungus against the tested plant (*Vigna radiata*) in an earlier report (Vinod *et al*. 2014).

EXPERIMENTAL

Materials

Trichoderma viride VKF3, isolated from sediment samples of Valanthakad mangrove, Kerala, India (Nathan *et al*. 2014a), was inoculated into potato dextrose broth (Hi Media, Mumbai, India) and incubated at 27 ± 2 °C for 3 days. The fungal biomass was filtered using a sterile mesh (100 µm). The filtrate was then further centrifuged at 5,000 rpm for 5 min to obtain a clear supernatant, which was used as a crude enzyme.

Xylanase assay

Xylanase activity was evaluated using a standard method described below (Bailey *et al*. 1992). A 0.5 mL of culture supernatant was mixed with 1.5 mL of 10% birch wood xylan (BWX) (Sisco Research Laboratories Pvt. Ltd., Mumbai, India**)** in 0.1 M phosphate buffer (pH 8.0) and incubated at 50 °C for 15 min. The reaction was terminated by an addition of 2.0 mL of dinitrosalicylic acid (DNSA) (Sisco Research Laboratories Pvt. Ltd., Mumbai, India**)** reagent and further incubated in boiling water for 10 min. The color formation was measured at 540 nm and quantified by comparing the D-xylose standard curve. The amount of enzyme to release 1 µmol of reducing sugars equivalent to the D-xylose released from xylan/min under standard assay conditions which was defined as one unit of xylanase activity.

Optimization of fermentation conditions

Optimization of various fermentation conditions, namely carbon source, nitrogen source, medium pH, and incubation temperature were performed as previously described (Nathan *et al*. 2014a) using a fungal basal medium (K_2HPO_4- 0.20 g/L, KH_2PO_4- 0.18 g/ L, $NaHPO_4$- 2.0 g/ L, $NaNO_3$- 3.80 g/ L, H_3BO_3- 0.057 mg/ L, $MnSO_4.H_2O$- 5.5 µg/ L, $CuSO_4.7\ H_2O$- 2.5 µg/ L, $ZnSO_4.7\ H_2O$- 0.5 mg/ L, $Fe(SO_4)_3$. 6 H_2O- 5 µg/ L, $MgSO_4.7\ H_2O$- 5.5 µg/ L, NH_4NO_3- 0.60 g/ L, $(NH_4)_6MoO_{24}$- 0.025 mg/ L) (Jayant *et al*. 2011). The 3% fungal inoculum was added into the sterilized medium and incubated on a rotary shaker at 150 rpm for 3 days.

The xylanase activity was quantified from the 3rd day to the 11th day to understand the productive enzyme kinetics. The identified best carbon source was further used for nitrogen source optimization. Four different pH values (3, 5, 7, and 9) and incubation temperatures (25 °C, 35 °C, 45 °C, and 55 °C) were tested and evaluated for optimum xylanase production (best pH with best temperature).

Solid state fermentation for xylanase production

Enzyme production was evaluated on abundant solid substrates to reduce the production cost as described previously (Nathan *et al*. 2014a). The substrates were moistened with a basal fungal medium excluding the carbon and nitrogen sources. Substrates were maintained with 20%, 30%, and 50% of moisture with a basal medium and inoculated with a 3% fungal inoculum. The solid-state cultures were incubated at 28 ± 2 °C for 7 days.

Enzyme extraction was performed by the addition of phosphate buffer [pH 6.8] to the solid substrate followed by incubation for 3 h through filtration using a sterile mesh (100-µm). The recovered enzyme was subjected to partial purification by ammonium sulphate precipitation. The effect of metal ions, reducing, and oxidizing agents on enzyme activity was also tested and residual activity was expressed by a percentage.

Methods

Molecular and physico-chemical characterization of xylanase

A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the standard method reported earlier (Laemmli 1970). A 12% acrylamide gel was loaded with crude enzyme and protein mid-range markers. Following the electrophoresis, the gel was stained using Coomassie Brilliant Blue staining solution followed by destaining to visualize the protein bands and to determine the molecular weight of the enzyme fractions. For the zymogram analysis, Native PAGE was performed with 12% acrylamide gel supplemented with 1% (w/v) birch wood xylan. After electrophoresis, the gel was stained with 0.1% (w/v) Congo Red solution at room temperature for 30 min. Band of clearance was visualized at the fraction possessing xylanase activity. To evaluate the effect of metal ions on xylanase activity, the enzyme obtained was assayed for xylanase activity in the presence of known concentrations (5 mM, 10 mM, and 15 mM) of various metal ions, such as K^{+}, Mn^{2+}, Co^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, and Hg^{2+}. An Arrhenius plot was constructed and the activation energy of the enzyme was calculated.

Deinking experiments

The deinking experiment was performed using the oven-dried newspaper waste pulp that was dispensed into distilled water to obtain the optimized consistency. The crude enzyme was added in different dosages (20%, 30%, and 50% w/v) and incubated for 1 h or 4 h. After the enzyme treatment, the pulp was heat inactivated at 121 °C for 15 min and washed thoroughly in tap water. The recycled paper was made using deinked paper pulp in a Uni-Vat machine (TARA Machines, New Delhi, India) and shade-dried. Dried papers were subjected to calendaring and further analyzed for their improved brightness, tensile strength, pH of 5% solution, alkalinity, and opacity. A scanning electron microscopy (SEM) (JEOL JSM-6400, Peabody, MA, USA) analysis of the enzyme-treated and untreated paper pulp was performed to compare the morphological changes as described earlier (Nathan *et al.* 2014b).

Determination of hexenuronic acid and Kappa number

The hexenuronic acid (HexA) and Kappa number of paper pulp are considered as critical factors in understanding the efficiency of enzymatic bio-bleaching. Hexenuronic acids (HexA) are unsaturated compounds formed during kraft pulp cooking, by conversion of 4-*O*-methylglucuronic acid present in xylans to its corresponding unsaturated hexenuronic acid. In the recent years, HexA have attracted much attention owing to their adverse effects on pulp properties after bleaching. They are known to contribute to Kappa number, brightness reversion, oxalic acid formation, bleaching agent consumption and metal ion retention. Hence determination of HexA release during biobleaching is critical.

Hexenuronic acid of the oven-dried pulp was determined by a method described below. Briefly, 0.05 g of oven-dried pulp was hydrolyzed with 10 mL of hydrolyzing solution that contained 0.6% $HgCl_2$ and 0.7% sodium acetate. The reaction mixture was incubated in a water bath at 60 °C for 30 min. Absorbance of the solution was read at 260 nm and 290 nm. The Hexenuronic acid content of the pulp was calculated by Eq. 1 (Carvalho *et al.* 2000; Chai *et al*. 2001),

$$C = 0.287 \times \frac{(A260 - 1.2\ A290)}{w} \times v \quad (1)$$

where *v* is the volume of hydrolysis solution (mL) and *w* is the weight of oven-dried pulp (g). The Kappa number is the volume of 0.1 N potassium permanganate solution consumed by 1 g of moisture-free pulp under standard conditions. The Kappa number is proportional to HexA and hence tend to increase based on HexA concentration. The delignification and bleaching ability of the pulp was expressed in terms of Kappa number change. It was detected using the TAPPI T236 om-99 (2004) standard.

RESULTS AND DISCUSSION

Xylanase Production Optimization using *Trichoderma viride* VKF3

Trichoderma viride VKF3 was earlier identified as a potential cellulase-producing isolate (Nathan *et al*. 2014a) that also has potential for xylanase production. After the 3rd day of incubation, an enzyme assay was performed using the culture filtrate, and sucrose was found to be the suitable carbon source. However, dextrose could achieve a comparable activity on the 7th day of incubation itself at room temperature. Xylose, when used as carbon source, yielded low enzyme activity. It was noted that xylan as a carbon source or as an inducer had a less noticeable increase in xylanase activity by *T. viride* (Simoes *et al*. 2009), in *Aspergillus* sp. (Gawande and Kamat 2000), *Trichoderma harzianum* (Ahmed *et al*. 2003), and *Streptomyces* sp. (Rawashdeh *et al*. 2005). Glucose and xylose, when used as a carbon source, affected the enzyme synthesis negatively (Battan *et al*. 2006). This inhibition may be due to the catabolite repression mechanism occurring in other enzymes (Kermnicky and Biely 1998). However, there are many strains such as *T. viride* showing high xylanase activity with glucose and maltose as carbon sources (Simoes *et al*. 2009). Sucrose gave the highest xylanase activity at the end of the 7th day of incubation, by which time there was a decline in enzyme activity in other carbon sources tested (Table 1).

Supplementation with urea as a nitrogen source could achieve a maximum xylanase activity by the 5th day of incubation, but there was a steep decline of activity by the 7th day. Other nitrogen sources also achieved maximum enzyme activity by the 5th day, but the values were low compared to the urea containing medium. During the pH optimization, it was found that 7 days of incubation could obtain maximum xylanase activity. There was an exponential increase in xylanase activity from the 3rd day, and a slight decline of enzyme activity was observed following the 7th day. Hence, it was found that the optimal recovery time of xylanase was on the 7th day of incubation. The maximum xylanase activity was achieved using *T. harzianum* on the 7th day of incubation (Seyis and Aksoz 2005). The declining trend observed in enzyme activity might be due to the depletion in macro- and micronutrients in the fermentation medium, which induced stress on fungi, thereby inactivating the enzyme secretory pathway (Simoes *et al*. 2009).

Incubation temperature is an important factor determining the enzyme production (Seyis and Aksoz 2005). The incubation temperature of 50 °C achieved a maximum xylanase activity at the 5th day of incubation followed by a decline of activity (Table 1). This showed the thermophilic nature of the enzyme compared to the previous reports.

A maximum xylanase activity of 93.5 U/mL was found at 25 °C (Simoes *et al*. 2009). Simoes *et al*. (2009) reported that the production of xylanase by *T. viride* started from the 2nd day achieving maximum activity on the 6th day, followed by a decline on day 7. Sater and Said (2001) observed maximum activity by 8th day for the xylanase obtained from *T. harzianum.*

Table 1. Optimization of Factors for Xylanase Production using *T. viride* VKF3 under Submerged Fermentation (Xylanase Activity in U/mL)

Parameters	3rd Day	5th Day	7th Day	9th Day	11th Day
Carbon Sources					
Dextrose	0.66 ± 0.21	0.90 ± 0.15	0.85 ± 0.24	0.55 ± 0.21	0.45 ± 0.20
Sucrose	0.80 ± 0.02	0.97 ± 0.07	1.78 ± 0.32	0.43 ± 0.13	0.00 ± 0.00
Xylose	0.55 ± 0.17	0.95 ± 0.11	0.90 ± 0.31	0.49 ± 0.09	0.00 ± 0.00
CMC	0.00 ± 0.00	0.47 ± 0.19	0.99 ± 0.26	0.58 ± 0.17	0.00 ± 0.00
Nitrogen Sources					
Peptone	3.00 ± 0.12	9.70 ± 0.11	27.50 ± 0.05	7.40 ± 0.15	0.00 ± 0.00
Beef Extract	6.75 ± 0.16	7.55 ± 0.18	4.05 ± 0.08	3.95 ± 0.23	0.00 ± 0.00
Sodium Nitrate	7.40 ± 0.03	8.90 ± 0.07	10.20 ± 0.16	6.10 ± 0.18	6.00 ± 0.11
Ammonium Nitrate	0.00 ± 0.00	3.00 ± 0.04	10.00 ± 0.11	6.25 ± 0.21	0.00 ± 0.00
Medium pH					
3	12.20 ± 0.17	17.00 ± 0.06	13.50 ± 0.11	7.00 ± 0.07	1.50 ± 0.11
5	32.50 ± 0.25	22.00 ± 0.12	7.50 ± 0.13	3.50 ± 0.04	0.00 ± 0.09
7	27.00 ± 0.32	35.00 ± 0.02	11.00 ± 0.18	0.00 ± 0.09	0.00 ± 0.00
9	13.00 ± 0.11	17.00 ± 0.09	12.00 ± 0.21	6.00 ± 0.02	1.20 ± 0.17
Incubation Temperature					
25 °C	22.5 ± 0.18	21.5 ± 0.15	11.5 ± 0.10	5.5 ± 0.15	5.0 ± 0.17
30 °C	26.5 ± 0.23	23.4 ± 0.13	3.5 ± 0.24	0.0 ± 0.00	0.0 ± 0.23
40 °C	15.5 ± 0.31	18.5 ± 0.11	24.8 ± 0.19	0.0 ± 0.00	0.0 ± 0.00
50 °C	23.5 ± 0.09	28.5 ± 0.07	2.5 ± 0.05	0.0 ± 0.00	0.0 ± 0.00

[mean ± S.D values of triplicate experiments]

Solid State Fermentation (SSF) for Xylanase Production

Operation simplicity, high productivity, low feasible contamination, and high concentrated product formation are the major factors that attract industries to convert to SSF instead of submerged fermentation (Gupta *et al*. 2001). The SSF plays an important role in solid waste management, where the solid substrates used are usually agro-wastes. Hence, the utilization or degradation of the solid matter is very important in this process. During SSF, fungal biomass utilizes solid waste for growth, and further as an energy source for enzyme production.

In the present study, coconut oil cake was found to be a reliable substrate for xylanase production, which was evident from its higher activity (Table 2). However, there was a positive correlation with enzyme production and moisture content. There was a high enzyme activity achieved in SSF compared to submerged fermentation due to the low catabolite repression, which might be attributed due to the slow diffusion process because of low water activity (Gupta *et al*. 2001).

Table 2. Xylanase Production using *T.viride* VKF3 under SSF Utilizing Various Substrates

Substrates	Moisture (%)	Xylanase Activity (IU/mL)	Protein Concentration (µg/mL)
Coconut Oil Cake (COC)	20	1.321 ± 0.14	204.6 ± 0.321
	30	1.934 ± 0.11	197.3 ± 0.115
	50	3.045 ± 0.23	284.8 ± 0.175
Groundnut Oil Cake (GOC)	20	0.783± 0.27	105.4 ± 0.121
	30	0.657 ± 0.18	213.8 ± 0.203
	50	2.310 ± 0.16	193.7 ± 0.321
Neem Oil Cake (NOC)	20	0.213 ± 0.32	109.3 ± 0.112
	30	0.895 ± 0.22	189.0 ± 0.321
	50	0.734 ± 0.25	197.0 ± 0.127
Rice Bran (RB)	20	0.923 ± 0.09	111.2 ± 0.184
	30	1.227± 0.15	171.4 ± 0.061
	50	1.872± 0.17	198.7 ± 0.117
Sugarcane Bagasse (SB)	20	0.812± 0.24	122.3 ± 0.078
	30	1.112 ± 0.21	249.0 ± 0.129
	50	2.108± 0.32	272.6 ± 0.011

[mean ± S.D values of triplicate experiments]

Enzyme Characterization

After the incubation period, the substrate was flooded with the phosphate buffer and filtered through a sterile mesh to obtain a crude enzyme. Partial purification of the enzyme was performed using ammonium sulfate, and 40% ammonium sulfate yielded maximum enzyme activity with a yield of 84% (Table 3).

Table 3. Purification Yield of Xylanase from *T.viride* VKF3

Ammonium Sulphate (%)	Enzyme Activity	Specific Activity	Purification Fold	Yield (%)
40	3.054	93.53	2.5	84

Xylanase obtained from *T.viride* VKF3 exhibited various bands at approximately at 66 kDa, 52 kDa, 35 kDa, 22 kDa, and 14 kDa (Fig. 1). A zymogram analysis was performed using gel with birch wood xylan as the substrate stained with Congo red. This approach has been widely used for confirmation of xylanolytic activity and to identify the fraction possessing the activity. From the analysis, two distinct zones of clearance were observed between 43 kDa to 66.0 kDa and at 14 kDa. In a previous study, the molecular masses of the purified xylanase estimated by SDS-PAGE and gel filtration were, respectively, 19 kDa and 14 kDa for Xyl I, 21, 14.6 kDa for Xyl II (Silva *et al*. 2015). The activation energy of xylanase was calculated using the Arrhenius equation and found to be 60.877 KJ mol^{-1}.

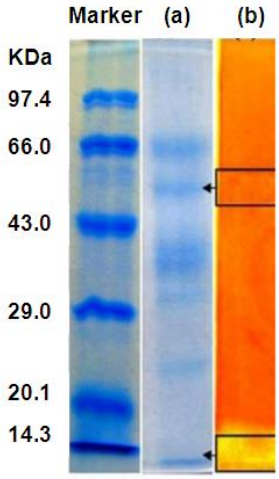

Fig. 1. SDS-PAGE and zymogram analysis of xylanase from *T.viride* VKF3; Lane a) SDS-PAGE and b) zymogram with birch wood xylan as substrate

Table 4. Residual Xylanase Activity in the Presence of Metal Ions

Metal Ions	Concentrations (mM)	Residual Activity (%)
Control	0	100 ± 0.14
K^+	5	100 ± 0.21
	10	88 ± 0.23
	15	81 ± 0.15
Mn^{2+}	5	98 ± 0.11
	10	90 ± 0.08
	15	81 ± 0.17
Co^{2+}	5	99 ± 0.13
	10	92 ± 0.11
	15	91± 0.05
Fe^{3+}	5	97 ± 0.10
	10	90± 0.14
	15	86 ± 0.24
Cu^{2+}	5	100 ± 0.18
	10	96.4 ± 0.22
	15	79 ± 0.14
Zn^{2+}	5	100 ± 0.19
	10	100 ± 0.27
	15	92 ± 0.13
Mg^{2+}	5	100 ± 0.11
	10	91 ± 0.20
	15	67 ± 0.32
Hg^{2+}	5	64 ± 0.07
	10	52 ± 0.14
	15	49 ± 0.12

[mean ± S.D values of triplicate experiments]

Effect of Metal Ions on Xylanase Activity

Xylanase from *T. viride* VKF3 experienced reduction in its relative activities with the addition of metal ions. Lower concentrations of K^+, Cu^{2+}, Zn^{2+}, and Mg^{2+} had no effect on xylanase activity (Table 4). However, in previous studies, certain metal ions such as

Zn^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Ca^{2+} were positive for xylanase activity (Guan *et al*. 2016). In the present study there was a low reduction in xylanase activity in the presence of Cu^{2+}. However, some xylanases were completely inhibited with the Cu^{2+} (Guan *et al*. 2016). Basically, the metal ion inhibitions or enhancements are directly related with the interaction with the bonds in the protein structure. Certain metal ions also influence the conformation of proteins and hence tend to change their enzymatic actions. It was previously observed that the metal ions may not affect only the active site of the xylan-binding xylanase but also the noncatalytic xylan-binding region, which is involved in the efficient hydrolysis of the substrate thereby a reduction in enzyme activity could be observed (Chauvaux *et al*. 1995; Choi and Ljungdahl, 1996; Spurway *et al*. 1997).

Bio-bleaching of Newspaper Pulp using Xylanase

Xylanase was found to be a potential bio-bleaching agent in the deinking process. The deinking efficiency of the microbial enzymes is usually determined by the Hex-A release and the Kappa number change of the paper pulp. Xylanase obtained from *T. viride* VKF3 was used for understanding the bio-bleaching potential. The same pulp was treated with the enzyme for different time periods. The morphology of the paper pulp was changed during the enzymatic treatment. This was depicted through the SEM analysis of xylanase-treated and untreated pulp as the control (Fig. 2). Defibrillations and crack formations were visible on the fiber surface, which was in agreement with the observation made by Chutani and Sharma (2015). The xylanase facilitated maximum Hex-A release with 30% enzyme dosage within 1 h and 4 h of incubation. However, 4 h of incubation was considered most efficient (Fig. 3). The Kappa number of the paper pulp is also a factor to understand the deinking efficiency of enzymes. In the case of the xylanase treatment, the Kappa number tended to decrease with increased enzyme dosage and incubation time. Generally, the Kappa number exhibited an inverse relationship with the Hex-A concentration. In 2010, Valls *et al*. demonstrated the efficiency of xylanase in the bleaching of eucalyptus kraft pulp. There was a 10% increase in Hex-A removal after the chlorine dioxide treatment. Xylanases exhibited an increased delignification of 9% and brightness of 3% ISO.

Hexenuronic acids (HexA) of hemicellulosic heteroxylan were shown to play an important role in brightness development of chemical pulps during xylanase-aided bio-bleaching (Shatalov and Pereira 2009). The elimination of HexA in kraft pulps was shown to be also beneficial to Totally Chlorine Free (TCF) pulp bleaching operations, thereby achieving higher brightness values. Another parameter is Kappa number, which is an indication of the residual lignin content or bleachability of wood pulp. The Kappa number varies according to the raw material of the pulp and ranges from 25-30, 45-55, and 60-90 for bleachable pulps, sack paper pulps, and corrugated fiberboard, respectively. Moreover, the oxygen delignification efficiency is rather low for low-kappa pulps containing high Hex A concentrations (Eiras and Colodette 2003; Colodette *et al.* 2006) since oxygen does not react with HexAs (Vuorinen *et al.* 1996). There is an inverse relation with Hex A and Kappa number and the assumption is that 10 mmol/kg pulp of HexA is equivalent to 1 kappa unit (Vuorinen *et al.* 1996).

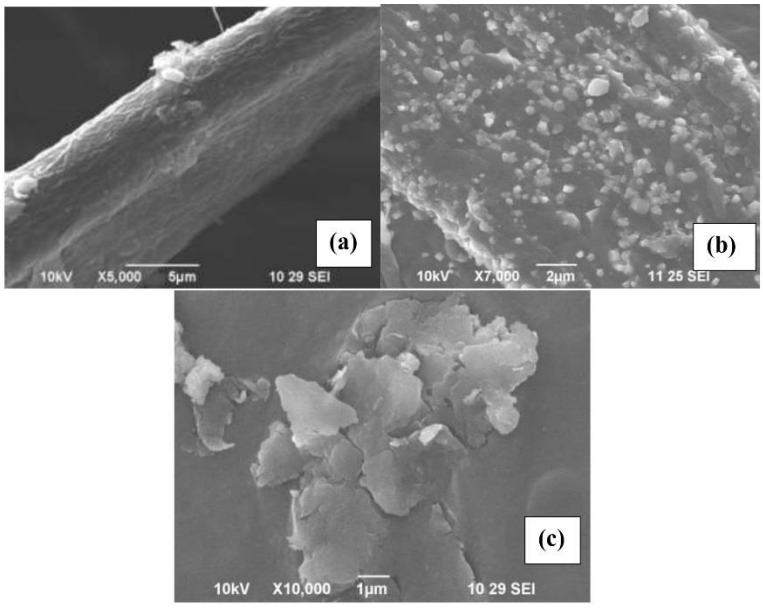

Fig. 2. Scanning electron micrograph of paper pulp treated with xylanase from *T. viride* VKF3: a) control-untreated pulp, b) treated pulp, and c) crack formation on fiber surface

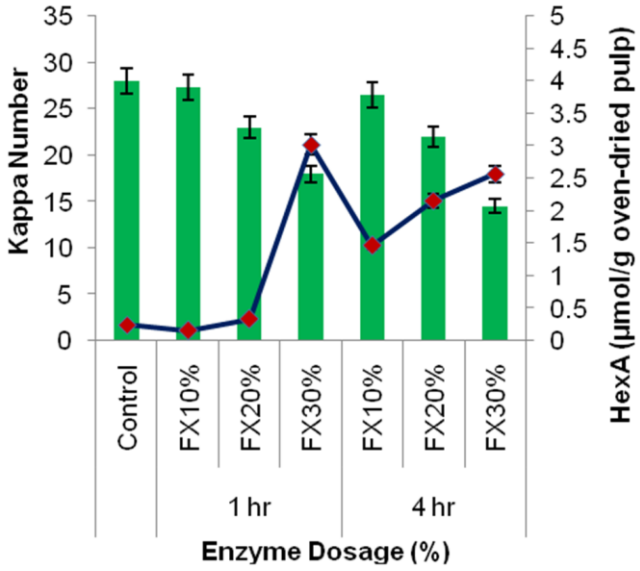

Fig. 3. Hex-A content and Kappa number of paper pulp treated with xylanase enzymes produced by *T. viride* VKF3

During the enzymatic treatment, the brightness of the recycled paper made from deinked pulp was found to be higher than that of the control (untreated) pulp (Table 5). There was a Δ brightness of 9% and 11% following 1 h and 4 h enzymatic treatments, respectively. There was an increased tensile strength observed following 1 h of incubation but it declined after 4 h incubation. This was supported by a report where deinked newspaper pulp samples showed a brightness 9.6% higher than its control sample (Chutani and Sharma 2016). Opacity of the paper was also improved compared to the control paper. The pH and alkalinity had a slight shift during the enzymatic treatment; however, they were no substantial change. The crude enzyme-deinked pulp showed 23.6% higher deinking efficiency and 3.2% higher brightness than chemically-deinked pulp. Strength properties

like tensile, burst indices, and folding endurance were also observed to increase by 6.7%, 13.4%, and 10.3%, respectively, for enzyme-deinked pulp. However, the tear index decreased 10.5%. The freeness of the pulp also increased 21.6% with a 13.9% reduced drainage time (Pathak *et al*. 2014).

The efficiency of xylanase in the bleaching of kraft pulp from eucalyptus was described by Valls *et al.* (2010). However, it was noted that xylanase did not greatly bleach the pulp, but there was a 10% increase in Hex-A removal after the chlorine dioxide treatment. Between the two xylanases studied, one exhibited an increased delignification of 9% and brightness of 3% ISO. A similar observation was also reported by Maity *et al*. (2012) and Woldesenbet *et al*. (2012).

Maity *et al.* (2012) observed that, after the enzymatic deinking using the bacterial xylanase from *Bacillus* sp., the appearance properties of the pulp, such as brightness and ERIC values, were enhanced, whereas the pulp opacity was much reduced when compared to the control. Woldesenbet *et al*. (2012) also reported that microwave irradiation could enhance xylanase mediated bio-bleaching with increased brightness of 1% ISO, decreased Kappa number by 14.3%, and 20% reduction in chlorine consumption. In (2013), Woldesenbet *et al*. reported that alkalophilic bacterial xylanase could achieve a 40.7% reduction in the Kappa number and 38.3% Hex-A content with increased brightness of 31.5% ISO. There was an increase in breaking length by 39%, burst factor by 20.6%, tear factor by 20.2%, and pulp viscosity by 12.1%. Similarly, the deinking of old newspaper pulp with a combination of xylanase and laccase enzyme resulted in an increase in brightness to 21.6% ISO, breaking length (16.5%), burst factor (4.2%), tear factor (6.9%), viscosity (13%), and cellulose crystallinity (10.3%), along with a decrease in the Kappa number (22%) (Virk *et al*. 2013). The present xylanase was found to be suitable for the bio-bleaching of newspaper waste with enhanced quality and brightness compared to the previous reports.

Table 5. Bio-bleaching Efficacy of Xylanase Produced by *T. viride* VKF3

Parameters	Control	Treatment Time	
		1 h	4 h
Brightness (% ISO)	27	36.6	38.0
Breaking length/Tensile strength (m)	485	690	400
Opacity (%)	90	97	99
pH of 5% Solution	7.6	7.8	7.2
Alkalinity (ppm)	2000	2400	2000

CONCLUSION

1. *T. viride* VKF3 was found to be a potential isolate for production of the xylanase enzyme for the application of a biological deinking process.
2. The solid-state fermentation offered a cost effective and easy production of the enzyme.

3. The enzyme was found to achieve a better brightness to old newspaper pulp after treatment for 4 h.
4. The process also sustained the properties of recycled paper compared to the untreated control even after enzymatic treatment.
5. The Kappa number and Hex-A exhibited an inverse relation and contributed to achieve a maximum brightness of 38% during enzymatic deinking.

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