THE SCREENING AND SELECTION OF *TRICHODERMA* SPECIES CAPABLE OF PRODUCING EXTRACELLULAR CELLULOLYTIC ENZYMES FROM SOIL OF DECAYING PLANT MATERIALS

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(Received: October, 2013; Accepted: April, 2013)

ABSTRACT

The study of the screening and selection of *Trichoderma* species capable of producing extracellular cellulolytic enzymes from soil of decaying plant materials and investigation of the optimum conditions for their production of the enzymes was undertaken in order to obtain organisms with cellulolytic capability. The soil samples were collected using sealed cellophane bags and a sterile spatula by digging twenty centimetres deep from the soil surface and fungal isolation was carried out immediately. Isolation and identification of fungal isolates were performed using standard procedures and then screened for cellulase production. Optimization studies of enzymes production for selected *Trichoderma* species were conducted. Five *Trichoderma* species were isolated from the soil samples viz: *Trichoderma koningii, T. reesei, T. longibrachiatum, T. harzianum* and *T. viride*. Three out of the five *Trichoderma* species isolated namely *Trichoderma longibrachiatum, T. harzianum* and *T. viride* produced appreciable amount of cellulase activity and were used for further studies. Maximum production of cellulase was obtained on the 11th day in all isolates. Commercial carboxymethyl cellulose (CMC) was the best substrate for cellulase production for all the isolated *Trichoderma* species giving the highest values of 786.67 reducing sugar equivalent per minute per millilitre of enzyme (units/ml) for *T. longibrachiatum* while potassium nitrate ($KNO_{3)}$ was the best nitrogen compound for cellulase production with *T. viride* giving the highest value of 846.67 units/ml. Optimum production of cellulase was attained at a concentration of 10 mg/ml for carboxymethyl cellulose and 1.0 mg/ml for KNO_3 for all the three *Trichoderma* species. The study concluded that *T. longibrachiatum*, *T. harzianum* and *T. viride* isolated from soil of decaying plant materials produced proteins, which exhibited appreciable cellulase activity and could be used in producing cellulases for industrial and biotechnological uses.

Keywords: Cellulolytic enzyme; Trichoderma species; Soil samples; Industrial purposes; Optimum production.

INTRODUCTION: The proportion of cellulose in plant tissues ranges from 20 - 45% of dry weight and to almost over 90% in cotton fibre (Stephen and Heichel, 1975). It is important to note that much of the cellulose exist as wastes such as straw, corn cobs, wood wastes, peat, bagasse and waste paper (Crueger and Crueger, 1990). Fungi play an important role in plant litter decomposition in forest ecosystems through nutrient recycling and humus formation in soil because they attack lignocelluloses matrix in litter that other organisms are unable to assimilate (Swift *et al.,* 1979; Kjoller and Struwe, 1982; Cooke and Rayner, 1984; Osono *et al*., 2003). During the process of decomposition, the decomposers provide food for themselves by extracting chemicals from the dead bodies or organic wastes; using these to produce energy (Couteaux *et al.,* 1995).

The vast amount of plant biomass waste produced by the agro-technological industry forms an attractive potential as a renewable source of energy and basic chemicals (Hahn-Hagerdal *et al*., 2006; Stricker *et al*., 2008). One of the bottlenecks in fully exploiting this potential is the efficient hydrolysis of the cellulose and hemicellulose fractions to its monomeric compounds. These monomeric constituents are mainly sugars such as D-glucose and D-Xylose (Stricker *et al*., 2008). Many microorganisms have the capacity to degrade plant biomass.

Trichoderma species are fungi that are present in substantial numbers in nearly all agricultural soil and in other environments such as decaying wood, compost or dead mushrooms (Singh *et al*., 2007). Filamentous fungi like *Aspergillus niger* and *Hypocrea jecorina (Trichoderma reesei)* have been shown to produce a wide spectrum of

polysaccharide-hydrolytic enzymes. The filamentous fungus, *Trichoderma* species has a long history in the production of hydrolytic enzymes widely used in the food and feed industries as well as textile, pulp and paper industries (Nakari-Setala and Penttila, 1995).

Among the hydrolases produced by *Trichoderma* species is the complete set of cellulolytic enzymes. Enzymatic hydrolysis of cellulose is an important reaction in nature for it marks the first step in the decay of cellulose, the most abundantly occurring organic material. Cellulase is an enzyme that can degrade cellulose and its derivatives which are the basic unit of structural framework of plant cell wall. Cellulase is produced by a number of bacteria and fungi though species of *Trichoderma* and *Aspergillus* are most reported (Zaldivar *et al.*, 2001). Some thermophilic fungi have been reported to be strongly cellulolytic (Tansey, 1971) and their role being of paramount importance in the colonization and degradation of cellulosic waste.

The use of microorganisms as commercial sources of enzyme production has been a common practice for decades (Adeleye, 1990, Adeleye and Lashebikan, 2003). Cellulolytic enzymes are generally induced by cellulose, but other inducers of the enzymes are known (Tan *et al.*, 1986). These include cellulose derivatives, cellobiose, sophorose, xylan, pectin and lactose (Mandels *et al.*, 1975; Mandels, 1981). Since growth of fungi as well as the enzyme production depends on the composition of the growth media, water activity (a_w), pH, temperature, light and the surrounding atmospheric gas mixture; optimization of culture conditions and culture medium influences the enzyme production (Prashanth *et al.*, 2008). In this study, the ability of *Trichoderma* species isolated from decaying plant materials in a farmland to produce cellulolytic enzyme will be examined with a view to exploiting the organisms as potential enzyme source for industrial purpose.

MATERIALS AND METHODS

Sample Collection, Isolation and Identification of Fungal Isolates: Soil samples were obtained from an abandoned farmland using sealed cellophane bags and a sterile spatula by digging twenty centimetres deep from the soil surface. Fungal isolation was carried out immediately using 10-fold serial dilution and pour plate technique. Typical fungal isolates were isolated and identified on Potato Dextrose Agar (PDA) [Cappuccino and Sherma, 1996]. Each fungal isolate was identified with the aid of illustrated handbook of fungi (Barnett and Hunter, 1972).

Preparation of basal medium for cellulase synthesis: The basal medium composition for production of cellulase by submerged fermentation was prepared based on the modified medium of Mandels and Weber (1969) as described by Olutiola (1976) containing: K_2HPO_4 (0.5 g); KNO_3 (9.9 g), KH_2PO_4 (2.0 g); Biotin (0.005 mg); Thiamine (0.005 mg); $MgSO_4.7H_2O$ (0.1 g) and $FeSO_4.7H_2O$ (1.0 mg) all dissolved in 1 L of distilled water. Sterile carbon source of carboxymethyl cellulose (high viscosity) was added to the sterile basal medium in 250 ml conical flask at 1% concentration. Each conical flask contained a final volume of 100 ml of growth medium.

Enzyme production by submerged fermentation: Submerged fermentation for enzyme production was carried out as outlined by Olutiola and Nwaogwugwu (1982). On daily basis, contents of each flask was filtered through glass fibre paper (Whatman GF/A). The filtrate was analyzed for cellulase activity until the peak of production of enzyme activity was attained. The filtrate then served as the crude enzyme preparation.

Optimization of production conditions: The effects of various nutritional and cultural factors on enzyme productions were studied. Various carbon sources (glucose, fructose, maltose, lactose, sucrose, galactose, CMC and starch) and different nitrogen sources (NH_4Cl, $NaNO_3$, $(NH_4)_2SO_4$, Aspartic acid, Lysine, KNO_3 and Glycine) as well as the concentration of Carbon & Nitrogen were investigated for their effect on cellulase production. The content of the flasks was filtered through glass fibre filter paper and used as crude enzyme source.

Effect of period of incubation on cellulase production: Conical flasks (250 ml) each containing 100 ml of growth medium for cellulase production and was inoculated with 1 ml of standard spore suspension of fungi. The flasks

were incubated at room temperature for a period ranging from 1 to 14 days. The culture flasks were each analyzed for enzyme activity.

Carboxymethyl cellulase activity: Cellulase activity was determined by estimating amount of reducing sugars released in a reaction mixture containing 1 ml of 0.6% (w/v) CM-cellulose in 0.1 M citrate phosphate buffer (pH 4.5) and 0.5 ml of the crude enzyme solution. The control tube contained the same amount of substrate and 0.5 ml of the enzyme solution heated at 100^0C for 15 minutes. The experimental and control tubes were incubated in a water bath at 35^oC for 1 h. The amount of reducing sugars released during the reaction was measured by the modified dinitrosalicyclic acid reagent method of Miller (1959) and Olutiola (1983). The absorbance was taken at 540nm. Standard dilutions of glucose were used to plot a standard graph and the unknown values of the reducing sugars in the samples were extrapolated from the standard curve. One unit of cellulase activity was defined as the amount of enzyme in 1 ml of the reaction mixture that liberated reducing sugar equivalent to 10 μg glucose per minute under the specified conditions of the reaction.

RESULTS AND DISCUSSION

Five *Trichoderma* species namely *Trichoderma longibrachiatum, T. harzianum, T. viride, T. reesei* and *T, koningii* were isolated and identified based on their cultural and morphological characteristics from soil samples of decaying plant materials in a farmland. Three of the isolates namely *Trichoderma longibrachiatum* (designated as T10); *T. harzianum* (designated as T12) and *T. viride* (designated as T15) produced appreciable amount of cellulase activity (Fig. 1) and were later used for further studies. The least enzyme activity of 3.33 units/ml was produced by *T. reesei* while the highest activity of 1130 units/ml was produced by *T. Viride*.

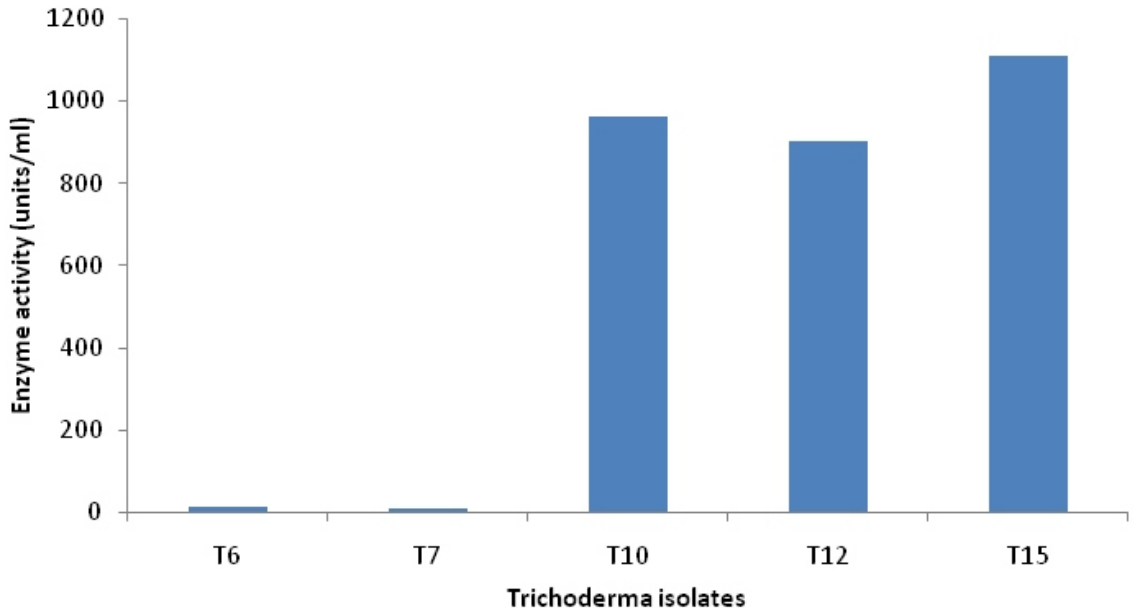

Figure 1: Production of Cellulase by *Trichoderma* Species Isolated from Decaying Plant Materials in a Farmland. T6, *T. koningii*; T7, *T. reesei*; T10, *T. longibrachiatum*; T12, *T. harzianum*; T15, *T.*

The results as presented in Fig. 2 showed the time course of cellulase production by the three isolates. The amount of enzyme produced increased up to the maximum level and then declined. Maximum production of cellulase was obtained on the 11th day in all the isolates while minimum was noted for all the isolates on the 5th day (Figure 2). Further incubation after this did not show any increment in the level of enzyme production probably due to increase in toxic unwanted wastes and depletion of nutrients in the medium which leads to decreased growth and enzyme (Haq *et al.*, 2005). Similar time course has been reported for xylanase production where the maximum production was observed after 6 days (Kavya and Padmavathi 2009) and for cellulose production by *Bacillus licheniformis* MVS1 and B. sp MVS3 where maximum cellulases production by both isolates was detected after 60 h incubation period using wheat and rice straw (Acharya and Chaudhary, 2012). Pothiraj *et al.*, (2006) recorded maximum FPase activity (0.46 IU/ml after 10 days of incubation period by *Rhizopus stolonifer* on Cassava waste).

The effect of different carbon sources on cellulase enzyme production by the three isolates showed that carboxymethyl cellulose (CMC) was the best substrate for cellulase production for all the three isolates while lactose and sucrose produced no detectable cellulase activity in the three isolates (Fig. 3). The result was in accordance with the work of Guatam *et al.*, 2010 who reported that good cellulose production can be obtained in CMC followed by cellulose for production of cellulose. Similar induction by *A. niger* in the presence of CMC substrates had been reported (Corel *et al.*, 2002). Likewise the results of Narasimha *et al.*, (2006) and Niranjane et al., (2007) revealed that CMC was best carbon source followed by cellulose for cellulose production. Low level of cellulolytic enzyme in the presence of glucose in this study could be attributed to repression of synthesis of cellulolytic enzymes involved in the utilization of cellulose by easily metabolisable carbon glucose that was demonstrated in many organisms (Suto and Tomita 2001; Ahmed *et al.*, 2009).

The effect of different nitrogen compounds on cellulase production by the three fungal isolates is presented in Fig. 4. Aspartic acid, potassium nitrate and sodium nitrate gave high cellulase yield with KNO_3 giving the highest value of 846.67 units/ml in *T. viride*. The least cellulase yield of

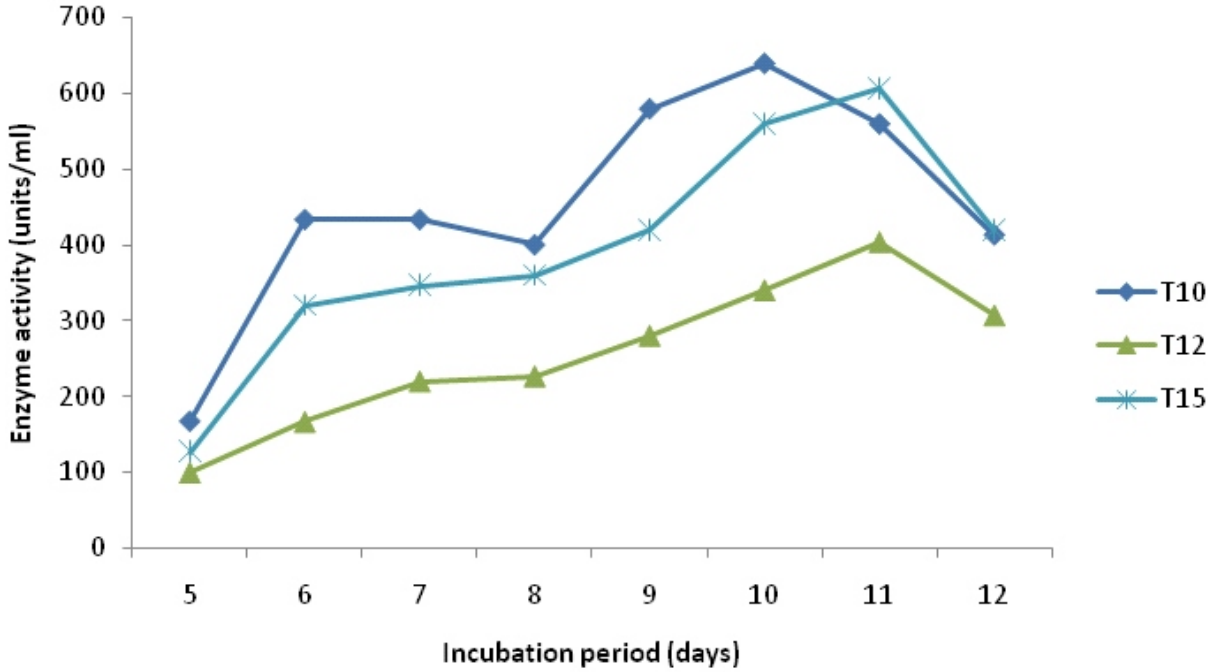

Figure 2: Effect of Incubation Period on Cellulase Production by *Trichoderma* Species Isolated from Decaying Plant Materials in a Farmland. T10, *T. longibrachiatum*; T12, *T. harzianum*; T15, *T. Viride*

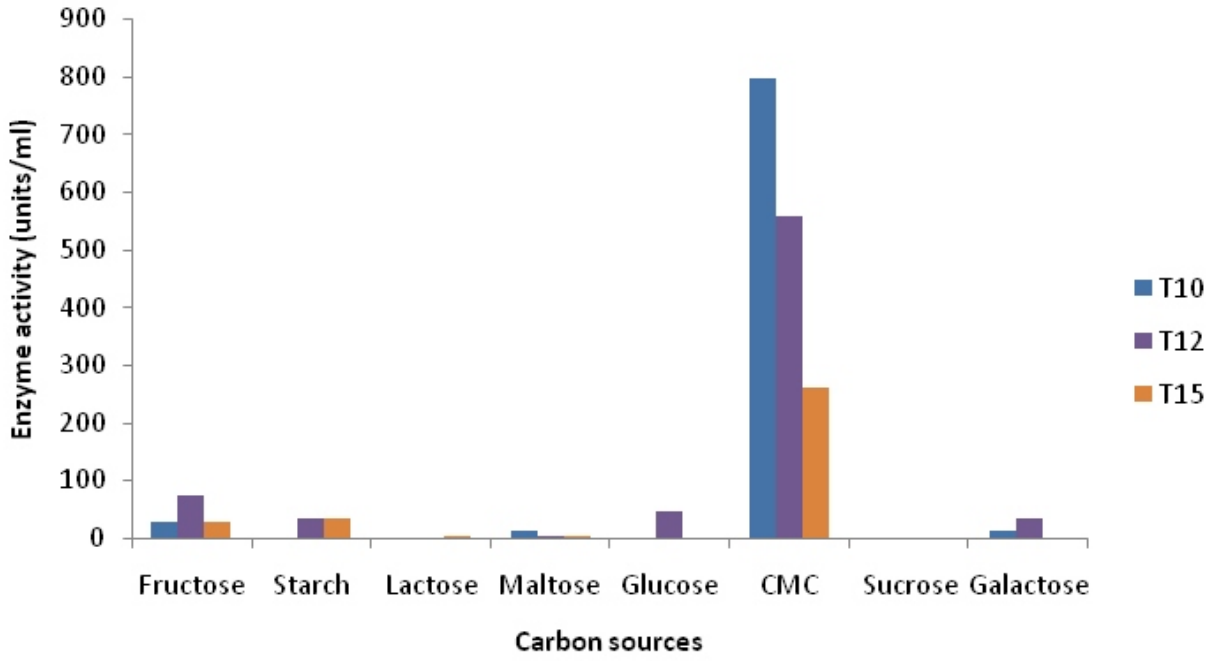

Figure 3: Effect of Different Carbon Sources on Cellulase Production by *Trichoderma* Species Isolated from Decaying Plant Materials in a Farmland. T10, *T. longibrachiatum*; T12, *T. harzianum*; T15, *T. Viride*

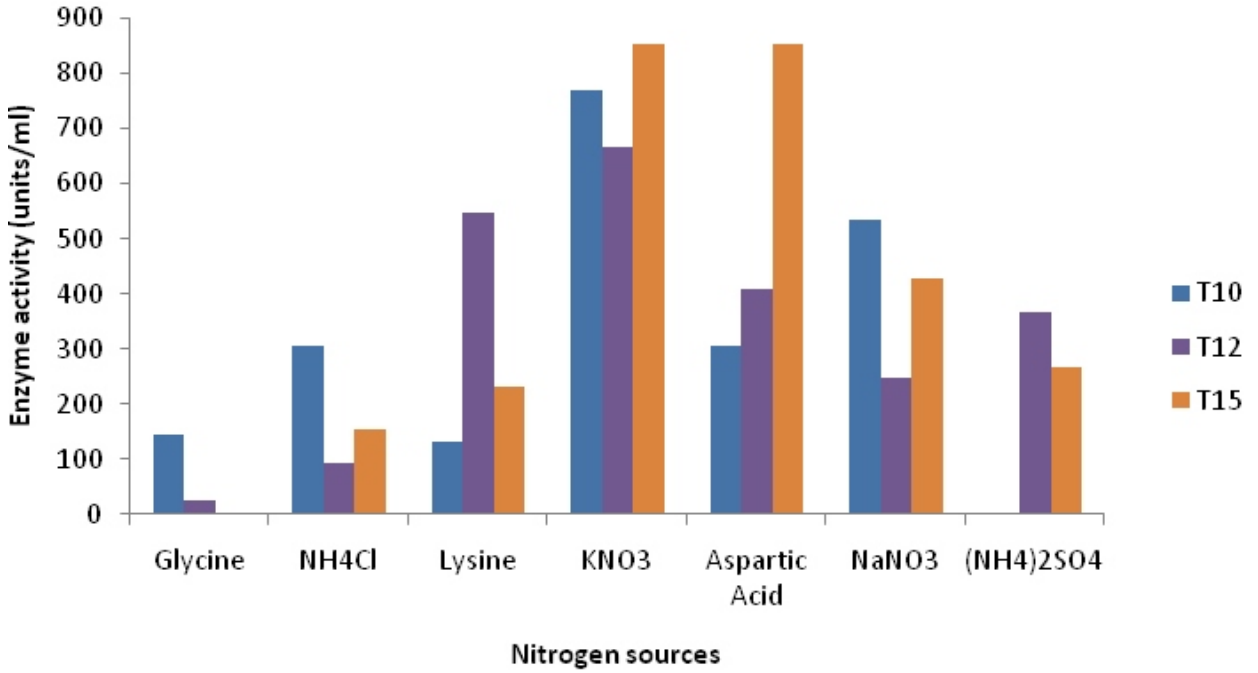

Figure 4: Effect of Different Nitrogen Sources on Cellulase Production by *Trichoderma* Species Isolated from Decaying Plant Materials in a Farmland. T10, *T. longibrachiatum*; T12, *T. harzianum*; T15, *T. Viride*

Production of microbial enzymes is dependent upon various nutritional and cultural parameters such as pH, temperature, carbon and nitrogen sources. These parameters have been studied in order to optimize the production of cellulolytic enzymes by selected fungal isolates. When these organisms were grown on basal medium lacking carboxymethyl cellulose (cellulase inducer) but containing either glucose, fructose, maltose, lactose, starch, sucrose or galactose as sole carbon source, all the *Trichoderma* species investigated released no appreciable quantities of cellulase. This showed that *T. longibrachiatum*, *T. harzianum* and *T. viride* could produce cellulase when a cellulosic material was present in the culture medium. This indicates that cellulase productions in these fungi are inducible and not constitutive. It has been reported that inducible enzymes are produced only in small amounts in the absence of inducers because the repressor system will not function as an absolute block, thus allowing constitutive production of cellulase which could easily yield soluble hydrolytic products (Eriksson, 1993). The soluble products of hydrolysis could then enter the cells and function as inducers because cellulose itself cannot enter the cell and act as an inducer as a result of its large size and complexity (Eriksson, 1993; Beguin *et al.*, 1977).

The effect of different concentrations of carboxymethyl cellulose (CMC) showed that cellulase production was highest at a CMC concentration of 10 mg/ml for all the isolates and was least at a CMC concentration of 2 mg/ml (Fig. 5). In all the isolates, cellulase activity increased with increase in CMC concentration until a CMC concentration of 10 mg/ml beyond which enzyme activity decreased gradually. Similar results was obtained in the work of Guatam *et al.*, 2010 who reported growth increased with increase in concentration of cellulose up to 1% (w/v) and further increase in cellulose concentration did not result in proportionate increase in yield of fungal biomass and cellulase production.

The amount of enzyme produced increased with increase in the concentration of potassium nitrate (KNO_3). However, optimum activity occurred at KNO_3 concentration of 1.0 mg/ml beyond which there was a gradual decrease in enzyme activity in all the isolates (Fig. 6).

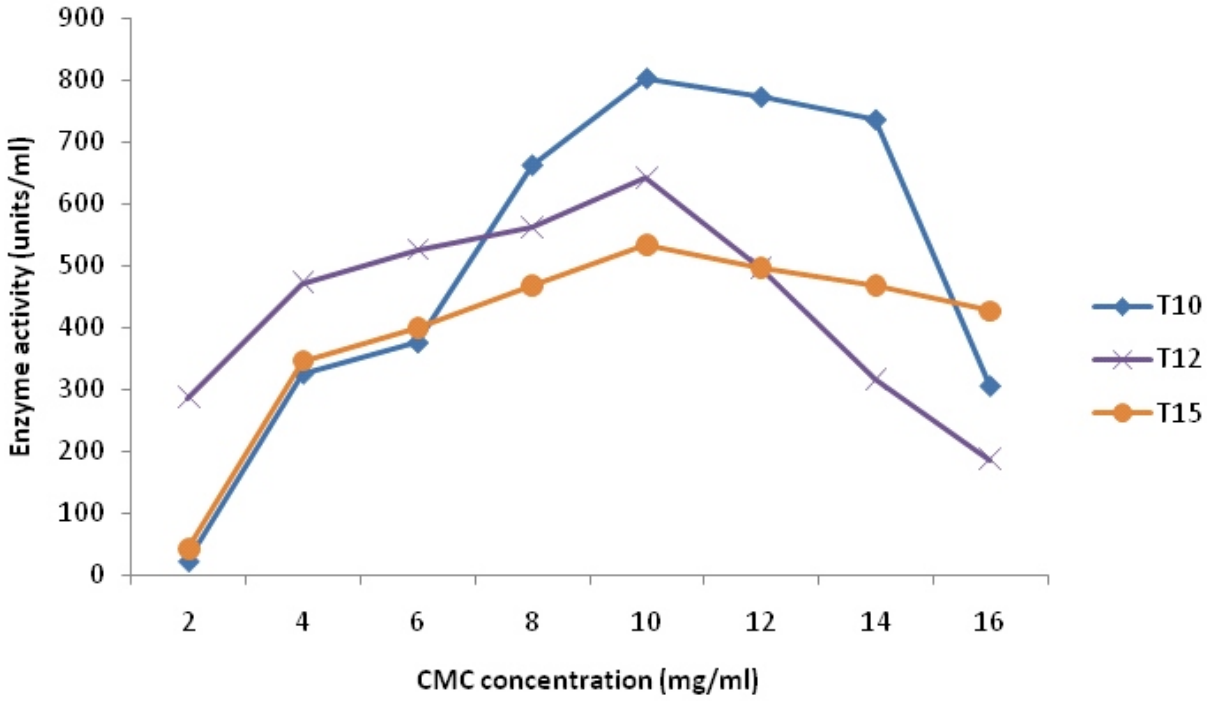

Figure 5: Effect of Different Concentrations of Carboxymethyl Cellulose (CMC) on Cellulase Production by *Trichoderma* Species Isolated from Decaying Plant Materials in a Farmland. T10, *T. longibrachiatum*; T12, *T. harzianum*; T15, *T. Viride*

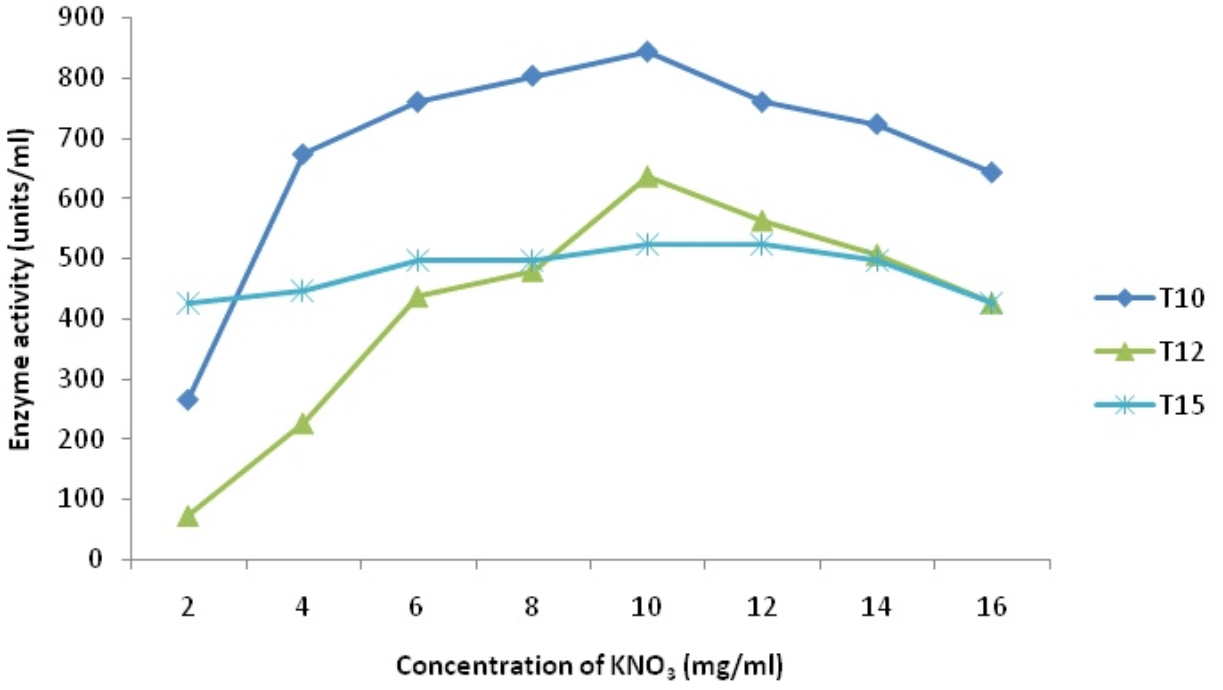

Figure 6: Effect of Different Concentrations of Potassium Nitrate (KNO_3) on Cellulase Production by *Trichoderma* Species Isolated from Decaying Plant Materials in a Farmland. T10, *T. longibrachiatum*; T12, *T. harzianum*; T15, *T. Viride*

The result of these investigations showed that *T. longibrachiatum*, *T. harzianum* and *T. viride* synthesized proteins which exhibited appreciable cellulase activity.

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