Influence of Chromium Nanoparticales on Activity of *Erwinia carotovora* and *Pseudomonas fluorescens*

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***Abstract*—** The present paper investigates to examine the *in vitro* antibacterial activities of the chromium nanopartical which prepared by -green synthesis techniques- reduction of potassium dichromate solution with *Arachis hypogaea* leaf extract containing reducing sugars which act as reducing agent. Chromium nanoparticles were shown to be an effective control of two phytopathogenic bacteria, namely *Erwinia carotovora* and *Pseudomonas fluorescens*, which cause several diseases in fruits and vegetables. Cr_2O_3 nanoparticales with different concentrations (15 µl, 25 µl and 35 µl) were showed a marked reduction of extracellular enzymes activity such as cellulase, pectinase and protease that produced by two phytopathogen organisms. The antibacterial effect of Cr_2O_3 nanoparticles against *Erwinia carotovora* and *Pseudomonas fluorescens* with different concentrations of Cr_2O_3 nano particles by using disc diffusion. It showed antibacterial activity against *Erwinia carotovora* (46 mm) and *Pseudomonas fluorescens* (39 mm).

***Keywords*—** Cr_2O_3 nanoparticles, Antibacterial, *Erwinia*, *Pseudomonas*.

I. INTRODUCTION

THE green synthesis techniques utilize relatively non-toxic chemicals to synthesize nanomaterials . Chromium is an essential micronutrient for living organisms. However, oxidized form of chromium [Cr(VI)] is extremely toxic , while Cr(III) is relatively inert, less mobile, less bioavailable and easily adsorbed on mineral surfaces [1]and the energy of interaction for Cr^{+++} is lower than other forms [2]

The *Arachis hypogaea* leaves posses biomolecules such as carbohydrates, amino acids and vitamins, which could be used as reducing agent to react with chromium ions and as scaffolds to direct the formation of Cr_2O_3 NPs in solution [3], there are also suggestive studies to show that Cr also improves cellular antioxidant capacity of cells [4]. The wall of plant cells is composed of pectin and hemicelluloses which constitute the most abundant polymers in nature [5].The phytopathogenic organisms ability to produce an array of enzymes capable of degrading the complex polysaccharides of the plant cell wall and membrane constituents. *Pseudomonas fluorescens* implicated in soft rot to produce pectic enzymes *in vitro* and *in vivo* [6].. *Erwinia carotovora*, a plant pathogen that causes soft-rot disease dependent on the production and secretion of a complex of plant cell wall degrading enzymes [7]. *Erwinia carotovora* subsp. *carotovora* and *Pseudomonas fluorescens* can be degrade theses polymers to simple sugars by secreting extracellular enzymes such as amylases, cellulases , pectinases and protease . They are required in the elicitation of tissue-macerating (soft-rotting) disease in a wide variety of plants and plant organs [8].. Chromium (III) complexes have antibacterial effect on strains of *E. coli* and *Bacillus subtilis* [2], [9], [10]. At the present research, Cr nanoparticles were formed from the reaction Arachis hypogaea leaf extract with potassium dichromate solution and screened *in vitro* antibacterial properties of Cr_2O_3 nanoparticles against *E. carotovora* subsp. *Carotovora* and *P. fluorescens.*

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II. MATERIALS AND METHODS

A. Culture media for Enzyme Production.

The medium has the following composition in (g/l): Ammonium nitrate 1.0g,Magnesium sulphate 0.18g, Dipotassium hydrogen phosphate 0.7 and Potassium chloride 0.15_ 2ml of stock solution containing: Zinc sulphate 2.85 mg /ml, Manganese sulphate 3010 mg/ml, Ferric chloride 8.65 mg/ml and Thiamine hydrochloride 0.1 mg [11].

B. Preparation of Stock Solutions for Enzyme.

To study, in vitro, the activity of some enzymes produced by *E. carotovora* and *Pseudomonas fluorescens* . The two organisms were grown at 28 ^oC for 48 hours in steril 100 ml – conical flask, each containing 30 ml medium, described previously and amended eith 0.5 g of 15 – day old cell walls as the carbon source. These media were further supplemented with the different concentrations of the sample of Cr_2O_3 nanoparticles solution (15 µl, 25 µl and 35 µl), beside the control. Three replicates were prepared for each treatment. Cells from the liquid cultures were harvested by centrifugation at 20000 g at – 5^oC for 20 minutes. The supernatant fluid of these cultures (*Erwinia carotovora* and *Pseudomonas fluorescens*) was saved for the estimation of the extracellular enzymes: cellulase and pectinase [11].

C. Cellulase Enzyme.

Cellulase was assayed according to Schoemãker and Brown [12].. The reaction mixture composed of 0.5 ml of 1 % CMC

(carboxymethyl cellulose) in o.o5 M phosphate – citrate buffer (pH 4.6) and 0.5 ml crude enzyme , was incubated at 50 ^{o}C for one hour. The reaction was stopped by dipping the tubes in boiling water for 3 minutes, and then the mixture was completed to a known volume. The increase in reducing end groups was measured photometrically using Nelson's method [13]. Results was expressed as µg reducing sugars liberated per ml medium.

D. Pectinase Enzyme.

Lytic cleavage of pectic substances was determined by following the increase in absorbance of reaction mixtures at 230 nm [14]. The increase in absorbance was followed in Lambad 3 UV/VIS spectrophotometer after addition of 0.1 ml or less of supernatant fluid to a reaction mixture containing 100 µ moles Tris buffer, (pH 8.8), 20 µ moles calcium chloride, 1 ml of 0.3 % sodium polypectate and water to a total volume of 3 ml. An increase in absorbance of 1.73 was considered to represent the formation of 1 µ mole of unsaturated uronide product in the reaction mixture [15]. One unit of enzyme is the amount that catalyzes the formation of unsaturated uronides at a rate of 1 µ mole / minute at ^{o}C.

E. Protease enzyme.

The activity of protease was assayed by Todd and yoo [16], techniques for measuring total initial and final amino acid content with little modification, 2.2 % casein solution was used as 1 ml of crude enzyme was mixed with 1ml phosphate buffer at pH 7.4, then incubated at 37 ^{o}C for 45 minutes. Another set of tubes was prepared but immediately boiled in water (as control) 1 ml of 1N TCA was added to precipitate the insoluble proteins. Centrifuged for 15 min. then completed to a known volume. The initial and final amino acid content of the experimental mixtures was assayed as follows:

1. Peptide nitrogen.

A sample of the reaction mixture was with 1 ml freshly mixed (in 1 : 1 ratio) solution of 2 % sodium carbonate and 4 % sodium hydroxide and 0.5 % copper sulphate solution in 10 % sodium potassium tartarate. The mixture stood 10 min. before addition of 0.1 ml Folin phenol and made up to volume. The optical density of the mixture was measured, after 30 min., at 700nm [17].

2. Free amino acids.

The protein - free sample was made slightly alkaline with phenolphthalin before addition of 1 ml of 1 % borax [18]. After shaking, another 1 ml of 0.25-0.3 %freshly prepared naphthoquinone-4-sulphonic acid was added. The mixture was kept in boiling water bath for 15 min. then rapidly cooled. Acid formalin and sodium thiosulphate were added and the shaken mixture stood 20 min., before estimation of its colour intensity, at 470nm.

F. Test for Antibacterial Assay.

After of bacterial inoculums preparation**.** The antibacterial activity of different concentrations of the sample of Cr_2O_3 nanoparticles solution (15 µl, 25 µl and 35 µl), beside the control were tested by the modified disc diffusion method. The bacterial inoculums (20h broth) *E. carotovora* and *P. fluorescens* were uniformly spread over the agar plates using a glass L-rod. A total of 0.2 ml of each concentration was aseptically added to the discs (0.5 mm diameter) and allowed to dry before being placed on the top of the agar plate. The plates were incubated at 37°C for 24h and the diameter of growth inhibition zone was recorded. Dist. water was used as negative control. Each extract was tested in triplicate for calculation of standard deviation [19].

III .Result And Discussion

A. Cellulase Enzyme.

It is evident from Figure 1 that the presence of nanoparticale of Cr_2O_3, of varying amounts, in the liquid medium induced highly significant inhibitory effect on the activity of cellulase enzyme produced by two organisms *E. carotovora* and *P. fluorescens*. The increase in amount of Cr_2O_3 NP was accompanied by a corresponding reduction the enzyme activity. Thus the highest amount of Cr_2O_3 NP (35µl) induced maximum inhibitory activity of cellulase.

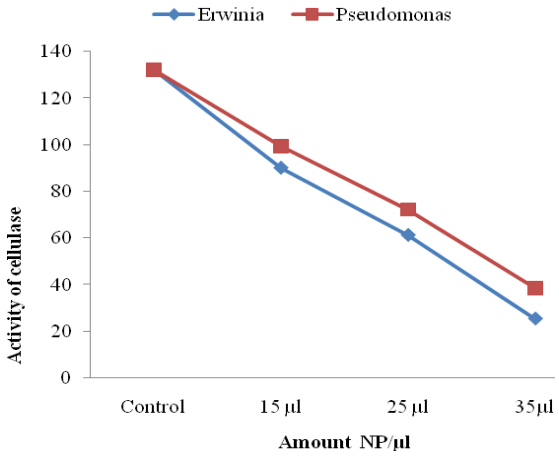

Fig. **1:**Effect of Cr_2O_3 NP on cellulase enzyme of *E. carotovora* and *P. fluorescens.*

B. Pectinase Enzyme

Results in Figure 2 showed that application of varying amounts (15 µl, 25 µl and 35 µl) of Cr_2O_3 nanoparticales induced variable change in the pectinase enzyme activity of *E. carotovora* and *P. fluorescens.* Pectinase was expressed as µg unsaturated uronoids / ml medium. The activity of the assayed enzyme in the control was greater than those of the same enzyme activity in all treated samples. The presence of low amount of Cr_2O_3 Nano-particale (15 µl) in the induced significant inhibitory effects on the activity of the activity of the preceding tested enzyme. The increase in Cr_2O_3 nanoparticale amount (25 µl) was accompanied by a corresponding reduction in the enzymatic activity.

We believe that Antibacterial activity of Cr2O3 nanoparticles dependent on the interaction between the chromium with natural protein as a natural polymer which have some functional groups as terminal (COOH), where the energy of interaction for Cr(III) through COOH is low [2].

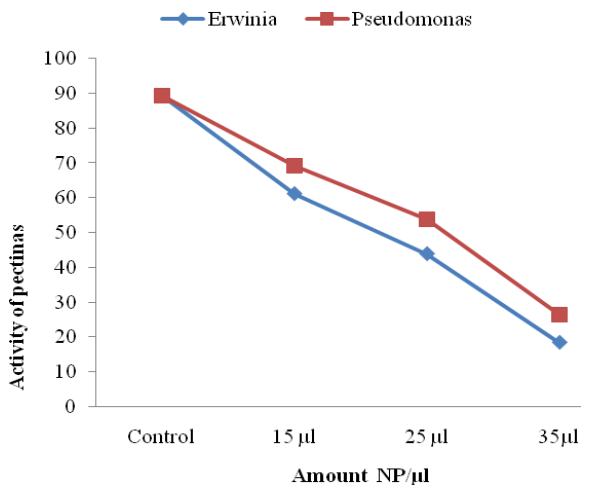

Fig. 2: Effect of Cr_2O_3 NP on cellulase enzyme of *E. carotovora* and *P. fluorescens.*

C. Protease Enzyme.

Protease activity was expressed as µg amino acids and µg peptides per 100 ml medium. The results obtained for the various amounts (15 µl, 25 µl and 35 µl) of Cr_2O_3 nanoparticales are presented in table I and II.

TABLE I
EFFECT OF Cr_2O_3 Nps ON PROTEASE ENZYME AS AMINO ACID

Cr_2O_3 nanoparticales/ µ	*E. carotovora*	*P. fluorescens*
control	211.46 ± 1.86 $_a$	211.46 ± 1.86 $_a$
15	164.45 ± 0.94 $_b$	178.65 ± 1.15 $_b$
25	110.34 ± 0.68 $_c$	158.67 ± 0.96 $_c$
35	62.66 ± 1.33 $_d$	98.75 ± 1.32 $_d$

-Protease for *E. carotovora* and *P. fluorescens* (as µg / 100 ml medium) after 48 hours incubation at 28 ^oC .
-Means followed by the different letters are significantly different at the 5 % level of significance using Duncan test.
-Each value is the mean of replication ± standard error

TABLE II
EFFECT OF Cr_2O_3 Nps ON PROTEASE ENZYME AS PEPTIDES

Cr_2O_3 nanoparticales/ µ	*E. carotovora*	*P. fluorescens*
control	269.97 ± 0.91 $_a$	269.72 ± 0.91 $_a$
15	174.45 ± 1.61 $_b$	182.81 ± 1.15 $_b$
25	114.34 ± 0.99 $_c$	134.97 ± 0.96 $_c$
35	66.85 ± 1.29 $_d$	86.54 ± 1.07 $_d$

-Protease for *E. carotovora* and *P. fluorescens* (as µg / 100 ml medium) after 48 hours incubation at 28 ^oC .
-Means followed by the different letters are significantly different at the 5 % level of significance using Duncan test.
-Each value is the mean of replication ± standard error

The results indicated that application of different amount of the Cr_2O_3 nanoparticles were highly significant inhibitory to the activity of this assayed enzyme. In the meantime, in control was greater than this of the same enzyme activity in treated samples ; the inhibition being greatest in protease (70.37 % and 75.24 % for amino and peptides respectively) which produced by *E. carotovora* and the inhibition of the activities of the assayed enzyme produced by *P. fluorescens* (53.30 % and 67.91 % for amino and peptides respectively).

D. Antibacterial Assay

The result shows the antibacterial activity of Cr_2O_3 nanoparticles was performed against *E. carotovora* and *P. fluorescens*. The result showed very high activity (inhibition zones (35 µl) used as antagonist in Figure 3, whereas (inhibition zones reach to 39 mm) when Cr_2O_3 nanoparticles (35 µl) used as antagonist against *Pseudomonas fluorescens* . The mean of three replicates of zone of inhibition (mm) around Cr_2O_3 nanoparticles is presented in the Table III.

TABLE III
IN VITRO ANTIBACTERIAL OF THE Cr2O3 NANOPARTICLES (µl)

Test organisms	Diameter of zones of inhibition (in mm)			
	water	15 µl	25 µl	35 µl
Erwinia carotovora	14	22	28	46
Pseudomonas fluorescens	19	27	32	39

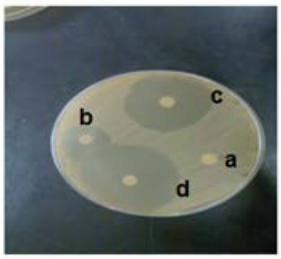

Fig. 3: Effect of Cr_2O_3 nanoparticles (µl) on growth of *Erwinia carotovora* and *Pseudomonas fluorescens* using
a- warer b- 15 µl c- 25 µl d- 35 µl

In the present investigation we found that the Cr_2O_3 nanoparticles showed better antibacterial against of Erwinia carotovora and Pseudomonas fluorescens [2], [20].

V. Conclusion

From the above study, it is concluded that the green chemistry for synthesis of Cr2O3 nanoparticles are valuable for cost effective, eco-friendly, highly stable and reproducible. The Cr_2O_3 nanoparticles have significant inhibitor effects on the cellulase and pectinase enzyme activity which secreted by Erwinia carotovora and Pseudomonas fluorescens and behavior of Cr2O3 nanoparticles reveals that it has effective antibacterial agent against Erwinia carotovora and Pseudomonas fluorescens.

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