JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2016. Vol. **10**(3), p. 2315-2321

Investigation on Production, Purification and Characterization of Protease and Xylanase from *Trichoderma koningii*

L. Prince

PG and Research Department of Microbiology,
Marudupandiyar College of Arts and Science, Thanjavur - 613 403, Tamil Nadu, India.

https://doi.org/10.22207/JPAM.10.3.73

(Received: 10 June 2016; accepted: 20 August 2016)

Soil contains number of diverse living organisms assembled in complex and varied communities, ecological niches and structure varied number that also vary from soil to soil, patch to patch. Totally 10 fungal species were isolated from sugarcane field soil in Thanjavur (Dt.). The species diversity was encounted with the fungal species belonging to the class Deuteromycetes. Production, optimization, purification and characterization of an extracellular Protease and Xylanase from *Trichoderma koningii*. The xylanase activity peaked at 55°C and pH of 7 on *Trichoderma koningii*. The protease activity of *Trichoderma koningii* peaked at 35°C and pH of 5. The role of *Trichoderma* protease and xylanase in biological control as well as their advantages in biotechnology. This enzyme is very helpful in industrial sector especially in pulp and paper industry, food industry, biocontrol and also in bioethanol production. Pilot scale production of this enzyme in industries can reduce the import cost of the enzyme and make the whole process cost effective.

Keywords: Soil, *Trichoderma koningii,* Protease, Xylanase, biotechnology, biocontrol.

Soil enzymes activities have been suggested as suitable indicators of soil quality because: (a) they are a measure of the soil microbial activity and therefore they are strictly related to the nutrient cycles and transformations; (b) they rapidly may respond to the changes caused by both natural and anthropogenic factors; (c) they are easy to measure (Nannipieri *et al.,* 2002).

Among a large number of non-pathogenic microorganisms capable of producing useful enzymes, filamentous fungi are particularly interesting due to their easy cultivation, and high production of extracellular enzymes of large industrial potential. These enzymes are applied in the industrialization of detergents, starch, drinks, food, textile, animal feed, baking, pulp and paper, leather, chemical and biomedical products. The use of starch degrading enzymes was the first large-scale application of microbial enzymes in the food industry.

Proteases are the most important class of industrial enzymes and comprise about 25% of commercial enzymes in the world (Narayana and Vijayalakshmi, 2008). Proteases are classified as acid, neutral and alkaline proteases. These enzymes are widely using in dairy industry as milk clotting agent and meat tenderizing agent in food industry. Reduction of tissue inflammation (clinical and medical) application (Djamel, *et al.,* 2009). Proteases are mainly produced by submerged fermentation; the microorganisms and the substrate are present in the submerged state in the liquid medium, where a large quantity in the form of solvent is present. Since the contents are in submerged state in the liquid medium, the transfer of heat and mass is more efficient, and is amenable for modeling the process (Sinha, 2009).

Xylanases have potential application in food, feed, paper, pulp and textile industries (Polizeli, *et al.,* 2005). These enzymes degrade plant

* To whom all correspondence should be addressed.
E-mail: prince7000.7000@gmail.com

fibers made of xylan hemicellulose producing xylose monomers. One of the most important xylanase applications is the pretreatment of pulps, prior to bleaching, in pulp and paper industries (Subramaniyan, *et al.,* 2012). These enzymes release lignin fragments by hydrolyzing residual xylan and the pretreatment with xylanase reduces the usage of chlorine as the bleaching agent. Not less important is the use of xylanases for bread-making and beer production (Harbak and Thygesen, 2002). On the other hand, in the industry of animal feed, the xylanases can be added to the ration increasing the intestinal absorption of nutrients (Twomey, *et al.,* 2003). In this study we have explored the biopotential of *Trichoderma koningii*, isolated from soil samples, for the production, purification and characterization of Protease and Xylanase enzymes.

MATERIALS AND METHODS

Collection of Sample

The present investigation was carried out by collection and examination of soil sample in sugarcane field from Thanjavur, Tamilnadu.

Isolation of fungi

The fungi were isolated by the methods reported by Kader *et al*. (1999). In a typical assay, 1g of soil sample was mixed with 9ml of sterile distilled water in a test tube shaken vigorously. Series of dilutions were made until 1:1000 dilution. After preparing the appropriate dilution, 0.1ml was pipetted, poured and dispersed by swirling on potato dextrose agar (PDA) and incubated at 28°C for five days. The various isolates were sub-cultured until single pure isolates were obtained.

Identification of fungi

Characterization method employed for the fungal isolates were made by both the inspection of colonial features, cellular characteristics at 100 X and 40 X microscopic magnification. Identification was done by employing the method of (Barnet and Hunter, 1972) and conventional techniques of isolating individual microorganisms and allowing them to grow and produce colonies

Enzyme production

Screening test for protease production fungus

Proteolytic fungus was screened on skim milk agar medium (Sharma *et al*., 2006). The medium is composed of Skim milk powder 10g, Peptone 0.5g and Agar 2g. The above contents were dissolved in 100 ml of distilled water and the pH was adjusted to 8. The medium was sterilized and it was poured in to a petriplates. After solidification the fungus were placed on the well made by the sterilized cork borer. After 72 hours, fungal isolates showing the zone of clearance was observed. It indicates that the fungi have the proteolytic activity.

Screening Test for Xylanase Production

Xylanase production was screened on oat spelt xylan agar medium (Takashi Nanmori *et al.,* 1990). The medium is composed of Yeast extract (0.1%), Xylan (1%), Agar (2%), KH_2PO_4 (0.4%), NaCl (0.2%), $MgSO_4$ $7H_2O$ (0.1%), $MNSO_4$ (0.005%), $FeSO_4$. $7H_2O$ (0.005%), $CaCl_2$. $2H_2O$ (0.2%) and NH_4Cl (0.2%). The above contents were dissolved in 100ml of distilled water and the pH was adjusted to 7.0. The medium was sterilized and it was poured into a sterilized petriplates. After solidification, the well was made by sterilized cork borer. The isolated culture was placed on the well After 48 hours at 37°C the plates were stained with 1% Congo red and incubated for 15 minutes and then the plates were destained with 1 m NaCl (Teather, 1982). The zone of clearance around the strains indicates the presence of xylanase produced by the fungus.

Assay for Protease and Xylanase

Protease activity can be assayed using casein as a substrate by spectrophotometer method of Kaey *et al*. (1970). The Xylanase activity has be assayed by the method of Miller (1952).

Estimation of protein

The amount of protein present in the protease and xylanase from isolated fungus was estimated by the method of Lowry's *et al*. (1951).

Extraction of Protease and Xylanase by Ammonium Sulphate Salt precipitation

An extra cellular protease and xylanase from isolated fungus was extracted by the method of Deutscher *et al*. (1990). The crude enzymes were centrifuged at 10000 rpm for 10 minutes and the supernatant was collected and purified by 20 % ammonium sulphate precipitation. First measured the volume of enzymes and added 20 % weight of ammonium sulphate slowly with constant stirring under cold condition for 2 hours. After the precipitate was formed and centrifuged at 10,000 rpm for 10 minutes. Retained the supernatants and

the pellet was discarded. It was followed by the addition of 80% ammonium sulphate in a drop wise manner and incubated for overnight at 4°C to get the precipitate. Centrifuged and retained the pellet and dissolved it using minimum quantity of phosphate buffer for protease (pH6.2) and citrate buffer for xylanase (pH5.8).

Purification of Protease and Xylanase

The extracted Protease and Xylanase were subjected to purification first by Dialysis followed by Ion-exchange chromatography. The Protease and Xylanase enzymes from isolated fungus were dialyzed by the method of Scope (1994). The solution contains a lot of residual Ammonium sulphate, which was bound to the enzyme. One way to remove this excess salt was to dialyze the enzyme against a buffer in low concentration. Extracted enzyme solution was placed in a dialysis bag and placed the dialysis bag in large volume of buffer and stirred for many hours, which allows the solution inside the bag to equilibrate with the solution outside the bag with respect to salt concentration. The process of equilibration was repeated for several times by replacing the external solution with low salt concentration each time. Mainly the dialysis process was done to get rid of excess salt.

Table 1. List of fungi isolated from sugarcane field soil

No	Name of the fungal isolates
1.	*Colletotrichum falcatum* Went
2.	*Curvularia geniculata* (Tracy and Earle) Boedijn
3.	*Botrytis cinerea* Persoon
4.	*Trichoderma glaucum* Abbott
5.	*T. harzianum* Rifai
6.	*T. koningii* Oudemans
7.	*Penicillium chrysogenum* Thom
8.	*P. citrinum* Thom
9.	*P. expansum* (Link) Thom
10.	*P. janthinellum* Biourge

Ion-Exchange Chromatography

The dialyzed enzymes were again purified by column chromatography by the method of Truter (1963). The chromatography column was washed using distilled water one or twice. DEAE cellulose was kept in boiling water bath for 10 minutes before packing. The column was packed with DEAE cellulose. Added 0.1N HCl to the column till the pH reaches 1. Again the pH was equilibrated to 7 with distilled water. Added NaOH till the pH reaches to 14. pH was equilibrated to 7 with distilled water. Then phosphate buffer was added to the protease column and citrate buffer for the Xylanase column. Loaded to the column with the appropriate enzymes and eluted the enzyme using the same buffer. The elutants were collected in the test tubes separately. Fractions showing enzyme activity were pooled and used for further studies.

Purification table

Measurement was taken for each purification step.

1. The volume of the enzymes solution (ml)
2. The protein content of the solution ($mg.ml^{-1}$)
3. The activity of the enzymes solution ($U.ml^{-1}$)

Total amount of enzymes (u)

Activity ($U.ml^{-1}$) ´ Volume (ml)

Specific activity ($u.mg^{-1}$)

Activity ($U.ml^{-1}$)/ protein content ($mg.ml^{-1}$)

Yield (%)

Total amount of enzymes after purification step / total amount of enzymes before purification step.

Table 1. Purification of Protease from *Trichoderma koningii*

PurificationLevel	Volume (ml)	Activity (U/ml)	Specific Activity (U/mg)	Protein (mg/ml)	Yield (%)	Purification Fold
Crude enzyme extract	100	12.8	71.2	90.2	100	1
After ammonium sulphate fraction	40	12.39	95.44	65	95.97	1.3
After dialysis	25	10.41	271.2	19.1	83	3.6
After ion exchange chromatography	15	8.41	273.2	15.4	65	3.7

Purification fold

Specific activity of enzymes after purification step / specific activity before purification step.

Optimization of pH for protease and xylanase activity

The activity of protease and xylanase was evaluated at different pH values, such as 4, 5, 6, 7 and 8 under assay condition. The fermentation media was prepared each pH. The particular pH was adjusted to 4, 5, 6, 7 and 8 by using 1.0N HCl /1.0 NaOH. Then the flask were autoclaved at 121°C (15 lb /15 minutes). After the sterilization were inoculated with 0.2ml 2% fungus spore suspension. The flask incubated for room temperature at 160 rpm, 30°C for 7 days.

Optimization of temperature for Protease and Xylanase activity

To determine the effect of temperature on protease and xylanase activity, the reaction was carried out at different temperatures such as 20°C, 30°C, 40°C, 50°C and 60°C. The fermentation media prepared for temperatures. After sterilization were inoculated with 0.02ml 2% V/N fungus spore suspension. The 40°C for room temperature and 50°C, 60°C for shaking incubator, 70°C, 80°C were incubated on boiling water bath for 7 days. Absorbance for measured at 530nm.

Optimization of different carbon source for Protease and Xylanase activity

Effect of various carbon compounds viz., cellulose, glucose, lactose, maltose and sucrose

Table 2. Purification of Xylanase from *Trichoderma koningii*

PurificationLevel	Volume (ml)	Activity (U/ml)	Specific Activity (U/mg)	Protein (mg/ml)	Yield (%)	Purification Fold
Crude enzyme extract	100	43.27	49.1	87.18	100	1
After Ammonium Sulphate Fraction	40	42.06	109	38.36	97	2.2
After Dialysis	25	30	138.1	21.72	69	2.7
After Ion exchange chromatography	15	15	160.0	9.36	34	3.1

Table 3. Enzyme activity at different pH

S.No	Different pH	Enzyme activity (IU/ ml) Protease	Xylanase
1	4	3.28	2.37
2	5	5.26	3.25
3	6	7.19	4.29
4	7	4.38	6.29
5	8	5.46	5.39

Table 4. Enzyme activity at different temperature

S. No	Different Temperature °C	Enzyme activity (IU/ ml) Protease	Xylanase
1	20	4.97	4.26
2	30	6.35	7.19
3	40	7.98	3.98
4	50	5.72	5.23
5	60	6.45	6.18

Table 5. Enzyme activity at different carbon sources

S. No	Different carbon sources	Enzyme activity (IU/ ml) Protease	Xylanase
1	Cellulose	2.38	3.21
2	Glucose	7.91	3.98
3	Lactose	5.61	4.17
4	Maltose	6.31	6.25
5	Sucrose	3.71	8.02

Table 6. Enzyme activity at different nitrogen sources

S.No	Different nitrogen sources	Enzyme activity (IU/ ml) Protease	Xylanase
1	Ammonium chloride	3.19	3.23
2	Ammonium nitrate	8.95	4.17
3	Ammonium sulphate	6.81	7.27
4	Potassium nitrate	4.82	5.67
5	Calcium nitrate	7.63	8.72

were used for studying. The broth was distributed into different flasks and 3.0 % of each carbon sources were then added before inoculation of the strain and after culture inoculation, the flasks were incubated for 7 days at 30°C.

Optimization of different nitrogen source for Protease and Xylanase activity

In the present study, the appropriate nitrogen source for protease and xylanase production by fungus was analyzed. The influence of ammonium chloride, ammonium nitrate, ammonium sulphate, calcium nitrate and potassium nitrate were studied. The fermentation medium was supplemented with nitrogen sources at 3.0% level, replacing the prescribed nitrogen source of the fermentation medium.

Effect of immobilization for the activity of Protease and Xylanase

The enzyme was immobilized by the technique of entrapment according to the method of Laidler *et al.* (1980).

Preparation of beads

10ml of 1% sodium alginate solution was heated for two minutes and cooled. To this added 500µl of enzyme and mixed well. 1 ml of above mixed solution was added to $CaCl_2$ solution in a drop wise manner to get the beads.

SDS - Polyacrylamide Gel Electrophoresis

Molecular weight of Protease and Xylanase were determined by SDS-PAGE electrophoresis adopted by the method of Laemmli (1970).

RESULTS AND DISCUSSION

Totally 10 fungal species were isolated from sugarcane field soil in Thanjavur (Dt.) (Table 1). Besides the above, number of species diversity was encounted with the fungal species belonging to the class Deuteromycetes.

Enzyme production from *Trichoderma koningii*

Production, optimization, purification and characterization of an extracellular Protease and Xylanase from *Trichoderma koningii* were studied. *Trichoderma koningii* is potential enzymatic activity compared to other isolates so it was selected for further studies. *Trichoderma koningii* was screened for protease production and the zone formation was clearly evident in the organism which may be due to hydrolysis of protein in skim milk agar medium. From the above result, it was evident that the species carry protease gene. Xylanase producing *Trichoderma koningii* formed the zone due to the hydrolysis of xylan in the oat spelt xylan agar medium. The results indicate that the species carry xylanase gene. The fermentation medium of protease and xylanase. The fungus was inoculated in the medium for enzyme production. The medium contains the substrates (casein and xylan). Due to the presence of substrate, the fungus produced the enzyme for hydrolysis.

Genus *Trichoderma* lives in soil and plant root system. Some strains establish long-lasting colonization of root systems and penetrate into the epidermis (Yedidia *et al.,* 1999) using hydrolytic enzymes as cellulases, chitinases, glucanases and proteases (De la Cruz *et al.,* 1993). The antagonistic action of *Trichoderma* species against phytopathogenic fungi might be due to either by the secretion of extracellular hydrolytic enzymes (Schirmbock *et al.,* 1994) or by the production of antibiotics (Howell, 1998).

Tables 1 and 2 represents the volume, activity, specific activity, protein content, yield and purification fold of enzyme after extraction and purification of protease and xylanase from *Trichoderma koningii.* From the table, it could be clearly understood that the volume, activity, protein content and yield markedly decreased after purification. After purification specific activity and purification fold was increased. Xiao-Yan *et al.* (2006) where antimicrobial metabolites were produced by *Trichoderma koningii* SMF2 and exhibited antimicrobial activity against a range of gram-positive bacterial and fungal phytopathogens. The active metabolites proved to be three known peptaibols: Trichokonin VI, VII and VIII. Trichokonins were insensitive to proteolytic enzymes.

The optimization of pH, temperature, carbon and nitrogen sources of protease and xylanase production were carried out. The activity of protease and xylanase at different pH (4 to 8) was analysed. The protease and xylanase exhibited maximum activity at pH 6 and 7 respectively (Table 3).

Xylanases have been produced by a variety of microorganisms, including filamentous fungi and bacteria (Knob and Carmona, 2010). Fungal xylanases are more interesting from

industrial point of view because their extracellular activities are much higher than those of yeast and bacteria (Polizeli *et al.,* 2005). A number of fungal species are known for the production of xylanases such as *Aspergillus niger*, *Chaetomium thermophilum*, *Humicola lanuginosa,* and *Trichoderma harzianum* (Ahmed *et al.,* 2009a).

The effect of various temperatures (20°C to 60°C) on the enzyme activities was studied. The optimum temperature of protease and xylanase from *Trichoderma koningii* was analysed. The enzyme activity was maximum at 40°C for protease and 30°C for Xylanase (Table 4).

The effect of various carbon sources on the enzyme activities was studied. The enzyme activity was maximum at glucose carbon source for protease and sucrose carbon source for xylanase (Table 5).

The activity of protease and xylanase at different nitrogen sources was studied. The protease production was optimum in fermentation medium containing ammonium nitrate and xylanase exhibited maximum activity in fermentation medium containing ammonium sulphate (Table 6).

Immobilization of enzymes

The immobilization of enzymes by entrapment method using sodium alginate was carried out.

SDS-PAGE

Delgado-Jarana *et al.* (2000) detected several acidic, neutral and basic extracellular proteases by IEF in the case of *T. harzianum.* Antal *et al.* (2001) examined and compared the extracellular enzyme profiles of *mycoparasitic T. aureoviride, T. harzianum* and *T. viride* strains by Sephadex G150 gel filtration chromatography. Multiple proteases bands with high-molecular weights (higher than 70 kDa) were detected by gelatin zymography. Patterns of protease bands were inducer-specific. According to this study, *Trichoderma* strains may be able to degrade fungal pathogen cells, and proteases are suggested to play a role in this process. In the present study, the molecular weight of the enzymes protease and xylanase from *Trichoderma koningii* were determined by SDS-PAGE. The molecular weight of the protease enzyme was determined by SDS-PAGE using standard protein markers ranging from 14.4kDa to116KDas. The molecular weight of the protease enzyme from *Trichoderma koningii* found to be 45kDa. The molecular weight of the Xylanase enzyme from *Trichoderma koningii* found to be 39kDa .

CONCLUSION

From this study it is found that the protease and xylanase isolated from *Trichoderma koningii* has a great scope of medicinal, diagnostic, biocontrol and industrial applications. The molecular weight of the protease (45kDa) and Xylanase (39kDa). Further investigation on strain improvement studies will provide better yield of enzyme and a promising strain for the industrial production of these enzyme.

REFERENCES

1. Halpern, S.D., Ubel, P.A., Caplan, A.L. Solid-organ transplantation in HIV-infected patients. *N. Engl. J. Med.*, 2002; **347**(4): 284-7.
2. Ahmed, S., Riaz, S., and Jamil, A.. Molecular cloning of fungal xylanases. An overview. *Applied Microbiology and Biotechnology.*, 2009a.; **84**(1): 19-35.
3. De la Cruz, J., Rey, M., Lora, J.M., Hidalgo-Gallego, A., Dominguez, F., Pintor-Toro, J.A., Llobell, A., Benitez, T. *Arch. Microbiol*. 1993; **159**: 316-322.
4. Deutscher, Murray P. *Methods in Enzymology,* Guide to Protein Purification, New York: Academic Press, 1990; **182**: 285-306.
5. Djamel, C., Ali, T. and Nelly, C. *European J. Scientific Res.*, 2009; **25**(3): 469-477.
6. Harbak, L. and Thygesen, H.V. Safety evaluation of a xylanase expressed in *Bacillus subtilis. Food Chem. Toxicol.,* 2002, **40**:1.
7. Howell, C.R.. The role of antibiosis in biocontrol. In: Harman GE, Kubicek CP (eds) *Trichoderma & Gliocladium*. *Taylor & Francis, Padstow*., 1998; 173-184.
8. Kader, A. J., Omar, O. and Feng, L. S. Isolation of cellulolytic fungi from the Bario highlands, Sarawak. ASEAN Review of Biodiversity and Environmental Conservation (ARBEC). 1999.
9. Kaey, L. and Wildi, B.S. Protease of the genus bacillus 1 neutral protease. *Biotechnol. Bioeng*., 1970; **12**: 179-212.
10. Knob, A. and Carmona, E.C. Purification and characterization of two extracellular xylanases from *Penicillium sclerotiorum*: A novel acidophilic xylanase. *Applied Biochemistry and Biotechnology.,* 2010; **162**(2): 429-443.

11. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), 1970; **227**: 680-685
12. Laidler, K.J., Ngo, T.T. and Yam, C.F. Kinetics of acetylcholinesterase Immobilized on polyethylene tubing. *Can. J. Biochemistry.,* 1980; **57**: 1200-1203.
13. Lowry, O.H., Rosebrough, N.H., Farr, A.L. and Randall, R.J., Protein measurement with folin phenol reagent. *J.Biol. Chem.,* 1951; **193**(1): 265-275.
14. Narayana, K.J.P. and Vijayalakshmi, M. *Asian Journal of Biochemistry*, 2008. (3)3: 198-202.
15. Polizeli, M.L.T.M., Rizzatti, A.C.S., Monti, R., Terenzi, H.F., Jorge, J.A. and Amorim, D.S. Xylanases from fungi: properties and industrial applications. Review. *Appl. Microbiol. Biotechnol.,* 2005; **67**: 577-591.
16. Schirmbock, M., Lorito, M., Wang, Y.L., Hayes, C.K., Arisan-Atac, I., Scala, F., Harman, G.E. and Kubicek, C.P. Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. *Appl. Environ. Microbiol*., 1994; **60**: 4364-4370.
17. Scope, Methods in enzymology, 1994; pp 1-765.
18. Sinha, S., Sinha, S., *International Journal of Food Engineering,* 2009; **5**: 1.
19. Subramaniyan, S. and Prema, P. Biotechnology of microbial xylanases: enzymology, molecular biology, and application. *Crit. Rev. Biotechnol.,* 2002; **22**(1): 33-64.
20. Truter, Methods in enzymology., 1963; **2**: 1227–1232.
21. Twomey, L.N., Pluske, J.R., Rowe, J.B., Choct, M., Brown, W., Mcconnell, M.F. and Pethick, D.W.The effects of increasing levels of soluble non-starch polysaccharides and inclusion of feed enzymes in dog diets on faecal quality and digestibility. Anim. Feed *Sci. Technol.,* 2003; **108**(1-4): 71-82.
22. Yedidia, I., Benhamou, N. and Chet, I. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. *Appl. Environ. Microbiol.,* 1999; **65**: 1061-1070.

© The Author(s) 2016. **Open Access**. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License which permits unrestricted use, sharing, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.