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EVALUATION OF ENZYMATIC ACTIVITIES AND DEGRADATION ABILITIES OF ANTAGONISTIC MICROORGANISMS ASSOCIATED WITH COMPOST

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ABSTRACT

During the present studies, thirteen antagonistic compost inhabiting bacteria and fungi selected from *in vitro* studies against soil borne pathogens were evaluated for their amylase, cellulase and pectinase activities. Most of the antagonists showed the enzymatic activities. The antagonists were also inoculated in to the grass clippings to see their degradation abilities. All the antagonists showed the ability to degrade the grass clippings but with varied rate of degradation. The highest rate of degradation shown by *Bacillus licheniformis* and *Aspergillus fumigatus* followed by *Bacillus subtilis*, *Trichoderma harzianum*, *Bacillus cereus*, *Bacillus megaterium*, *Penicillium citrinum*, *Bacillus pumilus*, *Micrococcus varians*, *Trichoderma virens*, *Acrophialophora fusispora*, *Stachybotrys charatum* and *Pseudomonas fluorescens*.

Key words: Amylase, cellulase, pectinase, compost inhabiting bacteria and fungi, degradation abilities.

INTRODUCTION

Compost is a solid mature product obtained from composting process. Composting is the recycling of solid waste. By the action of bacteria and fungi, organic waste change in to humus (Adediran *et al*., 2003). The composting material contains complex compounds and degradation of these compounds requires the use of extracellular enzymes produced by microorganisms (Miyatake and Iwabuchi, 2005). Many of the extracellular enzymes common in both bacteria and fungi convert complex compounds in to simpler. Beneficial microbes *viz.*, *Bacillus*, *Pseudomonas*, *Aspergillus*, *Penicillium* and *Trichoderma* species associated with compost (Ryckeboer *et al*., 2003). Many of them produce lytic enzymes that can hydrolyze a variety of compounds, including chitin, proteins, cellulose and hemicelluloses (Tripathi *et al*., 2008).

Amylase enzymes that play a major role in the utilization of polysaccharides (Zoltowska, 2001) occur in various bacteria, fungi, plants and animals (Da Lagea *et al*., 2007). α-amylase and glucoamylase are the two major classes of amylase enzymes (Gomes and Steiner, 2004). Commercially about 25-35% of the world enzymes consist of α-amylase produced by different microorganisms (Nguyen *et al*., 2002). The utilization of agriculture waste materials reduced the pollution and upgraded the materials. Agriculture waste used for both liquid and solid fermentation consists of carbon and nitrogen sources necessary for the growth and metabolism of organisms (Tang-Yao, 2002). These nutrients sources include corn, orange waste, wheat and rice as flours used for amylase production (Djekrif-Dakhmouche *et al*., 2006). It is also used in the fermentation industries for the conversion of starch in to simplest sugars (Farid *et al*., 2002).

Cellulose is one of the most important sources of carbon (Nowak *et al*., 2005). Cellulolytic enzymes include endoglucanases, exoglucanases and β-glucosidases that play an important role in the hydrolysis of cellulose and biomass utilization (Bhat and Bhat, 1997; Subramaniyan and Prema, 2000). Cellulolytic microorganisms degrade the cellulose into simplest sugar by producing cellulase enzyme (Kasana *et al*., 2008). During organic degradation, several microorganisms such as species of *Aspergillus*, *Penicillium* and *Trichoderma* have been reported to generate cellulolytic enzymes (Gautam *et al*., 2010b; Wilson, 2011). Many bacteria including species of *Bacillus*, *Pseudomonas*, *Streptomyces* and *Staphylococcus* are also capable to degrading cellulose (Gautam *et al*., 2010c). At industrial scale the cost of enzyme production can be reduced by obtaining the enzymes from microorganisms.

Pectin is a polysaccharide present in the cell wall of plants (Alphons *et al*., 2009). Various bacteria, fungi and higher plants produced pectinolytic enzymes that catalyze the degradation of pectic substances (Chinedu *et al*., 2008). The most important commercial pectinase is polygalacturonase. Several bacterial and fungal strains have potential to produce pectinolytic enzymes (Pericin *et al*., 2007; Nitinkumar and Bhushan, 2010). Pectinases are also produced from several agriculture pectin containing wastes (Reda *et al*., 2008), such as citrus peel (Mamma *et al*., 2008). The composting process can be speed up by microbial inoculations (Sarkar *et al*., 2010). The microbes generate enzymes and increase the degradation of organic materials (Ghaffari *et al*., 2011). The microbial

inoculation efficiency is usually affected by competition with indigenous microorganisms (Xi *et al*., 2005. Selection of suitable microbes is, therefore, an important factor for effectiveness of inoculation. The aim of the present study is to find out the beneficial microbes which can accelerate the composting process by producing extra cellular enzymes.

MATERIAL AND METHODS

Antagonistic microorganisms

Antagonistic microorganisms isolated from compost and check their antagonistic activity against soil borne pathogens by dual culture plate assay.

Amylase activity

A 5mm inoculum disc from pure culture of each antagonistic fungus was inoculated in the centre of Petri plates containing Starch Peptone Agar (SPA) medium (Starch 5g, peptone 10g, agar 20g, distilled water, 1000ml) amended with penicillin and streptomycin. The antagonistic bacteria were streaked on SPA medium without antibiotics. There were three replicates of each bacterium and fungus. The plates were incubated at their respective temperatures for 2-3days before exposer to iodine vapours. Formation of a clear zone around the colonies showed amylolytic activity and indicated a positive reaction. Medium where starch was not utilized turned blue when exposed to iodine vapours.

Cellulase activity

A 5mm inoculum disc from pure culture of each antagonistic fungus was inoculated in the center of Petri plates containing Cellulose agar (CA) medium (Cellulose 10g, agar 20g, distilled water, 1000ml) amended with penicillin and streptomycin. The antagonistic bacteria were streaked on CA medium without antibiotics. There were three replicates of each bacterium and fungus. The plates were incubated at their respective temperatures for 2-3 days. The plates were then exposed to iodine vapours. Formation of clear zone around the colonies showed the cellulolytic activity and indicated a positive reaction. Medium where cellulose was not utilized turned pinkish when exposed to iodine vapours.

Pectinase activity

A 5mm inoculum disc from pure culture of each antagonistic fungus was inoculated in the center of Petri plates containing Hanskin's medium. To prepare the medium, mineral salts *viz.*, $(NH_4)_2SO_4$ 2g, K_2HPO_4 4g, Na_2HPO_4 6g, $FeSO_4.7H_2O$ 0.2g, $CaCl_2$ 0.001g, H_3BO_3 0.00001g, $ZnSO_4$ 0.00007g, MoO_3 0.00001g, all dissolved in 100ml distilled water and volume makeup to 500ml; Yeast extract 1g, pectin 5g, agar 20g, distilled water 500ml and then both the 500ml solutions mixed to get 1000ml medium. Penicillin and streptomycin added to the medium for inhibition of bacteria. The antagonistic bacteria were streaked on Hanskin's medium without antibiotics. There were three replicates of each bacterium and fungus. The plates incubated at their respective temperatures for 2-3days and then flooded with 1% aqueous solution of hexa-decyle-tri-methyl ammonium bromide. Formation of a clear zone around the colonies showed the pectinase activity and indicated a positive reaction**.** Medium where pectin was not utilized showed no change in colour.

Degradation abilities of antagonists

Four ml water was added to 10g of grass (*Cynodon dactylon* L.) clippings in 100ml flasks. The flasks were plugged with cotton and autoclaved. After autoclaving, the flasks were allowed to cool down and then inoculated with antagonist. For each antagonist there were three replicates. Flasks without antagonist served as control. Time that each antagonist took to degrade grass clippings was recorded.

RESULTS

Antagonistic microorganisms

Thirteen microorganisms (six fungi and seven bacteria) were selected by dual culture plate assay.

Amylase activity

Five fungi *viz.*, *Aspergillus fumigatus*, *Penicillium citrinum, Stachybotrys charatum, Trichoderma harzianum, T. virens* and five bacteria *viz., Bacillus cereus, B. licheniformis, B. megaterium, B. subtilis* and *Micrococcus varians* showed amylolytic activity (Table 1; Fig. 1).

Cellulase activity

All fungi *viz.*, *Acrophialophora fusispora, Aspergillus fumigatus, Penicillium citrinum, Stachybotrys charatum, Trichoderma harzianum, T. virens* and six bacteria *viz., Bacillus cereus, B. licheniformis B. megaterium, B. pumilus, B. subtilis* and *Micrococcus varians* showed cellulolytic activity (Table 1; Fig. 2).

Pectinase activity

Four fungi *viz.*, *Acrophialophora fusispora, Aspergillus fumigatus, Penicillium citrinum, Trichoderma harzianum* and seven bacteria *viz., Bacillus cereus, Bacillus licheniformis, B. megaterium, B. pumilus, B. subtilis, Micrococcus varians* and *Pseudomonas fluorescens* showed pectinolytic activity (Table 1; Fig. 3).

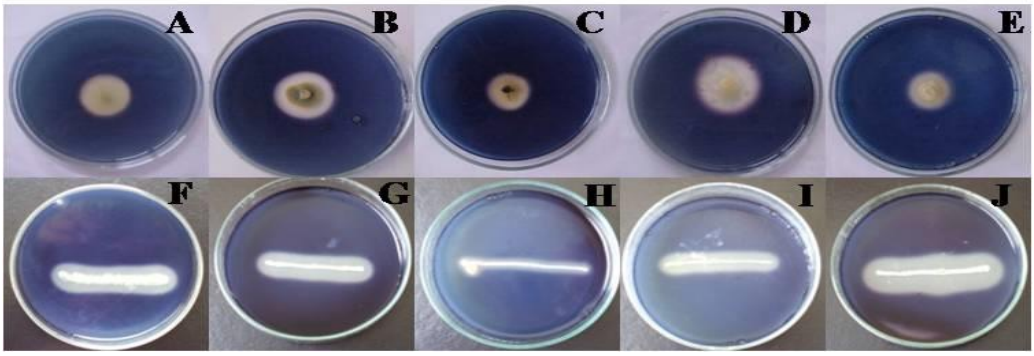

Fig. 1. Amylase activities of antagonists

A= *Aspergillus fumigatus*, B= *Penicillium citrinum*, C= *Stachybotrys charatum*, D= *Trichoderma harzianum*, E= *Trichoderma virens*, F= *Bacillus cereus*, G= *Bacillus licheniformis*, H= *Bacillus megaterium*, I= *Bacillus subtilis*, J= *Micrococcus varians.*

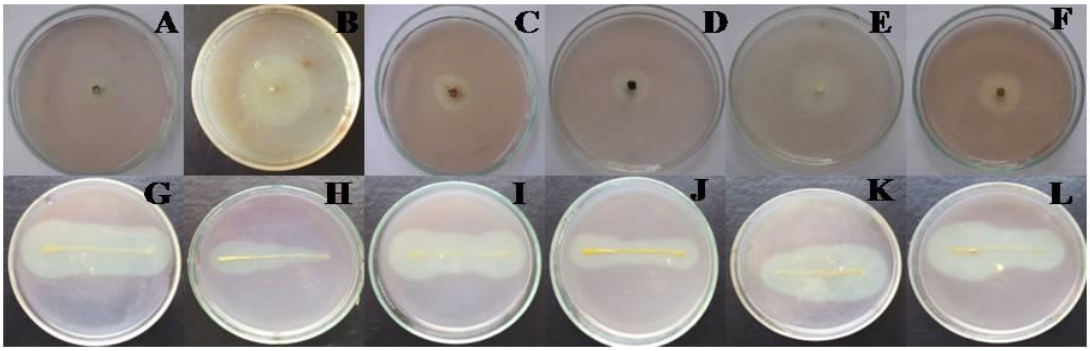

Fig. 2. Cellulase activities of antagonists

A= *Acrophialophora fusispora*, B= *Aspergillus fumigatus*, C= *Penicillium citrinum*, D= *Stachybotrys charatum*, E= *Trichoderma harzianum*, F= *Trichoderma virens*, G= *Bacillus cereus*, H= *Bacillus licheniformis*, I= *Bacillus megaterium*, J= *Bacillus pumilus*, K= *Bacillus subtilis*, L= *Micrococcus varians.*

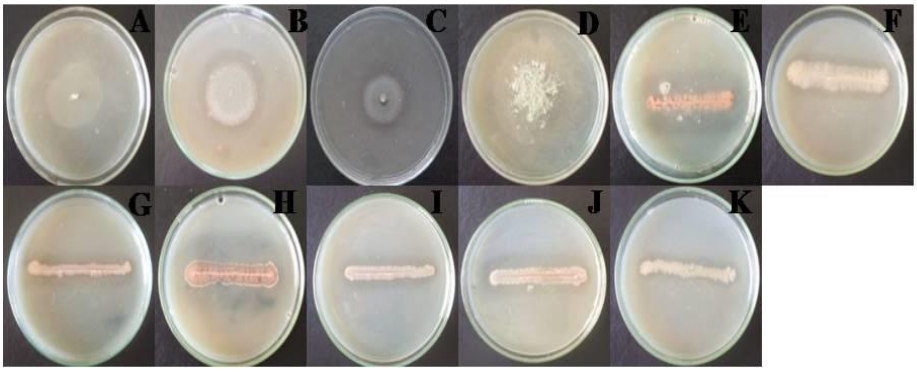

Fig. 3. Pectinase activities of antagonists

A= *Acrophialophora fusispora*, B= *Aspergillus fumigatus*, C= *Penicillium citrinum*, D= *Trichoderma harzianum*, E= *Bacillus cereus*, F= *Bacillus licheniformis*, G= *Bacillus megaterium*, H= *Bacillus pumilus*, I= *Bacillus subtilis*, J= *Micrococcus varians*, K= *Pseudomonas fluorescens.*

Degradation abilities of antagonists

All the antagonists showed ability to degrade the grass clippings. However, the microorganisms showed varied rate of degradation. The highest rate of degradation was showed by *Bacillus licheniformis* and *Aspergillus fumigatus* followed by *Bacillus subtilis, Trichoderma harzianum, Bacillus cereus, Bacillus megaterium, Penicillium citrinum, Bacillus pumilus, Micrococcus varians, Trichoderma virens, Acrophialophora fusispora, Stachybotrys charatum* and *Pseudomonas fluorescens* (Table 2).

Table 1. Enzymatic activities of antagonists.

S.No.	Test organisms	Amylase	Cellulase	Pectinase
	Fungi			
1	*Acrophialophora fusispora*	-	+	+
2	*Aspergillus fumigatus*	+	+	+
3	*Penicillium citrinum*	+	+	+
4	*Stachybotrys charatum*	+	+	-
5	*Trichoderma harzianum*	+	+	+
6	*Trichoderma virens*	+	+	-
	Bacteria			
7	*Bacillus cereus*	+	+	+
	Bacillus licheniformis	+	+	+
9	*Bacillus megaterium*	+	+	+
10	*Bacillus pumilus*	-	+	+
11	*Bacillus subtilis*	+	+	+
12	*Micrococcus varians*	+	+	+
13	*Pseudomonas fluorescens*	-	-	+

Table 2. Degradation of grass clippings by biocontrol agents.

S.No.	Name of microorganisms	Days of incubation	Degradation
1	*Acrophialophora fusispora*	11	++
2	*Aspergillus fumigatus*	5	+++
3	*Bacillus cereus*	6	++
4	*Bacillus licheniformis*	5	+++
5	*Bacillus megaterium*	6	++
6	*Bacillus pumilus*	7	++
7	*Bacillus subtilis*	6	+++
8	*Micrococcus varians*	8	++
9	*Penicillium citrinum*	6	++
10	*Pseudomonas fluorescens*	12	+
11	*Stachybotrys charatum*	14	++
12	*Trichoderma harzianum*	6	+++
13	*Trichoderma virens*	8	++

+ = Good Degradation
++ = Better Degradation
+++ = Best Degradation

DISCUSSION

Fungi and bacteria are involved in the cycling of nutrients since they have ability to produce enzymes that degrade the complex organic molecules into simplest, that are unavailable to many organisms (Orth *et al*., 1993). Bacteria secrete many extracellular enzymes that are involved in assortment recognition and important for symbiosis (Van Workum Wat *et al*., 1998). Enzymes such as chitinase, B-1,3 glucanases and cellulases produce by *Trichoderma harzianum* (Lorito *et al*., 1994; Di Pietro, 1995) help in degradation of the cell wall of pathogens and therefore, biological activity increases.

During the fermentation of starch many bacteria and fungi produce extracellular amylases (Adeniran and Abiose, 2009). The microbes produced amylase enzyme convert the starch into the oligosaccharides with quick reduction in blue colour and appearance of clear zone (Najafi *et al*., 2005). In starch industries, α- amylase play a major role (Pandey *et al*., 2000). The antifungal activity of *Trichoderma* species is due to the greater variety of lytic enzymes. These lytic enzymes play a great role in biocontrol (Kubicek *et al*., 2001). During the present studies, amylase enzymes produced by antagonists *viz., T. harzianum, T. virens, A. fumigatus, P. citrinum, S. charatum, B. cereus, B. licheniformis, B. megaterium, B. subtilis* and *Micrococcus varians*. Similarly report made by (Das *et al*., (2004), Mohamed *et al*. (2011), and Hamuel (2011). Some species of *Bacillus* produced enzymes in stationary or some in exponential phase. Stability of enzymes is sensitive to pH and temperature (Declerk *et al*., 2003).

The cellulase producing fungi and bacteria were selected on the formation of clear zone around their colonies on coboxymethyl cellulose agar plates (Immanuel et al., 2006). During the present studies *T. harzianum, T. virens, A. fumigatus, A. fusispora, P. citrinum, S.charatum, B. cereus, B. licheniformis, B. megaterium, B. pumilus, B. subtilis* and *M. varians* showed cellulase activity. Mishra *et al*. (1982) isolated different cellulolytic fungi *viz., Chaetomium globossum, Fusarium solani, Paecilomyces variotii* and *Penicillium chrysogenum* from soil and compost. Cellulolytic fungi such as *Aspergillus, Chaetomium, Penicillium* and *Trichoderma* hasten composting and reduce the composting period for one month (Dubey and Maheshwari, 2005). *B. cereus* (Lah *et al*., 2012), *B. pumilus* (Ariffin *et al*., 2006), *B. licheniformis* (Fujimoto *et al*., 2011), *B. subtilis* (Kim *et al*., 2012), *A. fumigatus*, (Kumari *et al*., 2011) have been reported earlier to produced cellulases.

Pectic substances are the main component of cell wall. These substances are degraded by the enzyme pectinase. The major degrader of fruit wastes are the pectinolytic bacteria (Rolz *et al*., 2011). Many workers observed pectin production using agricultural wastes as substrate (Castilho *et al*., 2000). *T. harzianum, P. citrinum, A. fumigatus, A. fusispora, M. varians, P. fluorescens* and thermotolerant species of *Bacillus* showed pectinase activity during present studies. Bhardwaj and Garg, (2010) observed similar results. Different workers isolated pectinase from bacteria and fungi *viz., A. fumigatus* (Phutela *et al*., 2005), *T. harzianum* (Nabi *et al*., 2003), *Bacillus* species (Marcia *et al*., 1999). Through bacteria and fungi important enzymes could be produced cheaply and it will reduce the cost of final products.

All the species that produced enzymes are important from the biotechnological point of view and in the decomposition of agricultural residues (Singh and Hayashi, 1995). The abundant growth in the fermentation substrate indicated the ability of fungi and bacteria to produce enzymes, which degraded the substrate. Enzymes can be produced on both solid and liquid substrates. However, solid-state fermentation has many advantages as compared to liquid state fermentation (Aguilar and Huitron, 1990). To reduce the cost of fermentation, agricultural waste is used for fermentation. Mostly bacteria and fungi are involved in the waste bioconversion. These wastes mostly consist of carbon and nitrogen sources. In the present studies, grass was used as a substrate. For decomposition of grass clippings, spore suspensions of selected biocontrol agents were added to grass clippings. The degradation of grass clippings was much faster in inoculated grass as compared to non-inoculated grass clippings. *A. fumigatus* and *B. licheniformis* showed the fastest degradation of grass clippings within five days as compared to other biocontrol agents, when inoculated singly. There are many microbiological agents involved in the biodegradation. *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp., produced cellulase enzyme during the degradation of organic waste (Gautam *et al*., 2010b). The microbes present in compost produced a variety of enzymes, which help in the degradation. Vargas-Garcia *et al*. (2005) reported that waste from the agriculture sources could be used as substrates for the growing and preservation of lingo-cellulolytic fungi.

Normally natural composting process takes five to six months (Jilani, 2007), but this period can be shortened by inoculating the beneficial microbes with high enzymatic activities in to the composting material. Biswas and Narayanasamy (2002) inoculated *Trichoderma* and *Aspergillus* species into the composting material and they observed that composting period was shortened and maturity of compost occurs within one month. Composts represent an optimal substrate for biocontrol agents, thus encouraging their establishment into the soil environment (Leandro *et al*., 2007).

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