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EFFECT OF FUNGICIDES AND PHENOLIC COMPOUNDS ON THE PRODUCTION OF PROTEASES BY *BACILLUS SUBTILIS*

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Addition of fungicides and phenolic compounds to the basal medium caused marked changes in the production of neutral as well as alkaline proteases by *Bacillus subtilis*. All the fungicides tried were found to inhibit the production and activity of proteases. Brassicol and Bavistin were most effective. Phenolic compounds other than Ferulic Acid wefe observed to have an inhibitory effect on enzyme production.

Key words: Fngicides, Phenolic compounds, Proteases.

INTRODUCTION

Bacillus subtilis produces both neutral and alkaline proteases in synthetic medium [1] and Czapek Dox medium [2]. Production of different enzymes such as pectic enzyme cellulases have been suppressed by various fungicides as reported by Mehta [3]. Production of cellulose and its growth was inhibited by the addition of different phenolic compounds such as catechol. Gallic and phloretin by the culture *Fusarium* oxysporum and *Fusarium vasinfectum* as reported by Reddy [4].

The present paper describes the effect of various fungicides and phenolic compounds on growth and production of neutral and alkaline proteases by *Bacillus subtilis*.

MATERIALS AND METHODS

Organism. The strain of *Bacillus subtilis* WRL-1036 was used in the present studies. The culture was maintained on the agar medium consisting of (g/1) glucose 10, Agar, 20, Beef extract 2.0, yeast extract 2.0, peptone 1.0, NaCl 1.0 g. The cultures were grown at 30 ± 2°C for two days and then stored in a refrigerator.

Inoculum preparation. Vegatative inoculum was used. The composition of the inoculum medium was (g/1) glucose 10.0 beef extract 2.0, yeast extract 2.0, peptone 1.0, NaCl 1.00. 24 ml of the inoculum medium was placed in 300 ml conical flask and inoculated by transferring small mycelium from the agar slant asceptically. It was allowed to grow at 30°C for 24 hours on a rotary shaker.

Fermentation medium. 1 ml of the vegetative inoculum was transferred in 300 ml erlenmeyer flasks containing 24 ml of fermentation medium composed of (g/1) glucose 10 g. Ammonium citrate 2.80, KH_2PO_4 1.3, $MgSO_4.7H_2O$ 0.5, KCl 0.10, $CaCl_2.2H_2O$ 0.20. All the reagents are of analytical grade and glass distilled water used for the preparation of solutions. All media unless otherwise stated, were autoclaved at 121° for 15 minutes. The initial pH of the medium was 6.8. The flasks were shaken on a rotary shaker rotated at 125 rev/min.

Assay of proteolytic enzymes. The proteolytic activity was determined by the method of Kokichioshima [5] and protease unit was expressed as that amount of activity which hydrolyzed 5 mg of casien in one hour under specific conditions. The activity of the culture liquid was measured in tris-Buffer solution of pH 7.0 and 9.0 at temperature 40° for one hour.

Chemicals. Commercially available fungicides were employed. All these fungicides are obtained from the Agriculture Department Punjab, Lahore. Ferulic acid, Caffeic acid and catechol were from Sigma Chemical Co. U.S.A.

Tannic acid, Cinnamic acid, Salicylic acid were of analytical reagent grade.

RESULTS

Table 1 shows that all the phenolic compounds except ferulic and tannic acid decreased the growth of the culture when added to the nutrient medium. Different concentrations such as 0.05, 0.075 and 0.10 (g/100 ml) of every phenolic compound was investigated. It was observed that cinnamic acid and Catechol were quite effective on growth as well as production of the enzyme. All the phenolic compounds were responsible to increase the pH of the culture medium. Ferulic acid gave slightly better yield as compared to control culture. Produciton of proteases went on decreasing in case of ferulic and tannic acid with the increase of their concentration. Such behaviour was also

seen in other phenolic compounds. However, formation of enzyme and growth of culture was not found at all. Table 2 shows the effect of different fungicides. Different concentrations such as 0.05, 0.075, 0.1 % of fungicides were used for the production of proteases. Zincop and mildothane gave better results. It was observed that enzyme yield was quite satisfactory in case of zincop 2.0 and 4.0 units/ml as compared to mildothane 1.4, and 2.6 units/ml as neutral and alkaline proteases. Other fungicides did not response except morestan. Morestan, mildothane tended to increase the pH of the culture filtrate, whereas zincop, brassicol and bavistin caused no change in the initial pH. Both brassicol and bavistin suppressed completely the growth and production of proteases. Mildothane gave poor results while compared with Zincop and Morestan. Behaviour of the culture was quite different towards fungicides, which were responsible in changing the environmental conditions such as pH, surface area and structure of the cell. Optimum formation could be achieved by maintaining the set conditions of the specific culture.

DISCUSSION

The different phenolic compounds also showed variable inhibitory effects on protease production (Table 2). The neutral enzyme is more sensitive towards phenolic compounds than alkaline proteases. Although all the phenolic compounds investigated were found to decrease the growth of culture. Caffeic acid at 0.05 level was slightly stimulatory. All the phenolic compounds except caffeic acid inhibited the protease formation. Salicylic acid and catechol gave complete inhibition whereas. Caffeic acid was quite satisfactory in formation against alkaline proteases.

The results of Table 2 indicate that all the fungicides tried inhibit protease production. Brassicol and bavistin are the most inhibitory fungicides of protease prodcution. Brassicol was also reported to be inhibitory to polygalac Juronase production. Bavistin decreased completely the proteolytic and cellulytic enzyme formation by Fusarium solani [7].

Table 1. Effect of phenolic compounds on the production of proteases by *Bacillus subtilis.*

Phenolic comp concentration % age	Ferulic acid		Tannic acid		Caffeic acid		Cinnamic acid		Salicylic acid		Catechol	
	Neut	Alk	Neut	Alk	Neut	Alk	Neut	Alk	Neut	Alk	Neut	Alk
Control	1.8	2.40	1.8	2.40	1.8	2.4	1.8	2.4	1.8	2.4	1.8	2.4
0.050	2.0	2.80	1.6	2.2	1.8	2.5	1.6	2.3	1.4	2.0	1.0	2.0
0.075	0.6	0.80	0.4	0.6	0.20	0.30	0.15	0.20	0.10	0.10	0.10	0.10
0.100	0.10	0.10	0.10	0.20	0.10	0.20	0.00	0.00	0.00	0.00	0.00	0.00

Alk = Alkaline Proteases; Neut = Neutral Proteases

Table 2. Effect of fungicides on the production of proteases by *Bacillus subtilis.*

Fungicides concentration % age	Morestan		Zincop		Brassicol		Mildothane		Bavistin	
	Neut	Alk	Neut	Alk	Neut	Alk	Neut	Alk	Neut	Alk
Control	2.00	4.0	2.10	4.20	2.00	4.10	2.10	4.20	2.0	4.0
0.05	1.80	3.00	2.00	4.00	0.0	0.0	1.40	2.60	0.0	0.0
0.075	1.40	2.20	1.10	2.20	0.0	0.0	0.80	1.60	0.0	0.0
0.100	0.800	1.20	0.60	1.50	0.0	0.0	0.20	0.40	0.0	0.0

Alk = Alkaline Proteases; Neut = Neutral Proteases

The effect of fungicides and phenolic compounds on the production of proteases may be due to an instant effect on the physiology of the organism. Which causes the enzyeme to be synthesized in less amount or the secretion enzyme from the mycelia to be blocked [3].

Fungal oxidases react with phenolic compounds and change their structure and their toxicities as reported by Reddy [4]. It is quite clear that different phenolic compounds may be modified differently by the Bacterial culture. Thus, it is contributory factor in the variable degree of inhibition of enzyme production. Caffeic acid stimulated the production of proteases. Although it did not increase the growth. Molot reported [6] that P. caumaric acid enhanced the production of pectinases and cellulases. Amylase inhibition by various phenolic compounds has also been reported [8].

In conclusion, it may be stated the all the fungicides inhibit the production of neutral as well as alkaline proteases. Caffeic acid slightly increased the enzyme at low concentration. However, other phenolic compounds inhibited the growth and production of the proteolytic enzyme, but the degree of their effects was different.

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