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Production of Chitinase From Some Mixed Culture Bacterial Strains Grown on Seafood Wastes and Its Application.

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ABSTRACT

Chitinase is an enzyme responsible for degrading chitin resulted in producing renewable source. Fish and shrimp shells are marine wastes their main component is chitin. Some bacterial strain as *Bacillus subtilus*, *Pseudomonus fluorescene* and *Pseudomonas lindbergii* tested for their ability for degrading chitin in shell of fish and shrimp in comparison with chitin powder. Chitinase activity of separate and mixed bacterial culture was determined after 24 and 48 h. Mixed culture of *Bacillus subtilus* + *Pseudomonus fluorescene* were more effective in degrading chitin and produce high enzymatic activity after 48h when growing on shrimp shell produce 410U/ml followed by fish shell produce 340 U/ml then by chitin powder which produce 320 U/ml.
In vitro highly antifungal activity of chitinase in separate and mixed culture of bacteria observed after 48h. when growing on shrimp shell. In vivo treated soil with mixed culture of *B. subtilus* + *P. fluorescene* grown on shrimp shell after 48 h. of incubation before sowing on bean plants resulted in reducing root rot disease under green house condition.

Keywords: Chitinase production ,Sea food wastes , chitin , bacterial strains

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INTRODUCTION

Chitin considered as the chemical analogue of cellulose , it is different from cellulose in the acetylation of the hydroxyl groups of each glucoside residues. Chitin is a linear biopolymer which exists widely in shellfishes, arthropod exoskeletons ,insect cuticles , mammals , fungal cell walls and plants. The total body weight of shellfishes is composed of about 70% chitinous substances and are often discarded which hydrolyze the chitin to its monomer N-acetyl glucosamine by breaking the glycosidic as wastes (Xu *et al*., 2008; Wang *et al*.,1997). Shrimp and crab shell (SCS) contain chitin, protein, and inorganic compounds, which are mainly composed of calcium carbonate (Wang *et al*.,2010)

Crustaceans contain the highest amount of chitin with respect to dry weight ,for this reason, crustacean shells are regarded the main source of chitin for the chemical industry (Jeniaux *et al*., 1989). Chitinase belongs to glycosyl hydrolases which hydrolyse the chitin to its monomer N-acetyl glucosamine by breaking the glycosidic bonds(Fukamiso,2000). Environmental implication of traditional disposal methods of shell fish waste, coupled with the strengthening of environmental regulations in many countries has created an interest in alternative methods of disposal/utilization of this waste (Wang *et al*., 2010). This waste can be utilized as an economic source of chitin and its derivative chitosan which are considerably versatile and promising biomaterials (Domard , 2011; Abirami *et al*., 2016).

Chitinase split into two broad categories as endo-chitinases and exo-chitinases (Hamid *et al.,2013)* . Some bacterial strains as *Pseudomonas* sp. and *Bacillus* sp. effectively use shrimp shell and seafood wastes to produce chitinase (Wang *et al* .,2010).

Chitinase have multiple applications as biocontrol agent, morphogenesis, bioconversion of waste containing chitin, pollution degradation, mosquito control, fungal biomass estimation, protoplast isolation and bio pesticides. Some of the commonly used sources for chitinase production are insects, plants ,mammals ,bacteria and fungi (Sally *et al*.,2015). The diversty of chitinolytic bacteria is quite rich in soil and bacteria from *bacillus*, *Pseudomonas*, *Streptomyces*, *Serratia* and *Aeromonas* genera are frequently in soil (Das *et al*.,2010) . Soil bacteria are the major source of chitinases and could be used for agricultures (Bhaktacharya *et* al.,2007 ; Kishore *et al* .,2005). Biological control of some soil-borne fungal diseases has been correlated with chitinase production (Buxton *et al*.,1993) bacteria-producing chitinases exhibit antagonism in vitro against fungi (Fridlender and Inbar,1993;Schlumboum *et al*.,1986)

Bacillus subtilis NPU 001, excreted chitinase when cultured in a medium containing Shrimp and Crab shell powder ,the chitinase produced inhibited hyphal extension of the fungus *Fusarium oxysporum* (Chang *et al*.,2010).*Bacillus subtilis* W-118 produced chitinolytic activity when cultured in a Shrimp and Crab Shell powder medium and chitinase produced inhibited the growth of plant pathogenic fungi(Wang *et al*.,2006).

The aim of this work was production of chitinase from chitin , Shrimp shell or fish shell wastes using three different bacterial strains and its antifungal activity in vitro and in vivo.

MATERIALS AND METHODS

Microorganisms

Bacillus subtilis, Pseudomonas fluorescens and Pseudomonas lindbergii were isolated from rhizosphere of healthy bean plants and identified in Plant Pathology department at(NRC). Soil born fungal strains (*Fusarium solani*, *Rhizoctonia solani and Sclerotium rolfsii) which* are the causative agents of bean damping-off and root-rot diseases, were isolated from bean roots that showed the disease symptoms. The fungi in pure culture were identified after pathogenicity test according to the keys given by Barnett and Hunter (1972) and Nelson *et al*. (1983) affiliated to the Plant Pathology Department, National Research Centre, Giza, Egypt. Cultures were kept on nutrient agar media slants and kept at 4 ◦C.

Sea food wastes

Fish and shrimp shells were collected , washed with tap water , dried in oven at 70 °C . The dried shells were ground into fine powder added and mixed by shaking.

Fermentation media

Batch fermentation was carried out in Erlenmeyer flask containing 50 ml of nutrient broth media in addition of one gram of chitin ,fish shell and shrimp shell in each flask . The flasks were incubated for 24-48 h. at 200 r.p.m.

Preparation of colloidal chitin

Colloidal chitin was prepared from chitin powder (Sigma Co.) according to the method described by Ried and Ogryd-Ziak (1981).

Ten grams of chitin powder suspended in 100 ml of 85 % phosphoric acid (H_3PO_4) and stored at 4°C for 24 h., then blended in 2 liter of distilled water using a warring blender. Then the suspension was centrifuged. This washing procedure was repeated twice. The colloidal chitin suspension was adjusted to pH 7.0 with 1 N NaOH and recentrifuged. The pelleted colloidal chitin was stored at 4°C until used.

Chitinase assay

Determination of enzyme activity was carried out according to the method of Monreal and Reese (1969).

One ml of 1 % colloidal chitin in citrate phosphate buffer (pH 6.6) in test tubes. One ml of culture filtrate was added and mixed by shaking.

Tubes were incubated in a 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 dinitrosalysilic acid (DNS). Optical density was measured at 540 nm.

Determination of chitinase production using selected bacteria strain

The ability of the selected bacterial strains for chitinase production was carried out by measuring the activity of the enzyme in fermentation media inoculated with one strain of selected bacteria (*B. subtilus, P. fluorescens, P. lindbergii*) individually or with mixed culture of three different bacterial strains as follows *B. subtilus+ P. flourescens*, *B. subtilus+ P. lindbergii* and *P. flourescens+ P. lindbergii* after 24 and 48 hours incubation period.

In vitro: Antifungal activity

In vitro: Evaluation of antifungal activity of *Bacillus subtilis, Pseudomonas fluorescens and Pseudomonas lindbergii* .

Bacillus subtilis, Pseudomonas fluorescens and Pseudomonas lindbergii grown on fish shells, shrimp shells and chitin powder were screened for their antifungal activity (inhibit mycelium growth) using the well-plate diffusion method as described by Boubaker *et al.,*(1996). Pathogenic fungi over laid on PDA plate and after 30 min. three wells (5mm. in diameter) were made in each plate and inoculate with 20ml of extracts of *B. subtilis ,P. fluorescens* and *P. lindbergii separately* and (water)as a control. The plates were kept for 2h.at 4°C to allow diffusion of the extract then incubated at 28°C for 5days. Diameter of inhibition zone was measured

In vivo : Pots Experiments

Preparation of antagonistic bacteria

Bacillus subtilis, Pseudomonas fluorescens and Pseudomonas lindbergii were prepared by grown sea food waste and counted by plate technique (108 cfu /ml.) Mojica *et al*.,(2008). Pot experiment was designed under green house conditions using plastic pots 25cm, diameter containing sterilized loamy clay soil were infested with *Fusarium solani* and *Rhizoctonia solani and Sclerotium rolfsii* separately grown on barley grain at rate 5g/Kg soil before sowing. Infested pots were irrigated for 10 days before sowing. The mixed cultures of *B. subtilis, P. fluorescens and P .lindbergii* were formulated on sea food waste added to soil as cell suspension at the rate of 50 ml/pot. Ten bean seeds were grown in each pot five replicate pots. Pots were kept under green house conditions till the end of the experiment.

Disease assessment for incidence of pre and post emergence

Percentage of root rot incidence at the pre-emergence stage was calculated as the number of absent emerged seedlings in relative to the total number of sown seeds. Meanwhile, percentage of post- emergence root rot was calculated as the number of bean plants showing disease symptoms in relative to the total number of emerged seedlings.

Statistical analysis

Tukey test for multiple comparisons among means was utilized(Neler *et al*.,1985).

RESULTS AND DISCUSSION

Effect of different bacterial strain on chitinase production using sea food wastes and chitin after 24 & 48 h.

Three different bacterial strains were tested for their ability for chitinase production in presence of chitin , fish and shrimp shell in fermentation media after 24 and 48 h. of incubation . Results in Fig. (1&2) showed that chitinase activity was high in media containing fish shell inoculated with *B. subtilus* after 24 h. produce 160 U/ml and shrimp shell produce 140 U/ml ,While after 48 h. maximum chitinase activity was at shrimp shell inoculated with *Bacillus subtilus* produce 270 U/ml followed by *P. fluorescens* produced 220 U/ml , minimum activity of chitinase produced in medium containing chitin powder. Maria *et al*., (2010) showed that chitinase activity of planktonic bacteria observed in a medium containing chitin was very high followed by media containing shrimp shells . The majority of bacteria were able to break down chitin to synthesize chitinase and use it as a source of carbon and energy Shubakov *et al.,* (2004). Certain strains of bacteria produced higher chitinolytic activity in presence of shrimp shells El-Tarabily *et al*., (2000).Sabry (1992) observed that *Bacillus subtilis* exhibited the highest chitinolytic activity in the presence of colloidal chitin, and *Bacillus amyloliquefaciens* and *Bacillus megaterium* produce higher chitinase activity in a medium containing shrimp shells. *Lactobacillus plantarum* inoculated on media containg shrimp shell as low cost carbon source able to produce high chitinase activity after 60 hour of fermentation Khorrami *et al*., (2012).

Effect of co-culture of three different bacterial strains on chitinase production

Mixed culture of three different bacterial strains showed different ratios of chitinase activity after 24 and 48 h. incubation period as shown in Fig. (3&4), shrimp shell produce maximum chitinase activity after 24 as well as after 48 h. when media inoculated with *B.subtilus+ P. fluorescens* followed by *P. fluorescens+ P. lindbergii* while inoculated media with *B. subtilis + P. lindbergii* produce the lowest activity. Fish shell produce maximum activity at media inoculated by *P. fluorescens+ P. lindbergii* after 24 h. of incubation while after 48 h. maximum chitinase activity with media inoculated with *B. subtilus+ P. fluorescens*. From results obtained shrimp shell was most effective in chitinase production than other wastes. Tesfaw *et al*.,(2014) illustrated that , efficiency of microorganisms increased when grown together , many enzymes produced at high amount with mixed culture. Some bacterial strain as *Pseudomonas sp*. and *Bacillus sp.* use shrimp shells as substrate for chitinase production which become more efficiently than colloidal chitin Sally *et al*., (2015)

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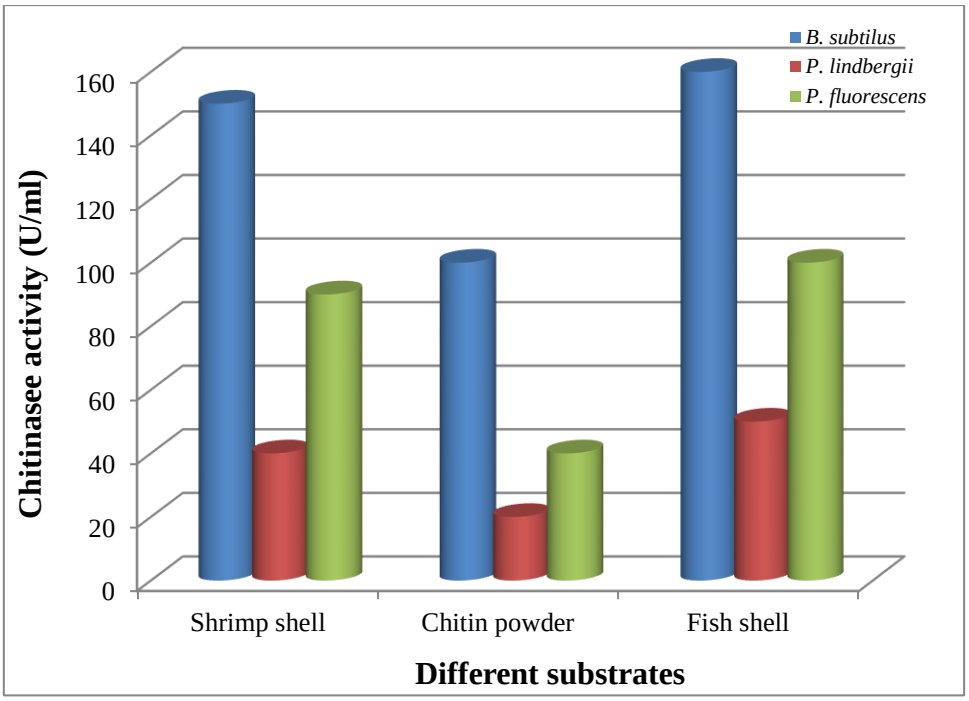

Fig. (1): Effect of different bacterial strains on chitinase production using sea food wastes and chitin after 24 h.

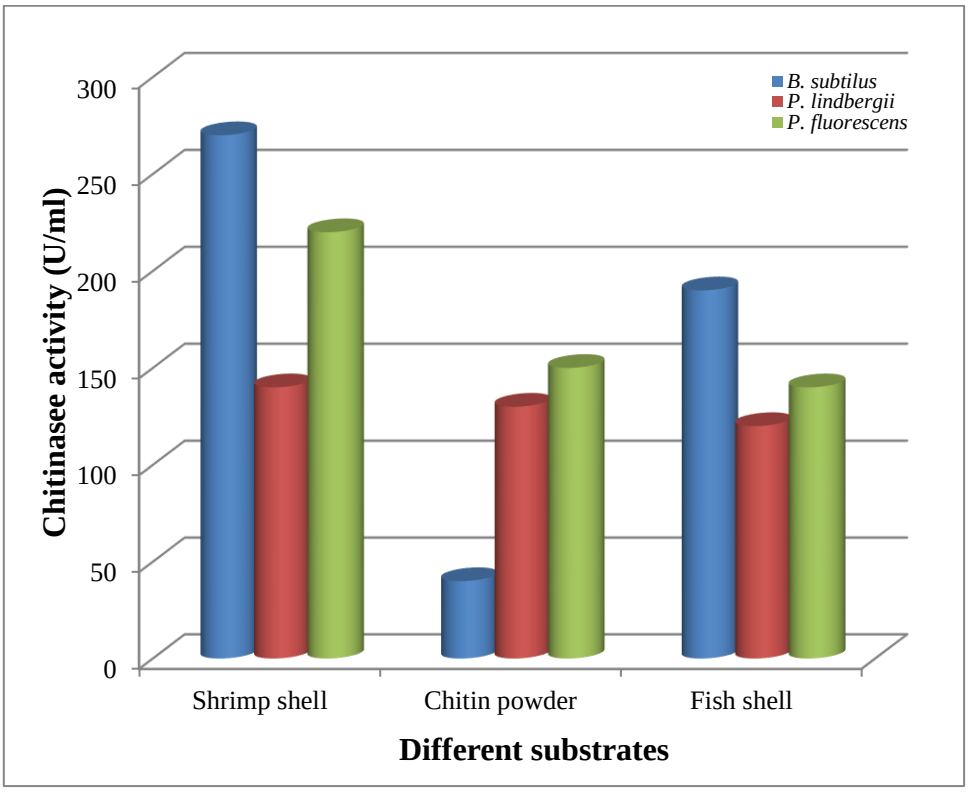

Fig. (2): Effect of different bacterial strains on chitinase production using sea food wastes and chitin after 48 h.

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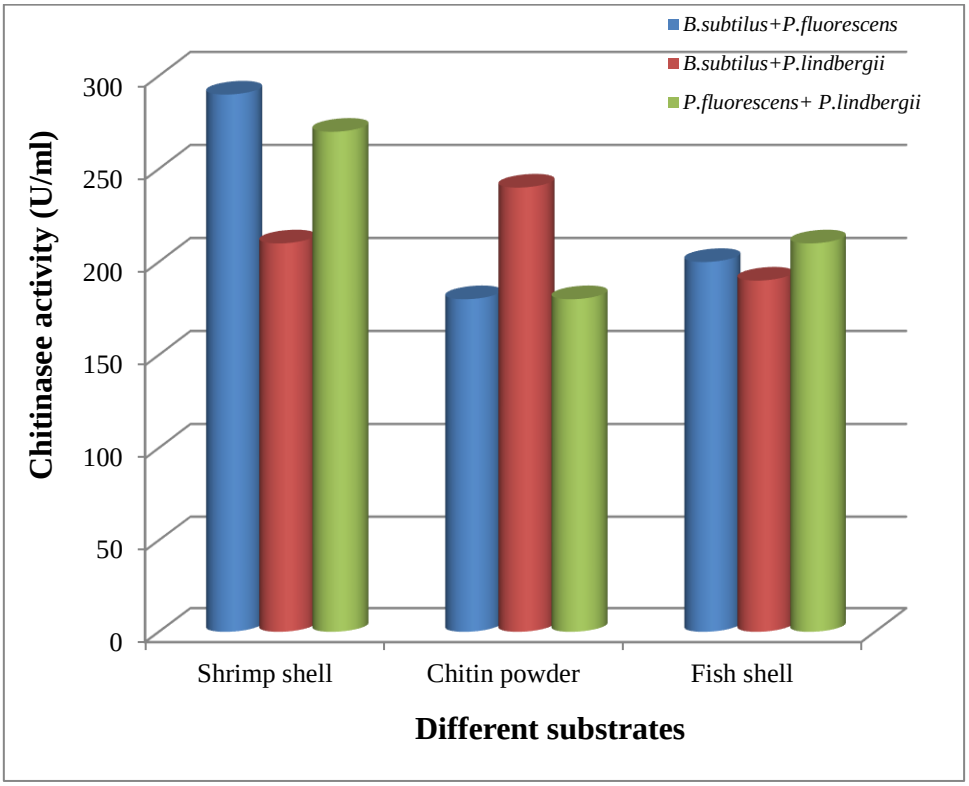

Fig.(3): Effect of co-culture of different bacterial strains on chitinase production using sea food wastes and chitin after 24 h

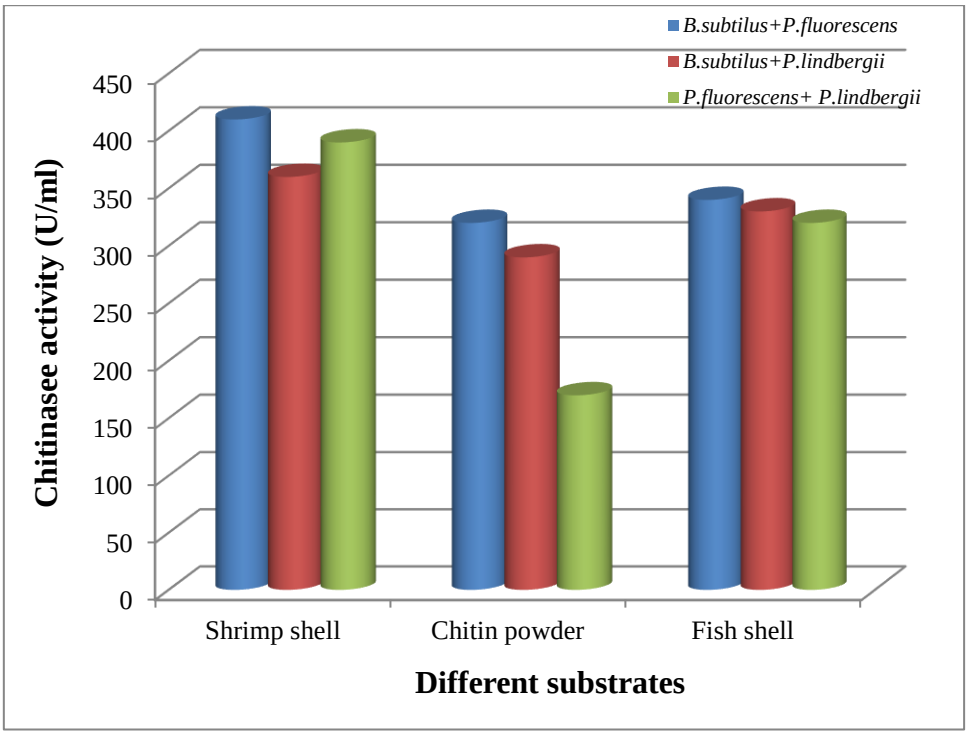

Fig.(4): Effect of co-culture of different bacterial strains on chitinase production using sea food wastes and chitin after 48 h

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In vitro: Evaluation of antifungal activity of *B. subtilis, P .fluorescens* and *P. lindbergii* grown on seafood wastes and chitin after 48h.from incubation period against bean soil borne fungi

Results in Table (1) revealed that the isolates of bacteria proved their ability to antagonize pathogenic fungi *F. solani , R.* solani and *S.rolfsii*. The highest effective of zone of inhibition was found by treatment with all bioagents which grown on shrimp shell which ranged from (2.4 to 3.0 cm) followed by grown on fish shells, chitin and control. The least effect was recorded with treatment with *P.lindbergii* on *F.solani ,R.solani and S. rolfsii* (1.8,1.5 and 1.3 cm) respectively. The zone of inhibition in the growth of the pathogen could be attributed to antibiosis, hyper-parasitism or production of chitinase, β-1, 3 glucanase and β-1, 4 glucanase enzymes which degrade the cell wall leading to lyses of mycelium of the pathogen Weyens *et al*.,(2009).

Table(1):Antagonistic effect of *B .subtilis, P. fluorescens* and *P. lindbergii* grown on seafood wastes and chitin after 48h.from incubation period against bean soil borne fungi

Organisms / Seafood waste	Zone of inhibition (cm.)								
	B.subtilis			*P. fluorescens*			*P. lindbergii*		
	F.solani	*R.solani*	*S.rolfsii*	*F.solani*	*R.solani*	*S.rolfsii*	*F.solani*	*R.solani*	*S.rolfsii*
Fish shell	2.8	2.5	2.4	3.0	2.8	2.6	2.3	2.0	2.1
Shrimp shell	3.0	2.8	2.7	3.3	2.8	2.7	2.5	2.3	2.4
Chitin powder	2.0	1.9	1.7	2.5	2.0	2.2	1.8	1.5	1.3
Control	1.5	1.3	1.3	2.0	1.8	1.6	1.5	1.3	1.0

In vitro: Evaluation of antifungal activity of mixed cultures

Data in Table (2) revealed that the most effective treatment mixed culture *B. subtilis+ P. fluorescens* grown on shrimp shells against *F. solani, R. solani and S. rolfsii* (4.5,4.3and 4.0 cm)respectively , moderate effect with treatment by *B. subtilis+ P. lindbergii* (3.5,3.0 and 2.9 cm) respectively**.** Pleban *et al.,(* 1997) found that crude extracellular chitinase of an entophytic bacterium *B .cereus* 65 decreased spore germination of *F. oxysporum*. Chitinolytic enzymes have been considered as important biocontrol agents of soil borne pathogens because of its ability to degrade fungal cell walls, whose major component is chitin Miller *et al*., 1986.

Table(2): Antagonistic effect of mixed culture grown on seafood waste after 48h. against bean soil borne fungi

Organisms / Seafood waste	Zone of inhibition (cm.)								
	B.subtilis + P.fluorescens			*B.subtilis + P.lindbergii*			*P. fluorescens + P.lindbergii*		
	F.solani	*R.solani*	*S.rolfsii*	*F.solani*	*R.solani*	*S.rolfsii*	*F.solani*	*R.solani*	*S.rolfsii*
Fish shell	4.2	4.0	3.8	3.2	3.1	3.0	2.5	2.4	2.2
Shrimp shell	4.5	4.3	4.0	3.5	3.0	2.9	2.8	2.5	2.5
Chitin powder	3.8	3.5	3.3	2.8	2.5	2.6	2.0	2.0	1.7
Control	2.5	2.8	2.7	2.2	2.0	2.1	1.8	1.6	1.5

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In vivo :Effect of soil treatments with different mixed culture bioagents formulated of sea food waste on bean plant (under green house condition).

Treated soil with different mixed culture of bioagents before sowing bean plants resulted in reducing root rot disease under green house conditions. Results in Table (3) showed that all applied soil treatments reduced the incidence of root rot caused by *F. solani, R. solani* and *S. rolfsii*. The highest reduction in disease incidence was observed with the treatment by *B. subtilis* + *P. fluorescens* formulated on shrimp shell reduced the pre-emergence by(55.6,70.0 and 58.3)respectively at pre-emergency . Also at post-emergence the same trend. Also data showed that treatment with *B. subtilis+ P. lindbergii* or *P. fluorescens* + *P. lindbergii* were significantly reduced pre and post –emergence compared with control.

The same work supported by Wang *et al*.,(2006) *Bacillus subtilis* W-118, a strain excreted chitinase enzyme when cultured in a medium containing shrimp and crab shell powder as the major carbon source. It is an inexpensive method for the production of chitinase enzyme. In addition, the oligosaccharides prepared by acidic hydrolysis might be toxic because of chemical changes during conversion Wang and Chang (1997). Therefore, there is a growing interest for enzymatic hydrolysis of chitin from shrimp shell wastes.

Table (3) Incidence of Bean root rot diseases in response to soil treatment with different bioagents formulated on sea food waste under greenhouse condition

Treatments	Pre-emergency %		Post-emergency	
	Disease incidence	Reduction	Disease incidence	Reduction
Fusarium solani	**36.0a**	---------	**31.25a**	--------
F. solani+ B.subtilis+P.fluorescens	**16.0b**	**55.6**	**19.0b**	**39.2**
F. solani+ B.subtilis+P.lindbergii	**20.0c**	**44.4**	**25.0c**	**20.0**
F. solani+ P.fluorescens+P.lindbergii	**24.0d**	**33.3**	**21.0b**	**32.8**
Rhizoctonia solani	**40.0a**	--------	**53.3a**	----------
R. solani B.subtilis+P.fluorescens	**12.0b**	**70.0**	**22.7b**	**57.4**
R. solani B.subtilis+P.lindbergii	**20.0c**	**50.0**	**30.0d**	**43.7**
R. solani P.fluorescens+P.lindbergii	**24.0d**	**40.0**	**26.3c**	**50.6**
Sclerotium rolfsii	**48.0a**	----------	**53.8a**	----------
***S. rolfsii B.subtilis+P.*fluorescens**	**20.0b**	**58.3**	**10.0b**	**81.4**
S.* rolfsii *B.subtilis+P.lindbergii	**28.0d**	**41.7**	**22.2c**	**58.7**
S.* rolfsii *P.fluorescens+P.lindbergii	**24.0c**	**50.0**	**26.3d**	**51.1**

Figures with the same letters are not significant (P ≤ 0.05)

CONCLUSION

Chitinase activity of separate and mixed bacterial strain *B. subtilus*, *P. fluorescene* and *P. lindbergii* was determined after 24 and 48 h. Mixed culture of *Bacillus subtilus* + *Pseudomonus fluorescene* were more effective in degrading chitin and produce high enzymatic activity after 48h when growing on shrimp shell .In vitro highly antifungal activity of chitinase in separate and mixed culture of bacteria observed after 48h. when growing on shrimp shell. In vivo treated soil with mixed culture of *B. subtilus* + *P. fluorescene* grown on shrimp shell after 48 h. of incubation before sowing on bean plants resulted in reducing root rot disease under green house conditions

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