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Cell immobilization of hydrolytic enzymes on olive mill wastes by *Trichoderma harzianum* NRC 12 and its antifungal activities aganist soil born fungi

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ABSTRACT

Cell immobilization of Trichoderma harzianum NRC 12 on chitin by covalent binding was effective for production of hydrolytic ezymes endoglucanase (endo-GN; EC 3.2.1.4.) and endopolymethylgalacturonase (endo-PMG;EC3.2.1.15) using 60 g/l of olive mill waste as substrate. Optimization conditions for free and immobilized T.harzianum NRC 12 proved the use of immobilized one. Antifungal activity and green house experiment for immobilized T. harzianum NRC 12 was most effective than free.

Key words: olive mill wastes, cell immobilization, antifungal activity.

INTRODUCTION

In Egypt, the annual average production of olive is 330.000 tones per year. About 80% of the total production is consumed as table olive and the residual (20%) is used for oil production (Central administration for public mobilization and Statics, 2006).The production of olive oil generates three phases and two wastes: olive oil (20%), solid waste (30%) and aqueous liquor (50%).These olive mill wastes are produced in significantly large quantities during short periods of time. Disposal of olive wastes from olive oil mills is already a major environmental issue in several olive growing countries in the world. Spreading the solid waste on farm lands causes enormous pollution to the land and air [1].

Raw (untreated) olive mill waste (OMW) has broad spectrum toxicity against bacteria and human cells [2], fungi [3], algae [4], plants [5 &6] and insects [7].

Endoglucanase and Endo-polygalacturonase both hydrolytic enzymes which considerable commercial value produced by fungi and bacteria. Endoglucanase used in the food industry, for baking and fruit and vegetable processing, breakdown of agricultural waste, in the manufacture of animal feed, e.g., a monogastric animal feed, such as a swine or poultry (e.g., chicken) feed, in pulp and paper production, textile manufacture, household, industrial cleaning agents. Endoglucanases are also important for the digestion of cellulose, a beta- 1,4- linked glucan found in all plant material. Endo-polygalacturonase hydrolyzes the a-1,4 glycosidic bonds of non-esterifies portions of pectic substrates, which are major components of plant cell walls [8] .

Cell immobilization emerged as an alternative for enzyme immobilization [9, 10&11]. Immobilization of cells containing specific enzymes has further advantages such as elimination of long and expensive procedures for enzymes separation and purification and it is vital to expand their application by enabling easy separation and purification of products from reaction mixtures and efficient recovery of catalyst [12&13]. In comparison with immobilized en-

zymes, immobilized cells provide new possibilities since they can be used as natural, water-insoluble carriers of required enzyme activities [14]. In the case of the immobilization of microbial cells, their field of application spreads from industrial to environ-mental process. Microorganisms retained on a carrier can be used in continuous and semi-continuous production processes allowing for significant cost decrease, as the biocatalyst does not need to be refilled [15, 16&17].This technique is based on the physical interaction between the microorganism and the carrier surfaces, while frequently reversible is simple, cheap and effective.

The aim of the present work was production of endoglucanase (endo-GN; EC 3.2.1.4.) and endopolymethylgalacturonase (endo-PMG;EC3.2.1.15) from free and immobilized *Trichoderma harzianum* NRC 12 under optimum condition and the effectiveness in reducing percentage of infection of soil borne fungi under green house conditions.

MATERIALS AND METHODS

Microorganism:
Trichoderma harzianum NRC 12 was collected from obtained culture collection unit of Plant Pathology Department, National Research Centre, Dokki ,Cairo, Egypt. These bioagent proved high antagonistic effect against wide spectrum of plant pathogens in many previous works at the same department and was tested for their ability to produce hydrolytic enzymes.

Media and culture condition:
Dry olive mill residue was collected from olive oil manufacturer in Egypt. *Trichoderma harzianum* NRC 12 was grown on Czapek' s Dox agar medium at 28 °C and stored at 4° C. The culture media was composed of (g/l): $NaNO_3$ 2.0, K_2HPO_4 0.5, KCl 0.5, $MgSO_4.7H_2O$ 0.5, sucrose was replaced by 20 g/l of olive mill waste. Two discs (6 mm in diameter) from 7 days old cultures were transferred to 250 ml Erlenmeyer conical flasks each containing 50 ml fermentation medium. The inoculated flasks were incubated on a rotary incubator shaker at 180 r.p.m for 7 days at (28-30° C). At the end of incubation period, cultures were centrifuged at 8000 r.p.m. The cell free supernatant was used as a crude hydrolytic enzyme for further determinations of endoglucanase (endo-GN; EC 3.2.1.4.) and endopolymethylgalacturonase (endo-PMG; EC3.2.1.15)

Enzyme assay:
All hydrolytic enzymes were assayed according to [18] using carboxymethylcellulose for endoglucanase (endo-GN; EC 3.2.1.4.) and citrus pectin for endopolymethylgalacuronase (endo-PMG;EC3.2.1.15).

The reaction mixture contained 1 ml of 0.5 % substrate in 50 mM citrate phosphate buffer pH (4.8) with 0.2 ml enzyme , incubate at 50° C for 30 min , then add 1ml of DNS ,reducing sugar was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that converts one micromole of reducing sugar per minute reaction under the described condition.

Cell immobilization :
Cell immobilization was carried out by the method of (Woodward, 1988).Half gram of the carriers (natural sponge, synthetic sponge, loaf, chitin, sawdust, foam) were inoculated with counted cells (175 x 10^3 spores /ml) for 2 h. Then added to Erlenmeyer flask containing 50 ml fermentation media then incubated for 7 days at 28-30 °C and 200 r.p.m.

Antimicrobial activity of *T. harzianum* NRC 12 free and immobilized:
One ml of *T. harzianum* NRC 12 free and immobilized were inoculated on PDA plate in four wells inoculated with the pathogenic fungi (*Fusarium solani, Fusarium oxysporum ,Rhizoctonia solani and Sclerotium rolfsii*) at the center of the plate. Three replicates were used for each treatment, the inoculated plate with only pathogenic fungi were used as control. The tested plates were incubated for 7 days at 28 °C. The percentage of inhibition zone and reduction was measured.

Determination of Aflatoxins (AFs) by HPLC:
Derivatization:
The derivatives of samples and standard were done as follow:100µl of trifluoroacetic acid (TFA) was added to samples and mixed well for 30 second and the mixture stand for 15 min. 900µl of water: acetonitrile (9:1 v/v) were added and mixed well by vortex for 30 second and the mixture was used for HPLC analysis. The HPLC system consisted of Waters Binary Pump Model 1525, a Model Waters 1500 Rheodyne manual injector, a Waters 2475 Multi-Wavelength Fluorescence Detector, and a data workstation with software Breeze 2. A phenomenex C_{18} (250 x 4.6 mm i.d.), 5µm from Waters corporation (USA). An isocratic system with water: methanol: acetonitrile 240:120:40.

The separation was performed at ambient temperature at a flow rate of 1.0 ml/min. The injection volume was 20µl for both standard solutions and sample extracts. The fluorescence detector was operated at wavelength of 360 nm for excision and 440 nm for emission. Samples were prepared by incubating the media which contain dry olive mill residue with the tested fungus (*Trichoderma harzianum* NRC 12) free and immobilized [19].

Green house experiments:
Evaluation of bioagents which grown free or immobilized was carried out in a sandy loam soil artificially infected with pathogenic fungi. Inocula of pathogenic fungi were individually grown on autoclaved sand barlly medium (1:1, v: v+40% water for two weeks at 25 ˚C [20]. Soil infested with different pathogens at rate 5%w.w.and filled in plastic pots (30cm. in diameter) and irrigated every day for one week before sowing.

The evaluated of *T .harzianum* NRC 12 free or immobilized as a seed soaking seeds of tomato c.v. Castel Rock was used in the present study was sterilized using 3% sodium hypochlorite for 5 min., then picked up and air dried and soaked in sticky suspensions (1 ml of Arabic gum/100 suspension) from *T. harzianum* NRC 12 free or immobilized for one hr., then left for air dried. Soaked seeds were sown as five seeds per pot, five pots per replicates in each treatment. Another pots of soil infested with pathogenic fungi only kept as control. The average of growth stages was recorded up to 15 and 60 days of sowing date, respectively, Pre-emergence (%) was based on the number of unemerged seeds in relation to the number of sown seeds, while post- emergence (%) was based on the number of plants showing disease symptoms in relation to the number of emerged seedlings.

Statistical analysis :
Tukey test for multiple comparison among means was utilized [21].

RESULTS AND DISCUSSION

Different olive mill waste concentration:
Different concentrations of olive mill ranged from (20-100) g/l were examined for their ability of production of hydrolytic enzymes .Results in Fig. (1) showed that maximum enzymes activity was at 60 g/l for both enzymes followed by 40 g/l while 100g/l of olive mill wastes produce lowest activity . This results was not coincided with [22] found that 25 g/l of olive mill wastes was the optimum for production of hydrolytic enzymes by saprophytic fungi.

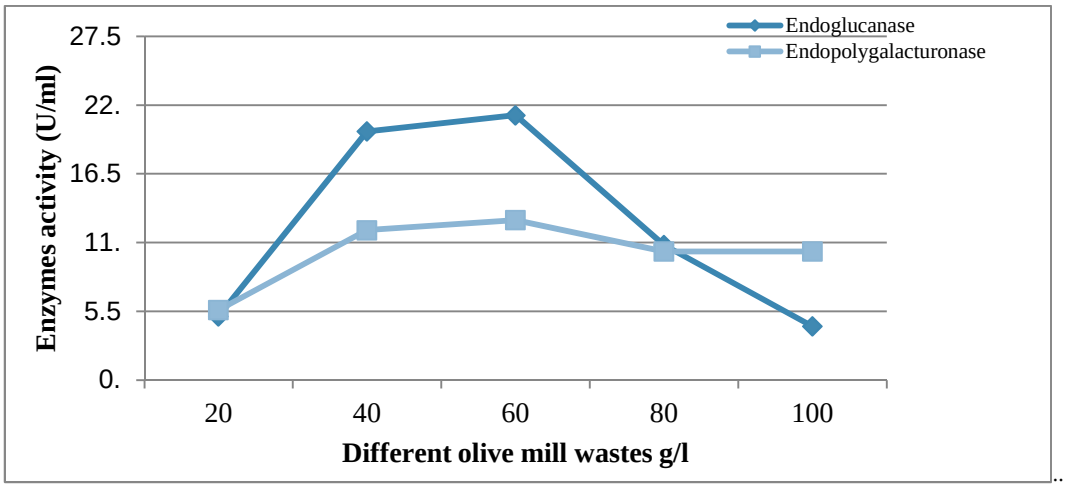


Fig.(1) : Effect of different olive mill waste concentration

Cell immobilization by covalent binding:
Results in Fig. (2) showed that while using different carriers , chitin was the most suitable for production of endoglucanase (endo-GN; EC 3.2.1.4.) and endopolymethylgalacturonase (endo-PMG;EC3.2.1.15) produce (34.7 &33.9 U/ml respectively) followed by prawn shells and Loafa cylinderica .Other carriers produce moderate to low enzymes activity. *Trichoderma harzianum* NRC 12 was a good producer for hydrolytic enzymes by cell immobilization using chitin as carrier .

Microorganisms retained on a carrier can be used in production processes allowing for significant cost decrease, as the biocatalyst does not need to be refilled [15,16&17] . Inorganic carriers were selected to immobilize microorganisms because they can resist microbial degradation [23 &24].

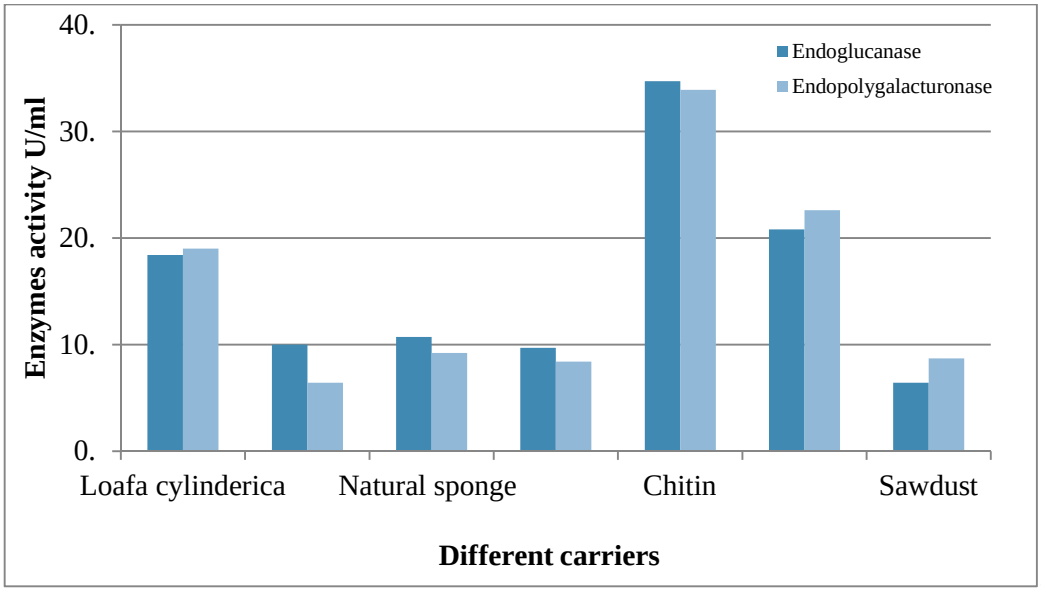


Fig. (2): Cell immobilization by covalent binding

Optimization of cell immobilization of *T. harzianum* NRC12

Different chitin concentrations:

Different chitin concentrations ranging from (0.25- 0.5) g/l were tested for enzyme production. Results in Fig. (3) showed that increase in chitin concentration lead to increase in enzymes activities till reached 0.5g/l, for endoglucanase produce 34.7 U/ml and 33.9 U/ml for endopolygalacturonase then enzymes activities begin to decrease, 2.0 g/l of chitin produce the lowest activities for both enzymes. *T .harzianum* was affected by the concentration of chitin as described by [25]. The highest enzyme production was obtained at 1.3 % chitin, while further increase in chitin concentration reduced enzyme production. Filamentous growth of cell immobilization on porous carriers allowed strong biomass retention [26].

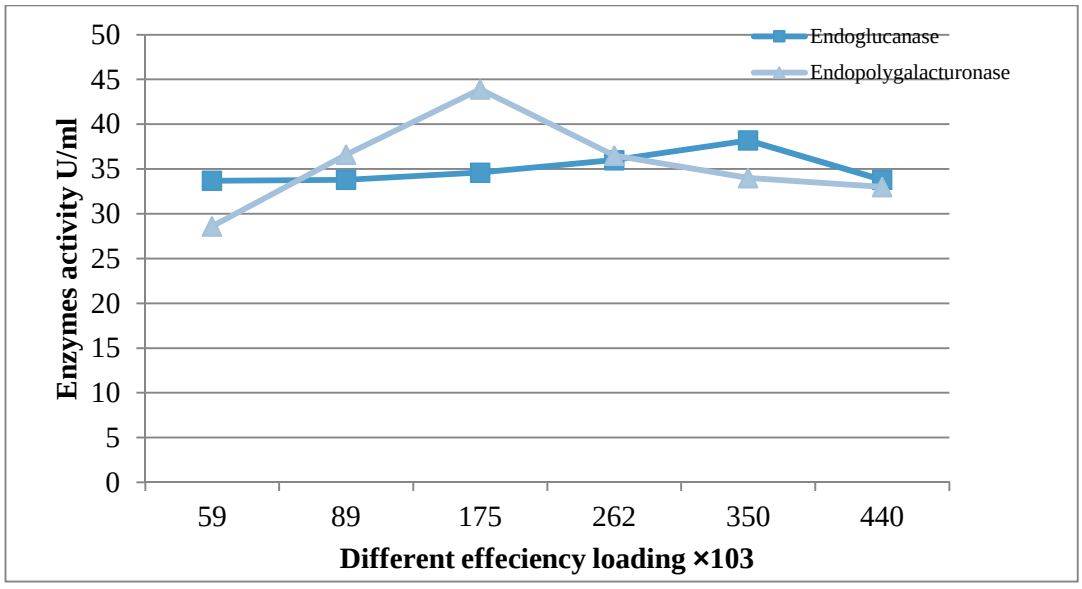


Fig. (3): Effect of different chitin concentrations

Effect of biomass loading (efficiency loading):

Half gram of chitin was mixed with different spore concentrations (59,89,175, 263,350,440) x 10^3 spores / ml / g chitin. Results in Fig. (4&5) indicated that there was an increase in the enzymes activities with increasing cells load up to (175 x 10^3 spores / ml/ g carrier), where by the maximal enzyme yield was attained 43.8 U/ml for endoglucanase and 38.2 U/ml for endopolygalacturonase. Further increase in spores led to a decrease in enzymes activities. The lower inoculums size leads to insufficient microbial growth and enzyme synthesis while an increased level of

inoculums size causes rapid proliferation of microbial biomass and enzyme synthesis [27]. After a short time, this enzyme production becomes ceased due to the depletion of nutrients and too much biomass is formed and the metabolic activities of the microorganism drop off. Spore concentration of 1.5 x 10^9 was optimum for cellulase production by *T. viride* GIM 3.0010 using banana peel as a substrate in solid-state fermentation [16]. Spore concentrations of 10^5 and 10^6 per milliliter did not prove significant differences in cellulase activities by *Trichoderma longibrachiatum* (GHL) [28].

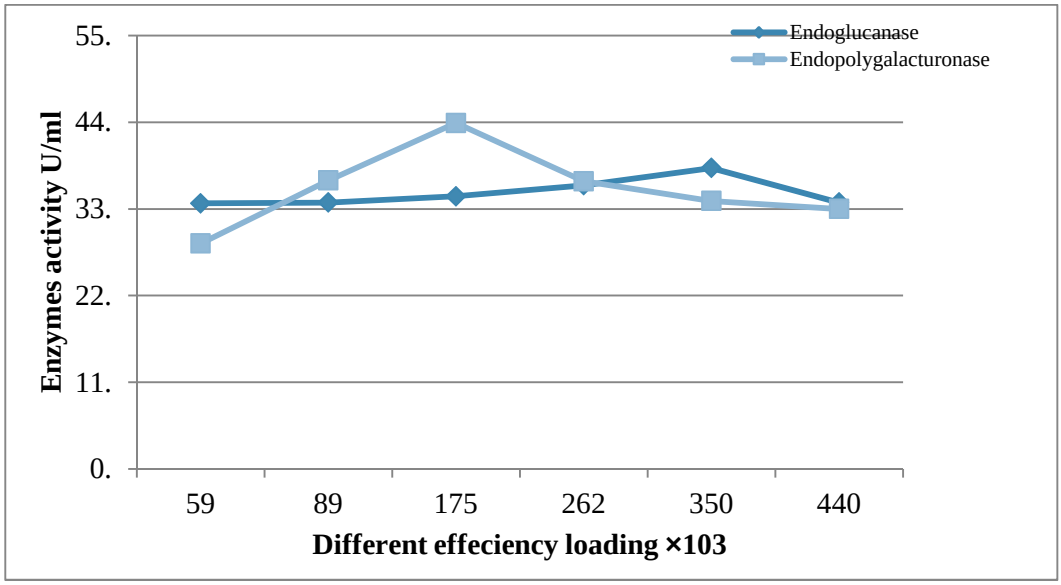


Fig. (4): Effect of biomass loading (efficiency loading) on enzyme activity on immobilized enzymes

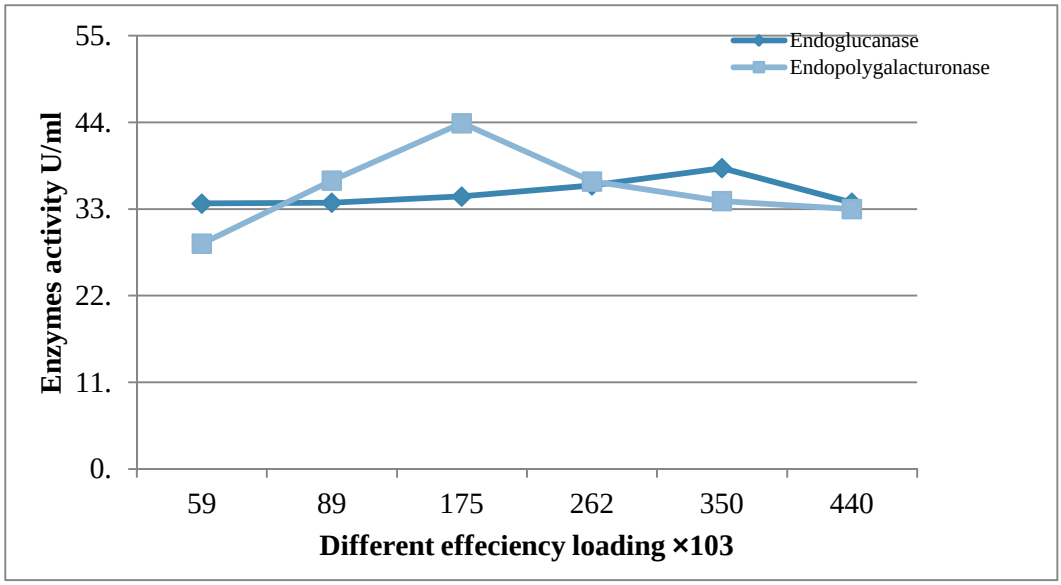


Fig. (5): Effect of biomass loading (efficiency loading) on enzyme activity on free enzymes

Different pH values

The effect of reaction pH on the activity of native, immobilized enzymes was investigated using citrate phosphate buffer (3.5- 8.0). Results in Fig. (6&7) indicated that the optimum pH for immobilized cells was 6.5 for endoglucanase and 6.0 for endopolygalacturonase produce 43.8 &39.6 U/ml respectively. While for free the optimum pH was at 6.0 for endoglucanase and 6.5 for endopolygalacturonase produce 29.7 &38.6 U/ml respectively.Increase and decrease of pH lead to decrease of enzyme activity. This results were in agreement with [29] which reported that initial medium pH of 5 and 5-7 was optimum for CMCase production by immobilized cells of *Trichoderma harzianum.*

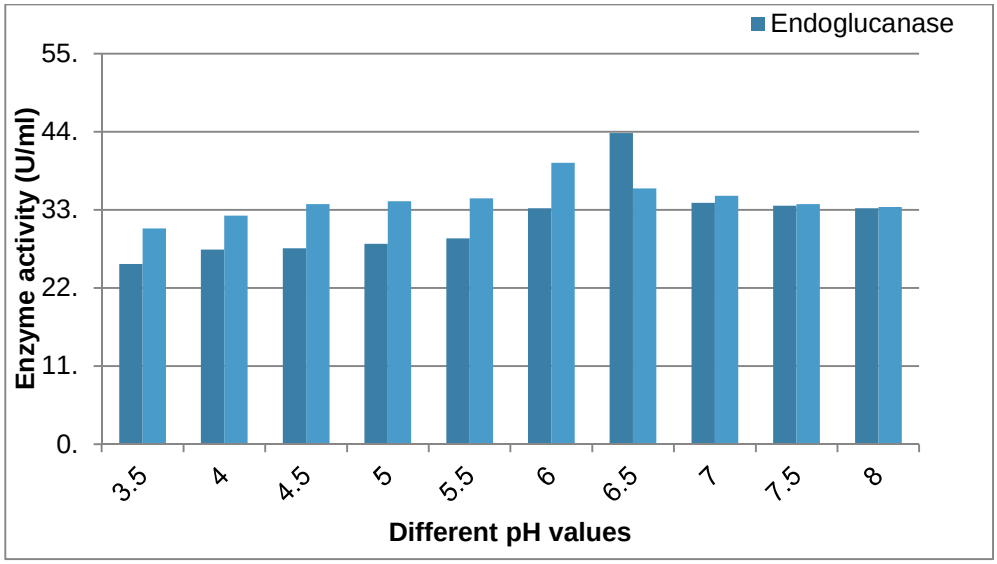


Fig. (6): Effect of different pH values on immobilized enzymes

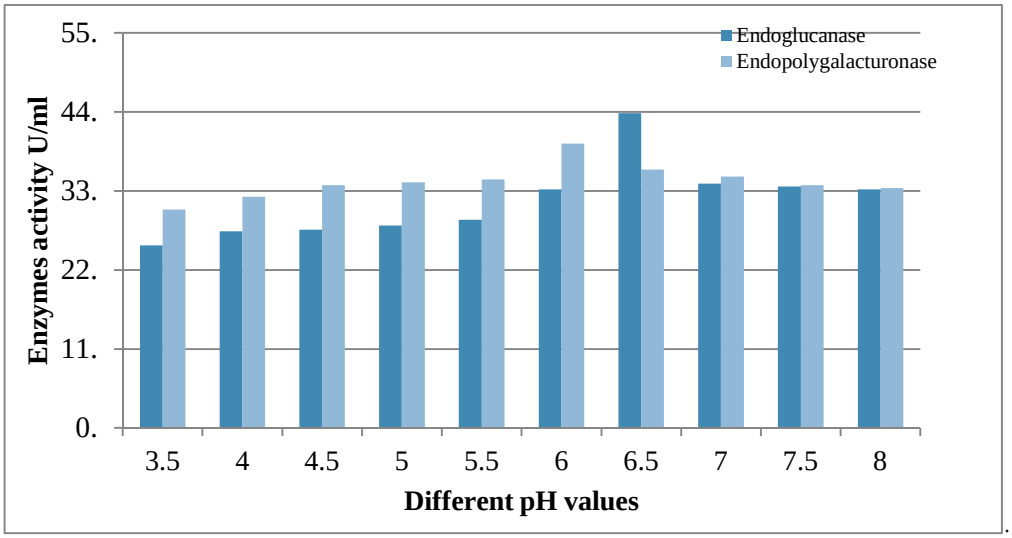


Fig (7): Effect of different pH values on free enzymes

Antifungal activity of free and immobilized *T. harzianum* NRC12 :

The data in Table (1) showed that the highest inhibitory effect on the growth of all pathogenic fungi with *T. harzianum* NRC12 immobilized on chitin followed by *T. harzianum* NRC12 free and control. The reduction on the growth of pathogens were 99.4% while treatment with *T. harzianum* NRC12 free against *R. solani* ,*S .rolfsii*, *F. solani and F. oxysporum* (88.9,83.3,72.2 and 66.7) respectively. Organic matter amendments potentially increased fungal association and hence might increase growth and yield of chickpea plants in the field [30].

Table (1): Effect of *T. harzianum* NRC 12 free and immobilized on the linear growth of pathogenic fungi

Test organisms	Control	*T. harzianum* NRC12			
		Free		immobilized	
		L.G.	%R.	L.G.	%R.
F. oxysporum	90.0 a	30.0 b	66.7	0.5 f	99.4
F. solani	90.0 a	25.0 c	72.2	0.5 f	99.4
R. solani	90.0 a	10.0 e	88.9	0.5 f	99.4
S. rolfsii	90.0 a	15.0 d	83.3	0.5 f	99.4

Figures with the same letters are not significant ($P \leq 0.05$).

Aflatoxins productions of free and immobilized cells:

Results in Table (2) showed that immobilization of *T. harzianum* NRC12 on chitin led to in complete inhibition of total aflatoxins (AFG1,AFB1, AFG2, AFB2)in comparing with free cells sample which had detected aflatoxins,0.229 (mg/ml).Olive waste proved to be rich in polyphenol compounds and could be used as low cost edible natural antioxidants for protection against aflatoxicosis in animal and human

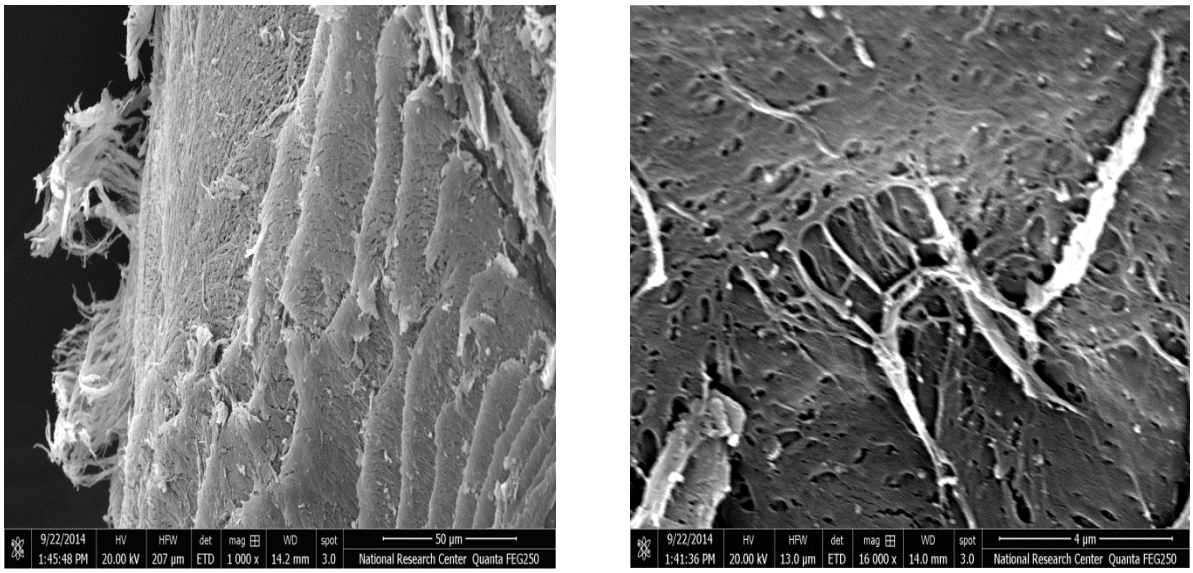
Aflatoxin B1 (AFB1) was the most prevalent aflatoxin usually found in cases of aflatoxicosis, and was responsible for acute toxicity, chronic toxicity, carcinogenicity, teratogenicity, genotoxicity, hepatotoxcisity and immunotoxicity [19].

Table (2): Comparison of aflatoxins productions of free and immobilized cells

Samples	Concentrations of aflatoxins (mg/ml)				Total AFs (mg/ml)
	AFG_1	AFB_1	AFG_2	AFB_2	
Immobilized cells	ND	ND	ND	ND	ND
Free cells	ND	ND	0.229	ND	0.229

Scanning Electron Microscopy:

Microscopic Photograph of adhered *T. harzianum* NRC 20 on one piece of chitin about (2 mm x 2 mm x 0.05ml) loaded with fungal cells placed on a scanning electron microscope sample holder and coated with gold .The gold-Coated samples were viewed with electron microscope (model Quanta FEG 250) .Photo (1) illustrate that chitin pores in which fungal spore imbedded in , this groves protect the spores from scattering during shaken process, consequently much spore formation multiplication had occurred. Immobilization of microbial cells has received increasing interest in the field of waste treatment [31 &32] .The synergistic action of chitin resulted in chitin - chitosan containing fibrils that crystallized to form the main structural mesh of the cell walls which could also be involved in deacetylation of chitin oligosaccharides during autolysis after action of on the cell wall [33]. The advantage of the process based on immobilized biomass include enhancing microbial cell stability, allowing continuous process operation and avoiding the biomass - liquid separation requirement [34].



Photo(1):Scanning electron miccroscope of *T. harzianum* NRC 12 immobilized on chitin

Green house experiments:

The results of the greenhouse experiment which applied of *T. harzianum* NRC12 either free or immobilized caused significant effect on root disease incidence at both grown stages of tomato comparing with control, when applied as a seed soaking treatment. Data in Table (3) showed that the treatment at pre-emergence with *T.harzianum* NRC12 immobilized on chitin was the most effective treatment compared with control and *T.harzianum* NRC12 free. The reduction was recorded as (70.0, 60.0, 50.0, and 50.0%) on chitin followed by *T.harzianum* NRC12 free showed less effect than *T. harzianum* NRC12 immobilized on chitin (50.0, 50.0, 40.0, and 30.0%) which observed after 15 days

(pre-emergency). At post-emergency treatment with *T.harzianum* imobilized on chitin was the most effective which recorded 100% in case of *F.solani* and R.solani , while recorded 80% in case of *F.oxysporum* and *S.rolfsii*. OMW significantly reduced the growth of important soil borne plant pathogens as *F. oxysporum* f.sp. *lycopersici*, *Pythium* spp., *S. sclerotiorum* and *V. dahlia* according to [35&36]

Table (3): Influence of treatment with *T.harzianum* NRC12 free and immobilized on the percentage of average disease incidence of tomato plants (under greenhouse condition)

Treatments	Pre-emergence root diseases incidence (%)			
	% PRE EMERGENCY	% Reduction	% Post EMERGENCY	% Reduction
F. oxysporum	36.0 b	----	31.3 a	----
F. oxysporum +A	20.0 d	50.0	15.0 b	40.0
F.oxysporum +B	16.0 d	60.0	9.5 c	62.0
F. solani	48.0 a	----	15.4 a	----
F. solani+A	20.0 d	50.0	5.0 c	80.0
F .solani+D	12.0 e	70.0	0.0 d	100.0
R .solani	52.0 a	----	25.0 a	----
R. solani+ A	24.0 c	40.0	10.0 b	58.0
R. solani+ B	20.0 d	50.0	0.0 d	100.0
S. rolfsii	56.0 a	----	27.2 a	----
S. rolfsii+ A	28.0 c	30.0	11.1 b	55.6
S. rolfsii+ B	20.0 d	50.0	5.0 c	80.0
Control	40.0 a	----------	25.0 a	--------

Figures with the same letters are not significant ($P \leq 0.05$).

N.B.: *A: T. harzinum NRC12 free & B: T. harzinum NRC12 immobilized. DI – disease incidence , R- reduction over control*

CONCLUSION

Cell immobilization of *T. harzianum* NRC 12 on chitin was most effective on hydrolytic enzymes activity using olive mill wastes as substrate.

REFERENCES

[1] M.O. Amal, and A. M. Mahmoud **.** *Agriculture and Biological Sciences* **2012,** 8(1): 55-61**.**
[2] R. Capasso, A., Evidente, L. Schivo , G. Orru andG. Marcialis . *Journal of Applied Bacteriology* **1995.** 79: 393-398.
[3] A.S. Fodale, R. Mule, and B. Bati . *Acta Horticulturae* **1999**, 474: 753-756.
[4] G.M. Della , P. Monaco, G. Pinto, A. Pollio and L. Previtera .*Bulletin of Environmental Contamination and Toxicology* **2001**, 67: 352-359.
[5] R. Capasso ,G. Cristinzio, A. Evidente and F. Scognamiglio. *Phytochemistry* **1992**, 31: 4125-4128.
[6]R. Capasso, A. Evidente ,E. Trembaly , A. Sala , C. Santoro ,G. Cristinzio and Scognamiglio F. *Journal of Chemical Ecology* **1994,** 20:1189-1199.
[7] R. Casa, A. Annibale, F. Pieruccetti, and S.R. Stazi**.**. *Germinability Chemosphere*, **2003** 50: 959-966.
[8]L.S. Robert, S. Allard, J. Gerster . *Plant Mol.Biol*. **1993,** 23:1273-1278.
[9] P.S.J. Cheetham, K.W.Blunt, C. Bucke . *Biotechnol. Bioeng*. **1979,** 21:2155-2168.
[10]G.L.P. Parascandola, V. Scardi. *Appl. Microbiol. Biotechnol*. **1980,** 11:6-7.
[11]J.Woodward, *Methods* **1988.,** 8:91-102.
[12]G.A. Junter, and T. Jouenne. *Biotechnol*. **2004,** 22(8):633-58.
[13]I.Stolarzewicz,E.BialeckaFlorjañczyk,E.Majewska, J. Krzyczkowska, *Chem. Biochem. Eng*. **2011,**25:135-144.
[14] V. Vojtisek and V. Jirku, V. *Folia Microbiol*. **1983,** 28:309-340.
[15]M. Wada, J. Kato, I.Chibata. *Appl. Microbiol. Biotechnol*. **1979,** 8:241-247.
[16]J. K. Park, and H.N. Chang**.** *Biotechnol. Adv*. **2000,**18:303-319.

[17]S. Mrudula and N. Shyam **.** *Braz. Arch. Biol. Technol*. **2012,** 55:135-144.
[18]Y.H. Percival,H. Jiong and Y.E. Xinhao. *Methods in molecular biology* **2009,**581
[19]S.M. Sherif ,R.M. Sherif ,A.A. Assem ,M.N. Khayria , H.H. Mustafa, and A.O. Nahla. *Int. J. Curr. Microbiol*. App. **2014,** 3(4): 141-152
[20]El-Gamal N G, Eman H R. **2003.** *Egypt J. Microbial*. 38(4):351–361.
[21]J. Neler, W. Wassermann and M.H. Kutner . Regression, analysis of variance and experimental design: 2nd ed. Richard, D. Irwin Inc. Homewood, Illinois. **1985.**
[22]E. Aranda, I.Sampedro , J.A. Ocampo and I. Garc_a-Romera,I**.** *Appl Microbiol Biotechnol* **2004**,64:132–135.
[23]P.E. Nelson , T.A. Toussoun and W.F.O. Marasas . USA: Pennsylvania University Press. **1983,** 193 p.
[24]M. Verma, S.K. Brar, J.F. Blais, R.D. Tyagi, and R.Y. Surampalli. *Waste Manage*. **2006,**10:264-276.
[25]H. EL-K ,Momein, M.H. Ahmed , M.S. Gehan and M.EL-K Hesham**.***Food Technol. Biotechnol*. **2003,** 41 (3) :219–225.
[26]V. Nikoly, F. Massimilino, F. Fedrico, and A. Rosari**..** *Process Biochemistry* **1997,** 32 (7):617-620.
[27]P. Kashyap, A. Sabu, A. Pandey, G. Szakacs, P. Kashyap, A. Sabu, A. Pandey and G. Szakacs . *Biochem*. **2002,** 38: 307-312.
[28]H. Leghlimi, M. Zahia, L. Hayet Boukhalfa,C. Estelle and D. Francis. *African Journal of Biotechnol*. **2013,** 12 (5):465-475.
[29]I. H. Attitalla, and B. Salleh . *Biotechnology*. **2010 ,**9 (4):529-532.
[30]Assaf, T.A., Turk ,M.A. and Hameed ,K.M**.2009.** *Australian Journal of Crop Science*, 3(1):6-12
[31]Y. Cohen**.** *Technol*. **2001,** 77:257-274
[32]S.A. Ahmad, N.A. Shamaan, M.A. Noorliza, G.B. Koon, M.Y.A. Shuko and M.A. Syed . *World J. Microb*. Biot. **2012** ,28:347-352**.**
[33]V. Ghormade, S. Kulkarni, N. Doiphode, P.R. Rajamohanan and M.V. Deshpande**.** Current research, technology and education topics in applied microbiology and microbial biotechnology. Formatex Research Center,Badajoz, Spain **2010**, 1054–1066.
[34]C.S.M. Suzana, M. Claudia, M.C.G.F. Larissa and T.S. Sandra T. S. *African Journal Of Biotechnology* **2013 ,**12(28):4412-4418.
[35]A. D'Annibale, R. Casa, F. Pieruccetti, M. Ricci and R. Marabottini, *Chemosphere* **2004** , 54:887–894.
[36]H. Vagelas, A. Kalorizou, , Papachatzis and M. Botu. *Biotechnol. &Biotechnol*.Eq. **2009 ,**23(2): 1217-1219.