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Efficacy of Chitinolyic Enzyme Produced by Some Soil Fungi *(Candida Albicans* and *Aspergillus Fumigatus*) in Biological Control of Cattle Ticks

Atef A. Hassan*, Noha, H. Oraby

Departments of Mycology and Mycotoxins
**atefhassan2000@yahoo.com*

M.A.Rashid

Biochemistry
Animal Health Research Institute,
Agricultural Research Center, Egypt

M.M. Minshawy

Faculty of pharmacy
Misr Univ. for Science and Technology

Abstract: *The present study was undertaken to evaluate the use of fungal chitinase enzyme in control of cattle ticks. Fifty soil samples were collected from animal houses at Giza Governorate and examined for mycological contamination. The incidence rates of A.fumigatus and C.albicans were 24 % and 20%, respectively, whereas, other species were also obtained at the rate of 80%. Chitinase enzyme was produced and extracted from cell walls of Candida albicans and Aspergillus fumigates isolates that were recovered from soil and assayed for control of cattle tick's infestation (Boophilus microplus) in comparison with one of the most commonly used arsenical pesticide. These fungi yielded active chitinase enzymes at mean levels of (3.42±0.02 and 2.35±0.03 U/ml), which showed the ability to eradicate 90% and 80% of active ticks, respectively. It is concluded that the chitinase enzymes produced by fungal species could be used for biological control of cattle tick infestation to avoid the use of carcinogenic chemical.*

Keywords: *Candida albicans, A.fumigatus, Chitinase enzyme, Biopesticid, Ectoparasites, Cattle ticks.*

1. INTRODUCTION

Fungi are found frequently in the environment, particularly in nesting places and soil. Animal feces fertilize the soil in such a way as to give the fungus competitive advantage over other soil microorganisms. Mycotic diseases are acquired by inhaling the spores of fungi from environment and soil. They tend to dominate in soils containing high proportion of organic matter and low pH. It has long been known that the soil is the main reservoir of fungi. In recent years, the number of fungal infection in human has increased, which trigger an interest to examine the source and reservoir of such fungi and how they causevarious mycotic infections in man and animals **(1)**

The arthropod parasites (ectoparasites) are a major cause of production losses in livestock throughout the world and many arthropod species act as vectors of diseases for both animals and humans **(2-3)**. The cattle ticks caused huge economic losses due to their effects on milk production and body weight of buffaloes and cattle as well as the transmission of diseases. When tick eradication is controlled by regular pesticides, the cost of chemicals, equipment and production losses associated with treatment plus the high serious effect of pesticides on human and animal health as well as economic impact are major drawbacks **(4-6).** Therefore, the search for alternatives to chemical pesticides gained attention of scientists. Insect killing fungi have high potential in controlling agriculturally harmful pest, but their slow progress and high variation in killing insect are major impediments to their successful industrialization **(7).** So, there is a great demand to introduce this technology in veterinary practices as well as outcome its manufactured problems for controlling animal external parasites. To achieve this goal, a novel fungal chitinases-based biopesticide production was studied. Chitin is a polysaccharide composed of 1, 4 N-acetyl-D-glucosamine units. It is widely distributed in nature, as a constituent of insect exoskeletons, shells of crustaceans and fungal cell walls. The main commercial sources for chitin production are crustacean wastes due to abundance and disponibility **(8).** Chitinases are digestive enzymes that

breaks down glycosidic bond in chitin. Chitin is a component of the cell walls of fungi and exoskeletal elements of some animals (including worms and arthropods), while chitinases are generally found in organisms that either need to reshape their own chitin or to dissolve and digest the chitin of fungi or animals **(8-9).**The fungus of *Metarhizium anisopliae* produces chitinolytic enzymes which had been implicated in digestion of the cuticles of the host insect during the infection process **(10)**. Man has never synthesized an insecticide that has a mode of action not found in nature. Most synthetic insecticides have modes of action similar to those found in plants, animals, minerals, and microbes. Chitinases are enzymes that degrade the chitin contained in an insect's shell-like outer covering called the exoskeleton and the insect's exoskeleton and the thin, threadlike filaments of fungi both possess chitin. Chitin and plant cellulose are both composed of repeating sugar units but differ in how these are connected to each other in forming the large complex molecules known as polymers (**1**1). The chitinases are present in chitin-containing microorganisms and plants with a diversity of roles, such as chitin metabolism in growing cells, defense mechanisms in response to pathogens and a biotic stress, in nutrition and parasitism **(12-13)**. Apart from the application of chitinases as inhibitors and biopesticides, the chitinases have been used for production of single cell protein for animal and aquaculture feed **(14)**. Therefore, the aim of this study was to isolate ***Candida albicans*** **and** ***Aspergillus fumigates*** from soil of animal houses and to evaluate the activity of chitinase enzyme prepared from fungal cell wall in eradication of cattle tick (*Boophilus microplus*) .

2. MATERIALS AND METHODS

1. **Soil samples:** Fifty soil samples were collected from cattle farms in Giza Governorate and transported to the laboratory in sterile plastic bags. The samples were subjected for mycological examination.
2. **Parasites**: *Boophilus microplus*, ectoparasite (cattle tick) were used for laboratory evaluation of fungal chitinase.
3. **Pesticides:** ARSENICAL pesticides, water-soluble forms of arsenic and arsenic-containing compounds, usually As_2O_3, have been used for many years in dipping vats to control ticks, especially ticks of the genus *Boophilus*. It was purchased from El Kahira Chemical Company, Egypt. It was used as control.
4. Isolation and identification of fungi from soil samples

10 gm of soil samples were diluted in 90 ml sterile normal saline and thoroughly mixed by using a shaker for 30 minutes. One ml of the last dilution was directly streaked into three Sabouraud's dextrose agar plates with chloramphenicol as illustrated by **(15, 16)**. The isolated *C. albicans* and *A. fumigatus* strains (10 isolates of each) were maintained on Sabouraud's dextrose agar (Oxoid) and grown in Sabouraud's dextrose broth **(15, 16).**

2.1. Production of Fungal Chitinase

The isolated *C. albicans* and *A. fumigatus* from, soil samples were grown on modified chitinase-detection agar (CHDA) prior to their use for inoculums' preparation. The medium consisted of colloidal chitin as a carbon source and a mixture of yeast extract and peptone as a nitrogen source (2 g/L colloidal chitin, 3.5 g/L bacteriological peptone, 1.5 g/L yeast extract, 1.6 g/L $NaNO_3$, 1 g/L K_2HPO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4.7H_2O$, 0.01 g/L $FeSO_4.7H_2O$ (**17).** The final pH of the fermentation medium was adjusted to 7.0 before the inoculation. The fermentation process was carried at 150 rpm and temperature at 45°C for 24 h. and then centrifuged at 7000 rpm for 5 min. The supernatant was then used for residual NAG (N-acetyl D-glucosamine) and chitinase enzyme assays.

2.2. Measurement of Produced Chitinases Enzymes (18)

Enzyme activity was determined colorimetricaly by detecting the amount of reducing sugar liberated from the hydrolysis of the chitin polymer to the simpler forms of N-acetyl-D-glucosamine monomers (NAG). One unit of chitinase activity is defined as the amount of enzyme required to form 1 μmol of NAG in an hour at 50°C. The reducing sugar released (as NAG) was measured by the DNS method **(19)** and the amount of monomer released was extrapolated from the standard graph of NAG.

2.3. In Vitro Insecticide Activity of Prepared Chitinases from Fungal Cell Walls (20)

One hundred engorged female cattle ticks were used in the experiment and 50 ticks remained as control. The experiment was carried out in Petri dishes of 10 cm diameter and 1.5 cm high. Two sheets of filter paper were soaked in the solution of the tested extracts (separately), one of which was put in the Petri dishes on which the cattle ticks **(Boophilus microplus)** were placed and covered with the other filter paper. The ticks were left for 10 minutes after which they were removed to be dried on filter paper, put in breeding tubes (one for each) and kept in growth chamber at 25 °C and 70 RH.

The ticks were observed every 2 hours during the day of experiment; then observed daily to record activity and mortality rates. Judgment on dead ticks was based on pedol reflex, preovi position, ovipositor period, pre-hatching period, hatching period and egg output per female. Hatching percentage was calculated after counting the un-hatched eggs after 2day from hatching of eggs.

2.4. Statistical Analysis

The obtained data were computerized and analyzed for significance. Calculation of standard error and variance according to **(21)**.

3. RESULTS AND DISCUSSION

Most mycoses that affect animals occur through the soil. This group of diseases named saprozoonoses and the ability of infectious agent to grow saprophytically and replicate in a biotic environment (soil, water, and decaying matter) are the most important characteristics of a spronotic microbe **(22).** The species of *A. fumigates* were recovered in many studies from soil samples as reported by **(23)** at the incidence of 4% of 150 soil samples and **(24)** 1.6% of 100 soil samples .while, **(25)** recovered *A. fumigatus*, from house dust samples at the rate of 10.78%.While, the most frequent yeast fungi were *C. albicans* (38.96%).

On the other hand, **(26)** demonstrated that the most saprophytic fungi isolated from soil containing poultry droppings were species of *Candida.* Whereas, **(27)** recorded prevalence of *Candida spp.*at the rate of 29.7%.

Similler findings were obtained in our work, where *A.fumigatus and C.albicans* were recovered from samples collected from soil of animal farms .The rate of incidence of *A.fumigatus* was 24 % and *C.albicans* was recovered from 20% of the samples . Whereas, other species were also obtained at the rate of 80% (Table, 1).

Table1. *The incidence of A.fumigatus and C.albicans in soil of animal houses (50 soil samples)*

Fungal species	Incidence of isolates	
	Number	%
A.fumigatus	12	24
Candida albicans	10	20
Other fungi	40	80

The current results in (Table, 2) demonstrated that *Candida albicans* and *Aspergillus fumigatus* yielded active chitinase enzyme in amounts of 3.42±0.02 and 2.35±0.003 µ/ml, respectively.

Table (2) Chitinases enzyme concentration (U/ml) in different prepared fungal or bacterial extract.

Organism	Source	Chitinases enzyme (µ /ml)
Fungal strains	*C. albicans* (10 strains)	3.42±0.02
	A. fumigates (10 strains)	2.35±0.03

However, the data in (figures 1 a, b, c) showed that no ticks died during the experimental period in control group, while using arsenicals caused 100% death in ticks after 3 days. On the other hand, the prepared chitinases from *C. albicans* resulted into paralysis of 70% of ticks and 90% of test ticks were dead after 7 days. Whereas, the extracted chitinases from *A. fumigatus* caused paralysis in 60% after 3days and 80% at end of the experiment.

The demonstrated results in Fig. (1) Showed the mean time effect per day of every tested extract compared with arsenicals, where it was 4.3, 6.8, 1.5 days for, *Candida albicans, Aspergillus fumigatus* and arsenicals, respectively.

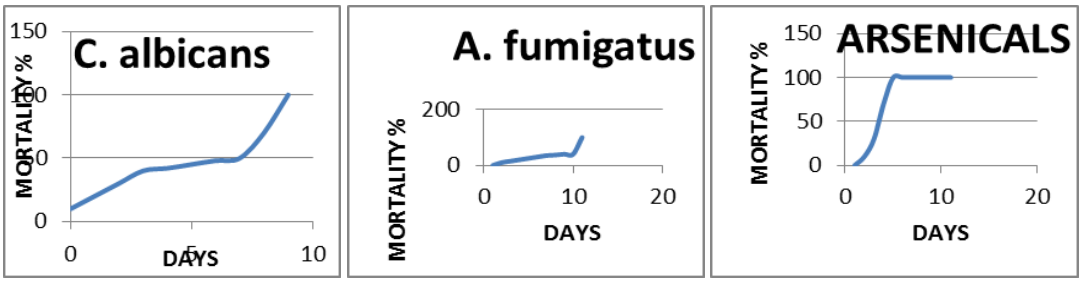

Fig1. *Effects of fungal chitinases (C.albicans & A.fumigatus) on ticks compared with Arsenicals.*

The extraction procedure of chitinases were compared with the aim of isolating sufficient quantities from fungal cell walls for future purification studies and for characterization of the potential of these enzymes in inhibition of harmful parasites particularly ticks. The microorganisms able to degrade chitin are widely distributed in nature **(28)**. Among the producers are fungi like Mucor, *Candida albicans, Aspergillus fumigatus* and *Pencillium chrysogenum* **(18)(28-3**3). In the present work the *chitinases production by the fungal species of C. albicans was 3.42±0.02* and *A. fumigatus* was 2.35±0.003 U/ml,.

The prepared chitinases from tested fungi were tested for *in vitro* study for eradication of ticks. The results revealed that the activity of extracted enzymes from *C. albicanse* (3.42±0.02 µ/ml) caused death of 40% of live active ticks at 3rd day of their treatment with this extract, whereas, the mortality rate reached to 90% at 7th days post application of this enzyme for eradication of ticks. However, the extracted enzymes from cell walls of *A. fumigatus* (2.35±0.003ug/ml U/ml) required 7 days to kill 40% of live active ticks and the mortality rate reached 80% on the 8th day. The higher elevation in the activities of *C. albicans* than *A. fumigatus* extracts is due to the slight high level of enzyme obtained from yeasts of *C. albicans* than filamentous fungus of *A. fumigatus* where during the budding of yeast cells, the degradation of chitin deposit at the bud sit leads to cell separation and involves the activity of extensively glycosylated endochitinase . This led to abnormal spore wall biosynthesis which caused more digestion and utilization of exogenous chitin and yielded higher enzyme activities than other filamentous fungi.These results confirm the findings previously reported by **(5-6)** and **(34-38)**.However**,** yeasts and fungi with yeast-like growth forms have low numbers of chitinases, *Candida albicans* has four, but filamentous fungi have, in general, between 10 and 20 different Glycoside hydrolase (GH) family 18 proteins **(39).** Mycoparasitic and entomopathogenic fungi have even 30 or more (GH) family 18 proteins (**40-41).** It should be noted that for most of these proteins, biochemical evidence verifying that they are active chitinases is still missing. However, based on aa-similarities and the presence of conserved aa-residues that are essential for catalytic cleavage, it can be assumed that most of these GH family 18 proteins are indeed chitinases **(42).** As has been discussed in a recent review (**43)**, suggest that the same chitinases can participate in self- and non-self cell wall degradation

Apart from the application of chitinases as inhibitors and biopesticides, the chitinases have been used for production of single cell protein for animal and aquaculture feed **(14),** for the isolation of fungal and bacterial protoplasts, preparation of bioactive chito-oligosaccharides and for phytopathogen inhibition, reinforcement of animal defense **(44)**. Among the major benefits of incorporating biopesticides into agriculture system that it are generally more environmentally friendly and do not damage the soil, water supply or wildlife-including the beneficial insects. Their safety to beneficial organisms is one area where biopesticides are definitely having a fit. The mean time effect per day give an indication on the efficacy of pesticide, the shorter the time the better is efficacy of the product. Our results proved that these times was 4.3, 6.85, 1.5 days for, *Candida albicans, Aspergillus fumigates* and Arsenicals respectively. So, further studies must be carried out to decrease this period for increasing the efficacy of prepared biopesticides. Recent advances toward understanding the roles of chitinases in fungal biology, as well as substrate-binding properties and new mechanistic insights in different chitinases should include biotechnological implications of these findings. In aggressive fungi and parasites, e.g. mycoparasites and ectoparasites of invertebrates, chitinases are involved in the attack of other

fungi and insects. Over-expression of single chitinases in mycoparasitic, insects and entomopathogenic fungi and/or addition of a chitin-binding domain to these chitinases was shown to improve the mycoparasitic potential and insect virulence, respectively, of these fungi (**45).**

4. CONCLUSION

From the foregoing results, it is become apparent that the fungal chitinases showed a successful role in the processes of eradication of parasites which would be suitable biological control agents in agricultural applications. The use of chitinases solves many problems associated with application of chemical carcinogenic bio-pesticide in control of ecto-parasites infestation in farm animals.

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