

OPTIMIZATION OF BIOTECHNOLOGICAL PARAMETERS AND CHARACTERIZATION OF CATALYTIC PROPERTIES OF THERMOSTABLE POLYGALACTURONASE FROM A CHROMIUM TOLERANT STRAIN OF *TRICHODERMA PSEUDOKONINGII*

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Abstract

The hexavalent chromium tolerant strain of *Trichoderma pseudokoningii*, isolated from chromium enriched tannery effluent was found to produce extracellular polygalacturonase even in presence of chromium. Although there was a negative correlation between the amount of hexavalent chromium present in the cultivation medium and amount of polygalacturonase produced, moderate amount (300 U/ml) of enzymes was found to be produced even in presence of high amount of hexavalent chromium (0.80 gm/ml) in medium. The strain was found to synthesise maximum level of polygalacturonase when the culture medium was supplemented with 1.5 % (w/v) pure pectin as sole carbon source at pH 7.0 and 37°C. The highest level of extracellular enzyme synthesis could be achieved at 72nd hour of growth. The enzyme was stable under a wide range of pH (5.0-8.0) and up to 80 % of enzymatic activity was retained even after exposure to 90°C for 30 minutes.

Keywords: Chromium tolerant fungi, *Trichoderma pseudokoningii*, thermostable polygalacturonase

Introduction

Pectin, a structural heteropolysaccharide of galacturonic acid units present in the primary cell walls of terrestrial plants can be hydrolysed by pectinases, a group of enzymes that include polygalacturonase, pectin lyases and pectin methyl esterase (Pedrolli *et al.*, 2009). Pectinolytic enzymes having great industrial importance are required for food processing industries, especially for extraction and clarification of fruit juices,

extraction of oils, flavors and pigments from plant materials, textile (Phugare *et al.*, 2011), pharmaceutical, leather, detergent and paper (Reid and Ricard, 2000). Among the pectinases, polygalacturonase (EC 3.2.1.15) hydrolyses the 1, 4 α -D galacturonic acid linkages of pectin. It has attracted the attention of biotechnologists for its significant role in phytopathogen invasion, fruit ripening as well as potential anti microbial drug target (Agüero-Chapin *et al.*, 2009). Although

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commercially, pectinase has a share of 25% in the global sales of food enzymes (Jayani *et al*, 2005) and 10% of total enzyme production (Mukesh kumar *et al*, 2012), higher cost of the production is perhaps the major constraint in commercialization of new sources of enzymes (Siddiqui *et al*, 2012). At present almost all the pectinolytic enzymes used for industrial applications are produced by fungi (Dey *et al*, 2011) and a number of report is available on polygalacturonase producing strains of *Trichoderma* (Markovič *et al*, 1985; Mohamed *et al*, 2003, Mohamed *et al* 2009; Arotupin and Ogunmolu, 2011), no report is available from any chromium resistant strain of *Trichoderma pseudokoningii*. Research is going on heavy metal removal by various *Trichoderma* strains (Morales-Barrera and Cristiani-Urbina 2008; Singh *et al*, 2012), but so far the literature study is concerned, this may be the first report of the production of extracellular polygalacturonase from a chromium resistant strain.

Present study includes the optimization of production parameters and characterisation of catalytic properties of the extracellular polygalacturonase produced by a chromium resistant strain of *Trichoderma pseudokoningii*.

Materials and methods

Chemicals

All chemicals used were of analytical grade purchased from Sigma chemicals Co. Merck, Germany and Himedia, India.

Microorganism

The fungal strain was isolated from the chromium enriched tannery effluent mixed soil around the leather industry at Kolkata, India. The strain was identified as *Trichoderma pseudokoningii* by Agharkar Research Institute, Pune, India. The stock cultures were maintained on PDA agar, at 4°C.

Cultivation for enzyme biosynthesis

The strain was cultivated statically in 100 mL Erlenmeyer flasks each containing 10 mL Basal Medium (BM) composed of (g l⁻¹): peptone 0.9;

(NH₄)₂HPO₄ 0.4; KCl 0.1; MgSO₄·7H₂O 0.1, and pure pectin 15 (pH 7.0) for 48 hours at 28°C. To estimate the relation between chromium tolerance and polygalacturonase production, the cultivation media were supplemented with various concentration of K₂Cr₂O₇ (0 - 0.35 g l⁻¹).

Enzyme Assay

The biomass was centrifuged (REMI C-24, India) at 10,000 rpm for 10 min and the supernatant was used as the crude enzyme. To measure the activity of polygalacturonase, the assay mixture (1 ml) containing an equal volume of enzyme and 1% (w/v) pectin dissolved in 0.1M phosphate buffer (pH 7.0) was incubated at 55°C for 10 min. The reducing sugar released was measured by the dinitrosalicylic acid method (Bernfeld, 1955). Control samples were prepared with inactivated enzymes. One unit of enzymatic activity (U) was defined as one μmol of galactunoric acid released per minute (Trejo-Aguilar *et al*, 1996). For further characterization, the enzyme was subjected to 60-80% (w/v) ammonium sulphate precipitation followed by overnight dialysis against in 0.1M phosphate buffer (pH 7.0).

Optimization of enzyme production parameters

The concentrations of pectin used as sole carbon source were varied from 0.5%-3% to optimize the substrate concentration of submerged culture of *Trichoderma pseudokoningii*. The optimum pH was determined by adjusting the initial pH of the fermentation media at a range from pH 4.0-9.0. Most favourable production temperature was studied by incubating the culture medium at different temperatures (7- 55°C). Similarly, the effects of various nitrogen sources namely peptone, gelatine, ammonium sulphate, tryptone, potassium nitrate, and urea 0.9% (w/v) and various additives (10 mM) were tested. The time course of growth and enzyme production by the strain under optimized culture conditions were studied by checking the enzyme production kinetics for 0 to 120 hours at 37°C.

Characterization of catalytic properties of the enzyme

The effect of pH on the activity of the extracellular enzyme was tested by assaying it at various pHs adjusted by 0.1 mM suitable buffers like acetate buffer (pH 4.0 and 5.0), phosphate buffer (pH 6.0 – 8.0), Tris-HCl buffer (pH 9.0). Similarly, the stability of the enzyme at different pH values was studied by keeping the enzyme at various pHs overnight, followed by the estimation of the residual enzyme activity. Temperature optimum was determined by incubating the enzyme with substrate (pure pectin) at temperatures ranging from 27-90°C at pH 7.0

Thermoinactivation kinetics was determined by exposing the enzyme at various temperatures (45°C to 90°C), at pH 7.0 for 30 minutes in water bath and then estimating the residual activity.

The effect of metal ions and thiol compounds were measured by incubating the enzyme at 28°C and pH 7.0, for 30 minutes with the additives at a concentration of 10mM followed by the assay of enzyme activity in usual procedure.

All experiments are done in triplicate and the values are averaged.

Result and discussion

The studied fungal strain was found to produce extracellular polygalacturonase even in presence of hexavalent chromium and there was a distinct negative correlation between the amount of chromium present in the medium and production of extracellular polygalacturonase. Although the enzyme production was found to reduce in presence of hexavalent chromium, the studied strain could produce around 300 U/ml crude enzyme even in presence of 0.80 gm/litre hexavalent chromium (Fig. 1), such tolerance was much higher than the chromium (VI) reducing ability of *Arthrobacter aureus* (Horton *et al*, 2006) and *Pseudomonas aeruginosa* (Chatterjee *et al*, 2011).

Enzyme production was found to increase gradually with increase in pure pectin

concentration and highest production was achieved at 1.5% (w/v) of pure pectin (Fig. 2), an observation in agreement with that obtained by MohdAsif Siddiqui *et al*, 2013 for production of polygalacturonase with a *Rhizomucor pusillus* strain. Further increase in substrate concentration resulted in drastic reduction in enzyme production probably due to catabolic repression of biosynthesis (Omojola *et al*, 2008) or as a result of reduced mass transfer of oxygen by higher amount of solid substrate (Ghosh and Ray, 2011).

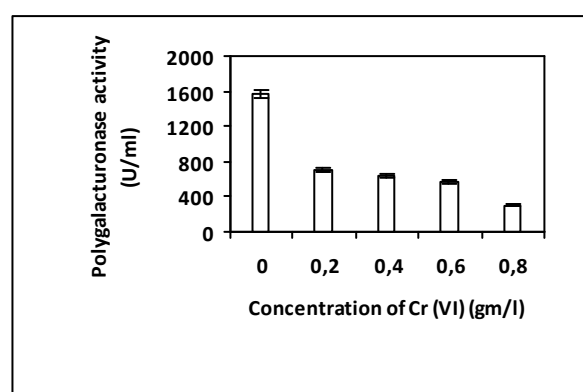


Figure 1. Effect of hexavalent chromium concentration on extracellular polygalacturonase production by *Trichoderma pseudokoningii*

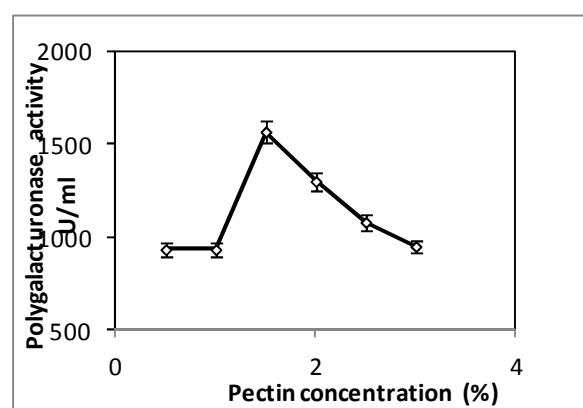


Figure 2. Effect of pectin concentration on extracellular polygalacturonase production by *Trichoderma pseudokoningii*

Maximum extracellular polygalacturonase production could be achieved by the studied *Trichoderma pseudokoningii* strain when the pH was adjusted to 7.0 (Fig. 3), a higher pH than required by *Rhizomucor pusillus* (Siddiqui *et al*, 2013, Phutela *et al*, 2005) but almost similar to

bacterial pectinase production by *Bacillus sp.* MFW7 (Mukesh Kumar *et al*, 2012) and *Bacillus sp.* AD1 (Dey *et al*, 2011). A sharp decrease in enzyme production was noted at pH 9.0.

Trichoderma pseudokoningii could synthesise maximum polygalacturonase at a temperature of 37°C (Fig. 4) with a rapid decrease of enzymatic activity at 55°C. But a lower optimal temperature of 30°C was reported in case of *Aspergillus sp.* (Galiotou-Panayotou *et al*, 1997) and *Peacilomyces clavosporus* (Soares *et al*, 1999) and higher optimal temperatures of 45-50°C were reported from *Aspergillus fumigates* (Phutella *et al*, 2005) and *Rhizomucor pusillus* (Siddiqui *et al*, 2013).

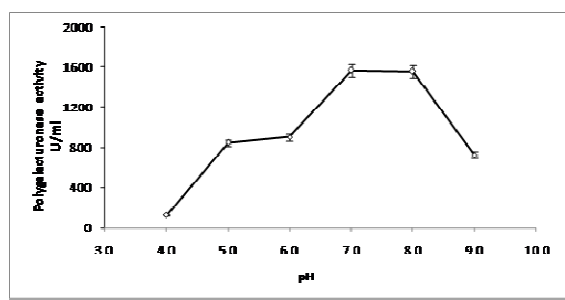


Figure 3. Effect of pH on extracellular polygalacturonase production by *Trichoderma pseudokoningii*

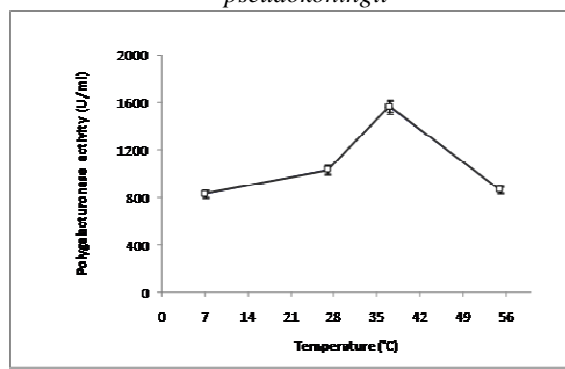


Figure 4. Effect of temperature on extracellular polygalacturonase production by *Trichoderma pseudokoningii*

The enzyme production kinetics of *Trichoderma pseudokoningii* (Fig. 5) indicated that the enzyme production increased rapidly after 24 hours and reached its maximum at 72 hours. The enzyme activity decreased gradually after 96 hours and

about 70.2% activity was retained even at 120 hrs of submerged cultivation. Similar report was found in case of *Aspergillus sp.* (Freitas *et al*, 2006 and Phutella *et al*, 2006).

Among various nitrogenous sources, peptone showed maximum enzymatic activity, followed by gelatine, urea and potassium nitrate (Fig. 6). The boosting effect of peptone for growth and polygalacturonase synthesis was also noted for *Trichoderma viride* BITRS-1001 (Arotupin and Ogunmolu, 2011).

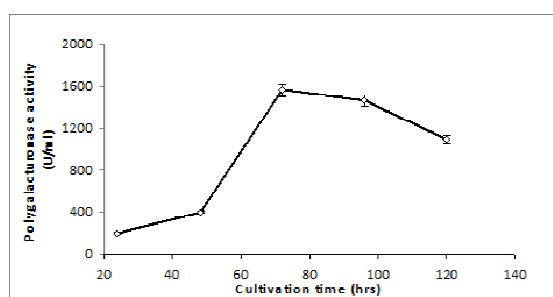


Figure 5. Effect of cultivation time on extracellular polygalacturonase production by *Trichoderma pseudokoningii*

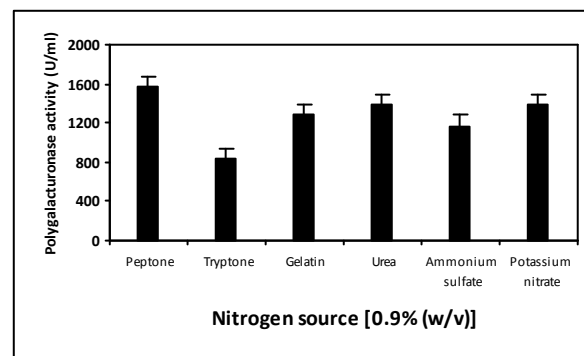


Figure 6. Effect of various nitrogenous sources on extracellular polygalacturonase production by *Trichoderma pseudokoningii*

Potassium ion, when added exogenously brought about 1.51 times enhancement in enzyme production (Fig. 7), which might be attributed to the enhanced K^+ -ATP ase activity but this enhanced mycelia growth and enzyme production in presence of potassium ions in neutral pH did not go in agreement with the observations of Wuyep *et al*, 2003.

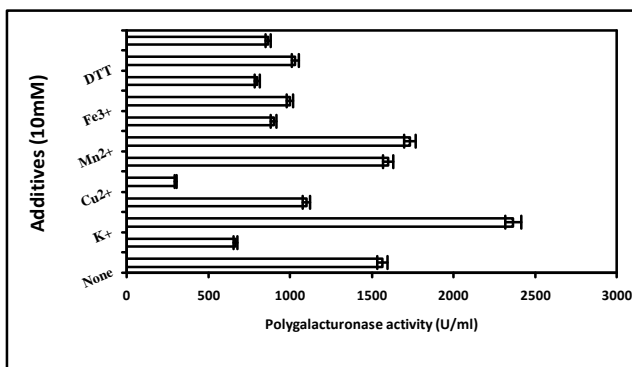


Figure 7. Effect of various additives on extracellular polygalacturonase production by *Trichoderma pseudokoningii*

Addition of copper ion significantly slashed down the enzyme production indicating the presence of thiols at active site of the enzyme.

Characterization of the enzyme

The maximum activity of the enzyme was found to be at pH 7.0 and 75% of activity was found to be retained at a broad pH range of 5.0-9.0 (Fig. 8). A much lower peak of optimum pH was reported from polygalacturonase of *Trichoderma reesei* (Marković *et al*, 1985), *Trichoderma harzianum* (Mohamed *et al*, 2006) and *Aspergillus fumigates* (Phutela *et al*, 2005).

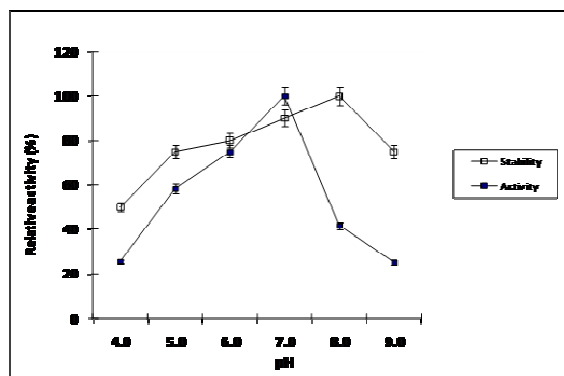


Figure 8. Effect of pH on the stability and activity of extracellular polygalacturonase synthesised by *Trichoderma pseudokoningii*

The enzyme showed optimum activity at 55°C and pH 7.0, almost similar observation were reported from other fungal polygalacturonases from *Rhizomucor pusillus* (Siddiqui *et al*, 2012), and *Trichoderma harzianum* (Mohamed *et al*, 2009). The thermo inactivation kinetics of polygalacturonase enzyme showed 80% of enzyme

activity was retained even after exposure to 90°C at pH 7.0 for 30 minutes (Fig. 9) indicating the thermostable nature of the enzyme. On the contrary, the stability of PGase of *Rhizomucor pusillus* (Siddiqui *et al*, 2012) was found to decrease rapidly above 60°C.

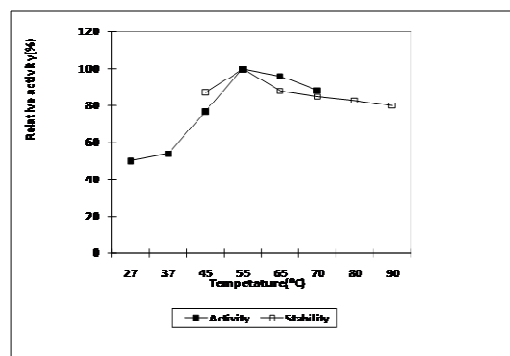


Figure 9. Effect of temperature on the stability and activity of extracellular polygalacturonase synthesised by *Trichoderma pseudokoningii*

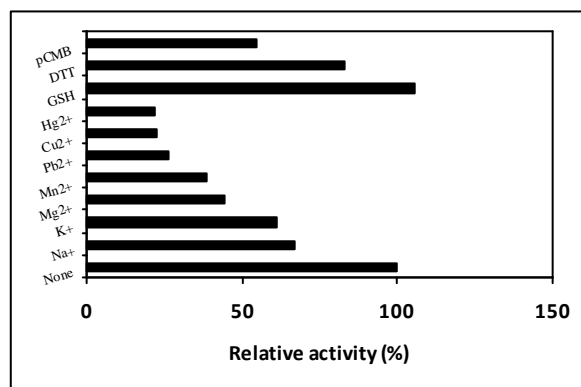


Figure 10. Effect of various additives on the activity of polygalacturonase

Among various metal ions (mono and divalent) Na⁺ and K⁺ were found to retain about 66% of enzymatic activity, but enzyme protein was denatured after exposure to the heavy metals. The exogenous thiols like DTT (Dithiothreitol), and GSH (Reduced Glutathione) could retain 83% to more than 100% of activity of the enzyme, emphasising the presence of thiol group at the active site of the enzyme.

Conclusion

Although a number of microbes are known to produce polygalacturonase, this is the first report

of production and this is the first report of production and characterisation of polygalacturonase enzyme from a chromium tolerant *Trichoderma pseudokoningii* strain. The enzyme is important for biotechnological processes adopted in food industries as it is essential for maceration of fruits and vegetables and can be used in extraction of juice from vegetables (like beet root, carrot and sweet potato) and fruits (guava and pineapple) and clarification and depectinization of these juices. Each application requires unique properties with respect to specificity, stability, temperature, and pH dependence of the concerned enzyme. The convenience in production and enzyme production in presence of toxic metal (hexavalent chromium) and the pH and the pH (5.0-8.0) and thermostability of obtained fungal enzyme depicted its efficacy in industrial utilization for commercial purpose.

References

- Agüero-Chapin G., Varona-Santos J., de la Riva G.A., Antunes A., González-Villa T., Uriarte E., González-Díaz H. (2009) Alignment-Free Prediction of Polygalacturonases with Pseudofolding Topological Indices: Experimental Isolation from *Coffea arabica* and Prediction of a New Sequence. *Journal of Proteome Research*, 8, 2122–2128.
- Arotupin D.J., Ogunmolu F.E. (2011) Effect of carbon and nitrogen sources on polygalacturonase production by *Trichoderma viride* (BITRS-1001) isolated from tar sand in Ondo State Nigeria. *Malaysian Journal of Microbiology*, 7, 164–169.
- Bernfeld P. (1955) Amylases α and β . *Methods of Enzymology*, 1, 149-58.
- Chatterjee S., Ghosh I., Mukherjee K.K. (2011) Uptake and removal of toxic Cr(VI) by *Pseudomonas aeruginosa*: physico-chemical and biological evaluation. *Current Science*, 101, 645-652.
- Dey A., Karmakar M., Ray R.R. (2011) Extracellular Thermostable Polygalacturonase from *Bacillus* sp. AD 1. *Der Pharmacia Lettre*, 3(2), 358-367.
- Freitas P.M., Martin N., Silva D., Silva R., Gomes E. (2006). Production and partial characterization of poly galacturonases produced by thermophilic *Monascus* sp. N8 and by thermotolerant *Aspergillus* sp. N12 on solid-state fermentation. *Brazilian Journal of Microbiology*, 37, 302-306.
- Galiotou-Panayotou M.P.R., Kapantai M., (1993) Enhanced polygalacturonase production by *Aspergillus niger* NRRL-364 grown on supplemented citrus pectin. *Letters in Applied Microbiology*, 17, 145–148.
- Ghosh B., Ray R.R. (2011) Current Commercial Perspective of *Rhizopus oryzae*. *Journal of Applied Science*, 11(14), 2470-2486.
- Horton R.N., Apel W.A., Thompson V.S., Sheridan P.P. (2006) Low temperature reduction of hexavalent chromium by a microbial enrichment consortium and a novel strain of *Arthrobacter aurescens*. *BMC Microbiology*, 6,5.
- Jayani R.S., Saxena S., Gupta R. (2005) Microbial pectinolytic enzymes: A review. *Process Biochemistry* 40(9), 2931–2944
- Markovič O. A., Slezárik A., Labudová I. (1985) Purification and characterization of pectinesterase and poly galacturonase from *Trichoderma reesei* *FEMS Microbiology Letters*, 27 (3), 267–271.
- Morales-Barrera L., Cristiani-Urbina E. (2008) Hexavalent Chromium Removal by a *Trichoderma inhamatum* Fungal Strain Isolated from Tannery Effluents. *Water, Air, Soil Pollution*, 187(1-4):327-336.
- Mohamed S.A., Al-Malki Abdulrahman L., Kumosani Taha A. (2009) Characterization of a Poly galacturonase from *Trichoderma harzianum* Grown on Citrus Peel with Application for Apple Juice. *Australian Journal of Basic & Applied Sciences*, 3(3), 2770- 2777.
- Mohamed S.A., Christensen T.M.I.E., Mikkelsen, J.D. (2003) New polygalacturonases from *Trichoderma reesei*: characterization and their specificities to partially methylated and acetylated pectins. *Carbohydrate Research*, 338 (6), 515–524.

Mohamed S.A., Farid N.M., Hossiny E.N., Bassuiny R.I. (2006) Biochemical characterization of an extracellular polygalacturonase from *Trichoderma harzianum*. *Journal of Biotechnology*, 127(1), 54-64.

Mukesh Kumar D.J., Saranya G.M., Suresh K., Andal Priyadharshini D., Rajakumar R., Kalaichelvan P.T. (2012) Production and Optimization of Pectinase from *Bacillus* sp. MFW7 using Cassava Waste, *Asian Journal of Plant Science and Research*, 2 (3), 369-375.

Omojasola P.F., Jilani O.P. (2008). Cellulase production by *Trichoderma longi*, *Aspergillus niger* and *Saccharomyces cerevisiae* cultured on waste materials from orange. *Pakistan Journal of Biological Science*, 11(20), 2382 – 2388.

Pedrolli D.B., Monteiro A.C., Gomes E., Carmona E.C. (2009) Pectin and Pectinases: Production, Characterization and Industrial Application of Microbial Pectinolytic Enzymes. *The Open Biotechnology Journal*, 3, 9-18.

Phugare S.S., Kalyani D.C., Patil A.V., Jadhav J.P. (2011) Textile dye degradation by bacterial consortium and subsequent toxicological analysis of dye and dye metabolites using cytotoxicity genotoxicity and oxidative stress studies. *Journal of Hazardous Material*, 186, 713-723.

Phutela U., Dhuna V., Sandhu S., Chadha B.S. (2005) Pectinase and polygalacturonase production by a thermophilic *Aspergillus fumigatus* isolated from decomposing orange peels. *Brazilian Journal of Microbiology*, 36(1), 63-69.

Reid I., Ricard M. (2000) Pectinase in paper making, solving retention problems in mechanical

pulps bleached with hydrogen peroxide. *Enzyme and Microbial Technology*, 26(2-4), 115-123.

Siddiqui M.A., Pande V., Arif M. (2013) Polygalacturonase production from *Rhizomucor pusillus* isolated from fruit markets of Uttar Pradesh. *African Journal of Microbiology Research*, 7(3), 252-259.

Siddiqui M.A., Pande V., Arif M. (2012) Production, Purification, and Characterization of Polygalacturonase from *Rhizomucor pusillus* Isolated from Decomposing Orange Peels. *Enzyme Research*, 2012, Article ID 138634, 8 pages

Singh G., Nema R., Khare S., Singh D., Jain P., Pradhan A., Gupta A., Naidu S. (2012) Tolerance and biodegradation capacity of *Trichoderma viride* with special reference to heavy metals (Cr, Cd). *Indo American Journal of Pharmaceutical Research*, 2(10), 1007-1014.

Soares M.M.C.N., Silva R.Da., Carmona E.C., Gomes E. (2001) Pectinolytic enzyme production by *Bacillus* species and their potential application on juice extraction, *World Journal of Microbiology and Biotechnology*, 17(1), 79-82.

Wuyep P.A., Khan A.U., Nok A.J., (2003) Production and regulation of lignin degrading enzymes from *Lentinus squarrosulus* (mont.) Singer and *Psathyrella atroumbonata* Pegler. *African Journal of Biotechnology*. 2 (11), 444-447.