INT. J. BIOL. BIOTECH., 8 (3): 377-381, 2011.

ASSESSING ENZYME ACTIVITY OF *TRICHODERMA* SPECIES

Amna Ali*[1], Sana Hanif [2], Rukhsana Bajwa[2] and Rasheda Jabeen[2]

[1]*First Fungal Bank of Pakistan (FCBP), Institute of Agriculture Sciences (IAGS), University of the Punjab, Lahore, Pakistan.*
[2]*Institute of Agriculture Sciences (IAGS), University of the Punjab, Lahore, Pakistan.*
**Corresponding author: write2amna@gmail.com*

ABSTRACT

Trichoderma is a ubiquitous fungus found in air, soil, plant materials and other substrates. Twenty four isolates of *Trichoderma* belonging to four species *T. harzianum, T. hamatum, T. koningii* and *T. pseudokoningii* were procured from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agriculture Sciences (IAGS), University of the Punjab, Lahore. All species were screened out for their enzyme production potential biochemically on different substrates. The isolates exhibited variable efficiency on producing amylase, cellulase, catalase, urease, lipase, pectinase enzymes. In addition, alkaloid estimation was also carried out through Ehrlich test. The results revealed that except for cellulase productivity of all other test enzymes was comparatively low. The highest amylase activity was recorded in *T. harzianum*; *T. koningii* and *T. pseudokoningii*. In alkaloids estimation test, *T. harzianum* showed high activity by producing violet ring around the plug of mycelium. In lipase and urease test, *T. harzianum, T. koningii* and *T. hamatum* displayed high enzymatic activity. In case of pectinase estimation, almost same trend was observed regarding different isolates as in case of lipase activity. The study clearly indicated that some strains of *Trichoderma* species like *T. harzianum* and *T. koningii* can be exploited in industrial production of fruit juices by cellulase enzyme activity along with further genetic engineering with the selected isolates.

Key words: *Trichoderma* species, isolates, media, enzyme potential

INTRODUCTION

Microorganisms have become increasingly important as producer of different industrial enzymes. Due to their biochemical diversity and the ease with which enzyme concentrations may be increased by environmental and genetic manipulation, attempts are now being made to replace enzymes, which traditionally have been isolated from complex eukaryotes. Biochemical analysis consists essentially of examining the distribution of chemical compounds such as secondary metabolites and enzymes in a series of related or supposedly related isolates (Azin *et al.*, 2007).

Trichoderma spp. are among the most studied fungal species as biological control agent (BCAs) and commercially marketed as biopesticides, biofertilizers and soil amendments (Vinale *et al.*, 2008). Similarly by selecting strains that produce a particular kind of enzyme, and culturing these in suspension, industrial quantities of enzyme can be produced. *Trichoderma*, being a saprophyte is adapted to thrive in diverse environmental situations and produces a wide array of enzymes. *T. reesii* is being used for the industrial production of cellulase and hemicellulase, *T. longibrachiatum* is used for xylanase and *T. harzianum* is used for chitinase (Harman *et al.*, 2004; Azin *et al.*, 2007; Felse and Panda, 1999). The biocontrol ability of *Trichoderma* seems to be due to multiple factors, as they have the ability to produce a variety of extracellular lytic enzymes i.e. chitinases, glucanases, and proteases and the production of many secondary metabolites and antibiotics (Kullnig *et al.*, 2000; Harman, 2006; Ghisalberti and Sivasithamparam, 1991). The present study will provided a comprehensive evaluation of local isolates of *Trichoderma* species regarding their enzymatic potential.

MATERIALS AND METHODS

Culture procurement, maintenance and preservation of *Trichoderma* species

The pure cultures of different morphotypes of *Trichoderma* species characterized for biochemical basis are enlisted in Table 1 and 2. These isolates were acquired from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agriculture Sciences (IAGS), University of the Punjab, Lahore. All isolates of various *Trichoderma* species were sub-cultured on MEA (malt extract agar) medium by inoculating the spores and were incubated at 25^{o}C for 7 days. All pure cultures of *Trichoderma* species were maintained at 4^{o}C for future use and reference. For biochemical assay, preserved cultures were inoculated on fresh ME (malt extract) broth media. Flasks were incubated at 20-24^{o}C for a week.

Amylase Activity Test

Spore suspensions of *Trichoderma* species were inoculated onto potato dextrose agar medium plates (PDA) (200g potato starch, 20g dextrose and 15g agar was dissolved in 1 liter of distilled water with pH 6.5). The

inoculated cultures were incubated for 5-7 days at 20-24^{o}C. The production of enzymes was determined by the extent of clear zone produced by each fungal isolate in the PDA agar plate around the growing colony. The radius of the clear zones was rated as (++++) highly producing isolates on 2.7-3.3 mm diameter, (++) moderate producing isolates on 1.5-2.6 mm diameter and (+) weak producing isolates on 0.9-1.4mm diameter (Abe *et al.,* 1988).

Cellulase Activity Test

The sterilized 2% suspension of cellulose azure in water was pipetted aseptically onto solidified basal MEA medium at a rate of 0.5 mL per tube and the caps were tightened. The medium was surface inoculated with loop full of mycelium and incubated for 5-7 days at 20-24^{o}C. Tubes were inspected periodically for evidence of dye release by examining basal layers for blue coloration. The appearance of blue dye into the basal layer of medium indicated that fungi has cellulolytic ability and highly active in the degradation of cellulose (Smith, 1977).

Catalase Activity Test

The fungal mycelium/spores were inoculated on MEA and incubated for 5-7 days at 20-24^{o}C. The 3-5mm disc of fresh fungal culture was taken by sterilized cork borer and was put into 3% H_2O_2 solution (3mL of 100% hydrogen peroxide was added in distilled water making final volume up to 100 mL). The evolution of bubbles was observed in solution for the presence of catalase activity (Bailey and Scott, 1994). High catalase activity was observed by the rapid production of numerous bubbles of O_2.

Urease Activity Test

The MEA medium was prepared with the addition of phenol red at 0.01 g/L of the medium. After sterilization at 121^{o}C for 15 min, the medium was poured in petri-plated aseptically and allowed to cool at room temperature. The fungal inoculum was placed on the basal medium. Plates were incubated for 5-7 days at 20-24^{o}C. The appearance of deep pink color indicated the positive reaction of the microbe (Finegold and Baron, 1986).

Lipase Activity Test

The lipase assay medium for lipase production was prepared in 1 liter distilled water by dissolving Peptone: 20g, NaCL: 10g and $CaCl_2.2H_2O$: 0.2g. The pH of the medium was adjusted to 6.0 then 20g/L agar was added. Medium was sterilized by autoclaving at 121ºC for 15 minutes. Tween 80 (1mL/100mL) used as a lipid substrate, was autoclaved separately and later added to sterile and cooled basal medium. The medium was surface inoculated with a disc of mycelium and incubated for 5-7 days at 20-24^{o}C. An opaque halo around the colonies appeared that indicated the lipase activity (Haba *et al.,* 2000).

Pectinase Activity Test

The medium was prepared by adding of Yeast extract: 1g, Ammonium sulfate: 2g, Na_2HPO_4: 6g, KH_2PO_4: 3g, Pectin: 5g and Agar: 20g in 1 liter distilled water. The medium was sterilized at 121^{o}C for 15 min. Then medium was poured in petri-plated aseptically and allowed to solidify at room temperature. The fungal inocula was placed on the basal medium and incubated for 5-7 days at 20-24^{o}C. After incubation, the fungal colonies with clear zones were taken as pectinase positive. The radius of the clear zones was rated as (++++) highly producing isolates on 2.7-3.3 mm diameter, (++) moderate producing isolates on 1.5-2.6 mm diameter and (+) weak producing isolates on 0.9-1.4mm diameter (Kobayashi *et al.,* 1999).

Ehrlich Activity Test

A round piece (1cm diameter) of wet filter was placed in the petriplates after dipping into the Ehrlich reagent (2g of 4-dimethylamino-benzaldehyde, 85mL of 96% ethanol and 15mL of 10N HCL). A four millimeter (mM) agar plug was cut out from the center of a freshly growing colony of *Trichoderma* species and was placed on wetted filter paper. Appearance of violet ring within 2-6 minutes indicated that the culture contained cyclopiazonic acid or other related alkaloids. If the reaction was delayed from 7-10 min, the results were regarded as less efficient (Lund, 1995).

RESULTS

Enzyme assays of isolates of *Trichoderma harzianum*

Trichoderma harzianum has shown a great variety of activities or potential in different biochemical tests. Table 1 represent results of amylase, cellulase, catalase, urease, lipase pectinase and ehrlich tests performed for 13 different isolates of *Trichoderma harzianum*. The five isolates displayed extremely high amylytic activity by the extent of producing expanded clear zone on solid agar plate. Five other isolates indicated moderate size of zone

while three isolates showed meager amylytic activity. Four isolates of *T. harzianum* indicated high cellulolytic activity, five isolates indicated moderate while four isolates exhibited low potential of cellulase enzyme production.

For determining catalase enzyme production potential, six isolates were specified as high exhibitors of catalase activity by speedily producing air bubbles. Five isolates displayed moderate level of catalase production. The other two isolates presented low catalase enzyme activity. In the case of urease enzyme production potential test, two isolates of *T. harzianum* indicated extremely high urease activity by the appearance of deep pink color of medium. Accordingly, six other isolates were specified as moderate producers showing rather medium pink color and five isolates exhibited low potential of producing urease enzyme. In lipase and pectinase production test, two isolates displayed extremely high activity in terms of extent of producing an opaque halo around the colonies. In addition, two other isolates showed no opaque halo, hence were marked as non enzyme producers. High amount of alkaloids were demonstrated by two isolates and five isolates exhibited moderate ability although three other isolates produced no ring of violet color hence marked as negative for alkaloids production.

Enzyme assays of isolates of *Trichoderma koningii*

Trichoderma koningii has shown a great variety of activities or potential in different biochemical tests. Table 2 represent results of amylase, cellulase, catalase, urease, lipase pectinase and Ehrlich test performed for 5 different isolates of *Trichoderma koningii*. The three isolates displayed extremely high amylytic activity while two isolates indicated moderate size of zone. In the case of cellulase enzyme only one isolate was specified as moderate producer. In catalase, lipase and alkaloids test, only one isolate displayed high level of activity whereas in the case of urease enzyme potential test, two isolates indicated low urease activity accordingly, another three isolates showed negative potential for producing urease enzyme. While in pectinase production potential, the two isolates particularly exhibited high levels of pectinase activity.

Enzyme assays of isolates of *Trichoderma pseudokoningii*

Trichoderma pseudokoningii studied through biochemical assays exhibited highly variable activities or potentials. The results of different biochemical tests are represented in Table 2. Only one isolate displayed extremely high amylytic activity even as in the case of cellulose and catalase enzyme production test, the two isolates indicated high activity. While in the case of urease and pectinase enzyme potential test, only one isolate indicated moderate activity and two isolates were showed moderate amount of alkaloids. All isolates displayed moderate lipolytic activity in terms of extent of producing an opaque halo around the colonies.

Table 1. Enzyme Activity Assessment of isolates of *Trichoderma harzianum*.

Accession#	*Source	Amylase test	Cellulase Test	Catalase Test	Ehrlich test	Urease test	Lipase test	Pectinase test
84	Rhizispheric soil	++++	+++	++++	+++	++	+++	++++
125	Decaying wood	+++	++++	+++	++++	+++	++++	-
139	Fruiting body of *Helvela elastica*	+++	++	++++	++++	+++	+++	+++
140	*C of *M. phaseolina*	++++	++++	++++	-	+++	+++	++
193	Air	++++	+++	+++	++	++	-	+++
210	Soil	++	++	+++	+++	+++	++	++
249	Polluted water	+++	++++	++++	+++	++	+++	++++
325	Mushroom *C	++	+++	++++	-	+++	+++	+++
496	Basidiocarp of *Hydnum* sp.	++++	++	+++	+++	++	-	+++
732	Air	+++	+++	++	+++	+++	++	-
755	Decaying Wood	++++	++++	++++	++	++++	+++	++
779	Leaf litter	++	++	++	-	++	+++	+++
860	*Mangifera indica*, rhizospheric soil	+++	+++	+++	++	++++	++++	+++

*C = contamination; ***Source = at the point of origin**

Key: High = ++++ Moderate = +++ Low = ++ Negative = -

Table 2. Biochemical Potential/Activity of isolates of *T. koningii. T. pseudokoningii* and *T. hamatum.*

Accession#	*Source	Amylase test	Cellulase Test	Catalase Test	Ehrlich test	Urease test	Lipase test	Pectinase test
Trichoderma koningii								
191	Citrus fruit	++++	++++	++++	++++	-	+++	+++
585	*M. indica* stem	++++	++++	+++	+++	++	+++	++++
692	Oat seeds	+++	++++	+++	++	-	++++	++++
747	Wood	++++	++++	++	++	++	+++	+++
765	*M. indica,* wood	+++	+++	++	++	-	+++	+++
Trichoderma pseudokoningii								
212	Green Chilli	++++	++++	++++	++	+++	+++	++++
213	Citrus fruit	+++	+++	+++	++	-	+++	+++
489	Tannery effluent	+++	+++	++++	+++	++	+++	+++
54	Green Cillies	++	++++	++	+++	-	+++	+++
Trichoderma hamatum								
769	*Syzygium cumini,* stem bark	+++	++++	++++	+++	++	+++	+++
908	Soil	+++	++++	+++	+++	++	++++	++++

***Source = at the point of origin**

Key: High = ++++ Moderate = +++ Low = ++ Negative = -

Enzyme assays of isolates of *Trichoderma hamatum*

Trichoderma hamatum exhibited a great variety of activities or potential in different biochemical tests which is apparent in Table 2. Two isolates displayed moderate amylytic activity and high amount of alkaloids. In the case of cellulase and urease enzyme production test, all isolates (substrates: *Syzygium cumini,* stem bark and soil mycoflora) indicated high cellulolytic activity by the appearance of blue dye into the basal layer of medium. In catalase, pectinase and lipase enzyme production assay, only one isolate (substrate: *Syzygium cumini,* stem bark) was specified for high level of catalase production while another isolate (substrate: soil mycoflora) displayed moderate level of catalase activity.

DISCUSSION

The integration of physiological and biochemical attributes, together with classic morphological criteria, has served to show enzymatic potential within *T. harzianum, T. hamatum, T. koningii and T. pseudokoningii* which can be related to different levels of biochemical activity (Grondona *et al.,* 1997). The component of medium, incubation time, pH and temperature of the medium has a direct influence on the growth of microorganisms. While working on *Penicillium caseifulvum* stated that low pH induces the production of secondary metabolites (Suhr *et al.,* 2002). Efficient production and utilization of enzyme potential depends upon substrate and capacity of the organisms to produce the extracellular enzymes required to degrade the main polymers of the substrate, cellulose, hemicellulose and lignin (Shafique *et al.,* 2009). The results showed that all the selected isolates produced capable quantities of cellulase on culture tube (Smith, 1977). However, the selection of more efficient cellulolytic strains depends upon biochemical traits (Elander, 1982). The ability of lipase potential of *Trichoderma* species presented remarkable variation in activity. The enzymatic activity by different species of *Pleurotus* has been studied (Elisashvili *et al.,* 2003). Considerable variations in results have been observed among different strains of same species analyzed. The results indicated that pectinase production in different strains showed high potential in *T. hamatum, T. koningii* and *T. pseudokoningii*. Such a phenomenon has been observed in other fungal taxa (Madhavi *et al.,* 2005) possibly due to differences in their biochemical potential. It was inferred from the results that such differences in the enzyme production of *Trichoderma* strains might be due to genetic variations. The maximum activity was achieved after incubation period of 5-7 days at 20-24^{o}C. Further incubation for a longer period of time did not show any boost in the enzyme production (Haq *et al.,* 2005). The data recommend that *Trichoderma hazianum* strain FCBP-84, 125, 140, 249, 755, *Trichoderma koningii* strain FCBP-191, 585, 692, *Trichoderma pseudokoningii* strain FCBP-212 and

Trichoderma hamatum strain FCBP-908 illustrated promising results by exhibiting increased amount of various enzymes like amylase, cellulase, catalase, urease, lipase and pectinase. The study clearly indicated that some local strains of *Trichoderma* species like *T. harzianum* and *T. koningii* can be exploited for the industrial production of amylase, cellulose and pectinase enzymes along with advanced genetic engineering with the selected isolates. At present many microbial, particularly fungal enzymes, are known to play vital role in food processing industries and their impact is going to be felt much more in coming years. In Pakistan the microbial dairy enzymes requirement has been limited till now. However, with the advent of technological processes for the manufacture of different varieties of milk products, the markets for the sale of such products in megacities and towns is slowly growing for the past two to three years. In the near future, the requirement for these enzymes is bound to increase by leaps and bounds, basically due to requirement of value-added industrial products in the country.

REFERENCES

Abe, J., F.W. Bergman, K. Obata and S. Hikuri (1988). Production of raw starch digesting amylase by *Aspergillus* K-27. *Appl Microbiol Biotech.*, 27: 447-450.

Azin, M., R. Moravej and D. Zareh (2007). Production of xylanase by *Trichoderma longibrachiatum* on a mixture of wheat bran and wheat straw Optimization of culture condition by Taguchi method. *Enzyme Microbial Technol.*, 40: 801-805.

Bailey, S. and F. Scott (1994). Diagnostic Microbiology. In: St. Louis, Mo., Mosby. (9th Ed) WEBCLS.

Elander, R.P. (1982). Traditional *versus* current approaches to the genetic improvement of microbial strains. In: Overproduction of Microbial products. (Eds.): V. Krumphanzl, B. Sikyta and Z. Vanek. 353-369.

Elisashvili, V.D., E. Chichua, N. Kachlishvili, S. Tsiklauri and T. Kharziani (2003). Lignocellulolytic enzyme activity during growth and fruiting of the edible and medicinal mushroom *Pleurotus ostreatus* (Jacq. Fr.) Kumm. (Agaricomycetideae). *Int J Med Mushrooms.*, 5: 193-198.

Felse, P.A. and T. Panda (1999). Production of xylanase by *Trichoderma longibrachiatum* on a mixture of wheat bran and wheat straw: Optimization of culture condition by Taguchi method. *Enzyme Microbial Technol.*, 40: 801–805.

Finegold, S. and E. Baron (1986). Diagnostic Microbiology. In: St Louis: CV Mosby Company. 897-899.

Ghisalberti, E.L. and K. Sivasithamparam (1991). Antifungal antibiotics produced by *Trichoderma* spp. *Soil Biol Biochem.*, 23: 1011–1020.

Grondona, I., R. Hermosa, M. Tejada, M.D. Gomis, P.F. Mateos, P.D. Bridge, E. Monte and I. Garcia-Acha (1997). Physiological and Biochemical Characterization of *Trichoderma harzianum*, a Biological Control Agent against soil borne fungal Plant Pathogens. *Appl Env Microbiol.*, 63(8): 3189–3198.

Haba, E., O. Bresco, C. Ferrer, A. Marques, M. Basguets and A. (2000). Manresa Isolation of lipase secreting bacteria by deploying used frying oil as selective substrate. *Enzyme Microbial Technol.*, 26: 40-44.

Haq, I., U. Hameed, K. Shahzadi, M.M. Javed, S. Ali and M.A. Qadeer (2005). Cotton saccharifing activity of cellulases by *Trichoderma harzianum* UM–11 in shake flask. *Int Jour Bot.*, 1(1): 19-22.

Harman, G.E. (2006). Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathol.*, 96: 190–194.

Harman, G.E., C.R. Howell, A. Viterbo, I. Chet and M. Lorito (2004). *Trichoderma* species - opportunistic, avirulent plant symbionts. *Nature Rev Microbiol.*, 2: 43–56.

Kobayashi, T., Y. Hatada, N. Higaki, D. Lusterio, T. Ozawa, K. Koike, S. Kawai and S. Ito (1999). Enzymatic properties and deduced amino acid sequence of a high alkaline pectatelyase from an alkaliphilic *Bacillus* isolate. *Biochemica et Biophysica Acta.*, 1427: 145-154.

Kullnig, C.M., L. Robert, M. Lorito and C.P. Kubicek (2000). Enzyme Diffusion from *Trichoderma atroviride* (*T. harzianum* P1) to *Rhizoctonia solani* is a prerequisite for triggering of *Trichoderma ech42* gene expression before mycoparasitic contact. *Appl Environ Microbiol.*, 66(5): 2232–2234.

Lund, F. (1995). Diagnostic characterization of *Penicillium palitans, P. commune* and *P. solitum*. *Lett Appl Microbiol.*, 2: 60-64.

Madhavi, K.J., M. Sujatha, D.R. Reddy and C. Rao (2005). Biochemical characterization of resistance against *Alternaria helianthi* in cultivated and wild sunflowers. *HELIA.*, 28(43): 13-24.

Shafique, S., R. Bajwa and S. Shafique (2009). Cellulase biosynthesis by selected *Trichoderma* species. *Pak J Bot.*, 41(2): 907-916.

Smith, R.E. (1977). Rapid tube test for detecting fungal cellulase production. *Appl Environ Microbiol.*, 33(4): 980-981.

Suhr, K.I., I. Haasum, L.D. Steenstrup and T. Larsen (2002). Factors affecting growth and pigmentation of *Penicillium caseifulvum*. *Jour Dairy Sci.*, 85: 2786-2794.

Vinale, F., K. Sivasithamparam, E.L. Ghisalberti, R. Marra, S.L. Woo and M. Lorito (2008). *Trichoderma*–plant–pathogen interactions. *Soil Biol Biochem.*, 40: 1–10.

(*Accepted for publication July 2011*)