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In Vitro Action of *Streptomyces griseolus* Proteases as Bio-Control on *Fasciola* gigantic Eggs

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Abstract

This work evaluated the *in vitro* bio-control action of actinomycetes isolated from Egyptian soil against *Fasciola gigantica* eggs. In this respect, the identified *Streptomyces griseolus* was the best parasite-control agent which gave the highest mortality percent applying three methods; the first method was the application of the grown bacterial disks with the eggs at zero time on the medium (2% w/v water-agar) and incubated for 21 days (75.5% mortality), the second method was the incubation of the bacterial disks using the same medium for 5 days before eggs inoculation, after 21 days of incubation the mortality percent was 80.8%. The application of the third method showed a good effect by using all bacterial culture filtrates. *S. griseolus* filtrate which had the highest lytic enzyme and proteolytic activities reached 2.0 and 660.7 U/ml, respectively, gave the highest mortality (95.5%) of *F. gigantica* eggs compared to the other bacterial filtrates. The crude culture filtrate and the diluted culture filtrate (1.33 fold) of *S. griseolus* gave the highest mortality percent against the parasite´s eggs (95.8 and 94.5%, respectively) compared to the higher diluted filtrates. The culture filtrate of *S. griseolus* grown on two different media and its partially purified enzyme (precipitated by 60% ammonium sulfate) were tested for their proteolytic activity and inhibitory effect against *F. gigantica* eggs. Medium no. 2 showed the highest proteolytic activity (1030.3 and 1138.1 U/ml) for the crude and the partially purified enzyme, respectively. While the highest inhibitory effect reached 97.6 and 89.9%, respectively. Finally, the application of crude enzyme filtrate was better than the partially purified one. *Streptomyces griseolus* was proved as a potential biological control agent for this helminth.

Keywords: Biological control; Actinomycetes; *Streptomyces griseolus*; *Fasciola gigantica* eggs; Proteases

Introduction

Actinomycetes are gram positive mycelium-forming soil bacteria. They have the ability for decomposition of many macromolecules because they synthesize of various extracellular hydrolases including lytic enzymes which degrade chitin, cellulose, proteins, xylan, lignin, starch, lipids, pectin, and keratin [1]. Also they can produce antibiotics and other useful compounds of commercial interest [2,3]. Furthermore, they play an important role in the biological control of insects and parasites [4,5]. Finally, Pirali-Kheirabadi reported that *Streptomyces avermitilis*, produces toxins collectively called "avermectins" which are highly effective against several invertebrates from the classes Insecta, Arachnida and Nematodes [6].

Lytic enzymes such as α and β-glucanases, proteases, peptidases, cellulases, chitinases and lipases have been proposed as the key enzymes in the lysis of pathogenic bacterial and fungal cell wall. Proteases known as proteinases or proteolytic enzymes which occur naturally in all organisms, they act on the peptide bonds formed by specific amino acids to hydrolyze them [7]. They represent one of the three largest groups of industrial enzymes and find application in detergents, leather industry, food industry; pharmaceutical industry and bioremediation processes [8]. Also, Proteases from a variety of sources (viruses, bacteria, fungi, plants, and insects) have toxicity towards insects; Harrison and Bonning used the insecticidal proteases as potential pesticides. *Streptomyces* species producing protease include *S. griseus, S. rimosus* and *S. thermovulgaris* [9-12].

Fasciolosis, caused by the trematode liver fluke *Fasciola gigantica*, is the cause of considerable loss in domestic ruminants' production systems in tropical and subtropical parts of Asia and Africa [13]. It is also emerging as a significant zoonotic infection of humans [14]. In Egypt, high infection rates had been described in livestock [15]. Such infection rates induced important economic problems. The annual loss in milk and meat due to fasciolosis was being estimated to be 30%. Also, the number of human cases had dramatically increased since 1980, especially in the Nile Delta region [16]. On the other hand, one adult liver fluke produces between ten and 20,000 eggs per day that are expelled to the outside along with the stool. In the right humidity, temperature, and light conditions in the pasture, the eggs hatch and release miracidia, initial larval forms that will infect the *Lymnaea spp.* freshwater mollusk [17]. Although humans and domestic ruminants become infected through water consumption, most infections in ruminants occur in animals during the dry season, when they graze in contaminated areas and become infected orally by ingestion of metacercaria; the infectious form [18]. At present, effective and commercial vaccines are not yet available; therefore anthelmintic drugs are the main method employed for controlling the fluke infection [19]. However, due to emergence of resistance and the cost of animal treatment, especially for small ruminants, the use of new control alternative measures is important, especially of the present eggs in the pasture, such as biological control [20]. The use of bacteria

appears therefore as a viable and promising control alternative for constantly infected animals.

The major objective of the present study is screening of the soil actinomycetes isolates for their ability to degrade or lysis the eggs of *F. gigantica*, then the most promising organism was applied as a bio-control agent against the eggs under laboratory conditions.

Materials and Methods

Microorganism

a. Isolation: The soil samples were collected from three different locations in Egypt (Kafr Elzayat city, Tanta city and El mansoura city) air dried and powdered. The samples were diluted in sterile saline solution (0.89% w/v). The diluted samples were plated onto sterile starch nitrate plates (pH 7) containing (g/l) Starch 20; KNO_3 1; K_2HPO_4 0.5; $MgS0_47H_20$ 0.5; NaCl 0.5; Fe SO_4 0.01; and agar 20 and were incubated at 30°C [21]. After 7 days, the isolated actinomycetes colonies were subcultured in fresh plates and then the single uniform colonies were transferred into slants of the same medium and preserved in the refrigerator at 4°C until use.

b. Reference microorganisms: *Streptomyces griseolus* was isolated from the soil and identified by biochemical tests in National Research Center [22]. *Streptomyces griseus* and *Streptomyces vlavogriseus* were isolated from the soil and identified previously by 16S rRNA sequencing data collection [23].

c. Collection and counting of *F. gigantica* eggs: Immature *F. gigantica* eggs were obtained from the gall bladders of naturally infected buffaloes slaughtered in El Warak abattoir, Giza, Egypt, known by the presence of adult *F.* gigantic worms in the bile passages of the livers of these buffaloes. The eggs were processed according to the method of Hegazi et al. [14]. Number of immature eggs per 1 ml of solution was determined and kept in the refrigerator at 4°C until used.

Screening for biological activity against *F. gigantica* eggs

All the isolated actinomycetes included the three identified *streptomyces* were screened for biological activity against *F. gigantica* eggs by three methods

Screening by using antagonistic effect: This was done using two methods as follows, the first: Fifteen ml of the assay medium (2 %w/v agar) were poured in Petri dishes till solidification. One cm diameter disks were taken from 9-day-old cultures of actinomycetes and placed on the solidify assay medium containing five hundred immature *F. gigantica* eggs; after one week of their collection. The second method was the same as the previous method except that the bacterial disks were incubated with the assay medium for 5 days before the inoculation of the eggs. The Petri dishes were incubated at 30°C for 21 days. The eggs were collected from each plate containing the microorganism and from the control (without actinomycetes) and examined. The cultured eggs were examined every 3 days until the eggs approached hatching, as evidenced by movement of the miracidia within the eggs [24]. The day of hatching was the day in which most of the eggs with developed active miracidia hatch after exposure to artificial light for 15 min.

Screening by using culture filtrate: The inoculum was prepared by growing the organisms on soy bean slants for 9 days; then a bacterial suspension of optical density 0.6 at 660 nm was made by using sterile saline solution. Two ml of the suspension was inoculated on 48 ml liquid medium which contain (g/l): 0.7, K_2HPO_4; 0.3, KH_2PO_4; 0.5, $MgSO_4$; 0.01, $FeSO_4$; 0.001, $ZnSO_4$ and 7, baker's yeast; modified from Reynolds [25]. Thereafter, the flasks were incubated for 4 days at 30°C in a shaking incubator (200 rpm). The culture medium was centrifuged at 6000 rpm for 10 min under the sterile conditions and the supernatant was assayed for lytic enzyme and proteolytic activities. Thereafter, five ml of the supernatant was poured on test tubes containing five hundred immature *F. gigantica* eggs. The tubes were incubated for three days on a shaking incubator adjusted at 100 rpm, then the eggs washed several times with sterile distilled water and placed in Petri dishes. The dishes were covered and incubated at 26°C, and the eggs were examined every 3 days until the eggs approached hatching, as above.

Inhibitory activity of tested microorganisms and enzymes on immature *F. gigantica* eggs: At the end of the incubation period, the rate of egg hatching in both exposed and control eggs were evaluated according to Hegazi et al. [14]. The reduction percentage of *F. gigantica* egg hatching was estimated using the following formula:

$$\text{Inhibitory activity} = \frac{\text{Hatching \% of control eggs - Hatching \% of exposed eggs}}{\text{Hatching \% of control eggs}} \times 100$$

Production media

Two different types of media were used for production of lytic enzymes (especially protease). The compositions of these media g/l were as follows:

Medium 1: 0.7, K_2HPO_4; 0.3, KH_2PO_4; 0.5, $MgSO_4$; 0.01, $FeSO_4$; 0.001, $ZnSO_4$ and 7, baker yeast; modified from Reynolds [25].

Medium 2: 30 glucose; 0.5, NaCl; 2, KNO_3; 1, K_2HPO_4; 0.5, $MgSO_4$; 3, $CaCO_3$; 0.01 for each of $FeSO_4$, $ZnSO_4$ and $MnCl_2$.

Preparation of dead enzyme

The crude culture filtrate was autoclaved at 121°C, 1.5 atm for 5 minutes.

Assay of lytic enzymes

Preparation of cell wall suspension: The residual cell walls of *Saccharomyces cerevisiae* after sonication were centrifuged and washed then lyophilized and stored according to Beaulieu et al. [26].

Turbidimetric method: This was carried out according to the method described by Tominaga and Tsujisaka with some modifications in the volumes of the reaction mixture (enzyme solutionandcell wall suspension) [27].

Quantitative assay of protease

Protease activity in the culture filtrate of *S. griseolus* was assayed by method of Tsuchida et al. [28] by using casein as the substrate. One protease unit is defined as the amount of enzyme that releases 1µg of a tyrosine per ml per minute under the above assay conditions.

Protein concentration

The protein concentration was determined by the method of lowry et al. [29] with bovine serum albumin as a standard.

Precipitation by ammonium sulfate

Finley powdered ammonium sulfate was added to the culture filtrate of *S. griseolus* maintained at 4°C with stirring until the required saturation of ammonium sulfate was reached (60%). The mixture was lift to stand overnight at 4°C. The precipitate protein was separated by centrifugation at 10.000 rpm for 15 min. at 4°C. The precipitate was dissolved in 0.2 M phosphate buffer pH 7 and dialyzed against the same buffer, and dried then stored at -18°C. This concentrated enzyme solution is called Partially Purified Enzyme (PPE).

Statistical analysis

Data were statistically represented in terms of mean and Standard Deviation (SD) of five independent astrocytic culture preparations. Comparison among different groups in the present study was done using One-way ANOVA Test as multiple comparisons. Probability value (p-value) less than or equal to (0.01) was considered highly significant. All statistical calculations were done using computer program SPSS (Statistical Package for Social Science) statistical program version (16.0).

Results

Screening of actinomycetes for ovicidal activity

The isolates including the three previously identified *Stereptomyces* were screened for ovicidal activity against *F. gigantica* eggs using water agar medium (Table 1). The first two methods exhibited the antagonistic effect of all actinomyctes after 21 days of incubation. The most potent isolate, *S. griseolus*, exhibited a maximum inhibitory effect reached 75.5% by using method no. 1 and 80.8% by using method no. 2 at 30°C compared to the other isolates. The data obtained from method no. 3 showed the highest inhibitory effect against the eggs compared to the other two methods (Table 1). *S. griseolus* gave the highest lytic enzyme and protease activities reached 2.0 and 660.7 U/ml, respectively, with the highest inhibitory effect reached 95.5% compared to the other organisms. On the other hand, isolate no. 8 gave the lowest inhibitory effect (5.0, 0.0 and 47.3% by using the three methods, respectively) and the lowest enzymatic activities (0.9 and 264 U/ml for lytic enzyme and protease, respectively).

Isolate no.	Inhibitory effect (%)			Lytic enzyme activity (U/ml)	Protease activity (U/ml)
	Method no. 1	Method no. 2	Method no. 3		
I1	61.5 ± 5.0	0.0 ± 0.0	75.0 ± 6.0	1.1 ± 0.10	448.1± 10.5
I2	75.0 ± 4.7	80.3 ± 2.1	93.8 ± 1.1	1.6 ± 0.12	524.2 ± 12.9
I3	47.5 ± 7.0	63.3 ± 5.0	94.8 ± 0.8	1.8 ± 0.11	616.0 ± 14.3
I4	61.3 ± 3.0	43.8 ± 8.0	92.0 ± 2.5	1.5 ± 0.14	492.1 ± 9.5
I5 (S. griseolus)	75.5 ± 3.2	80.8 ± 2.2	95.5 ± 1.0	2.0 ± 0.14	660.7 ± 13.7
I6	42.5 ± 10.0	0.0 ± 0.0	81.8 ± 3.0	1.3 ± 0.09	460.9 ± 8.9
I7 (S. vlavogriseus)	10.0 ± 6.2	0.0 ± 0.0	74.2 ± 7.2	1.4 ±0.10	470.0 ± 7.5
I8	5.5 ± 3.5	0.0 ± 0.0	47.3 ± 6.2	0.9 ± 0.09	264.0 ± 7.3
I9 (S. griseus)	62.8 ± 13.0	66.0 ± 6.0	90.5 ± 4.3	1.3 ± 0.07	520.8 ± 11.2
I0	42.5 ± 10.2	25.0 ± 5.0	76.8 ± 4.0	1.2 ± 0.11	488.6 ± 13.4
F value	33.779	229.570	37.709	29.847	270.123
P value	0.000	0.000	0.000	0.000	0.000

Table 1: Screening of actinomycetes isolates for ovicidal effect on *Fasciola gigantica* eggs by using three methods at 30°C. Data indicated that there is highly significant difference among all isolates at 0.01 levels ($p \leq 0.01$).

Ovicidal activity of enzyme protein concentration

The ovicidal effect of the protein concentrations of extracellular lytic enzymes (especially protease) produced by *S. griseolus* was performed to find out the minimum inhibitory concentration which gave the maximum ovicidal effect after 3 days at 30°C of incubation at 100 rpm. Different concentrations of enzyme protein ranged from 0.03-0.28 mg/ml were tested (Figure 1). The results revealed that the ovicidal effect increased with increasing the protein concentration and protease activity. The maximum inhibitory effect which ranged from 89.8 to 95.8% was at concentrations ranged between 0.14 to 0.28 mg/ml, respectively, and had the highest enzyme activity ranged from 356.6 to 663.3 U/ml, respectively.

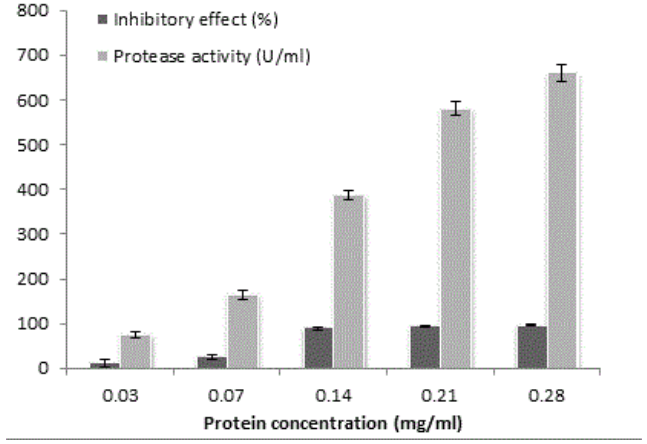

Figure 1: Effect of different concentrations of enzyme protein on *Fasciola gigantica* eggs after 3 days of incubation at 30°C using *S. griseolus*

Comparison between the inhibitory effect of crude, partially purified and dead enzymes on *F. gigantica* eggs

The enzyme production varied greatly with the culture media used. So, the effect of crude, partially purified and dead enzymes secreted by *S. griseolus* grown on two different media (M 1 and M 2) on *F. gigantica* eggs was tested at 30°C after 3 days of incubation at 100 rpm

(Table 2). The results indicated that the inhibitory effect (97.6 crude, 89.9 partially purified and 25.5% dead enzyme) and protease activity (1030.3 crude, 1138.1 partially purified and 0.0 U/ml dead enzyme) of enzyme produced in medium no. 2 were higher than those of medium no. 1 (95.1 crude, 86.6 partially purified and 19.5% dead enzyme) and (647.0 crude, 718.8 partially purified and 0.0 U/ml dead enzyme), respectively. Based on the data, it indicated that the organism could produce protease by using different substrates. The highest activity of protease (crude and partially purified) was observed by using medium no. 2. The most potent enzyme form was the crude one. At the meantime, 60% ammonium sulfate partially purified enzyme had slightly lower ovicidal activity.

Effect	Protease activity (U/ml)	Inhibitory effect (%)
Crude enzyme (M1)	647.0 ± 5.0	95.1 ± 2.0
Crude enzyme (M2)	1030.5 ± 14.0	97.6 ± 1.5
Partially purified enzyme (M1)	718.6 ± 8.0	86.0 ± 3.0
Partially purified enzyme (M2)	1138.0 ± 5.2	89.9 ± 3.0
Dead enzyme (M1)	0.0 ± 0.0	19.5 ± 5.0
Dead enzyme (M2)	0.0 ± 0.0	25.5 ± 4.0
F value	8443.476	350.317
P value	0.000	0.000

Table 2: Comparison between the effect of crude, partially purified and dead enzyme on *Fasciola gigantica* eggs after 3 days of incubation at 30°C using *S. griseolus*. Data indicated that there is highly significant difference among all isolates at 0.01 levels ($p \leq 0.01$). M1= medium no. 1. M2= medium no. 2

Discussion

Actinomycetes are considered as important natural bacteria which exhibit diverse modes of action: these include parasitizing and producing of toxins, antibiotics, or enzymes, they act synergistically on the parasite through the direct suppression of it or by promoting the microbial antagonists [5]. Among various actinomycetes, the genus *Streptomyces* always hold special significance in the research because this genus is known to produce a vast array of compounds with diverse biological properties. In the present study, all screened *Streptomyces* showed ovicidal activity against *F. gigantica* eggs. This might be due to that the isolates had lytic effect on the eggs wall without morphological damage. In this sense, Braga et al. [20] found that the fungi *Duddingtonia flagrans* and *Monacrosporium sinense* had shown lytic effect on *F. hepatica* egg wall without its morphological damage reached 66.5 and 73.4%, respectively. Also, the same author reported the same effect on eggs of *Ascris lumbricoides*. Furthermore, *Artobotrys robusta* and *A. conoides* had the same effect against *Toxocara cains* eggs [30,31]. Moreover, Hussain et al. [4] used actinomycetes as a biological control against the house fly *Musca Domestica*, they found that the mortality of larval and pupal stages, were very high reaching up to 90%. Also, Sundarapandian et al. [32] used actinomycetes effectively against *Culex quinquefasciatus*. In the present study, *S. griseolus* was the most potent isolate that exhibited a maximum inhibitory effect reached 75.5 by using method no. 1 and 80.8% by using method no. 2 at 30°C compared to the other isolates. The culture filtrates of the ten isolates grown on medium no. 1 were collected after 4 days of incubation and were tested for ovicidal effect against *F. gigantica* eggs after 3 days of incubation at 30°C and 100 rpm. The medium no. 1 was chosen because it contained baker's yeast that consisted of biopolymers which had a stimulating influence on synthesis of lytic enzymes [33].The culture filtrates were collected after 4 days because the lytic enzymes reached its maximum production at the stationary phase of growth. In this study, the third method was the most effective method for killing the eggs by all *Streptomyces* used but with variable potentialities; *S. griseolus* gave the highest inhibitory effect reached 95.5% compared to the other organisms. This might be due to that the culture filtrates of the actinomycetes, contained extracellular lytic enzyme systems varied in their activities. These results appeared to be in line with Shubakov and Kucheryavykh who reported that the activities of extracellular lytic enzymes in the tested fungi varied [34]. The antagonistic activity of *Streptomyces* was related to the production of extracellular hydrolytic enzymes and antifungal compounds [35]. The lytic enzyme system including protease acts on the peptide bond of protein. Since the egg shell of the parasite consists largely of protein and related compounds; sclerotin or quinone tanned proteins, like other living matter does, it was postulated that protease whether alone or in a mixture with other lytic enzymes could be involved in parasite control [36,37]. In these findings, Lysek and Sterba found that the fungi *Verticillium chlamydosporium* and *Verticillium* spp. were shown to be able to degrade the egg shell of *Ascaris lumbricoides* enzymatically and infect the eggs [38]. Also, Gadelhak et al. [39] found that the actinomycetes *Actinoplanes philippinensis*, *Actinoplanes missouriensis* and *S. clavuligerus* were capable of reducing the insect (*Drosophila melanogaster*) population by their effective production of chitinase under controlled laboratory conditions. Meanwhile, the inability of the autoclaved preparation of each chitinase producing isolate to kill the insect indicated that the reduction in the insect population by the chitinase-producing isolates might be associated with their chitinase production where, the cuticle of insect species consisted largely of chitin, it was postulated that chitinase produced by these isolates could be involved in insect control. Furthermore, *Streptomyces saraceticus* and *Pasteuria psenetrans* had the best antagonistic abilities, which reduced egg masses number of nematode (*Meloidogyne incognita*) on roots of banana and citrus from 207.8 to 90.1 and 83.6, respectively [40]. This was attributed to a secreted chitinase that dissolved the chitin layer of the plant parasitic nematode egg shell [41].

The ovicidal effect of the protein concentrations of extracellular lytic enzymes produced by *S. griseolus* increased with increasing the protein concentration and protease activity, this might be due to that the increasing of the enzyme activity, and this was in accordance with Vetrivel and Dharmalingam who reported that the increase in enzyme activity was due to increased synthesis of the enzyme protein [42]. The maximum inhibitory effect which ranged from 89.8 to 95.8% was at concentrations ranged between 0.14 to 0.28 mg/ml, respectively. Indeed, Prapagdee et al. [43] mentioned that the decrease in the degree of fungal growth inhibition corresponded to the decrease in concentration of the culture filtrates. In *Streptomyces*, the enzyme production varied greatly with the culture media used. So, the effect of crude, partially purified and dead enzymes secreted by *S. griseolus* grown on two different media (medium no. 1 and medium no. 2) on *F. gigantica* eggs was tested at 30°C after 3 days of incubation. This study revealed that the organism could produce protease by using different substrates. The highest activity of protease (crude and partially purified) was observed by using medium no. 2, this might be due to

induction of enzyme secretion by glucose and potassium nitrated in medium no. 2 compared to baker's yeast in medium no. 1. In these findings, El-Shafei et al. [44] observed that glucose (1.25%) was the best carbon source for protease production by *S. albidoflavus*. Also, Johnvesly and Naik noticed that 1% (w/v) of sodium nitrate and potassium nitrate seemed to be good nitrogen sources for protease production [45]. The most potent enzyme form was the crude one. The partially purified enzyme which precipitated by 60% ammonium sulfate had slightly lower ovicidal activity, however it had a high specific activity, this might be due to the presence of compounds produced by *Streptomyces* at the stationary phase (after 4 days) in the crude culture filtrate and when precipitated by ammonium sulfate, these compounds were separated. Prapagdee et al. [43] suggested that extracellular hydrolytic enzymes in the exponential and stationary culture filtrate and secondary compounds in the stationary culture filtrate of the strain *S. hygroscopicus* played an important role in the inhibition of the growth of some pathogenic fungi. On the other hand, the dead enzyme gave inhibitory effect however, it had no activity. In this sense, Gadelhak et al. [39] found that the autoclaved spores of *Actinoplanes philippinensis*, did reduce the amount of pupa formed by *Drosophila melanogaste*, at a time, its active spores gave the lowest pupal formation percentages.

This was the first study on the effect of actinomycetes on *F. gigantica* eggs. *S. griseolus* might be a potential candidate for biological control of this fluke.

References

1. El-Shanshoury AR, El-Sayed MA, Sammour RH, El-Shouny WA (1995) Purification and partial characterization of two extracellular alkaline proteases from Streptomyces corchorusii ST36. Can J Microbiol 41: 99-104.
2. Saugar I, Sanz E, Rubio MA, Espinosa JC, Jiménez A (2002) Identification of a set of genes involved in the biosynthesis of the aminonucleoside moiety of antibiotic A201A from Streptomyces capreolus. Eur J Biochem 269: 5527-5535.
3. Basilio A, González I, Vicente MF, Gorrochategui J, Cabello A, et al. (2003) Patterns of antimicrobial activities from soil actinomycetes isolated under different conditions of pH and salinity. J Appl Microbiol 95: 814-823.
4. Hussain AA, Mostafa SA, Ghazal SA, Ibrahim SY (2002) Studies on antifungal antibiotic and bioinsecticidal activities of some actinomycete isolates. African J Mycol Biotechnol 10: 63-80.
5. Tian B, Yang J, Zhang KQ (2007) Bacteria used in the biological control of plant-parasitic nematodes: populations, mechanisms of action, and future prospects. FEMS Microbiol Ecol 61: 197-213.
6. Pirali-Kheirabadi K (2012) Biological Control of Parasites, Parasitology, Dr. Mohammad Manjur Shah (Ed.), ISBN: 978-953-51-0149-9.
7. Tunga R, Shrivastava B, Banerjee R (2003) Purification and characterization of a protease from solid state cultures of Aspergillus parasiticus. Process Biochem 38: 1553-1558.
8. Gupta R, Beg QK, Lorenz P (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59: 15-32.
9. Harrison RL, Bonning BC (2010) Proteases as insecticidal agents. Toxins (Basel) 2: 935-953.
10. Yeoman KH, Edwards C (1994) Protease production by Streptomyces thermovulgaris grown on rapemeal-derived media. J Appl Bacteriol 77: 264-270.
11. Hadeer A (1999) Optimization of Streptomyces griseus proteinase Production by solid substrate fermentation. Iraq J Sci 40B: 15-26.
12. Yang S, Wang J (1999) Protease and amylase production of Streptomyces rimosus in submerged and solid state cultivation. Bot Bull Acad Sin 40: 259-265.
13. Anuracpreeda P, Songkoomkrong S, Sethadavit M, Chotwiwatthanakun C, Tinikul Y, et al. (2011) Fasciola gigantica: production and characterization of a monoclonal antibody against recombinant cathepsin B3. Exp Parasitol 127: 340-345.
14. Hegazi AG, Abd El Hady FK, Shalaby HA (2007) Inhibitory effect of Egyptian propolis on Fasciola gigantica eggs with reference to its effect on Clostridium oedematiens and correlation to chemical composition. Pak J Biol Sci 10: 3295-3305.
15. Degheidy NS, Shalaby HA (2010) Scanning electron microscopic observations of adult Fasciola gigantica after immunization with glutathione S-transferase in goats. Res J Parasitol 5: 79-89
16. Hussein AA, Khalifa RMA (2010) Fascioliasis prevalences among animals and human in Upper Egypt. Journal of King Saud University 22: 15-19
17. Dalton JP (1999) In: Dalton, J.P. (Ed.), Fasciolosis. CABI Publishing, CAB International, Wallingford, Oxon OX10 8DE, UK.
18. Pile E, Santos JAA, Pastorello T, Vasconcellos M (2001) Fasciola hepatica em búfalos (Bubalusbubalis) no município de Maricá, Rio de Janeiro, Brasil. Braz J Vet Res An Sci 38: 42-43.
19. Saowakon N, Tansatit T, Wanichanon C, Chanakul W, Reutrakul V, et al. (2009) Fasciola gigantica: anthelmintic effect of the aqueous extract of Artocarpus lakoocha. Exp Parasitol 122: 289-298.
20. Braga FR, Araújo JV, Campos AK, Araújo JM, Carvalho RO, et al. (2008) In vitro evaluation of the action of the nematophagous fungi Duddingtonia flagrans, Monacrosporium sinense and Pochonia chlamydosporia on Fasciola hepatica eggs. World J Microbiol Biotechnol 24: 1559-1564.
21. Kuester E, Williams ST (1964) Selection of Media for Isolation of Streptomycetes. Nature 202: 928-929.
22. Maany DA, Kheiralla ZMH, El-Diwany AI, Abdel-Hady MA (2004) Microbiological and biochemical studies on the mycolytic enzymes of some microorganisms. M.Sc. Thesis submitted to Botany Department, Faculty of Girls for Arts, Sci. and Edu., Ain shams University, Egypt.
23. El-Gammal EW, El-Hadedy DE, Saad MM, Moharib SA (2012) Optimization of the environmental conditions for alkaline protease production using Streptomyces griseus in submerged fermentation process. Aust J Basic Appl Sci 6: 643-653.
24. Roweliffe SA, Ollerenshaw (1959) Observations on bionomics of the eggs of F. hepatica. Ann Trop Med Parasit 54: 172-181.
25. REYNOLDS DM (1954) Exocellular chitinase from a Streptomyces sp. J Gen Microbiol 11: 150-159.
26. Valois D, Fayad K, Barasubiye T, Garon M, Dery C, et al. (1996) Glucanolytic Actinomycetes Antagonistic to Phytophthora fragariae var. rubi, the Causal Agent of Raspberry Root Rot. Appl Environ Microbiol 62: 1630-1635.
27. Tominaga Y, Tsjisaka Y (1981) Investigation of the structure of Rhizopus cell with lytic enzymes. Agric Biol Chem 45: 1569- 1575.
28. Tsuchida OY, Yamagata T, Ishizuka T, Arai J, Yamada M, et al. (1986) An alkaline protease of an alkalophilic Bacillus sp. Curr Microbiol 14: 7-12.
29. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.
30. Braga FR, Araújo JV, Campos AK, Carvalho RO, Silva AR, et al. (2007) Observação in vitro da ação dos isolados fúngicos Duddingtonia flagrans, Monacrosporium thaumasium e Verticillium chlamydosporium sobre ovos de Ascaris lumbricoides (Lineu, 1758). Revista da Sociedade Brasileira de Medicina Tropical 40: 356-358.
31. Araújo JV, Santos MA, Ferraz S (1995) Efeito ovicida de fungos nematófagos sobre ovos embrionados de Toxocaracanis. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 47: 37-42.

32. Sundarapandian S, Sundaram MD, Tholkappian P, Balasubramanian V (2002) Mosquitocidal properties of indigenous fungi and actinomycetes against Culex quinquefasciatus. Say J Biol Control 16: 89-91.
33. Yamamoto S, Fukuyama J, Nagasak S (1974) Production, purification, crystallization and some properties of yeast cell lytic enzyme from a species of fungi imperfection. Agr Boil Chem 38 : 329- 337.
34. Shubakov AA, Kucheryavykh PS (2004) Chitinolytic activity of filamentous fungi Appl Biochem Microbiol 40: 445- 447.
35. Mukherjee G, Sen SK (2006) Purification, characterization, and antifungal activity of chitinase from Streptomyces venezuelae P10. Curr Microbiol 53: 265-269.
36. Ramalingam K (1973) The chemical nature of the egg-shell of helminths. I. Absence of quinone tanning in the egg-shell of the liver fluke, Fasciola hepatica. Int J Parasitol 3: 67-75.
37. Cordingley JS (1987) Trematode eggshells: Novel protein biopolymers. Parasitol Today 3: 341-344.
38. LÃ½sek H, StÄ•rba J (1991) Colonization of Ascaris lumbricoides eggs by the fungus Verticillium chlamydosporium Goddard. Folia Parasitol (Praha) 38: 255-259.
39. Gadelhak GG, EL-Tarabily KA, AL-Kaabi FK (2005) Insect control using chitinolytic soil actinomycetes as biocontrol agents. Int J Agri Biol 4: 627–633.
40. Tsay T, Chen P, Wu W (2006) A new method for isolating and selecting agents with high antagonistic ability against plant parasitic nematodes. Plant Pathol Bull 15: 9-16.
41. Lee MD, Tsay TT, Lin YY (1996) Physiological characteristics and effect of Streptomyces saraceticus on hatching ratio of southern root-knot nematode. Plant Prot Bull 38: 235-245.
42. Vetrivel KS, Dharmalingam K (2000) Isolation of a chitinase overproducing mutant of Streptomyces peucetius defective in daunorubicin biosynthesis. Can J Microbiol 46: 956-960.
43. Prapagdee B, Kuekulvong C, Mongkolsuk S (2008) Antifungal potential of extracellular metabolites produced by Streptomyces hygroscopicus against phytopathogenic fungi. Int J Biol Sci 4: 330-337.
44. El-Shafei HA, Abdel-Aziz1 MS, Ghaly MF, Abdalla AAH (2010) Optimizing some factors affecting alkaline protease production by marine bacterium Streptomyces albidoflavus. Proceeding of fifth Scientific Environmental Conference Zagazig University, 125-142.
45. Johnvesly B, Naik GR (2001) Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic Bacillus sp. JB-99 in a chemically defined medium. Process Biochem 37: 139-144.