

Effect of Essential Oil of *Eucalyptus Rostrata* on the Production of Some Enzymes by *Trichoderma virens* and *Fusarium solani*

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Abstract: Effect of volatile oils from *Eucalyptus rostrata* on the production of α -amylase, cellulases and chitinases from two pathogenic fungi *Trichoderma virens* and *Fusarium solani* was investigated using a liquid culture. The production of these enzymes by *Trichoderma virens* was increased by increasing the concentrations of the volatile oils to certain limit. *Trichoderma virens* and *Fusarium solani* showed a slightly high enzymes production on the first stage of growth, then, the enzymes level production were decreased with the inhibition of the fungal growth.

Key words: Eucalypt oils, α -amylase, cellulases, chitinases, *Trichoderma virens*, *Fusarium solani*.

INTRODUCTION

Many fungi produce extracellular enzymes that enable them to breakdown polysaccharides and proteins into sugars and amino acids that can be assimilated easily. These enzymes are of considerable industrial importance. α -Amylase and protease are mainly fungal and eubacterial products. The possibility of using streptomyces for enzyme production has recently investigated (Yang and Wang, 1999). Cellulase and amylase are two important industrial enzymes whose need is on the increase. Cellulase and amylase are used in paper, textile, food industries and chemical industries (Emmanuel and Saleh, 2004).

During the last twenty years, a great interest has been brought to enzymatic hydrolysis of cellulose. Cellulolytic enzymes are synthesized by a numerous number of microorganisms, though fungi are generally 50-1000 times more cellulolytic than the most active bacteria. The production of cellulases from fungi has been extensively studied and the most active cellulases are presently derived from species of *Trichoderma* and *Aspergillus* (Tahoun and Ibrahim, 1999). Eriksson and Wood (1985) and Philippidis and Smith (1995) reported that the distinguished feature of the cellulases that can solubilize crystalline cellulose is that it contains, (1) Endoglucanases, which randomly attack cellulose chains to produce short length of polysaccharides, (2) Exoglucanase, which attack to the non-reducing ends of those shorter chains and remove cellobiose moieties, (3) β -glucosidase, which hydrolyze cellobiose and other oligosaccharides to glucose.

Chitinases are digestive enzymes that break down glycosidic bonds in chitin (Jollès and Muzzarelli, 1999). Because chitin composes the cell walls of fungi and exoskeleton elements of some animals (including worms and arthropods), chitinases are generally found in organisms that either need to reshape their own chitin (Sami *et al.*, 2001) or to dissolve and digest the chitin of fungi or animals. Fungi, such as *Trichoderma* sp. (Marco *et al.*, 2003), *Aspergillus niger* (Emmanuel and Saleh, 2004) are known to possess chitinases. This may be related to their typical role as detritivores and also to their potential as arthropod pathogens. Chitin like cellulose has been thought of as abundant but difficult to digest (Akaki and Duke, 2005). It is typically considered unavailable carbohydrate in animal diets, though certain fish can digest chitin to sugar (Gutowska *et al.*, 2008) and dogma suggests that just as ruminants need bacteria to digest cellulose and they digestion would also require symbiosis and lengthy fermentations.

The present study was therefore carried out to investigate the effect of *Eucalyptus rostrata* volatile oils on the production of three industrial enzymes (namely α -amylase, cellulases and chitinases) from two pathogenic fungi *Trichoderma virens* and *Fusarium solani*.

MATERIAL AND METHODS

The volatile oil of *Eucalyptus rostrata* was extracted from the above ground air dried parts (Shoot system) using steam-distillation apparatus for 3 hours. The oils obtained were separately dried over anhydrous sodium

sulphate (Baratta *et al.*, 1998). The crude oil was tested at different concentrations for the enzyme productions with the two tested fungi *Trichoderma virens* and *Fusarium solani* which procured from NRC Microbial collection unit. 20 μ l of volatile oil was mixed with Tween-80 (0.05%) and diluted with 20ml of distilled water to make a 30 ppm stock solution. This was further diluted with 30 ml water to give concentrations of 2.5, 5, 7.5, 10, 15, 20 and 30 ppm. 1ml of each concentration was added to 20 ml of Czapek - Dox agar medium and mixed thoroughly. Thereafter, a mycelial disc of approximately 5 mm diameter, cut plate (Ramezani *et al.*, 2002). Tween-80 (0.05) mixed with distilled water instead of oils served as the control for each treatment. Three replicates were maintained in a completely randomized design. These Petri plates were then incubated at 25 + 2°C and observations were recorded on the 8th day.

Enzymes Assay

α -Amylase:

α -Amylase was assayed by adding 1ml of enzyme (filtrate) to 1ml soluble starch (1%) in acetate buffer pH 5 and incubating at 50°C for 15 min. The reaction was stopped by the addition of 2 ml of 3, 5-dinitrosalicylic acid reagent (Bernfeld, 1955). The absorbance was measured at 550 nm. One enzyme unit (v/ml) is defined as the amount of enzyme which releases 1 μ M glucose.

Exoglucanase (FPase):

FPase was measured according to Mandels and Sternberg (1976). The reaction mixture contained 0.5ml of 0.05 M acetate buffer at pH 4.8, 0.5ml culture filtrate and one filter paper strip 1x3 cm (25mg). The mixture was incubated for 60 min at 50°C. The reaction was terminated with 1ml dinitrosalicylic acid reagent (Miller, 1959) and boiling for 5 min in a boiling water bath, then, 10ml distilled water was added and absorbance was measured at 540nm. One unit of enzyme activity was defined as the amount of enzyme required to release reducing sugars equivalent to 1.0 μ M of glucose under the standard assay condition.

Carboxy methyl cellulase activity (CMCase):

CMCase activity was measured as the above mentioned method for determination of FPase activity except that 0.5 ml of 1% carboxy methyl cellulose in acetate buffer was used as substrate; incubation period was 30 min only at 50°C. Blank tube was prepared by the same method, where enzyme solution was boiled for 5 min before used. One unit of enzyme activity was defined as the amount of enzyme required to release reducing sugars equivalent to 1.0 μ M of glucose under assay conditions.

β - Glucosidase activity:

β -Glucosidase activity was assayed with 0.67 mM (0.02%) p-nitrophenyl β -D glucosidase as substrate in 0.05 M acetate buffer pH 4.8. Reaction mixture containing 10 μ M culture filtrate and complete to 1ml with buffered substrate was incubated at 50°C. The reaction was terminated after 10min with 3ml 0.1 M NaOH. The absorbance was measured at 400nm. Standard curve was obtained with p-nitrophenol. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μ M p-nitrophenol (Reczey *et al.*, 1990).

Chitinase activity:

Colloidal chitin was prepared from chitin powder (Sigma Co.) according to the method described by Reid and Ogryd-Zia (1981). Twenty five grams of chitin powder suspended in 250 ml of 85% phosphoric acid (H₃PO₄) and stored at 4°C for 24h., then blended in 2 liter of distilled water using a warring blender. The suspension was centrifuged. This washing procedure was repeated twice. The colloidal chitin suspension was adjusted to pH 7.0 with (1N) NaOH and recentrifuged. The pelleted colloidal chitin was stored at 4°C until used. Determination of enzyme activity was carried out according to the method of Monreal and Reese, (1969). One ml of 1% colloidal chitin in 0.05 M citrate phosphate buffer (pH 6.6) in the tubes. One ml of enzyme extract was added and mixed by shaking. Tubes were incubated in water bath at 37°C for 60 minutes, then cooled and centrifuged before assaying. Reducing sugars were determined in 1 ml of the supernatant by dinitro-salicylic acid (DNS). Optical density was determined at 540 nm.

RESULTS AND DISCUSSION

Based on literature data it can be concluded that essential oils originating from plants have highly antimicrobial activities (Lee *et al.*, 2004). The effect of essential oil of *Eucalyptus rostrata* on *α - amylase* production by *Trichoderma virens* and *Fusarium solani* is shown in Fig. (1). All samples tested induced *α - amylase* production at

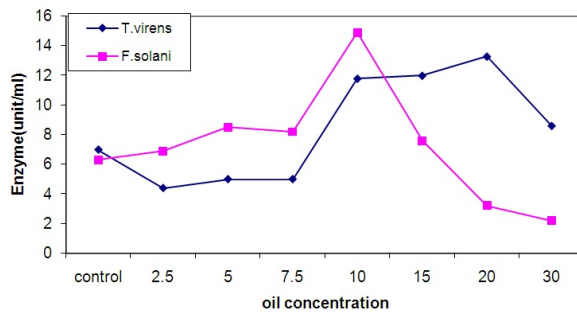


Fig. 1: Effect of essential oil of *Eucalyptus rostrata* on α - Amylase activities produced by *Trichoderma viren* and *Fusarium solani*.

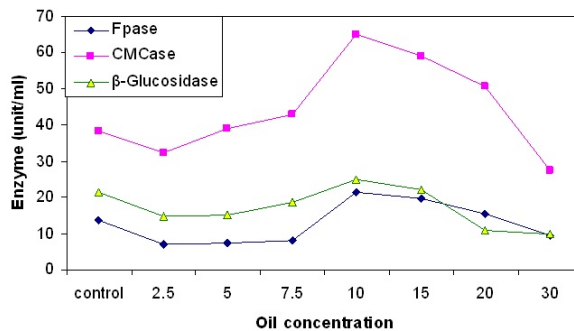


Fig. 2: Effect of essential oil of *Eucalyptus rostrata* on cellulase activities produced by *Trichoderma virens*.

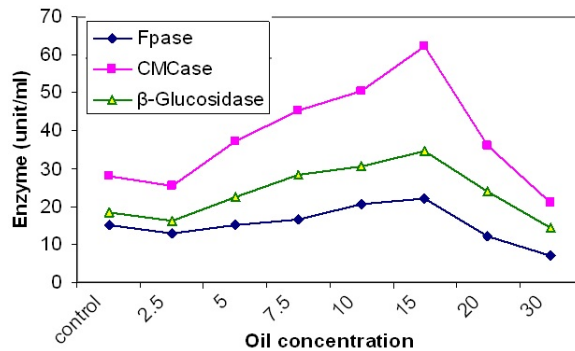


Fig. 3: Effect of essential oil of *Eucalyptus rostrata* on cellulase activities produced by *Fusarium solani*.

different oil concentration. α - Amylase production increased gradually until 20 ppm (13.3 unit/ml) of oil concentration in the culture medium of *Trichoderma virens* but at values lower than the control (at oil concentrations 2.5, 5, 7.5 ppm) then decreased at 30 ppm of oil concentration. On the other hand, α - amylase production by *Fusarium solani* is increased at oil concentration 10ppm (14.9 unit/ml) after this concentration α - amylase production has been decreased. These results agreed with the findings of Ellen *et al.* (2008) who stated that the orange essential oil at concentrations greater than 1% (v/v) was strongly inhibited fungal growth of *Botryosphaeria rhodina* and more greatly reduced laccase and pectinase enzyme productions.

Trichoderma spp. and *Fusarium* spp. are known to produce cellulases (Nevalainen and Penttila 1995). Dekker *et al.* (2001) stated that the ascomyceteous fungi described as ligninolytic, produces enzymes degrading the lignified plant cell wall, including cellulases.

The initial attack of crystalline cellulose is investigated to be by the endoglucanase creating nicks in the linear cellulose chains. The action of endoglucanase is followed by attack of the cellobiohydrolase at these nick sites resulting in the release of cellobiose. The continued combined action of cellulose to cellobiose and small oligosaccharides which converted to glucose by β - glucosidase (Ryu and Mandels 1980). In accordance, *Trichoderma virens* was found to produce substantial amounts of these enzymes.

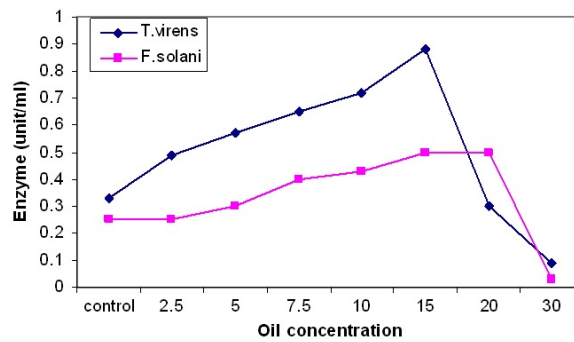


Fig. 4: Effect of essential oil of *Eucalyptus rostrata* on chitinase activities produced by *Trichoderma virens* and *Fusarium solani*.

The results of exoglucanase (FPase), endoglucanase and β -glucosidase activities were shown in Fig. (2). However, although the highest exoglucanase (21.3 U/ml), endoglucanase (65.2 U/ml) and β -glucosidase (25.0 U/ml) activities produced by *Trichoderma virens* was obtained at concentration (10ppm) of eucalyptus essential oil, this activity was decreased gradually with the increase of oil concentration. Ellen *et al.* (2008) discussed that if high enzyme production are required, then it is necessary beforehand to remove the essential oils fraction from plant materials used.

The results of exoglucanase (FPase), endoglucanase and β -glucosidase activities produced by *Fusarium solani* were shown in Fig. (3). The highest exoglucanase (22.2 U/ml), endoglucanase (62.3 U/ml) and β -glucosidase (34.6 U/ml) activities was produced at concentration (15 ppm) of eucalyptus essential oil, this activity was decreased gradually with the increase of oil concentration.

Fungal cell walls are complex structures constituted almost exclusively of polysaccharides such as glucan (cellulose, β -1, 3- and β -1, 6-glucan) and chitin (Chet and Baker, 1980). These polysaccharides may well act as inducers of hydrolytic enzymes. Both *Trichoderma virens* and *Fusarium solani* tested produced and secreted substantial amounts of chitinolytic enzyme under the same growth conditions (Fig. 4). Maximal activity of chitinase production was (0.88 U/mL) by *Trichoderma virens* at oil concentration 15 ppm and (0.5 U/ml) by *Fusarium solani* at oil concentration 20 ppm respectively. After this concentrations of oil the production of enzymes is decreased. It has recently been reported by Wilkins *et al.* (2007), that d-limonene strongly interfered with the growth of the yeast *Saccharomyces cerevisiae*.

The essential oil of eucalypt oil exerts fungitoxic effect (Hamido and El Shamy, 2007). This oil is rich in terpenes and aromatic compounds. Terpenoids can be active against fungi and bacteria as antimicrobial agents (Cowan, 1999).

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